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].

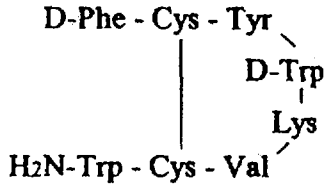

FIG. 1. Structure of RC-160.

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.

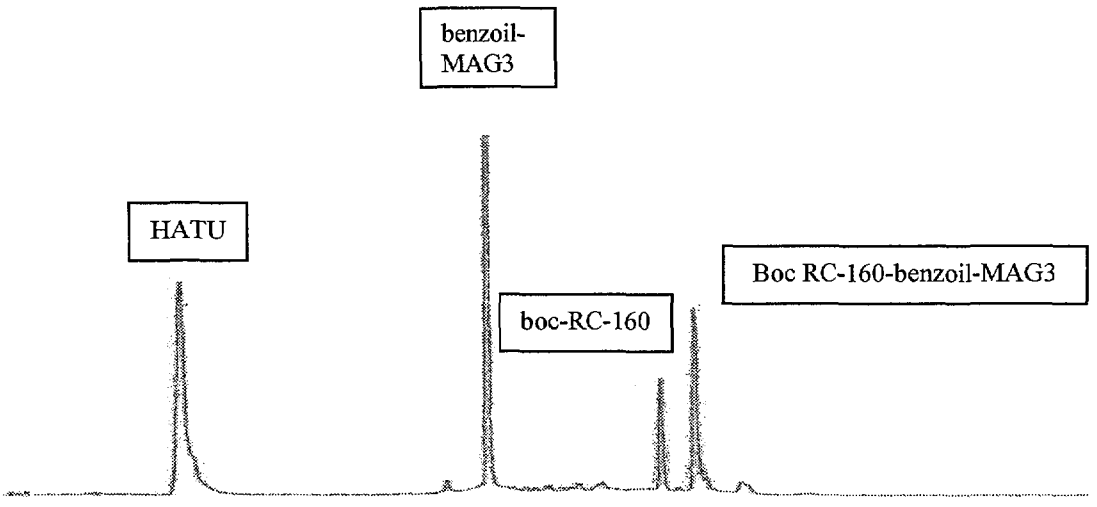

FIG. 2. Chromatogram profiles of the reactives and the conjugate.

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

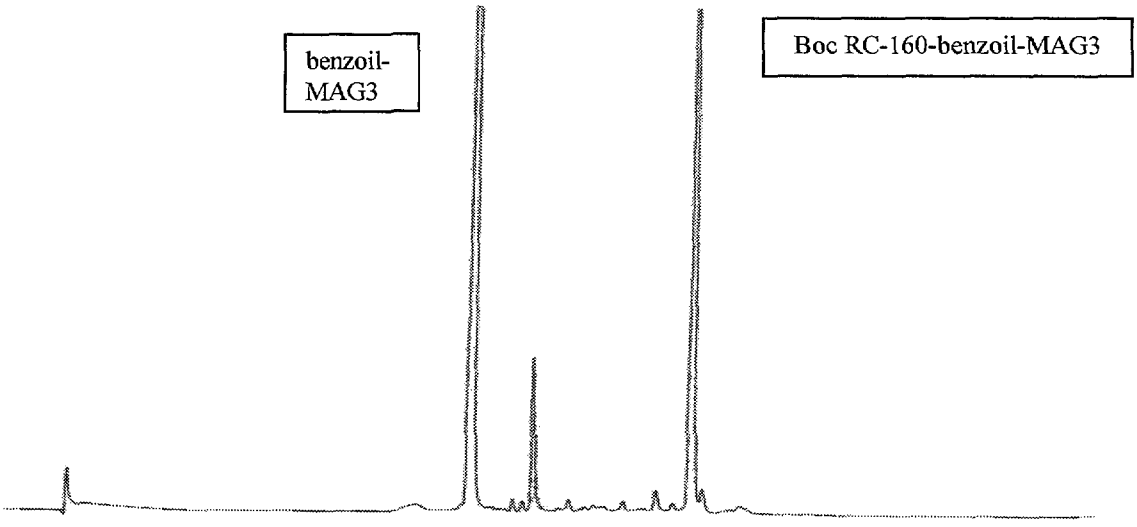

FIG. 3. Chromatogram of the acetonitrile fraction eluted from the SEPPAK.

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 82°C 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 37°C. 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 37°C 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 4°C.

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.

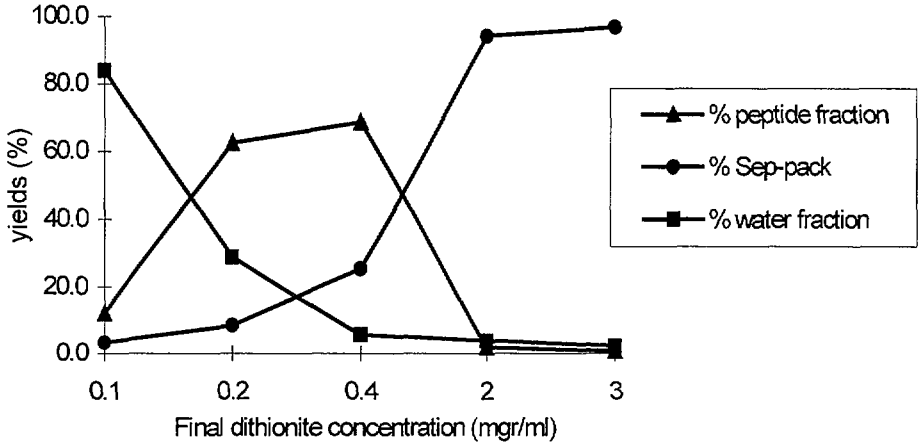

FIG. 4. Fraction yields after SEPPAK purification.

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

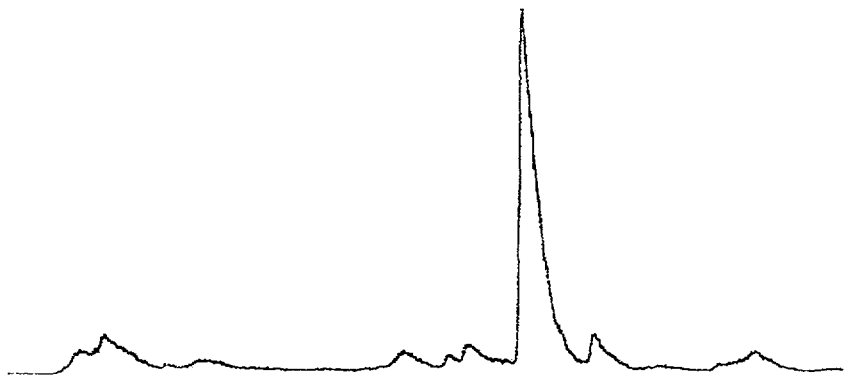

FIG. 5. Chromatogram profile of directly labelled RC-160.

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.

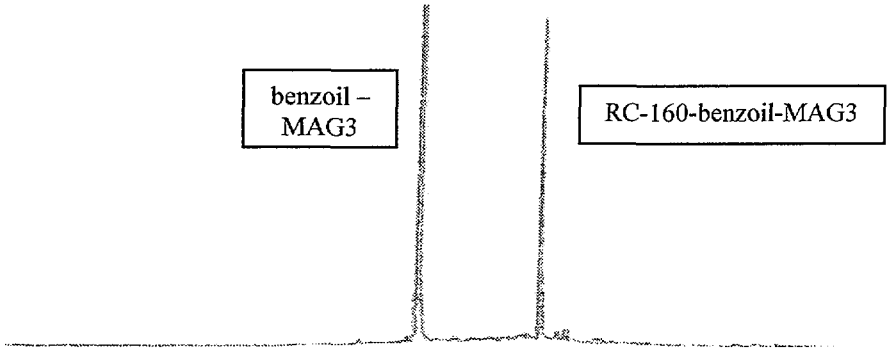

FIG. 6. HPLC analysis of deprotected RC-160 conjugate.

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.

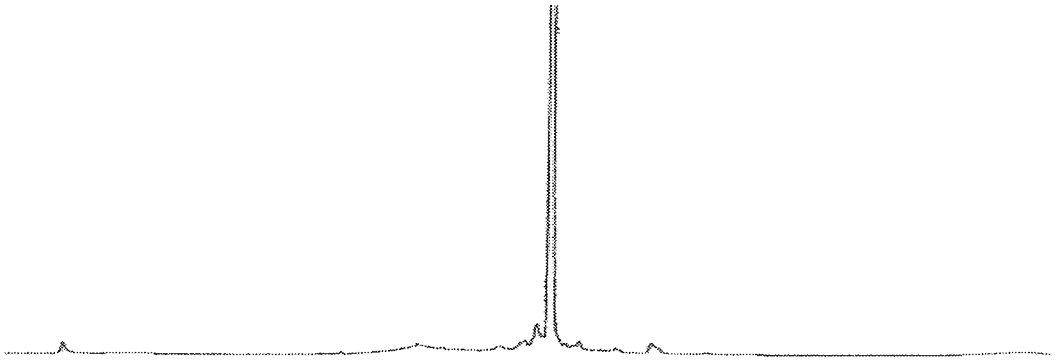

FIG. 7. HPLC profile of the pure peptide HYNIC-TOC conjugate.

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.

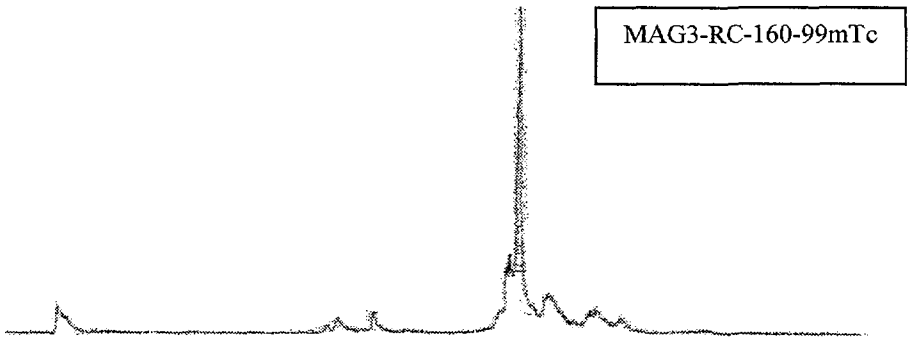

FIG. 8. HPLC profile of labelled MAG-3-RC-160.

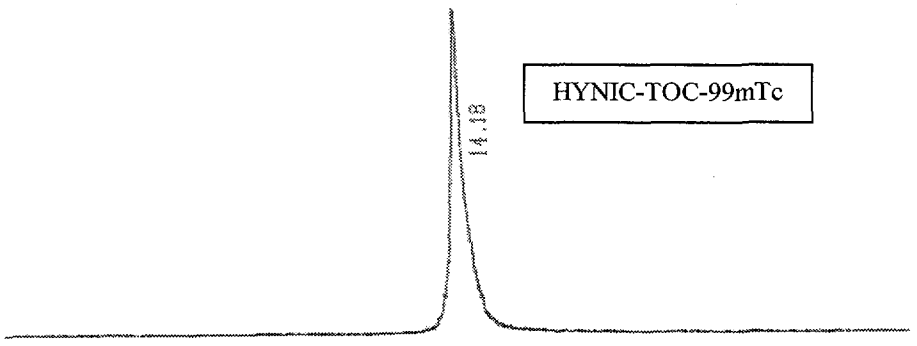

FIG. 9. HPLC profile of labelled HYNIC-TOC.

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.

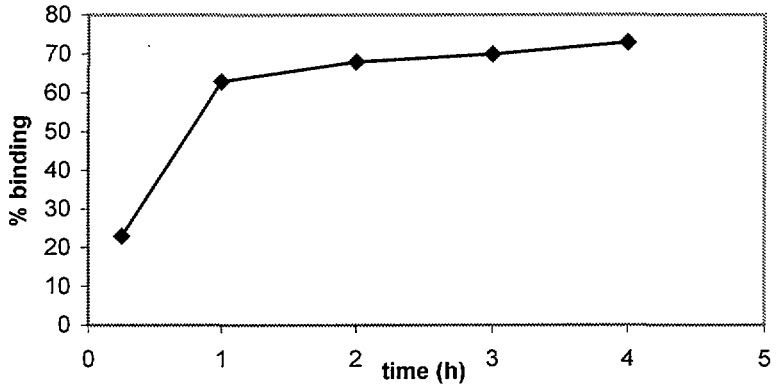

FIG. 10. Protein binding versus time.

2.6. Biodistributions

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

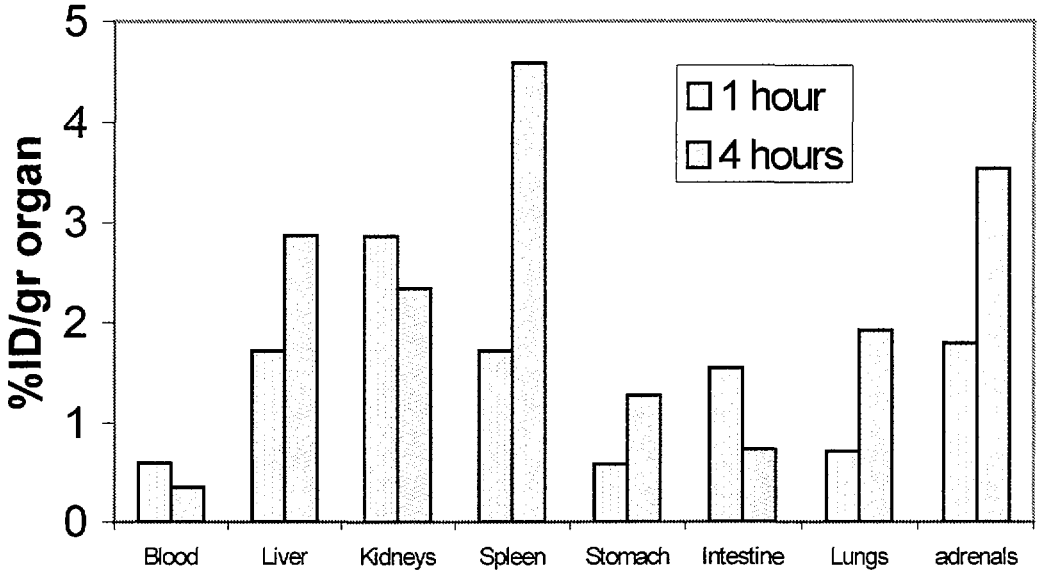

FIG. 11. Biodistribution at 1 and 4 h of MAG-3–*RC–160–*^{99m}*Tc.*

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.

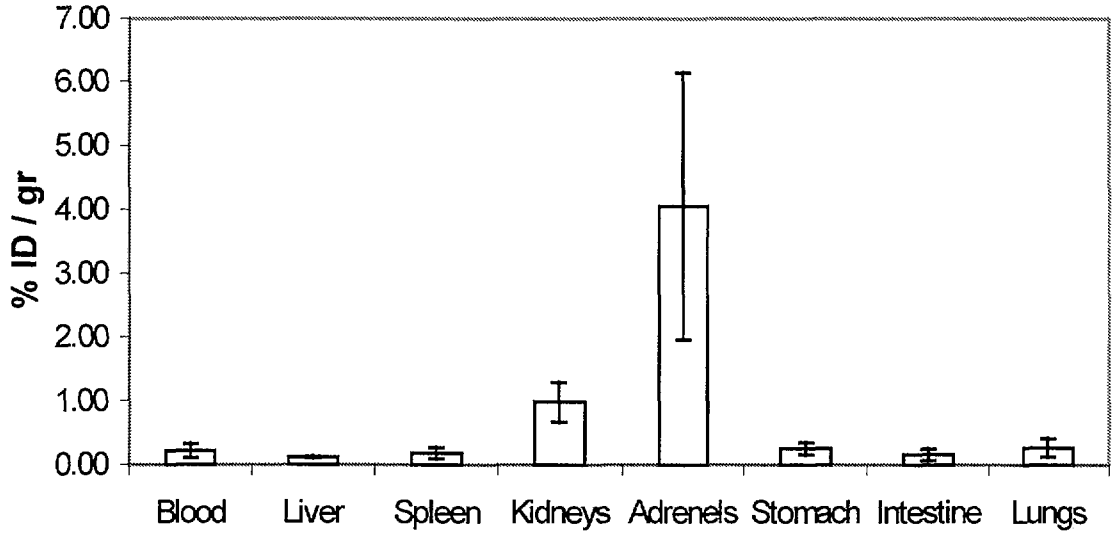

FIG. 12. Biodistribution at 4 h of HYNIC-TOC-^{99m}Tc.

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.

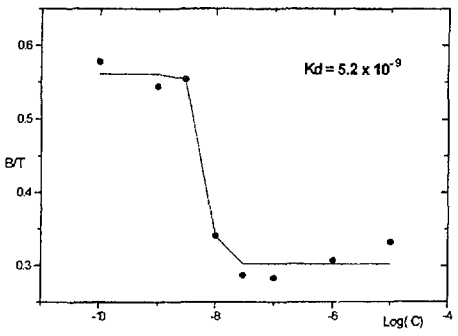

FIG. 13. Binding assay.

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.

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