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^{99m}Tc labelled peptides for imaging of peripheral receptors

Final report of a co-ordinated research project
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FOREWORD

^{99m}Tc radiopharmaceuticals have remained the workhorse of diagnostic nuclear medicine over the last three decades ever since the introduction of the gamma camera as the main imaging instrument. Due to the near ideal nuclear properties such as gamma energy, half-life, lack of beta radiation and easy availability as a convenient generator system at an affordable cost of ^{99m}Tc, it can be reasonably anticipated that ^{99m}Tc will continue to retain this position in the foreseeable future. To a large extent this has been possible because of the successful development, over the years, of ^{99m}Tc radiopharmaceuticals as substitutes for other clinically well established agents. Examples of these success stories are ^{99m}Tc substitutes for ^{131}I hippuran and rose bengal, ^{201}Tl and ^{123}I brain perfusion agents, which have come to be known collectively as 'second generation ^{99m}Tc radiopharmaceuticals'. It should be acknowledged that each one of these developments was a result of innovative and sustained research and development efforts by scientists from different parts of the world. Concurrently these research efforts have made significant contributions to better understanding of the radiochemistry and co-ordination chemistry of ^{99m}Tc. The radiopharmaceutical scientists are now in a much better position to design, prepare and evaluate ^{99m}Tc complexes for specific applications. Building on this capability, the next step is development of ^{99m}Tc substitutes for receptor specific radiopharmaceuticals, which have established clinical potential. Efforts in this direction are already ongoing and the work during the last decade on ^{99m}Tc labelling of monoclonal antibodies can be considered the beginning of these 'third generation ^{99m}Tc radiopharmaceuticals'. The International Atomic Energy Agency (IAEA) had organized two co-ordinated research projects (CRPs) in the past covering ^{99m}Tc second generation agents and ^{99m}Tc monoclonal antibodies, and the results were published in two IAEA-TECDOCs.

Even though a few ^{99m}Tc monoclonal antibodies are now used for diagnostic imaging, it is generally acknowledged that receptor specific peptides will provide better carriers for ^{99m}Tc for *in vivo* receptor imaging. The possible peptides are, however, too numerous and information from other sources, such as the pharmaceutical industry, has to be relied upon to narrow down the choice for labelling and evaluation. One such peptide, octreotide, an analogue of the neuropeptide somatostatin, developed by the pharmaceutical industry for therapy of neuroendocrine tumours, was used as a carrier for ^{123}I and ^{111}In for imaging such tumours. ^{111}In-octreotide soon became a regular, commercially available and probably the first peptide based radiopharmaceutical successfully used for imaging somatostatin positive tumours. There are several obvious advantages of using ^{99m}Tc in place of ^{111}In and soon development of a ^{99m}Tc labelled octreotide analogue attracted the attention of several radiopharmaceutical research groups. Keeping in mind the possibility of a wider reach for a ^{99m}Tc octreotide agent and based on the recommendations of an Advisory Group meeting, the IAEA initiated a CRP in 1995 on ^{99m}Tc Labelled Peptides for Imaging Peripheral Receptors. The techniques and methodologies involved in ^{99m}Tc labelling of peptides with high specific activity and their evaluation are also more complex and their mastering could be expected to pave the way for further research and development of other ^{99m}Tc labelled peptides for various applications in Member States. Fourteen laboratories from Europe, Latin America, the United States of America and Asia took part in the CRP which was concluded at the end of 1999. Efforts in the CRP were focused on evaluating different methods of ^{99m}Tc labelling of a few selected octreotide derivatives and evaluating their receptor specificity by biochemical techniques. Based on the experience of a number of the participants, it could be concluded that ^{99m}Tc labelled HYNIC conjugate of tyrosine-3-octreotide is comparable to ^{111}In-octreotide and a potential candidate for further evaluation. The standardized protocols for its preparation and quality control are included in this report. They are expected to provide practical guidance to any radiopharmaceutical laboratory to initiate work on ^{99m}Tc labelled

octreotide and ^{99m}Tc labelling of peptides in general. One participating laboratory has developed ^{99m}Tc labelled vasoactive intestinal peptide, another potentially useful peptide for tumour imaging and its details are also included in the report.

The IAEA wishes to thank all the participants in the CRP for their valuable contributions. The IAEA officers responsible for this CRP were H. Vera Ruiz and D.V.S. Narasimhan of the Division of Physical and Chemical Sciences.

EDITORIAL NOTE

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1. INTRODUCTION

In recent years the range of radiopharmaceuticals for diagnostic use has been enlarged with a new class of radiolabelled compounds based on analogues of naturally occurring peptides of high receptor specificity. When administered *in vivo* they bind to the sites expressing particular receptors. This mechanism of action allows characterization of the pathologically changed tissue at a cellular level. In addition, unlike monoclonal antibodies, peptides are usually small, flexible molecules which clear rapidly from the circulation to interact efficiently with the high affinity receptors on the surface of cells with much less likelihood of immunoreactivity. They can be synthesized in large quantities with high purity and can also be characterized fully. Synthesis of suitable bifunctional chelates, radiolabelling, purification and evaluation are likely to be more amenable with peptides.

Despite these advantages, the development of peptide based radiopharmaceuticals can proceed rapidly only if the right peptides with affinity to specific antigenic sites are identified. This is not easy considering the fact that each antigenic site can interact with thousands of potential small peptides whose synthesis, radiolabelling and experimental screening is probably beyond the limited resources of the radiopharmaceutical groups researching in this field. Consequently, a small number of specific peptides from other sources such as the pharmaceutical industry have formed the starting point for carrying out research in this domain. The usefulness of images taken with radiolabelled peptides has been proven in oncology, in the diagnosis of a variety of tumours. Among the various peptides reported, somatostatin analogues whose receptors are expressed by a variety of tumours, specially of neuroendocrine origin, have shown the maximum potential for tumour scintigraphy. The ^{111}In labelled somatostatin analogue, ^{111}In-DTPA-octreotide is the first commercially available and widely clinically used product of this new class of radiopharmaceuticals. Successful application of ^{123}I labelled octreotide analogue has also been reported. Unfortunately, these two radioisotopes are cyclotron produced, available only in a limited number of centres and expensive.

Therefore, to make somatostatin receptor scintigraphy universally available, development of a ^{99m}Tc labelled analogue is imperative. ^{99m}Tc is the most widely used radioisotope in nuclear medicine, commonly available and much less expensive when compared to ^{111}In or ^{123}I. With this objective in mind the IAEA organized a Co-ordinated Research Project (CRP) on ^{99m}Tc Labelled Peptides for Imaging Peripheral Receptors, based on the recommendations of an Advisory Group meeting held in Vienna earlier. In addition to the expectation that the project would lead to the development of a useful ^{99m}Tc somatostatin analogue, the other motivating factor for the CRP was that the participants, particularly from developing countries and with varied experiences, will have the opportunity to work in an area which marks an important new direction in radiopharmaceutical development and to acquire new skills including reverse phase high performance liquid chromatography (HPLC) analysis and purification strategies based on solid phase extraction. Another important aspect in which the participants will gain experience is the capability to study *in vivo* and *in vitro* receptor binding assays. These will help them in the future for further research and development on peptide based radiopharmaceuticals for diagnosis as well as therapy. Fourteen laboratories from Asia, Europe, North and South America took part in this four year CRP. The first research co-ordination meeting (RCM) to plan the work programme was held in Sacavem, Portugal in March 1996. It was decided to pursue the direct labelling approach for ^{99m}Tc using RC-160 as a model peptide. The second RCM was held in Warsaw, Poland, in December 1997 to review the progress and discuss future work plan. It was decided in the meeting to work with ^{99m}Tc labelling of bifunctional chelates of another peptide, Tyr^3-

Octreotide (TOC) as direct labelling of RC-160 was not very promising. The third and final RCM was held in Buenos Aires, Argentina, in November 1999 to assess the progress of the CRP and decide on the final report.

2. CONCLUSIONS OF THE CRP

2.1. Technical conclusions

All participating centres have made significant progress acquiring and applying the techniques required in this programme including: radioiodination of peptides, direct labelling of peptide with ^{99m}Tc, bifunctional chelate conjugation, purification of conjugates and ^{99m}Tc labelling of conjugates and purification. Valuable experience was also gained on the applications of analytical techniques like HPLC, TLC/ITLC, electrophoresis for the separation and detection of different components. Methods for measuring radioligand binding assays & biodistribution were also acquired. However more work is needed in some laboratories to conclude their finding. Areas which created greatest difficulty in some laboratories were direct labelling of peptides, acquisition and interpretation of reproducible HPLC chromatograms and development of quantitative binding assays.

2.2. Scientific conclusions

2.2.1. Conjugate preparation and labelling

(1) The peptide RC-160 can be readily radioiodinated using either iodogen or chloramine-T techniques to produce reagents which remain stable for several weeks.

(2) Direct labelling of RC-160 could be achieved in some centres but not others. Limited opportunity was available for the optimization of this procedure owing to limited availability of the unprotected peptide. Labelling efficiencies up to 90% were achieved, but significant technical difficulties were found with this method and a decision was taken during the course of the CRP to concentrate on bifunctional chelate approaches.

(3) The three technetium complexing agents used were successfully synthesized in some centres and the ease of synthesis was found to be HYNIC>MAG-3>tetramine.

(4) The HATU system is a convenient and reproducible method for conjugation of these complexing agents to peptides. The use of N-hydroxysuccinimide esters was comparatively less successful.

(5) All three technetium complexing systems can be used to prepare radioligands with high radiochemical purity and high specific activity (>40 GBq/μmole).

(6) SEPPAK purification is, in most cases, a convenient and simple method for the purification of peptides and their conjugates from hydrophilic impurities.

(7) In general higher labelling efficiencies are obtained when HYNIC conjugates are labelled using tricine and tricine/nicotinic acid than with EDDA, but >95% labelling efficiencies are possible with EDDA if tricine is used as a transfer ligand.

(8) Convenient one-step 'kits' of HYNIC-conjugates and co-ligands can be prepared producing preparations with high labelling efficiency.

2.2.2. In vitro studies

(1) A reproducible system of HPLC analysis is essential for monitoring the conjugation and labelling of these peptides. The systems described in the protocols were found to be applicable in most participating laboratories.

(2) Interpretation of the, sometimes complex, HPLC chromatograms obtained can be difficult and the use of defined quality assurance (QA) programme and known standards is essential.

(3) Different mobile-phase systems are needed to identify heterogeneity in radioconjugate preparations.

(4) Once validated, TLC/ITLC and electrophoretic techniques are a convenient means of measuring the labelling efficiency of these compounds and should be used in combination with HPLC in particular to identify insoluble components.

(5) Development of radioligand binding assays requires considerable effort. In particular the quantitative analysis of lipophilic compounds such as RC-160 conjugates, which show a high degree of non-specific binding is very difficult. When successfully performed, these assays show that all the radiolabelled peptide conjugates studied retain a high affinity for the somatostatin receptor.

(6) All the radiolabelled peptides are stable in aqueous solution and show only limited transchelation to challenge agents such as cysteine.

(7) Incubation studies in human plasma show little evidence of proteolytic degradation or instability of the radiolabel (except N4-RC160). However, varying degrees of protein binding were seen, in particular in the HYNIC system, EDDA and tricine/nicotinic acid show lower protein binding than tricine in this system.

2.2.3. In vivo studies

(1) All ^{99m}Tc labelled peptides studied show rapid clearance from the blood. The uptake in receptor-rich tissues which can be blocked to varying degrees by co-injection of cold octreotide varied from compound to compound.

(2) RC-160 conjugates are more lipophilic and show greater hepatobiliary clearance and lower receptor-mediated uptake than TOC conjugates.

(3) MAG-3 conjugation results in peptides which are relatively lipophilic and show greater uptake and excretion by the hepatobiliary system.

(4) HYNIC, when used in conjunction with appropriate co-ligands, results in more hydrophilic radioconjugates which show a more desirable pattern of biodistribution.

(5) The tetramine (N4) core is an alternative system which also produces hydrophilic radioconjugates with a promising pattern of biodistribution.

(6) Tricine, EDDA and the mixed tricine/nicotinic acid system are all useful co-ligands for use with HYNIC conjugates. EDDA and tricine/nicotinic acid show lower uptake in non-target tissues than tricine.

(7) The HYNIC-TOC conjugates are excreted unchanged in the urine while RC-160 conjugates appear to be more unstable *in vivo* with more metabolites being excreted.

2.3. Areas of future research

(1) Structural characterization of the peptide-technetium-complexes, determination of charge, co-ligand valency;

(2) Exploration of renal clearance and metabolism in particular tubular re-absorption mechanisms and blocking systems;

(3) Acute toxicological studies;

(4) Studies in different tumour models;

(5) Alternative peptides, co-ligands and labelling methods.

3. ACHIEVEMENTS OF THE CRP

The technology for radiolabelling of the peptides, bifunctional chelate conjugation, conjugate purification, analysis using specialized techniques, and use of receptor positive animal models have been established in the participating centres. For many participants this provided the first opportunity for them to work in this field. This know-how can support future development of other new diagnostic and therapeutic agents in radiopharmaceutical research.

It has been demonstrated that ^{99m}Tc labelled somatostatin analogues can be prepared with high radiochemical purity and specific activity, which retained affinity for the receptor, sufficient receptor-mediated uptake in target tissues and acceptable pattern of biodistribution. Protocols for these techniques have been drawn up, validated and published.

The studies carried out under this CRP have culminated at such a point from where clinical applications of ^{99m}Tc-HYNIC-TOC can be initiated.

Interactive collaboration and co-operation have been established between many of the CRP participants, which have enabled the transfer of materials and information. These collaborations are expected to continue beyond the completion of the CRP and an interest group has also been established.

4. RECOMMENDED PROCEDURES AND PROTOCOLS

4.1. Preparation of peptide conjugates for ^{99m}Tc labelling

4.1.1. HYNIC conjugate

4.1.1.1. Preparation of Boc protected HYNIC-peptide conjugate

Prepare following solutions in dry glass vials:

(1) 1,3 mg 6-Boc-Hydrazinpyridine-3 carboxylic acid in 100 µL dry dimethyl formanide (DMF)

(2) 2,0 mg HATU in 100 µL DMF

(3) 10 µL Diisopropylethylamine in 300 µL DMF.

— Add each 50 µL of solution 1, 2 and 3 to a dry glass vial (reaction solution) and allow to react for 15 min;

— Prepare a solution of 1 mg Boc-peptide in 20 µL DMF and 5 µL water in a 5 mL reaction vial;

— To this solution add 60 µL of the reaction solution after the 15 min reaction time;

— Allow the solution to react for 1 h;

— Activate a SEPPAK Mini Cartridge by washing with 5 mL ethanol followed by 5 mL water. Dry the cartridge by pushing 5 mL of air through the cartridge;

— After a minimum of one hour reaction time add 1 mL of water to the reaction vial and slowly load the solution into the SEPPAK cartridge, slowly wash the reaction vial and cartridge with additional 3–4 mL of water. Dry the cartridge by pushing 5 mL of air through it;

— Slowly elute the cartridge with 0,5 mL of 100% acetonitrile and collect the eluate in a 1 mL Eppendorf vial;

— Reduce the acetonitrile solution on the Speed-Vac (low temperature) to a volume of about 100 µL.

4.1.1.2. Deprotection of Boc-HYNIC-peptide conjugate

— Add 10 µL thioanisole and 300 µL TFA to the 100 µL acetonitrile solution in Eppendorf vial prepared in 5.1.1.1;

— Allow to react for maximum of 5 min;

— Put Eppendorf vial in Speed-Vac and evaporate to dryness (about 30–45 min. at low temperature);

— Dissolve the residue in 50 µL ethanol, then add 50 µL water, and load the solution on the HPLC;

— Wash vial and injection syringe with 2 × 30 µL 50% ethanol;

— Purify the solution by HPLC using TFA gradient;
  – Reverse Phase column: Beckman Ultrasphere ODS 5 um, 4,6 × 250 mm;
  – 0,1% TFA/water, flow 1 mL/min, 220 nm;

– Gradient: 0–3 min 0% ACN, 3–13min 0–50% ACN, 13–23 min 50%A CN, 23–26 min 50–70% ACN, 26–27min 70–0%CAN;

— Collect the peak of the deprotected HYNIC-peptide conjugate at about Rt 15 min in a glass vial.

— Close glass vial with rubber and aluminium cap, seal it and purge with nitrogen for five min;

— Store the solution at –20°C.

REMARKS:

Provided stoichiometric ratios are maintained, the amounts of peptide can be changed easily without loss of yields; however, larger quantities of peptide may require the use of larger SEPPAK cartridges to avoid losses.

4.1.2. Benzoyl MAG-3 conjugate

4.1.2.1. Preparation of Boc-benzoyl MAG-3-peptide conjugate

Prepare following solutions in dry glass vials:

(1) 2,2 mg benzoyl MAG-3 in 100 µL dry DMF
(2) 2,0 mg HATU in 200 µL DMF
(3) 10 µL Diisopropylethylamine in 300 µL DMF.

— Add 100 µL of solution 1, 50 µL of 2 and 20 µL of 3 to a dry glass vial (reaction solution) and allow to react for 15 min;

— Prepare a solution of 0,4 mg Boc-RC-160 in 40 µL DMF and 5 µL water in a 5 mL reaction vial;

— To this solution add 40 µl of the Reaction solution after the 15 min reaction time;

— Allow the solution to react for one hour;

— Activate a SEPPAK Mini Cartridge by washing with 5 mL ethanol followed by 5 mL water, dry the cartridge by pushing 5 mL of air through it;

— After a minimum of one hour reaction time add 1 mL of water to the reaction vial and slowly load the solution onto the SEPPAK cartridge, slowly wash the reaction vial and cartridge with additional 3–4 mL of water;

— Dry the cartridge by pushing 5 mL of air through it;

— Slowly elute the cartridge with 0,5 mL of 100% acetonitrile and collect the eluate in a 1 mL Eppendorf vial;

— Reduce the acetonitrile solution on the Speed-Vac (low temperature) to a volume of about 100 µL;

— Deprotect the Boc-benzoyl MAG-3 peptide conjugate as described above.

4.2. ^{99m}Tc labelling of peptide conjugates

4.2.1. HYNIC conjugate using tricine as co-ligand

METHOD 1: Two vial labelling

Prepare following in rubber stoppered vials:

(1) 140 mg tricine (Sigma T-0377) in 2 mL water
(2) 3 mL 0,1N HCl
(3) 10 mg dry stannous chloride dihydrate.

— Purge each vial with nitrogen for a minimum of 5 min and dissolve the stannous chloride in 2 mL of purged 0,1N HCl;

— Add up to 300 MBq sodium [^{99m}Tc] pertechnetate in 2 mL to a rubber sealed vial;

— Add subsequently: 0,5 mL tricine solution and 20 μL stannous chloride in HCl and allow to react for 10–15 min;

— Add 10 μg peptide conjugate solution to a sealed vial;

— Add 1 mL ^{99m}Tc-tricine solution to the peptide solution and let it react for 20 min.

METHOD 2: One vial labelling

Prepare the following in rubber stoppered vials:

(1) 100 mg/mL tricine in 25 mM Succinate buffer pH5 (>1 mL);
(2) 10 mg stannous chloride dihydrate in a 10 mL vial;
(3) 10 mL 0,1N HCl.

— Purge all solutions with nitrogen for at least 5 min;

— Add subsequently in a 3 mL rubber closed vial: 10 μg peptide conjugate, 0,5 mL tricine solution; ^{99m}Tc pertechnetate in 0,5 mL;

— Dissolve stannous chloride in 10 mL 0,1N HCl;

— Add 25 μL of this solution to start the reaction;

— Incubate for 30 min.

4.2.2. HYNIC conjugate using nicotinic acid as ternary ligand

— Prepare HYNIC/tricine compound as described in Method 2 above;

— Prepare 1 mL nicotinic acid solution (40 mg/mL in 25 mM succinate buffer pH5);

— Add 0.1 mL of this solution to the reaction vial;

— Heat at 100°C for 15 min.

4.2.3. HYNIC conjugate using EDDA as co-ligand

Prepare the following:

(1) 30 mg EDDA in 1.5 mL 0,1N NaOH;
(2) 60 mg tricine in 1.5 mL 0,2N phosphate buffer pH7.2;

(3) 6 mg stannous chloride dihydrate in a 10 mL vial;
(4) 10 mL 0,1N HCl.

— Purge each vial with nitrogen for a minimum of 5 min and dissolve the stannous chloride in 6 mL of purged 0,1N HCl;

— Heat water bath to 70°C;

— Mix 1 mL EDDA solution and 1 mL tricine solution (10 mg/mL EDDA, 20 mg/mL tricine);

— Add 20 μg of HYNIC-TOC into an evacuated vial;

— Add 1 mL EDDA/tricine solution;

— Add 1 mL ^{99m}Tc pertechnetate (up to 600 MBq);

— Add 15 μL of the stannous chloride solution;

— Heat for 30 min in the water bath (70°C).

4.2.4. Benzoyl-MAG-3-conjugate

— Turn on water bath to boiling temperature;

— Prepare following materials in rubber stoppered vials: 200 mg sodium tartrate in 2 mL 0.1M phosphate buffer pH7.2; 3 mL 0,1N HCl; 10 mg dry stannous chloride dihydrate;

— Purge each vial with nitrogen for a minimum of 5 min and dissolve the stannous chloride in 2 mL of purged 0,1N HCl;

— Add conjugate solution containing 10 μg of the conjugate to a rubber sealed vial;

— Add up to 300 MBq of ^{99m}Tc pertechnetate in 2 mL;

— Add subsequently: 0,5 mL tartrate solution; 20 μL stannous chloride in HCl;

— Place the vial for 15 min in a boiling water bath.

4.2.5. Reverse-phase HPLC of peptide conjugates

A gradient HPLC system is required with online UV and, preferably, radiometric detection. UV detection should be performed at 220 nm. A suitable C-18 (ODS) reverse-phase column (e.g. 'Jupiter' Phenomonex) 25 cm in length is recommended. A flow-rate of 1 mL/min should be used.

The mobile-phase needed depends upon the particular application envisaged.

METHOD 1 is suitable for:

(a) monitoring the conjugation procedures
(b) monitoring the deprotection step
(c) purification of the peptide conjugate
(d) assessing the radiolabelling efficiency/stability of the labelled peptide.

METHOD 2 is suitable for:

(a) determining the presence of isomers in the radiolabelled preparations
(b) separating labelled from unlabelled peptide conjugate.

METHOD 1:

Solvent A is acetonitrile; solvent B is 0.1%TFA/water: 0–3min 0%A, 3–10min 0–40%A, 10–20min 40%A, 20–23 min 40–70%A, 26–27 min 70–0%A

METHOD 2:

Solvent A is acetonitrile; solvent B is 0.01M phosphate buffer pH6.2: 0–3 min 0%A, 3–10 min 0–25%A, 10–20 min 25%A, 20–23 min 25–70%A, 26–27 min 70–0%A

4.3. Biodistribution studies on radiolabelled somatostatin analogues

These studies may be performed in either normal or tumour bearing animals, normally rats or mice. The purpose of the study may be to determine:

— rate of blood clearance
— rate and route of excretion
— *in vivo* stability of the peptide complex
— degree of uptake and retention in receptor-negative tissues
— degree of uptake and retention in receptor-positive tissues and the extent to which this may be blocked by co-administration of unlabelled peptide.

Somatostatin receptor positive tumour models include:

(1) AR4-2J Rat pancreatic tumour in nude mice
(2) AtT-20 Mouse pituitary tumour in nude mice
(3) CA20948 Rat pancreatic tumour in normal rats.

If these or other suitable tumour models are not available then the adrenals and pancreas are suitable receptor-positive tissues for study in normal animals.

A wide variety of procedures may be employed for biodistribution studies. The following is a protocol which has been found to be useful for comparing the biodistribution of a series of ^{99m}Tc labelled somatostatin analogues in nude mice bearing AR4-2J xenografts.

Nude mice are injected subcutaneously in the flank with 5×10^6 AR4-2J cells. Tumours grow to a useable size (approx. 5 mm diameter) in about two to three weeks. For each study eight animals are required, divided into two groups. One group is injected intraperitoneally with 50 μg of octreotide (Sandostatin, Novartis, Basel) and the other group with the same volume of sterile saline for injection. Thirty minutes later all the animals are injected intravenously via a tail vein with 0.1 μg of peptide conjugate radiolabelled with approximately 1 MBq of ^{99m}Tc in a volume of about 50 μL. Syringes should be measured before and after injection in order to accurately determine either the weight or the activity of material injected. After the desired time period has elapsed (most commonly 4 h) the animals are killed and samples of organs of interest (typically, blood, muscle, liver, kidneys, spleen, lungs, stomach, intestines, pancreas, adrenals, tumour and tail) are collected and placed in pre-weighed counting tubes. These are then counted in a gamma counter together with a

standard prepared from a known dilution of the injected material (preferably prepared at the time of injection). The total activity injected into each mouse is determined and the activity remaining in the tail is subtracted. The uptake of the radiopharmaceutical in each tissue is calculated in terms of % ID/g and the uptake in the two groups of mice (blocked and unblocked) is compared by means of a suitable statistical test such as Student's *t* test.

4.4. Somatostatin receptor binding assay

4.4.1. Preparation of membranes from cell-lines

4.4.1.1. Reagents

Dilution buffer: 20 mM HEPES pH7.3 containing 10 mM $MgCl_2$ and 10μM Bacitracin. (This should be freshly prepared every two weeks, and stored at 4°C. Stock solutions of 10 M $MgCl_2$ (2 g/mL) and 14 mM Bacitracin (20 mg/mL) can be prepared and stored for some months at 4°C).

Somatostatin receptor positive cells, e.g. AR4-2J, AtT-20): Ideally $>10^8$ cells should be used. Approximately 15×10^6 cells are required per assay.

4.4.1.2. Preparation

(1) Place the cells in culture media in a 50 mL centrifuge tube on ice. Half fill a sufficient number of tubes to contain all the available cells;

(2) Spin cells down (20 min. 1500 G) and resuspend in ~20 mL cold dilution buffer/tube;

(3) Crack cells by holding the tube in an Ultraturrax homogenizer for ~10sec/tube. Keep the cell preparations on ice;

(4) Spin down the membranes again and discard the supernatant. Combine all the membranes into one tube, resuspending in dilution buffer and repeat the Ultraturrax treatment;

(5) Repeat step 4 twice more finally resuspending the membranes in 1 mL of buffer;

(6) Measure the protein concentration of the membranes using a suitable assay (e.g. Bradford or Biorad Protein assay);

(7) Resuspend membranes in dilution buffer, to a concentration of about 5 mg/mL;

(8) Homogenize with Ultraturrax for 10 seconds (s);

(9) Divide into suitable size aliquots for the binding assay (a 24 sample assay requires about 1 mg of membranes). Freeze at –70°C or in liquid nitrogen until required for use.

4.4.1.3. Protocol for Somatostatin binding assay (competition assay)

(a) Prepare the following solutions:

Radioligand stock solution: dissolve 370 kBq 125I-SST14 (Amersham) in 1,5 mL 50% ethanol water. Make aliquots of 50 μL (12 kBq, one aliquot is normally sufficient for one binding assay, depending on the calibration date).

Dilution buffer: 500 mL 20 mM HEPES pH7,3 (store in the refrigerator, renew every two weeks).

Washing buffer: 2000 mL TRIS buffered saline: 15 mM TRIS, 139 mM sodium chloride, pH7,4 (store in the refrigerator, renew every two weeks).

$MgCl_2$ Solution: 10 M (stable for several months).

Bacitracin solution: 14 mM (stable for several months).

— Prepare 1% BSA in 40 mL dilution buffer (requires 10 min shaking). Add 40 µL $MgCl_2$- and 40 µL Bacitracin solution *(Buffer A)*;
— Add 20 µL $MgCl_2$- and 20 µL Bacitracin solution to 20 mL dilution buffer *(Buffer B)*;
— Put buffers A and B on ice.

(b) Dilution of competitor

Note that for a good assay, at least eight concentrations of competitor are necessary. Make sure that the mean concentration is about in the range of the expected IC50 value.

— Prepare a stock solution of 40 µM competitor in buffer A;
— For an expected IC_{50} of 1 nm, dilute the competitor in 1 mL Eppendorf vials using buffer A to prepare the following concentrations: 0,04/0,4/1,2/4/12/40/400/4000 nm;
— Vortex thoroughly in between dilutions;
— These will produce final assay concentrations of: 0,01/0,1/0,3/1/3/10/100/1000 nm.

(c) Dilution of Radioligand

— Dilute the radioligand with buffer A so that the expected concentration is about 20000–30000 cpm/50 µL;
— Measure 50 µL in a gamma counter. If, more or less 20 000–30 000 cpm is obtained, adjust the concentration accordingly.

(d) Assay procedure

— Turn on the water bath (15°C);
— Remove an aliquot of cell membrane stock solution from the freezer and put on ice;
— Prepare 24 polypropylene tubes (Sarstedt 3 mL) and label them according to the dilution of the competitor in triplicate;
— Pipette 3 × 50 µL of each competitor dilution into the tubes, change pipette tips for each concentration;
— Pipette 50 µL radioligand solution into each vial;
— Dilute the membrane stock solution to 400–500 µg/mL using buffer B, thoroughly homogenize the solution using the multi pipette, and add 100 µL to each tube (40–50 µg membranes/tube);
— Cap and vortex each tube, place the tubes in the water bath and incubate for 2 h.

If using the 8-position Whatman vacuum manifold for filtration:

— Put 26 glass fibre filters (Whatman GF/C) in buffer A;
— Label 24 counting vials in the same way as the Sarsdedt tubes;
— Place 250 mL washing buffer on ice and prepare the multi pipette;
— Prepare the filtration sample manifold for use;
— Take tubes out of the water bath and place on ice, put pre-soaked filters on the sample manifold and start the vacuum;

— Place the first 12 tubes in a rack and remove caps;
— Quickly pipette 2 mL ice cold washing buffer into each tube, draw up the contents of the tube and apply to the filtration manifold;
— Wash each tube twice with 3 mL washing buffer and filter the washings;
— Wash each filter in the sample manifold with 1 × 3 mL washing buffer;
— Put the filters into the counting vials and repeat the procedure with the last 12 tubes;
— Count filters in the gamma counter and calculate IC_{50} values using Microcal ORIGIN.

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REPORTS BY PARTICIPANTS IN THE CO-ORDINATED RESEARCH PROJECT

SOMATOSTATIN ANALOGUES LABELLED WITH ^{99m}Tc

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Abstract

The aim of the present work was to study the biological and radiochemical behaviour of two somatostatin analogues, the RC-160 and Tyr3Octreotide(TOC) peptides when labelling with ^{99m}Tc by two methods: direct and indirect using S-benzoyl- mercaptoacetyl triglycine (MAG-3) and hydrazinonicotinamide (HYNIC) as chelating agents. RC-160 was labelled with 125I (30% labelling yield) in order to examine its receptor specificity and to study the biodistribution in normal animals. A total binding of 30% and a non specific binding lower than 10% was obtained. On the other hand, the RC-160 was labelled with ^{99m}Tc by a direct method (70% labelling yield), using sodium ascorbate and dithionite in order to reduce the peptide and ^{99m}Tc, respectively. The synthesis of RC-160 with S-benzoyl MAG-3 and TOC with HYNIC, for labelling with ^{99m}Tc are also described. The conjugates were prepared on a small scale and labelled with the radionuclide using tricine as co-ligands for HYNIC conjugates. Chromatographic studies were performed using HPLC system and radiochemical purities higher than 75% and 95% were obtained respectively. Biodistributions studies in normal Wistar rats were performed and results were correlated with chromatographic and protein binding properties. Lower lipophilicity of the labelled conjugates resulted in a higher renal excretion. HYNIC-TOC complex showed promising results when labelling with ^{99m}Tc using tricine as co-ligand although higher stability should be found for ternary co-ligands compared to tricine.

1. INTRODUCTION

Somatostatin is a peptide hormone containing 14 aminoacids with a short biological half-life of only 2 min and which exhibits a wide spectrum of biological and oncological actions [1]. The expression of large numbers and high-affinity somatostatin receptors by certain tumours makes it attractive but unsuitable for diagnostic application in nuclear medicine. For this reason several analogues have been synthesized which are more potent and long acting than somatostatin itself [2, 3, 4]. RC-160 (Cyclic D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2) is a synthetic peptide analogue of the native somatostatin and has affinity for different somatostatin receptor subtypes and might be used for targeting breast, ovary, prostate and colon tumours [5]. TOC (D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Cys-Thr(ol)) is another somatostatin analogue that has been used in nuclear medicine labelled with ^{123}I [2].

Although the radiolabel of choice for tumour imaging would be ^{99m}Tc, experience with suitable labelling methods is not yet as great as for other radionuclides. Various analogues have been proposed for scintigraphic purposes, among them, Lanreotide, which has been labelled with ^{90}Y and ^{111}In [6]. Breeman, et al. [7] noted that somatostatin receptor subtypes on human prostate cancer bind RC-160 differently from Octreotide. RC-160 has also been labelled with ^{188}Re and ^{99m}Tc [8].

A direct labelling method after reduction of disulfide bridge has been developed for labelling RC-160 with ^{99m}Tc [5]. A widely used method for labelling small peptides is by conjugation of bifunctional chelators to the peptide and several attempts have been made using Hydrazinonicotinamide (HYNIC) and N_3S compounds (S-benzoyl MAG-3) [9, 10, 11].

The purpose of this investigation was to label RC-160 with ^{125}I and ^{99m}Tc and evaluate its suitability as an agent for *in vivo* use. Labelling with ^{99m}Tc was carried out using a direct

method and after conjugation of the peptide with S-benzoyl-MAG-3 in comparison with labelled TOC after its conjugation with HYNIC.

1.1. Materials

Cold peptides, HYNIC, HATU, EDDA were provided by IAEA. S-benzoyl MAG-3 was provided by CGM Nuclear , Chile. Tricine and other chemicals were purchased from Sigma Chemical Company.

1.2. Methods

1.2.1. Radioiodination of RC-160

RC-160 was labelled with ^{125}I by the iodogen method: 1 mg of iodogen was dissolved in 25 mL of dichoromethane and 500 µl were added into 1,5 mL polypropylene tubes. The tubes were stored in a dessicator at –20°C. 100 µCi of ^{125}INa, 10 µg RC-160 in saline and 100 µl phosphate buffer pH7,4 were mixed and incubated at room temperature for 30 minutes. SEPPAK purification using C-18 Waters cartridges was carried out. The cartridge was washed successively with 5 mL. 70% ethanol and 5 mL. 2-propanol . The reaction mixture was loaded and the cartridge washed with 5 mL deionized water, 5 mL 0.5 molar acetic acid and 5 mL. 95% ethanol. The eluted peptide was collected in three fractions of equal volume and ethanol was evaporated using nitrogen and the residue was dissolve in 200µl of 0.9% NaCl.

Radiochemical purity was analysed by HPLC using a Nova-Pack C-18 column, 3.9 × 150 mm. Gradient: solvent A: methanol, solvent B: 0.9% NaCl; 0 min: 40% A, 60% B; 10–20 min: 80% A, 20% B; 30 min: 48% A, 52% B. Paper electrophoresis was also carried out in Whatman 3M 35cm., in Veronal buffer, 500 V, 2 mA during 1.5 h.

1.2.2. Labelling RC-160 with ^{99m}Tc

RC-160 was labelled using the method developed by Thakur, et al. [5].

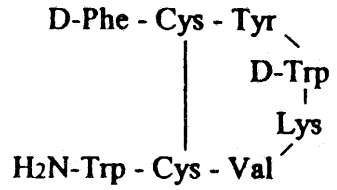

FIG. 1. Structure of RC-160.

A mixture was made of (a) 100 µL of RC-160 (0.1 mg/mL) in acetate buffer 0.1M pH4.2, (b) 100 µL of sodium ascorbate (10 mg/mL) in H_2O adjusted to pH6.5 with ascorbic acid, (c) a required amount of $^{99m}TcO_4Na$ in 100 µL, and (d) a solution of dithionite (10 mg/mL) in acetate buffer 0.1M pH7.2, such that the final concentration of dithionite was 0.2–0.4 µg/µL. The reaction mixture was heated in boiling water for 15 min. The purification step was performed by SEPPAK C-18 cartridge using the same procedure as in ^{125}I labelling. The

ethanol fraction containing the labelled peptide was evaporated using nitrogen and the residue was dissolved in 0.9% NaCl containing 2 mg/mL glycine.

Radiochemical purity was analysed by HPLC using Delta Pack C-18 column, 3.9 × 150 mm gradient — solvent A: methanol, solvent B: saline, 0 min: 48% A, 52% B; 10 min: 72% A, 28% B; 30 min: 72% A, 28% B; 40 min: 48% A, 52% B.

1.2.3. Labelling of RC-160 with ^{99m}Tc using benzoyl-MAG-3

1.2.3.1. Synthesis of peptide conjugate

Boc-RC-160 was conjugated with benzoyl-MAG-3 using O-(7-azabenzotriazolyl)-1,1,3,3-tetramethyluranium hexafluorophosphate (HATU) [10]. The following reaction mixture was prepared : 100 µL of a solution of benzoyl-MAG-3 (2,2 mg/100 µL dry DMF), 50 µL of a solution of HATU (2 mg/200 µL DMF) and 20 µL of a solution of diisopropylethylamine (DIPEA) (10 µL/300 µL DMF).

This reaction mixture was incubated during 15 min, and 40 µL of a solution of RC-160(400 µg) in DMF was added and allowed to react for 1 h. The solution was passed through a C18 SEPPAK cartridge (Waters) previously activated using 5 mL ethanol, followed by 5 mL water, and 5 mL air . The conjugate was eluted with acetonitrile. The organic solvent was evaporated under nitrogen until 100 µL of volume.

HPLC: reverse phase, column: Delta Pack: 3.9 × 150 mm, 0.1%TFA/water, flow 1mL/min., UV 220, gradient: 0–3': 0%CAN, 3–13': 0–50%CAN, 13–23': 50%CAN, 23–26': 50–70% CAN, 26–30': 70–0% CAN. The chromatogram profiles of the reactives and the conjugate are shown in Fig. 2.

Retention times in minutes : benzoyl-MAG-3 = 13.1, BocRC-160 = 19.0, HATU = 6.92, BocRC-160benzoylMAG-3 = 21.16.

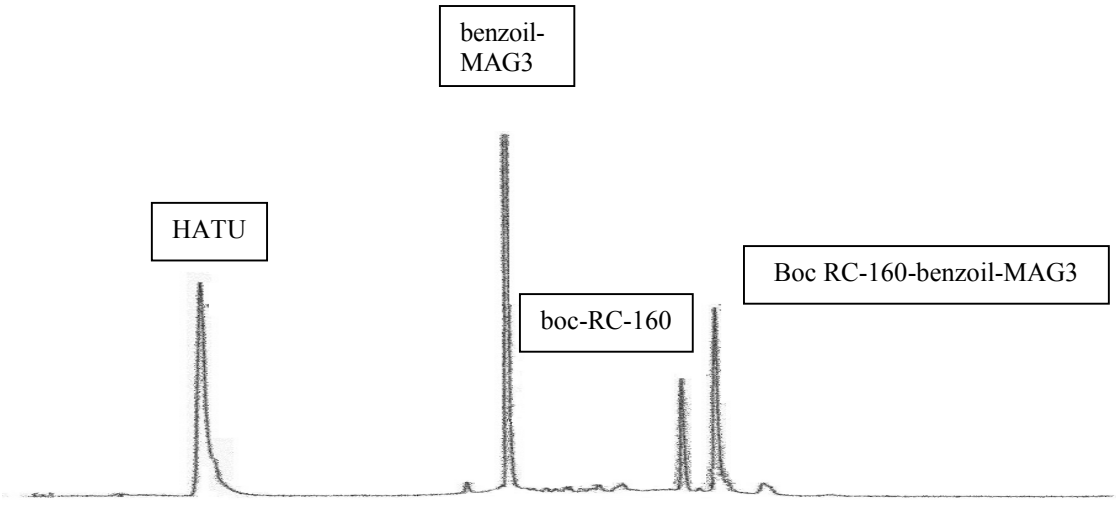

FIG. 2. Chromatogram profiles of the reactives and the conjugate.

Figure 3 shows the chromatogram of the acetonitrile fraction eluted from the SEPPAK.

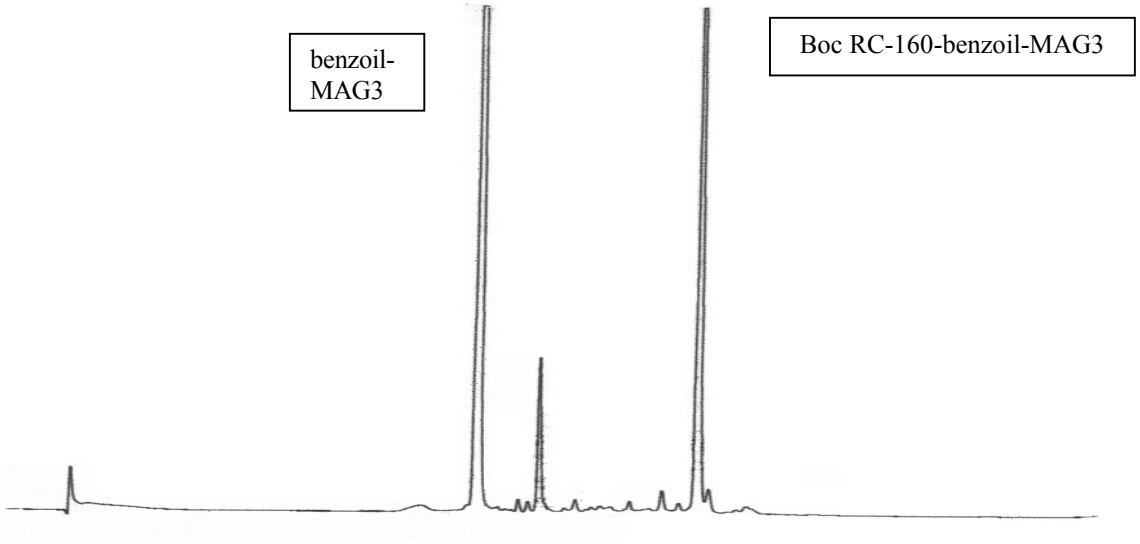

FIG. 3. Chromatogram of the acetonitrile fraction eluted from the SEPPAK.

The peptide conjugate was deprotected with 10 µL of thioanisole and 300 µL of TFA during 10 min. The solution was evaporated to dryness and the residue dissolved in 200 µL of 50% ethanol and purified on HPLC, and a fraction was collected at Rt = 17.67 min.

1.2.3.2. Radiolabelling

S-benzoyl-MAG-3 was heated with 500 µl sodium tartrate (100 mg/mL in 0,1M phosphate buffer pH7,2), 1 mL $^{99m}TcO_4^-$ solution (110 MBq) and 20 µL tin (II) solution (2 mg/mL 0,1 HCl) at 82ºC during 12 min. The radiochemical purity was evaluated with the above HPLC system, Rt = 17,63 min.

1.2.4. Labelling of TOC with ^{99m}Tc using HYNIC

1.2.4.1. Synthesis of HYNIC-TOC

HATU was used in order to conjugate the blocked peptide and BocHYNIC [11]. 1,3 mg Boc-HYNIC, 254 µL of 2,09 mg HATU in 290 µL DMF and 6,5 µL of diisopropyethylamine (DEA) were reacted for 15 min. 132 µl of this solution was added to 1 mg Boc-TOC and allowed to react for 1 h. To stop the reaction, 1 mL of water was added and the resulting solution was passed through a C 18 SEPPAK cartridge (Waters) and eluted with acetonitrile. Deprotection and purification through HPLC were carried out as described for S-benzoyl MAG-3-RC-160.

1.2.4.2. Radiolabelling

The conjugate peptide was labelled with ^{99m}Tc using tricine as co-ligand. 10 µg of HYNIC-TOC was incubated with 0,5 mL fresh eluted $^{99m}TcO_4^-$ solution (185 MBq), 0,5 mL

tricine solution (100 mg/mL in 25 mM succinate buffer pH5,0) and 15 µl tin (II) solution (10 mg/5 mL HCl 0,1 N) for 30 minutes at RT. Radiochemical purity was tested by HPLC.

1.2.5. Biodistribution

Normal Wistar rats were given intravenous (i.v.) injection of 0,37 MBq ^{99m}Tc labelled peptide. Animals were sacrificed after 4 h, samples of different organs were obtained and counted, and results were expressed as %DI/g.

1.2.6. In vitro studies

1.2.6.1. Plasma stability

Plasma stability was determined after 4 hour incubation of the ^{99m}Tc labelled peptide in human plasma at 37ºC. After precipitation of plasma protein with acetonitrile the incubation mixture was analysed by HPLC.

1.2.6.2. Protein binding

Protein binding of ^{99m}Tc labelled peptide was determined after 15 min, 1, 2, 3 and 4 hours incubation in human plasma at 37ºC using Centricon 30 (Amicon) devices. The radioactivity in the filter and filtrate were counted after centrifugation at 5000 rpm for 20 min. Protein bound peptide was calculated as the percentage measured in the filter.

1.2.6.3. Receptor binding

Rat brain cortex membrane was chosen as a source of somatostatin receptors. Tissues were prepared using a modified method of Raynor and Resine. The labelled ^{125}IRC-160 was diluted in order to achieve 25000 cpm in 70 µL. Membrane fractions were incubated for 30 minutes with labelled peptide in the absence (total binding) or presence of increasing concentrations of cold ligand ranging from 10^{-5}M to 10^{-10} M. After filtration through millipore 0.22 µ Millex, GV filters, radioactivity associated with the filters and filtrates was counted and data were computed.

2. RESULTS

2.1. Labelling RC-160 with ^{125}I

The reaction mixture, after labelling with ^{125}I, was passed through SEPPAK purification and eluted fractions were analysed by electrophoresis studies. The fraction containing the labelled peptide showed a major peak at the origin and a small peak of radioiodine at fraction 15, which could not be removed completely by the SEPPAK washing procedure. Before purification, the reaction mixture showed the same peaks described above and a peak at fraction 9 that appeared also in the electrophoresis of fraction eluted with water. A labelling experiment was carried out without peptide, and electrophoresis shows the same radiochemical species at fraction 9 and 15. The HPLC profile showed one peak at Rt = 2.91 and two peaks at Rt = 23.1 and Rt = 24.7 separated by 1.6 min. The radiochemical purity was higher than 80%. It seems that the iodide is not being removed completely by SEPPAK

washing procedure. Radiochemical purity results were the same for the labelled peptide after one week at 4ºC.

2.2. Labelling RC-160 by a direct method

Labelling experiments with ^{99m}Tc were carried out using different amounts of dithionite. Fig. 4 shows the percentage of radioactivity in peptide fraction eluted with ethanol, the percentage of radioactivity retained in the cartridge and the percentage of radioactivity eluted with water versus different final concentrations of dithionite. All those percentages are relative to the radioactivity loaded on the SEPPAK. The highest result in peptide fraction was obtained with a final concentration of sodium dithionite between 0.2–0.4 μg/μL.

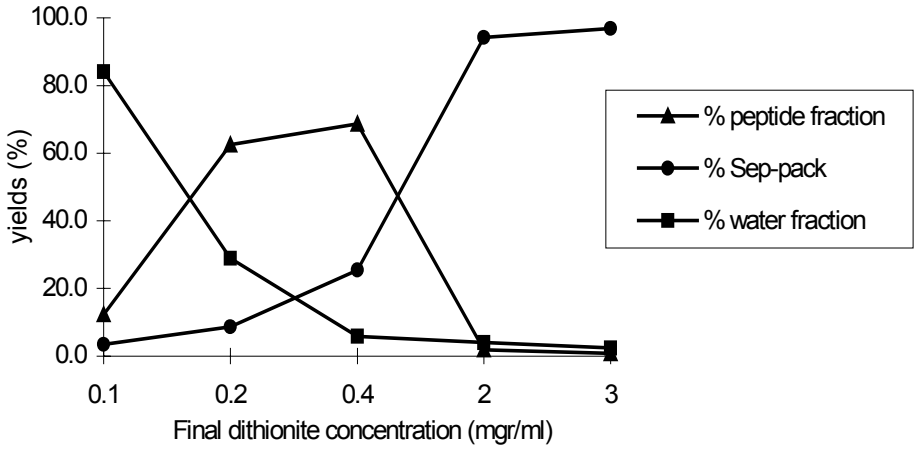

FIG. 4. Fraction yields after SEPPAK purification.

The chromatographic control showed a major peak with a Rt = 22.1 min as it is shown in Fig. 5.

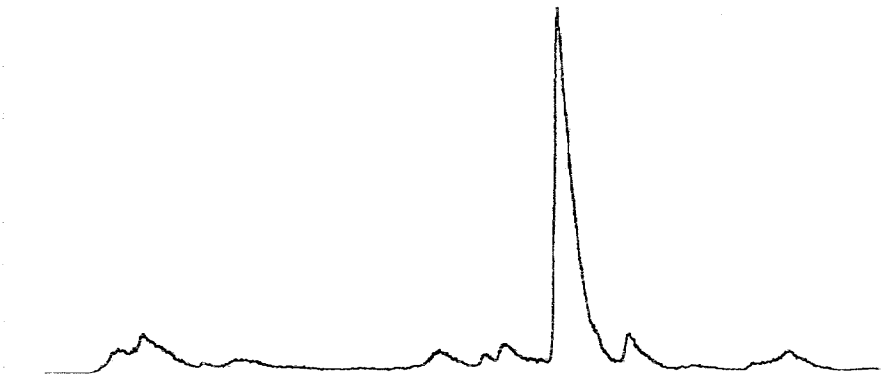

FIG. 5. Chromatogram profile of directly labelled RC-160.

2.3. Synthesis of peptide conjugates

HPLC analysis of RC-160 conjugate after deprotection resulted in one major peak for S-benzoyl-MAG-3RC-160, Rt = 17.7, as shown in Fig. 6.

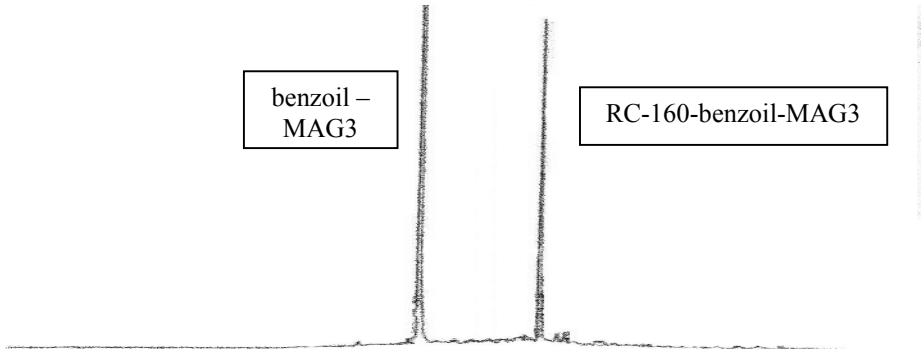

FIG. 6. HPLC analysis of deprotected RC-160 conjugate.

Deprotection and HPLC purification through HPLC resulted in an efficient separation of the HYNIC-TOC conjugate. Fig. 7 shows the HPLC profile of the pure peptide conjugate, Rt = 16.3 min.

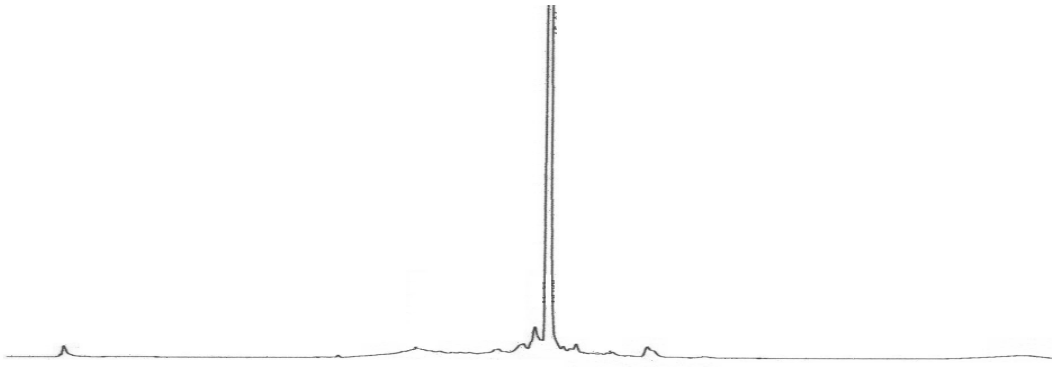

FIG. 7. HPLC profile of the pure peptide HYNIC-TOC conjugate.

2.4. ^{99m}Tc labelling

Labelling yields of RC-160 and TOC conjugates were 85 ± 4.8 and 98.5 ± 1.3, respectively. Figs 8 and 9 show the HPLC profiles of both labelled products. Rt MAG-3-RC-160-^{99m}Tc = 16.1 min. Rt HYNIC-TOC-^{99m}Tc = 14.2 min. ^{99m}Tc labelled TOC conjugate showed lower retention time on HPLC as compared to labelled RC-160 conjugate, that is indicating lower lipophilicity of this peptide.

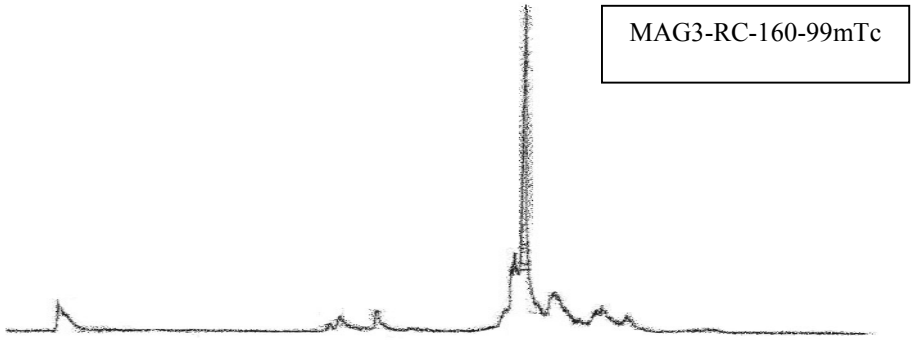

FIG. 8. HPLC profile of labelled MAG-3-RC-160.

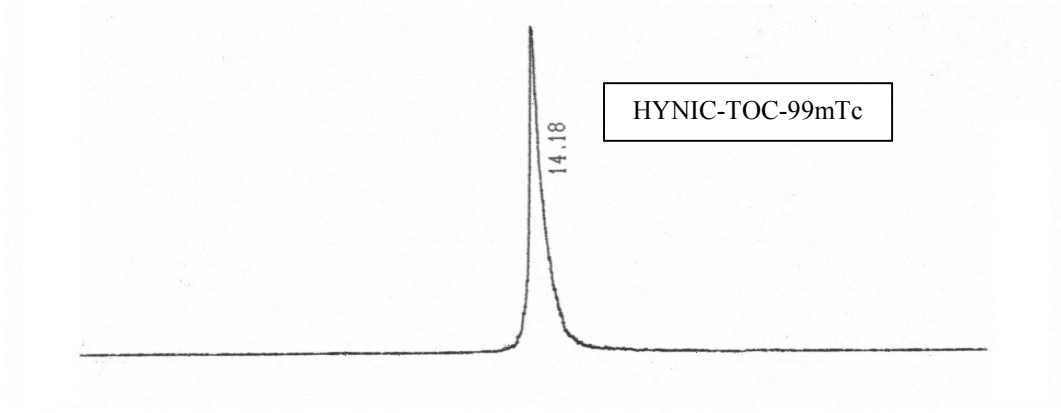

FIG. 9. HPLC profile of labelled HYNIC-TOC.

Lipophilic impurities were found in the MAG-3 conjugate labelling mixture. The HPLC method allowed efficient separation of the ^{99m}Tc complex from the cold conjugate to yield carrier free technetium complexes.

	Cold peptide Rt	Labelled peptide Rt
S-benzoyl-MAG-3-RC-160	17.7	16.1
HYNIC-TOC	16.3	14.2

2.5. Stability

HPLC analysis, after 4 h incubation in human plasma, showed no degradation of the radiolabelled peptide after precipitation with acetonitrile. Retention times were 16.1 and 14.2 min for labelled RC-160 conjugate and labelled TOC conjugate,` respectively.

Protein binding, as determined by Centricon 30 ultrafiltration, showed a significant increase over time for labelled TOC conjugate using tricine as co-ligand, from 20% after 15 min to 67% after 4 h, as shown in Fig. 10. These data are in agreement with the literature [9].

The authors have stabilized tricine with pyridines as ternary ligands, and a stable protein binding was found.

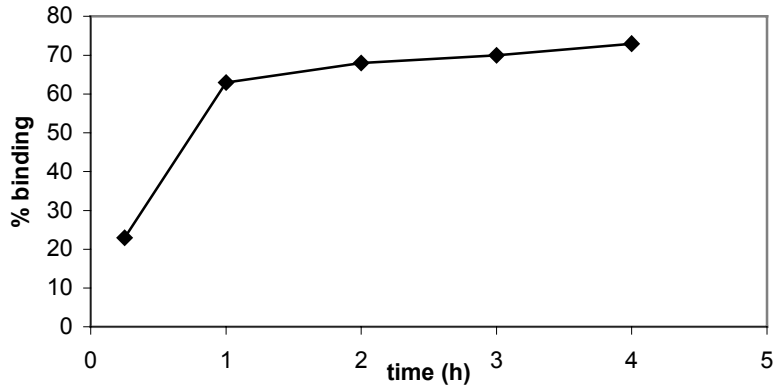

FIG. 10. Protein binding versus time.

2.6. Biodistributions

Fig. 11. shows biodistribution results at 1 and 4 h with MAG-3-RC-160-^{99m}Tc.

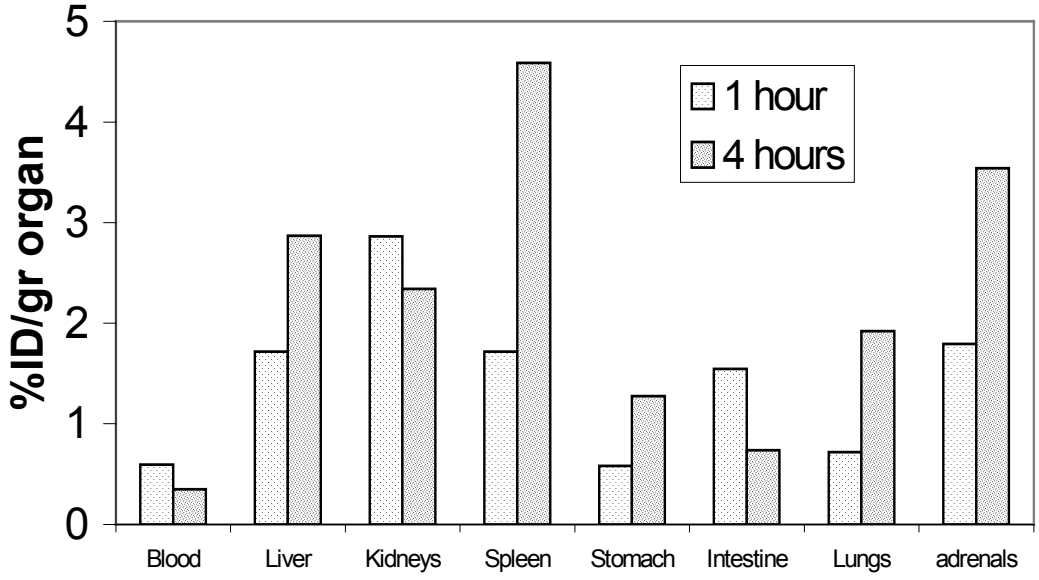

FIG. 11. Biodistribution at 1 and 4 h of MAG-3–*RC–160–*^{99m}*Tc.*

The labelled HYNIC-TOC conjugate when tricine was used as co-ligand, showed a tendency towards renal excretion and lower levels of activity in liver and intestine (Fig. 12).

Results showed a good correlation between lipophilicity, HPLC retention times and biodistribution. Greater lipophilicity causes increased liver activity and retention time.

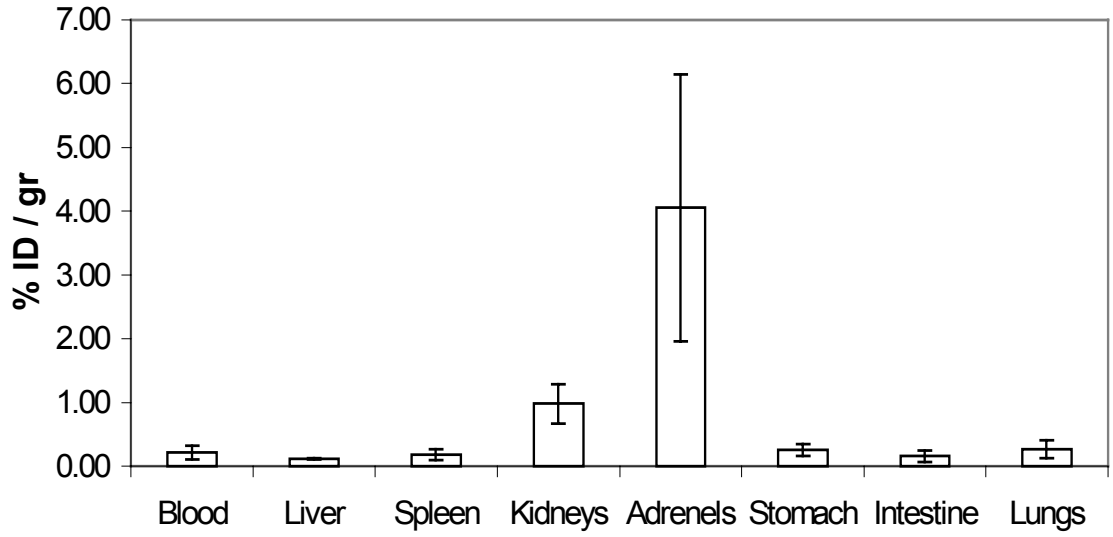

FIG. 12. Biodistribution at 4 h of HYNIC-TOC-^{99m}Tc.

2.7. Binding assay

A fitted curve was obtained (Fig. 13) showing the activity of unlabelled peptide to compete with labelled peptide for binding to membrane receptors. The dissociation constant (Kd) value for RC-160 labelled with ^{125}I was 4.8×10^{-9} and it was calculated as the peptide concentration at B/T equal to 0.5.

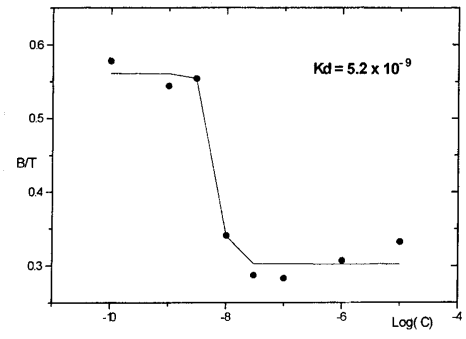

FIG. 13. Binding assay.

3. CONCLUSION

Two different labelling methods were used and two conjugates were prepared for labelling the peptides with ^{99m}Tc. HYNIC-TOC complex showed promising results when labelled with ^{99m}Tc using tricine as co-ligand although higher stability should be found for ternary co-ligands compared to tricine.

ACKNOWLEDGEMENTS

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^{99m}Tc LABELLED PEPTIDES FOR IMAGING PERIPHERAL RECEPTORS

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Abstract

Radiolabelling of Somatostatin analogues as RC-160 and TOC with ^{99m}Tc, using direct and bifunctional chelating methods as well as quality control and evaluation methods, has been accomplished following the techniques and recommendation of the first and second RCMs. Synthesis of bifunctional chelating agents, such as Bz-MAG-3, is routinely produced in our Laboratory. Synthesis of HYNIC and HYNYC-MAG-3 is in progress. Radioiodination of RC-160 using chloramine-T and iodogen methods were also studied in order to get experience with the different techniques used to evaluate the labelled peptides.

1. INTRODUCTION

Radiopharmaceuticals based on biologically active peptides present a wide range of potential applications for diagnostic imaging and therapy. They offer advantages over monoclonal antibodies because of their smaller size, their low probability of immune response, can be produced synthetically and can, therefore, be less inexpensive.

Somatostatin is an endogenous peptide that inhibits the secretion of many hormones, such as growth hormone, thyrotropin, gastrin. It also acts as a neurotransmitter and neuromodulator. Many tissues express somatostatin receptors. This include pituitary, gut, pancreas and endocrine tumours associated with these tissues. Because of the short half-life of naturally occurring somatostatin in blood, somatostatin itself is not effective as a diagnostic agent. Analogues of somastostatin, have been developed. Among them Octreotide possesses the same pharmacological properties as somastostatin but clears more slowly from the blood. The goal of the radiopharmacist during the last ten years has been radiolabelling of these agents with high specific activity using metal radionuclides such as ^{99m}Tc, permitting the peptide to carry the radionuclide-binding group in its structure while maintaining high-affinity binding to the receptor site.

Other synthetically produced Somatostatin analogue peptides such as RC-160 (Vapreotide) and TOC (both developed by Sandoz) have been used in this CRP in order to develop the radiolabelling techniques and quality control methods. ^{99m}Tc, because of its ideal physical characteristics, was the radiometal of choice.

2. MATERIALS AND METHODS

2.1. ^{125}I peptide labelling methods

The octapeptide RC-160 can be labelled with radioiodine because of the Tyr group present in the molecule.

2.1.1. Chloramine — T method

Solutions:

Chloramine T: 1 mg/mL 0.05M phosphate buffer pH7.4

Peptide: 1 mg/mL 0.005M acetic acid pH4.2

Methanol: 100%

^{125}I peptide labelling

(1) To an Eppendorf tube containing 42.5 µL phosphate buffer 0.05M, pH7.5
(2) Add 5 –10 µg of RC-160 dissolved in 5–10 µL of 0.05M acetic acid pH4.2 mix with Vortex
(3) Add 200 µCi (7400 KBq) ^{125}I/10 µL
(4) Add 2.5 µl of chloramine-T solution
(5) After 60 s incubation time at RT, add 40 µL of methanol and immediately purify by SEPPAK C-18 cartridge.

2.1.2. Iodogen method

Solutions:

Iodogen: 1 mg/25 mL of CH2Cl2

Peptide: 1 mg/10 mL buffer phosphate pH7.4

Saline: pH4.5 acidified using CH3COOH

Saline: NaCl 0.9% MeOH 85%.

Methanol: 100%

Iodogen tube preparation

(1) Add the necessary amount of iodogen solution in 1.5 mL Eppendorf tubes (25, 50, 100 µg/tube)
(2) Evaporate to dryness under N_2 stream in a dark fume cupboard.
(3) Keep at –20ºC under dry conditions.

^{125}I peptide labelling

(1) Add 200 µCi (7400 KBq) of ^{125}I to the iodogen tube.
(2) Add, as fast as possible, 100 µL of peptide solution (10 µg). Mix well.
(3) Incubate 30 min at room temperature
(4) Transfer the reaction solution to a clean tube
(5) Wash the labelling tube with 500 µl of saline solution (pH4.5) and add to the reaction mixture.

2.1.3. Purification of labelled ^{125}I peptide

Anion exchange column Dowex 1X8

Thoroughly wash 5 g of the resin with HCl 0.1 M for 10 min. Rinse three 3 times with deionized water and once with saline (pH4.5). Load the column with resin (0.5–1.0 mL), wash with 2 mL of saline pH4.5. Purify the labelled peptide by passing it down the column and elute with 4 × 0.5 mL aliquots of saline (pH4.5).

SEPPAK C-18 purification

SEPPAK activation: Wash the SEPPAK successively with 5 mL ethanol and 5 mL of water.

Elution: Load the reaction mixture and wash with 5 mL of water and elute with 5 mL of methanol 95%. Evaporate the solvent under N_2 stream and re-dissolve with saline.

2.1.4. Radiochemical purity determination of ^{125}I peptide

Determination of radiochemical purity was achieved by:

Chromatography: ITLC/methanol 85%

Rf. $^{125}I^-$: 1.0

Rf. ^{125}I peptide: 0.0

2.2. ^{99m}Tc peptide labelling using bifunctional chelating agent

2.2.1. Preparation of RC-160 and TOC conjugates

HYNIC CONJUGATE

Preparation of Boc protected HYNIC RC-160/TOC — prepare the following solutions in dry glass vials:

A. 1,3 mg 6-Boc-Hydrazinpyridine-3-carboxylic acid (Boc-HYNIC) in 100 μL dry DMF
B. 2,0 mg HATU in 100 μL DMF
C. 10 μL Diisopropylethylamine in 300 μL DMF
D. 0,4 mg Boc-RC-160 or Boc-TOC in 20 μL DMF and 5 μL water in a 5 mL reaction-vial.

(1) Add 50 μL each of solution A, B and C to a dry glass vial (reaction solution) and allow reacting for 15 min
(2) Add 60 μL of the reaction solution to solution D (peptide solution) prepared in step 1 above after the 15min reaction time
(3) Allow the solution to react for 1 h
(4) Activate a SEPPAK C-18 Mini Cartridge by washing with 5 mL Ethanol followed by 5 mL water; dry the cartridge by pushing 5 mL of air through the cartridge

(5) After a minimum 1 h reaction time add 1 mL of water to the reaction vial and transfer the solution on the SEPPAK cartridge, wash vial and cartridge with additional 3-4 mL of water; dry the cartridge by pushing 5 mL of air through it
(6) Elute the cartridge with 0.5–1.0 mL (1.5 mL, when required) of pure acetonitrile and collect the eluate in a 1.5 mL Eppendorf vial
(7) Reduce the acetonitrile solution under N_2 stream (low temperature) to a volume of about 100 μL.

Deprotection of Boc-HYNIC-RC-160/Boc-HYNIC-TOC

(1) Add 10μL of Thioanisole and 300 μL TFA to 100 μL of acetonitrile solution in Eppendorf tube containing protected peptide
(2) Allow reacting for maximum of 5 min
(3) Evaporate to dryness under N_2 stream
(4) Dissolve the residue in 50 μL ethanol, them add 50 μL water, and load the solution on the HPLC column. Wash vial and injection syringe with 2 × 30 μL 50% ethanol
(5) Purify the solution on HPLC using TFA gradient:
(6) Column: RP Delta Pack: 5um, 4,6 × 150mm.
(7) Flow 1 mL/min at 220 nm
(8) Gradient: 0.1% TFA/A CN and 0.1% TFA/water
(9) 0–3 min: 0% CAN
(10) 3–13 min: 0–50% CAN
(11) 13–23 min: 50% CAN
(12) 23–26 min: 50–70% CAN
(13) 26–30 min: 70–0% CAN
(14) Collect the deprotected HYNIC-RC-160 or HYNIC-TOC with an approximate 15 min to 20 min retention time in a glass vial
(15) Close glass vial with rubber, seal it and purge with nitrogen for 5 min
(16) Store the solution at –20ºC.

Bz-MAG-3 CONJUGATE

Preparation of Boc protected Bz-MAG-3 RC-160/TOC — prepare the following solutions in dry glass vials:

A. 2,2 mg Bz-MAG-3 in 100 μL dry DMF
B. 2,0 mg HATU in 200 μL DMF
C. 10 μl Diisopropylethylamine in 300 μl DMF
D. 0,4 mg Boc-RC-160 or Boc-TOC in 40 μL DMF and 5 μL water in a 5 mL reaction vial.

(1) Add 100 μL of solution A, 50 μL of solution B and 20 μL of solution C to a dry glass vial (Reaction solution) and allow to react for 15 min
(2) To solution D (peptide solution) add 40 μL of the Reaction solution prepared in 1 after the 15 min reaction time
(3) Allow the solution to react for 1 h
(4) Activate a SEPPAK C-18 Mini Cartridge by washing with 5 mL Ethanol followed by 5 mL water, dry the cartridge by pushing 5 mL of air through the cartridge
(5) After a minimum 1 h reaction time add 1mL of water to the reaction vial and transfer the solution on the SEPPAK cartridge, wash vial and cartridge with additional 3–4 mL of water. Dry the cartridge by pushing 5 mL of air through it

(6) Elute the cartridge with 0.5–1.0 mL of pure acetonitrile and collect the eluant in a 1.5 mL Eppendorf vial
(7) Reduce the acetonitrile solution under N_2 stream (low temperature) to a volume of about 100 μL.

Deprotection of Boc-Bz-MAG-3-RC-160/Boc-Bz-MAG-3-TOC

The same procedure as for HYNIC conjugate.

2.2.2. ^{99m} Tc radiolabelling of conjugate RC-160/TOC

^{99m}TC-HYNIC CONJUGATE USING TRICINE AS CO-LIGAND

Solutions:

(1) Tricine 100 mg/mL in 25 mM Succinate buffer pH5
(2) Stannous chloride 1 mg/mL 0,1N HCl in purged water and all solutions with nitrogen.

Add subsequently in a 5 mL rubber closed vial:

— 10 μg HYNIC conjugate
— 0,5 mL tricine solution
— 30 mCi (1100 MBq) ^{99m}Tc pertechnetate in 0,5 mL
— Add 25 μL of tin (II) solution to start the reaction
— Incubate for 30 min.

^{99m}TC-BZ-MAG-3 CONJUGATE

Solutions:

(1) Sodium tartrate 100 mg/mL in 0.1M Phosphate buffer pH7.2
(2) Stannous chloride 1 mg/mL 0,1N HCl in
(3) Purge water and all solutions with nitrogen.

Add subsequently in a 5 mL rubber closed vial:

— 10 μg Bz-MAG-3-conjugate
— 0,5 mL Tartrate solution
— 30 mCi (1100 MBq) in 0.5 mL of ^{99m}Tc pertechnetate
— Add 40 μL of tin solution to start the reaction
— Heat at 100ºC for 15 min.
—

2.3. ^{99m}Tc peptide labelling using direct methods

Direct labelling methods using ascorbate to reduce disulfide peptides group and dithionite or GH-Sn to reduce ^{99m}Tc pertechnetate were also used.

ASCORBATE/DITHIONITE

10 mg/mL Ascorbate solution pH6.2 was used as peptide reducing agent and dithionite as pertechnetate reducing agent.

ASCORBATE/GH-SN(II)

10 mg/mL Ascorbate solution pH6.2 was used as peptide reducing agent and GH/Sn lyophilized kit containing 100 mg of Glucoheptonate and 1 mg of Sn_2Clx2H_2O as pertechnetate reducing agent. GH-Sn kit was labelled with 3.0 mL of ^{99m}Tc pertechnetate saline solution.

2.4. Purification of ^{99m}Tc labelled peptide

Purification of the labelled peptide-conjugate was accomplished using SEPPAK C-18 mini cartridge activated with ethanol and water. After loading the labelled peptide it was washed with water and eluted with 80% acetonitrile, which was evaporated under nitrogen stream and re-dissolved with saline.

2.5. Radiochemical purity determination of ^{99m}Tc peptide

Radiochemical purity of ^{99m}Tc-HYNIC-RC-160, ^{99m}Tc-HYNIC-TOC, ^{99m}Tc-Bz-MAG-3-RC-160 and ^{99m}Tc-Bz-MAG-3-TOC were determined using HPLC RP Gradient method in a Water System with on line UV(220 nm) and radiometric detectors analysed by a multichannel S-100 Card. A Water Delta Pack C 18 Column 5µ 300 Aº and 15 mm was used.

Results:

^{125}I LABELLED PEPTIDE

Radioiodination was carried out using oxidation methods with chloramine-T and iodogen, in order to produce ion iodonium (I^+) using commercial ^{125}INa. Labelled peptide with ^{125}I using chloramine-T method gave better yield using the iodogen method. The best result with iodogen method was obtained when using 50 µg of iodogen per tube. With chloramine-T labelling, yield was around 50% and purification was carried out immediately after 1 min of reaction time, using reverse phase SEPPAK C-18 cartridge (Table I). ^{125}I Peptide was obtained in methanol fraction and $^{125}I^-$ in water fraction.

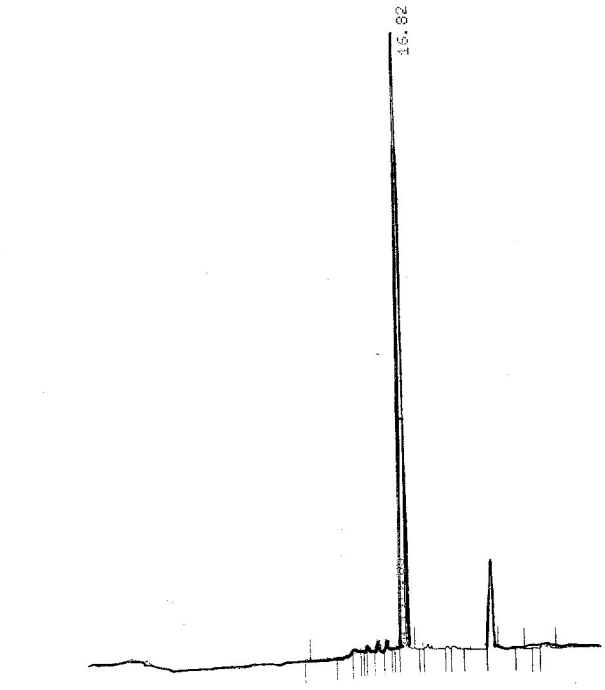
TABLE I. ^{125}I-PEPTIDE RADIOCHEMICAL PURITY

	Before purification	After purification
chloramine-T	50 ± 12%	92 ± 4.5
iodogen	26 ± 3.2	-

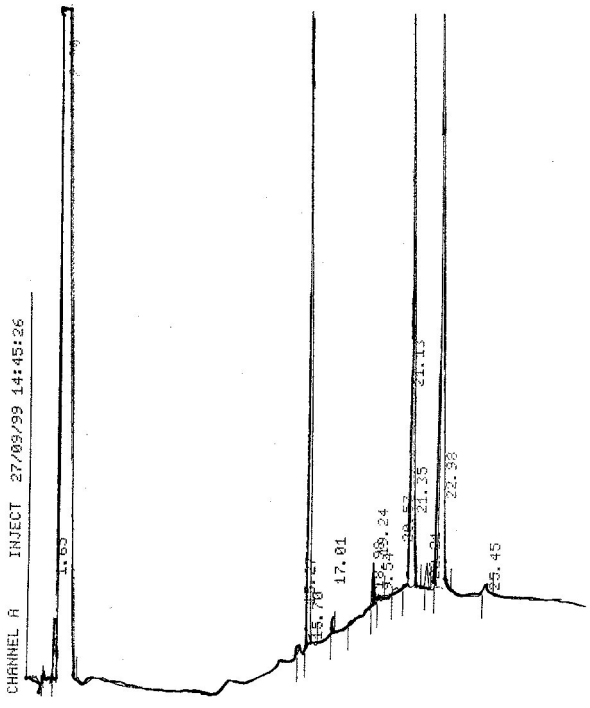
^{99m}TC PEPTIDE LABELLING

(1) ^{99m}Tc peptide labelling using bifunctional chelating agent

Conjugate preparations were done using HATU and dry DMF as solvent. When DMF is not dry the peptide precipitates and the conjugate might be lost. Both peptides were conjugated and after deprotection with TFA and thioanisole the conjugates HYNIC-RC-160, Bz-MAG-3-RC-160 and HYNIC TOC, and Bz-MAG-3-TOC were purified by HPLC by gradient methods. HPLC Chromatograms are shown in Figs 1 and 2.

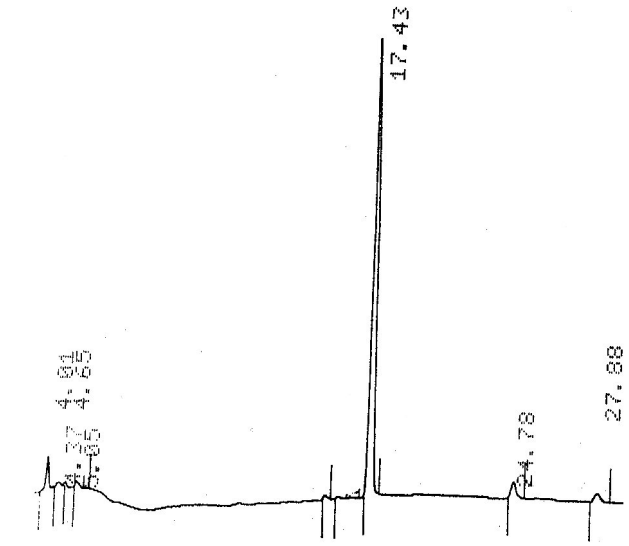


HYNIC-RC-160



Bz-MAG3-RC-160

FIG. 1. HPLC conjugate chromatograms.



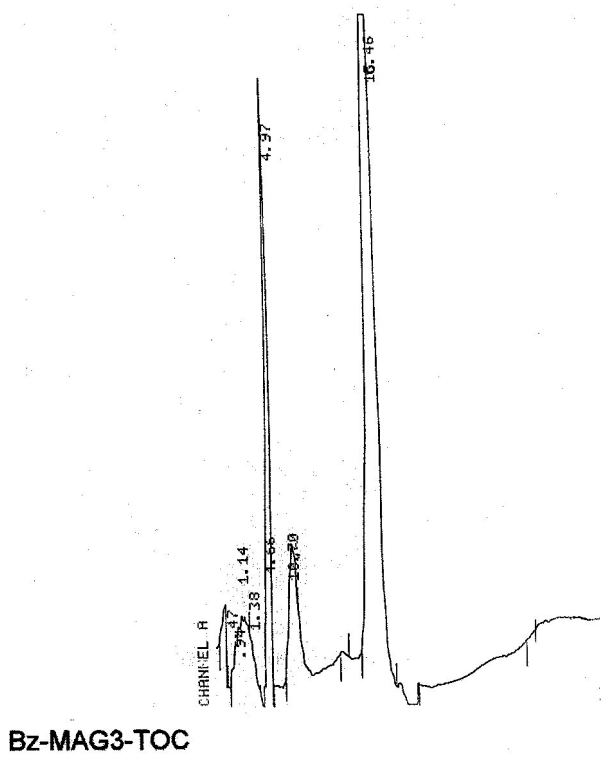


FIG. 2. HPLC conjugates chromatograms.

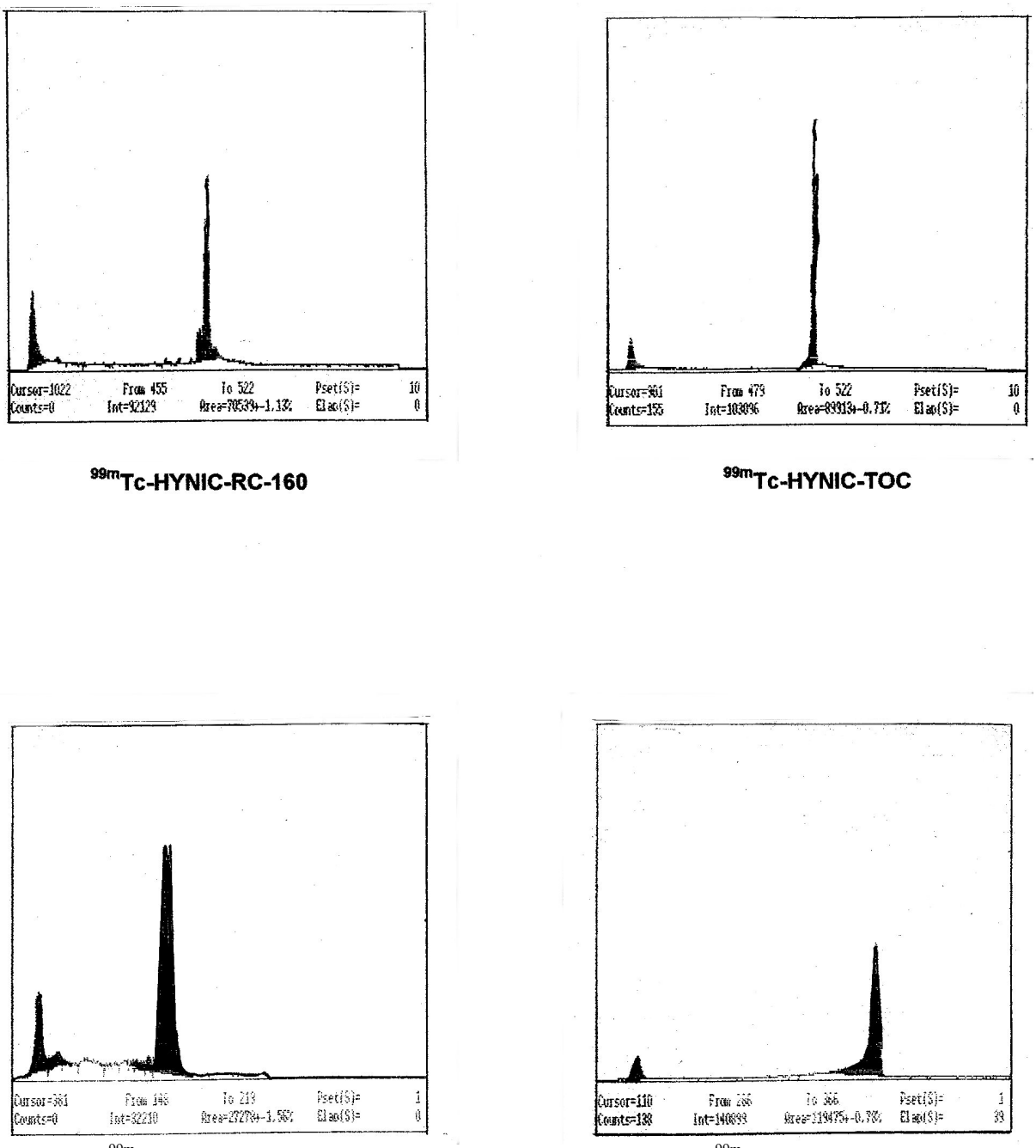


FIG. 3. Radiochromatograms.

Labelling with ^{99m}Tc using Sn(II) as $SnCl_2$ x2H_2O as reducing agent of pertechnetate and tricine as co-ligand was carried out. ^{99m}Tc-tricine labelling was over 90%. When labelling tricine all solutions were nitrogenated in order to avoid presence of oxygen because of the low amount of Sn(II) used.

Radiochromatograms of all radiolabelled conjugates are shown in Fig. 3 and labelling yields are given in Table II. With a bigger amount of peptide the SEPPAK cartridge might be overloaded and may be a changed to a bigger sized type such as Plus SEPPAK could be necessary.

TABLE II. ^{99m}Tc PEPTIDE RADIOCHEMICAL PURITY

Peptide	Conjugate	Labelling
RC-160	HYNIC	90 ± 4.5
RC-160	Bz-MAG-3	88 ± 5.2
TOC	HYNIC	92 ± 5.2
TOC	Bz-MAG-3	90 ± 2.6

(2) ^{99m}Tc peptide labelling using direct labelling methods

Direct labelling methods using ascorbate to reduce disulphide bridges and dithionite or GH-Sn(II) as ^{99m}Tc pertechnetate reducing agents were also used but reproducible results were not achieved. During the second period of the CRP we did not work on these techniques.

Labelling yields using different amounts of ascorbate solution (50; 100; 150; 200 and 400 μL) with 1 mL of ^{99m}Tc-GH solution and different amount of ^{99m}Tc-GH (0; 25; 50; 75; 100; 300; 500; 700 and 1000 μL) with 150 μL of ascorbate solution were studied. Maximum yields were 30%. Results are shown in Table III.

TABLE III. ^{99m}Tc RC-160 LABELLING STUDIES AT DIFFERENT ASCORBATE AND GH-Sn CONCENTRATIONS

RC-160 μL [100μg/mL]	ASCORBATE μL [10 mg/mL]	*GH/Sn μL	^{99m}Tc-RC-160 %	^{99m}Tc-GH %	$^{99m}TcO_4^-$ %
100	50	1000	7.04	92.96	----
100	100	1000	2.78	97.22	----
100	150	1000	30.45	50.20	19.35
100	200	1000	22.28	69.50	8.22
100	400	1000	10.1	73.8	16.1
100	150	0	0.75	4.48	94.76
100	150	25	19.53	----	80.47
100	150	50	16.74	----	83.26
100	150	75	27.33	----	72.67
100	150	100	19.31	25.55	55.14
100	150	300	13.65	29.37	56.98
100	150	500	5.95	11.34	82.71
100	150	700	2.78	18.28	78.95

* Lyophilized kit + 4 mL pertechnetate.

3. CONCLUSION

The use of bifunctional chelating agents to label this kind of peptides allow to obtain good results but still we must improve them by using other bichelating agents and other co-ligands. Also, we must try again to get better labelling with the direct labelling methods. We need to get more experience with Receptor Binding Assays in order to evaluate binding affinity to Somatostatin receptors.

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STUDY OF PHARMACOKINETICS AND BIODISTRIBUTION OF RADIO-LABELLED RECEPTOR SPECIFIC PEPTIDES IN LABORATORY ANIMALS

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Abstract

Somatostatin analogues labelled with different radionuclides could be employed for visualization or treatment of somatostatin receptor-positive tumours. An octapeptide ^{111}In [DTPA] octreotide is a synthetic radiolabelled somatostatin analogue which is currently in clinical use for detecting small neuroendocrine tumours and metastases not detectable by conventional means. However, several other somatostatin analogues have been under development and testing. The aim of this study was to radiolabel selected somatostatin receptor-binding octapeptides by different radionuclides and to report the results of their biodistribution in rats. The study was focused on the direct labelling of vapreotide (RC-160) with ^{99m}Tc, on the conjugates of octreotide with DFO (desferrioxamine) for labelling with ^{67}Ga, and on the conjugates of octreotide and TOC with DOTA (tetraazacyclo-dodecane tetraacetic acid) for labelling with ^{88}Y. In the present study, ^{88}Y isotope instead of ^{90}Y was used as a label as ^{88}Y exhibits a longer half life of decay and emits gamma radiation which can be much more easily detected in biological samples than beta emission. The labelling of octreotide analogues with metal radionuclides using derived bifunctional chelates was simple, straightforward and consistently resulted in high radiochemical purity of the product. On the other hand, the application of the direct labelling method for labelling of RC-160 with ^{99m}Tc was difficult because all procedures had to be made under nitrogen atmosphere and an attainment of high yield proved to be highly dependent on the accurate observation of reaction conditions. The labelling efficiency makes an immediate use of the radiolabelled RC-160 for biological studies impossible and it is necessary to involve the purification step into the labelling procedure. All radiolabelled receptor specific peptides under study exhibited rapid radioactivity clearance from the blood and most organs and tissues. On the other hand, long-term retention and high radioactivity concentrations in the kidneys and somatostatin receptor-rich organs, such as the pancreas and adrenals, were found. The results indicated significantly higher concentrations of ^{88}Y-DOTA-TOC in organs with a high density of somatostatin receptors in comparison with the other agents. High and long term uptake of radioactivity in kidneys was probably due to partial degradation of the peptides under study and consequent accumulation of radiolabelled fragments in the renal cells. Elimination studies showed relatively rapid renal excretion of radiolabelled peptides under study, about three quarters of the administered radioactivity was eliminated in the urine within 2 h after administration. Analysis of the elimination mechanism by employing rat kidney perfusion and rat liver perfusion showed that all peptides under study were eliminated in the kidneys mostly by glomerular filtration, the bile elimination being relatively low. The radiolabelled peptides under study form a part of a new series of diagnostics and therapeutics which could be invaluable in the clinic for management of patients with cancer if some mechanism to decrease the kidney retention is provided. Another part of the study was focused on the preparation of ^{99m}Tc labelled tetrapeptides, namely acetyl-Gly-Gly-Cys-Gly, acetyl-Ser-Ser-Cys-Gly, and acetyl-Gly-Gly-Cys-Lys, and determination of their biodistribution and analysis of elimination mechanism in rats. The peptides were formed by amino acid sequences capable of chelating technetium useful as universal chelators in “hybrid” peptides composed of receptor specific

part and the part chelating technetium [1]. Biodistribution studies in rats showed that all agents were rapidly cleared from the blood. No specific accumulation of radioactivity in different organs and tissues was found. Analysis of renal elimination mechanism of radiolabelled peptides under study by using perfused rat kidney *in situ* showed that all agents were eliminated by both glomerular filtration and tubular secretion. The results obtained will assist in the design of optimal biocompatible tetrapeptides as chelators for formation of hybrid receptor specific peptides.

1. MATERIALS

- DTPA-octreotide was obtained from the Faculty of Pharmaceutical Sciences, Kyoto University, Japan (Prof. K. S. Horiuchi).
- DFO-octreotide, DOTA-TOC and DOTA-octreotide were obtained from the Institute of Nuclear Medicine, University Hospital, Basel, Switzerland.
- RC-160 [*D*-Phe-Cys-Tyr-*D*-Trp-Lys-Thr-Cys-Thr(ol)] was obtained from the IAEA, Vienna.
- Tetrapeptides acetyl-Gly-Gly-Cys-Gly, acetyl-Ser-Ser-Cys-Gly, and acetyl-Gly-Gly-Cys-Lys were obtained from St. Bartholomew´s Hospital, London.

2. METHODS

2.1. Radiolabelling and quality control of receptor-specific peptides under study

2.1.1. ^{111}In DTPA-Octreotide

^{111}In DTPA octreotide was prepared by adding 1 mL of $^{111}InCl_3$ (37 MBq) in 20 mM HCl to the kit vial containing 10 μg of the peptide and citrate buffer. After standing for 1 h at room temperature, the preparation was subjected to reversed phase gradient HPLC.

2.1.2. ^{99m}Tc-RC-160

Labelling of RC-160 with ^{99m}Tc was based on reduction of disulphide groups of the peptide by sodium dithionite in ascorbate to sulfhydryls (strong binding groups for technetium):

10 μg of RC-160 was dissolved in 100 μL of 0.1 mol/L acetate buffer pH4.2 in a siliconized glass test tube and 1 mg (100 μL) of ascorbate solution pH6.2 was added. After mixing of the solution, required activity of ^{99m}Tc (37–74 MBq) was added, and later a carefully calculated amount of $Na_2S_2O_4$ solution in 0.1 mol/L acetate buffer pH7.2 was added in such a way that the final dithionite concentration was 2 μg per mL of solution. The reaction mixture was heated in a boiling water bath for 15 min and after cooling of solution the purification process was performed on a SEPPAK C-18 cartridge. All reagents were kept and all reactions were made under N_2 atmosphere.

SEPPAK purification

The cartridge was gradually washed with 5 mL of 70% ethanol and 5 mL of 0.1mol/L acetic buffer pH4.2. The reaction mixture was slowly loaded on the washed cartridge. The cartridge was then gradually washed with 5 mL of deionized water, 5 mL of 0.5 mol/L acetic acid and by 5 mL of 95% ethanol. Eluants were collected to the test tubes and the

radioactivity of each fraction and the remaining radioactivity bound to the cartridge was determined.

2.1.3. ^{67}Ga-DFO-octreotide

^{67}Ga-DFO-octreotide was prepared by adding of 4 μl of 1 mM [DFO]-octreotide (in 0.1% acetic acid) to 100 μL of 0.1 M amoniumacetate pH5.6 together with 10 μL of Ga-67-$(NO_3)_3$ (2.14 GBq/mL in 0.04 M HCl). Molar-concentration ratio of [DFO]-octreotide-to-no carrier added Ga^{3+} was 32.05. After 60 min incubation the labelling efficiency was determined by HPLC analysis with gradient elution.

2.1.4. ^{88}Y-DOTA-Octreotide and ^{88}Y-DOTA-TOR

^{88}Y-DOTA-Octreotide and ^{88}Y-DOTA-TOC were prepared by adding 5 μL of $^{88}YCl_3$ in 50 mM HCl to 200 μl 0.4M acetate buffer pH5 with 7.4 mg of gentisic acid and 10 μg of peptide. After 25 min incubation at 95°C, the quality control was determined by gradient HPLC analysis.

2.1.5. ^{99m}Tetrapeptides (acetyl-Gly-Gly-Cys-Gly, acetyl-Ser-Ser-Cys-Gly, and acetyl-Gly-Gly-Cys-Lys)

For labelling with ^{99m}Tc, a conventional transchelation from ^{99m}Tc-gluconate was used and radiolabelled peptides were purified by filtration on Whatman microfilters 12 kD.

For radiochemical purity analysis, HPLC Pharmacia LKB with Gradient Master GP 962 (UOCHB Prague) performed with Sepharon TM SGX C18 reversed phase column (7 μm, 3.3 × 150 mm Tessek, Prague) were used.

2.2. Biological experiments

Male Wistar rats weighing 180–220 g were used. The radiolabelled peptides were administered intravenously into the tail vein in a volume of 0.2 mL. For analysis of elimination mechanisms, a recirculating rat kidney *in situ* preparation was employed as described previously [2]. For analysis of bile excretion, a perfused rat liver *in situ* preparation was used.

3. RESULTS

3.1. Quality control of radiolabelled peptides under study

The results of HPLC analysis of the individual peptides is shown in Figs 1–5. By means of the described procedure, products with high radiochemical purity were obtained.

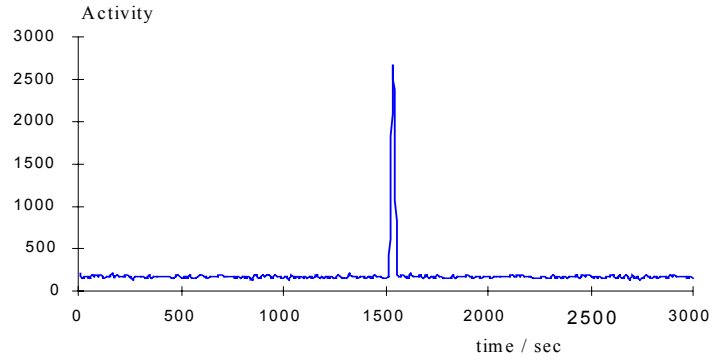

FIG. 1. Radioactivity profile of ^{111}In DTPA-Octreotide on HPLC.

Mobil phases: 0.05 M acetate buffer pH5.5 as solvent A and methanol as solvent B.

Gradient: 0–15 min: 5% B, 15–25 min: 5–55% B, 25–50 min: 55% B

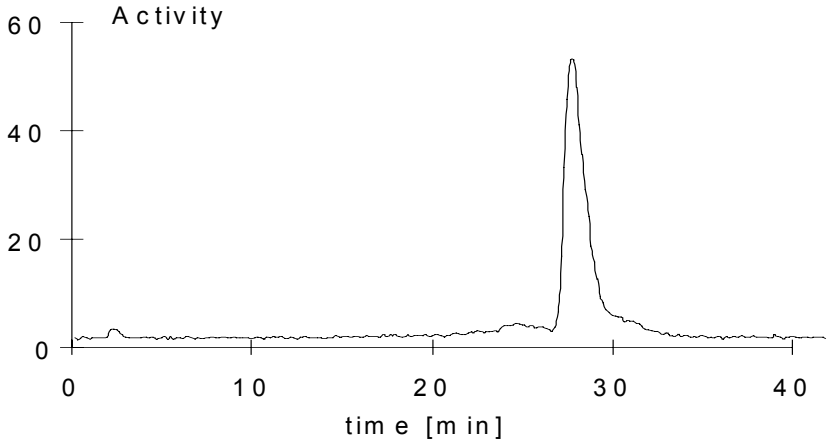

FIG. 2. A typical HPLC profile of ^{99m}Tc-RC-160.

Mobil phases: 0.15 mol/L NaCl as solvent A and 80% methanol in 0.15 mol/L NaCl as solvent B. The gradient was programmed as follows: the elution was started at 50% B reaching to 60% in 5 min and to 90% in the next 35 min.

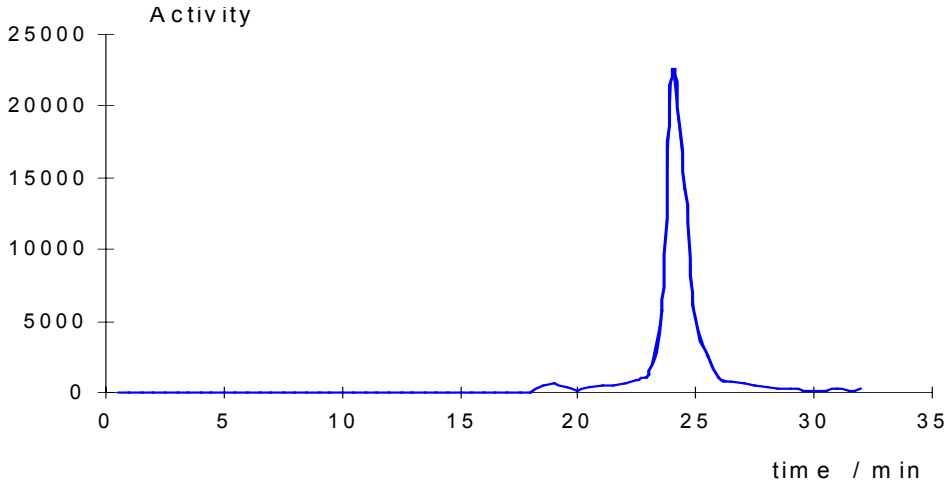

FIG. 3. An example of HPLC profile of ^{67}Ga-DFO-octreotide.

Mobil phase: 20 mM ammonium acetate pH4.5 as solvent A; acetonitrile as solvent B.

Gradient: 0–10 min: 0%B, 10–15 min: 0–45% B, 15–35 min: 45% B.

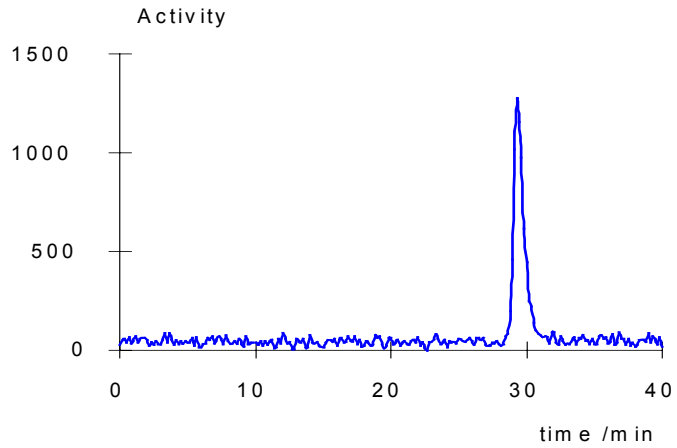

FIG. 4. Radioactivity profile of ^{88}Y-DOTA-Tyr-3-Octerotide on HPLC.

Mobil phases: 0.1% TFA as solvent A and acetonitrile as solvent B.

Gradient: 0–5 min: 0% B, 5–25 min: 0–30% B, 25–50 min: 30% B, 30–35 min: 30–100% B, 35–40 min: 100% B.

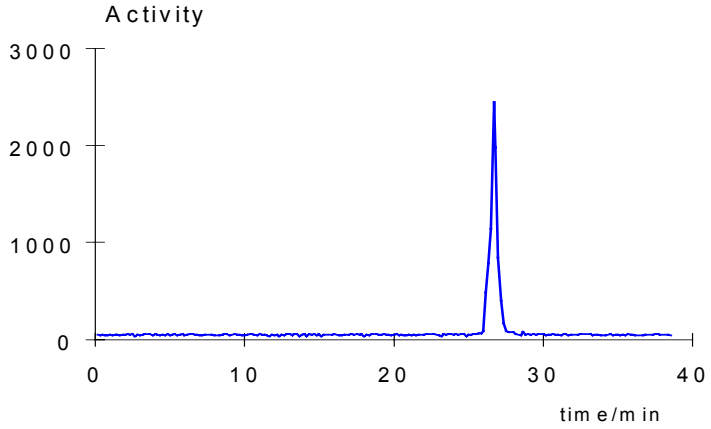

FIG. 5. Radioactivity profile of ^{88}Y-DOTA-octerotide on HPLC.

Mobil phases: 0.1% TFA as solvent A and acetonitrile as solvent B.

Gradient: 0–5 min: 0% B, 5–25 min: 0–30% B, 25–30 min: 30% B, 30–35 min: 30–100% B, 35–40 min: 100% B.

3.2. Distribution and elimination of radiolabelled peptides under study in rats

3.2.1. Receptor-specific octapeptides

Distribution of ^{111}In DTPA-octreotide, ^{67}Ga-DFO-octreotide, ^{88}Y-DOTA-octreotide and ^{88}Y-DOTA-Tyr^{3}octreotide in selected organs of rats in time intervals 5 min, 1 h, 2 h, 24 h and 48 h is presented in Figs 6 and 7.

A progressive clearance of radioactivity from the blood and most organs and tissues was clearly demonstrated for all compounds under study. The main differences in the uptake of radioactivity in somatostatin receptor-rich organs (adrenals and pancreas) were determined. For these organs, long residence time and relatively high uptake were found, the radioactivity concentration for 88Y-DOTA-TOC in the adrenals and pancreas were, however, about three times higher than that for the other compounds. A relatively high and long term radioactivity accumulation in the main excretion organ, i.e. the kidneys, was also determined for all peptides under study. Kidney accumulation was possibly due to partial re-absorption of peptides by the cells of proximal tubules by means of pinocytosis [3]. In consequence of this process, the agents may be transferred to lysosomes and digested by proteolytic enzymes.

The major excretion route for all radiolabelled peptides was the kidney but, a partial accumulation of radioactivity in the bowels of rats suggests that a small portion of the agents was cleared also by the hepatobiliary pathway. The results of biodistribution studies were confirmed by the elimination experiments. Most radioactivity was eliminated via the urine in the first two hours after dosing. A relatively small amount of radioactivity in the faeces was found. Analysis of renal handling of the agents showed that the peptides were eliminated mainly by glomerular filtration as the value of their renal clearance corrected to protein binding was close to the value of glomerular filtration rate in the experiments with perfused rat kidney. Bile clearance of radioactivity in perfused rat liver experiments was negligible for all radiolabelled peptides under study.

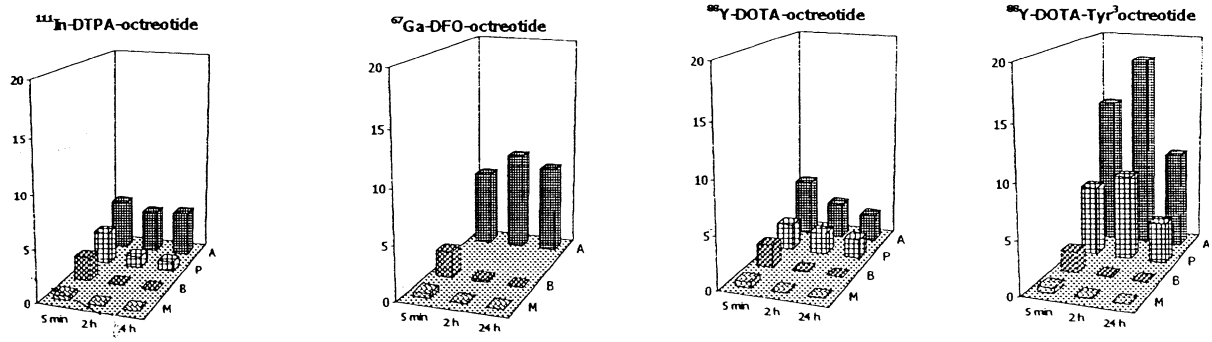

FIG 6. Concentrations of radioactivity (% dose/1% body weight) of radiolabelled octapeptides in selected organs and tissues of rats (M-muscle, B-blood, P-pancreas, A-adrenals).

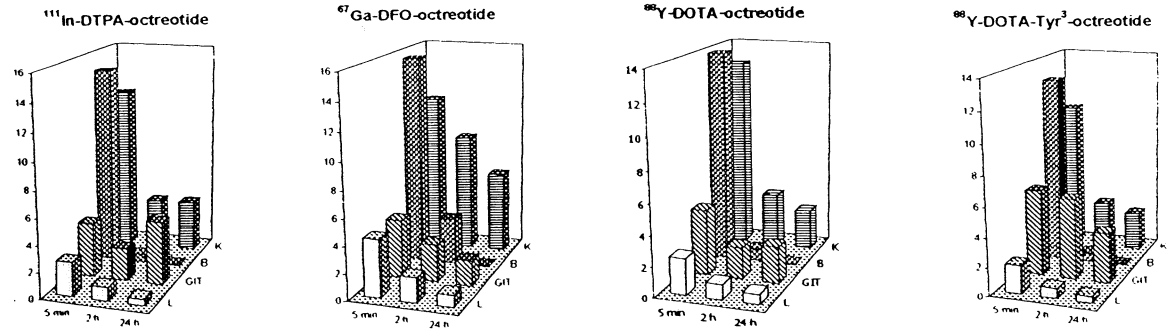

FIG. 7. Distribution of radioactivity (% dose/organ) of radiolabelled peptides in selected organs of rats (L-liver, GIT-gastrointestinal tract, B-total blood, K-kidney).

3.2.2. Tetrapeptides

Biodistribution studies in rats showed that all agents were rapidly cleared from the body mostly via urine, but some part of administered radioactivity also in the faeces was found. The later route of elimination was decreased in the order acetyl-Gly-Gly-Cys-Gly > acetyl-Ser-Ser-Cys-Gly > acetyl-Gly-Gly-Cys-Lys. Blood radioactivity time decrease was more rapid for acetyl-Gly-Gly-Cys-Gly and the slowest for acetyl-Gly-Gly-Cys-Lys. No specific accumulation of radioactivity in different organs and tissues was found, the high radioactivity in the kidneys and liver at the shortest time interval was due mainly to partial elimination of the agents by the kidneys to urine and by the liver to bile. At 1 hour after dosing, a major part of administered agents was excreted by elimination through urine and bile and only tracer radioactivity remained in blood and other organs. An analysis of renal elimination mechanisms of radiolabelled peptides under study by using perfused rat kidney in situ showed that all agents are eliminated by both glomerular filtration and tubular secretion, but the intensity of their elimination by tubular secretion was lower than that for ^{99m}Tc-MAG-3.

5. CONCLUSIONS

Somatostatin analogues labelled with suitable radionuclide can be used for diagnosis of somatostatin receptor-positive tumours and metastases. ^{111}In DTPA-octreotide is the only agent commercially available for somatostatin receptor scintigraphy, but some other somatostatin analogues have been reported to be very promising for the detection of variety of neuroendocrine tumours. Labelling procedures have employed strong chelating groups such as DTPA, DOTA and DFO which are covalently attached to the protein molecules so that they may be labelled with radiometal. A new and fascinating application is the use of labelled octreotide analogues for radionuclide therapy since the labelled radionuclide could be changed to deliver beta radiation to ablate the malignant tumour. Probably the most promising agent in this field is DOTA-Tyr^{3}octreotide, which can be labelled either with ^{111}In or with ^{90}Y. For its efficient clearance from the blood most organs and its significantly higher affinity to somatostatin receptor-rich tissues in comparison with other radiolabelled octreotide analogues, ^{90}Y-DOTA-octreotide could be invaluable in the clinic. ^{111}In DOTA-TOC could be administered prior to therapeutic ^{90}Y-TOC to localize possible somatostatin receptor-positive tumours. On the other hand, high and long-term retention of radioactivity of all radiolabelled somatostatin analogues in the kidneys represents the main obstacle in the potential use of these peptides in therapy of patients due to their possible nephrotoxicity.

ACKNOWLEDGEMENT

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^{99m}Tc LABELLED PEPTIDE FOR IMAGING OF PERIPHERAL RECEPTORS

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Abstract

Conjugates of somatostatin analogues, RC-160 with different bifunctional chelators to label with ^{99m}Tc, were synthesized. Conjugates with hydrazinonicotinamide (HYNIC) and compounds (benzoyl MAG-3 and CITC-DTPA) were prepared on a small scale with high purity and evaluated as different types of chelators on RC-160. Stability studies performed under physiological conditions showed high stability. Peptide conjugates could be labelled at high specific activities (307mCi/umol) with ^{99m}Tc and different transchelator were used for the HYNIC conjugates. The resulting radiolabelled with (^{99m}Tc and ^{125}I) complexes were highly stable and showed binding affinity to somatostatin receptors in the nanomolar range. The radioconjugates were administered to rabbits and mice in order to study their *in vivo* stability, biokinetics and biodistribution.

1. MATERIALS

Reagents were purchased from Aldrich Sigma Chemical Co. except where otherwise stated and used as they were received. 6-Boc-hydrazinopyridine-3-carboxylic acid (Boc-HYNIC) was synthesized and characterized at INMAS. 2,3,5,6,-Tetrafluorothiophenyl-S- (1-ethoxyethyl)mercaptoacetamido-adipoylglycylglycine was provided by the IAEA. N-Hydroxy-succinimide-8-acetyl-mercaptoacetyltriglycin [S-acetyl-NHS-MAG-3 was a gift from Donald J. Hnatowich (University of Massachusetts, Worcester, MA). S-benzoyl-mercapto-acetyltriglycine (S-benzoyl-MAG-3) was prepared at INMAS Delhi, India. [Lys5-Boc-RC-160, RC-160, and [Tyr3, Lys-Boc] octreotide was provided by the IAEA.

2. METHODS

2.1. HPLC

Waters Millipore with UV detector 2487 waters was used for reversed-phase HPLC analysis and preparation. A Beckman Ultrasphere ODS 5 μm, 4.6 × 250 mm column, flow rates of 1 mL/min, and UV detection at 220 nm and 368 nm were employed together with the solvent systems: Solvent B 0.05% acetonitrile Solvent A 0.05%TFA-water. Gradient: 0-3 min 0% B; 3–13 min 0 to 50% B; 13–23 min 50% B, 23–26 min 50 to 70% B, 26–27 min 70 to 0% B was used for analyses of RC-160 conjugates.

2.2. TLC

Instant TLC on silica gel (ITLC-SG, Gelman Sciences) was performed using different mobile phases. 2-Butanone was used to determine the amount of free $^{99m}Tc0_4^-$ (Rf = 1), and the PBS buffer was used to determine non-peptide-bound ^{99m}Tc colloid (Rf = 1) and $^{99m}TcO_4^-$ (Rf = 1), 50% acetonitrile/water for ^{99m}Tc colloid (Rf = 0).

2.3. SPE purification

For purification of the radiolabelled peptide for stability studies, a solid-phase extraction (SPE) method was used. The radiolabelling mixture was passed through a C18-

SEPPAK mini cartridge (Waters, Milford, MA). The cartridge was washed with 5 mL of water (six times with flow rate 1 drop/6 s), the radiolabelled peptide eluted with 50% 0.05% TFA acetonitrile, and the organic solvent evaporated under N_2 stream. This method efficiently removed all hydrophilic impurities ($^{99m}TcO_4^-$, ^{99m}Tc co-ligand) and ^{99m}Tc colloid to a concentration of less than 2% when tested by HPLC or TLC.

2.4. Receptor binding assay

The binding affinity of ^{125}I-RC-160 conjugates was tested in a competition assay against cold peptide on cells expressing somatostatin receptors.

2.5. Synthesis of peptide conjugates

2.5.1. Synthesis of HYNIC-RC- 160

A total of 0.3 mg of Boc-HYNIC, 2.0 mg of O-(7-azabenzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), and 5 mg of diisopropylethylamine (DEA) in 300 μL of DMF (anhydrous) were reacted for 15 min. Boc-peptide in 20 μL of DMF 5 μL of water was added and allowed to react for 45 min. To stop the reaction, 1 mL of water was added, and the resulting solution was passed through a C18-SEPPAK cartridge (Waters, Milford, MA) washed with additional 5 mL of water and finally eluted with 1 mL of 100% acetonitrile. The acetonitrile solution was removed under N_2 stream to a volume of 100 μL. A total of 300 μL of TFA and 10 μL of thioanisole were added and reacted for 10 min. The solution was evaporated to dryness, and the residue dissolved in 200 μL of 50% ethanol and purified on HPLC using above gradient. The peak of HYNIC-RC-160 was collected and stored in the HPLC eluent at –20°C under nitrogen. Further characterization was performed by HPLC analysis.

2.5.2. Synthesis of S-Acetyl-MAG-3-RC-160

A total of 0.3 mg of Lys-Boc RC-160, 1.0 mg of S-acetyl-NHS-MAG-3, and 1 mg of DEA were reacted for up to 3 h. SEPPAK purification and deprotection were carried out as described for HYNIC-RC-160.

2.5.3. Synthesis of S-benzoyl-MAG-3-RC-160

A total of 2.2 mg of S-benzoyl-MAG-3, 1.0 mg of HATU, and 1 mg of DEA in 170μL of DMF were reacted for 15 min. A total of 40μL of this solution was added to 0.4 mg of Lys-Boc-RC-160 in 20 μL of DMF/5 μL of water and allowed to react for 1 h. SEPPAK purification and deprotection were carried out as described for HYNIC-RC-160.

2.6. ^{99m}Tc labelling of HYNIC conjugate

2.6.1. Tricine as co-ligand

In a rubber sealed vial, 10 μg of HYNIC conjugate was incubated with 0.5 mL of tricine solution (100 mg/mL in 25 mM succinate buffer, pH5.0), 0.5 mL of $^{99m}TcO4^-$ solution (100–

1000 MBq), and 25 μL of tin(II) solution (10 mg of $SnCI_2.2H_20$ in 10 mL of nitrogen purged 10%acetic acid) for 30 min at room temperature.

2.6.2. Ethylendiaminediacetic acid (EDDA) as co-ligand

10 μg of HYNIC-RC-160 was incubated with 0.5 mL of solution of EDDA (10 mg/mL, pH7.0), 0.5 mL of $^{99m}Tc0_4^-$ solution (100–1000 MBq), and 5–l0 μL of tin (II) solution (10 mg of $SnCI_2.2H_20$ in 10 mL of nitrogen purged 0.1 N HCI) for 60 min at room temperature.

2.7. ^{125}I Radiolabelling

Radiolabelling of RC-160 with ^{125}I was performed using the chloramine T technique. After labelling, the peptide was purified using a SEPPAK C-18 cartridge (Millipore) by successive injection of the labelling solution, 5 mL of trifluoroacetic acid 0.05% in water (Aldrich) and 5 mL of a 50% 0.05% TFA acetonitrile. In these conditions, free ^{125}I was eluted in acid aqueous phase and the radiolabelled RC-160 in the first 2 mL of the acetonitrile phase. The radiochemical purity of the purified solution was measured both by thin layer chromatography on silica gel (Kieselgel 60 F_{254}; Merck) using methanol as migration solvent. The specific binding of the ^{125}I labelled RC-160 was determined by competition against unmodified RC-160 in a similar assay on cell lines, which expresses the somatostatin receptors. Analysis of the different chromatograms obtained was performed after exposure of the bands on a phosphorus screen using IPLab-Gel software (Analytics Corp., 1995).

2.8. Animal studies

Animal Model: Female normal mice 25–30 g (Balb C) 4 months old were used for biodistribution studies. Rabbits 2–3 kg and rats (Sprague Dawley) 200–250 g and nude mice xenografted with PTC tumour were used for imaging and *in vivo* studies of ^{99m}Tc-RC-160.

2.9. *In vitro* serum stability studies

Labelled peptides after quality control were incubated at 37°C in fresh human serum at concentration 100nM/mL. Stability was assessed by size exclusion (Sepharose SW 3000 column) HPLC shows that the metal ion is intact under physiological conditions. It loses less than 0.5% in 8 hours.

2.10. Blood clearance

10 MBq (150 mL) of labelled peptides in aqueous solution was injected intravenously to rabbits weighing ~2.5 kg through the dorsal ear vein. Blood samples were drawn from the other ear using sterile syringe at 5, 15, 30 min and 1, 2, 4, and 24 h after i.v. injection. All samples were weighed, and their activity measured in a gamma counter and compared with a standard.

2.11. Biodistribution

The labelled peptides ^{99m}Tc-Rc-160 were diluted in sterile saline at a concentration of (52.5MBq) before being distributed into syringes where radioactivity was determined before and after injection. A volume of 0.1 mL (3.7MBq) of each solution was injected into a lateral

tail vein of each animal. The mice were sacrificed by cervical dislocation after anaesthesia. The study of ^{99m}Tc peptides was performed in 15 animals in three groups of five mice and 24 h after administration. Blood and normal tissues (liver, kidney, spleen, lungs, heart, bone, intestine and muscles) were removed from each animal and weighed before radioactivity was measured in a gamma counter and compared with the standard. The results are expressed as a percentage of injected dose per gram (% ID/g) of tissues. Urine was collected at 1, 4, 24 h post injection (p.i.).

2.12. Imaging

Imaging of animals was carried out at different time intervals after administering 18.5 MBq of the labelled compound intravenously using a planner gamma camera fitted with parallel collimator (ECIL, INDIA).

3. RESULTS

In this study, reaction mixture of peptide conjugates of RC-160 was purified with overall yield of 60% for both the conjugates. Mass spectrometry data of the HYNIC conjugate and MAG-3 was found as M+1 in FAB$^+$ mode. Conjugates of RC-160 produce stable and reproducible complexation with ^{99m}Tc and receptor binding with ^{125}I. Radiolabelled peptide was analysed and found to be easily and reproducibly labelled with ^{99m}Tc and ^{125}I. Labelling efficiencies were generally greater than 95% as determined by TLC and HPLC. TLC was performed by heat treated ITLC-SG strips using saline or 85% ethanol as mobile phase. Reverse phase (RP) chromatography using C_{18} SEPPAK confirmed high labelling efficiency, as did analytical RP-HPLC.

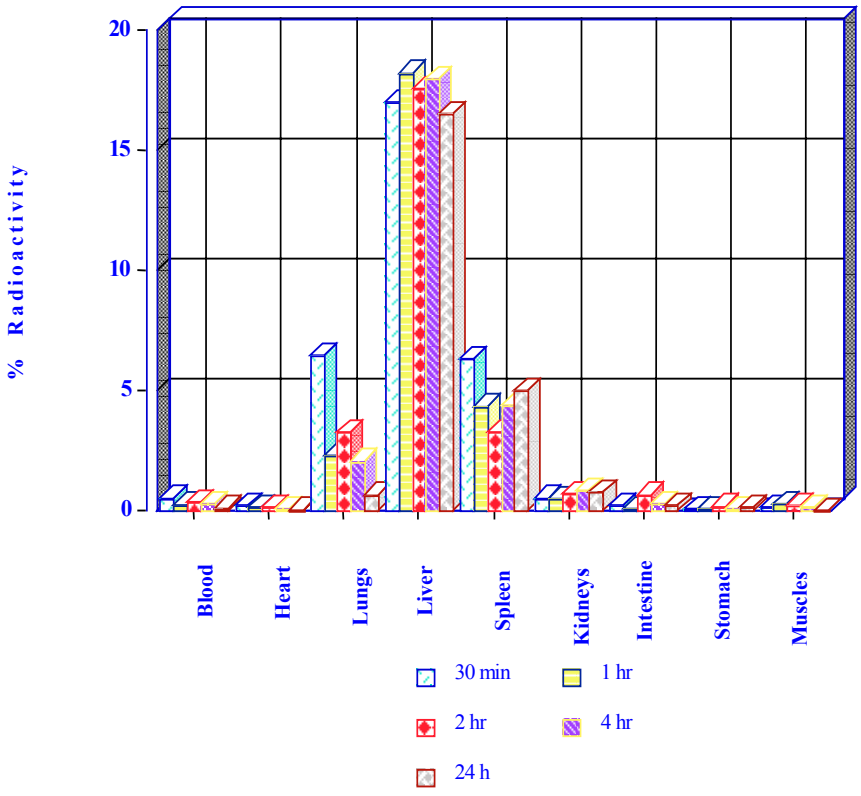

FIG. 1. Biodistribution of ^{99m}Tc-RC-160 conjugate at different time intervals.

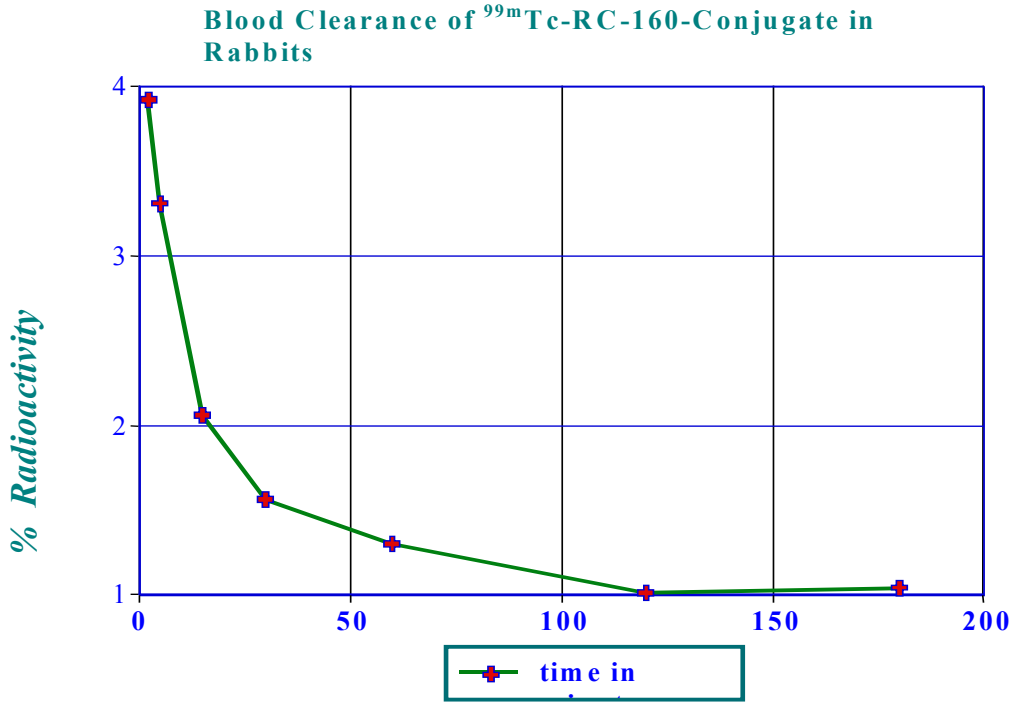

FIG. 2. Blood clearance of ^{99m}Tc-RC-190 conjugate in rabbits.

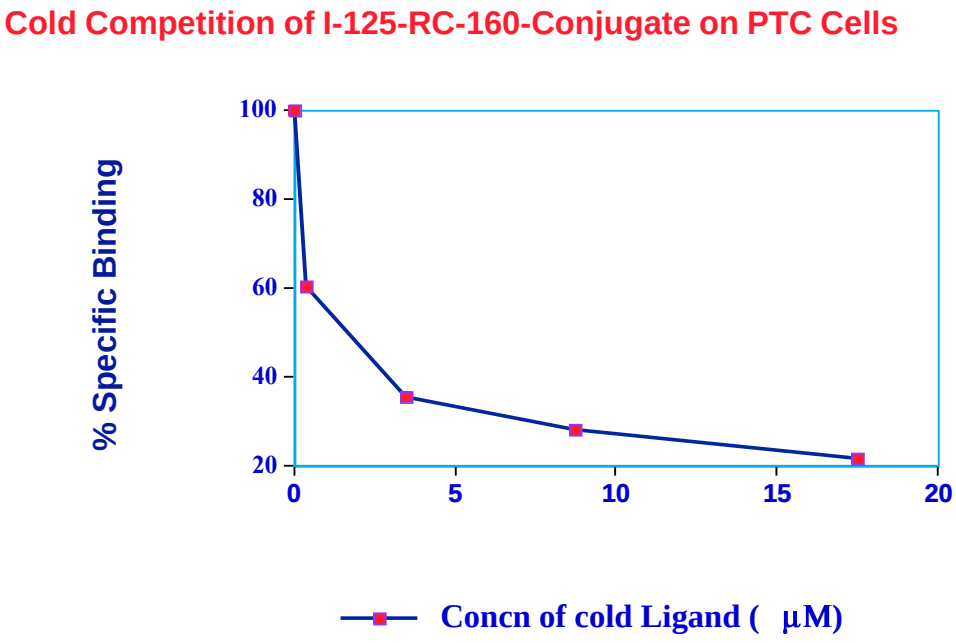

FIG. 3. Cold competition of ^{125}I-RC-190 conjugate on PTC cells.

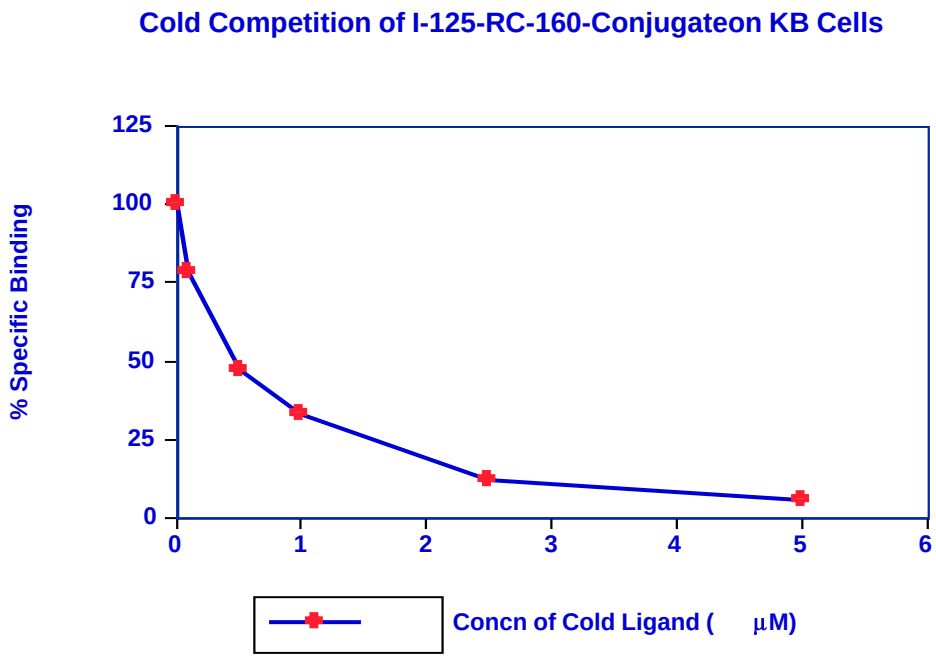

FIG. 4. Cold competition of ^{125}I-RC-190 conjugate on KB cells.

The *in vitro* dissociation of radiolabelled tracer with human serum proteins was determined by both gel electrophoresis and gel filtration (size exclusion). Both radiolabelled conjugates are stable under physiological conditions. It shows the rate of decomposition is about 0.57% in 8 hours.

All subsequently described animal studies were performed under an approved animal protocol.

In biodistribution studies (mice), the data was calculated as the per cent injected dose per gram of organ. The biodistribution of radiolabelled peptide preparations were evaluated in normal mice at different time intervals (30min, 1h, 2h, 4h, and 24h) after injection (Fig. 1). Imaging technique in normal rabbits substantiated the results. ^{99m}Tc labelled peptide conjugates cleared rapidly from the blood circulation (Fig. 2) to the liver.

Imaging studies in rabbits substantiated the observation in biodistribution regardless of the radiolabelled peptide conjugates.

Receptor binding and tumour targeting were performed at PTC and KB cell lines and xenografted nude mice respectively. ^{125}I peptide conjugates showed high- affinity specific binding to somatostatin receptors (Fig. 3) with no relevant differences in binding constants (IC_{50} and K_d values calculated using Biosoft programs). Imaging was performed with ^{99m}Tc-RC-160-MAG-3 conjugate showed maximum accumulation in liver and spleen. The tumour uptake obtained at 2h with peptide conjugate was low regardless of the radionuclide used (1.6%ID/g for ^{99m}Tc; 2.4% ID/g for ^{125}I).

The compounds show promising future for tumour targeting studies. Further experiments also have to perform to avoid liver uptake either modifying the RC-160 amino

acid (Lysine) sequences to improve the efficacy of peptide. Conjugation chemistry must be performed in solid phase to facilitate the radiolabelling procedure and purification steps. However, all the complexes showed good binding affinity to somatostatin receptors.

ACKNOWLEDGEMENT

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DEVELOPMENT OF ^{99m}Tc LABELLED SOMATOSTATIN ANALOGUES WITH HIGH AFFINITY FOR SOMATOSTATIN RECEPTORS

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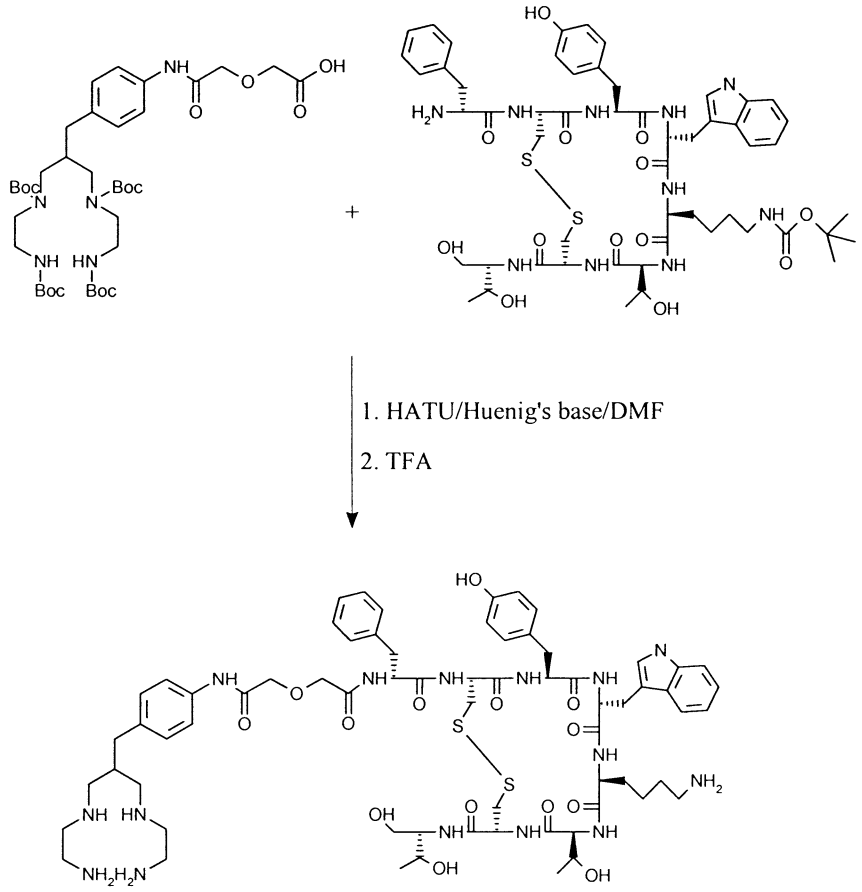
Abstract

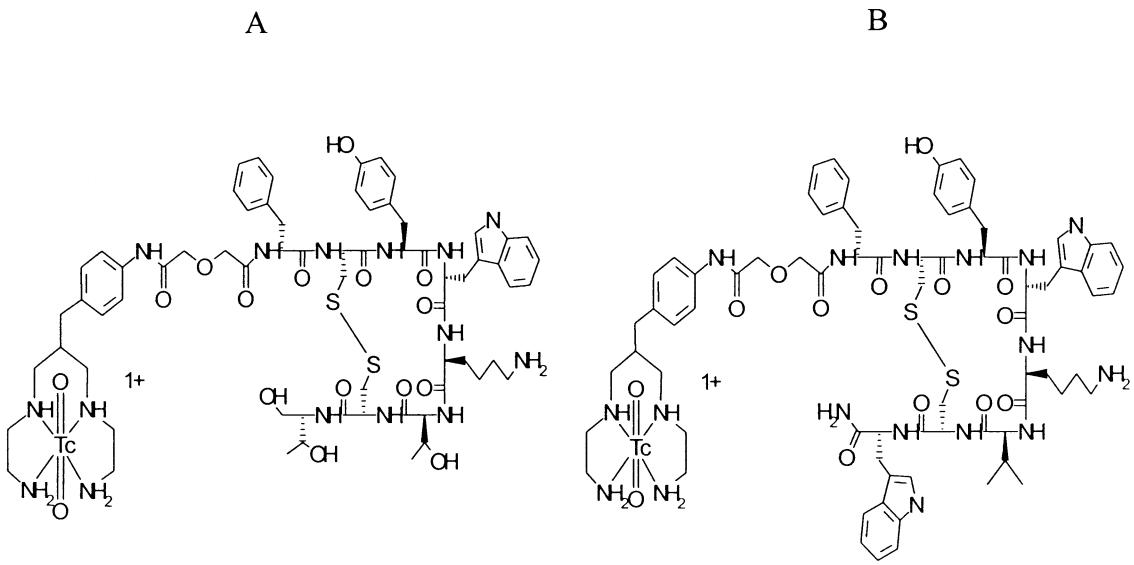
A recent development in oncology involves the use of metabolically stabilized peptide hormone analogues labelled with metallic radionuclides for the diagnosis or therapy of malignant disease. This approach was successfully applied for the first time in the visualization of somatostatin positive tumours and their metastases with ^{111}In DTPA-octreotide. In an effort to obtain a ^{99m}Tc somatostatin receptor affine radioligand we describe herein the synthesis, radiochemistry and preliminary biological evaluation of two novel ^{99m}Tc labelled somatostatin analogues, N_4-TOC and N_4-RC-160. In these compounds a tetraamine bifunctional unit was covalently attached to the N-terminal (D)Phe^1 of the peptide chain using Boc-protection strategies. The peptide conjugates were purified by high performance liquid chromatography (HPLC) and characterized by UV/Vis and ES-MS spectroscopies. As revealed by HPLC, ^{99m}Tc labelling was quantitative under mild conditions, leading to a single ^{99m}Tc species in high specific activities. Affinity of ^{99m}Tc N_4-TOC for the somatostatin receptor, as determined by *in vitro* binding assays in rat brain cortex membranes, was found unaffected by the presence of the bulky metal chelate. The binding properties of ^{99m}Tc N_4-RC-160 could not be determined by this assay due to an extremely high non-specific binding of this radioligand, and will be shortly investigated by other methods. Tissue distribution in healthy mice revealed that ^{99m}Tc N_4-TOC is clearing mainly through the kidneys and the urinary tract whereas ^{99m}Tc N_4-RC-160 shows a high accumulation in the liver as a result of its lipophilicity. Analysis of urine samples by HPLC showed that ^{99m}Tc N_4-TOC is excreted integer from the body of mice, while ^{99m}Tc N_4-RC-160 is totally transformed to an unidentified hydrophilic metabolite *in vivo*. The location of this metabolism is currently investigated. *In vivo* blocking experiments using animals pre-treated with 50 μg octreotide prior to the injection of the radioligand demonstrated that accumulation of the radioactivity in somatostatin receptor rich organs like the pancreas and adrenals, is *in vivo* specific and mediated by the somatostatin receptor.

1. MATERIALS

The somatostatin peptide analogues RC-160 and TOC or protected with Boc at the ε-NH_2 group of Lys^5 were provided by BACHEM through the IAEA. Octreotide used for *in vivo* blocking experiments was kindly offered by Novartis-Hellas. Reagent grade chemicals were purchased from Aldrich or Fluka and used without further purification. ^{99m}Tc was obtained as an Na^{99m}TcO$_4$ eluate in physiological saline from a commercial ^{99}Mo/^{99m}Tc generator (Cis Int.). Solvents for HPLC were filtered through membrane filters and degassed with He flux prior to use. For HPLC purification a preparative HPLC system from Waters was used (Waters Prep LC 4000) coupled to a Waters 996 Photodiode Array UV Detector. The Millennium Software by Waters was applied for controlling the HPLC system and processing the data. For separations a Prep Nova-Pak HR C-18 cartridge (25 mm × 100 mm, 6 μ) from Waters was used. HPLC analyses were performed on a Waters ChromatograpHefficient with a 600 solvent delivery system and coupled to the above PDA-UV detector along with a gamma GABI detector from Raytest. The conditions for the analyses are given for individual products below. Competition binding assays were performed on a Brandel-48 Cell Harvester.



*Scheme 1: Synthesis of **1**.*



Scheme 2: Somatostatin analogues labelled with ^{99m}Tc.

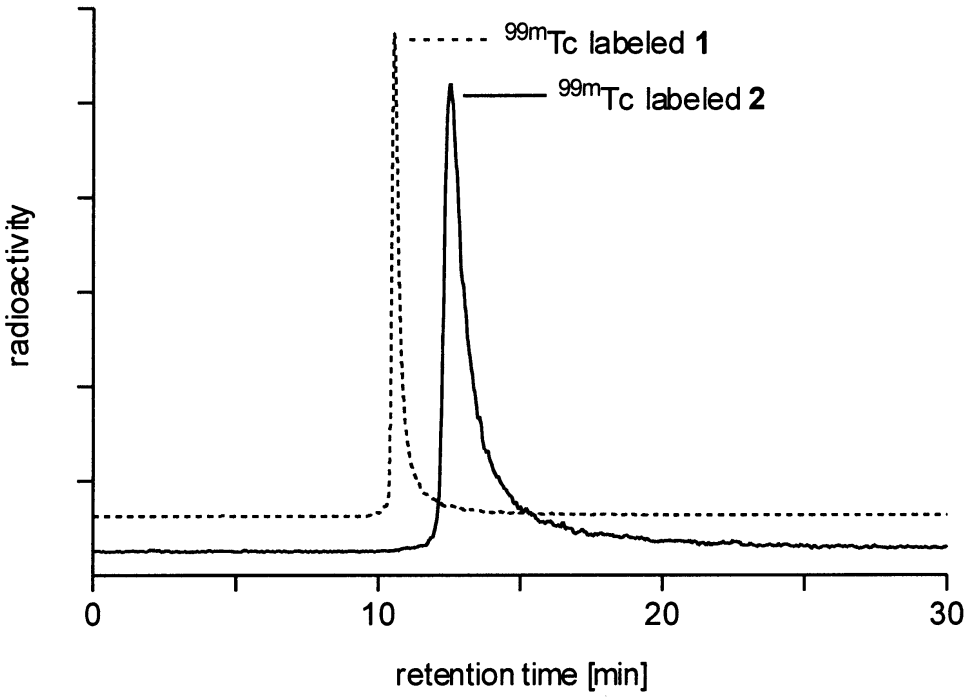


*FIG. 1. Representative radiochromatograms of ^{99m}Tc labelled **1** and **2**.*

2. METHODS

2.1. Preparation of the tetraamine-coupled somatostatin analogues

The protected $(Boc)_4$-N_4-diglycolic acid bifunctional chelating agent was reacted in a threefold molar excess with the corresponding Boc protected ε-Lys^5 SMS peptide analogue in CH_2Cl_2/DMF medium in the presence of HATU and Hünig's base. The mixture was left to react at room temperature for about 1.5 h and then 1 N HCl and H_2O were added. The Boc protected conjugate was extracted in ethyl acetate and the organic phase washed several times with H_2O. The organic phase was concentrated to a small volume and the product purified over a small silica gel column using $CHCl_3$/MeOH 10/1 as the eluent.

Deprotection was completed at ambient temperature within 25 min in TFA medium containing 5% H_2O and 4% thioanisol. The reaction was quenched by introducing the mixture in 0.5N NaOH to pH3. The reaction mixture was rinsed with $CHCl_3$ and concentrated to a small volume. The product was purified by preparative HPLC. For **1**: The column was eluted at 10 mL/min with the following gradient system: 0 to 25 min 40 to 80% 0.1% TFA in MeOH and 60 to 20% 0.1% aqueous TFA solution. t_R: 12 min. For **2**: The column was eluted at 10 mL/min with the following gradient system: 0 to 30 min 20 to 80% 0.1% TFA in MeOH and 80 to 20% 0.1% aqueous TFA solution. t_R: 25 min.

*Analytical data for N_4-diG-TOR, **1**:* ES-MS calculated for $C_{67}H_{95}N_{15}O_{14}S_2$: 1397.7, found: 1398.8 (MH^+); t_R (Merck Lichrospher RP-18 column, 10 µ, 250 mm × 4 mm; 1 mL/min; 0 to 20 min 40 to 80% 0.1% TFA in MeOH and 60 to 20% 0.1% aqueous TFA solution): 14.7 min; UV/Vis (λ_{max}, nm): 248, 283, 290.

*Analytical data for N_4-diG-RC-160, **2**:* ES-MS calculated for $C_{73}H_{89}N_{17}O_{12}S_2$: 1558.8, found: 1559.6 (MH^+); t_R (as for **1**): 18 min; UV/Vis (λ_{max}, nm): 248, 282, 290.

2.2. Labelling of somatostatin analogues with ^{99m}Tc

To a $Na^{99m}TcO_4$ generator eluate (730 μL) 0.5M phosphate buffer pH11.0 (200 μL) was added followed by 0.1M citrate solution (10 μL), an aqueous solution of the **1** or **2** (10 nmol, 40 μL) and a freshly prepared $SnCl_2$ solution in EtOH (20 μl, 20 μg). The labelling reaction mixture was incubated for 30 min at ambient temperature and adjusted to pH7.0 with 1N HCl. Labelling yields were monitored by HPLC analysis on an RP-Amide C16 Discovery column from Supelco (5 μ, 150 mm × 3.9 mm). The column was eluted at a 1 mL/min flow rate with the following gradient system: from 0 to 10 min 10% to 60% MeCN and 90% to 40% 0.2% ammonium phosphate buffer pH7.0. t_R for ^{99m}Tc **1**: 10.6 min; t_R for ^{99m}Tc **1**: 12.2 min (Fig. 1).

2.3. Competition binding assays of ^{99m}Tc labelled somatostatin analogues

The binding affinity of ^{99m}Tc labelled peptides **1** and **2** were determined in rat brain cortex membrane homogenates according to reported protocols. The membranes were diluted to 50 μg protein per assay tube by Tris buffer (10mM, pH7.4) containing $MgCl_2$ (10mM), bacitracin (10 μM) and 0.1% BSA. The assay tubes contained each ^{99m}Tc labelled peptide (30 000 cpm, 70 μL), buffer or increasing concentrations of somatostatin analogues (30 μl) and membrane suspension (200 μl, 50 μg). The tubes were incubated in triplicates for 30 min at ambient temperature. Incubation was stopped by rapid filtration on a Brandell Cell Harvester through GF/C glass fibre filters and rinsing with Tris buffer (10 mM Tris, 150 mM NaCl, pH7.4, 4ºC). The filters were counted in a well type gamma counter and specific radioligand binding was defined as total binding minus binding in the presence of 1 μM somatostatin analogue (= non specific binding). The data from the displacement experiments were analysed according to a one-site model.

2.4. Tissue distribution of ^{99m}Tc labelled somatostatin analogues in healthy mice

For tissue distribution experiments Swiss albino mice (30 ± 5 g) were used in groups of four. Mice were injected each with a bolus of labelled peptide phosphate buffer solution pH7.4 (100 μl, 2.5–4 mCi) through the tail vein. Animals were sacrificed at 30 min, 1 h, 2 h and 4 h after injection (pi) of the radioligand by heart puncture. Blood and urine samples were immediately collected and organs of interest were excized, weighed and their radioactivity content measured in a well-type gamma counter along with proper standards. An additional group of animals received each intraperitoneally 50 μg octreotide 35 min prior to the intravenous injection of the radiolabelled peptide (blocked animals). These animals were sacrificed at 30 min p.i. and the same procedure was followed as before. Biodistribution data were calculated as per cent injected dose per gram tissue (%ID/g) according to a known algorithm. Mouse body composition data were used in the calculation of%ID/g values for blood and muscle.

3. RESULTS

3.1. Preparation of peptide conjugates

The synthesis of N_4-TOC and N_4-RC-160 proceeded following these steps: The BOC protected N_4 bifunctional ligand was coupled to the Boc ε-Lys^5 peptide in the presence of HATU and Hünig's base. After purification on a small silica column the BOC protected conjugate was reacted shortly with TFA and purified with preparative HPLC affording the

respective product **1** or **2** in a pure form. The purity of **1** and **2** was confirmed by analytical HPLC, whereas ES-MS findings were consistent with the expected formulae. The synthetic route followed for **1** is depicted in Scheme 1.

3.2. Preparation and radiochemical analysis of ^{99m}Tc labelled peptides

Labelling of **1** and **2** with ^{99m}Tc was quantitative using $SnCl_2$ as reductant and citrate as transfer ligand. High specific activities were reached using as low as 10 nmol total peptide under very mild conditions. The radioligands (Scheme 2) were stable in phosphate buffer over 24 h, as revealed by HPLC analysis. As shown in the representative chromatograms of Fig. 1, ^{99m}Tc labelled **1** is more hydrophilic than **2**. However, the introduction of the hydrophilic $(TcO_2N_4)^+$-diglycolic acid residue in the RC-160 peptide backbone led to a substantially more hydrophilic radioligand as compared to the very lipophilic ^{125}I-RC-160. The trend in the hydrophilicity is reflected in the relative biological characteristics of the two somatostatin analogues.

3.3. Characterization of ^{99m}Tc labelled somatostatin analogues binding properties

Competition binding assays were performed on rat brain cortex membrane homogenates using ^{99m}Tc labelled **1** and **2** as the radioligand, and native somatostatin-14 as competing ligand. Binding of ^{99m}Tc labelled **1** to the rat cortex somatostatin receptors was inhibited by somatostatin-14 in a monophasic manner. The level of non-specific binding, as determined in the presence of 1 μM somatostatin-14 was less than 20% of total binding. The binding properties of ^{99m}Tc labelled **2** could not be determined by this model, given that the non-specific binding was extremely high. Alternative assays are currently studied for this purpose. A representative displacement curve for ^{99m}Tc labelled **1** is shown in Fig. 2 revealing that this analogue binds with high affinity to somatostatin receptors (IC_{50} for **1**: 2.8×10^{-10}; IC_{50} for TOC: 3.0×10^{-10}).

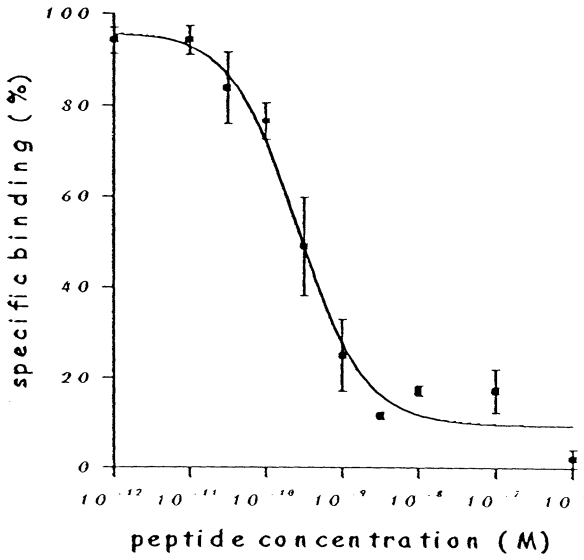


FIG. 2. Competition binding of ^{99m}Tc labelled 1 against various concentrations of somatostation-14 for somatostatin receptors in rat brain cortex homogenates.

3.4. Tissue distribution of ^{99m}Tc labelled somatostatin analogues in healthy mice

Biodistribution data of ^{99m}Tc labelled analogues **1** and **2** in healthy mice is presented as %ID/g in Figures 3 and 4, respectively. Results of biodistribution measurements for blocked (non-specific) groups of animals are also included in the diagrams for comparison. Both radioligands are cleared rapidly from the circulation and excreted mainly through the kidneys into the bladder, with **1** exhibiting a faster body clearance and **2** showing also a high liver uptake. As evidenced by HPLC analysis of urine samples, while peptide **1** is washed into the urine integer evading *in vivo* transformation, peptide **2** is totally converted to a hydrophilic unidentified metabolite within 30 min. The liver is the suspected location of this *in vivo* metabolism and incubation of ^{99m}Tc labelled **2** in liver homogenates are currently studied. Preliminary experiments of ^{99m}Tc labelled **2** incubates in murine plasma show that the major part of the radioactivity co-elutes with plasma proteins. High uptake of activity is exhibited by the adrenals and the pancreas, tissues known to be rich in somatostatin receptors, but also throughout the gastrointestinal tract. The activity uptake into these organs is found significantly lower in the animals, which had received a high dose of octreotide prior to the radioligand injection. These findings demonstrate that the *in vivo* localization of ^{99m}Tc labelled peptides **1** and **2** is specific for somatostatin binding sites.

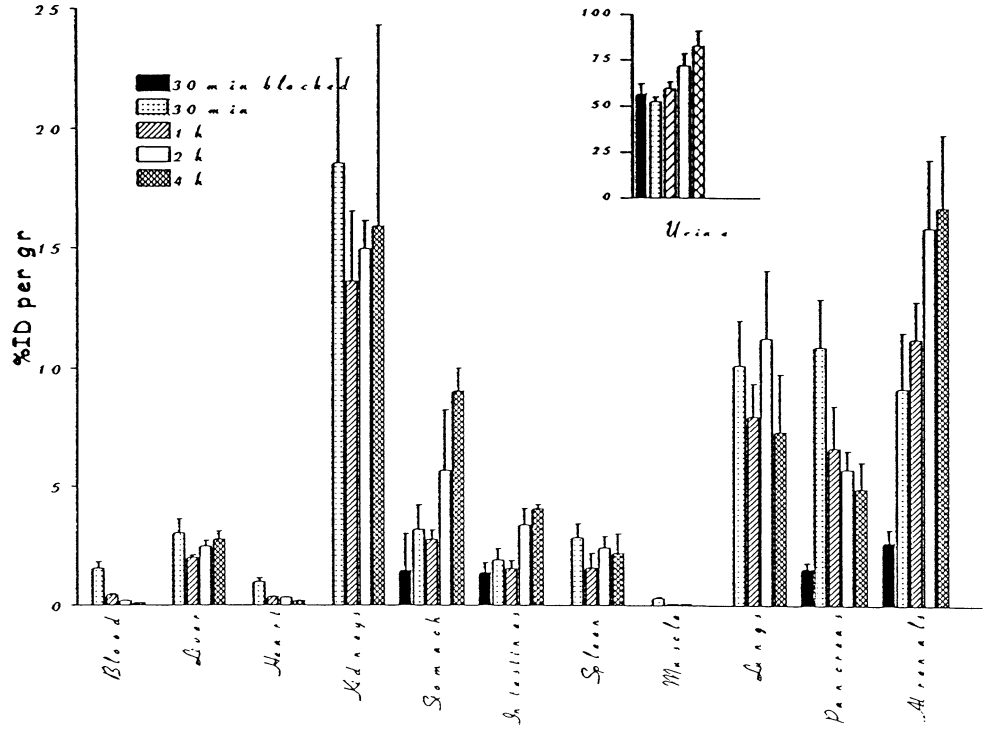


*FIG. 3. Biodistribution of ^{99m}Tc **1** in healthy mice as % ID/g at 30 min, 1 h, 2 h and 4 h time intervals p.i. accumulation of activity in the urine over time as % ID/organ is shown in the inset.*

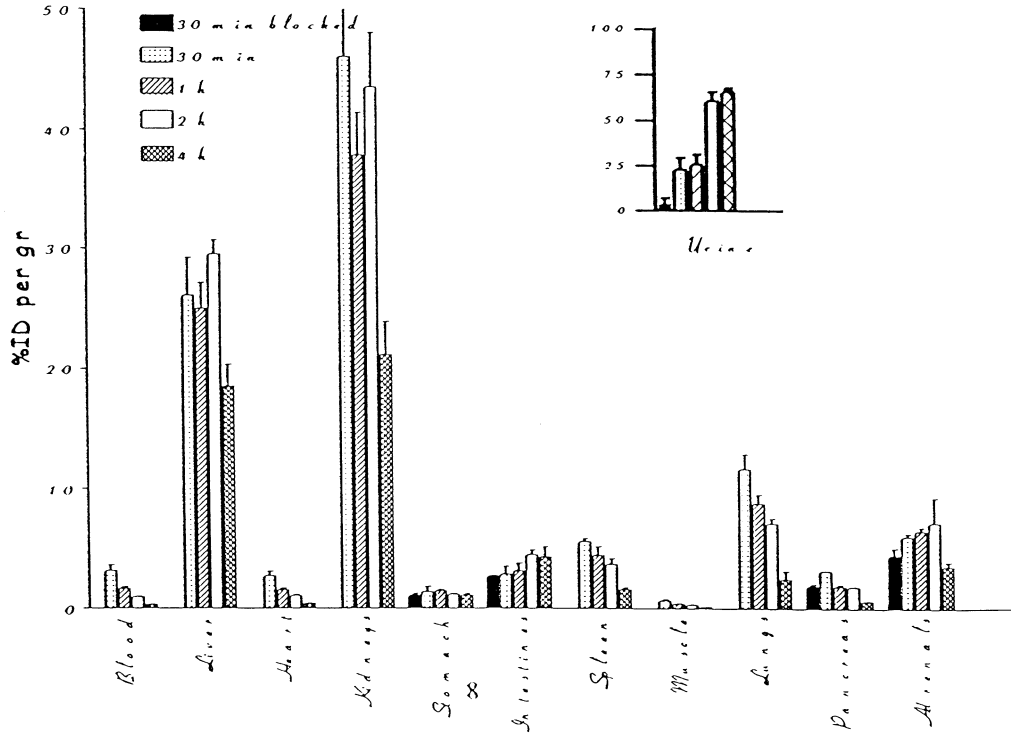


*FIG. 4. Biodistribution of ^{99m}Tc **2** in healthy mice as % ID/g at 30 min, 1 h, 2 h and 4 h time intervals p.i.; accumulation of activity in the urine over time as % ID/organ is shown in the inset.*

4. CONCLUSION

Two novel stabilized somatostatin analogues carrying a tetraamine residue through a diglycolic acid spacer, N_4-TOR (**1**) and N_4-RC-160 (**2**), were synthesized. Labelling of **1** and **2** with ^{99m}Tc proceeded in high yields in alkaline aqueous medium and using $SnCl_2$ as reductant in the presence of citrate. A single hydrophilic ^{99m}Tc species formed from each analogue in buffer, as revealed by HPLC analysis. Displacement experiments in rat brain cortex membranes demonstrated, that ^{99m}Tc labelled **1** binds with high affinity and specificity to somatostatin receptors *in vitro*, but were not possible to perform for ^{99m}Tc labelled **2** due to excessive non specific binding. Biodistribution of ^{99m}Tc labelled peptides in healthy mice demonstrated high and specific accumulation of radioactivity in somatostatin binding sites *in vivo*. Furthermore, the radiolabelled peptides exhibited pharmacokinetics related to their hydrophilicity, with ^{99m}Tc labelled **1** excreted mainly through the kidneys into the urine and ^{99m}Tc labelled **2** exhibiting a high liver uptake. The two newly developed ^{99m}Tc labelled analogues may provide a useful tool for the detection of somatostatin receptor positive tumours *in vivo* and are currently under further evaluation in tumour bearing animals.

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APPROACHES IN THE DESIGN OF ^{99m}Tc BASED PEPTIDE RADIOLABELLING FOR TUMOUR TARGETING

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Abstract

One of the major drawbacks in diagnostic and/or therapeutic uses of peptides radiolabelled with radiometals via bifunctional chelating agents (BCA) is their accumulation in excretory organs such as liver or kidney. Thus, the aim of the project is centred in the search for chemical and radiochemical approaches to reduce radioactivity accumulated in excretory organs while preserving the *in vivo* receptor binding affinity of the peptide. During the first stage a suitable procedure using the F-moc-chemistry (solid phase) was developed and synthesis of DTPA-D-Phen1-Octreotide and DTPA-L-Phen1-Octreotide was carried out. During the synthesis, the need to improve the yield demanded the synthesis of a DTPA derivative holding only one reactive carboxylic group to avoid side intermolecular reaction. The availability of both isomeric conjugated octreotide led to their radiolabelling with ^{111}In. Their metabolic studies in animals indicated that the degradation rate of the peptide containing the natural aminoacid, ^{111}In DTPA-L-Phen1-Octreotide, was slightly higher than the corresponding D-aminoacid derivative, as expected. Stability of the peptide during radiolabelling with ^{99m}Tc was then studied, requiring the use of variable agents such as ascorbic acid, dithionite and stannous ion. The selected peptide, RC-160, was provided by the IAEA and, as reference compounds, corresponding iodinated and radioiodinated peptides were synthesized. Demonstration of the stability of the peptide was carried out using disodium 2-nitro-5-thiosulfobenzoate (NTBS) and the lack of Bunte salt formation served as an indication of the stability of the disulfide bond under various mild conditions required for the future radiolabelling with ^{99m}Tc. The knowledge gained served in moving to the next stage of ^{99m}Tc radiolabelling using HYNIC as the BCA and tricine as co-ligands. The biodistribution studies demonstrated great accumulation on excretory organs. This led us to look for a model protein. Neogalactoalbumin (NGA), a specific protein that was incorporated by hepatic parenchymal cell via receptor-mediated endocytosis immediately after administration, was selected as a biological tool. The fate of the radiolabelled NGA after lysosomal proteolysis in hepatocytes was studied in liver homogenates. Subcellular distribution and identification of radiometabolites were performed using multiple analytical methods such as electrophoresis, TLC, and size exclusion and reverse phase HPLC. The data indicated that the chemical bonding between ^{99m}Tc and HYNIC remains stable in the lysosomes but the persistent liver localization was due to a radiometabolete identified as ^{99m}Tc-HYNIC-lysine (tricine)2, demonstrated by the similar HPLC data obtained with the synthesized and radiolabelled metabolete. Better co-ligands or new ligands or new design of the peptides are required in order to reduce the residence time of radioactivity in non-target tissue for future peptide use in radiodiagnosis or radiotherapy.

1. INTRODUCTION

The development of Tc based biological targeting with peptides constitutes nowadays, an important research goal. In recent years, the radiolabelled octapeptide, octreotide, a synthetic derivative with similar bioactive structure as the parent tetradecapeptide somatostatin (SM), with ^{123}I and ^{111}In (^{123}I -Octreotide and ^{123}In DTPA-Octreotide), was used for the SM receptor positive tumour imaging. Also, another analogue RC-160 or vapreotide has recently been found to have affinity for different subtypes of somatostatin receptors. Nevertheless those radiopharmaceuticals have registered high liver and kidney uptake, a drawback in diagnostic and/or therapeutic uses. Thus, the aim of the project is centred in the search of chemical and radiochemical approaches for reducing radioactivity accumulated in excretory organs while preserving the *in vivo* receptor binding affinity of the peptide. The gathered data will be used for building up the drug design basis of Tc peptide analogues.

The research programme was carried out in three stages, as follows:

Stage I. Basic studies on Tc labelled octreotide analogues
Stage II. Studies on integrity of the disulphide bond in RC-160
Stage III. Intracellular metabolic fate of radiolabelled RC-160.

STAGE I. BASIC STUDIES ON Tc LABELLED OCTREOTIDE ANALOGUES

At the initial stage, back in 1995, there was lack of commercially available peptide Octreotide or RC-160. This forced us to look for a method to synthesize them. A suitable procedure using the F-moc chemistry (solid phase) was developed and the synthesis of DTPA-D-Phe1-Octreotide and DTPA-L-Phen1-Octreotide was accomplished first. In this task, the synthesis of a monoreactive DTPA (m-DTPA) (Fig. 1) played an important role; the m-DTPA could be easily deprotected by TFA and its suitability for F-moc chemistry became evident.

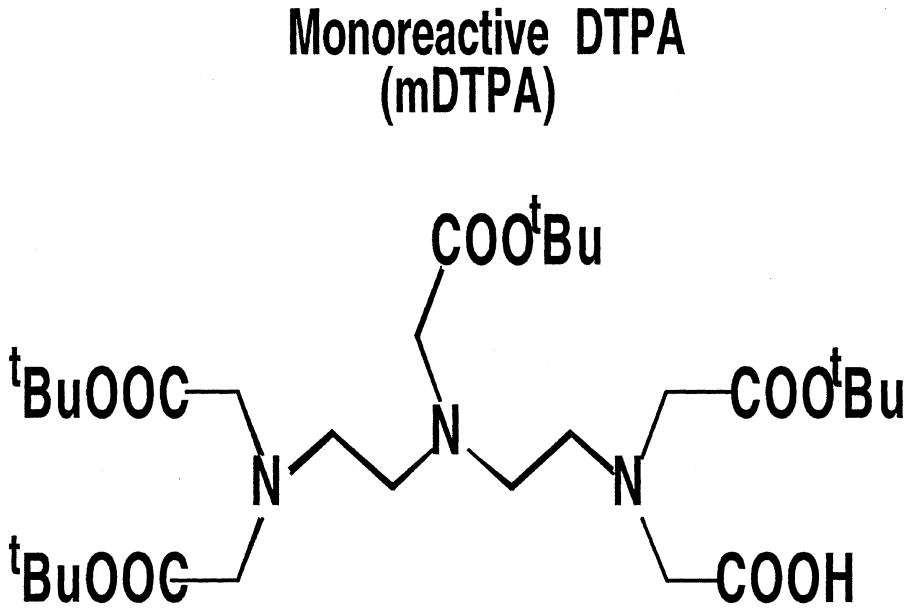

FIG. 1. mDTPA.

The availability of the L and D forms of octreotide conjugated to DTPA, offered approaches for learning about the effect of those isomers on the renal metabolism. Although the ^{99m}Tc labelled conjugate could not be formulated, the study was carried out with ^{111}In labelled octreotides conjugates. In the animal studies with mice, no dramatic differences were observed between the L and the D forms, but the residence time of radioactivity in the kidney of the D form was slightly higher than the corresponding L form. Analysis of the kidney homogenate excized from animals at 1 h, 3 h and 24 h p.i. indicated higher rate production of the end product ^{111}In DTPA-L-Phe than the ^{111}In DTPA-D-Phe. Those studies showed that the degradation rate of the peptide derivative containing the natural aminoacid, ^{111}In DTPA-L-Phen-Octreotide, was, as expected, slightly higher than the D-aminoacid containing derivatives.

Moreover, in co-operative studies with Alice Laznickova from the Czech Republic some pharmacokinetic studies of those radiolabelled peptides were carried out in rats and a paper was submitted for publication.

Part of the work was published in Bioconjugate Chemistry **9**: 662–670, 1998: "Renal metabolism of ^{111}In DTPA-D-Phe1-Octreotide *in vivo*" by H. Akizawa, Y. Arano, T. Uezono, et al.

STAGE II. STUDIES ON INTEGRITY OF THE DISULPHIDE BOND IN RC-160

During the first stage the synthesis of RC-160 was initiated using F-moc chemistry. Time was dedicated to learn about its synthesis and the preparation of its monoiodinated derivative (cold reference), including its radioiodinated counterpart to be used as reference for the *in vitro* or *in vivo* studies on either the radioiodinated $^{131/125}$I RC-160 or the technetium labelled ^{99m}Tc RC-160 peptide. Thus, during this second term, studies on the integrity of the disulfide bond in the cyclic RC-160 analogue during the radiolabelling reaction were carried out. The effect of Tc radiolabelling parameters such as ascorbic acid, dithionite and stannous ions were screened and the assessment of disulfide bond integrity was demonstrated by using disodium 2-nitro-5-thiosulfobenzoate (NTBS), according to the Thannhauser method (Anal. Biochem. 138:181, 1984); the lack of Bunte salt formation served as an indication of the stability of the disulfide bond.

STAGE III. INTRACELLULAR METABOLIC FATE OF RADIOLABELLED RC-160

After learning about the basic conditions necessary for handling the peptide RC-160, we proceeded to our main research interest, i.e. the metabolic fate of radiolabelled peptides. As previously mentioned, the greatest drawback in diagnostic and/or therapeutic uses of peptides radiolabelled with radiometals via bifunctional chelating agents has been their accumulation in excretory organs, such as liver or kidney. As described in the last report, in the third term of the CRP, studies were to be centred on the mechanism responsible for the excretory organs localization of the RC-160 based radiolabelled product. Since the ultimate objective of this project are in the development of ^{99m}Tc based peptides, namely Tc labelled RC-160, our work was centred in using HYNIC as the bifunctional chelating agent (BCA). The hydrazino nicotinate, or HYNIC has been reported as one attractive BCA, but the study with its radiolabelled protein conjugate ^{99m}Tc-HYNIC-protein(tricine)$_2$ have shown persistent localization of radioactivity in non-target tissue such as liver and kidney [1].

With the supply of RC-160 by the IAEA, *in vitro* and *in vivo* works were performed with ^{99m}Tc-(HYNIC-RC-160)(tricine)$_2$ and the radioiodinated ^{125}I-RC-160. However, some difficulties and discrepancies were observed on the interpretation of gathered data (Table I, Fig. 2). The biodistribution data showed greater accumulation of ^{99m}Tc-(HYNIC-RC-160)(tricine)$_2$ radioactivity in the non-target liver than the biodistribution of ^{99m}Tc (HYNIC-IgG)(tricine)$_2$, holding a bigger biomolecule and also higher than its radioiodinated reference (Tables II–III). Moreover, the RP-HPLC analysis of ^{99m}Tc (HYNIC-RC-160)(tricine)$_2$ and ^{125}I RC-160 samples incubated at 37°C with mouse plasma revealed the generation of multiple number of peaks increasing with the time of incubation. This adverse circumstance indicated to us that a more systematic study on the HYNIC-peptide/protein conjugate is required.

Factors related to the multiple number of peaks or to the long residence time of radioactivity in those excretory organs might involve the conjugated BCA side and/or the biomolecule itself. In other words, decomposition or exchange reaction of the ^{99m}Tc-HYNIC-tricine mixed ligand complex and/or metabolism of protein and peptide can be accounted for. Since lysosomes are the principal sites of intracellular metabolism of proteins and peptides [2], metabolic analysis of the radiolabelled protein/peptides in the lysosomal compartment of the liver and the kidney was estimated to provide insights into the responsible mechanism.

TABLE I. BIODISTRIBUTION OF RADOIACTIVITY AFTER INTRAVENOUS ADMINISTRATION OF [^{99m}Tc](HYNIC-IgC)(tricine)$_2$ [a]

	Time after Administration					
Tissue	10 min	30 min	1 h	3 h	6 h	24 h
	[^{99m}Tc](HYNIC-IgG)(tricine)$_2$					
Liver	14.68 (1.00)	14.24[d] (0.78)	13.31[c] (0.33)	13.25[d] (0.74)	12.22[c] (0.41)	9.80[c] (0.58)
Kidney	5.57 (0.52)	5.59[e] (0.34)	5.86 (0.30)	5.29 (1.18)	5.24[c] (0.38)	4.48[e] (0.61)
Intestine	0.61 (0.07)	0.97 (0.07)	1.26[d] (0.08)	1.90 (0.20)	2.35 (0.37)	1.54[e] (0.31)
Spleen	5.60 (0.67)	5.36 (0.26)	5.36 (0.49)	5.06[d] (0.41)	5.92[c] (0.35)	4.78[c] (0.55)
Stomach[b]	0.33 (0.06)	0.37 (0.09)	0.37[d] (0.07)	0.75 (0.22)	0.68 (0.10)	0.65[e] (0.05)
Urine[b]						4.57[c] (0.66)
Faeces[b]						1.71 (0.49)

[a] Expressed as % injected dose per gram. Each value represents the mean (s.d.) for five animals at each interval.

[b] Expressed as % injected dose.

[c] Significance determined by unpaired *t* -test; $p < 0.001$.

[d] Significance determined by unpaired *t*-test; $p < 0.01$.

[e] Significance determined by unpaired *t*-test; $p < 0.05$.

The strategy used for the first part of this study on the metabolic fate of radiolabelled peptides using HYNIC as the BCA was to incorporate *a model protein*, NGA. The use of NGA as a biological tool for pursuing the fate of radiolabelled protein with cDTPA or SCN-Bz-EDTA has been reported [3, 4]. NGA has been characterized as a specific protein, incorporated rapidly by hepatic parenchymal cells via receptor-mediated endocytosis immediately after administration. Thus, the ^{99m}Tc HYNIC labelled NGA was prepared by using tricine as co-ligands [^{99m}Tc HYNIC-NGA(tricine)$_2$] and the biodistribution and subcellular localization of the radioactivity in the murine liver was investigated. Moreover, since in recent studies, the administration of ^{111}In DTPA-NGA has induced the generation of an ^{111}In DTPA adduct of lysine (final radiometabolite), in the present work, ^{99m}Tc HYNIC - lysine (tricine)$_2$ was also synthesized. The factor affecting the long residence time of radioactivity in non-target tissues after administration of ^{99m}Tc HYNIC-protein/peptides was discussed.

2. MATERIAL AND METHODS

[^{99m}Tc]pertechnetate ($^{99m}TcO_4^-$) was eluted from Daiichi Radioisotope Laboratory generators (Chiba, Japan). Sodium triphenylphosphine-3-monosulfonate (TPPMS) from Tokyo Kasei Kogyo Co. Ltd. (Tokyo), and other reagents were of reagent grade and used as received. Succinimidyl 6-hydrazinopyridine-3-carboxylate hydrochloride (SHNH or HYNIC) was synthesized according to the procedure of Abrams et al. (*1*).

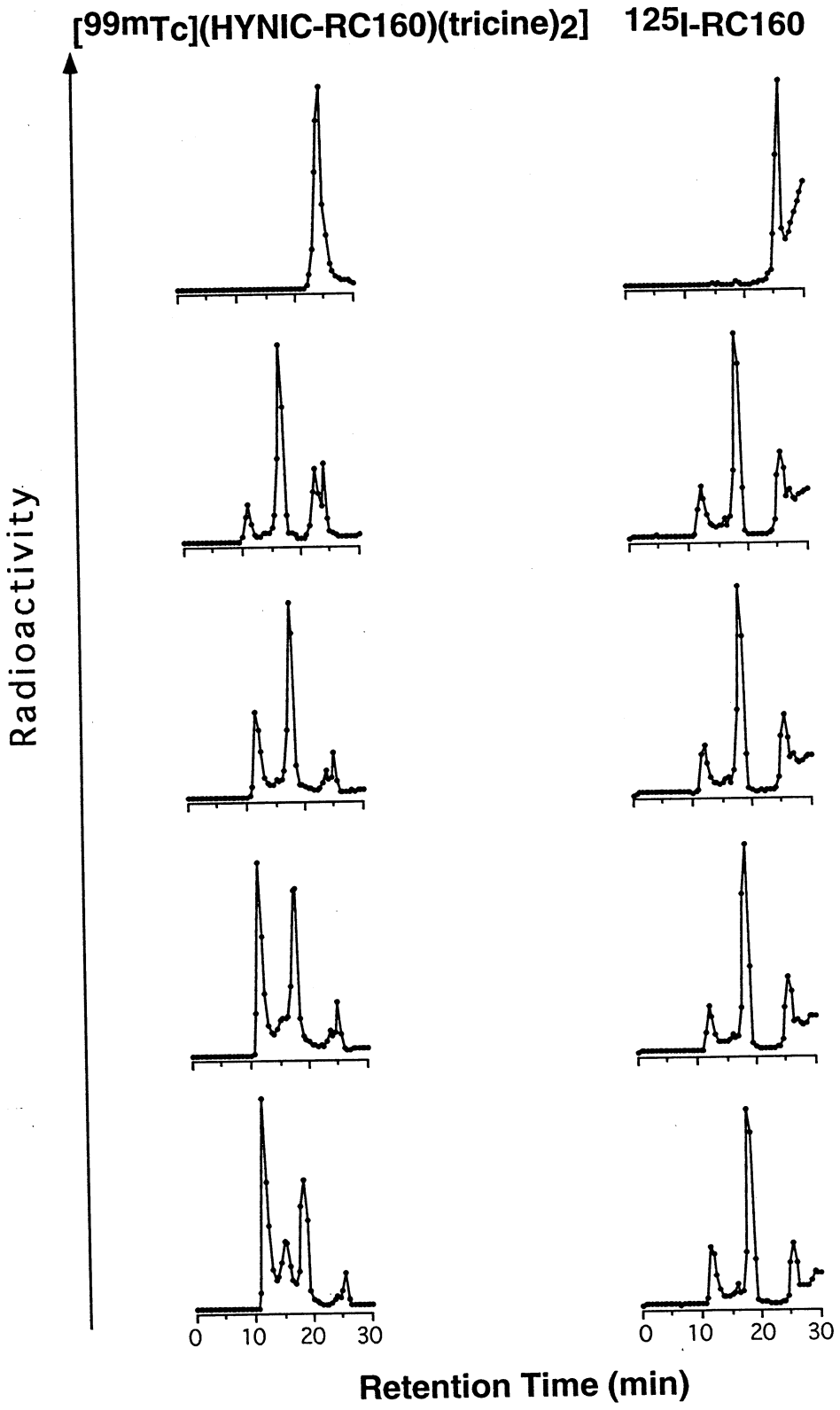

FIG. 2. Stability of ^{125}I-Rc-160 versus ^{99m}Tc-HYNIC-RC-160(tricine)$_2$ in the presence of plasma.

TABLE II. BIODISTRIBUTION OF RADIOACTIVITY AFTER INTRAVENOUS ADMINISTRATION OF [^{99m}TC](HYNIC-rc-160)(TRICINE)$_2$ AND ^{125}I-RC-160 IN MICE [A]

	Time after Administration					
Tissue	10 min	30 min	1 h	3 h	6 h	24 h
	[^{99m}Tc](HYNIC-RC160)(tricine)$_2$					
Liver	36.34[d] (3.43)	31.95[c] (3.39)	31.56[c] (3.42)	30.48[c] (2.69)	17.03[c] (1.93)	13.66[c] (2.31)
Kidney	13.24[c] (1.02)	11.13[c] (1.71)	8.30[c] (0.33)	6.37[c] (0.53)	4.98[c] (0.59)	2.53[c] (0.22)
Intestine	1.52[c] (0.13)	1.75[c] (0.15)	3.12[c] (0.80)	6.60[c] (0.39)	12.04[e] (1.18)	4.41 (3.30)
Spleen	14.02[c] (3.44)	8.93[c] (2.10)	9.66[c] (1.60)	7.60[c] (1.75)	9.54[c] (1.99)	3.65[c] (0.62)
Pancreas	2.23[c] (0.10)	1.64[d] (0.81)	1.87[c] (0.11)	1.32[c] (0.24)	1.31[c] (0.12)	0.53[c] (0.14)
Stomach[b]	0.28[d] (0.07)	0.70 (0.16)	0.96[d] (0.25)	1.18[d] (0.09)	1.78[d] (0.39)	0.63[c] (0.34)
Urine[b]						27.84[d] (0.13)
Faeces[b]						6.86[c] (1.76)
	^{125}I-RC160					
Liver	25.76 (3.63)	8.04 (0.81)	7.88 (0.65)	5.38 (1.05)	1.79 (0.20)	1.15 (0.08)
Kidney	4.63 (0.68)	2.18 (0.36)	2.30 (0.15)	2.45 (0.28)	1.17 (0.17)	0.76 (0.12)
Intestine	9.95 (1.35)	12.24 (1.10)	15.90 (1.60)	21.55 (2.16)	9.36 (1.45)	4.80 (2.13)
Spleen	1.92 (0.15)	0.54 (0.08)	0.69 (0.12)	0.81 (0.13)	0.54 (0.19)	0.38 (0.14)
Pancreas	0.29 (0.07)	0.21 (0.07)	0.25 (0.03)	0.16 (0.04)	0.15 (0.05)	0.07 (0.02)
Stomach[b]	0.48 (0.07)	0.87 (0.64)	2.08 (0.57)	2.60 (0.71)	3.57 (1.52)	4.37 (0.38)
Urine[b]						23.24 (2.20)
Faeces[b]						26.09 (5.57)

[a] Expressed as % injected dose per gram. Each value represents the mean (s.d.) for five animals at each interval.

[b] Expressed as % injected dose.

[c] Significance determined by unpaired *t*-test; $p < 0.001$.

[d] Significance determined by unpaired *t*-test; $p < 0.01$.

[e] Significance determined by unpaired *t*-test; $p < 0.05$.

TABLE III. BIODISTRIBUTION OF RADIOACTIVITY AFTER INTRAVENOUS ADMINISTRATION OF [^{99m}TC](HYNIC-NGA)(TRICINE)$_2$ IN MICE [A]

	Time after Administration					
Tissue	10 min	30 min	1 h	3 h	6 h	24 h
Blood[b]	0.27 (0.02)	0.18 (0.01)	0.09 (0.02)	0.13 (0.02)	0.08 (0.02)	0.01 (0.01)
Liver	92.30 (2.65)	88.71 (1.44)	84.56 (2.48)	75.10 (4.07)	63.29 (5.26)	38.77 (4.10)
Intestine	0.36 (0.09)	4.34 (0.62)	7.61 (0.91)	14.54 (0.97)	20.88 (2.14)	2.23 (0.52)
Kidney	0.30 (0.05)	0.33 (0.03)	0.38 (0.05)	0.45 (0.09)	0.53 (0.11)	0.38 (0.04)
Spleen	0.08 (0.04)	0.08 (0.01)	0.08 (0.01)	0.09 (0.03)	0.06 (0.01)	0.04 (0.03)
Stomach	0.31 (0.16)	0.55 (0.28)	0.58 (0.25)	0.70 (0.15)	0.71 (0.33)	0.42 (0.35)
Faeces						38.27 (3.80)
Urine						13.67 (0.59)

[a] Expressed as % injected dose. Each value represents the mean (s.d.) for five animals at each interval.

[b] Expressed as per cent dose per gram.

2.1. Analytical methods

2.1.1. HPLC

— Reversed phase HPLC (RP-HPLC) was performed with a Cosmosil 5 C_{18}-MS column (4.6 × 150 mm, Nacalai Tesque, Kyoto) at a flow rate of 1 mL/min with:

— Solvent system 1: gradient mobile phase starting from 100% A (0.1% aqueous trifluoroacetic acid) to 100% B (acetonitrile with 0.1% trifluoroacetic acid) in 30 min. Solvent system 2: gradient mobile phase starting from 10% acetonitrile in 0.05 M phosphate buffer (pH7.0) to 90% acetonitrile in the same buffer in 20 min. The final solvent maintained for a further 10 min.

— Size-exclusion HPLC was performed using a 5 Diol-300 column (7.5 × 600 mm) [Guard column, 5 Diol-300 (7.5 × 50 mm)], Nacalai Tesque, eluted with 0.1 M phosphate buffer (pH6.8) at a flow rate of 1 mL/min.

2.1.2. TLC

— TLC analyses were performed with silica plates (Merck Art 5553) in 10% aqueous ammonium chloride-methanol (1:1) or saline.

2.2. Synthesis

2.2.1. Synthesis of HYNIC RC-160

To a solution of Lys_5-Boc-RC-160 (30 mg, 26 mmol) in 1 mL DMF was added a solution of Boc-HYNIC (9.3 mg, 26.4 mmol) [1] in 1 mL of DMF, and the reaction mixture was stirred for 4 h at room temperature. The resulting reaction mixture was purified by a preparative RP-HPLC (50 × 250 mm, $5C_{18}$-AR, Nacalai Tesque) using a mixed solution of acetonitrile and water, and the fractions containing the desired products were collected and lyophilized to afford Boc-HYNIC-Boc-Lys_5-RC-160 in yields of 30.7%. FAB-MS calculated for $C_{73}H_{91}N_{15}O_{14}S_2$, $[MH^+]$, m/z 1466, found 1466. A mixture of 5% anisole and 95% TFA was added to Boc-HYNIC-Boc-Lys_5-RC-160, and the reaction mixture was stirred for 30min at room temperature. After removing TFA in vacuo, ether was added to the residue to precipitate TFA salt of HYNIC-RC-160 as a white solid in yields of over 90%. FAB-MS calculated for $C_{63}H_{75}N_{15}O_{10}S_2$, $[MH^+]$ m/z 1266, found 1266.

2.2.2. Synthesis of HYNIC-lysine

Boc protected HYNIC (500 mg, 1.43 mmol), prepared by the method of Abrams, et al. [1] in 15 mL of dry acetonitrile was added to a solution of Boc-L-lysine (352 mg, 1.43 mmol) in saturated NaHCO3 (5 mL) at 0°5C. The reaction mixture was stirred at room temperature for 6 h. After filtration to remove white precipitates, the filtrate was cooled to 0°C and the solution was acidified to pH2–3 with concentrated H_2SO_4 before extraction with ethyl acetate (20 mL × 4). The organic layers were combined and dried over anhydrous calcium sulfate. After removing the solvent *in vacuo*, the oily residue was chromatographed on silica gel using a mixture of chloroform-methanol-acetic acid (10:1:0.1) as the eluent to produce Boc-HYNIC-a-Boc-L-lysine as white crystals (532 mg, 75.9%). Boc-HYNIC-a-Boc-L-lysine dissolved in 2 mL dry ethyl acetate was added to a solution of 4N HCl prepared in 2 mL dry ethyl acetate. The reaction mixture was stirred at room temperature for 1 h. The HCl salt of HYNIC lysine precipitated as white crystals (160 mg, 91.4%).

2.2.3. Synthesis of galactosyl-neoglycoalbumin (NGA)

Cyanomethyl-2,3,4,6-tetra-*O*-acetyl-1-b-galactopyranoside, synthesized according to the procedure of Lee, et al. [5], was conjugated with human serum albumin (HAS, A-3782; Sigma Co. St. Louis, MO), according to the procedure of Stowell, et al. [6]. The phenol-sulphuric acid reaction indicated that 43 galactose units were attached to each HAS molecule.

2.2.4. Conjugate Preparation: HYNIC-IgG and HYNIC-NGA

The conjugation reaction of HYNIC with IgG or NGA was performed according to the procedure of Abrams, et al. [1] with slight modifications as follows: Briefly, 10 μL of SHNH (HYNIC) (8.3 mg/100 μL) in dimethylsulfoxide (DMSO) was added dropwise to stirred IgG or NGA solution (20 mg/mL) in 0.15 M borate buffer (pH8.5). The solution was stirred gently for 2 h at room temperature protected from the light. The conjugate was purified by Sephadex G-50 (Pharmacia Biotech Co. Ltd., Tokyo) column chromatography (1.8 × 40 cm) equilibrated and eluted with 10 mM citrate buffer (pH5.2). The protein fractions were subsequently concentrated to 5 mg/mL by ultrafiltration (8 MC model, Amicon Grace, Tokyo).

2.2.5. Determination of HYNIC groups attached per molecule of IgG, NGA

Determined by measuring the hydrazino groups with *p*-nitrobenzaldehyde, according to the method of King, et al. [7].

2.3. Radiolabelling methods

2.3.1. ^{99m}Tc Labelling of HYNIC-IgG and HYNIC-RC-160

To a solution of HYNIC-IgG or HYNIC-RC-160 (100 μL; 100 μg/mL) in 10 mM citrate buffer (pH5.2) was added an equal volume of ^{99m}Tc-tricine$_2$, and the mixture was incubated for 1h at room temperature. Radiochemical yields of [^{99m}Tc](HYNICRC-160)(tricine)$_2$ were determined by RP-HPLC and TLC developed with saline.

2.3.2. ^{99m}Tc Labelling of HYNIC-NGA and HYNIC-lysine

To a solution of HYNIC-NGA, HYNIC-lysine (100 μL; HYNIC-NGA = 5.0 mg/mL, HYNIC-lysine = 2.0 mg/mL) in 10 mM citrate buffer (pH5.2) was added an equal volume of ^{99m}Tc-tricine$_2$ prepared by the method of Larsen, et al. [8], and the mixture was incubated for 1 h at room temperature (HYNIC-lysine incubated at 37ºC). [^{99m}Tc-(HYNIC-NGA)(tricine)$_2$] was purified by the centrifuged column procedure using a Sephadex G-50 column equilibrated and eluted with 0.1 M phosphate buffer (pH7.0) [9]. Radiochemical yields of [^{99m}Tc-(HYNIC-NGA)(tricine)$_2$] were determined by size-exclusion HPLC and TLC developed with saline. In a control study, unmodified NGA was labelled with ^{99m}Tc-tricine$_2$, according to the procedures described above. Radiochemical yields of ^{99m}Tc-(HYNIC-Lys)(tricine)$_2$] assessed by size exclusion HPLC, RP-HPLC and TLC developed with a mixture of 10% aqueous ammonium chloride methanol (1:1).

2.3.3. [^{99m}Tc-(HYNIC-lysine)(tricine)(TPPMS)] Ternary Mixed Ligand Complex

A solution of [^{99m}Tc-(HYNIC-lysine)(tricine)$_2$] (100 μL) prepared as described above was mixed with 100 μL of TPPMS solution (5 mg/mL) in 0.1 M acetate buffer (pH3.0). The solution was acidified to pH3 with 0.1 N HCl and incubated at 37°C for 1 h. The reaction mixture was analysed by RP-HPLC, size exclusion HPLC and TLC developed with a mixture of 10% aqueous ammonium chloride methanol (1:1).

2.3.4. $^{131/125}$I-RC-160

Radioiodination of RC-160 was performed according to the procedure of Bakker, et al. [10] with slight modifications. To a solution of Na(^{125}I)I (20 μL in 0.01 M NaOH) was added successively: 20 μL of 0.05 M phosphate (pH7.5) and 1.5 μg of RC-160 in 20 μL of 0.05 M acetic acid. Radioiodination was initiated by adding 1.6 μg of freshly prepared chloramine-T in 20 μL of 0.05 M phosphate buffer (pH7.5), and the reaction mixture was vortexed for 1 min. Since degradation of (^{125}I)I-RC-160 was observed after purification of the reaction mixture by RP-HPLC, 20 μL of non-radioactive I-RC-160 (8.8×10^{-5} M) in 0.01 M acetate buffer (pH4.2) was added to the reaction mixture prior to RP-HPLC purification. Fractions containing (^{125}I)I-RC-160 were evaporated to dryness, and the residue was redissolved in 20 μl of 0.01 M acetate buffer (pH4.2). Aliquots of [^{125}I]I-RC-160 were diluted with 8.8×10^{-5} M

of nonradioactive I-RC-160 in 0.01 M acetate buffer (pH4.2 or 6.0) to adjust the radioactivity to 0.3 μCi/50 μL for subsequent studies.

2.4. *In vitro* studies

To evaluate the stability of Tc complex in plasma, [^{99m}Tc](HYNIC-IgG)(tricine)$_2$, and [^{99m}Tc](HYNIC-RC-160)(tricine)$_2$ were diluted 20-fold with 20 mM phosphate buffered saline (PBS) (20 mM PBS, pH7.4) containing 1000-fold excess cysteine of IgG, and RC-160, or in freshly prepared murine plasma for [^{99m}Tc-(HYNIC-NGA)(tricine)$_2$]. Solutions were incubated at 37°fC for 24 h. After 1, 3, 6, and 24h of incubation, 50 μL aliquots of the samples were drawn, and the radioactivity was analysed by size-exclusion HPLC or RP-HPLC, and TLC developed with saline. Some experiments were performed by double tracer method using each ^{125}I labels.

2.5. *In vivo* mice studies

Biodistribution studies were performed by the intravenous administration of mixed solution of ^{99m}Tc-HYNIC labels and ^{125}I labels of IgG or RC-160 to a 6 week old male ddY mice (27–30 g). The polypeptide concentration of IgG was adjusted to 200 μg/mL of RC-160 to 1 μg/mL with saline. Groups of five mice each were administered with 20 μg of radiolabelled IgG (^{99m}Tc: 1–2 μCi, ^{125}I: 0.2–0.3 μCi) or 0.1 μg of radiolabelled RC-160 (^{99m}Tc: 1–2 μCi, ^{125}I: 0.2–0.3 μCi). The protein concentration of [^{99m}Tc-(HYNIC-NGA)(tricine)$_2$] was adjusted to 90 μg/mL with saline and 0.1 mL per mice 9 μg (1–1.5 μCi) was injected. Tissues of interest were removed, weighed and radioactivity counts were determined with an auto well gamma counter (ARC 2000). To determine the amounts and routes of excretion of radioactivity from the body, mice were housed in metabolic cages for 24 h after administration of [^{99m}Tc-(HYNIC-NGA)(tricine)$_2$], and urine and faeces were collected, and radioactivity determined.

2.6. Subcellular radioactivity distribution

The subcellular distribution of radioactivity in the murine liver was investigated using a pre-*in situ* perfused organ at 1 and 24 h post-injection of [^{99m}Tc-(HYNIC-NGA)(tricine)$_2$]. Perfused medium was a cold 0.25 M sucrose buffered with 10 mM phosphate buffer (pH7.4). Then the isolated organ was minced with scissors, suspended in 4 volumes of the same buffer prior to homogenization by hand with a Dounce homogenizer (20 strokes) and proceeded as previously described [12, 13]. The isolated supernatant was then layered on top of iso-osmic (0.25 M sucrose) 37.5% Percoll (9 mL: Pharmacia Biotech Co. Ltd.) at a density of 1.08 g/mL. After centrifugation at 20 000 *g* (RP 30 rotor; Hitachi Co. Ltd., Tokyo) for 90 min at 4°C, the gradients were collected in 14 fractions; b-galactosidase was used as a marker enzyme for lysosomes and its activity in each fraction was determined using *p*-nitrophenyl b-galactopyranoside as the substrate. Density and radioactivity counts of the respective fraction were also determined.

2.7. Identification of radiolabelled metabolites

(a) *Liver tissue:* The radiolabelled species remaining in the liver at 1 and 24 h post-injection and excreted in the urine and faces, 24 h post injection of [^{99m}Tc-(HYNIC-NGA)(tricine)$_2$] (133–266 kBq) were analysed. The murine liver was perfused *in situ* and then homogenized as previously reported [12, 13]. The supernatant was separated from the pellet, and the radioactivity counted.

(b) *Faecal sample:* In a procedure similar to that used for liver tissue, faecal samples were homogenized in the presence of 0.1 M phosphate buffer (pH6.0) before centrifugation at 10 000 *g* for 20 min at 4ºC. The liver, faeces and urine samples were analysed immediately by size-exclusion HPLC and TLC after filtration through a polycarbonate membrane with a pore diameter of 0.45 μm (Nacalai Tesque). Each sample was also analysed immediately by RP-HPLC after ultrafiltration with a 10 kDa cut-off membrane (Microcon-10, Amicon).

(c) ©*Radio-metabolites analysis using TPPMS:* Since TPPMS forms a ternary mixed ligand complex with [^{99m}Tc-(HYNIC-peptide)(tricine)$_2$] [14], the liver homogenates were reacted with TPPMS and the reaction products were analysed. Homogenates, faecal and urine samples (100 μL each) were mixed with 100 μL solution of TPPMS (5 mg/mL) in 0.1 M acetate buffer (pH3.0) after filtration through a polycarbonate membrane with a pore diameter of 0.45 μm. After acidification to pH3 with 0.1 N HCl, the reaction mixture was incubated at 37ºC for 1 h. The reaction mixture (pre-filtrated, as above) was analysed by size-exclusion HPLC, RP-HPLC and TLC.

3. RESULTS

3.1. *In vitro* studies

HYNIC-NGA conjugate was prepared by reaction of the active esters of SHNH with e-amine residues of the NGA. Three HYNIC groups were attached per molecule of NGA as determined by measuring the hydrazine groups. After purification by the centrifuged column procedure, [^{99m}Tc-(HYNIC-NGA)(tricine)$_2$] was obtained with radiochemical yields over 95% as determined by size-exclusion HPLC and TLC. The reaction of ^{99m}Tc-tricine$_2$ with unmodified NGA resulted in only 5.4% of the radioactivity associated with protein. Fig. 3 shows size-exclusion radiochromatograms of [^{99m}Tc-(HYNIC-NGA)(tricine)$_2$] before and after incubation in freshly prepared murine plasma for 24 h. The radioactivity associated with NGA fractions was unchanged before (98.7%) and after 24 h incubation (97.6%). Similar results were obtained by TLC analyses.

3.2. *In vivo* studies

The biodistribution of radioactivity after intravenous administration of [^{99m}Tc-(HYNIC-NGA)(tricine)$_2$] is summarized in Table II. At 10 min post-injection, more than 92% of the injected radioactivity was accumulated in the liver. The radioactivity was gradually eliminated from the liver by hepatobiliary excretion as the major excretion route. At 24 h post-injection, over 38% of the injected radioactivity was still retained in the liver. During the same post-injection period, 38% and 14% of the injected radioactivity was excreted in the faeces and urine, respectively.

3.3. Subcellular radioactivity distribution studies

The Percoll density gradient centrifugation profiles of radioactivity in the liver at 1 and 24 h post-injection of [^{99m}Tc-(HYNIC-NGA)(tricine)$_2$] are illustrated in Fig. 4. Each liver homogenate showed a major radioactivity peak at a density of ca. 1.10 g/mL, which correlated well with the b-galactosidase activity profiles.

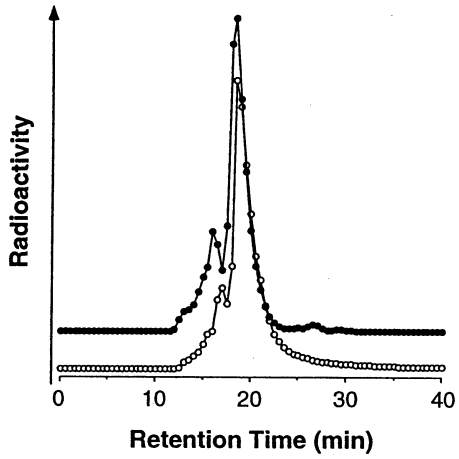

FIG. 3. Effect of plasma incubation. Size-exclusion radiochromatogram of ^{99m}Tc-HYNIC-NGA)tricine$_2$.

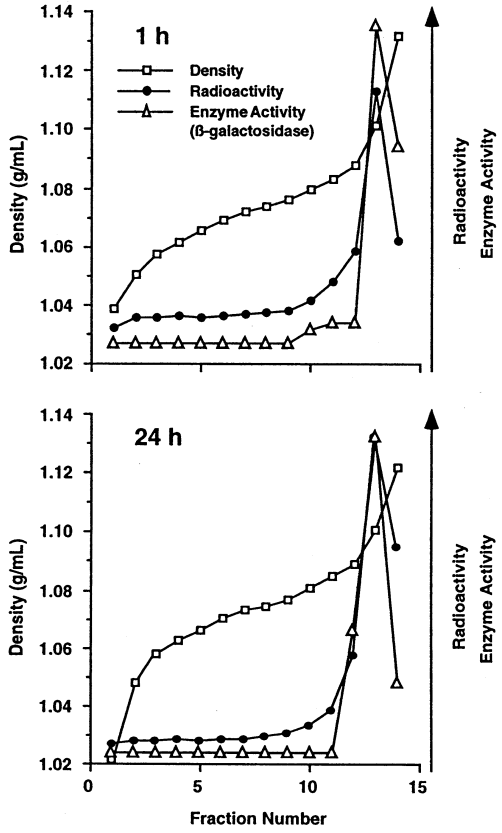

FIG. 4. Subcellular distribution using Percoll gradient density centrifugation.

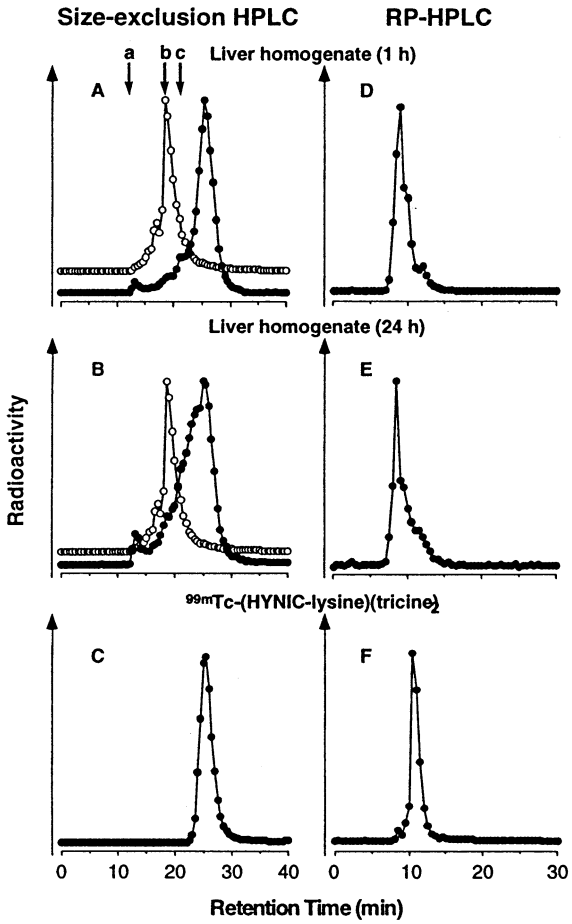

FIG. 5. Analysis of liver homogenates injected with ^{99m}Tc-(HYNIC-NGA)(tricine)$_2$. Comparative studies using size exclusion HPLC (left) and RP-HPLC (right).

3.4. Identification of radiolabelled metabolites

The supernatants of the liver homogenates were obtained with radiochemical efficiencies of over 90%. After the filtration through polycarbonate membrane, the recovered radioactivity of the filtrate was reduced to 86.1 and 92.3%, in liver supernant at 1 and 24 h p.i. injection, respectively. When analysed by size-exclusion HPLC, while the liver supernatant at 1 h depicted a major radioactivity peak at a retention time close to that of [^{99m}Tc-(HYNIC-lysine)(tricine)$_2$], [Fig. 5C] some radioactivity could also be detected in fractions over 10 kDa [Fig. 5A] and this fraction increased in sample taken at 24 h post-injection. The parental [^{99m}Tc-(HYNIC-NGA)(tricine)$_2$] had a retention time of 18 min [Fig. 5B]. On TLC analyses, the liver homogenates had radioactivity at the origin with a broad peak at Rf values of 0.5–0.65 [Figs 5A and 5B]. On the other hand, the liver supernatant filtrated through a 10 kDa cut-off ultrafiltration membrane was recovered in the filtrate with 76.3% and 54.3% efficiency from 1 h and at 24 h post-injection samples, respectively. Their RP-HPLC radiochromatograms using the solvent system 1 are shown [Figs 5D and 5E]; both supernatants showed similar radiochromatograms with a major radioactivity peak at a retention time of 8–9 min and a shoulder at a retention time of 11 min. The peak of a reference ^{99m}Tc-(HYNIC-lysine)(tricine)$_2$ compound registered at 11 min [Fig. 5F]. On the other hand, the RP-HPLC analyses of those liver homogenates using instead the solvent system 2 showed a radioactivity peak at a retention time of 4 min [Fig. 7A and 7B].

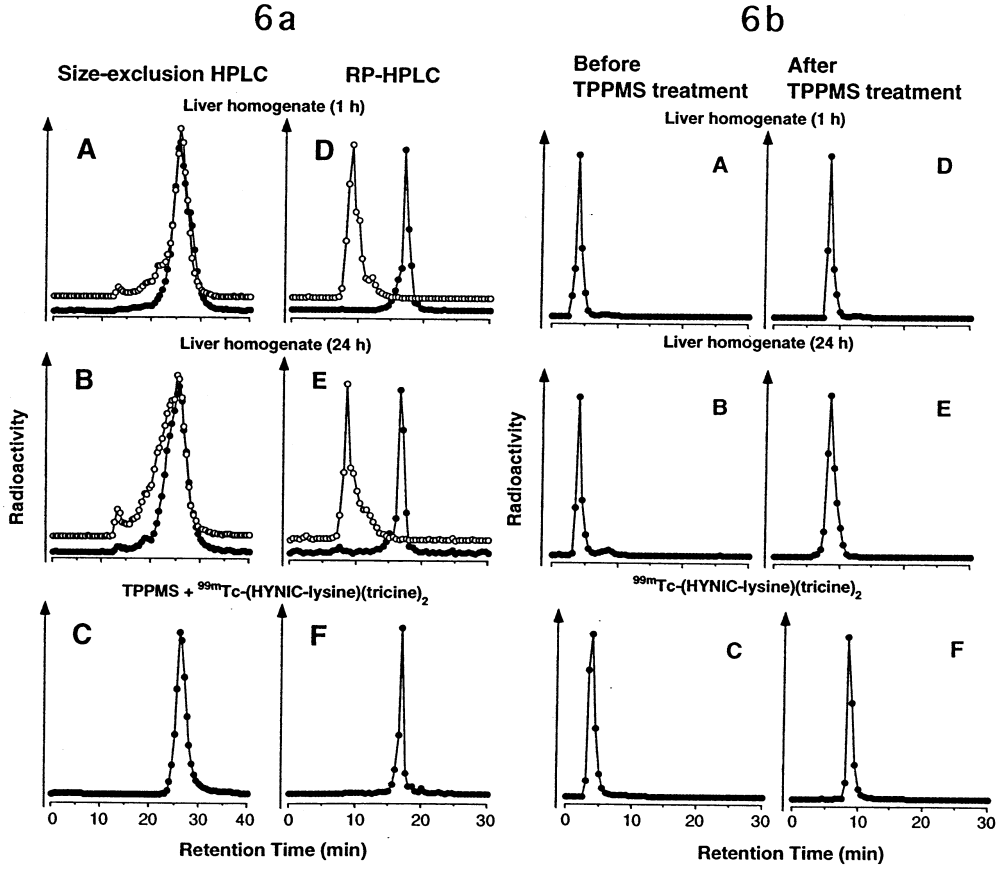

FIG. 6. Effect of TPPMS treatment on liver homogenates using RP-HPLC
6a) Analysis using solvent system 1
6b) Analysis using solvent system 2.

3.5. Radio-metabolites analysis using TPPMS

Filtration of those TPPMS treated homogenates through polycarbonate membranes yield samples with 91.1 and 88.6% recovery at 1 and 24 h post-injection. On size exclusion HPLC, a significant decrease in the radioactive fractions over 10 kDa was observed with TPPMS-treated liver homogenate at 24 h post-injection compared with the earlier 1 h sample [Figs 6 and 7]. The reaction product of [^{99m}Tc-(HYNIC-lysine)(tricine)$_2$] with TPPMS showed a single radioactivity peak at a retention time of 26 min on size exclusion HPLC [Fig. 6aC]. Filtration through a 10 kDa cut-off membrane yield higher recovery than those previous non-TPPMS treated homogenates, ranging from 89.2% to 91.1% at 1 and 24 h post-injection samples, respectively. Then, the RP-HPLC using solvent system 1 showed a sharp radioactivity peak at a retention time of 17 min for both TPPMS treated liver homogenates filtrates. This peak at 17 min was identical to that of the reaction product of [^{99m}Tc-(HYNIC-lysine)(tricine)$_2$] and TPPMS [Fig. 6aF]. This was confirmed by co-chromatographic analyses (data not shown). Under these conditions, the reaction products of ^{99m}Tc-tricine$_2$ and TPPMS showed a radioactivity peak at a retention time of 13 min on RP-HPLC. Similar results were obtained on RP-HPLC analyses of the liver homogenates using solvent system 2 where both TPPMS treated liver homogenates and the [^{99m}Tc-(HYNIC-lysine)(tricine)$_2$] showed single radioactivity peak at a retention time of 8.5 min [Fig. 6b]. Those two samples showed also similar radioactivity peaks on TLC analyses at Rf value of 0.7.

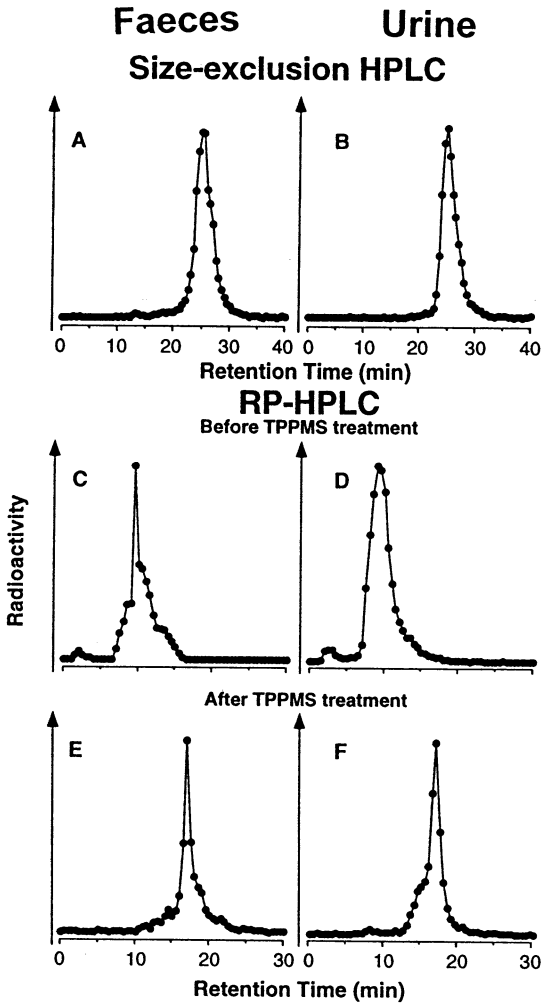

FIG. 7. Analysis of faeces and urine sample (size exclusion HPLC and RP-HPLC, solvent system 1).

Supernatants of faeces samples were obtained with an efficiency of 54.6%. The recovered radioactivities after filtration through the polycarbonate and the ultrafiltration membrane were 66.7% and 82.5% for faeces, and 96.9% and 98.3% for urine samples, respectively. Both faecal and urine samples showed a single radioactivity peak on size-exclusion HPLC at a retention time of 26 min [Figs 7A and 7B]. On RP-HPLC, a broad radioactivity profile at a retention time of around 9 min was observed with both preparations using solvent system 1 [Figs 7C and 7D]. After TPPMS treatment, both faeces and urine preparations showed migration of radioactivity peaks to a retention time of 17 min, which was identical to that of the reaction product between [^{99m}Tc-(HYNIC-lysine)(tricine)$_2$] and TPPMS [Figs 7E and 7F]. Similar results were obtained by TLC analyses, where both preparations showed a radioactivity peak at an Rf value of 0.7.

4. CONCLUSION

^{99m}Tc-HYNIC labelled galactosyl-NGA was prepared using tricine as a co-ligand to investigate the fate of the radiolabel after lysosomal proteolysis in hepatocytes. When injected into mice, over 90% of the injected radioactivity was accumulated in the liver after 10 min

injection. At 24 h post-injection, ca. 40% of the injected radioactivity still remained in liver lysosomes. Size exclusion HPLC analyses of liver homogenates at 24 h post-injection showed a broad radioactivity peak ranging from molecular weights of 50 kDa to 500 Da. RP-HPLC analyses of liver homogenates suggested the presence of multiple radiolabelled species. However, most of the radioactivity migrated to lower molecular weight fractions on size exclusion HPLC after treatment of the liver homogenates with sodium TPPMS. The TPPMS treated liver homogenates showed a major peak at a retention time similar to that of [^{99m}Tc-(HYNIC-lysine)(tricine)(TPPMS)] on RP-HPLC. Similar results were obtained with urine and faecal samples. These findings suggest that the chemical bonding between ^{99m}Tc and HYNIC remains stable in the lysosomes and following excretion from the body. The persistent localization of radioactivity in the liver could be attributed to the slow elimination rate of the final radiometabolite, [^{99m}Tc-(HYNIC-lysine)(tricine)$_2$], from lysosomes, and subsequent dissociation of one of the tricine co-ligands in the low pHenvironment of the lysosomes in the absence of excess co-ligands, followed by binding proteins present in the organelles. Further studies, with the ^{99m}Tc-(HYNIC-RC-160)(tricine)$_2$ and ^{125}I-RC-160 are required. However, the findings in this study also suggest that the development of appropriate co-ligands capable of preserving stable bonding with the Tc centre might be essential to reduce the residence time of radioactivity in non-target tissues after administration of ^{99m}Tc-HYNIC labelled peptides and also proteins.

From the gathered data using NGA as the model protein, it was learnt, that the chemical bonding between ^{99m}Tc and HYNIC remain stable in the lysosomes but the persistent liver localization was probably due to a radiometabolite, the ^{99m}Tc-HYNIC-lysine (tricine)$_2$. However, there is still a great need, in the following stage, for research to analyse the metabolic fate of the peptide RC-160 conjugated to HYNIC [^{99m}Tc-HYNIC-RC-160-(tricine)$_{2]}$ in order to learn about the basis for future of BCA design.

The CRP has provided great incentive for learning about the differential metabolic fates of proteins and peptides, and the need to find still better co-ligands or new ligands to reduce the residence time of radioactivity in non-target tissue for future peptide use in radiodiagnosis or radiotherapy.

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^{99M}Tc LABELLED PEPTIDES FOR IMAGING OF PERIPHERAL RECEPTORS

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Abstract

Several peptides are being used as radiopharmaceuticals for receptor imaging scintigraphy. The peptide receptors are found in the tumours of various sites [1] in the human body. Somatostatin is one of those, which is expressed by a variety of tumours say in brain cortex, medullary carcinoma of thyroid, adrenal glands, pancreas and gut [2]. Therefore neuropeptides based on somatostatin analogues are labelled with different radionuclide, ^{123}I and ^{111}In [3]. Efforts are underway to label RC-160 (an analogue of somatostatin) with ^{99m}Tc because of its favourable radiation dosimetry, short half-life, low price, high count rate and better diagnostic efficacy. In this project various methods of labelling RC-160 with different radionuclides ^{125}I and ^{99m}Tc have been studied in detail. Radioiodination of Rc-160 was tried with ^{125}I using the iodogen method [4] as directed and then with Chloramine T method [5]. Labelling of RC-160 peptide with ^{99m}Tc was done using two different aspects [7]. Direct labelling with ^{99m}Tc and indirect labelling with ^{99m}Tc using double chelating agents. Radiochemical quality control was carried out applying instant thin layer chromatography using ITLC-SG strips in 85% of methanol[5]. Later the HPLC analysis was used for its evaluation. To label RC-160 with ^{99m}Tc the approach of direct labelling was attempted first. 46% labelling could be achieved with 95% of radiochemical purity. The biodistribution of ^{99m}Tc-RC-160 complex in rats has also been studied to determine uptake in various sites of somatostatin receptors [6]. Eventually, attempt was made to synthesize biomolecule by conjugating Boc protected RC-160 with benzoyl MAG-3. As a result 80% of Boc-RC-160 went under conjugation with benzoyl MAG-3.

1. MATERIALS

RC-160 peptide (3.5 mg) and Boc-RC-160 (72.5 mg) were obtained from IAEA. HATU, DIPEA, iodogen and chloramine-T were obtained from Prof. Dr. Helmut Mäcke, Institute of Nuclear Medicine, Kantonspital Basel, Switzerland. Benzoyl MAG-3 was synthesized locally at PINSTECH. ^{125}I was obtained from Amersham, (90–100 mCi/mL). Ascorbic acid (Aldrich), Na dithionite (Aldrich) and HAS from Sigma were used, while the ^{99m}Tc generator used was from Amersham and Mallinckrodt.

2. METHODS

Radioiodination was done according to the procedures recommended by Mather and Mäcke [4,5]. Direct labelling of RC-160 with ^{99m}Tc was carried out as prescribed by Thakur [7].

2.1. Radioiodination of RC-160 using iodogen method

2.1.1. Preparation of Reagents

— Iodogen (1,3,5,6-tetrachloro-3a, 6a-diphenylglycouril) (1 mg) was dissolved in 25 mL of dichloromethane. 20 μg/500 μL of the prepared solution was pipetted out into polypropylene tubes. The tubes were left in dark fume hood for 2 h and were then stored in a freezer at –8°C.

— RC-160 (100 μg) was dissolved in 0.1 M acetate buffer pH4.2 (1 mL). Each 100 μL aliquot was dispensed in 1 mL siliconized glass vials and stored in a freezer at –8°C.
— Preparation of normal saline solution acidified with 0.1M acetic acid to pH4.5.
— Preparation of phosphate buffer 0.5 M pH7.5.

2.1.2. Protocol of ^{125}I labelling

Radioiodine ^{125}I (200 μCi), 100 μL of RC-160 (10 μg) and phosphate buffer 0.5M pH7.5(100 μL), were pipetted into 20 μg of iodogen tube in rapid succession. The reaction mixture was shaken well in a vortex mixer for 30 s and incubated for 30 min at room temperature. Normal saline acidified with acetic acid to pH4.5 (300 μL) was added to the reaction mixture and was again shaken in a vortex mixer for another 30 s.

2.1.3. Preparation of SEPPAK C-18 column

The C-18 cartridge was washed five times with 70% ethanol (5 mL) and then with 2-propanol (5 mL).

2.1.4. Purification of the iodine labelled peptide

The reaction mixture was loaded on the washed column and slowly eluted through the cartridge by pushing the syringe. The filtrate was collected in a vial No.1. The column was washed successively with a) 5 mL deionized water, b) 5 mL 0.5 M acetic acid, and then washed with three fractions of 9 mL 95% ethanol with the eluants collected into separate labelled tubes. At the end of each elution the air was pushed through the cartridge for complete extraction.

2.1.5. Radiochemical quality control

The radiochemical purity of the product was checked with ITLC-SG strips 5 × 0.7 cm developed in 85% methanol, once before purification and then after passing through SEPPAK and Dowex IX ion exchange resin. After SEPPAK purification the HPLC analysis was carried out using C-18 reverse phase column, 5 μm 4.5 × 250 mm, at a flow rate of 1 mL/min using solvent A: AcCN and solvent B:0.1% TFA solution. A gradient used was from 0–3min 0% AcCN, 3–13 min 50% AcCN, 13–23 min 50% AcCN, 23–30 min 50% AcCN and 30 to 35 min was again 0% AcCN.

2.2. Radioiodination of RC-160 using chloramine-T method

2.2.1. Preparation of reagents

(1) *284 μM chloramine-T solution:* 6.5 mg of chloramine-T was dissolved in 20 mL of phosphate buffer pH7.5, 0.5 M. The pHof chloramine-T solution was then lowered to 5.8 with 0.5 M acetic acid solution and the total volume was adjusted to 100 mL.
(2) *Solution of RC160:* RC-160 (100 μg) was dissolved in 0.5M acetate buffer pH4.2 (1 mL). Each 50 μL aliquot was dispensed in 1 mL siliconized glass vials and was stored in a freezer at–8°C.
(3) *7.5% HAS solution:* 7.5 mg of HAS was dissolved in 10 mL of phosphate buffer 0.5 M, pH7.2.

2.2.2. Protocol of labelling

To 50 μL of RC-160 (5 μg, 50 μM) radioiodine ^{125}I (200 μCi/100 μL) was added, followed by freshly prepared chloramine-T solution (20 μL = 284 μM, pH5.8). The reaction mixture was shaken well in a vortex mixer for 30 s. Reaction was stopped two minutes later by adding 7.5% HAS solution (100 μL). Then SEPPAK filtration was carried out as described above in the iodogen method.

2.2.3. Radiochemical quality control

The radiochemical purity was checked with ITLC-SG strips 5 × 0.7 cm developed in 85%, after the SEPPAK purification.

For the HPLC analysis a bit higher activity was required to label RC-160. Radioiodine ^{125}I (8 m Ci200 μL) and freshly prepared chloramine-T solution (800 μL = 284 μM, pH5.8) were to 2 mL of RC-160 (200 μg, 2 mM). The reaction mixture was shaken well in the vortex mixer for 30 s. The reaction was stopped 2 min later with the addition of 7.5% HAS solution (4 mL). Then SEPPAK filtration was carried out as described above in the iodogen method.

2.3. ^{99m}Tc labelling of RC-160

2.3.1. Preparation of Reagents

The formulation was carried out under nitrogen. Deoxygenated distilled water was used for the preparation of all reagents:

— 0.1 M Sodium Acetate buffer pH4.2
— 0.1 M Sodium Acetate buffer pH7.2
— Sodium ascorbate solution pH6.2 (ascorbic acid 10 mg/mL in 1 N, NaOH solution)
— Sodium dithionite solution in Acetate buffer pH7.2 (6 mg/1 mL, i.e. 600 μg/100 μL).
— 100 μg of RC-160 was dissolved in 1 mL of 0.1 M acetate buffer, pH4.2.

2.3.2. Labelling protocol

100 μL aliquot of RC-160 (10 μg) was pipetted out into a siliconized glass vial. Sodium ascorbate solution (1 mg/100 μL) was added to it. The vial was shaken in a vortex mixer. Na pertechnetate solution was pipetted into a reaction vial (50 mCi/600 μL) followed by an addition of sodium dithionite (2400 μg/400 μL). The vial was heated in a boiling water bath for 15 min. Then the vial was cooled to room temperature and SEPPAK purification was carried out.

2.3.3. Radiochemical analysis

The radiochemical purity of the product was checked with HPLC after SEPPAK purification. The ethanol fractions eluted were injected on HPLC to find the radiochemical purity of the complex. Bondapak, C-18 ODS 5 12 μm 4.5 × 250mm reversed phase column was used at a flow rate of 1 mL/min, Solvent A was 99.9% acetonitrile and solvent B was 0.1% TFA. A gradient used was from 0–3 min 0% AcCN, 3–13 min 50% AcCN, 13–23 min 50% AcCN, 23–30 min 50% AcCN and 30 to 35 min was again 0% AcCN.

2.4. Biodistribution studies of ^{99m}Tc RC-160 complex

RC-160 was labelled with a specific activity of 500 ?Ci per ?g of RC-160. and was diluted to a concentration of 2 5 ?L. The selected group of Sprague Dawly rats was not injected with a cold peptide to block the somatostatin receptors the tumour was not induced with AR-4-2J cell tumours. A group of 3 × 3 non-fasting male rats with an average body weight of 100 g were injected with 20 ?Ci.200 ?Lof the complex. One group was sacrificed at 1 h p.i. and the second group was sacrificed at 2.5 h p.i. The percentage of activity was found high in liver spleen, kidney and gut, while low in blood, pancreas, heart and adrenal gland. The activity in the organs rich in the somatostatin receptors, i.e. brain, adrenals and pancreas was also determined.

2.5. Conjugation reaction between Boc-RC-160 and MAG-3

2.5.1. Preparation of Boc-benzoyl MAG-3 RC-160

Following solutions were prepared in dry glass vials under nitrogen. Solvents used in the conjugation reaction were free of oxygen and water contents.

A: 55 mg of benzoyl MAG-3 in dry DMF (2.5 mL).

B: 12.5 mg of HATU[8] in dry DMF (2.5 mL).

C: 20 ?L Diisopropylethylamine in dry DMF 600 ?L.

Solution I: To a solution A (2.5 mL) in a dry glass vial were added solution B 2.5 mL and C (500 ?L), and these reactants were allowed to react for 15 min.

Solution II: 10 mg of Boc-RC-160 was dissolved in (1.13 mL) of DMF in a siliconized glass vial. To this solution was added Solution I (1 mL) after the 15 min reaction time. The combined solution was allowed to react for 18 h.

An aliquot from the reaction mixture was injected to the HPLC system using Solvent system B at UV-220 nm to find the occurrence of the reaction. 80% of the Boc-RC-160 got conjugated with benzoyl MAG-3. Then the peak for Boc-RC160-MAG-3 complex was isolated through HPLC with the same reverse phase column mentioned above (C-18, 5 ?m 4.5 × 250 mm). The elution collected at 25? 5 min was concentrated on rotary evaporator and was again injected on HPLC. The reaction peak at 25 min appeared in addition to the other peaks, which were not collected.

The SEPPAK mini cartridge was activated by washing with 5 mL of ethanol followed by 5 mL of water. 5 mL of air was pushed through the cartridge to dry it off. The conjugation reaction was repeated followed by HPLC analysis. The isolation of the reacted Boc-RC-160-MAG-3 complex was then undertaken. 1 mL water was added to the reaction vial and then transferred the solution on the SEPPAK cartridge, The reaction vial and the cartridge was washed with 3–4 mL of water and 5 mL air was pushed through the cartridge. Then the cartridge was eluted with two fractions of acetonitrile (1 mL). The acetonitrile was then reduced on rotary evaporator to 500 μL. To it 450 ? L of TFA and 50 μL of water was added and allowed to react for 5 min. Then TFA was dried off on rotary evaporator by adding three fractions 500? L of acetonitrile in every 10 min.

3. RESULTS

Iodine labelling of RC-160 was done using iodogen method (Mather). The radiochemical purity of the complex was determined before purification of the reaction mixture and was found to be 20? 5%.

To get rid of unreacted ^{125}I various methods of purification were tried. It was found that purification done by using Dowex IX ion exchange resin did not give good results as no activity was recorded in the filtrate and the maximum activity was retained on the column. However when SEPPAK C-18 cartridge was used, the results were found satisfactory because very good purification was observed and 100% yield of ^{125}I-RC-160 could be obtained (Table I.). The radioiodination of RC-160 was tried with chloramine-T method to improve the labelling yield. A comparison of the iodination yield by the two methods is given in Table II.

TABLE I. ACTIVITY REGISTERED AFTER SEPPAK PURIFICATION AT VARIOUS STEPS

	Iodogen method	Chloramine-T method
Activity in vial	210 μCi	198 μCi
Activity left in empty vial and syringe.	17 μCi	14 μCi
"Total activity" filtered in C_2H_5OH fraction C	185 μCi	174 μCi

TABLE II. RADIOCHEMICAL ANALYSIS OF ^{125}I RC-160 WITH ITLC IN 85% METHANOL

Sep Pak C18 cartridge	Activity	% of free ^{125}I & its R_f	% of ^{125}I RC-160 & its R_f
Iodogen method Filtrate (t.1) (8% of the total activity)	14.5μCi	100/1	0/0
chloramine-T method No activity (t.1)	-	-	-
Iodogen method Deionized H_2O (t. 2) (12% of total activity)	22 μCi	100/1	0/0
chloramine-T method 6% of total activity (t.2)	10 μCi	100/1	0/0
Iodogen method 0.5M acetic acid (t.3)	-	0 cpm	0 cpm
chloramine-T method 7% of total activity (t.3)	12 μCi		
Iodogen/chloramine-T method 95% ethanol A, B fr. (t.4,5)	0cpm	0 cpm	0 cpm
Iodogen method 95% ethanol C (t.6) (75% of the total activity)	135 μCi	0/1	80/0
chloramine-T/86% of total activity (t.6)	150 μCi	0/1	90/0

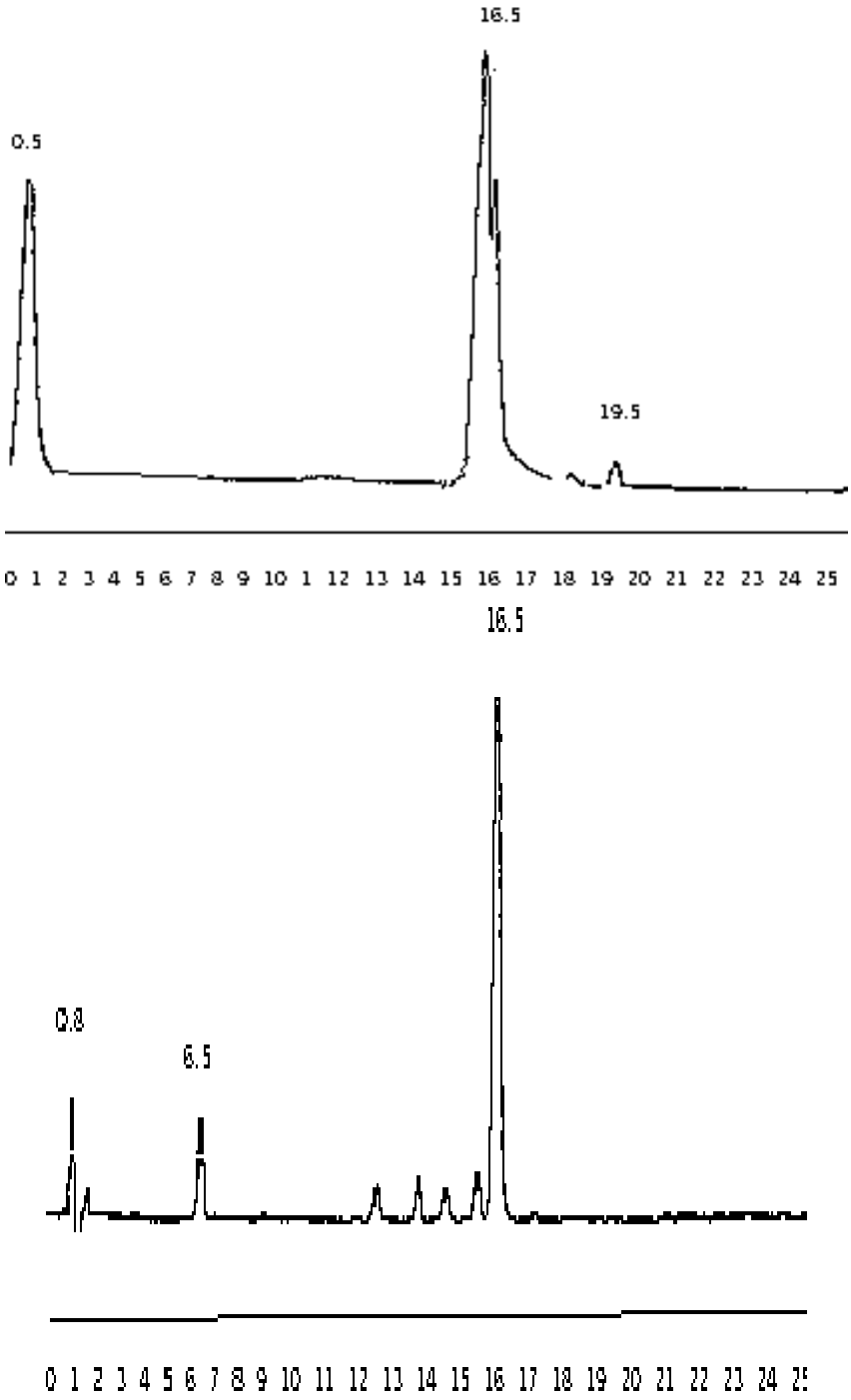

FIG. 1. Comparison of the HPLC Chromatogram of ^{125}I RC-160 labelled with iodogen/chloramine-T methods.

A direct labelling method was attempted to label RC-160 with ^{99m}Tc. The results of instant thin layer chromatography in Table III are found to be good. But this method did not differentiate between hydrolyzed TcO_2, colloid complex and ^{99m}Tc RC-160 complex. Therefore, HPLC was finally used to evaluate the radiochemical purity of the complex. Before HPLC analysis, SEPPAK purification of the labelled complex was done. Direct ^{99m}Tc labelling was carried out using high specific activity followed by the SEPPAK purification. The results in Table IV show 46% labelling yield. However, HPLC analysis gave 95% radiochemical purity (ethanol fractions).

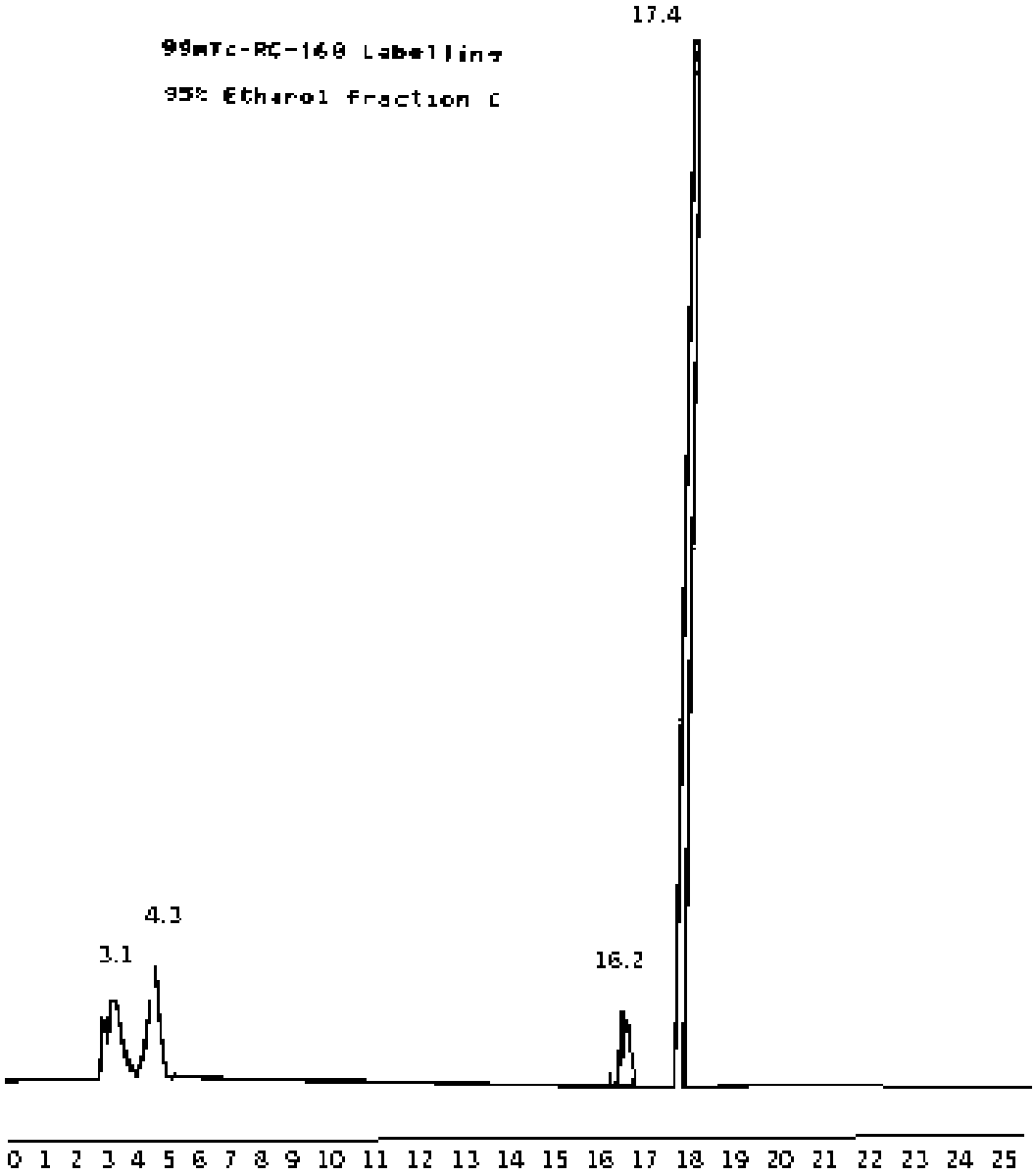

FIG. 2. HPLC Chromatogram of ^{99m}Tc-RC160 in ethanol fraction C.

In order to increase the labelling yield, indirect labelling through bifunctional chelates was attempted. The first step was the conjugation of Boc RC-160 with benzoyl-MAG-3. Several experiments were carried out to achieve conjugation of benzoyl-MAG-3 with Boc-RC-160 and then deblocking of conjugate benzoyl-MAG-3-Boc-RC160. HPLC analysis of the reaction mixture showed that the conjugate benzoyl MAG-3-Boc-RC160 was formed (Fig. 3). HPLC purification of the benzoyl-MAG-3-Boc-RC160 was done. However, conjugation could not be achieved in total and some starting material left unreacted as scan in chromatogram. Hence efforts are underway to achieve better conjugation.

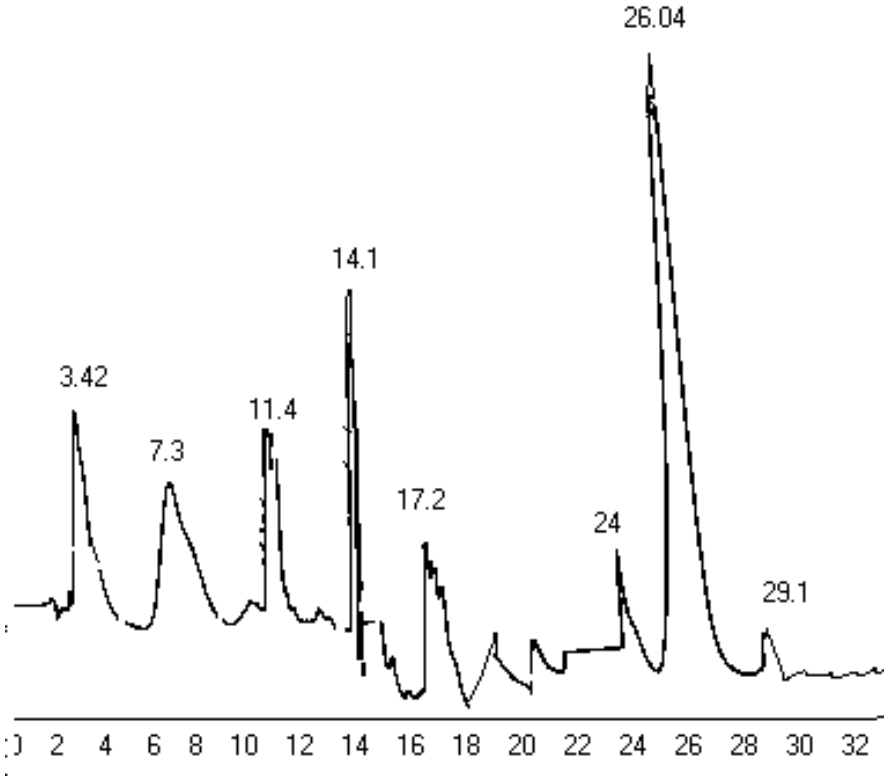

FIG. 3. HPLC chromatogram after conjugation reaction prior to purification (benzoyl MAG-3-Boc-RC-160 complex at 26 min).

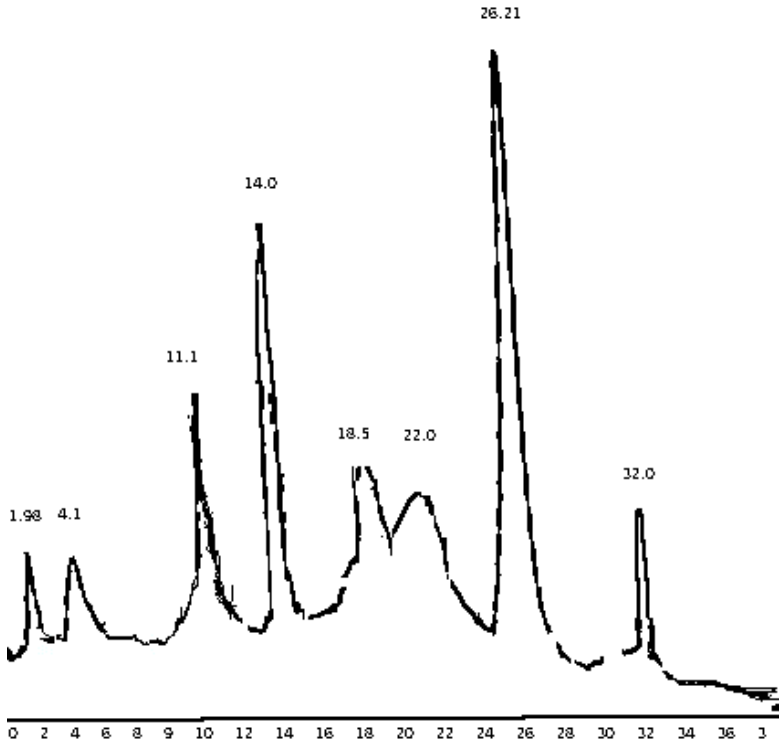

FIG. 4. HPLC chromatogram of conjugation reaction after purification (benzoyl MAG-3-Boc RC-160 complex at 26 min, Boc RC-160 at 22 min).

TABLE III. ^{99m}Tc RC-160 USING ITLC IN 85% METHANOL B BEFORE SEPPAK PURIFICATION

Instant thin layer chromatography ITLC-SG	% of free $^{99m}TcO^-_4/R_f$	% of ^{99m}Tc-RC-160/R_f
mini strip 85% methanol	1.3/1	98.7/0
14 cm 85% methanol	1.8/1	98.2/0

TABLE IV. ACTIVITY REGISTERED FOR ^{99m}Tc-RC-160 AFTER SEPPAK PURIFICATION AT VARIOUS STEPS

Total activity in vial	50 mCi
Activity left in emptied vial	11 mCi
Activity left in emptied syringe	1.7 mCi
Activity left in SEPPAK filter after elution	12 mCi
Filtrate (t.1)	2.2 mCi
Deionized H_2O (t. 2)	1.1 mCi
0.5M acetic acid (t.3)	0 mCi
95% ethanol A (t.4)	1.1 mCi
95% ethanol B (t.5)	1.9 mCi
95% ethanol C (t.6)	20 mCi

4. CONCLUSION

It can be concluded from the results obtained that labelling of RC-160 with ^{125}I can be achieved satisfactorily in >95% yield. However, direct labelling of RC-160 with ^{99m}Tc gave only 46% labelling yield with some lipophilic impurities which resulted in increased background of liver; however, purification of the reaction mixture through SEPPAK column yields a fraction with 90% ^{99m}Tc labelled RC-160. This fraction will be used for biodistribution studies in the future. For carrying out indirect labelling through bifunctional chelators, it was necessary to have the conjugate of Boc RC-160 with any of the chelators like MAG-3, HYNIC or adipic ester. We tried to make conjugate of MAG-3 with Boc RC-160 but 100% conjugation could not be achieved so efforts are underway to improve the conjugation yield and then it will be labelled with ^{99m}Tc.

ACKNOWLEDGEMENTS

We would like to thank the IAEA for inviting us to participate in this CRP. We are also thankful to Professor Helmut Mäcke of the University Hospital, Basel, Switzerland for his help and for providing us with HATU.

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^{99m}Tc LABELLED PEPTIDES FOR IMAGING OF PERIPHERAL RECEPTORS

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Abstract

The first trials of ^{99m}Tc labelling by direct method using dithionite as a reducing agent (prepared in the freeze-dried form) gave the yields of around 30%. RC-160 labelling with ^{125}I by chloramine -T method resulted in 40–80% labelling yield. Our efforts were focused on BFC approach. HYNIC-TOC and HYNIC-RC-160 conjugates obtained in our laboratory were successfully labelled with ^{99m}Tc with the yields over 90%. HPLC and TLC methods were applied for quality control (QC) of the labelled preparation. Methods of *in vitro* (stability and protein binding) testing of the labelled preparations were adopted to our laboratory conditions. First attempts on dry kit formulation based on HYNIC-TOC conjugates with tricine, tricine/nicotinic acid and EDDA were described. Various amounts of tin (II) (as $SnCl_2$) were added to the kits. Incubation conditions (time, temperature) were investigated. The kits were tested for labelling yield and radiochemical purity. It was shown that the results are at the same level or better than obtained in liquid phase but the procedure of labelling is significantly easier. Kit produced with tricine as co-ligand was labelled with 97% labelling yield after 30 min of incubation at room temperature, which is considered acceptable for diagnostic radiopharmaceutical preparation. Tricine/nicotinic acid kit requires heating to get labelling of around 95%. Similarly EDDA kit gives around 70% labelling after 30 min incubation at 80^{o}C. Further experiments on optimal kit composition and stability are required. Results of DOTA-RC-160 labelling with ^{90}Y show that this isotope, manufactured by Radioisotope Centre POLATOM, can be successfully used for medical applications.

1. MATERIALS

The peptides available for the experiments were obtained from the following sources: RC-160 from Bachem UK Ltd., and from the Faculty of Pharmacy, Medical Academy of Lodz, Poland, RC-160 (Boc) and TOC (Boc) from Bachem UK Ltd., DOTA-RC-160 and HYNIC-TOC from the University Hospital, Basel, Switzerland.

Reagents: tricine, TFA, acetonitrile, thioanisole, diisopropylethylamine (DIEA) from Merck, Germany. Dimethylformamide (DMF), nicotinic acid from Sigma-Aldrich, US., ethylenediaminediacetic acid (EDDA), 6-Boc-hydrazionpyridine-3-carboxylic acid (Boc-HYNIC) and O-(7-azabenzotriazolyl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HATU) distributed under the CRP; SEPPAK cartridges C-18 from Merck, Germany. Micro-spin^{TM} G-50 columns from Amersham Pharmacia Biotech. Inc., ^{125}I from NEN Dupont, ^{99m}Tc and yttrium-90 from the Radioisotope Centre POLATOM.

2. METHODS

Iodination with ^{125}I and the ^{99m}Tc labelling of peptides were performed according to the procedures recommended under the CRP. Kit preparations were manufactured using Christ ALPHA freeze-drier (liophilization at –40^{o}C for 16 hours) according to procedures applied at POLATOM.

Thin layer chromatography methods used in the work:

(1) Silica gel on aluminium foil, 5748 Merck, 0.01M PBS
(2) Silica gel on aluminium foil, 5748 Merck, 13.6% sodium acetate
(3) Silica gel on aluminium foil, 5748 Merck, ethyl acetate: ethyl alcohol (1:1 v.v.)
(4) Silica gel on aluminium foil, 5748 Merck, Pyridine: AcOH: H_2O (5:3:1.5 v.v.v.).

Systems 1–3 were used for separation of free pertechnetate-^{99m}Tc which moves with solvent front ($^{99m}TcO_4^-$, R_f = 1.0) while the labelled peptide and reduced forms of ^{99m}Tc remain at the origin ($^{99m}TcO_2$, R_f = 0). System 4 separates the labelled peptide and free $^{99m}TcO_4^-$ which moves with a solvent (R_f = 0.5 – 0.7) from the starting point in which reduced forms of ^{99m}Tc remain ($^{99m}TcO_2$ R_f = 0).

HPLC column: Lichrospher WP300, RP C-18 5 μm 250 × 4mm, Merck
UV and radioisotope detectors, Beckman
Mobile phase: A–0.1% TFA in water, B–0.1% TFA in CAN, flow 1.0 mL/min.

Gradient 1		Gradient 2		Gradient 3	
0–50	0% B	0–5	0% B	0–3	0% B
25–32	100% B	25–32	30% B	13–23	50% B
34–36	0% B	34–36	0% B	23–26	70% B
				26–27	0% B

2.1. Iodination of RC-160 by chloramine T-method

Iodination of RC-160 was done by chloramine-T method. The peptide was dissolved in distilled water and dispensed into Eppendorf vials in portions containing 20 μg of RC-160 (1 μg/μL). To one vial about 37 MBq of ^{125}I solution was added and 20 μL of chloramine-T (1μ g/μL in 0.1 M phosphoric buffer pH7.5). The mixture was vortexed for about 60–75 s. The reaction was stopped by adding 20 μL of sodium metabisulfite (1μg/μL in 0.1 M phosphoric buffer pH7.5). The reaction mixture was then analysed by HPLC (gradient 1).

2.2. ^{99m}Tc-labelling of RC-160 by direct method

For the direct labelling of RC-160 with ^{99m}Tc [2, 4] two kit compositions, A and B, were prepared as shown in Table I.

TABLE I. KIT FORMULATION FOR DIRECT METHOD LABELLING

Batch A	Batch B
10 μg of RC-160	200 μg of RC-160
2 mg glycine	20 mg sodium potassium tartrate
2 mg myo-inositol	4 mg potassium hydrogen phtalate
2 mg $Na_2B_4O_7$*$4H_2O$	2.7 mg stannous tartrate
2 mg sodium ascorbate	20 mg lactose
1 mg $Na_2S_2O_4$	

Kits were labelled by adding 4.5 mL of sodium pertechnetate-^{99m}Tc with total activity of about 200 MBq and incubated at 90ºC for 30 min.

2.3. [Tyr^3](Boc)Octreotide conjugate with (Boc)HYNIC for ^{99m}Tc labelling

The method of BFC conjugation to the peptide was described in CRP recommendations [3] and consisted of 2 steps: conjugation of (Boc)HYNIC to the Boc protected peptide and deprotection of the obtained conjugate.

Deprotected HYNIC-TOC was collected at about 16 min Rt in a glass vial and closed with rubber stopper and aluminium cap after purging the solution with nitrogen for 5 min. The solution was stored at –20 °C.

2.4. ^{99m}Tc-labelling of HYNIC conjugates with different co-ligands

The obtained HYNIC conjugate was labelled with ^{99m}Tc using tricine, tricine/nicotinic acid and EDDA systems following the published procedures [5].

2.5. ^{90}Y labelling of DOTA-RC-160 [8]

To the 50 μL of DOTA-RC-160 (20-1 μg) dissolved in 0.4 M sodium acetate buffer pH5.0 aliquots of 50 μL ^{90}Y (37 MBq) in 0.05 M HCl and 50 μL of gentisic acid in 0.05 M acetic acid (20 mg/mL) were added and incubated at 95^{o}C for 25 min.

2.6. Protein binding and stability studies

^{99m}Tc labelled peptide was mixed with human serum in the ratio of 1:10, control sample of ^{99m}Tc labelled peptide was mixed with 0.01 mM PBS buffer in the same ratio. Samples were incubated at 37°C and tests made within 15 min, 1 h, 2 h and 3 h or 6 h after labelling. Mini columns G-50 were prespun at 2000 × g for 1 min; a 20 μL sample was loaded and column centrifuged again at 2000 × g for 2 min. The activity of eluate collected and retained on the column was measured using NaI-scintillation counter.

2.7. Cysteine stability of ^{99m}Tc-HYNIC-TOC

In vitro stability of the labelled peptide was tested by incubation in cysteine solution (1.36 μmol cysteine: 8.4×10^{-3} μmol peptide) at 37ºC over 4 h after labelling. Control aliquots of the mixture were analysed by HPLC.

2.8. Animal studies

For animal studies 0.2 mL of obtained ^{99m}Tc-HYNIC-TOC preparation were injected into the tail vein of mice. Activity of blood, lung, liver, kidneys, intestine and stomach, tumour, muscle and excreted with urine was measured and percentage accumulated in organ and per gram of tissue calculated. Normal and breast cancer tumour bearing mice were used for the experiment.

2.9. Kit formulation

Using the HYNIC-TOC conjugate obtained as described in 2.3. and on the basis of our results of conjugate labelling with ^{99m}Tc as described in 2.4., several kit formulations were produced. Their detailed composition is shown in Table II.

TABLE II. KIT FORMULATIONS FOR ^{99m}Tc LABELLING

I	II	III
20 μg HYNIC-TOC 40 μg $SnCl_2$ in 0.1 N HCl 50 mg tricine (in water) pH5.5	20 μg HYNIC-TOC 25 μg $SnCl_2$ in 0.1 N HCl 40 mg tricine in 25 mM succinate buffer 4 mg nicotinic acid pH5.3	10 μg HYNIC-TOC 15 μg $SnCl_2$ in 0.1 N HCl 5 mg EDDA in 0.1 N NaOH pH5.5

Kits were labelled with ^{99m}Tc (400 MBq in 1 mL of sodium pertechnetate-^{99m}Tc). Incubation conditions, time and temperature were studied. The HPLC and TLC methods were applied for radiochemical purity control of the preparation.

3. RESULTS

3.1. ^{125}I radioiodination

Radioiodination of RC-160 was made to test the analytical conditions of labelling and HPLC control. The labelling yield varied in the range from 40 to 80%. Fig. 1 shows the HPLC profile of the ^{125}I iodinated RC-160.

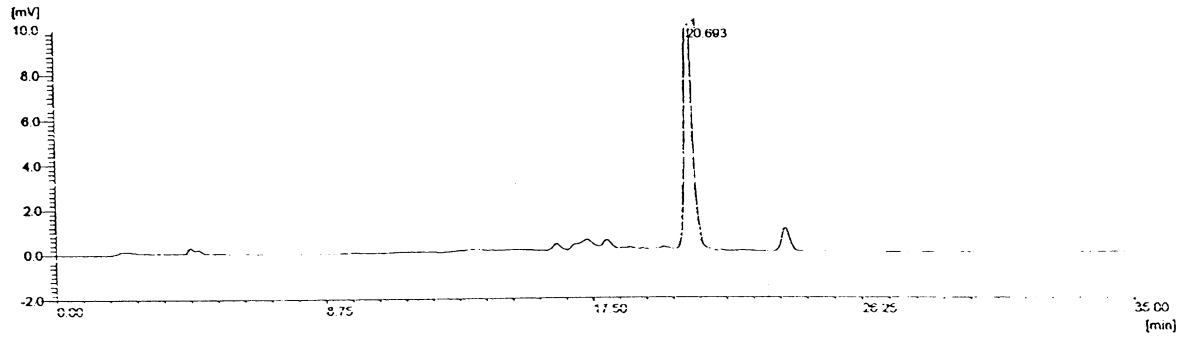

FIG. 1. HPLC profile of ^{125}I-RC-160 (about 80% radiolabelling yield).

3.2. ^{99m}Tc-RC-160 labelled by direct method

Kits of Batch A showed the labelling yield varying from 15 to 45% during the first 3 h after labelling. Trials of SEPPAK purification on the labelled product failed. Biodistribution test in normal rats of the unpurified preparation showed behaviour similar to free sodium pertechnetate-^{99m}Tc distribution. Labelling of Batch B kits gave very low, negligible yield.

3.3. HYNIC coupled RC-160 and TOC

After several experiments of (Boc)HYNIC conjugation to the peptide we could produce the conjugate which was than separated and labelled with ^{99m}Tc. Reaction steps were controlled by HPLC. Fig. 2 shows typical HPLC profiles obtained during this process.

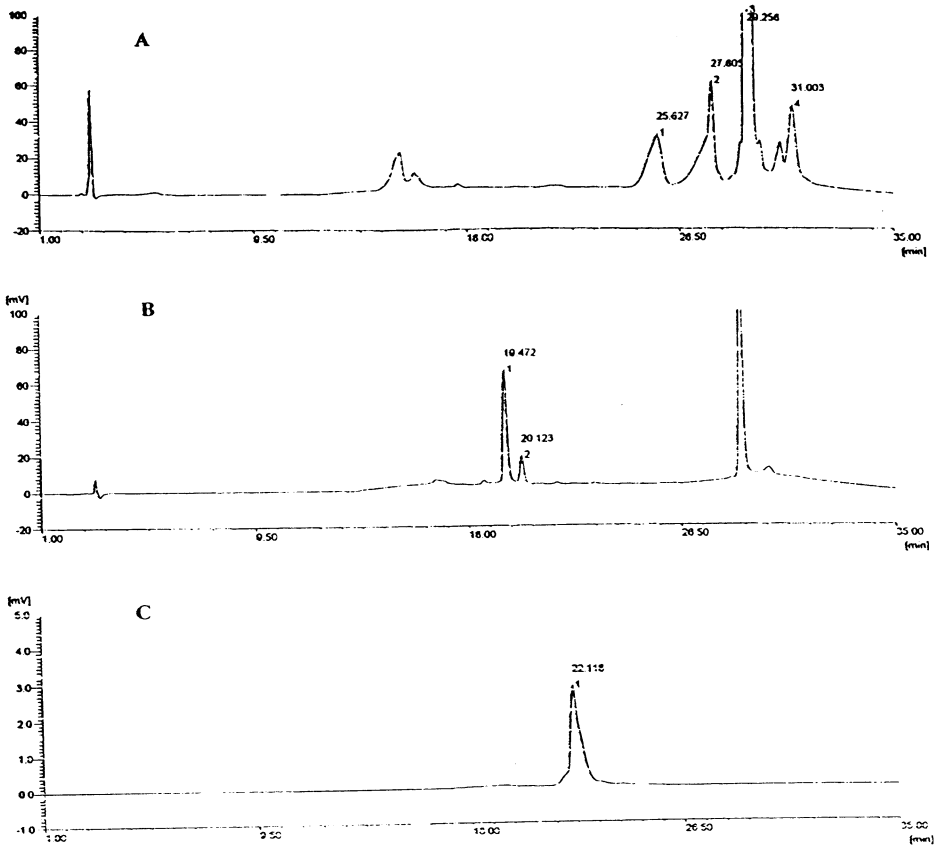

FIG. 2. HPLC profiles of the HYNIC-TOC: A reaction mixture of (Boc)HYNIC-(Boc)TOC; B-deprotected HYNIC-TOC conjugate; C–^{99m}Tc-HYNIC-TOC.

Labelling yields of HYNIC-TOC labelling with ^{99m}Tc using co-ligands and methods described in 2.4. are presented in Table III. In Table IV comparison of radiochemical purity results of ^{99m}Tc-HYNIC-TOC (of the conjugate produced in our hands and a reference conjugate from Prof. H. Mäcke) with tricine and nicotinic acid in 25 mM succinate buffer as ternary co-ligands, which shows the agreement of TLC methods and HPLC used for QC of the obtained complex.

TABLE III. LABELLING YIELD OF ^{99m}Tc-HYNIC-TOC WITH DIFFERENT CO-LIGANDS

Co-ligand	Labelling yield [%]					Mean yield [%]
tricine in H_2O	83.6	98.3	92.1	90.2		91.05 ± 5.24
tricine in succinate buffer	99.4	97.3				98.35 ± 1.05
tricine + nicotinic acid in succinate buffer	96.5	99.0	99.4	99.6	99.7	98.82 ± 1.19
EDDA	72.86					72.86*

* One result of labelling only.

TABLE IV. RADIOCHEMICAL PURITY RESULTS OF ^{99m}Tc-HYNIC-TOR WITH TRICINE AND NICOTINIC ACID IN 25 MM SUCCINATE BUFFER

	TLC [%]				HPLC [%]
	System (1)	System (2)	System (3)	System (4)	
HYNIC-TOC conjugate (UH Basel)	98.4	99.02	99.4	99.7	99.3
HYNIC-TOC conjugate (POLATOM)	98.7	98.7	99.5	99.7	98.9

Labelled preparations were tested for stability and protein binding. Figs 3 and 4 show the results of the respective studies.

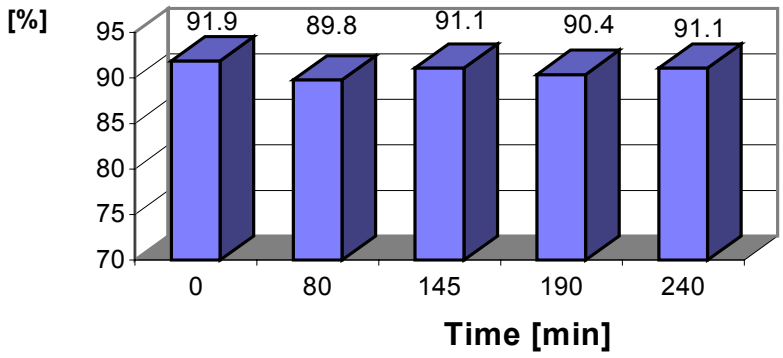

FIG. 3. Cysteine stability of ^{99m}Tc-tricine-HYNIC-TOC.

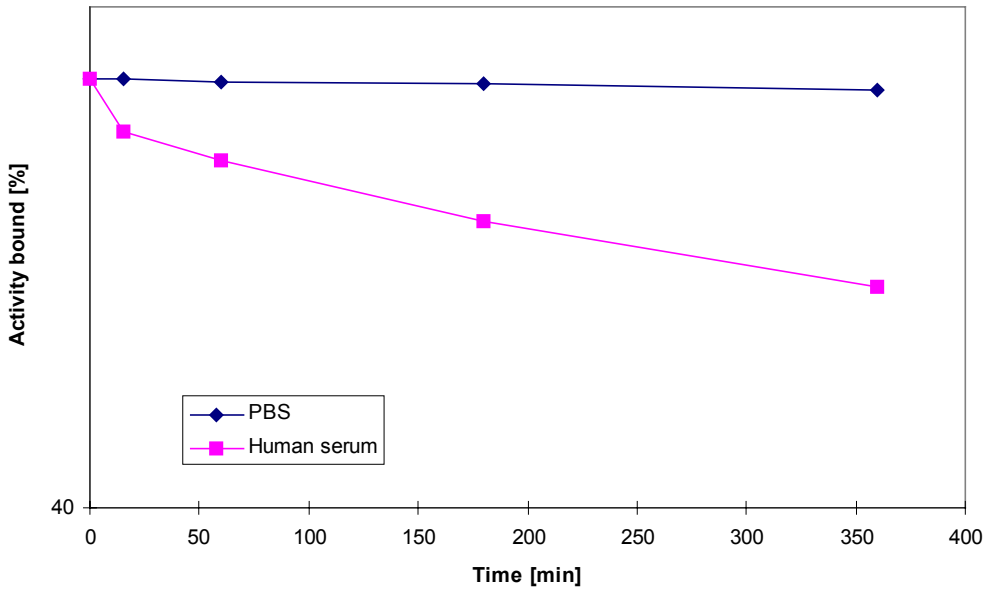

FIG. 4. Protein binding curve of ^{99m}Tc-tricine-nicotinic acid-HYNIC-TOC.

3.4. Results of ^{99m}Tc labelling of the produced kits

Labelling yields and respective incubation conditions obtained after ^{99m}Tc labelling of the kits produced at Radioisotope Centre POLATOM are presented in Table V.

TABLE V. LABELLING YIELD OF ^{99M}TC-HYNIC-TOC KITS

	Incubation conditions	Labelling yield [%]
Kit I	30 min 80°C	61.0*
	60 min 50°C	88.8
	30 min RT	97.0
Kit II	15 min 95°C	94.7
	60 min RT	68.0
Kit III	30 min 80°C	70.5

*Decomposition products visible in HPLC.

Preliminary biodistribution result of ^{99m}Tc-HYNIC-tricine-TOC in mice as presented in Fig. 5 showed that preparation was predominantly excreted by the kidney and some moderate tumour uptake was also present.

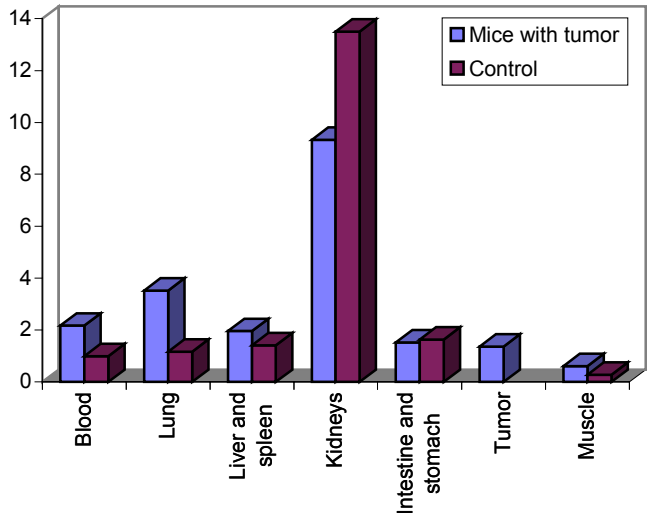

FIG. 5. Biodistribution of ^{99m}Tc-HYNIC-TOC in mice (tumour induced with breast cancer cells, activities measured in separated organs in 2 h pi).

The ^{90}Y used for labelling of biomolecules must be of high chemical purity to assure satisfactory labelling yields and specific activity of labelled preparations. In our Centre ^{90}Y is produced from ^{90}Sr by solid phase extraction method [8]. Its usefulness for medical application was tested by labelling varying amounts of DOTA-RC-160 (from 20 to 1 μg) with 37 MBq (1 mCi) activity of ^{90}Y. Results of this study are presented in Fig. 6. Labelling yields are over 98% in most cases (tested by HPLC, gradient 2), a decrease is observed only when the concentration of peptide goes down to 2 μg and falls to 5% with 1 μg of peptide. Such low ratios of ^{90}Y to peptide are not required in therapeutic applications.

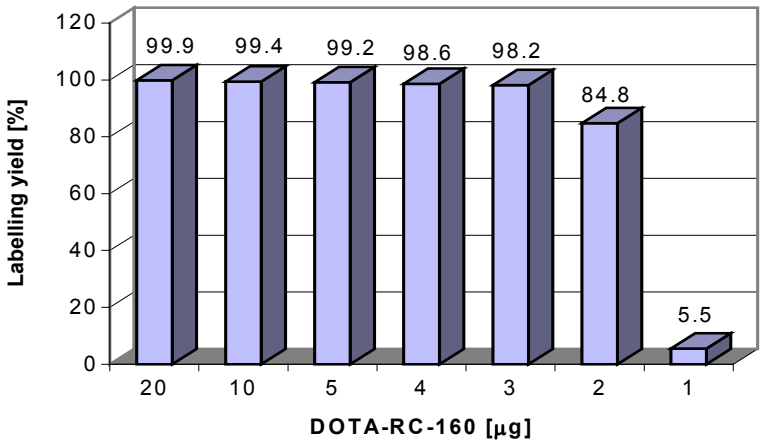

FIG. 6. ^{90}Y-DOTA-RC-60 labelling yields as function of amount of DOTA conjugate.

4. CONCLUSIONS

The preparation of bifunctional ligand conjugate with (Boc) protected peptides worked well in our hands. Also, the purified conjugates were labelled with ^{99m}Tc at the yields comparable to literature data. A method of protein binding studies by using Sephadex G50 columns with centrifugation was adopted to our laboratory conditions. Stability of the ^{99m}Tc labelled peptides in cysteine solution was also tested and preliminary animal experiments were carried out. The results of protein binding made on ^{99m}Tc-HYNIC-TOC with tricine and Nicotinic acid as co-ligands do not differ significantly from the reference literature data [5].

High labelling yields of kits produced with tricine when incubated at room temperature simplify the labelling procedures of radiopharmaceutical preparation. Heating is required to get the same labelling yield of the kit produced with tricine/nicotinic acid as co-ligands, due to low solubility of nicotinic acid. Poor labelling yield (at the level of 70%) of kit prepared with EDDA as co-ligand require further studies and optimization of kit composition. Medical usefulness of ^{90}Y (as $^{90}YCl_3$) produced in our Centre was proven, which encourages speed-up of work on radioisotope production at a commercial scale.

ACKNOWLEDGEMENTS

We thank the IAEA for inviting us to participate in the CRP. We are grateful to Professor Helmut Mäcke of the University Hospital, Basel, Switzerland for his help, personal communication and for providing us the reference conjugates, especially DOTA-RC-160, which enabled experiments with ^{90}Y labelling. We thank Professor Stephen J. Mather for accepting the scientific visit of our colleague, Alina Markiewicz, at the St. Bartholomew's Hospital, London, England.

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DEVELOPMENT OF ^{99m}Tc LABELLED SOMATOSTATIN ANALOGUES AND EVALUATION OF THEIR RADIOCHEMICAL AND BIOLOGICAL BEHAVIOUR

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Abstract

Conjugates of two somatostatin analogues, octreotide and RC-160, with HYNIC were synthesized, characterized and purified by reverse phase HPLC. Radiolabelling of the conjugates with ^{99m}Tc was achieved using tricine as co-ligand. High labelling efficiencies were obtained and ^{99m}Tc peptides with high radiochemical purity were found when analysed both by ITLC and HPLC. *In vitro* stability of ^{99m}Tc-peptides in human serum and towards cysteine challenge was determined by Cellogel electrophoresis and HPLC after ultrafiltration of serum solution through a 20 kDa cut off membrane. Biodistribution studies were performed in healthy mice at 5 and 30 minutes and 1, 2, 4 and 24 h post-injection. Urine and blood samples were collected at sacrifice time. Samples of urine and ultrafiltrate murine serum were analysed by electrophoresis and reverse phase HPLC in order to get some information about radiocompounds metabolism. Biological distribution of ^{99m}Tc octreotide was also assayed in mice pre-treated with an excess of unlabelled peptide. From our results we conclude that this labelling method led to stable ^{99m}Tc complexes both *in vitro* and *in vivo* when high specific activities (37–72 GBq/μmole) were used. Biodistribution studies of both ^{99m}Tc-peptides indicated a radioactivity distribution profile with significant differences especially in the liver uptake that is higher for ^{99m}Tc RC-160. However, a rapid blood clearance was obtained for both radiolabelled peptides, and the urine analysis indicated that ^{99m}Tc peptide is mostly excreted as the initial complex. Pre-treatment with unlabelled peptide did not affect renal excretion of ^{99m}TOC but pancreas and intestine radioactive uptake was significantly lower, indicating saturation of somatostatin receptors and selective uptake.

1. INTRODUCTION

Somatostatin, an endogenous peptide presents multiple pharmacological properties and inhibitory effects on various tumours which originated a great interest on its use for therapeutic purposes. Nevertheless its use was hampered by its short biological half-life and multiple biological and oncological actions. To overcome this inconvenience several analogues, with prolonged half-life, have been synthesized. Among these are the octapeptides octreotide and RC-160 that possess most of somatostatin biological properties. When radiolabelled with ^{111}In, they have proven to be a valuable tool for visualization of tumours that express somatostatin receptors with high sensitivity [1–7]. Because of the advantages of using ^{99m}Tc as radionuclide, efforts have been made to develop methods to obtain ^{99m}Tc somatostatin analogues with suitable biological behaviour to use as imaging agent [8–10]. Different radiolabelling methods have been proposed to label these peptides with ^{99m}Tc: direct labelling based on the previous reduction of disulphide bridge into free thiols and the indirect methods that require a bifunctional chelating agent to be coupled to the peptide.

This work was undertaken under the CRP to develop a radiolabelling method for the somatostatin analogues, octreotide and RC-160 with ^{99m}Tc. To achieve this goal, a method based on the conjugation of peptides with the bifunctional chelating agent, initially proposed by Abrams for protein radiolabelling [11], 6-HYNIC [12–13] was chosen. Studies to investigate radiochemical purity of labelled peptides, its *in vitro* and *in vivo* stability, biodistribution in mice and receptor mediated pancreas and intestine uptake were carried out.

2. MATERIALS AND METHODS

2.1. Materials

[Tyr3, Lys5-Boc] octreotide and [Lys5-Boc] RC-160 were synthesized by Bachem UK Ltd and supplied through the IAEA. The 6-Boc-hydrazinopyridine-3 carboxylic acid (HYNIC) and HATU used in the synthesis of the conjugates were also supplied through the CRP. All other reagents were purchased from Aldrich-Sigma Chemical Co and were of analytical grade. Sodium pertechnetate-^{99m}Tc was obtained from a commercial (Amertec II) ^{99}Mo/^{99m}Tc generator. The kit for labelling the conjugated peptide DTPA-D-Phe1-octreotide (Octreoscan) with ^{111}In was supplied by Mallinckrodt Medical Petten.

2.2. Synthesis of the HYNIC peptide conjugates

The HYNIC conjugate of peptides was prepared from Boc-hydrazinopyridine-3-carboxilic acid through HATU activation. HYNIC was activated by 15 minute incubation with diisopropylethylamin and 0–7-azabenzotriazolyl)-HATU in a molar ratio of approximately 1:3:1 and subsequently added to the Boc-protected peptide. After 2–3 hours of reaction time the conjugated peptide was extracted with ethyl acetate, successively washed with 5% $NaHCO_3$, 5% $NaHSO_4$ and saturated NaCl solution and evaporated to dryness. The conjugated peptides were Boc-deprotected with 92% trifluoroacetic acid and 6% thioanisole by 30 minute incubation time at room temperature. After deprotection the solution was immediately evaporated under high vacuum. The conjugated peptide in the residue was dissolved in water: ethanol, purified and characterized by reverse phase HPLC on a Nucleosil C18 (8×250 mm) column using 0.1% TFA/water and 0.1% TFA/acetonitrile as mobile phase, a flow rate of 0.5 mL/min and the following gradient: 0–5 min. 0% acetonitrile, 5–15 min. 0–80% acetonitrile, 15–25 min. 80% acetonitrile, 25–30 min. 80–0% acetonitrile. Purified fraction from the HPLC was lyophilized.

2.3. Radiolabelling of conjugated peptide with ^{99m}Tc

Radiolabelling of the conjugate with ^{99m}Tc was achieved using tricine as co-ligand. For that purpose, in a sealed 10 mL vial, 10 mg of tricine in 0.5 mL nitrogen purged water (pH $\simeq$ 5.0) and 35 μg stannous chloride (5mg/mL nitrogen purged 0.1N HCl) were added to 10 μg conjugated peptide dissolved in nitrogen purged water and 0.5 mL of pertechnetate (750–1500 MBq). The reaction mixture was allowed to stand at room temperature for 30 min.

The reaction sequence for the synthesis of the peptide conjugates, using the HYNIC octreotide as example, and the scheme of the respective radiolabelling reaction are summarized in Fig. 1.

2.4. Radiochemical purity and *in vitro* stability

Radiochemical purity and *in vitro* stability evaluation of ^{99m} Tc peptides in solution was accomplished by the use of different analytical methods: (1) instant thin layer chromatography using the following chromatographic systems: ITLC SG in saline or methylethylketone (MEK) or 50% acetonitrile as mobile phase. In saline labelled peptides and colloid remain at the origin while labelled tricine and pertechnetate migrate. In MEK, only pertechnetate migrate and, in acetonitrile, only colloid remains at the origin; (2) reversed phase — HPLC system coupled to UV at 220 nm and γ detectors, as described in the synthesis

of HYNIC peptide conjugates; (3) cellulose acetate electrophoresis using Veronal buffer, pH = 8.6 as electrolyte running at 200 V during 45 min.

In vitro stability of the ^{99m}Tc peptides in the presence of human serum was evaluated by incubation of 10 μL aliquots with 500 μl human serum for 1 to 24 h at 37ºC. Samples were analysed by reverse phase HPLC after ultrafiltration through a 20 kDa cut off membrane (Centrisart I, Sartorius GmbH). Total serum was tested by cellulose acetate electrophoresis

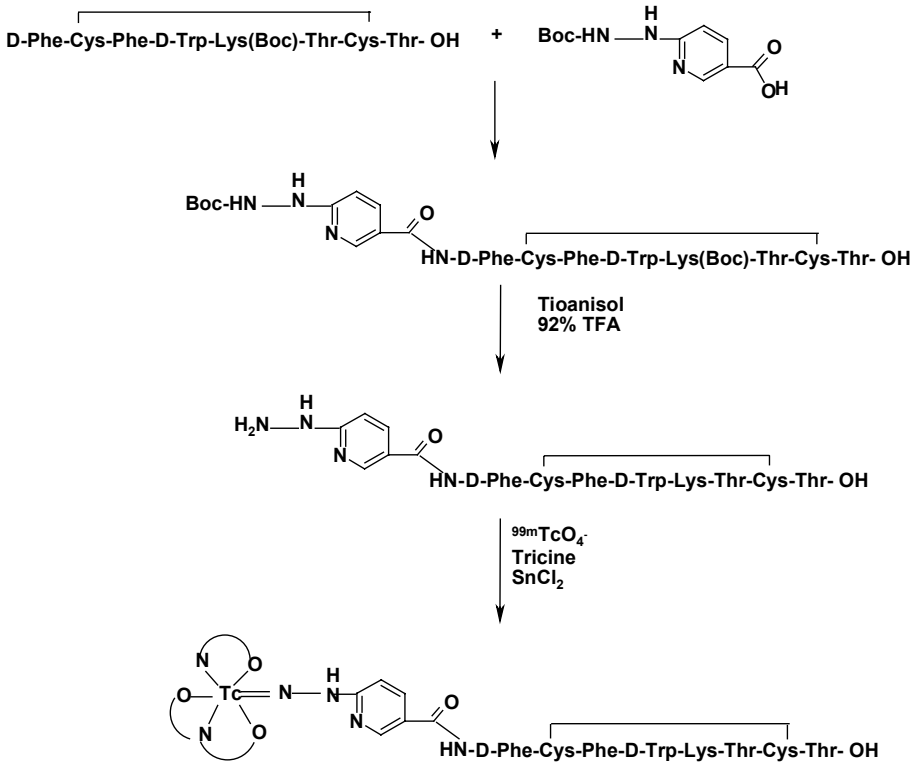

FIG. 1. Schematic representation of the synthesis of Hynic octreotide ^{99m}Tc.

Cystein challenge was studied with 100 and 1000-fold molar excess of fresh cystein solution in phosphate buffer saline, pH = 7.4. Mixtures were incubated up to 24 h at 37ºC. Samples of incubated mixture were checked by ITLC and HPLC.

2.5. *In vivo* studies

Biodistribution of labelled peptides was studied at 5 and 30 min., 1, 2, 4 and 24h after intravenous administration of ^{99m} Tc-octreotide into groups of six animals, 30–39 day old Charles River healthy female mice weighting approximately 25 g each. The animals were intravenously injected with 100 μL via the tail vein and were maintained on normal diet *ad libitum.* At sacrifice time, the animals were killed by cervical dislocation and the main organs removed for counting in a gamma counter. Blood samples were taken by cardiac puncture. Urine samples were also collected immediately before sacrifice. The results were expressed as per cent of injected dose per gram organ (% I.D./g organ).

Samples of urine were also analysed by HPLC and cellulose acetate electrophoresis. Murine serum was separated from total blood by centrifugation and analysed by cellulose

acetate electrophoresis. HPLC analysis of murine serum was also performed after ultrafiltration through a 20 kDa cut off membrane.

Biological distribution was also studied in mice pre-treated with 200 μg unlabelled peptide injected intravenously 30 min before ^{99m}Tc octreotide administration to saturate the receptors.

3. RESULTS

Synthesis of HYNIC peptide conjugates using HATU for activation of the free carboxylic acid was achieved within 2 h. As both peptides contain a lysine aminogroup protected by a t-Boc in order to keep their receptor affinity, after coupling reaction the peptides were Boc deprotected, HPLC purified and lyophilized. The overall yield (coupling, Boc deprotection reactions and liophilization) was *ca.* 30%. Reverse phase HPLC analysis of the purified compound revealed a single peak at retention times corresponding to the coupled peptide (Fig. 2).

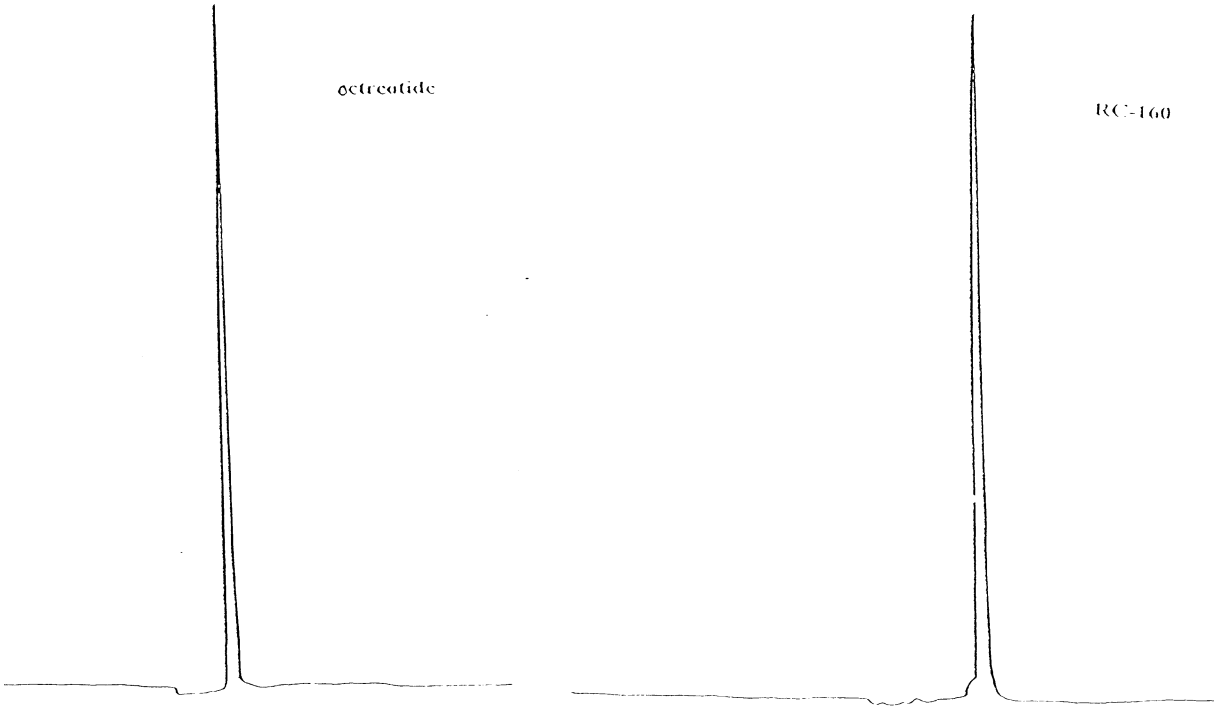

FIG. 2. RP-HPLC UV profile from the synthesis of octreotide (A) and RC-160 conjugates (B).

High labelling efficiency was obtained when HYNIC conjugated peptides were labelled with ^{99m}Tc using tricine as co-ligand to occupy the remaining sites in the technetium co-ordination sphere. Both ^{99m}Tc peptides, octreotide and RC-160 were obtained, at specific activities of 37–74 GBq/μmole, with high radiochemical purity when analysed both ITLC, HPLC and electrophoresis (as exemplified in Fig. 3). Recovery from HPLC was higher than 95%. The ^{99m}Tc compounds were stable in solution at least during 24 h.

When ^{99m}Tc peptides were incubated with fresh human serum for 24 h the HPLC analysis of the ultrafiltrate indicates that most of the radioactivity (>95%) was detected as intact ^{99m}Tc labelled peptides. However, by electrophoresis, some other small peaks were detected, probably resulting from serum protein competition towards labelled compound (Fig. 4).

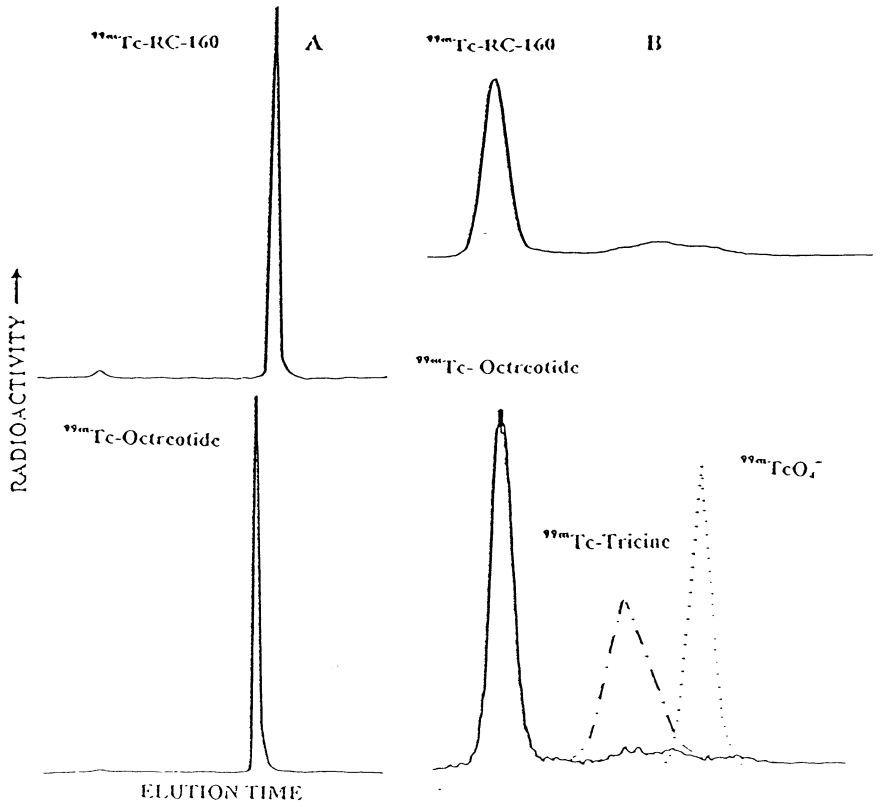

FIG. 3. Radiochemical purity of ^{99m}Tc octreotide and ^{99m}Tc RC-160. A–RP-HPLC radioactivity profile; B–Electrophoresis radioactivity profile.

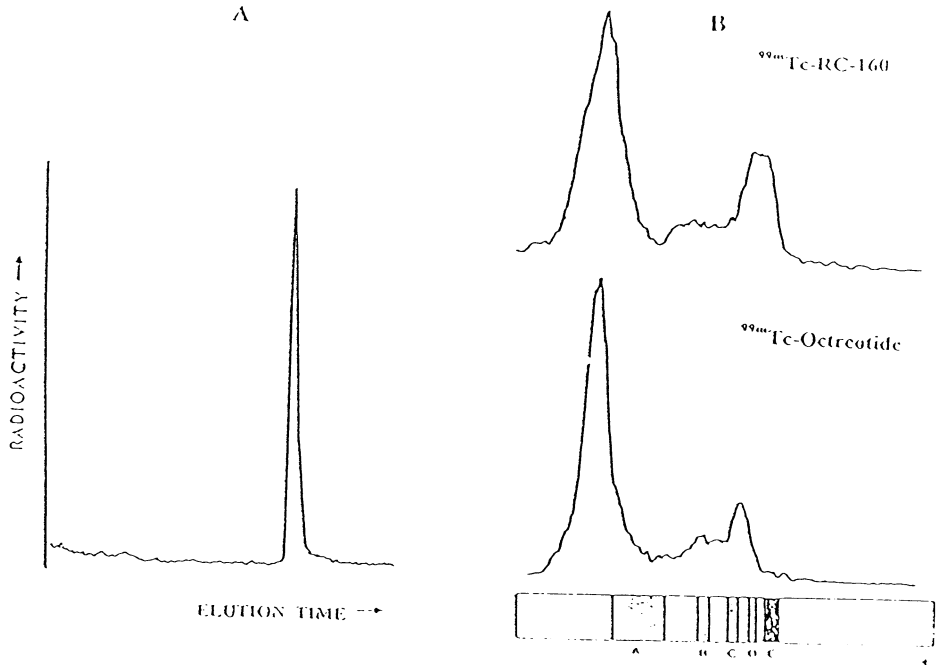

FIG. 4. In vitro stability in presence of human serum. A-RP-HPLC; B-electrophoresis.

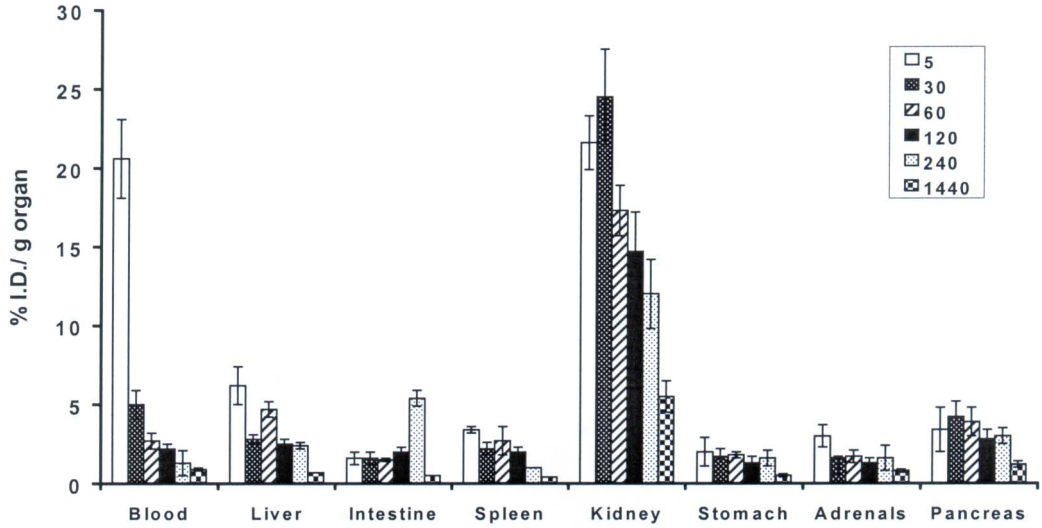

FIG. 5. Biodistribution of ^{99m}Tc-octreotide in mice.

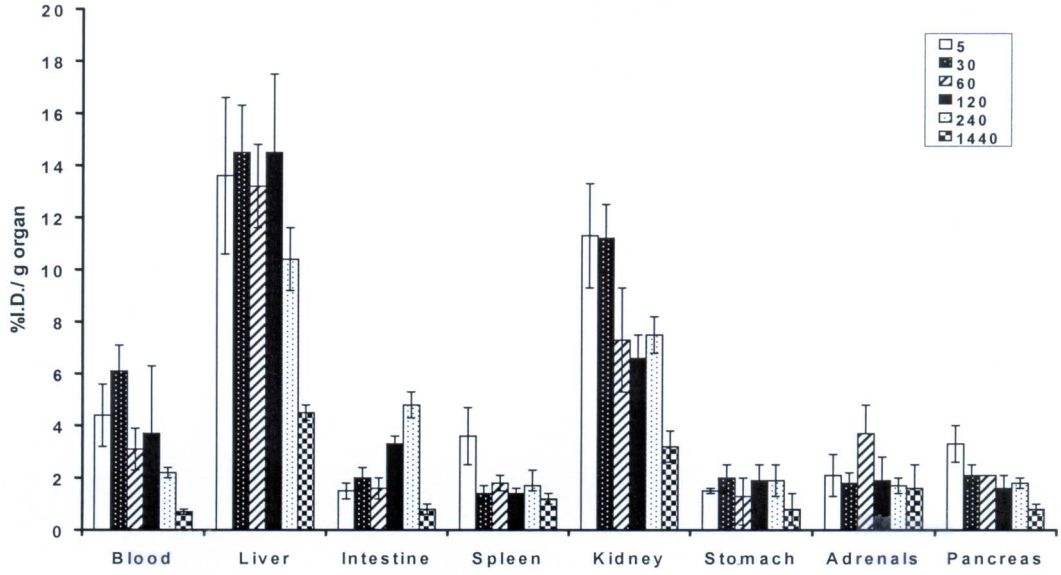

FIG. 6. Biodistribution of ^{99m}Tc RC-160 in mice.

Results of ^{99m}Tc peptide stability in the presence of 100 and 1000 fold molar excess of cysteine indicate that no significant degree (<5%) of transchelation was detected for any of the peptides.

Biological distribution data of labelled octreotide at 5 and 30 min, 1, 2, 4 and 24 h after i.v. administration are presented in histogram of Fig. 5. These biodistribution studies demonstrated that ^{99m}Tc octreotide is rapidly cleared from blood and that the tracer is mainly excreted via the kidneys with low uptake by the hepatobiliary system. The radioactivity excreted in urine was 14.6% + 3.9, 33.5% + 2.2, 45.6% + 1.3, 48.4% + 2.7, 52.3% + 0.5 and 71.8% + 2.1 of the injected dose, respectively, at 5 and 30 min, 1, 2, 4 and 24 h after administration.

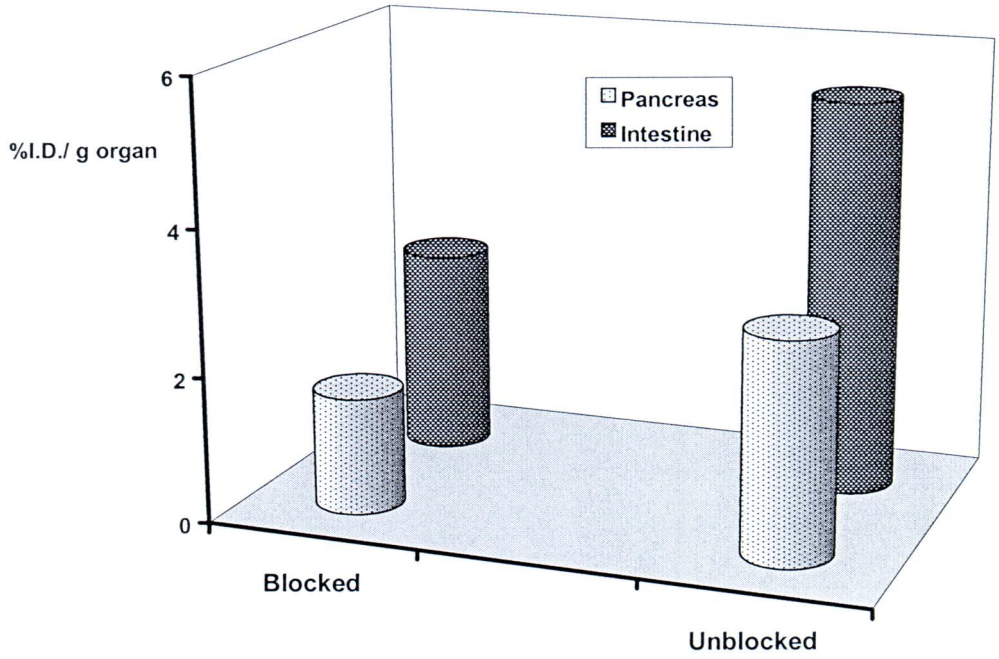

FIG. 7. Pancreas and intestine uptake 4 h after i.v. administration.

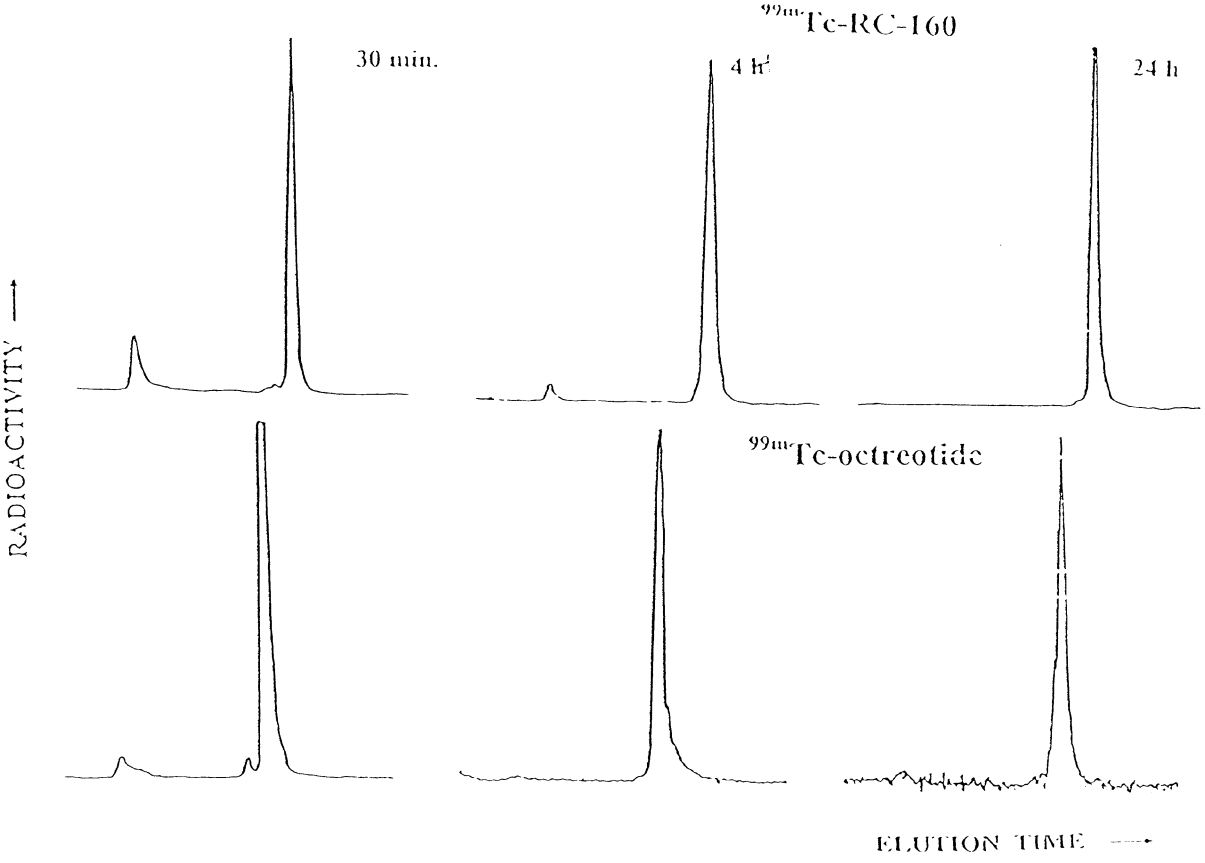

FIG. 8. Radioactivity profiles of urine samples obtained from ^{99m}Tc octreotide and ^{99m}Tc RC-160 injected mice by RP-HPLC at different times.

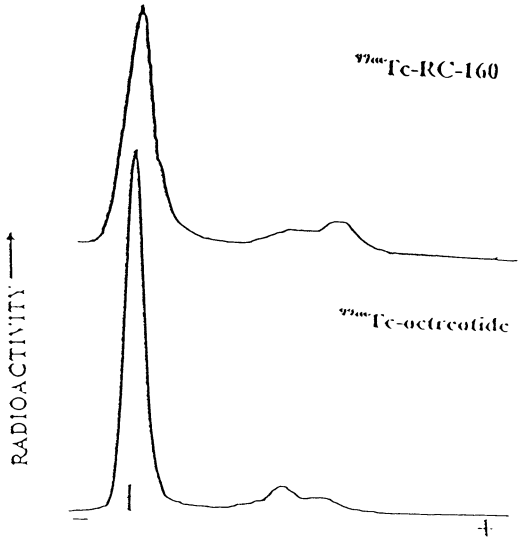

FIG. 9. Radioactivity profile of urine samples obtained from ^{99m}Tc octreotide and ^{99m}Tc RC-160 injected mice by electrophoresis.

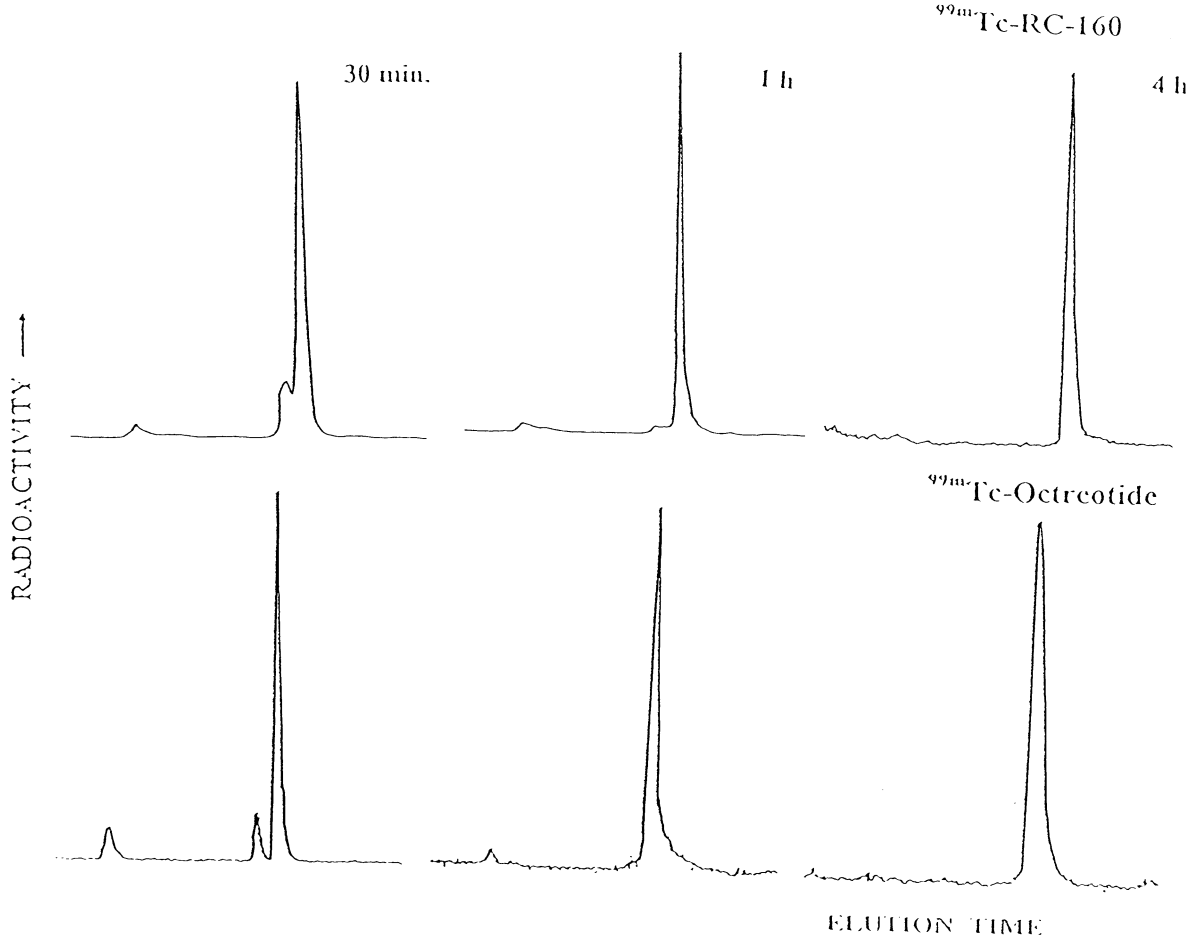

FIG. 10. Radioactivity profiles obtained by RP-HPLC of the ultrafiltrates of murine serum collected at 30 min, 1 and 4 h after sacrifice.

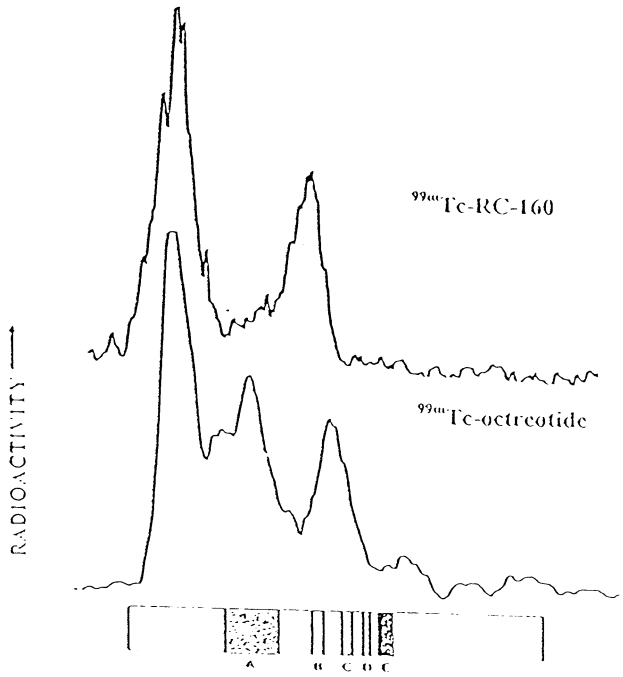

FIG. 11. Radioactivity profiles obtained by electrophoresis of total murine serum collected after ^{99m}Tc-octreotide and ^{99m}Tc RC-160 administration.

Biological distribution results obtained from the administration of ^{99m}Tc RC-160 at 5 and 30 min, 1, 2, 4 and 24 h after i.v. administration are presented in histogram of Fig. 6. Radioactivity distribution profile clearly presents a significant higher liver uptake when compared to the ^{99m}Tc octreotide. This is in accordance with its higher lipophilic character. Also a lower uptake in pancreas and adrenals was found. However, the radiolabelled peptide was also rapidly cleared from blood and presented a high per cent of radioactivity excreted via the urinary tract. The radioactivity excreted in urine was 4.3% + 0.2, 12.0% + 2.3, 19.0% + 0.5, 23.1% + 2.1, 36.3% + 0.1 and 58.6% + 2.8 of the injected dose, respectively, at 5 and 30 min, 1, 2, 4 and 24 h after administration.

Owing to the better biologic profile of ^{99m}Tc octreotide, specific tissue uptake in comparison with ^{111}In octreotide was only carried out with this labelled peptide. Pancreas, adrenals and intestines are known to be rich in somatostatin receptors. In order to get an insight on the mechanism involved on the ^{99m}Tc octreotide uptake we performed the biodistribution studies, 4 h after administration in parallel into two separated animal groups: a) without any treatment (unblocked) and previously i.v. injected with an excess of unlabelled peptide (blocked). For comparative purposes identical biodistribution studies were run with ^{111}In octreotide, a successfully used clinical radiopharmaceutical to image human tumours that express somatostatin receptors. Results found, expressed as % D.I./g organ, 4 h after administration are presented in Table I.

In the case of ^{99m}Tc octreotide the biodistribution pattern of untreated and pre-treated animals does not present significant differences except in the intestine and pancreas where the radioactivity accumulation decreases in the pre-treated animal group indicating a selective tissue uptake. Pancreas and intestine uptake is presented in the histogram of Fig. 7. These observations correlate well with the data obtained with ^{111}In octreotide although it has shown a higher radioactivity clearance from most of the tissues. Nevertheless, specific uptake in same organs (pancreas and intestine) was also identified.

TABLE I. BIODISTRIBUTION IN HEALTHY MICE OF ^{99m}Tc OCTREOTIDE AND ^{111}In DTPA-OCTREOTIDE

	^{99m}Tc octreotide		^{111}In octreotide	
Organ	Unblocked	Blocked	Unblocked	Blocked
Blood	1.3 ± 0.8	1.7 ± 0.4	0.1 ± 0.0	0.1 ± 0.0
Liver	2.4 ± 0.2	3.4 ± 0.2	0.2 ± 0.0	0.3 ± 0.1
Intestine	5.4 ± 0,5	2.8 ± 0.4	1.1 ± 0.4	0.4 ± 0.1
Spleen	1.0 ± 0.0	0.9 ± 0.3	0.1 ± 0.0	0.1 ± 0.0
Kidney	17.3 ± 2.5	20.8 ± 3.0	8.1 ± 2.1	8.8 ± 1.7
Stomach	1.6 ± 0.5	2.1 ± 0.9	-	0.4 ± 0.1
Adrenals	1.6 ± 0.8	2.1 ± 0.4	1.0 ± 0.3	0.6 ± 0.3
Pancreas	3.0 ± 0.5	1.6 ± 0.1	1.5 ± 0.4	0.3 ± 0.1

HPLC and electrophoresis analysis of urine indicated that ^{99m}Tc compounds is mostly excreted as non-metabolized form (Figs 8 and 9).

HPLC and electrophoresis analysis of murine serum at different times after i.v. administration showed high *in vivo* stability (Figs 10 and 11) although during the first hour the radioactivity profile of the total murine serum, obtained by electrophoresis, has revealed some radiochemical impurities, attributed to some ^{99m}Tc serum protein binding.

4. CONCLUSION

In conclusion, radiolabelling of HYNIC octreotide and HYNIC RC-160 conjugates with ^{99m}Tc, at specific activities of 37–74 GBq/μmole and high radiochemical purity, was achieved. *In vitro* stability studies indicated that the ^{99m}Tc-peptides are stable in the presence of human serum and do not undergo transchelation when challenged with an excess of cysteine.

Biodistribution studies in mice demonstrated that ^{99m}Tc-octreotide is rapidly cleared from blood and that the tracer is mainly excreted via the kidneys. In opposite the ^{99m}Tc RC-160 presented a markedly higher liver uptake, well correlated to its higher lipophilic character, conferring it a poorer biological profile.

HPLC and electrophoresis analysis of murine serum, at different times after i.v. administration, showed high *in vivo* stability for both ^{99m}Tc peptides and the analysis of urine indicated that ^{99m}Tc compounds are mostly excreted in non-metabolized form.

The lower uptake in pancreas and intestine, observed in the animals previously injected with unlabelled peptide, indicates that ^{99m}Tc octreotide uptake is somatostatin receptor-mediated.

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TOWARDS KIT FORMULATION OF ^{99m}Tc LABELLED SOMATOSTATIN RECEPTOR BINDING PEPTIDES OF HIGH SPECIFIC ACTIVITY FOR TUMOUR LOCALIZATION

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Abstract

The project aimed to develop ^{99m}Tc octreotide analogue for use in nuclear oncology. Several attempts to label SRIF analogues with ^{99m}Tc have used a direct labelling approach but, for this project, HYNIC was chosen as a technetium ligand. A comparison of two different SRIF analogues designed for high specific activity labelling with ^{99m}Tc was done. HYNIC-Octreotide and HYNIC-TOC were prepared and a kit formulation that can be labelled conveniently is currently being studied in a clinical setting.

1. INTRODUCTION

In the last decade strong efforts have been undertaken to establish radiopeptides in nuclear oncology [1–3].

The somatostatin-somatotropin release inhibiting factor (SRIF) receptors and its ligands are clearly the prototype for contrast enhanced diagnosis of neuroendocrine primary tumours and their metastases [4–6]. In addition vasoactive intestinal peptide as a radiolabelled version was successfully introduced into the clinic for *in vivo* localization of endothelial adenocarcinoma of the colon and ovarian cancer [7, 8]. *In vitro* autoradiographic studies demonstrating the high density and distribution of highly specific membrane receptors on tumours form the molecular basis of this application [9]. Today [^{111}In]-DTPA-(D)Phe^{1}-octreotide (OctreoScan-111) is routinely used in nuclear medicine for the diagnosis of SRIF receptor positive tumours [5]. Other radioligands based on somatostatin analogues have been developed for different purposes. ^{68}Ga [10, 11], ^{64}Cu [12, 13] and ^{18}F [14] for positron emission tomography and particularly ^{90}Y labelled to [DOTA]-(D)-Phe^{1}-TOR was successfully introduced into peptide receptor mediated radionuclide therapy [15, 18]. Despite the success with ^{111}In labelled octreotide analogues as imaging agents, somatostatin analogues incorporating the radionuclide ^{99m}Tc ($T_{1/2}$ = 6 h, monoenergetic radiation of 140keV) would be more desirable. The rapid blood clearance and the fast tumour accumulation of small peptides are in accordance with the use of shorter lived radionuclides. Moreover, ^{99m}Tc as a radionuclide used commonly in nuclear medicine has a wide availability in high quality and shows a higher yield of photon flux per unit radiation dose delivered to the patient than ^{111}In. Several attempts to label SRIF analogues with ^{99m}Tc have used a direct labelling approach after reduction of the disulfide bridge; thus forming high affinity binding sites for Tc(V) [19], or the bifunctional chelator approach including a bifunctional version of propylenediaminedioxime [20], acyclic [21] and macrocyclic tetraamines [22] and triamidomonothiol [23]. We have chosen 6-HYNIC as a technetium ligand. It shows great promise due to the likely monodentate character, which may allow the use of a variety of co-ligands [23, 24] leading to quite different biodistribution [25].

In this paper we compare two different SRIF analogues designed for high specific activity labelling with ^{99m}Tc: HYNIC-octreotide and HYNIC-TOC and describe a kit formulation which can be labelled conveniently and is currently being successfully studied in

a clinical setting [1]. In addition, we study the difference of the ^{99m}Tc labelled peptides with the gold standard of SRIF receptor imaging, ^{111}In DTPA octreotide (OctreoScan) in a tumour bearing animal model. HYNIC was shown to label antibodies and peptides very reliably and with high specific activity [26, 27] but a critical report by Hnatowich, et al. using HYNIC conjugated to oligonucleotides indicated that the radiolabel is unstable *in vivo* [27].

2. MATERIAL AND METHODS

2.1. Reagents

Unless stated otherwise, all chemicals and silica gel 60 were purchased from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany) and were used without further purification. The SRIF analogues (e-Boc)lys^5-octreotide, (e-Boc)lys^5-TOR and octreotide were obtained from Sandoz or Mallinckrodt. [O-(7-azabenzotriazol-1-yl)]-1,1,3,3-tetramethyluronium HATU was obtained from Hoffmann-LaRoche. Sephadex G50 was supplied by Pharmacia (Uppsala, Sweden). [^{111}In]-DTPA-(D)phe^1-octreotide (Octreoscan®) was purchased from Mallinckrodt Medical, Inc. (Petten, the Netherlands). ^{99m}Tc was eluted from a commercial ^{99}Mo/^{99m}Tc generator, Tecegen S (CIS, Saclay, France). Mice were obtained from the Institut für Labortierkunde (Zürich, Switzerland).

2.2. Instrumentation

HPLC was performed on a Hewlett Packard 1050 system with a variable wavelength UV monitor and a Raytest RSM 100 radioactivity monitoring analyser coupled to a computer using software HPLC Chemstation A 02.05 by Hewlett Packard. The ES/MS was performed on a TSQ/SSQ 7000 Atmospheric Ionization System from Finnigan MAT (CA, USA). Gamma counting was carried out on a Cobra Model 5003 from Packard Instrument Company (Meriden, CT, USA) with a NaI(Tl) detector.

2.3. Synthesis

2.3.1. HYNIC-TOC

6-BOC-hydrazinopyridine-3-carboxylic acid was synthesized according to Abrams, et al. [1]. A mixture of 3.9 mg (15.4 μmol) 6-[2-(tert-butoxycarbonyl)-hydrazinopyridine-3-carboxylic acid, 2.6 μl (15.4 μmol) diisopropylethylamin and 5.86 mg (15.4 μmol) HATU in 1 mL DMA was incubated for 15 min and added afterwards to a solution of 15 mg [12.8 μ mol) (e-(tert-butoxycarbonyl)]lys^5-TOC and 2.6 μl (15.4 μmol) diisopropylethylamin in 500 μ L water/DMF (1/1). After 7 h, 1 mL of a 5% aqueous $NaHCO_3$ solution and 1 mL of ethyl acetate were added. The $NaHCO_3$ solution was extracted three times with 1 mL ethyl acetate. The combined organic phases were washed four times with water and evaporated to dryness. The white solid was dissolved and incubated in 1 mL of 92% trifluoroacetic acid, 6% thioanisole and 2% water and precipitated with 5 mL of diisopropyl ether/petrolether (1/1) after eight min at room temperature. The peptide was centrifuged off and was dissolved another three times with the diisopropylether/petrolether solution and centrifuged. The peptide was dissolved in water and purified by HPLC with system A (VYDAC 218TP510 C_{18}, 250 × 10 mm; Solvent A: 0.1%TFA in water; Solvent B : AcCN; flow rate: 0.5 mL/min; gradient: 0 min 100% A, 0–30 min 50% A, 50% B; 30–35 min 100% B; l = 254 nm and 280 nm). After lyophilization 6.2mg (41%) of a white powder was obtained. The product was more than 95% HPLC pure and showed the expected peak of HYNIC-TOC in the ES-MS [586 (MH^{++}; 100%); 1170.5 (MH^+; 8%)].

The synthesis of 6-hydrazinopyridine-3-carboxylic amid-(D)phe^1-octreotide (HYNIC-Oct) was performed according to the procedure for the synthesis of HYNIC-TOC. It was obtained 3,0 mg (20%) of a white powder. The product was more than 95% HPLC pure and showed the expected peak in the ES-MS [578 (MH^{++}; 100%); 1154.7 (MH^{+}; 8%)].

2.4. Peptide radiolabelling

One or two step labelling procedures were studied for the ^{99m}Tc labelling of HYNIC-TOC and HYNIC-OCT using tricine or etylendiamine-N,N´-diacetic acid (EDDA). Briefly ^{99m}Tc-tricine was prepared by the addition of 75 μL aqueous $SnCl_2$ (2mg/mL) solution to a fresh generator eluate from a generator which was eluted 6 h before 3 GBq $^{99m}TcO_4$ in 1 mL 0.9% NaCl filled up to 3 mL with acetate buffer (pH5.2; 0.5 M) were added to 108 mg (6 mmol) tricine. The mixture was kept at room temperature for 5 minutes after which the radiochemical purity of the ^{99m}Tc-tricine complex was >97% as determined by paper chromatography (Whatman Nr. 3) using acetone and 0.9% NaCl as mobile phase solvents. One to ten μL of a 10^{-3} M (1 nmol-10 nmol) peptide solution were incubated for 1 h at room temperature with 250 μl (250MBq) of ^{99m}Tc(tricine)$_2$.

We used one step labelling method for the labelling of HYNIC-OCT and HYNIC-TOC with EDDA as co-ligand. To a solution of 250 MBq of $^{99m}TcO_4^-$ in 0.5 mL physiological NaCl, 0.5 mL of a 56.7 mM solution of EDDA (pH7.2) and 1–10 μL of a 10^{-3} M (1 nmol–10 nmol) peptide solution were added. The 1 h labelling procedure was started with the addition of 10 μL of a freshly prepared $SnCl_2$ solution (10 mg $SnCl_2x2H_2O$ in 10 mL 0,1 M HCl). For both labelling procedures, the radiochemical purity was 98% as shown by HPLC (System B: Nucleosil 120-3 C18, 250 × 4 mm; Solvent A: 0.1% TFA in water; Solvent B : AcCN; flow rate: 0.5 mL/min; gradient: 0–5 min 100% A, 0% B; 5–15 min 20% A, 80% B; 15–25 min 20% A, 80% B; radioactive detection). ITLC with pyridine/acetic acid/water (5/3/1.5) as mobile phase was used to determine colloidal TcO_2. Stability studies in physiological NaCl solution were performed up to 24 h.

2.5. Receptor binding studies and data analysis

Radioligand binding studies were performed according to a published procedure [10]. Briefly, rat cortex membranes were diluted to 50 μg protein/tube with binding buffer (50 mM Tris HCl pH7.5; 2 mM $MgCl_2$; 0.5% BSA; 5 μL/5 mL aprotinin). Binding assay consisted of 50 μL with 100 000 cpm radioligand and 50 μL buffer or increasing concentration of octreotide and 200 μL membrane suspension. The membranes were incubated for 60 minutes at room temperature with ^{99m}Tc labelled SRIF analogues per tube. Incubation was stopped by rapid filtration over glass fibre filters (Whatman GF/C, pre-soaked in 1% BSA) and subsequently washed with 4 mL of 154 mM NaCl/10 mM tris buffer (pH7.5, 4°C). Specific radioligand binding was defined as total binding minus non-specific binding. Triplicates were performed for each data point, averaged and the data were analysed by competition curve analysis.

2.6. Human serum incubation studies

25 μCi of the ^{99m}Tc labelled octreotide analogues in 100 mL physiological NaCl solutions were added to 5 mL human serum previously equilibrated in a 5% CO_2 (95% air) environment at 37°C. The solution was incubated in this environment and samples were removed and analysed at selected time intervals up to 24 h. The separation of labelled proteins

from low weight ^{99m}Tc compounds was performed by Sephadex G-50 gel filtration (1 × 19 cm) with PBS buffer as an elution medium and a flow rate of 1mL/min collecting 1mL fractions which were analysed with an automatic NaI(Tl) gamma counter. The amount of radioactivity recovered from the column was >95% of that expected. To precipitate the proteins 200 μL ethanol was added to 100 μL serum incubate and the solution was centrifuged for 15 minutes at 3300 g. The low molecular weight fractions found in the centrifugate of the precipitation were studied by HPLC for metabolites.

2.7. Animal studies

Animal studies were performed according to Swiss legislation and approved by a local animal ethical committee. Six week old female ICR nu/nu mice (about 25 g) were injected subcutaneously in the scapular region with 200 μL of cell suspension with 10^6 cells of the rat pancreatic tumour cell line AR4-2J. After three weeks the tumours were about 300 mg. The mice were co-injected intravenously with 12 μCi of the ^{99m}Tc labelled peptide and 5 μCi of [^{111}In]-DTPA-D-Phe^1-octreotide (Octreoscan). SRIF receptors of the tumour were blocked in a 4 h group of mice by i.p. of 50 mg cold octreotide 30 min before injection of the radiolabelled peptides. The mice were killed 4 and 24 h after injection of radiolabelled peptides by intraperitorial injection of 250 mg/kg pentobarbital. Samples of blood, urine and organs were removed and weighed; radioactivity was measured on an automatic NaI(Tl) gamma counter. The percentage injected dose per gram (%ID/g) for each organ and tissue was calculated. An unpaired t-test for the comparisons between the different conjugates and a paired t-test for comparisons between the different time points was performed on StatView 4.1 to determine significance of biodistribution of ^{99m}Tc/EDDA/HYNIC-OCT, ^{99m}Tc/tricine/HYNIC-OCT, ^{99m}Tc/edda/HYNIC-TOC, ^{99m}Tc/tricine/HYNIC-TOC and ^{111}In Octreoscan. The chemical form of the labelled peptide in urine was studied by HPLC.

2.8. Kit formulation

A kit formulation was developed for the labelling of HYNIC-TOC with ^{99m}Tc(tricine)$_2$. A 1 mL solution containing 15 mg (84 μmol) tricine and 17.5 mg (15 nmol) HYNIC-TOC and 50 μg $SnCl_2$ (10 μL of a 22.2mM $SnCl_2xH_2O$) was sterility filtrated through a 20 μm filter Millex-GS (Millipore, Molsheim, Frankreich) in a sterile glass vial. The solution was immediately frozen with liquid nitrogen afterwards. The frozen solution was lyophilized and was closed later under vacuum.

Labelling was performed by incubating up to 1.85 GBq (50 mCi) $^{99m}TcO_4^-$ in 1 mL physiological NaCl solution at room temperature for 60 min. Quality control was done by HPLC and TLC (silica gel 60 with pyridine/acetic acid/water 5/3/1.5 as eluent).

3. RESULTS

3.1. Synthesis

The peptide-HYNIC synthesis was performed in solution by coupling 6-Boc-hydrazinopyridine-3-carboxylic acid (Boc-HYNIC) to e-Lys^5(Boc)-octreotide or e-Lys^5(Boc), TOC with the coupling reagent HATU [28] and diisopropyethylamine as a base. The intermediate BOC protected HYNIC peptide was not isolated but deprotected using a mixture of TFA/thioanisole/water (92/6/2) as deprotecting mixture. HYNIC-OCT and HYNIC-TOC were synthesized in overall yields of 20% or 41%, respectively, and a purity of >95% based on HPLC. The exact mass was determined by electronspray mass spectroscopy.

3.2. Peptide radiolabelling and quality control

Both HYNIC peptide derivatives could be labelled using tricine or ethylenediamine-N,N'-diacetic acid (EDDA) as co-ligand with high radiochemical purity (>98%) at a specific activity of 2.4Ci/μmol for EDDA as co-ligand and 6.5Ci/μmol for tricine for both conjugates at room temperature; the kit labelling at 100°C allowed labelling at 16 Ci/μmol (Table I). The radiochemical purity was checked by HPLC for TcO_4^- and by TLC for TcO_2. Analysis of the ^{99m}Tc labelled peptide analogues using system A demonstrated radioactivity peaks for ^{99m}Tc/EDDA/HYNIC-OCT, ^{99m}Tc/tricine/HYNIC-OCT, ^{99m}Tc/EDDA/HYNIC-TOC, ^{99m}Tc/tricine/HYNIC-TOC with retention times of 15.4, 16.7, 14.9 and 15.5 min, respectively. The ^{99m}Tc/tricine/HYNIC-TOC showed with HPLC up to 5 broad peaks as the ^{99m}Tc/EDDA/HYNIC-TOC showed only one peak (data not shown). This indicates the existence of several isomers with tricine as co-ligand compared to EDDA. These isomers were observed by Liu, et al. also with their RGD peptides [24].

3.3. Binding studies

Binding affinities of the unlabelled HYNIC-OCT and HYNIC-TOC were determined by measuring the competition against ^{125}I-TOC on rat brain cortex membranes. The binding curves of HYNIC-OCT and HYNIC-TOC against ^{125}I-TOC were characteristic of the specific binding of a peptide to a single class of binding sites. HYNIC-OCT and HYNIC-TOC has a IC_{50} value of 1.07 ± 0.33 nM and 0.36 ± 0.08 nM (Table I). Binding studies show that the binding affinity of TOC is three times better than that of the octreotide analogue.

TABLE I. *IN VITRO* DATA OF THE SOMATOSTATIN ANALOGUES

Co-ligand	HYNIC-octreotide	Tricine	EDDA	HYNIC-TOC	tricine	EDDA
Specific activity (Ci/μmol)		6.4	2.4		6.5	2.5
Receptor affinity IC_{50} (nM)	1,07 ± 0,33	2,17 ± 0,49	1,71 ± 0,27	0,36 ± 0,08	0.68 ± ,13	0,40 ± 0,14
Activity transfer to proteins after 6 h		48%	12%		50%	10%

The IC_{50} values for ^{99m}Tc/tricine/HYNIC-OCT, ^{99m}Tc/EDDA/HYNIC-OCT, ^{99m}Tc/tricine/HYNIC-TOC and ^{99m}Tc/EDDA/HYNIC-TOC were evaluated by competition with octreotide. Fig. 1 shows competition experiments for the ^{99m}Tc labelled compounds. The IC_{50} values were 2.17 ± 0.49 nM, 1.71 ± 0.27 nM, 0.68 ± 0.13 nM and 0.40 ± 0.14 nM. No significant difference in the binding affinity of the two co-ligands can be seen. But there is a significant higher binding affinity for ^{99m}Tc-HYNIC-OCT compared to ^{99m}Tc-HYNIC-TOC. A high unspecific protein binding was found if tricine was used as co-ligand. More than 70% of 12% total binding of total radioactivity was found to be unspecific if ^{99m}Tc/tricine/HYNIC-OCT or HYNIC-TOC were used (Fig. 2). Whereas the unspecific binding shown in the experiments with ^{99m}Tc(EDDA) was 62% of 14% total binding of total radioactivity. The unspecific binding of ^{99m}Tc/tricine/HYNIC-OCT be lowered to 40% by adding 10 mM tricine to the binding medium (Fig. 2). The reason for this high unspecific uptake is a relatively high transfer of the activity to proteins.

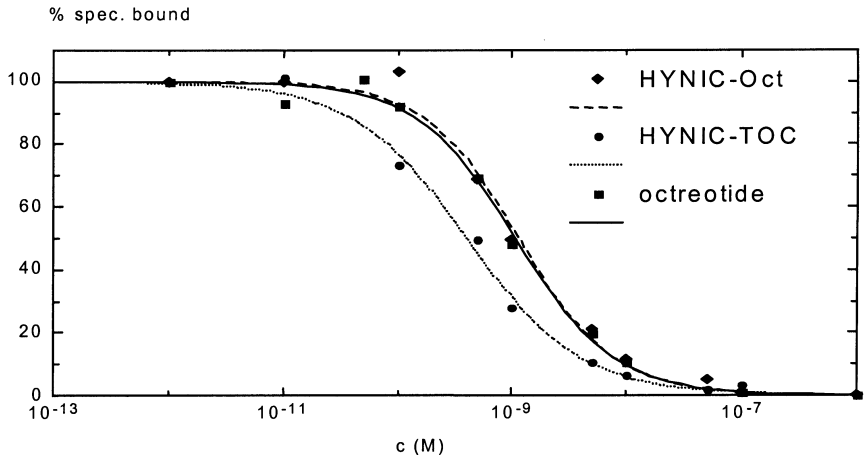

FIG. 1. Competition experiment on sstr2 positive rat cortex membranes with HYNIC-Oct, HYNIC-TOC and octreotide.

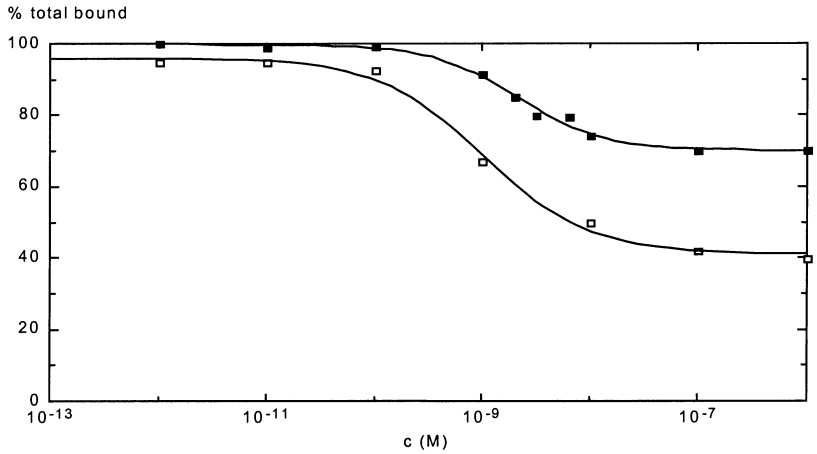

FIG. 2. Competition experiment of ^{99m}Tc/tricine/HYNIC-TOC on rat cortex membrane with the normal incubation media and with the incubation media/10 mM tricine.

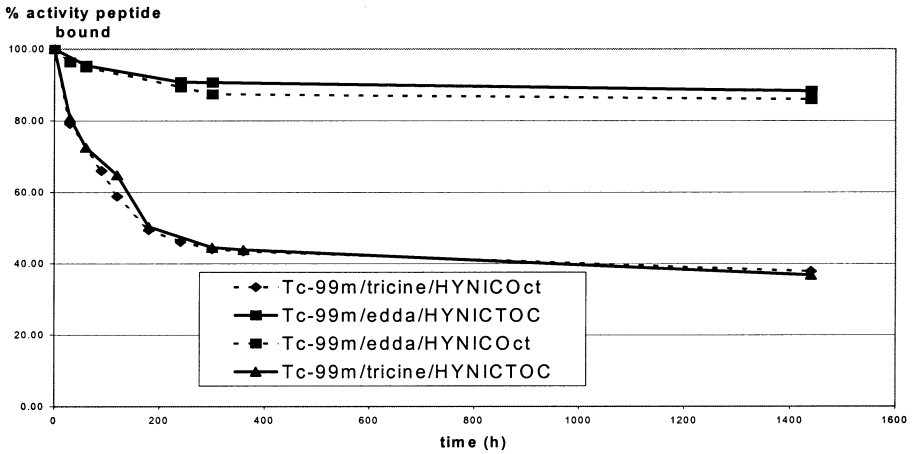

FIG. 3. Activity transfer to proteins of different ^{99m}Tc-HYNIC derivatives.

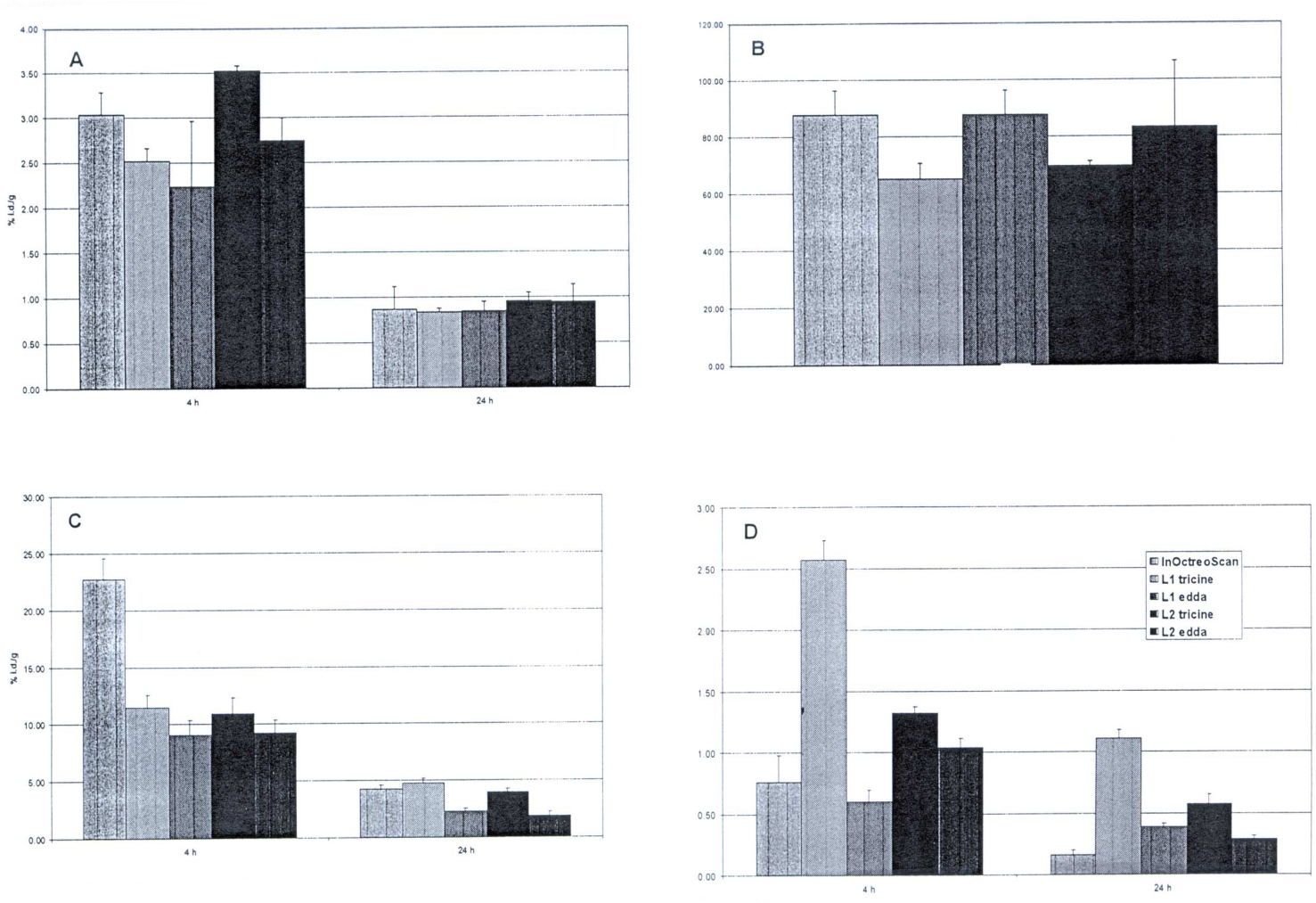

*FIG. 4. Uptake of the labelled octreotide derivatives in different organs in tumour bearing mice (**A** tumour; **B** specific uptake in the tumour; **C** kidney uptake; **D** liver uptake).*

3.4. Human serum incubation studies

Results of the human serum incubation studies with the four ^{99m}Tc labelled somatostatin analogues are shown in Table I/Fig. 3. There is a significant difference between the ^{99m}Tc labelled HYNIC-OCT and HYNIC-TOC with the same co-ligand. Both labelled peptides show a ^{99m}Tc transfer to the proteins of 48% with tricine as co-ligand and only about 10% with EDDA within 6 h. The choice of the co-ligand has a strong influence on the *in vitro* stability of the ^{99m}Tc labelled peptide. The HPLC chromatograms of the low molecular weight fraction of the precipitation show that there is no degradation of the peptide (data not shown).

3.5. Biodistribution

The results of biodistribution of the four ^{99m}Tc labelled somatostatin derivatives and OctreoScan-111 in tumour bearing nude mice at 4 h and 24 h post administration as well as the experiment with octreotide pre-injected animals at 4 h p.i. are presented in Table II and Fig. 4 as the percentage of the injected dose per gram.

TABLE II. BIODISTRIBUTION DATA OF THE FOUR ^{99m}Tc LABELLED SOMATOSTATIN DERIVATIVES AND OCTREOSCAN-^{111}In A TUMOUR BEARING NUDE MICE MODEL (4 H AND 24 H P.I.)

Octreotide analogue	HYNIC-TOC		HYNIC-TOC		HYNIC-TOC		HYNIC-TOC			Octreoscan-111
co-ligand	tricine		EDDA		tricine		EDDA		-	
time (p,i,)	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h
blood	1,02 ± 0,03	0,34 ± 0,02	0,54 ± 0,11	0,36 ± 0,02	0,73 ± 0,07	0,25 ± 0,04	0,78 ± 0,13	0,24 ± 0,00	0,40 ± 0,16	0,02 ± 0,003
liver	2,57 ± 0,16	1,11 ± 0,07	0,61 ± 0,9	0,39 ± 0,02	1,33 ± 0,10	0,58 ± 0,15	1,04 ± 0,08	0,29 ± 0,03	0,76 ± 0,22	0,17 ± 0,04
pancreas	0,75 ± 0,07	0,46 ± 0,03	0,46 ± 0,13	0,18 ± 0,01	1,68 ± 0,49	0,61 ± 0,19	1,55 ± 0,19	0,47 ± 0,03	0,78 ± 0,06	0,15 ± 0,02
spleen	0,65 ± 0,01	0,32 ± 0,05	0,38 ± 0,10	0,16 ± 0,05	0,55 ± 0,11	0,37 ± 0,08	0,36 ± 0,03	0,10 ± 0,02	0,36 ± 0,09	0,08 ± 0,01
adrenals	0,92 ± 0,09	0,61 ± 0,09	1,33 ± 0,21	0,27± 0,04	1,93 ± 1,15	0,71 ± 0,05	2,07 ± 0,33	0,48 ± 0,21	1,81 ± 0,13	0,66 ± 0,13
kidney	11,50 ± 1,06	4,74 ± 0,43	9,09 ± 1,31	2,33 ± 0,24	10,98 ± 1,38	0,25 ± 0,08	9,26 ± 1,11	1,89 ± 0,39	22,75 ± 1,81	4,28 ± 0,33
muscle	0,47 ± 0,05	0,20 ± 0,01	0,20 ± 0,07	0,18 ± 0,04	0,43 ± 0,06	0,20 ± 0,08	0,37 ± 0,04	0,14 ± 0,01	0,11 ± 0,02	0,03 ± 0,01
tumour	2,52 ± 0,14	1,46 ± 0,11	2,23 ± 0,73	0,85 ± 0,11	3,53 ± 0,13	0,96 ± 0,19	2,74 ± 0,26	0,95 ± 0,19	3,03 ± 0,25	0,87 ± 0,06
tumour blocked	0,88 ± 0,01		0,27 ± 0,06		1,07 ± 0,03		0,46 ± 0,22		0,37 ± 0,07	
tumour/bl	2,48	4,25	4,13	2,36	4,81	3,83	3,54	3,89	7,5	43,45
tumour/liv	0,98	1,32	3,54	2,18	2,66	1,66	2,65	3,28	3,98	5,19
tumour/m	5,31	7,38	11,15	4,72	8,18	4,86	7,32	6,59	27,99	26,21

The tumour uptake for all labelled somatostatin derivatives is reasonably high and specific ($p < 0.01$). The unspecific uptake for the ^{99m}Tc labelled compounds after 4 h with tricine as co-ligand is 30%-35%, with EDDA 12%–17% and for the ^{111}In labelled OctreoScan 12%. The ^{99m}Tc labelled HYNIC-TOC shows with both co-ligands a higher absolute tumour uptake 4 h p.i. (EDDA: 2.74 ± 0.26% i.D./g; tricine: 3.53 ± 0.13% i.D./g) than the corresponding labelled HYNIC-OCT conjugate (EDDA: 2.23 ± 0,73% i.D./g; tricine: 2.52% ± 0,14 i.D./g).

The main differences between the ^{99m}Tc and the ^{111}In labelled compounds can be seen in the kidney retention. It is in the same range (9.1%i.D./g–11.5%i.D./g) for all ^{99m}Tc labelled compounds after 4 h but is statistically significantly lower than Octreoscan-111 (22.8%i.D./g; $p<0.05$). The uptake is reduced to the same level after 24h for Octreoscan-111 and the two HYNIC peptides derivatives labelled with ^{99m}Tc(tricine)$_2$ but the corresponding HYNIC peptides labelled with ^{99m}Tc(EDDA) show a significantly lower retention (8.7%i.D./g; $P<0.03$).

The liver accumulation for the ^{99m}Tc labelled HYNIC-OCT analogues with tricine as co-ligand is significantly higher ($P < 0.09$) after 4 h and 24 h p.i. than the corresponding analogues labelled with EDDA as co-ligand. We observed also that the ^{99m}Tc labelled HYNIC-OCT analogue shows a significantly lower accumulation than the corresponding labelled HYNIC-TOC.

All these observations correspond to the results in the *in vitro* experiments. The labelling of HYNIC with ^{99m}Tc(tricine)$_2$ shows a higher activity transfer to proteins than labelled with ^{99m}Tc(EDDA) leading to a higher absolute but lower specific tumour uptake, a higher kidney retention and a higher accumulation in the liver as well as a slower blood clearance of ^{99m}Tc(tricine)$_2$ labelled compounds. The higher hydrophilicity and binding affinities of the TOC conjugates leads to a higher tumour uptake, a lower kidney and liver retention and faster blood clearance.

3. DISCUSSION

The aim of the study was the development of a clinically useful SRIF analogue chelator conjugate, which can be labelled with ^{99m}Tc and fulfils the criteria of stability, high binding affinity, a good target to non target ratio and favourable pharmacokinetics. We chose octreotide as the pharmacophoric peptide and HYNIC as the ^{99m}Tc binding unit. As a modification we replaced phenylalanine in three-position of octreotide by tyrosine, mainly because this modification leads to higher SRIF receptor binding affinity and higher hydrophilicity, i.e. more favourable pharmacokinetics. The two new conjugates were compared to each other and to the gold standard of SRIF receptor imaging ^{111}In DTPA-octreotide (OctreoScan-111) which was co-injected in the animal biodistribution experiments with ^{99m}Tc labelled peptides. In addition, the two new conjugates were tested for binding affinity and serum stability. The synthesis of HYNIC-OCT and HYNIC-TOC was accomplished using solution phase coupling of Boc-HYNIC to the a-aminogroup of D-Phe^1 of e-(Boc)Lys^5-octreotide and e-(Boc)Lys^5-TOC, respectively, with the powerful coupling reagent HATU. The synthesis resulted in our compounds with reasonable yields although only small amounts of starting material were used. The synthesis of HYNIC-OCT was described before [29]. Krois, et al. described an elegant [3+4] and [7+2] segment condensation as they did not succeed using a similar approach than the one described here. They noticed that the use of the N-hydroxysuccinimide ester gave only very small coupling yields indicating that the right choice of coupling reagent is of major importance. In addition

their conjugate lost binding affinity to the SRIF receptor (Virgolini abstract SNM 1996 or 1997).

Important conclusions can be drawn from the radiochemical, pharmacological and biodistribution studies: (1) the binding affinity of HYNIC-derivatized octreotide (HYNIC-OCT) and TOC is conserved and found to be in the nM range: Moreover, the TOC derivative is not only more hydrophilic but also shows higher SRIF receptor binding affinity by a factor of three, which is most likely due to the hydrogen bond forming potential of the hydroxyl group; (2) labelling of the two conjugates works equally well and shows a radiochemical yield of >97% at a specific activity of 6.4 Ci per μmole and 2.4 Ci per μmole peptide conjugate with tricine and EDDA as co-ligands, respectively. If the labelling is done by 10 min heating at 100ºC a specific activity of 18 Ci per μmole can be achieved with the kit formulated HYNIC-TOC; (3) the stability of the ^{99m}Tc-HYNIC-OCT and HYNIC-TOC is high in labelling solution with essentially no change within 12 h post labelling; (4) striking differences with regard to the stability in human serum was found if the two co-ligands were compared. With EDDA as co-ligand, the ^{99m}Tc complex showed much higher stability compared to tricine, i.e. lower transfer of the radionuclide to serum proteins indicating higher kinetic and thermodynamic stability of ^{99m}Tc-EDDA-HYNIC complex. This interpretation is corroborated by the fact that the performed ^{99m}TC-tricine-HYNIC complex converts to ^{99m}Tc-EDDA-HYNIC within 1 h even if only 0.3 equivalent EDDA compared to tricine is present in the labelling solution (data not shown).

HYNIC ligand, as a technetium complexation binding unit, was first used for infection imaging [26]. But, also, smaller molecules like chemotactic peptides were labelled with ^{99m}Tc [25, 26, 30, 31], DNA fragments [27] or cyclic peptides as a thrombus imaging agent [24, 25] with the help of HYNIC. Hnatowich, et al. [27] also observed with ^{99m}Tc labelled HYNIC oligonucleotides with glucoheptonate as a co-ligand high protein affinity. It could be shown with magnetic beads that weak co-ligand binding is responsible for the high protein affinity. Babich and Fischman [25] showed that different co-ligands for the ^{99m}Tc labelling of chemotactic peptides lead to total different clearance routes (hepatobiliary and renal) assuming it corresponds mainly to its radioactivity transfer to proteins. EDDA as co-ligand has a higher serum stability than tricine in our studies. One reason could be a lower exchange rate of the co-ligand for suitable structure of proteins. Liu, et al. [32] determined about more than ten converting isomers of a ^{99m}Tc labelled HYNIC-IIb/IIIa receptor antagonist as there are only three non-converting isomers with EDDA. Maybe the fast exchange rate between the isomers of the ^{99m}Tc(tricine)$_2$-HYNIC compound leads to a lower stability against the exchange of the co-ligand for other structures. The higher stability of the ^{99m}Tc(EDDA) labelled compounds compared with ^{99m}Tc(tricine)$_2$ leads to a superior biodistribution with a faster blood clearance, a lower accumulation in most tissues and better tumour to background ratios.

The biodistribution study of ^{99m}Tc/EDDA/HYNIC-TOC show comparable results to ^{111}In OctreoScan. The first study with a ^{99m}Tc labelled compound was done by Maina, et al. [20]. The chelator for the ^{99m}Tc was PnAO. Biodistribution studies were done in tumour bearing rats without comparison to ^{111}In OctreoScan. The labelled compound shows a high liver uptake due the lipophilicity of the ^{99m}Tc-PnAO complex. Thakur, et al. tried a direct labelling of Octreotide and Vapreotide with ^{99m}Tc via the reduced thiols of the disulfide bridge. They observed a low tumour uptake with a high liver and kidney accumulation resulting in tumour to liver or kidney ratio lower than 0.25.

4. CONCLUSION

The 6-hydrazino-nicotinic acid-octreotide and the Ty^3-analogue are peptides with high binding capacity for ^{99m}Tc. The resulting radiopeptides retain high receptor binding affinity but with a clear advantage for the Tyr^3-analogue. This advantage is also seen in the higher tumour uptake of this analogue. Despite the fact that EDDA as a co-ligand is superior to tricine we decided to develop a kit formulation with the latter because tricine is a pharmaceutical with low toxicity whereas EDDA has to be studied for toxicity first. The kit is being used successfully in the clinic at the university hospital in Bonn and performs favourably in comparison to OctreoScan.

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LABELLING AND QUALITY CONTROL OF ^{99m}TC LABELLED SOMATOSTATIN ANALOGUES

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Abstract

To standardize interlaboratory reproducibility, iodination of RC-160 with ^{125}I and direct labelling of RC-160 with ^{99m}Tc, quality control and binding assay were performed. Two conjugated peptides, HYNIC-RC-160 and MAG-3-RC-160, were synthesized. The conjugated peptides were radiolabelled with ^{99m}Tc via co-ligands; ^{99m}Tc-MAG-3-RC-160 via glucoheptonate, ^{99m}Tc-HYNIC-RC-160 via EDDA and tricine. Conditions for labelling were optimized. Analytical and purification methods for the labelled products were developed. Radiochemical purity test of ^{99m}Tc labelled peptides was performed by HPLC with gradient elution of 0.1%TFA/water and acetonitrile, or by ITLC-SG in saline and in 50%acetonitrile. The contaminants in ^{99m}Tc radiolabelled product were separated by elution from SEPPAK C-18 cartridge by 0.1% acetic acid and the pured product was eluted out of SEPPAK column by 50% acetonitrile with about 68% recovery. Stability of the purified ^{99m}Tc-MAG-3-RC-160 stored at –20°C was more than 72 h. ^{99m}Tc-MAG-3-RC-160 showed a high equilibrium dissociation constant with K_D of 26 pmole/mg protein and B_{max} of 7.9 mM.

1. INTRODUCTION

The objectives of our project were:

(1) Synthesis of HYNIC/MAG-3 conjugate of RC-160
(2) Optimizing labelling condition of the conjugated peptides
(3) Development of the purification and analytical methods for the ^{99m}Tc labelled peptide.

Attempts were made to synthesize the conjugated peptides with very high purity and yield. Chromatographic methods were used in analysing ^{99m}Tc labelled species and purification of the ^{99m}Tc labelled peptides.

2. MATERIALS AND INSTRUMENTS

RC-160 and Boc-RC-160 were prepared by Bachem UK Limited. Boc-HYNIC and HATU were distributed to participants by IAEA. S-benzoyl MAG-3 was prepared in our laboratory. Tricine, n,n-diisopropylethylamine and calcium glucoheptonate were products of Sigma. EDDA was a product of Aldrich. Thioanisol and trifluoroacetic acid were from Fluka . Sandostatin was from SANDOZ. ITLC-SG was from Gelman. SEPPAK C-18 cartridge was a product of Waters.

Waters-HPLC: 600E, UV 490E and LUDLUM model 220 with probe model 44.2 with Ultremex, reverse phase C-18, 5 μm , 4 × 250 mm. JASCO-HPLC: PU-980, UV-975 and γ-ram radioactivity detector with Crest Pak reverse phase C-18, 5 μm , 4.6 × 150 mm.

3. METHODS

3.1. Labelling of RC160 with ^{125}I

RC-160 was labelled with ^{125}I and chloramine-T was preferred for the iodination. The reaction mixture composed of 10 μg RC-160 in 0.5 M Phosphate buffer, pH7.4, 13.5 kBq of ^{125}I-NaI, and 20μl of chloramine-T (2 mg of chloramine-T in 1 mL of 0.5 M phosphate buffer pH7.4) was incubated at room temperature for 40 s. The reaction was stopped with a solution of 0.02 M TMAH in acetonitrile. Then the labelled sample was analysed by paper electrophoresis (350 volts, sodium barbitone buffer for 90 min). The labelled peptide was analysed for rat cortex membrane binding in the presence of 10 μM somatostatin analogue (Sandostatin). The experiment was carried out in triplicate.

3.2. Direct labelling of RC-160 with ^{99m}Tc

Dispense 100 μL ascorbate solution (10 mg/mL ascorbic acid and pHadjustment with 10 mg/mL sodium ascorbate) to 100 μL RC-160 (100 μg/μL 0.1 M acetate buffer pH4.2) then 40 μLof $Na^{99m}TcO_4$ was added, follow with a solution of. $Na_2S_2O_4$ in such a way that the final $Na_2S_2O_4$ concentration would be 2 μg/μL reaction mixture. The reaction mixture was heated in boiling water bath for 15 min, cooled to room temperature, checked for percentage of labelling then performed purification. SEPPAK C-18 cartridge was washed successively with 5 mL ethanol, 5 mL isopropanol, dried with 5 mL of air, then the labelled sample was loaded onto the washed cartridge and the cartridge was slowly washed successively with 5 mL deionized water, 5 mL 0.5M acetic acid and 5 mL 95% ethanol. The analysis method, i.e. ITLC-SG, in 85% MeOH, acetone, 0.9%NaCl and water and the paper electrophoresis in 0.05M sodium barbitone from 240 to 350 volts for many time intervals up to 90 min were studied.

3.3. Conjugation of bifunctional ligand to the Boc-peptide

3.3.1. Preparation of Boc-HYNIC-RC160

A solution of diisopropylethylamine, 1 μL (4.69 μmol) in 30 μL DMF, was added to a mixed solution of HATU, 0.6 mg (1.87 μmol) in 30 μl DMF, and Boc-HYNIC, 0.4 mg (1.56 μmol) in 30 μL DMF. The mixture became yellow and 80 μL of the mixed solution was transferred to the solution of Boc-RC160, 1.5 mg (1.2μmol) in 45 μL DMF/5 μL water, within 15 min. The mixture was allowed to react for 3 h, then 1 mL of water was added. The solution was transferred to the activated SEPPAK cartridge (5 mL ethanol followed by 5 mL water and dry by pushing 5 mL of air) and eluted with 0.5 mL acetonitrile. The acetonitrile solution was reduced to a volume of about 100 μL by nitrogen purging and purified again by HPLC (Ultremex C-18, 5 μm 4 × 250 mm.; 0.1%TFA/water (A) and acetonitrile (B); flow: 1 mL/min; 280 nm.; gradient:0-3 min 0%B, 13–23 min 50%B, 26–30 min 70%B, 32–40 min 0%B). The eluted product was collected and evaporated by lyophilization.

3.3.2. Preparation of benzoyl-MAG-3-Boc-RC160

Benzoyl-MAG-3-RC160 was prepared using the same procedure as HYNIC-RC160. Only the amount of reagents was changed as follows: 100 μL of solution of benzoyl-MAG-3 2.2 mg (1.5 μmol) in 100 μL DMF, solution of HATU, 0.5 mg (1.56 μmol) in 50 μL DMF, 20 μL of solution of diisopropylethylamine, 5 μL (2.25 μmol) in 150 μL DMF, and 160 μL of

the reaction mixture was added to a solution of 1.6 mg (1.28 μmol) Boc-RC-160 in 160 μL DMF/20 μL water.

3.4. Deprotection and purification of the final product

Add 10 μL of thioanisol and 150 μL of trifluoroacetic acid to the conjugated Boc-peptides and allow to react for 5 min. The solution was evaporated to dryness under a lyophilizer and redissolved in 100 μL of ethanol and 100 μL of water. The product was purified on HPLC using TFA gradient as mentioned above. The purified product was collected and dried under freeze dryer.

3.5. Determination of conjugated peptide using UV-Spectrophotometer

The dried conjugated peptide was reconstituted to final volume of 1 mL with distilled water. The solution was detected for the maximum absorption wavelength and 280 nm. was selected, The absorbance of sample was compared to standard curve of known octapeptide (Sandostatin) concentration.

3.6. Analytical methods for the ^{99m}Tc labelled peptide

The conjugated peptide (10 μg) was labelled with 135 MBq of $Na^{99m}TcO_4$. Glucoheptonate was used as a co-ligand for MAG-3-RC-160 while EDDA and tricine were used as co-ligands for HYNIC-RC-160. Samples of ^{99m}Tc-glucoheptonate, ^{99m}Tc-EDDA and ^{99m}Tc-tricine were also prepared. The labelled samples were analysed by ITL-/SG, paper chromatography (Whatman #1), paper electrophoresis and HPLC. Acetone, NSS, MEK, 85% MeOH, 50% acetonitrile, acidified ethanol (90%ethanol, 10% 0.01N HCl) and PBS were used as a mobile phase for paper chromatography and ITL-SG. Paper electrophoresis was performed in 0.05 M sodium barbitone. A gamma detector was equipped to HPLC and conditions of HPLC in analysis of labelled conjugated peptide were optimized.

3.7. Radiolabelling with ^{99m}Tc

3.7.1. MAG-3-RC160

Glucoheptonate kit, containing 10 mg of calcium glucoheptonate, 0.2 mg $SnCl_2.2H_2O$, was used in labelling. To optimize labelling conditions, the experiment was fixed with 10 μg of MAG-3-RC-160 and 185 MBq. of Tc activity of $Na^{99m}TcO_4$ for every sample. The effect of quantities of $SnCl_2.H_2O$ and glucoheptonate in glucoheptonate kit, labelling temperature, and reacting time were determined in order to obtain the highest radiochemical purity. Mole ratio of the conjugated peptide to the co-ligand was varied from 1:1 to 1:100. With the optimized mole ratio, labelling temperature was studied at room temperature up to 100° C. Finally, reacting time, from 0 to 180 minutes, was evaluated. The labelled samples were analysed by ITLC-SG using acetone, acid ethanol and saline as mobile phase.

3.7.2. HYNIC-RC-160

EDDA, tricine and glucoheptonate were studied as co-ligands for labelling. Amount of conjugated peptide, co-ligand, activity of $Na^{99m}TcO_4$ and $SnCl_2.2H_2O$ were optimized.

3.8. Purification

Four types of chromatography, i.e. SEPPAK C-18 cartridge, Sephadex G-25 column (1 × 5 cm.), Dowex 1 × 8 (0.25 mL in 1 mL syringe) and HPLC (reverse phase C-18), were evaluated for purification of labelled products. For each one, several elution systems were studied. Prior to sample loading, SEPPAK cartridge, Sephadex and Dowex were activated as follows: SEPPAK cartridge by 5 mL ethanol and 5 mL water, Sephadex by 10 mM sodium phosphate buffer pH6.8, Dowex by 0.1M HCl for 10 minutes, 3 changes of deionized water and 1 change of 0.9%NaCl pH4.5.

3.9. Stability of the radiolabelled peptide

The purified radiolabelled peptide was stored at –20°C and radiochemical purity was checked at many time intervals (0–72 h) using the standard analytical methods optimized in the above section.

3.10. Preparation of rat brain cortex membrane and Receptor Binding Assay

Rat brain cortex membrane was chosen as a source of somatostatin receptor. Three adult Sprague Dawley rats were sacrificed by decapitation, and the brains were quickly removed. The cortex was dissected and immediately placed in ice cold Hank's balanced salt solution (HBSS) pH7.5. HBSS was supplemented with 50 μL/mL stretomycin, 100 i.u./mL Ribonuclease complex (Gibco, USA) and with 10 000 Kallikrein inhibitor units/L (KIU/L) aprotinin. The cortex was then thoroughly rinsed twice with cold HBSS, cut into small pieces and minced with two surgical blades in 10 mL fresh HBSS on ice. The fine, uniform cell aggregate suspension was then transferred into two sterile 50 mL Sorvall (DuPont) test tubes and diluted with 40 mL ice cold HBSS. The tubes were then centrifuged at 500 × g for 10 min at 4ºC, supernatant was removed and placed on an ice bath. The pellet was resuspended in 20 mL homogenization buffer (25 mM Tris-buffer pH7.5) containing 0.3 M sucrose, 0.25 mM phenylmethyl sulfonyl fluoride (PMSF), 1 mM EGTA, and 10 000 KIU/L aprotinin. Using a 10 mL syringe the pellet was aspirated in and out several times and another 30 mL homogenization buffer was added. The homogenate was centrifuged as above and the pellet was homogenized in the same way for three more times, saving the supernatant after each centrifugation. The combined supernatants were then centrifuged at 48 000 xg for 45 min at 4ºC in Beckman RC-5 centrifuge using a Sorvall SS-34 rotor. Supernatant was discarded and the pellet was washed twice with 50 mM tris buffer pH7.5 containing 5 mM $MgCl_2$, 20 mg/L bacitracin, 0.25MPMSF, 100 000 KIU/L aprotinin and 1000 i.u./mL Rnase inhibitor. The final pellet was resuspended in 5 mL of washing buffer, separated into 50 μL aliquots (40 μg protein) and frozen on acetone/dry ice bath and stored at –80ºC. Membrane protein concentration was measured spectrophotometrically by Bradford's method.

3.10.1. Bradford's method

The unknown protein was aliquot in a variety of volumes then the final volume was adjusted to 0.8 mL with distilled water. Bradford's working solution (BIORAD PROTEIN ASSAY, Dye Reagent Concentrate), 200C, was added to the unknown protein solution, vortex, and incubated at room temperature for 5 min. The solution was detected by spectrophotometer at 595 nm. Concentration of protein was achieved by comparing the absorbance with standard curve of known protein concentration, Bovine serum albumin.

3.10.2. Competitive binding

The mixture, composed of 20 μg of rat cortex membrane in 10mM Tris-HCl buffer pH7.6 containing 10 mM $MgCl_2$, 30 000 cpm of radioligand, and volume, was adjusted to 100 mL with the same buffer or increasing concentration of somatostatin analogue (sandostatin). The sample was incubated at room temperature for 1 h and reaction was stopped by centrifugation at 12 000 rpm, at room temperature for 10 min. Pellets were counted by gamma counter and subsequent washed twice with 10 mM Tris-HCl containing 150 mM NaCl. Specific radioligand binding was determined by subtraction of total binding and non-specific binding. The experiment were carried out in triplicate.

3.10.3. Saturation binding

Rat cortex membrane were incubated in 10 mM Tris-HCl buffer pH7.6 containing 10 mM $MgCl_2$, increasing concentration of radiolabelled peptide, with or without 1 μM somatostatin analogue, and volume was adjusted to 100 or 200 μL. The samples were incubated at room temperature for 1 hour. The saturation radioligand binding was determined. Binding data was plotted by method of Scatchard. The maximum binding capacity (B_{max}) was calculated from the intercept on the abscissa and affinity (K_D) from the slope of the line.

3.11. Biodistribution

Biodistribution studies were conducted in rats (male, Sprague Dawley, 150 g) . The SEPPAK purified ^{99m}Tc-peptides were evaporated to dryness by nitrogen purging. The dried samples were redissolved with saline. About 4.4 MBq of the purified labelled peptide were injected intravenously to each animal. At 20, 60 and 120 min after injection, the rats were sacrificed and dissected.

4. RESULTS

4.1. Synthesis of conjugated peptides

The conjugated Boc-peptide was synthesized. Progress of reaction was detected by HPLC analysis. HPLC chromatogram of the reaction mixture was shown in Fig. 1. After three hours the reaction was almost completed. With only SEPPAK, some contaminants were still in the products therefore further purification by HPLC was done. Conjugated Boc-peptide was collected, deprotected, and purified by HPLC. The elution profile of the purified conjugated-RC-160 was shown in Fig. 2. The conjugated peptides were lyophilized, and stored at –20ºC. The reconstituted product was determined for peptide concentration by UV-spectrophotometer at 280 nm and the solution was also stored at –20ºC.

4.2. Analytical methods for the ^{99m}Tc labelled peptide

Tables 1 and 2 showed that ^{99m}Tc colloid and free $^{99m}TcO_4^-$ were at origin and solvent front, respectively, in every mobile phase used in this experiment. For paper chromatography, ^{99m}Tc peptide migrated to solvent front in all mobile phase as ^{99m}Tc co-ligand, except only in 50% acetonitrile/water, that ^{99m}Tc co-ligand could move at a rate of about 0.5. Chromatograms of samples by ITLC-SG in all kinds of solvent were almost the same, except for acetone, saline, PBS and acid ethanol. In acetone, there was only free $^{99m}TcO_4^-$ that moved to solvent front. In saline and PBS, ^{99m}Tc colloid and ^{99m}Tc peptide were still at origin while

^{99m}Tc co-ligand and free ^{99m} TcO_4^- moved to solvent front. With acid ethanol, there was only Tc colloid at the origin, the others moved to solvent front. This information was confirmed with analytical data of the filtered samples and purified products.

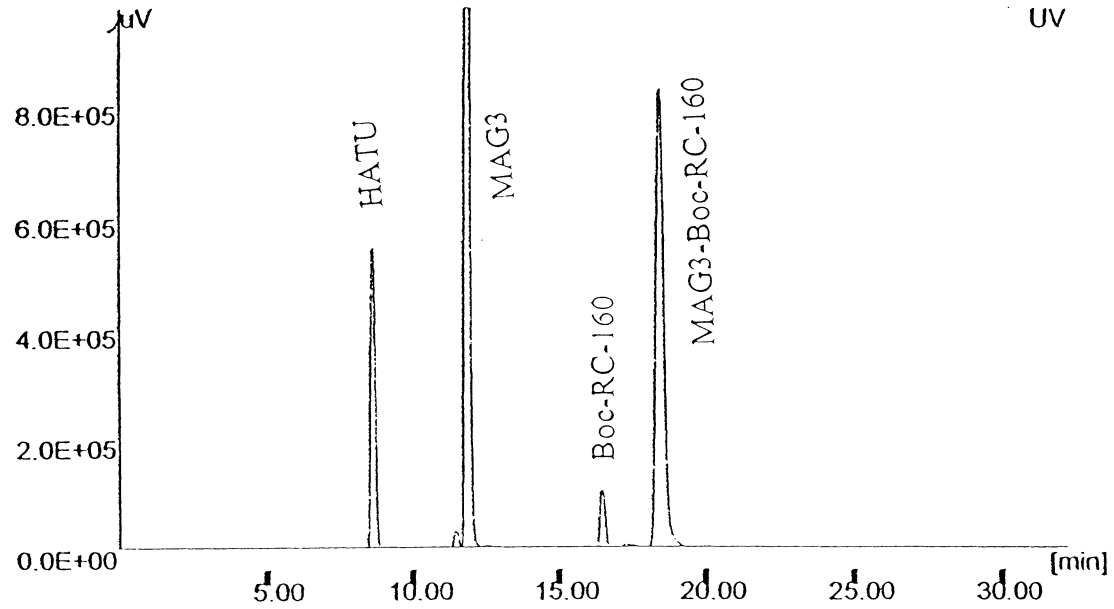

FIG. 1. HPLC chromatogram of reaction mixture of Boc-MAG-3-RC160 and Boc-HYNIC-RC160 (Crest Pak C-18, 5 μm , 4.6 x 150 mm).

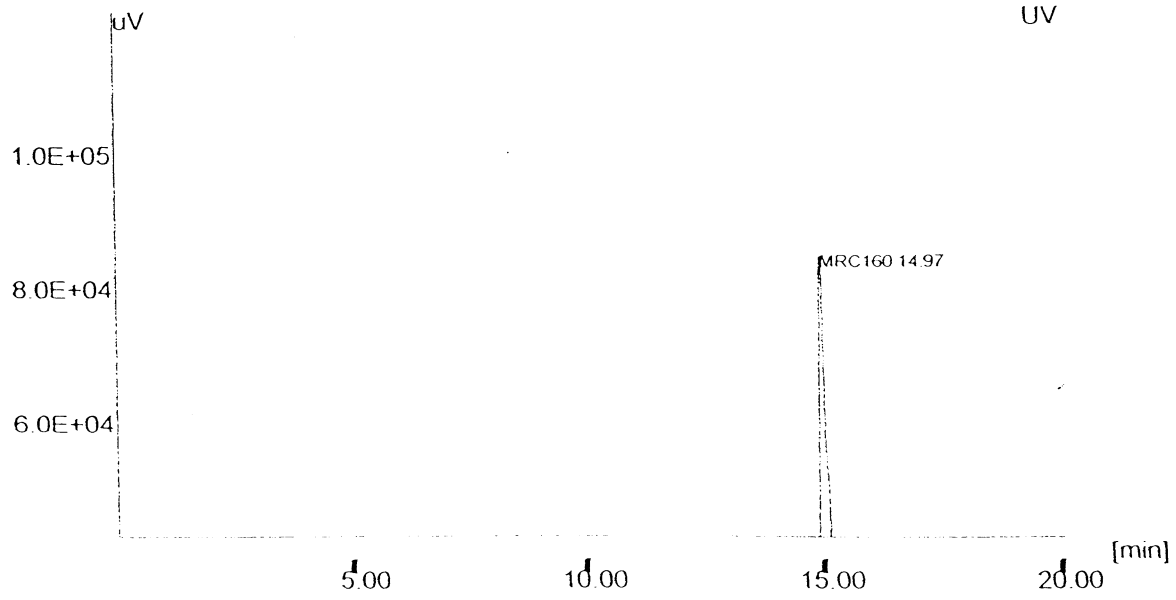

FIG. 2. HPLC chromatogram of deprotection MAG-3-RC160 (Crest Pak C-18, 5 μm , 4.6 × 150 mm).

4.2.1. Paper electrophoresis

Voltage and running time were varied. ^{99m}Tc colloid and ^{99m}Tc-peptide were still at origin while ^{99m}Tc co-ligand and free $^{99m}TcO_4^-$ moved at very close rate. It was found that at voltage of 350 V., 80 min of running time could completely separate ^{99m}Tc co-ligand and free $^{99m}TcO_4^-$ from each other as shown in Table III.

TABLE I. ANALYSIS OF LABELLED SAMPLES BY ITLC-SG

Solvent	Rf of sample			
	^{99m}Tc colloid	^{99m}Tc co-ligand	Na TcO_4	Tc conjugated Rc-160
50%CAN	0	1	1	1
Acid EtOH	0	1	1	1
CAN	0	1	1	1
Acetone	0	0	1	0
MEK	0	1	1	1
Methanol	0	1	1	1
Ethanol	0	1	1	1
PBS	0	1	1	0
Saline	0	1	1	0

TABLE II. ANALYSIS OF LABELLED SAMPLES BY ITLC-SG BY WHATMAN NO.1 PAPER

Solvent	Rf of sample			
	^{99m}Tc colloid	^{99m}Tc co-ligand	Na $^{99m}TcO_4$	^{99m}Tc conjugated Rc160
50%CAN	0	0.5	1	1
CAN	0	1	1	1
Acetone	0	1	1	1
MEK	0	1	1	1
Methanol	0	1	1	1
Ethanol	0	1	1	1
PBS	0	1	1	1
Saline	0	1	1	1

TABLE III. ANALYSIS OF LABELLED SAMPLES BY PAPER ELECTROPHORESIS (0.05M SODIUM BARBITONE, 350 V, 80 MIN)

Sample	Distance in cm		
Na $^{99m}TcO_4$	-	-	13
^{99m}Tc colloid	1	-	-
^{99m}Tc co-ligand	-	9	-
^{99m}Tc conjugated-Rc160	1	-	-

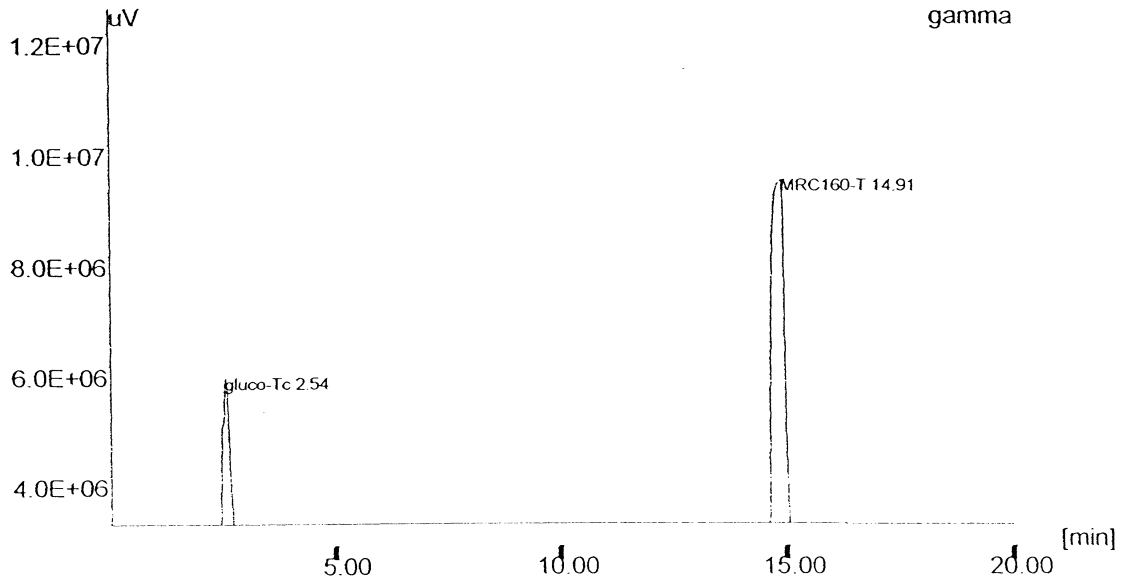

FIG. 3. Radiochromatogram of ^{99m}Tc-MAG-3-RC-160 (Crest Pak C-18, 5 μm, 4.6 × 150 mm).

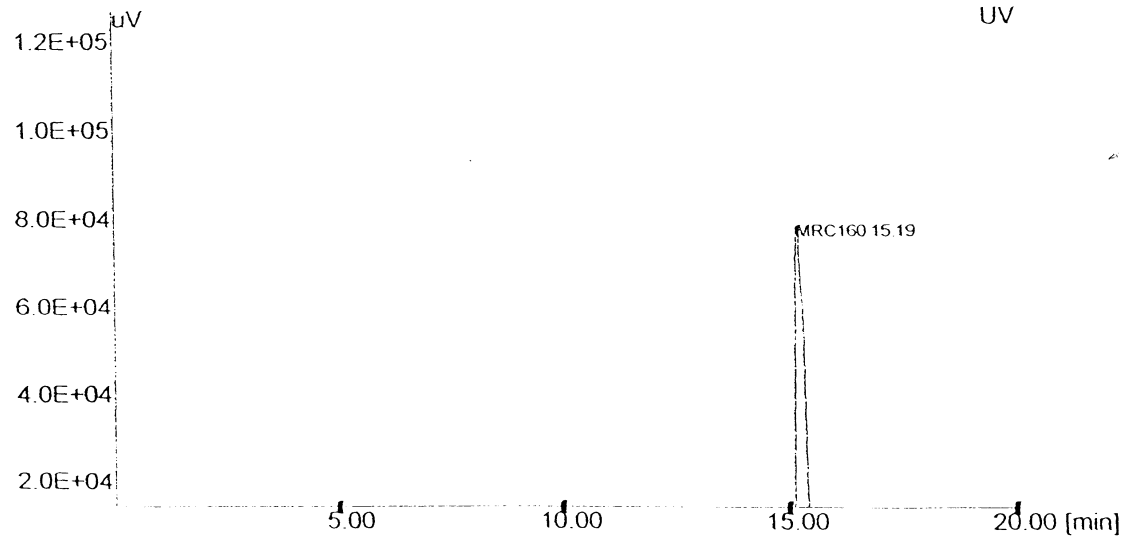

FIG. 4. UV-chromatogram of ^{99m}Tc-MAG-3-RC-160 (Crest Pak C-18, 5 μm, 4.6 × 150mm).

4.2.2. Analysis by HPLC

Variety mobile systems and gradients were studied. The satisfactory radiochromatogram of the labelled sample could be received in a gradient of 0.1% TFA/water and acetonitrile (flow: 1 mL/min, gradient: 0–3 min 0% CAN, 13–25 min 50% CAN, UV 280 nm.). The HPLC radiochromatogram and UV chromatogram were shown in Figs 3 and 4. ^{99m}Tc co-ligand and free $^{99m}TcO_4^-$ were eluted from the Crest Pak column at the same time (about 2.5 min). Although a UV detector was connected next to the HPLC column and followed with a gamma detector, the retention time of peptides as shown by the UV detector was a little bit longer than it was shown by the radioactivity detector. So there was the difference between elution of the ^{99m}Tc conjugated peptide and the conjugated peptide by this gradient system.

4.3. Optimum conditions in labelling of conjugated peptide with ^{99m}Tc

4.3.1. MAG-3-RC-160

In order to get highest percentage of labelling, the conditions for labelling were optimized. The amount of technetium species in each sample was analysed by ITLC-SG in acid ethanol (^{99m}Tc colloid), saline (^{99m}Tc colloid and ^{99m}Tc-peptide) and acetone (free TcO_4^-). Percentage of labelling of ^{99m}Tc-MAG-3-RC-160 was constant at 1 mole of MAG-3-RC-160 to 5 moles of calcium glucoheptonate as shown Fig. 5. It is seen in Fig. 6 that at room temperature ^{99m}Tc-glucoheptonate could transfer a little bit of technetium to the MAG-3-RC-160. The labelling yield was increased as the temperature increased until 80°C, after that it was decreased. The effect of reacting time is shown in Fig. 7. The labelling yield of ^{99m}Tc-MAG-3-RC-160 (at 80°C) was saturated in 30 min.

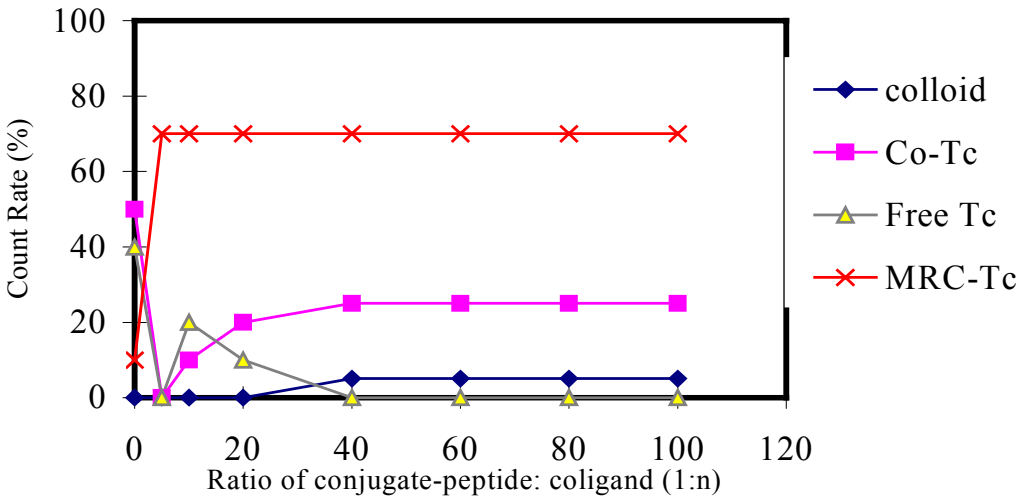

FIG. 5. Effect of mole ratio of MAG-3-RC-160 to glucoheptonate to the labelling yield.

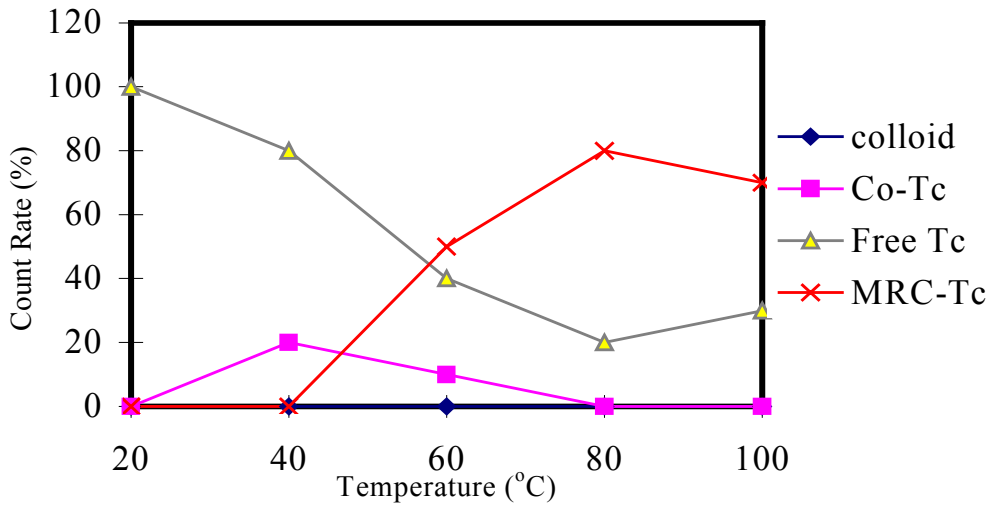

FIG. 6. Effect of temperature in labelling of MAG-3-RC-160 with ^{99m}Tc.

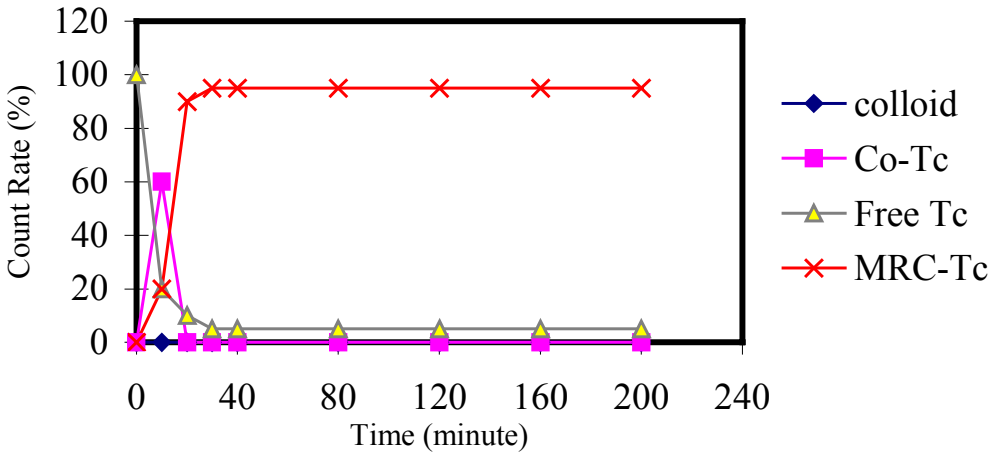

FIG. 7. Effect of reacting time in labelling of MAG-3-RC-160 with ^{99m}Tc.

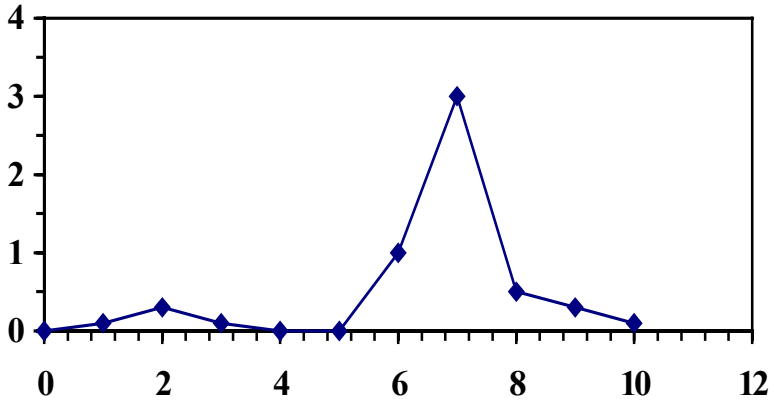

FIG. 8. Elution profile of purification by SEPPAK; AA = acetic acid and CAN = 50% acetonitrile in water.

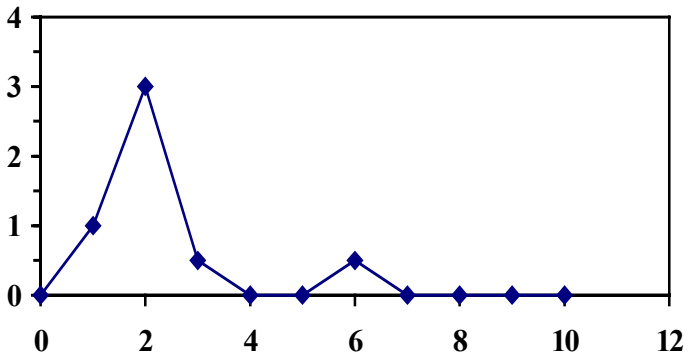

FIG. 9. Elution profile of the purification by Sephadex G-25.

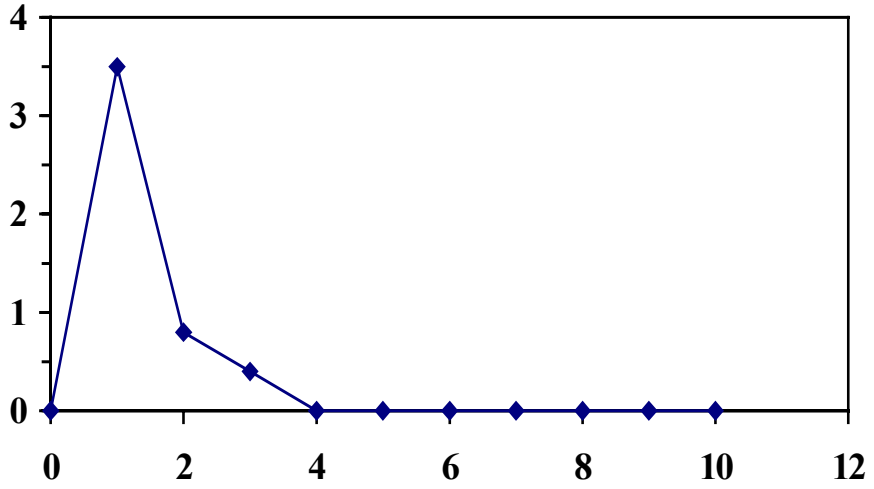

FIG. 10. Elution profile of the purification by Dowex.

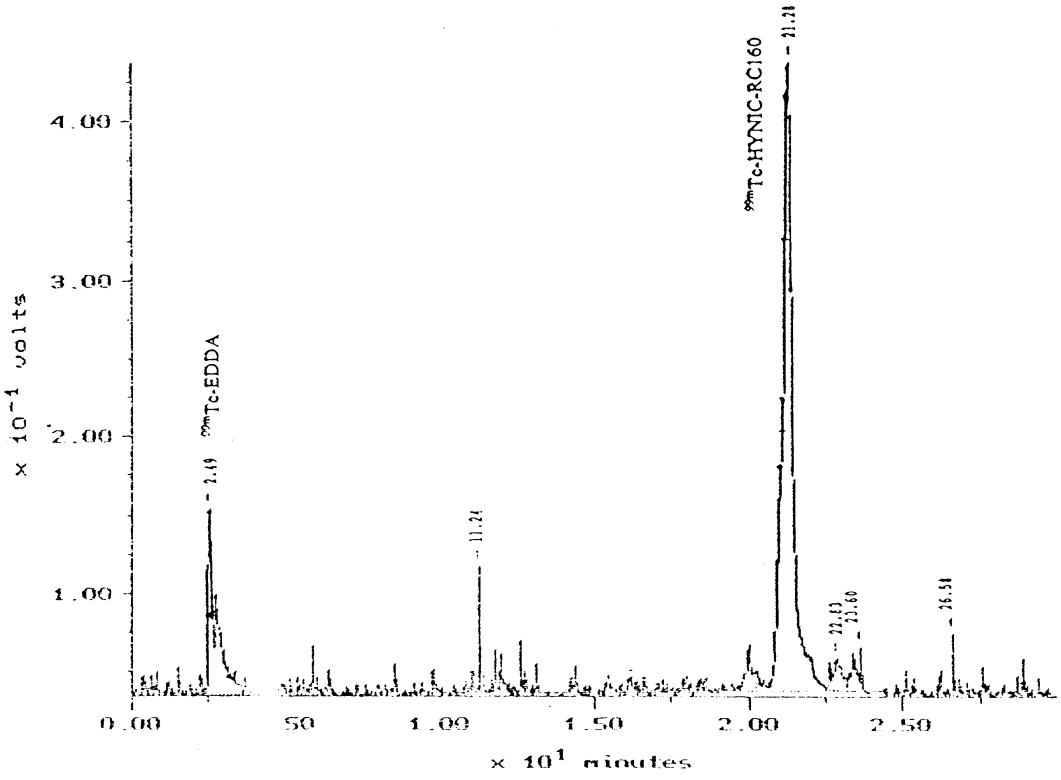

FIG. 11. Radiochromatogram of the HPLC purification of ^{99m}Tc-HYNIC-RC-160 (Ultremex C-18, 4 × 250 mm).

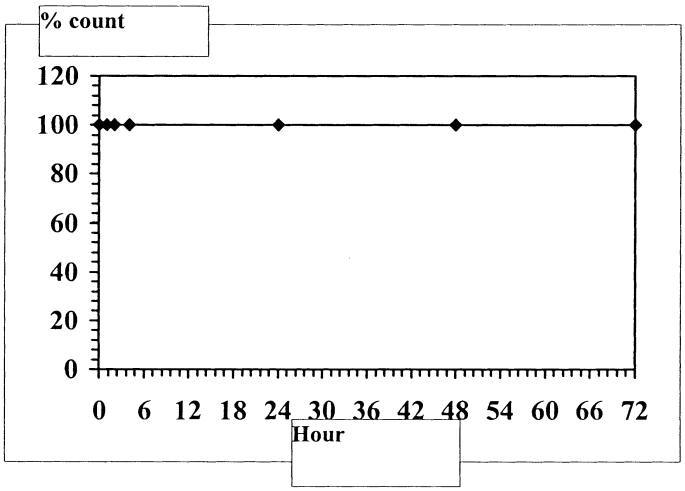

FIG. 12. Stability of radioligand were determined by HPLC.

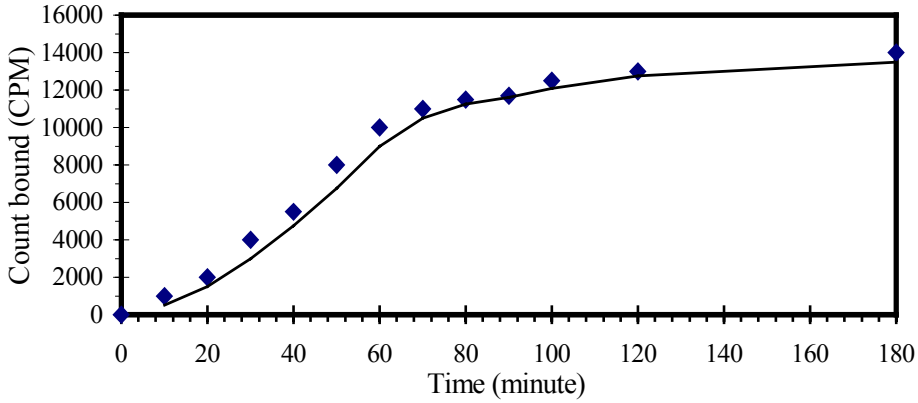

FIG. 13. Time course of specific binding of ^{99m}Tc-MAG-3-RC-160.

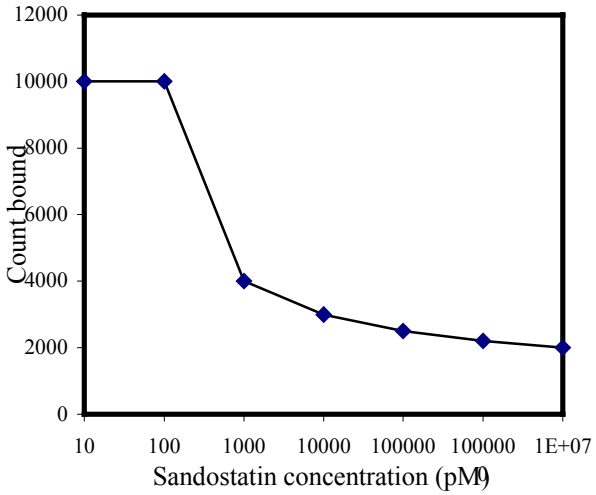

FIG. 14. Competitive binding of ^{99m}Tc-MAG-3-RC-160 in the presence of Sandostatin.

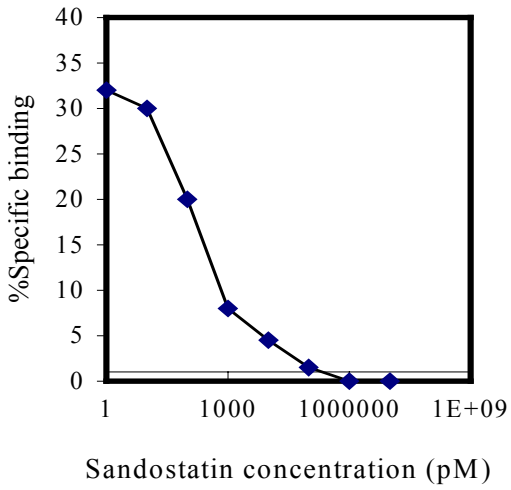

FIG. 15. Per cent specific binding of ^{99m}Tc-MAG-3-RC-160 in the presence of Sandostatin.

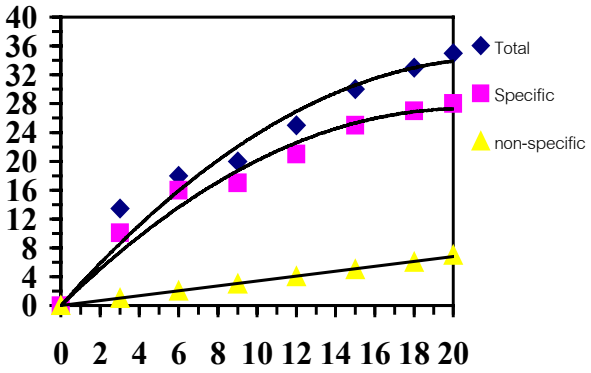

FIG. 16. Saturation curve of ^{99m}Tc-MAG-3-RC-160.

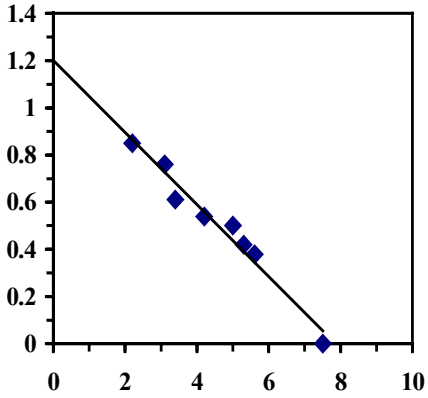

FIG. 17. Scatchard plot of data in to cortical membrane. incubated at room temperature 60 min in the presence of Sandostatin.

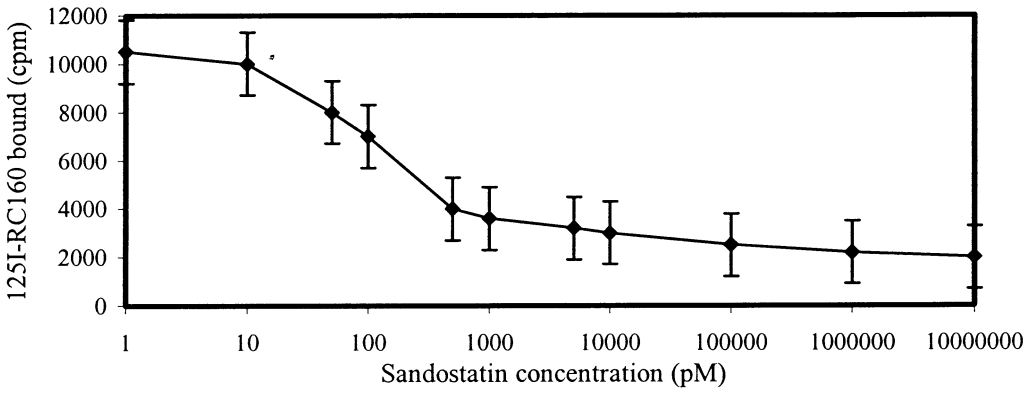

FIG. 18. Competition binding curve of 125I-RC160 to rat cortex membrane in the presence of Sandostatin.

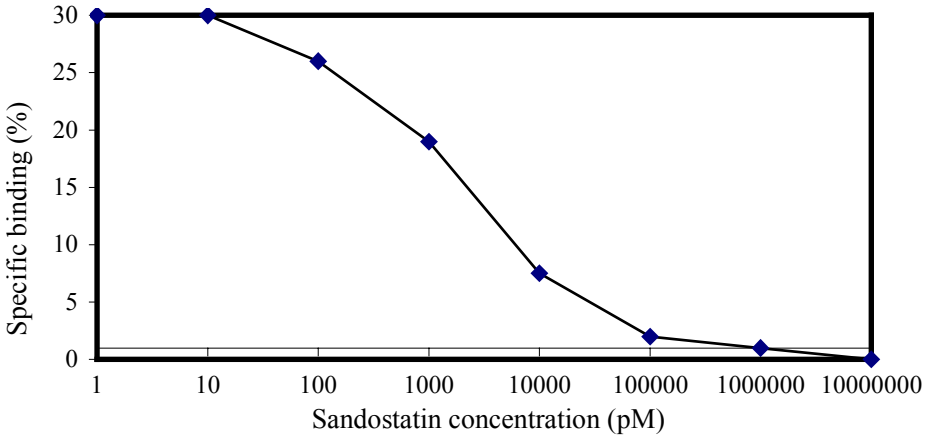

FIG. 19. %SRB of ^{125}I-RC-160 to rat cortex membrane in the presence of Sandostatin.

4.3.2. HYNIC RC-160

With the same amount of peptide (37.5 μg) and activity of sodium ^{99m}Tc pertechnetate (185 MBq) at various amounts of co-ligands, labelling temperature and reacting time. So far, the labelling yields were not satisfied, percentage of labelling of peptide with EDDA and tricine as co-ligands has been about 70% by using the following conditions: 4 μg $SnCl_2.2H_2O$, 3.2 mg EDDA or 21 mg tricine, at 80°C for 30 min.

4.4. Purification of the labelled product

The labelled products were purified on SEPPAK cartridge, Sephadex G-25 and Dowex. The eluents were analysed by ITLC-SG, PE and HPLC. The elution profile of SEPPAK purification is shown in Fig. 8. It was found that there were ^{99m}Tc co-ligand and free $^{99m}TcO_4^-$ in the 0.1% acetic acid/water eluent while it was only ^{99m}Tc peptide in the 50% acetonitrile/water eluent. The elution profile for the purification by Sephadex and Dowex are shown in Figs 9 and 10, respectively. ^{99m}Tc peptide and ^{99m}Tc co-ligand were eluted out of Sephadex column at 0–4 mL and free $^{99m}TcO_4^-$ was eluted at a volume of about 6 mL. Tc-

peptide and Tc co-ligand were eluted out of the Dowex column but the free $^{99m}TcO_4^-$ was stuck in the column.

HPLC purification was done in the same gradient conditions as for the analysis. The labelled sample was filtered to get rid of particulate contaminants. Each individual peak was collected separately and the analysis was done by ITLC-SG and paper electrophoresis. Fig. 11 shows the HPLC separation of ^{99m}Tc-HYNIC-RC-160. ^{99m}Tc-EDDA was eluted at 2.6 min and ^{99m}Tc-HYNIC-RC-160, at 21.28 min. Since there is only about 1 min difference in the retention time of the unlabelled and labelled conjugated RC-160, high specific activity product could be achieved with this gradient system.

The recovery yields of HPLC and SEPPAK purification were about 17.8% and 68%, respectively.

4.5. Stability of the purified product

The SEPPAK purified ^{99m}Tc-MAG-3-RC-160 was analysed by HPLC at various time intervals from 0 to 72 h. Prior to injection into the injector, the sample was filtered and the activity stuck on the membrane filter was checked. The stability curve of the purified product is shown in Fig. 12 . The radiochromatograms of the purified product showed only single peak which corresponded to a peak of ^{99m}Tc conjugated peptide. It could be seen that the ^{99m}Tc-MAG-3-RC-160 was stable up to 72 h.

4.6. Binding study

A representative time course of specific ^{99m}Tc-MAG-3-RC-160 binding to rat cortical membrane at room temperature was shown in Fig. 13. The non-specific binding in the presence of 10 μM of sandostatin was less than 20% of total binding. The amount of ^{99m}Tc-MAG-3-RC-160 specific binding was increasing with time and seemed to be constant within 60 min. Therefore in binding experiment performed to characterize the ^{99m}Tc-MAG-3-RC-160 binding site, incubation time of 60 min at room temperature was selected as optimum binding condition.

The competition binding curve of ^{99m}Tc-MAG-3-RC-160 to the receptor expressed by rat brain cortex membrane in the presence of sandostatin is shown in Fig. 14, with total binding about 30% (Fig. 15).

4.6.1. Saturation experiments

The concentration dependence of ^{99m}Tc-MAG-3-RC160 binding as shown in Fig. 16. ^{99m}Tc-MAG-3-RC-160 exhibited high affinity binding sites to rat cortex membrane. The Scatchad tranformations (Fig. 17) of the saturation curve was linear, demonstrates the presence of high affinity binding site for somatostatin receptor in rat cortex membrane. This experiment showed a high equilibrium dissociation constant with K_D was 26 pmole/mg protein and B_{max} was 7.9 mM.

4.7. Biodistribution studies

The biodistribution test of the sample was performed on the purified radioligands in normal rats. The biodistribution in normal rats expressed as % injected dose per organ and %

injected dose per gram of tissues (mean ± σ , n = 3) were shown in Table V and Table VI. The accumulation of ^{99m}Tc conjugated peptide in the liver up to 2 h was constant. Blood clearance was very fast. The adrenals uptake was very low in ^{99m}Tc-MAG-3-RC-160 and was negligible in ^{99m}Tc-EDDA-HYNIC-RC-160.

TABLE V. RESULTS OF BIODISTRIBUTION TEST OF ^{99m}Tc-MAG-3-RC-160

	20 min		60 min		120 min	
Organ	% per tissue	% per gram	% per tissue	% per gram	% per tissue	% per gram
Liver	34.07±1.54	4.67±0.37	29.66±0.70	4.21±0.18	26.06±0.75	4.19±0.28
Kidney	5.84±0.27	3.87±0.16	6.97±0.67	4.36±0.42	6.99±0.15	4.81±0.41
Bone	3.42±0.41	0.32±0.02	4.53±0.67	0.4±0.03	4.52±0.31	0.48±0.01
Lung	0.85±0.07	1.04±0.08	0.76±0.16	0.85±0.25	0.68±0.05	0.78±0.10
Blood	17.47±0.44	1.67±0.09	9.16±0.49	0.83±0.10	3.97±0.16	0.42±0.02
Urine	1.84±0.42	10.31±3.20	2.57±0.44	10.68±8.34	4.74±0.72	25.73±12.85
Stomach	0.32±0.05	0.16±0.01	0.20±0.02	0.10±0.02	0.20±0.02	0.18±0.01
Tot. GIT	16.72+0.78	1.32±0.01	28.6±1.80	2.30±0.19	33.69±0.46	2.93±0.21
Tail	0.93±0.01	0.04±0.00	0.91±0.16	0.20±0.04	0.76±0.13	0.18±0.08
Carcass	11.18±0.90	0.11±0.01	9.64±0.73	0.07±0.01	8.08±0.62	0.08±0.01
Spleen	0.69±0.15	1.50±0.12	1.09±0.18	2.11±0.24	1.33±0.15	0.18±0.18
Heart	0.24±0.04	0.37±0.05	0.31±0.02	0.43±0.05	0.12±0.01	0.18±0.02
Brain	0.07±0.01	0.04±0.00	0.06±0.02	0.03±0.01	0.02±0.01	0.01±0.00
Adrenals	0.02±0.00	0.43±0.07	0.03±0.00	0.70±0.16	0.00±0.00	0.00±0.00

TABLE VI. RESULTS OF BIODISTRIBUTION TEST OF ^{99m}Tc-EDDA-HYNIC-RC-160

	20 min		60 min		120 min	
Organ	% per tissue	% per gram	% per tissue	% per gram	% per tissue	% per gram
Liver	12.96±1.11	1.98±0.16	13.74±0.68	2.18±0.22	12.35±1.70	1.95±0.29
Kidney	6.95±0.63	4.59±0.38	3.18±0.29	2.14±0.20	5.05±1.31	3.61±1.07
Muscle	2.68±1.91	0.04±0.03	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Lung	7.94±0.80	8.33±0.97	8.86±0.98	10.0±0.91	8.69±2021	9.83±2.08
Blood	6.09±0.29	0.57±0.01	4.81±0.56	0.45±0.05	3.55±0.26	0.33±0.23
Urine	11.22±0.59	94.60±40.82	37.99± 2.63	139.27±6.27	22.33±2.23	169.08±97.09
Stomach	1.37±0.01	0.75±0.09	1.25±0.05	0.79±0.14	1.75±0.34	1.12±0.65
Tot.GIT	7.30±1.81	0.58±0.18	12.01± 1.72	0.96±0.12	16.83±0.68	1.32±0.05
Tail	1.22±0.51	0.36±0.21	1.01±0.63	0.24±0.14	1.01±0.38	0.25±0.09
Carcass	15.97±4.05	0.12±0.03	0.11± 0.01	4.60±6.38	10.74±3.95	0.08±0.03
Bone	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Heart	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Brain	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Spleen	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.70±0.20	1.45±0.41
Adrenals	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

4.8. Labelling of RC-160 with ^{125}I

The yield of labelling by chloramine-T was about 95%. In studying the binding assay, the inhibitory effect of sandostatin in competition to ^{125}I-RC-160 was determined. Figs 18 and 19 show that the non-specific binding and total binding of ^{125}I-RC-160 to rat cortex membrane were 10 μM and approximately 30%, respectively.

4.9. Direct Labelling of RC-160 with ^{99m}Tc

Most of technetium activity struck on the wall of reaction vial. The lower labelling yield at the longer storage time of dithionite indicated the decomposition of dithionite. The method for analysis of the labelled peptide should be by paper electrophoresis in 0.05M sodium barbitone, 350 volts, 90 min. Due to the very limited amount of RC-160, labelling could not be finished.

5. DISCUSSION AND CONCLUSION

In the iodination of RC-160 with ^{125}I, chloramine-T is preferred. The labelling yield is about 95% by paper electrophoresis (0.05M sodium barbitone, 350 V., 90 min). The total binding of ^{125}I-RC-160 to receptors expressed by rat brain cortex was about 30%.

All ^{99m}Tc species in the labelled sample could be identified by the following methods: (1) Tc colloid, ITLC-SG in 50% acetonitrile or acid ethanol (the developing time in the latter solvent is longer than in the former one. So, in analysing colloid, 50% acetonitrile is preferred.), (2) free pertechnetate ions, ITLC-SG in acetone (solvent front), (3) ^{99m}Tc co-ligand, paper electrophoresis, (4) ^{99m}Tc conjugated peptide, HPLC (1 mL/min, 0.1%TFA (A), acetonitrile (B), 0–3 min 0%B, 13–25 min 50%B). Therefore the radiochemical purity of ^{99m}Tc labelled peptides can be checked by HPLC in complement with membrane filtration or by ITLC-SG in saline and 50% acetonitrile (subtraction of percentages of count rate at origin of saline and 50% acetonitrile).

The optimum conditions in labelling of ^{99m}Tc-MAG-3-RC160 are 1 mole of MAG-3-RC160 to 5 mole of calcium glucoheptonate at 80°C for 30 min. EDDA and tricine seemed to be promising co-ligands for labelling of HYNIC-RC-160 with technetium. So far, about 70% and 50% of labelling yield were achieved with EDDA and tricine, respectively.

In general, SEPPAK is recommended as a purification method for the labelled product. For the high specific activity product, HPLC purification is preferred. The purified products should be stored at –20°C.

Amount of the conjugated peptides distributed in adrenals was very low. There might be something wrong with the product during injection. Since the labelled peptide was easily stuck to the wall of container, so what we injected into the animal was very low amount of peptide.

In binding study, the competition of ^{99m}Tc-MAG-3-RC-160 to the SIRF receptors expressed by rat cortex membrane was about 30% of the total binding. The dissociation constant was 26 pmole/mg protein and maximum binding was 7.9 mM.

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^{99m}Tc-HYNIC-SOMATOSTATIN ANALOGUES FOR IMAGING SST RECEPTOR POSITIVE TUMOURS, PRECLINICAL EVALUATION AND COMPARISON WITH ^{111}IN-OCTREOTIDE

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Abstract

HYNIC-TOC and HYNIC-RC-160 were prepared and radiolabelled at high specific activities using tricine, EDDA and tricine/nicotinic acid as co-ligand systems. Radioligand binding assays were performed on membranes prepared from receptor-positive cell lines. Biodistribution and tumour uptake were determined in AR42J tumour bearing nude mice and compared to ^{99m}Tc-MAG-3 –RC-160 and ^{111}In-DTPA-Octreotide. Specific tumour uptake between 2.4 and 9.6%ID/g was found for the ^{99m}Tc labelled HYNIC-conjugates compared to 0.2% for the ^{99m}Tc-MAG-3-RC-160 and 4.3% ID/g for ^{111}In-DTPA-Octreotide. RC-160 conjugates showed lower tumour uptake and greater hepatobiliary excretion than TOC. Tricine as co-ligand showed higher levels of radioactivity in muscle, blood and liver, while tricine/NA produced significant levels of activity in the GI-tract. EDDA showed the most promising overall biodistribution profile with similar tumour: liver and GI-tract ratios to those obtained with ^{111}In-DTPA-Octreotide, lower ratios in blood and muscle but considerably higher tumour/kidney ratios.

1. INTRODUCTION

^{99m}Tc labelling of somatostatin analogues has been previously extensively studied including attempts at direct labelling after reduction of the disulfide bridge [1, 2] and the use of bifunctional chelators such as propyleneaminoxime [1], triamidomonothiols [1], and tetramines [1, 2]. One analogue based on a carbocyclic peptide and a ^{99m}Tc N_3S-chelating moiety (P 829) have been studied extensively in clinical trials [7]. Recently Mäecke and Béhé described the use of the HYNIC core to prepare ^{99m}Tc labelled octreotide derivatives with binding affinity to SST receptors, and identified TOC as a particularly promising analogue. We have therefore evaluated this labelling approach in some detail [1, 2] for two peptides using different co-ligands based on aminocarboxylates [10] and tricine ternary ligand systems [11] and compared the results obtained with the well established N3S system. Herein we describe the preclinical evaluation of ^{99m}Tc-HYNIC-RC-160 and ^{99m}Tc-HYNIC-TOC labelled via three different co-ligands: tricine, ethylenediaminediacetic acid (EDDA) and a tricine/nicotinic acid ternary ligand system (tricine/NA), and compared the results obtained with ^{99m}Tc-MAG-3-RC-160 and ^{111}In-DTPA-Octreotide.

2. MATERIALS AND METHODS

All procedures were performed according to the standard protocols detailed elsewhere in this publication.

3. RESULTS

Radiolabelling of HYNIC-peptides could be readily performed at specific activities greater than 1Ci/μmol. Quantitative labelling (>98%) was achieved with tricine and tricine/NA as co-

ligand. When EDDA was used alone in the formulation, labelling yields of 60–70% were observed. However, incorporation of tricine as a transfer agent was able to increase the labelling efficiencies to greater than 95% (Table I). The resulting ^{99m}Tc complexes showed a high *in vitro* stability. Labelling and stability studies are described in greater detail elsewhere [8].

TABLE I. LABELLING CONDITIONS EMPLOYED AND EFFICIENCIES OBTAINED FOR ^{99m}Tc LABELLED PEPTIDE CONJUGATES. (TEN μG OF PEPTIDE LABELLED WITH 300 MBQ)

Peptide	Labelling	Labelling conditions	Mean [%]
RC-160	MAG-3	100°, 15 min	96,70
RC-160	HYNIC-tricine	RT	98,86
RC-160	HYNIC-tricine/NA	100°, 15 min	97,7
RC-160	HYNIC-EDDA	RT	69,38
TOC	MAG-3	100°, 15 min	96,02
TOC	HYNIC-tricine	RT	98,2
TOC	HYNIC-tricine/NA	100°, 15 min	98.70
TOC	HYNIC-EDDA	RT	63.60
		70°, 30 min	>95%

TABLE II. IC_{50} VALUES OBTAINED FOR UNLABELLED PEPTIDE CONJUGATES IN COMPETITION BINDING ASSAYS USING ^{125}I-SOMATOSTATIN AS RADIOLIGAND AND K_D VALUES FOR THE ^{99m}Tc LABELLED PEPTIDES IN SATURATION BINDING ASSAYS

Peptide conjugate	Co-ligand	IC_{50}	K_d
RC-160		0.38	
RC-160-MAG-3		0.36	1.46
RC-160-HYNIC		1.51	
	tricine		0.93
	tricine/nicotinic acid		1.32
	EDDA		2.25
TOC		0.50	
TOC-HYNIC		0.65	
	tricine		1.14
	tricine/nicotinic acid		2.11
	EDDA		2.65

TABLE III. BIODISTRIBUTION (% ID/GRAM, MEAN ± SD) AND TISSUE ACTIVITY RATIOS IN AR4-2J TUMOUR BEARING NUDE MICE 4 H AFTER INJECTION OF ^{99m}Tc LABELLED RC-160 CONJUGATES (N = 4)

Tissue	MAG-3-RC-160		tricine/HYNIC-RC160		tricine/NA/HYNIC		EDDA/HYNIC-RC160	
	Unblocked	Blocked	Unblocked	Blocked	Unblocked	Blocked	Unblocked	Blocked
Blood	0.30 ± 0.25	0.40 ± 0.08	1.77 ± 0.46	1.72 ± 0.17	1.05 ± 0.26	0.97 ± 0.42	0.67 ± 0.21	0.65 ± 0.08
Liver	22.37 ± 8.65	18.75 ±11.13	15.87 ± 5.01	15.42 ± 0.97	2.43 ± 0.57	3.23 ± 1.48	8.05 ± 1.44	7.49 ± 1.23
Kidney	0.94 ± 0.58	1.03 ± 0.21	13.18 ± 1.72*	18.35 ± 3.48	6.63 ± 2.71	7.89 ± 1.22	11.14 ±5.10	7.97 ± 3.34
Spleen	0.21 ± 0.15	0.27 ± 0.14	3.07 ± 1.65	3.21 ±0.70	1.25 ± 0.34	1.60 ± 0.52	2.62 ± 1.85	2.37 ± 0.61
Pancreas	0.37 ± 0.30	0.26 ± 0.19	1.46 ± 1.05	1.62 ± 0.49	0.60 ± 0.07	0.73 ± 0.54	0.43 ± 0.22	0.48 ± 0.10
Gut	34.11 ± 7.42	31.71 ± 1.99	6.27 ± 1.66	8.68 ± 1.95	9.52 ± 1.91	7.43 ± 1.39	2.80 ± 1.60	1.87 ± 0.23
Adrenals	2.35 ± 2.48	1.07 ± 0.79	2.34 ± 0.73	2.65 ± 0.06	3.72 ± 2.58	2.27 ± 2.17	1.40 ± 0.59	1.12 ± 0.20
Brain	0.03 ± 0.02	0.03 ± 0.01	0.12 ± 0.06	0.12 ± 0.03	0.08 ± 0.01	0.08 ± 0.05	0.05 ± 0.02	0.06 ± 0.02
Muscle	0.24 ± 0.20	0.38 ± 0.56	1.11 ± 0.38	1.04 ± 0.19	0.39 ± 0.27	0.69 ± 0.66	0.38 ± 0.22	0.37 ± 0.30
Tumour	0.22 ± 0.09	0.14 ± 0.08	3.54 ± 0.45 *	2.51 ± 0.38	3.25 ± 0.58*	2.24 ± 0.41	2.40 ± 1.09*	0.87 ± 0.26
TUMOUR:TISSUE RATIOS:								
blood	0.74		2.00		3.10		3.56	
liver	0.01		0.22		1.34		0.30	
GI tract	0.01		0.56		0.34		0.86	
muscle	0.95		3.18		8.29		6.36	

Note: Blocking with 50 µg Octreotide 30 min before injection. * indicates a significant difference between blocked and unblocked animals (student t-test $p < 0,05$).

TABLE IV. BIODISTRIBUTION (%ID/GRAM, MEAN ± SD) AND TISSUE RATIOS IN AR42J TUMOUR BEARING NUDE MICE 4 h AFTER INJECTION OF ^{99m}TC LABELLED PEPTIDE

Tissue	EDDA/HYNIC-TOC		NA-tricine/HYNIC-TOC		tricine/HYNIC-TOC		^{111}In-Octreotide	
	Unblocked	Blocked	Unblocked	Blocked	Unblocked	Blocked	Unblocked	Blocked
Blood	0.28 ± 0.04	0.36 ± 0.06	0.26 ± 0.08	0.28 ± 0.03	1.14 ± 0.10*	1.31 ± 0.09	0.07 ± 0.01	0.06 ± 0.03
Liver	1.06 ± 0.40	0.99 ± 0.40	0.75 ± 0.67	0.42 ± 0.19	2.08 ± 0.40	2.20 ± 0.39	0.47 ± 0.09	0.47 ± 0.19
Kidney	4.71 ± 1.38	6.67 ± 3.05	3.64 ± 0.52	4.40 ± 0.75	14.57 ± 3.42	18.15 ± 5.34	22.12 ± 6.53	23.5 ± 14.39
Spleen	0.40 ± 0.15	0.31 ± 0.12	0.18 ± 0.04	0.18 ± 0.06	1.07 ± 0.16	0.98 ± 0.04	0.16 ± 0.02	0.19 ± 0.07
Pancreas	0.45 ± 0.23*	0.26 ± 0.09	0.25 ± 0.03*	0.11 ± 0.02	1.66 ± 0.08*	0.80 ± 0.13	0.16 ± 0.07*	0.05 ± 0.02
Gut	1.58 ± 0.39	1.46 ± 0.73	3.25 ± 0.94	2.37 ± 0.51	2.32 ± 0.42	2.82 ± 0.86	0.55 ± 0.15	0.70 ± 0.50
Adrenals	0.86 ± 0.25*	0.43 ± 0.26	0.63 ± 0.23*	0.35 ± 0.08	1.80 ± 0.20*	1.24 ± 0.14	0.24 ± 0.07*	0.11 ± 0.08
Muscle	0.31 ± 0.28	0.41 ± 0.46	0.11 ± 0.08	0.08 ± 0.02	0.92 ± 0.14	0.73 ± 0.15	0.08 ± 0.07	0.03 ± 0.02
Tumour	9.65 ± 2.16*	1.82 ± 0.81	5.80 ± 2.31*	1.02 ± 0.19	9.58 ± 0.90*	3.04 ± 0.75	4.26 ± 1.00*	0.79 ± 0.25
TUMOUR:TISSUE RATIOS:								
blood	33.97		22.67		8.38		62.54	
liver	9.11		7.77		4.61		9.14	
GI tract	6.11		1.78		4.13		7.68	
muscle	31.29		51.14		10.46		52.28	
kidney	2.05		1.59		0.66		0.19	

Note: Blocking with 50µg Octreotide 30 minutes before injection. * indicates a significant ($P < 0.05$) difference between blocked and unblocked animals.

Binding assays of the unlabelled peptide conjugates in competition with ^{125}I-somatostatin performed on membranes prepared from either AtT-20 or AR42J cells showed that all peptide conjugates retained high binding affinity to the somatostatin receptor with IC_{50} values ranging from 1-2 nM as shown in Table II.

Saturation binding assays of the ^{99m}Tc labelled peptides on cell membranes prepared from AR42J cells showed that all of the radiolabelled peptide conjugates were able to bind to the somatostatin receptors with high binding affinity as shown in Table II.

Biodistribution data of ^{99m}Tc labelled RC-160 conjugates in AR42J tumour bearing nude mice are summarised in Table III. The labelled HYNIC complexes show considerably less hepatobiliary clearance than the MAG-3 conjugate which is cleared almost totally through the liver. Significant differences can also be seen between the three HYNIC labelled compounds with regard to retention in the blood and route of clearance. None of the RC-160 conjugates showed any specific binding in receptor positive normal tissues such as adrenals and pancreas, but all the HYNIC complexes showed significantly lower uptake in the tumours of animals which had been pre-treated with a blocking dose of cold Octreotide than untreated animals indicating specific receptor-mediated uptake in these tissues.

Biodistribution data of the ^{99m}Tc labelled HYNIC-TOC conjugates and ^{111}In-DTPA-Octreotide are summarized in Table IV. The uptake of these tracers was higher than RC-160 in the receptor positive tissues adrenal, pancreas and tumour and significant differences (t-Test, $p < 0,05$) in the uptake between blocked and unblocked animals were found for all the compounds tested. A higher specific, but also non-specific tumour uptake was found for all the ^{99m}Tc labelled TOC conjugates compared to ^{111}In-DTPA Octreotide.

The highest residual activity levels in all organs were found for tricine as co-ligand, especially in blood muscle and liver. EDDA and tricine/NA as co-ligands showed higher activity levels compared to ^{111}In-DTPA-Octreotide in blood, liver and gut, but considerably lower levels in kidneys. EDDA showed higher levels in muscle, liver and spleen compared to tricine/NA, but lower levels in the gut.

The lowest tumour:tisssue ratios were found for tricine as co-ligand. ^{111}In-DTPA-Octreotide showed the highest ratios especially for blood and muscle. while tumour/liver and tumour/GI-tract ratios were similar with EDDA as co-ligand). Tricine/NA showed high tumour/muscle ratios, but low tumour/GI-tract ratios. Tumour:kidney ratios were considerably higher for EDDA and tricine/NA than for ^{111}In-DTPA-Octreotide.

4. DISCUSSION

The aim of this study is the preparation of a ^{99m}Tc labelled analogue which is at least comparable to ^{111}In-DTPA-Octreotide in its ability to image somatostatin receptors *in vivo*. Such a goal requires preparation of a ^{99m}Tc tracer with good *in vitro* and *in vivo* stability, high affinity for the somatostatin receptors and a favourable pattern of biodistribution. This last parameter requires that the compound show a rapid blood clearance and low uptake in receptor negative tissues and organs of excretion. In particular, the radiopharmaceutical should be excreted predominantly through the renal system in order to avoid accumulation in the gastro-intestinal tract which would hinder the detection of tumour deposits in the pelvic area. We have previously demonstrated that both the nature of the peptide sequence and the bifunctional chelate system used can profoundly influence the pattern of biodistribution and the route of excretion [9] and we have also shown that this *in vivo* behaviour can be predicted to some extent by *in vitro* measurement of parameters such as stability, lipophilicity and protein binding. [12]

In the present study HYNIC showed a number of advantages over ^{99m}Tc labelling approaches using N_3S based ligands as a bifunctional chelator for labelling of small peptides for tumour imaging. We have shown that HYNIC peptide conjugates can be labelled at high specific activities >1Ci/μmol resulting in stable complexes with retained binding affinity to SST receptors in the nM range. The results confirm that complex stability is dependent on the co-ligand employed and also verifies our previous observation that patterns of biodistribution

can be partly predicted by the plasma protein binding behaviour. Thus, the use of tricine as a co-ligand showed the highest degree of plasma protein binding corresponding with high levels of activity in blood, liver, spleen and muscle. The two other co-ligands tested in this study showed much lower levels of protein binding, indicating a higher complex stability and correlating with considerably lower levels of activity especially in blood and muscle.

Small changes in the amino acid sequence of the peptide can also produce major changes in biodistribution. Thus, the substitution of two residues in the framework region of RC-160 with more hydrophilic amino-acids to produce TOC produces a tracer which shows a much improved pattern of biodistribution — lower hepatobiliary clearance and higher specific uptake in receptor rich tissues.

The use of EDDA as co-ligand produced the most promising pattern of biodistribution of all the ^{99m}Tc-TOC complexes explored, combining the highest degree of specific tumour uptake with the fastest blood clearance and the lowest levels of uptake in soft tissue and the gastrointestinal tract. In comparison with ^{111}In-DTPA-Octreotide a higher tumour uptake, but also higher levels in some receptor negative tissues especially blood, muscle, liver and spleen were seen. ^{99m}Tc-EDDA/HYNIC-TOC thus produced almost identical tumour/organ ratios to ^{111}In-DTPA-Octreotide in the liver and gastrointestinal tract, about half the ratios in blood and muscle, but a 10-fold higher ratio in the kidney. The lower renal accumulation of the technetium labelled conjugates may prove an advantage since, in clinical studies, the high kidney retention of ^{111}In-DTPA-Octreotide is a significant problem in imaging areas of interest in the region.

5. CONCLUSION

We have shown that the hydrazinonicotinamide conjugates of two octapeptide somatostatin analogues can be labelled with ^{99m}Tc to high specific activities and SST binding affinity. TOC was found to have a superior biodistribution to RC-160 and EDDA was found to be the most promising of all co-ligands tested. ^{99m}Tc-EDDA/HYNIC-TOC is a promising candidate for a more convenient alternative to ^{111}In-DTPA-Octreotide for imaging somatostatin receptor positive tumours in man.

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LABELLING AND QUALITY CONTROL OF SOMATOSTATIN ANALOGUES WITH ^{99m}Tc

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Abstract

Techniques and methodologies for labelling peptides with ^{99m}Tc and methods for their purification, chemical, radiochemical and biological controls were evaluated. With the purpose of gaining experience, labelling with ^{125}I was also studied. RC-160 was labelled with ^{125}I using iodogen as well as chloramine-T method. Higher yields were obtained with chloramine-T method (60%), rendering ^{125}I-peptide with 98% of radiochemical purity, with specific activity of 240 μCi/μg — 274 μCi/μg. The product was stable for five weeks (at –20ºC). For somatostatin receptors studies rat brain cortex membrane was prepared. Maximum binding capacity was 24.7% and Kaff for the binding of RC-160 to receptor was estimated as 2.0×10^{10} M^{-1}. Other peptides as ß-(2-Naphthyl)-$_D$Ala-Cys-Tyr-$_D$Trp-Lys-Val-Cys-Thr amide (N-9642, Sigma) and mouse epidermal growth factor (mEGF) were also labelled by means of limiting chloramine-T method. In case of mEGF the availability of membrane receptors allowed us to experiment in mice as well as *in vitro*. The reaction yields were up to 60% and 70% respectively. Biodistribution of ^{125}I-mEGF in a mouse with adenoma demonstrated preferential uptake in tumour (21,7% injected dose). The radioimmunoassay system gave 39% maximum binding (MB) and 50% displacement (ED_{50}) for 10 ng/mL unlabelled mEGF. Direct method and BFC's for labelling peptides with ^{99m}Tc were investigated and purification and quality controls studies were performed by TLC, HPLC (UV and gamma detection). RC-160 was labelled by a direct method using sodium dithionite as reducing agent with radiochemical purity >95%. The product was stable up to six hours (at RT). Considerable adsorption problems were observed. Biological behavior was in accordance with the compounds' lipophilicity. The synthesis of TOC conjugates with HYNIC as BFC was done with 45% ± 5% (n = 3) yield. Labelling of HYNIC-TOC with tricine as co-ligand was conducted with up to 90% yield. Studies of RC-160 labelling using ^{99m}Tc- tricine and benzoyl MAG-3 as BFC agent, were also initiated.

1. INTRODUCTION

Radiolabelled receptor specific biomolecules promise to play a major role in diagnostic as well as therapeutic applications of malignant tumors. There are several peptides which may serve as useful radiopharmaceuticals for imaging a wide range of major diseases.

As radiolabelling of somatostatin analogues have offered potential for tumor detection, the objective of this work is to develop ^{99m}Tc-based labelled somatostatin analogues. Therefore, special effort was made to gain experience in labelling techniques, mainly those involving conjugation of BFC to the peptide, as well as application of quality control procedures for evaluation of the different steps. This included preparation of rat brain cortex membranes and competitive binding as well as receptor assays.

Selected peptides were first labelled with ^{125}I and conventional quality control procedures as well as competitive binding assays were performed [1, 2].

Experimental procedures for labelling with ^{99m}Tc by direct method and BFC agents, were carried out following the experts' recommendations and IAEA lab. protocols [3-7].

2. EXPERIMENTAL PROCEDURES

2.1. Materials

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

6-Boc-Hydrazinopyridine-3-carboxilic acid (Boc-HYNIC), [Lys5-Boc] RC-160, RC-160 and [Tyr3,Lys5-Boc] Octreotide were synthesized by Bachem UK Ltd.

HATU and Tricine were provided by H. Mäcke (University Hospital, Basel, Switzerland) and by S. Mather (St. Bartholomew's Hospital, London, UK).

Iodogen was obtained from Pierce.

^{125}I-NaI high specific activity, highly concentrated, was obtained from Amersham and from CIS International.

^{99m}Tc-pertechnetate was obtained from commercial ^{99m}Mo/^{99m}Tc generator (CIS International).

S-benzoyl MAG-3 was a gift from C. Gil (Comisión Chilena de Energía Nuclear, Santiago, Chile)

Double distilled water was obtained from a bidistillation unit, Astell Scientific.

2.2. Instruments and Methods

HPLC

A solvent module (Varian Associates, model 5000) with UV detector coupled to an on-line NaI(Tl) detector, was used for HPLC analysis. In those cases in which on-line radioactivity determination was not possible, samples were collected and measured in a solid scintillation counter (Compac 120 or Ortec).

Method 1

C-18 stationary phase column (Micropak MCH-10, 4 × 300 mm, Varian Associates), at flow rate of 1 mL/min and UV detection at 280 nm were employed together with the following solvent systems:

A) MeOH/NaCl 0.15M. Gradient: 0–10 min 40%–80% MeOH, 20–25 min 80%–40% MeOH.

B) ACN/0.1%TFA/water. Gradient: 0–3 min 0% ACN, 3–13 min 0%–50% ACN, 13–23 min 50% ACN, 23–26 min 50%–70% ACN, 26–30 min 70%–0% ACN.

Method 2

C-18 stationary phase column (MCH-5 N cap, 4 × 150 mm), at flow rate of 1 mL/min and UV detection at 220 nm and 280 nm were employed together with the solvent system B) as previously indicated.

In all cases, HPLC controls were performed for the starting materials and for the products obtained in the different steps of labelling.

TLC. Instant thin layer chromatography on silica gel (ITLC-SG Gelman Sciences) was performed using different mobile phases: MeOH 85%, TCA 20%, NaCl 0.15N, MEK, ACN 50%, PBS.

Gel filtration. Sephadex G-25 Pharmacia, PD-10 columns were used for purification and/or control procedures.

Electrophoresis SDS-PAGE on 15% gels, 6 × 8 cm, 100V during 30, 45, 60 or 90 minutes. After development of electrophoresis, gels were cut and measured in a gamma well counter.

SPE purification. Solid phase extraction, using an activated C18-SEPPAK-Minicartridge (Waters Associates), was used for removal of impurities from the reaction mixtures. The sample is trapped by the cartridge matrix and after a washing with 5 mL of water, the peptide is eluted with an organic solvent.

Preparation, purification and quality control of ^{125}I labelled peptides

RC-160 was labelled by iodogen method according to the laboratory protocol prepared for the CRP. High purity nitrogen (dried by silica gel column) was used to allow evaporation of dichloromethane.

Limiting chloramine-T method was also used according to the following process. To the vial containing 1,4 µg of RC-160 (14 µL PBS 0.05M pH7.4), 10 µL PB 0,5M pH7,4 and 16,7 MBq (0,452 mCi) of ^{125}I-NaI solution, a freshly prepared chloramine-T solution (10 µL, 0,53 mM) was immediately added. The mixture was vortexed for 60 s. The reaction was stopped by the addition of 400 µL sodium acetate 0,005M. The same procedure was performed for radiolabelling the ß-(2-Naphthyl)-$_D$Ala-Cys-Tyr-$_D$Trp-Lys-Val-Cys-Thr amide (N-9642). In this case, 10 µg of peptide (10 µL PBS 0.05M pH7.4), 10 µL PB 0,5M pH7,4, 10 µL of 0,53 mM solution of chloramine-T and 9,75 MBq (0,264 mCi) of ^{125}I were used.

Ion exchange, gel filtration, electrophoresis SDS-PAGE, ITLC-SG chromatography, SPE using ethanol as organic solvent for peptide elution and HPLC (method 1A) were used as useful quality controls method for evaluation of radiolabelled products.

In order to conduct binding studies, rat brain membrane somatostatin receptors were prepared according to IAEA protocol method but with small modifications: 25mM Tris-Mg buffer was used without adding antibiotics. Tissue was dissected in cold environment, homogenization was done with a Polytron mixer and centrifugation at low g was used to avoid all big tissue debris. Further centrifugations were done at 10000 xg and 100000 xg to collect pellets for the experiments. Pellets were finally resuspended and aliquoted.

Determination of MBC was performed by challenging a fixed amount of ^{125}I-RC-160 (1×10^{-4} M, approx. 20 000 cpm) to various dilutions of receptor preparation (from 3 to 110 µL) in a reaction volume of 250 µL. Measurement of the bound activity were carried out after dilution with PBS 0,05M, pH7,4 and centrifugation at 3500 rpm during 30 minutes. NSB was estimated by evaluation of the bound activity in the presence of an infinite amount of cold peptide (1 and 50 µg).

Labelling of mEGF (MW 6053D) with ^{125}I was done by using chloramine-T method. Yield of labelling reaction was controlled by precipitation of a 1 µl aliquot with TCA 20%. Purification was performed by gel permeation. Binding capacity of the labelled molecule was analysed in a RIA system based on the use of a limiting first antibody against mEGF and second antibody as separation method. Stability was studied over three weeks.

Biodistribution was performed in a mouse bearing spontaneous adenoma. Two hours after i.v. injection of ^{125}I-mEGF the animal was sacrificed and dissected. Organs were weighted and radioactivity measured.

Selected quality control methods were applied to test *in vitro* stability of radiolabelled molecules at –20ºC and 4ºC storage temperature.

Preparation, purification and quality control of ^{99m}Tc- labelled peptides

Direct labelling of RC-160 was done using sodium dithionite as a reducing agent in the presence of ascorbic acid according to the labelling protocol (batch preparation) established for the CRP. Three different final sodium dithionite concentrations were tested: 0.02, 0.2 and 2 µg/µL. Freshly eluted pertecnetate solutions with concentrations of 12 mCi/mL-85 mCi/mL were used. Purification of the radiolabelled compound was performed by SPE in the same conditions mentioned for ^{125}I-RC-160. Quality control and *in vitro* stability (at RT) of the labelled molecule were performed by TLC with MeOH 85% and NaCl 0.15M as well as HPLC (method 1A). Biodistribution studies were performed in normal rats. The animals were injected intravenously and sacrificed one hour later. Whole organs and blood, muscle and skin samples were dissected out, weighed and counted. The results obtained were expressed as % ID/organ.

Conjugation of HYNIC-TOC was performed according to the IAEA protocol. Briefly the steps were: Boc-HYNYC (1 µmol), HATU (1,2 µmol), DEA (3,8 µmol) in 300 µL DMF were incubated for 15 min. 60µL of this solution were added to Boc-TOC (0,8 µmol) in 20 µL DMF/5 µL water and allowed to react up to one hour. To stop the reaction, 1 mL water was added and purification by SPE was done. Elution of the peptide was done with ACN (100%) in fractions of 0.5 mL. The fractions with significant evidence of conjugate were evaporated under N_2 atmosphere and concentrated to a volume of 100–150 µL. Afterwards 300 µL of TFA and 10 µL of thioanisole were added and reacted for 5–15 min. The solution was evaporated to dryness and the residue dissolved in 200 µL ethanol 50%. Analytic control of the different steps of the procedure was done using HPLC (method 1B and 2) while for purification step, HPLC method 2 was applied.

Preparation of ^{99m}Tc labelled HYNIC-TOC was done using tricine as co-ligand. The procedure was as follows: 0.5 mL Tricine (70mg/mL water), 20 µL $SnCl_2.2H_20$ (5mg/mL in HCl 0.1N) or SnF_2 (5 mg/mL in HCl 0.1N) and 1 mL $Na^{99m}TcO_4$ (20–50 mCi) were incubated for 15 min. To the peptide conjugate solution (5 µg or less) 0.5 mL of the ^{99m}Tc-tricine solution was added and allowed to react for 20 min. The product was examined by TLC and HPLC control (method 2).

Initial studies of conjugation of S-benzoyl-MAG-3-TOC was carried out as described for HYNYC-TOC as well as direct labelling of RC-160 (10 µg/100 µL) by reaction with 0.5 mL of ^{99m}Tc-tricine (30 mCi/mL) was prepared as described earlier.

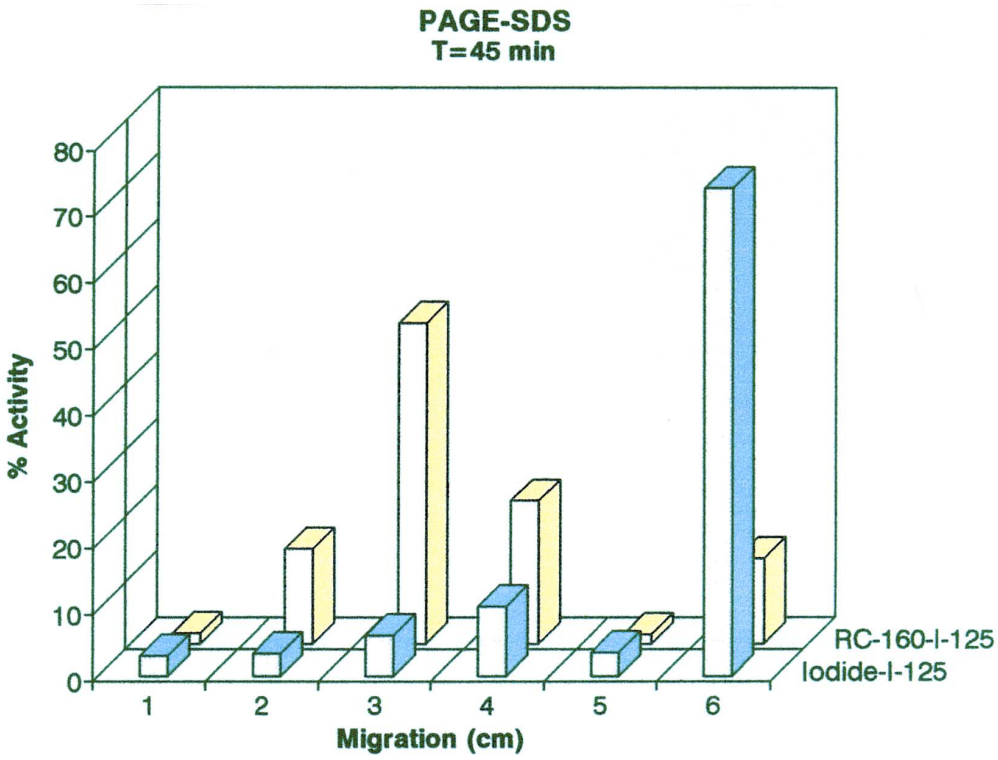

FIG. 1. Electrophoresis profile of ^{125}I-RC-160.

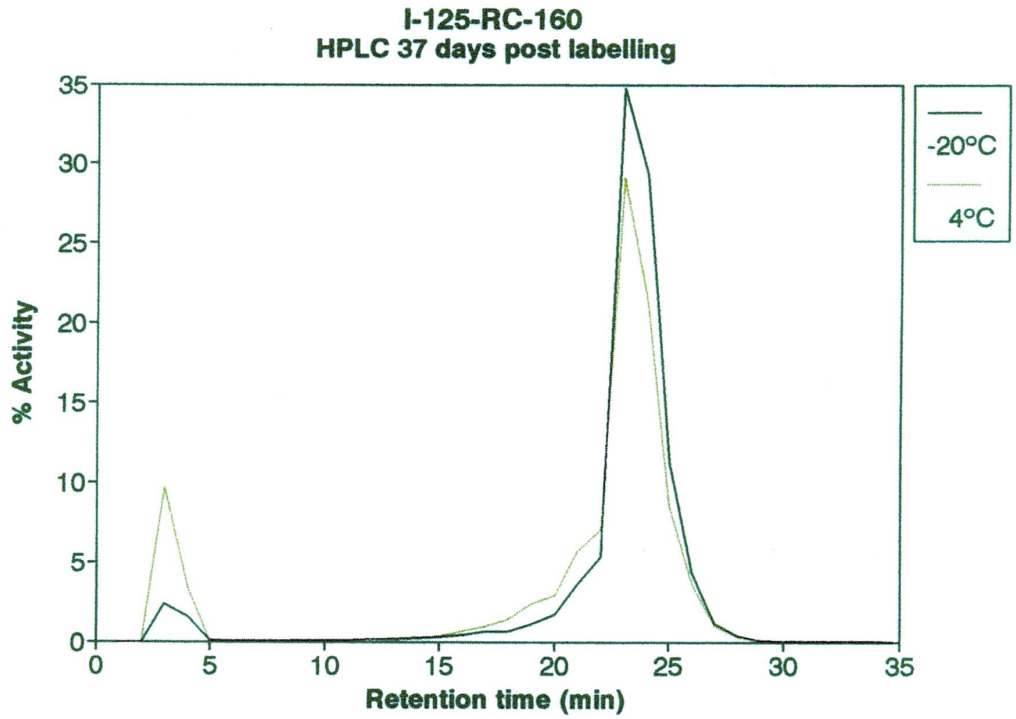

FIG. 2. Stability studies of ^{125}I-RC-160.

3. RESULTS

^{125}I-RC-160: SEPPAK cartridge was selected as the purification method. Comparable results were found for the different quality control methods except for TLC with methanol 85%. SDS-PAGE was useful to resolve labelled radioiodinated compound from free iodide with a 45 minutes run time (Fig 1). Considering the agreement among the other quality control methods, RP-HPLC and ITLC-SG/NaCl 0,9% were selected as methods (Rf $^{125}I^-$ = 0.9–1.0; Rf ^{125}I–RC160 = 0). Radioiodination of RC-160 showed higher yields when limiting chloramine-T method was used. The yields were 60% or more versus less than 50% for iodogen method. Specific activities were 240 μCi/μg–274 μCi/μg and the radiochemical purity was 98% (calculated by HPLC). Loss in the radiolabelling steps, mostly due to adsorption was up to 20%. Fig. 2 shows the typical HPLC profiles for the labelled molecule at different times post-labelling and storage conditions. It was verified that upon storage at –20ºC, the radioiodinated molecules do not have significant increase of hydrophilic impurities (4% ± 2%) during 5 weeks, but at 4ºC, an increase of up to 13% for the same elapsed time was observed.

Labelling of N-9642 peptide with ^{125}I was done with yields up to 60% with specific activity of 20 μCi/μg.

Labelling of ^{125}I-mEGF was done with yields higher than 70% (n = 4) and specific activities around 140 μCi/μg. Purification method selected for this product was gel permeation, which allowed excellent separation of free iodine. Binding assays done by means of the RIA system gave a maximum binding of 39% in absence of unlabelled mEGF and a displacement of 50% in the presence of mEGF (10 ng/mL). Stability of the labelled compound was demonstrated for longer than three weeks.

Biodistribution in a mouse with adenoma showed a preferential uptake at tumour site (21,7%ID) (Table I).

For ^{125}I-RC-160 used in binding studies on rat brain cortex receptors, NSB was estimated by evaluation of the bound activity in the presence of an infinite amount of cold peptide. Inverse of corrected bound activity was plotted against inverse of receptor volume. A straight line (r = 0.9833) was obtained and MBC of 24.7% was calculated.

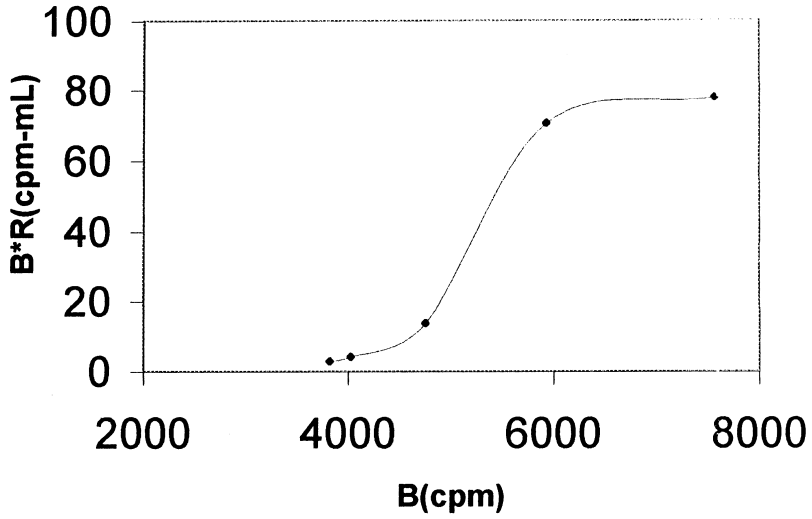

FIG. 3. Binding of ^{125}I-RC-160 to brain rat receptors.

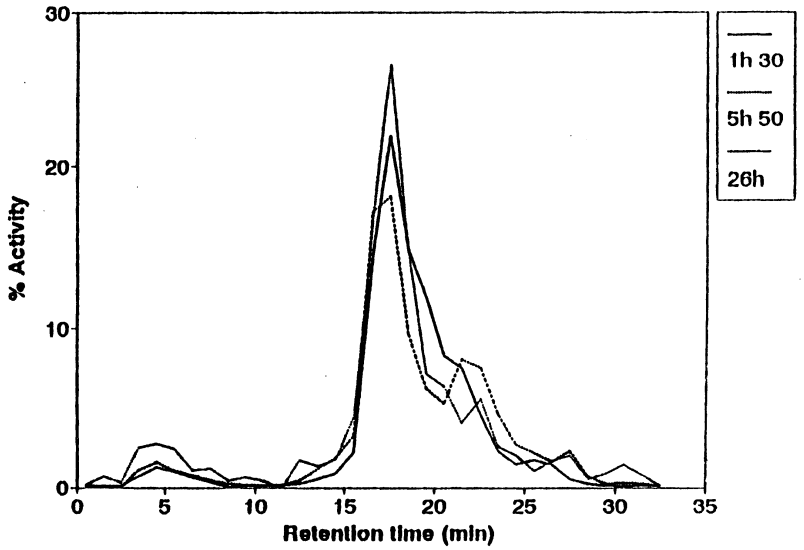

FIG. 4. Stability studies of ^{99m}Tc-RC160.

Affinity constant was estimated through saturation of receptors and application of mass action law. From the curve shown in Figure 3, saturation of the receptor was determined and 50% rate was calculated. Total bound activity corresponding to this point was determined and related to K_d. Thus K_{aff} for the binding of RC-160 to receptor was estimated as $2.0 \times 10^{10} M^{-1}$.

Labelling yield of RC-160 with ^{99m}Tc was better when high concentrations of radioactivity were used. From SEPPAK elution, more than 90% activity was recovered in the ethanolic fraction (second and third half milliliter) and less than 5% remained in water phase. The hydrophilic radiochemical impurities determined by HPLC were less than 4%, consistent with the results of TLC using NaCl 0,15M as solvent (Rf $^{99m}TcO_4^-$ = 1; Rf ^{99m}Tc–RC160 = 0). No significant difference was observed in radiochemical purity during 6 hours. At longer times, the hydrophilic species grew from less than 3% to more than 10% (24 h stored at room temperature) (Fig. 4).

Biodistribution of ^{99m}Tc-RC-160 showed rapid blood clearance (less than 0,10% ID/g at one hour) and high uptake in intestines (more than 80% of injected activity), which is in agreement with compound lipophilic characteristics (Table II). Very small or negligible activity was found in heart, thyroid, brain and adrenals.

From the experiences of conjugation of HYNIC-TOC, the HPLC method 2 was selected because column used in method 1 was unable to resolve the mixtures and UV detection at 220 nm was not reproducible.

Considering the UV detection and assuming that the retention time established for Boc-HYNIC-TOC/Boc-TOC/Boc-HYNIC are correct, yield of conjugation was 45% ± 5% (mean ± s.d.; n = 3) and this value was achieved with 20 minutes incubation time. Deprotection step analyzed by the same way demonstrated that Boc-HYNIC alone and Boc-TOC alone achieved less than 60% and 92% at 5 minutes of reaction. The analysis of mixture solution indicated that with 5 minutes incubation, Boc-HYNIC-TOC lead to less than 45% ± 5% of HYNIC-TOC (n = 3). So 15 minutes incubation time were used with more than 95% of yield. Representative RP-HPLC for the starting and final compounds are presented in Figs 5, 6, 7 and 8.

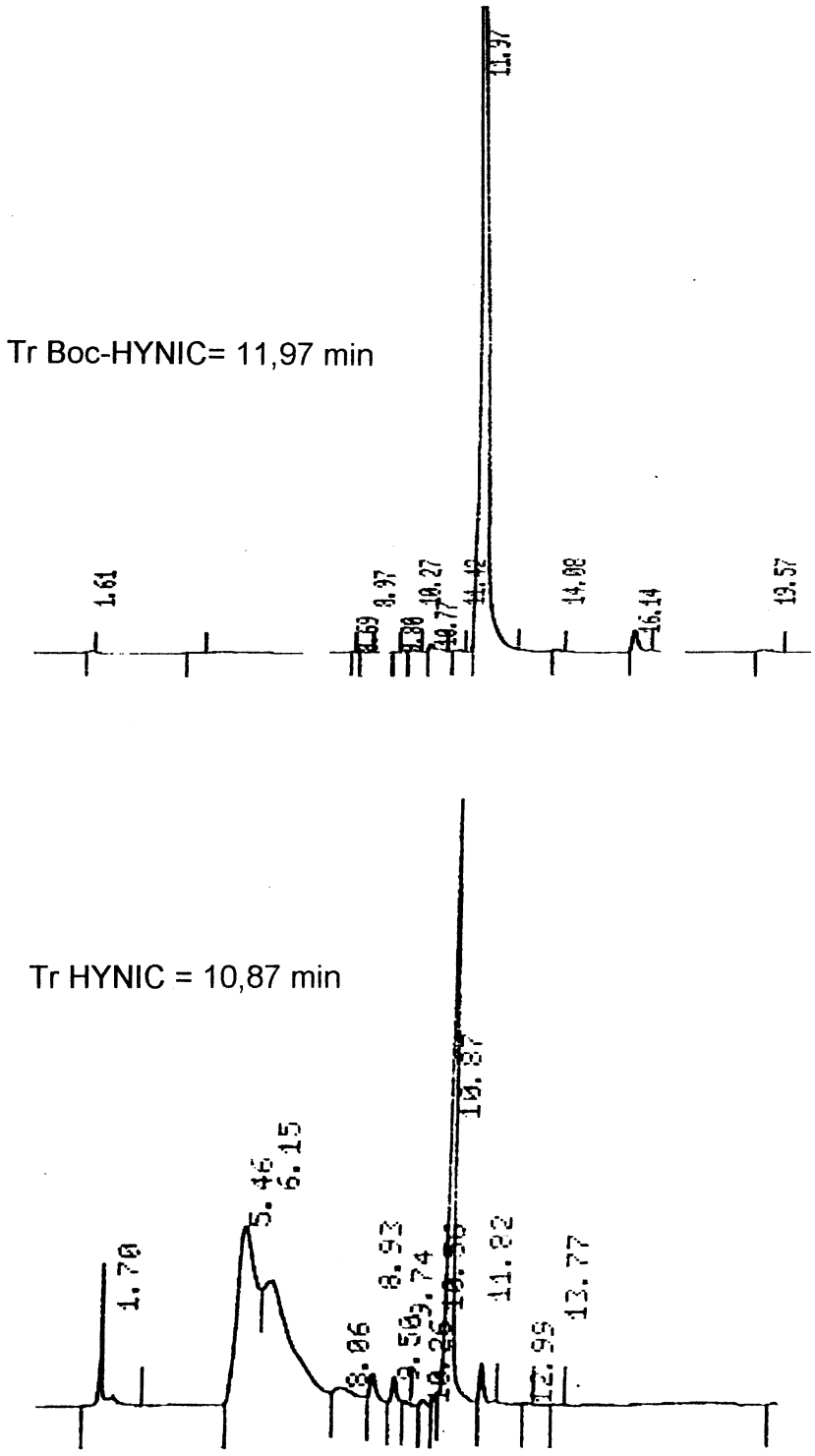

FIG. 5. HPLC profile of Boc-HYNIC and HYNIC (UV detection).

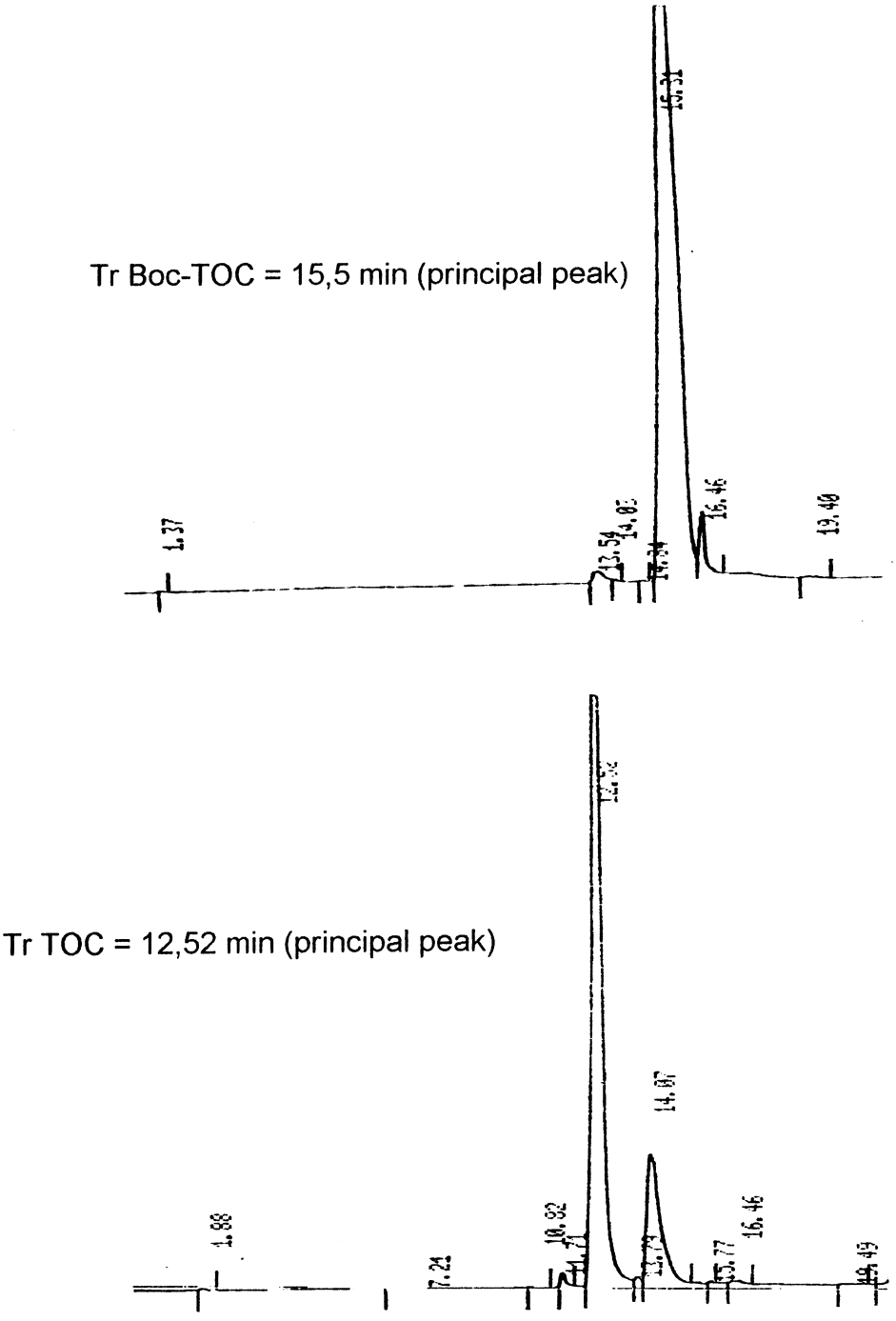

FIG. 6. HPLC profile of BOC-TOC and TOC (UV detection).

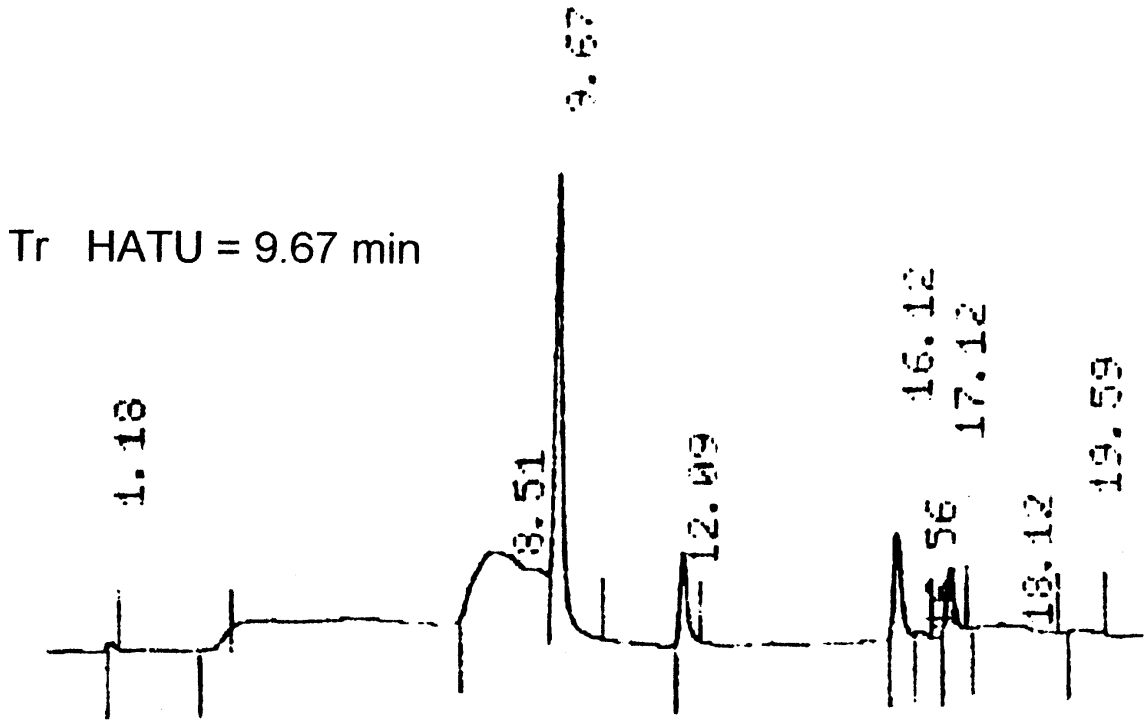

FIG. 7. HPLC profile of HATU (UV detection).

Labelling of HYNIC-TOC with tricine as co-ligand was better when stannous fluoride was used instead of stannous chloride. Therefore, TLC control with MEK of ^{99m}Tc-tricine must be done prior to peptide labelling. The yield was more than 90% with radiochemical purity about 70% (calculated by HPLC). TLC with MEK, ACN 50% and PBS were also selected for quality control of the labelled conjugate:

Rf	MEK	ACN 50%	PBS
$^{99m}TcO_4^-$	1	1	1
^{99m}Tc-tricine	0	1	1
^{99m}Tc-peptide	0	1	0
^{99m}Tc-colloid	0	0	0

Preliminary studies on direct labelling of RC-160 with ^{99m}Tc-tricine indicated that a labelled compound was obtained with TLC, which behaved as a peptide and had a HPLC retention time shorter than RC-160 (13.9 and 15.1 respectively; system 2). Future work must be done to improve and confirm that a ^{99m}Tc-labelled peptide is obtained and that binding affinity for somatostatin receptors is maintained.

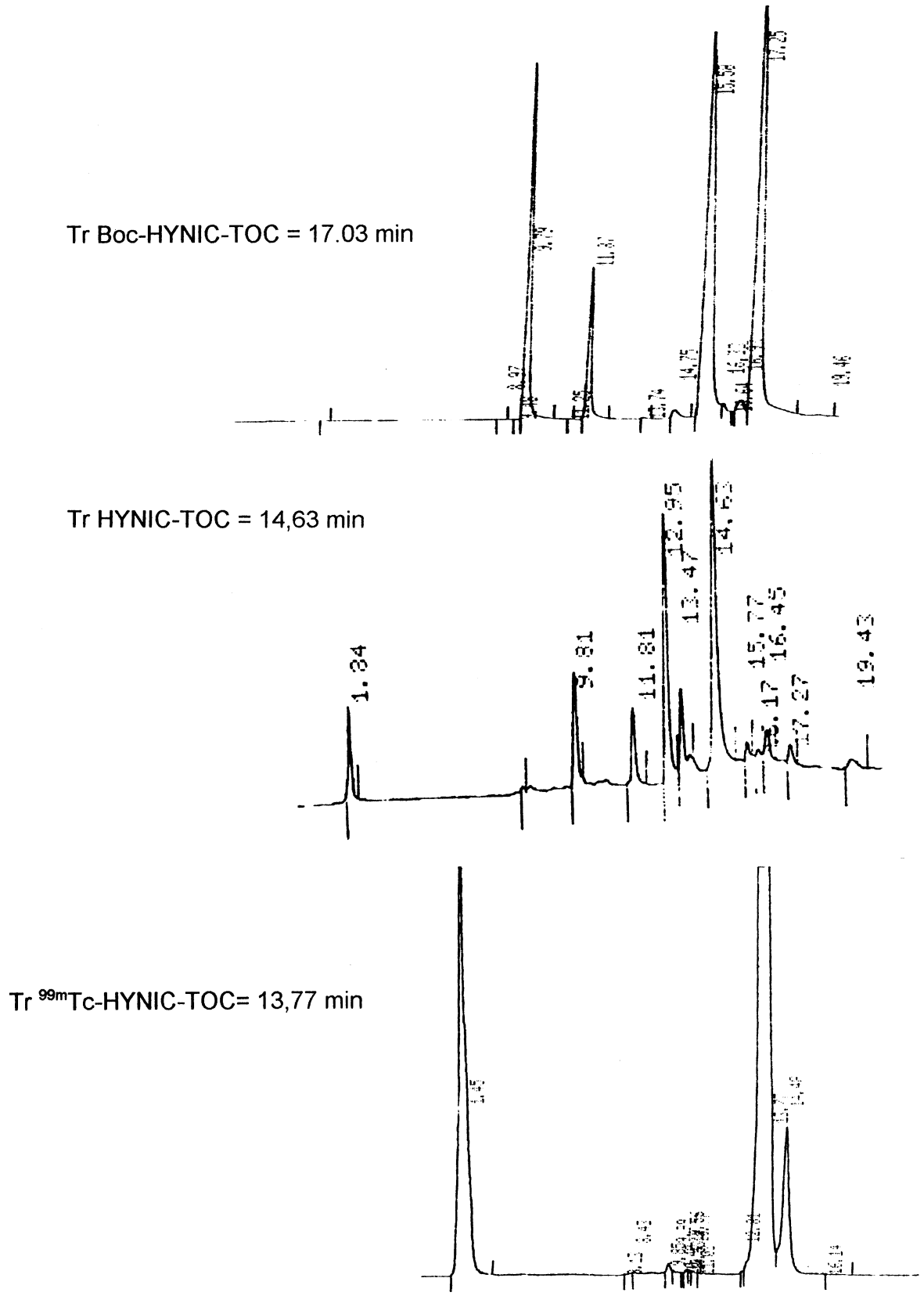

FIG. 8. HPLC profile of Boc-HYNIC-TOC conjugation (a) and deprotection (b) (UV detection) and labelling with ^{99m}Tc-tricine (c) (gamma detection).

TABLE I. BIODISTRIBUTION (% ID/ORGAN) IN A MOUSE WITH ADENOMA TWO HOURS AFTER INJECTION OF ^{125}I-MEGF

Tissue	%ID/organ
Blood	1.19
Liver	4.47
Lungs	0.99
Spleen	0.91
Kidneys	2.56
Urine	12.57
Intestine	8.91
Stomach (Content)	3.85 (27.51)
Submax	1.56
Tumour	21.70
Bladder	3.34
Uterus	1.34
Others	9.1

TABLE II. BIODISTRIBUTION (%ID/ORGAN; MEAN ± S.D. N = 4) IN NORMAL RATS ONE HOUR AFTER INJECTION OF ^{99m}Tc LABELLED RC-160

Tissue	Mean ± S.D.
Blood	1.52 ± 0.47
Liver	7.88 ± 3.2
Lungs	0.38 ± 0.08
Pancreas	0.04 ± 0.01
Kidneys	1.06 ± 0.4
Urine	2.93 ± 0.81
Muscles	2.45 ± 0.95
Intestine	81.7 ± 11.0

4. CONCLUSIONS

Different peptides were labelled with ^{125}I and/or ^{99m}Tc, leading to high radiochemical purity and high *in vitro* stability. In some cases, their biological characteristics were determined and forward to be unchanged.

At the same time, many problems in methodology procedures of peptide labelling were solved after hard laboratory work. This undoubtedly increases our knowledge in this field. Unfortunately other aspects like availability of chemicals and consumables were time consuming.

The interest created and the experience achieved through these activities encourages further research work in radiolabelling of peptides and in the field of biological and biochemical behavior.

ACKNOWLEDGEMENTS

We thank the IAEA for inviting us to participate in this CRP. We are grateful to S. Mather for the technical advice and discussions; to C. Gil for the possibility to attend a one week training course at the Comisión de Energía Nuclear, Santiago, Chile; to S. Castiglia, Comisión Nacional de Energía Atómica, Ezeiza, República Argentina and PEDECIBA-Química (Uruguay) for their support with reagents.

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^{99m}TECHNETIUM LABELLED VASOACTIVE INTESTINAL PEPTIDE ANALOGUE FOR RAPID LOCALIZATION OF TUMOURS IN HUMANS

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Abstract

In recent years, imaging tumours with receptor specific biomolecules has been the focus of increasing interest. VIP has a high affinity for specific receptors that are expressed in high density on a large number of malignant tumours. VIP was modified (TP 3654) without compromising its biological activity, and labelled with ^{99m}Tc. Pharmacokinetics and feasibility studies were performed in three normal volunteers and 11 patients with a history of cancer. Imaging was performed for up to two h post-injection. Within 24 h after injection of ^{99m}Tc-TP 3654 (10-15 mCi/5 μg), approximately 70% of the tracer cleared through the kidneys, and 20% through the liver. Blood clearance was rapid. No adverse reaction was noted in any subjects. All known tumours were clearly delineated within 20 min. Findings were compared with the results of ^{99m}Tc-MIBI, CT, MRI, or histology. There was concordance in nine patients. In the other two, only the VIP scan was positive for tumours known to express VIP receptors. The early results of imaging tumours with ^{99m}Tc-VIP are promising and warrant further studies.

1. INTRODUCTION

Vasoactive Intestinal Peptide is a 28 amino acid peptide first characterized more than a quarter of a century ago. A neuroendocrine mediator with a broad spectrum of biological activities, VIP stimulates secretion of various hormones and promotes growth and proliferation of normal and malignant cells. The action is mediated by cell surface membrane receptors, $VIPR_1$ and $VIPR_2$, which are expressed in much higher density on tumour cells of breast, prostate, colorectal, pancreas, brain, sarcomas and carcinoids, to name a few, than on normal cells [1].

VIP labelled with ^{123}I (t½–13.3 h, γ-159 KeV-84%) was given intravenously to cancer patients and all known and unknown lesions were detected by scintigraphic imaging [2]. ^{123}Iodine, however, is a cyclotron produced, relatively expensive radionuclide with limited availability, and must be ordered days ahead of the examination. The radiolabelling chemistry is also such that it requires HPLC purification and two to three hour preparation time. This is inconvenient and further limits its utilization. Imaging is performed two to three hours later.

^{99m}Tc, on the other hand, not only has excellent physical characteristics (t½–6 h, 140 KeV, 90%) for scintigraphic imaging but is also inexpensive and available throughout the world via a ^{99}Mo generator system. We have modified VIP (TP 3654) that permits us to rapidly label the peptide with ^{99m}Tc without compromising its biological characteristics or receptor specificity [3]. This study was undertaken to examine its pharmacokinetics [4] and to determine the feasibility of using ^{99m}Tc-TP 3654 as an agent for imaging tumours in humans.

2. MATERIALS AND METHODS

In order to be able to label VIP with ^{99m}Tc, the peptide was modified at the C-terminus by the addition of four amino acids. Of these, Gly^{30}-(D)-Ala^{32}-Gly^{31}-Gly^{33} (GAGG), served as a chelating moiety for strong chelation of ^{99m}Tc and Aba^{29} (4-amino butyric acid) as a spacer to minimize steric hindrance. The entire 33 amino acid peptide was synthesized, purified, characterized by mass spectroscopy, and named TP 3654 [3].

Ten μg of TP 3654, together with 25 μg $SnCl_2.2H_2O$ and 400 μL of 0.05 M Na_3PO_4 solution were lyophilized and stored under N_2 at –80ºC. At the time of preparation approximately 30 mCi ^{99m}Tc in 0.5 mL of 0.9% NaCl was added and incubated at 22ºC for 30 min. To the solution was then added 1 mL of 0.05 M NaH_2PO_4 solution, followed by the addition of 0.25 mL ascorbic acid (250 mg/mL). Routine quality control tests were performed [3]. Following approval from the Institutional Review Board and the Radioactive Drug Research Committee, three normal volunteers and eleven patients with a history of cancer were enrolled in this study. Each individual was given, intravenously, approximately 10 mCi ^{99m}Tc-TP 3654 (11×10^3 Ci/m mol) and serial spot views and/or whole body images were then obtained for up to two hour post injection. The vital signs of each patient were monitored. Scans were called positive when ^{99m}Tc-TP 3654 uptake in lesions exceeded radioactivity in the adjacent tissue. Findings were compared with the results of ^{99m}Tc-MIBI, computerized tomography (CT), magnetic resonance imaging (MRI) or histology.

3. RESULTS

The lyophilized kits were stable, and unbound ^{99m}Tc and colloid formation was less than 3%. No adverse reactions or changes in vital signs were noted in any of the subjects. Over a 24 h period, in the three normal volunteers, approximately 70% of the injected dose was eliminated in urine and 20% cleared through the liver [4]. Blood clearance of the tracer was rapid. Whole body images for up to 2 h post injection showed tracer uptake in the bladder, kidneys, and gallbladder, as well as faint uptake in the liver, spleen, and lungs [4].

The eleven patients were comprised of five patients with breast cancer, one with osteosarcoma, one with a history of colon cancer, one with spindle cell sarcoma in the neck, one with bowel obstruction due to an hemangiopericytoma, one with numeral spread of adenocarcinoma, and one with a small nodule in the left lung from a clear cell cancer of the uterus. Eight patients had positive scans within 20 min of injection. These results corroborated well with the findings of CT, MRI, ^{99m}Tc-SestaMIBI (SMM) scans, and/or histopathology. There was a normal VIP scan in a patient with previously removed colon cancer and no evidence of recurrence. One patient with a small left lung nodule seen by CT had a normal VIP scan, possibly due to small size of the nodule. One patient with right breast hyperplastic ductal epithelium with atypia had a normal ^{99m}Tc-MIBI scan as well as normal ^{99m}Tc-VIP scan. A 42 year old female with a history of right breast cancer, followed by mastectomy and silicon implant presented with left breast mass suspicious for malignancy. Because of dense tissue in the left breast, the mammogram was difficult to evaluate. SMM was normal, but ^{99m}Tc-VIP showed a large area of increased uptake. Biopsy revealed that it was ductal epithelial hyperplasia, which is known to express VIP receptors [1]. Autoradiographic studies of the biopsy tissues showed intense uptake of ^{99m}Tc-VIP in the ductal region, but not in the connecting tissue. The high grade spindle cell sarcoma was normal on the ^{99m}Tc MIBI scan, but intensely positive on the ^{99m}Tc-VIP scan. The history, diagnosis, and findings from ^{99m}Tc-TP 3654 studies of the eleven patients are given in Table I and two specific examples are described in Figs 1 and 2.

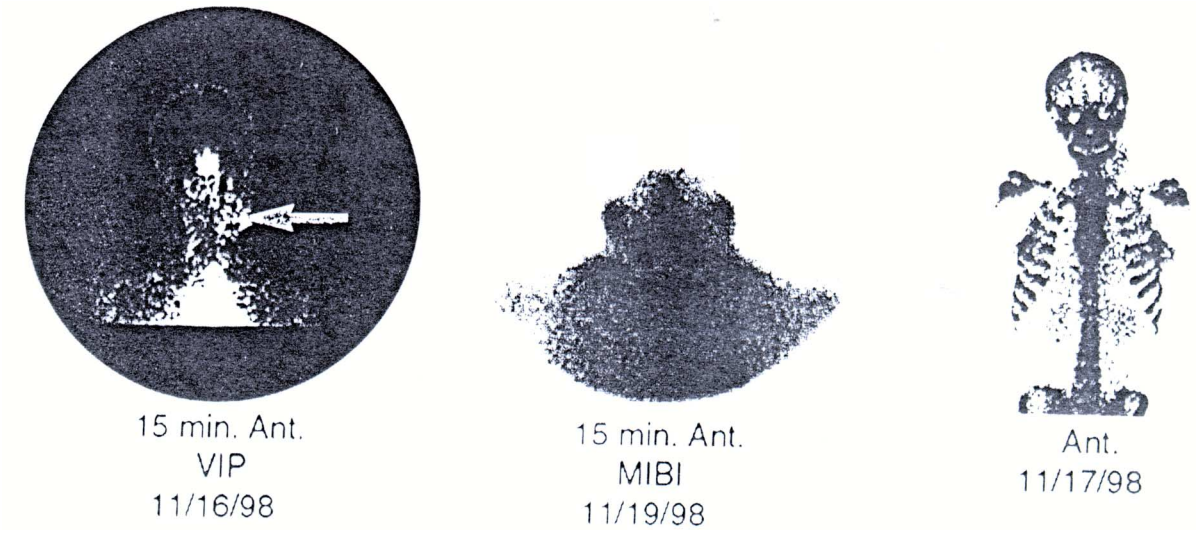

FIG. 1. A 42 YO female with prior L mastectomy presented with recurrence in the R breast and L operative site. The lateral images with ^{99m}Tc-SestaMIBI at the far left show uptake in the chest wall and R breast (arrows). The images in the middle and at the far right are the left and right lateral views obtained at 15 min and 1 h post injection of ^{99m}Tc-VIP. The same lesions perhaps with better intensity than the corresponding ^{99m}Tc-SestaMIBI images can be seen (arrows).

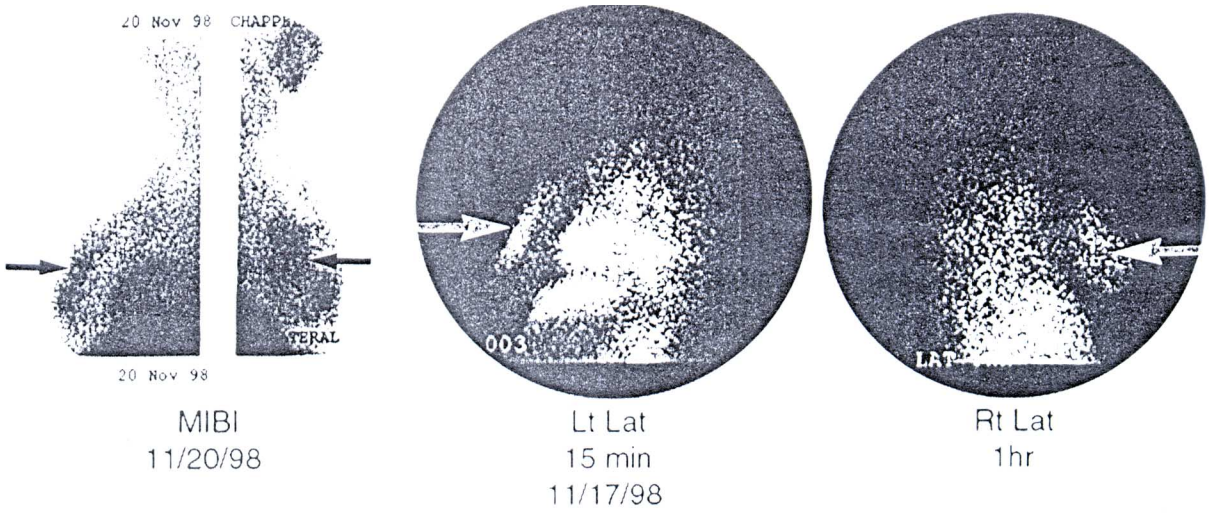

FIG. 2. A 20 YO female with a history of neurofibroma of the brain in childhood presented with a mass in the left neck evident for one month. Her ^{99m}Tc-MIBI scan (centre) was negative. Bone scan (right) showed faint blood pool. However, ^{99m}Tc-VIP scan (left) showed unequivocally positive uptake (arrow). Immunohistology of the lesion showed that it was a high grade spindle cell sarcoma.

TABLE I. SCINTIGRAPHIC RESULTS WITH ^{99m}TC-VIP AND FINAL DIAGNOSIS

Patient No.	Age	Gender	^{99m}Tc-VIP Results	History and Diagnosis
1/AO	44	F	L breast positive R breast positive	Bilaterally positive mammography and positive SMM 6 weeks previously. Lesions of the same shape and size as ^{99m}Tc-VIP.
2/PC	42	F	L chest wall positive R breast positive	L breast mastectomy for cancer. Presented w/recurrence in L chest wall and R breast. SMM positive for L chest wall and R breast. Lesions same shape and size with ^{99m}Tc-VIP.
3/GT	42	F	L breast positive	R breast mastectomy for cancer followed by silicon implant. Presented w/L breast palpable mass with ductal epithelial hyperplasia. Mammography was difficult to evaluate. SMM normal.
4/SS	39	F	L breast positive R breast positive	R breast cancer treated w/surgery, chemo- and radiotherapy. Mammography showed mass in R breast. SMM showed L breast mass and faint R breast uptake. ^{99m}Tc-VIP showed L breast uptake and greater R breast uptake than SMM. Histology showed malignancy.
5/GC	47	F	Normal breast scan	Biopsy for R breast mass w/hyperplastic ductal epithelium atypia. SMM normal bilaterally.
6/LE	27	M	Uptake close to bladder	Long history of bowel obstruction. CT showed a mass near bladder. ^{99m}Tc-MIBI inconclusive because of bladder activity.
7/JD	20	F	Positive for left neck mass	History of neurofibroma. Presented w/L neck mass. Bone scan showed faint blood pool activity. ^{99m}Tc-MIBI normal. ^{99m}Tc-VIP scan positive, consistent w/visible mass and histology showing spindle cell sarcoma.
8/IS	50	M	R shoulder positive	R shoulder adenocarcinoma. Bone scan showed involved bone. ^{99m}Tc-MIBI positive for R shoulder.
9/NT	18	M	Positive for R lower femur	Positive w/MRI, bone scan and ^{99m}TcIBI for osteosarcoma.
10/RC	59	F	Normal scan	Had surgical resection for colon cancer. No evidence of recurrence.
11/SL	43	F	Normal scan	History of clear cell cervical carcinoma. Small nodule in L upper lung seen by CT. No ^{99m}Tc-MIBI scan.

^{99m}Tc-VIP = ^{99m}Tc-TP 3654, SMM = ^{99m}Tc-SestaMIBI mammography; ^{99m}Tc-MIBI = ^{99m}Tc-SestaMIBI.

4. DISCUSSION

Cancer remains a major health problem of mankind. Although extensive new knowledge has led health care professionals to a changing philosophy in its management, one factor that remains of undisputed importance is early diagnosis. In recent years, CT and MRI have greatly improved the diagnostic abilities for anatomic localization of tumours. However, metabolic or receptor specific tumour imaging is advantageous since such imaging can demonstrate the state of malignancy and may distinguish malignant tumours from benign ones. Metabolic or receptor specific tumour imaging is more advanced with radionuclide imaging than with CT, MRI, or ultrasound. The complementary role of nuclear medicine imaging is therefore well recognized, and the search has continued for better and more specific imaging agents than available currently.

There are other tumour imaging agents, two of which are based upon tumour metabolism. The first, ^{18}F-deoxyglucose, is an excellent agent but needs ^{18}F, with a half-life of 110 min. This must be produced by a cyclotron, and requires PET or coincidence gamma camera for imaging. Currently, less than 1% of the nation's nuclear medicine centres can use ^{18}F-FDG. The second, a perfusion agent, ^{99m}Tc-SestaMIBI, is presumed to be taken up by mitochondria in tumours, and generally suffers from high background due to non-specific uptake in many normal tissues. Although it is 86% sensitive for palpable breast tumours, it detects only 63% of non-palpable breast tumours and does not obviate biopsy for histologic examinations [5]. Its utility for detecting other tumour types is not yet well established.

Frequently over expressed on the surface of malignant proliferative cells are certain characteristic biomolecules, the receptors, that specifically interact with a family of substances discovered during the last 25 years. These endogenous compounds or their analogues bind to the specific cell surface receptors in nanomole to femtomole concentrations and have been the focus of considerable interest, both in therapy and diagnosis of malignant tumours. The only commercially available neuropeptide, somatostatin receptor specific ^{111}In-[DTPA-D-Phe^{1}] Octreotide, is the third tumour imaging agent. However, the cyclotron produced ^{111}In is much more expensive than ^{99m}Tc and is not as easily available. Furthermore, somatostatin receptor density on most types of tumours is much lower than their VIP receptor density [1].

Our early data, obtained with ^{99m}Tc-VIP analogue in a limited number of patients, indicate that the agent is easy to prepare, convenient to use, and permits rapid imaging of tumours with planar gamma camera scintigraphy. In our current preparation we used approximately 5 μg of VIP. No adverse reaction of any kind was noted in any subject. The lung uptake of ^{99m}Tc-VIP was much less than that of ^{123}I VIP (2). The ^{99m}Tc-VIP was cleared from circulation via the kidneys and liver. All positive images were diagnostic in less than 20 min post-injection Although late images showed GI activity, the speed of its tumour uptake may permit us to image abdominal tumours. The rapid bladder uptake may make it difficult to image prostate cancer and may require continuous elimination of urine to succeed.

Most ^{99m}Tc-VIP images were similar to MIBI images, including a scan of hyperplastic atypia, which was negative on both VIP and MIBI. The receptor characteristics of hyperplastic atypia are unknown. However, it was interesting to note that the lesions with ductal epithelial hyperplasia and the high density spindle cell sarcoma, known to express VIP receptors, were both positive only with ^{99m}Tc-VIP scans and not with the other, commonly used imaging modalities. The patient with ductal epithelial hyperplasia had a mastectomy of the right breast for malignant cancer. The hyperplastic lesion presented in the left breast two

years later did not show malignant morphology. However, the ^{99m}Tc-VIP image of the lesion was intensely positive and suggested that the technique can demonstrate the biochemical changes by non-invasive means, prior to the characteristic changes of malignant morphology have occurred.

Thus, at the present time, the agent shows reliability, simplicity, speed, and the lack of toxicity. Further studies will determine if this agent can distinguish malignant tumours from benign ones and identify those lesions which are yet morphologically normal, but destined to become malignant.

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ABBREVIATIONS

ACN	acetonitrile
BCA	bifunctional chelating agent
BFC	bifunctional chelator
CT	computerized tomography
DEA	Diisopropylethlamine
DFO	Desferrioxamine
DMF	dimethyl formanide
DOTA	1,4,7,10-tetraazacyclo-dodecane tetraacetic acid
DTPA	diethylene triamine penta acetic acid
EDDA	Ethlendiamine diacetic acid
HATU	0.(7-azabenzotriazolyl) 1,1,3,3, tetramethyluronium hexafluorophosphate
HBSS	Hank's balanced salt solution
HPLC	high performance liquid chromatography
HYNIC	hydrazinonicotinanide
i.v.	intravenous
ITLC	instant thin layer chromatography
MAG-3	S-benzoyl-mercaptoacetyl triglycine
MBC	maximum binding capacity
mEGF	mouse epidermal growth factor
MRI	magnetic resonance imaging
NGA	Neogalactoabumin
NTBS	2-Nitro-5-thiosulfobenzoate
p.i.	post injection
PBS	phosphate buffer saline
PET	positron emission tomography
QA	quality assurance
QC	quality control
SB	specific binding
SMM	^{99m}Tc-SestaMIBI
SRIF	somatotropin release inhibiting factor
TFA	Trifluoroacetic acid
TLC	thin layer chromatography
TOC	Tyr^3-Octreotide
TPPMS	triphenylphosphine-3-monosulfonate
UK	United Kingdom of Great Britain and Northern Ireland
VIP	vasoactive intestinal peptide

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