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.

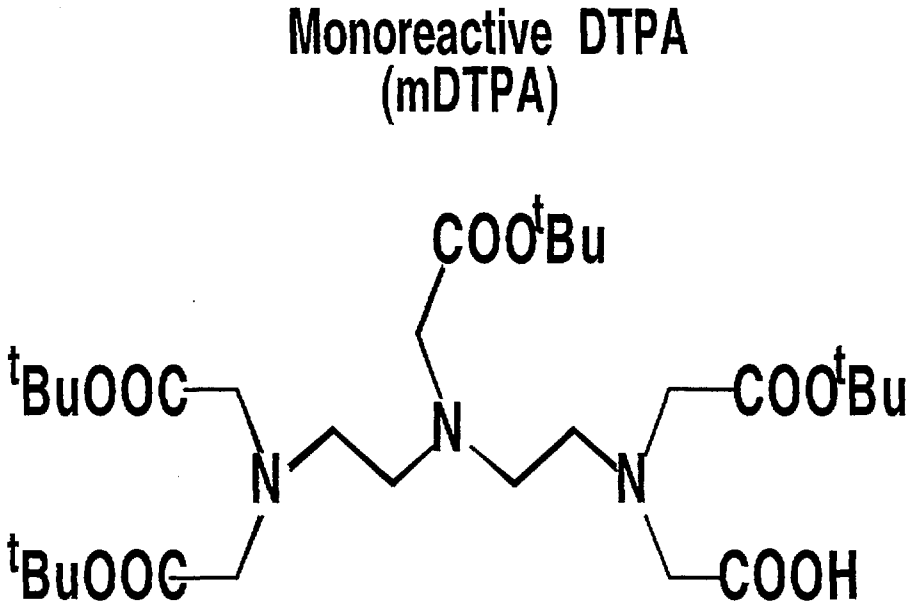

FIG. 1. mDTPA.

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*).

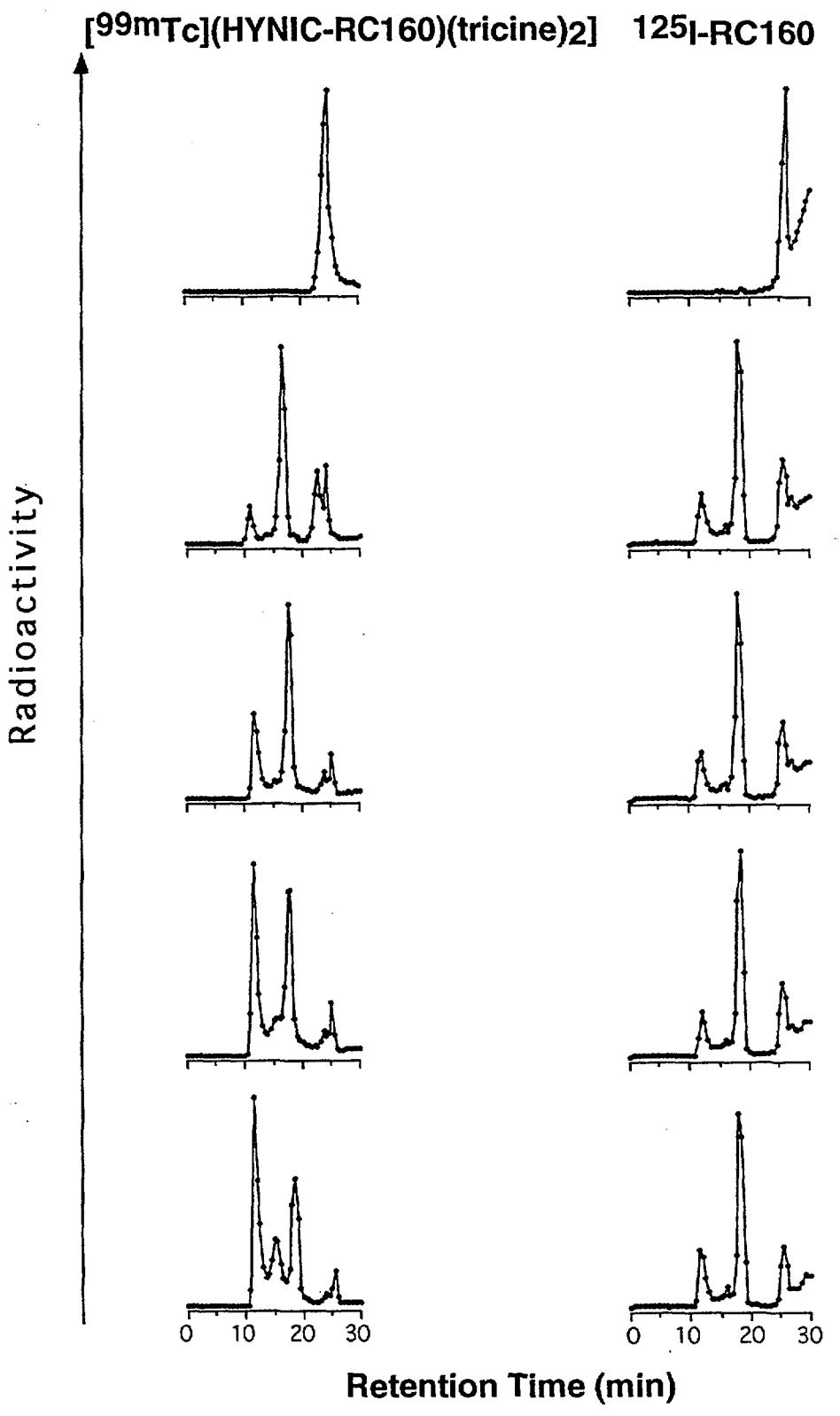

FIG. 2. Stability of ^{125}I-Rc-160 versus ^{99m}Tc-HYNIC-RC-160(tricine)$_2$ in the presence of plasma.

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 37°C). [^{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 4°C. 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 37°C 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.

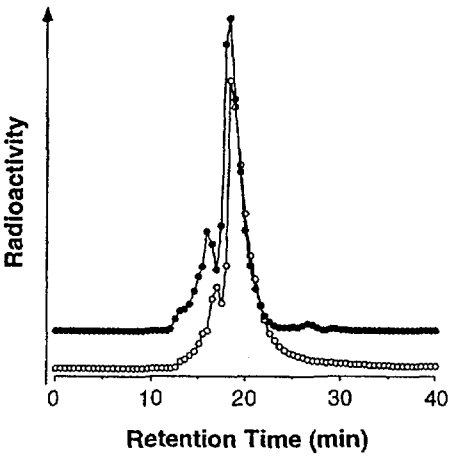

FIG. 3. Effect of plasma incubation. Size-exclusion radiochromatogram of ^{99m}Tc-HYNIC-NGA)tricine$_2$.

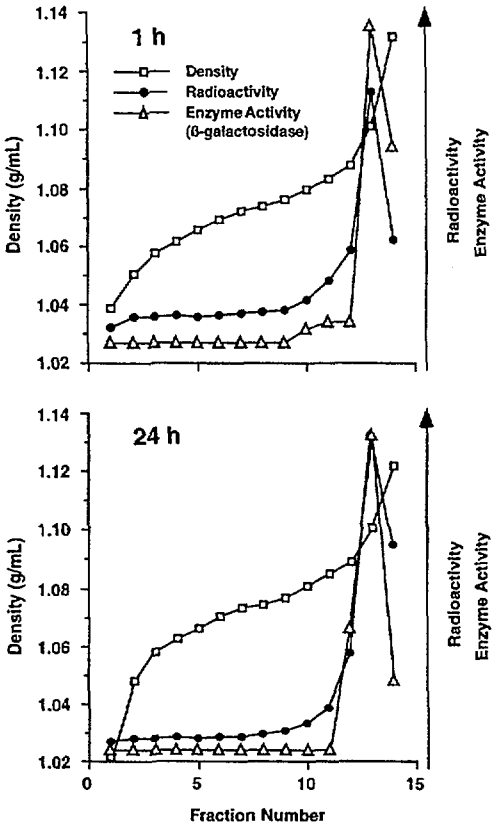

FIG. 4. Subcellular distribution using Percoll gradient density centrifugation.

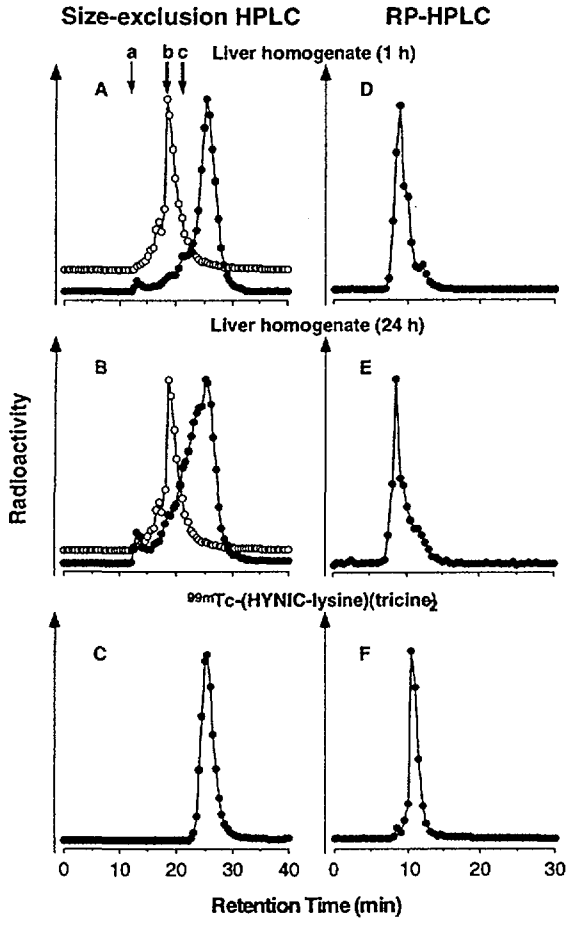

FIG. 5. Analysis of liver homogenates injected with ^{99m}Tc-(HYNIC-NGA)(tricine)$_2$. Comparative studies using size exclusion HPLC (left) and RP-HPLC (right).

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].

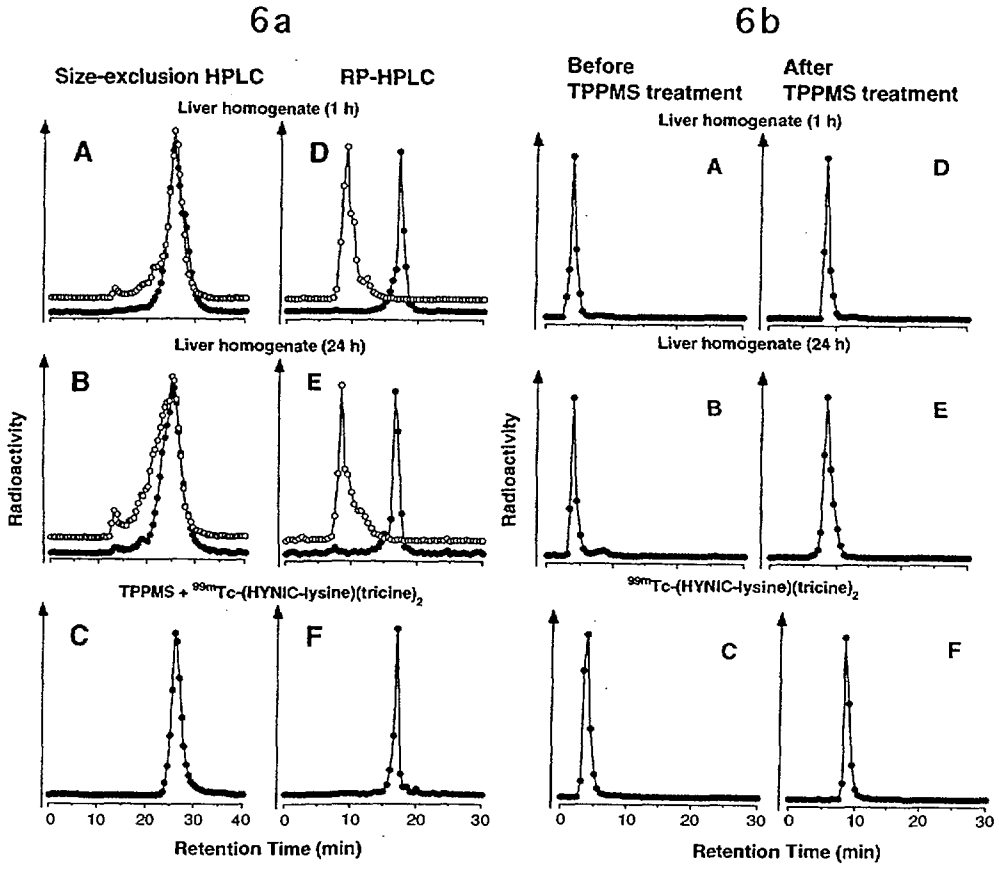

FIG. 6. Effect of TPPMS treatment on liver homogenates using RP-HPLC
6a) Analysis using solvent system 1
6b) Analysis using solvent system 2.

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.

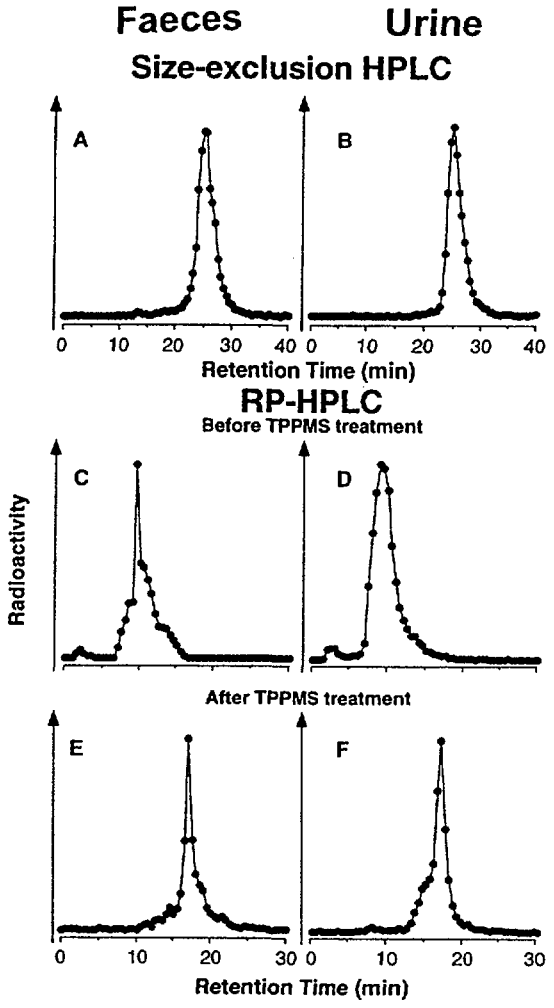

FIG. 7. Analysis of faeces and urine sample (size exclusion HPLC and RP-HPLC, solvent system 1).

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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