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

R. MIKOLAJCZAK, A. MARKIEWICZ, C.Z. DEPTULA, W. ZULCZYK,
G. BIRNBAUM, E. ZAKRZEWSKA, I. WOZNIAK
Radioisotope Centre POLATOM,
Otwock-Swierk, Poland

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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°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™ 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°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 90°C 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°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 37°C 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.

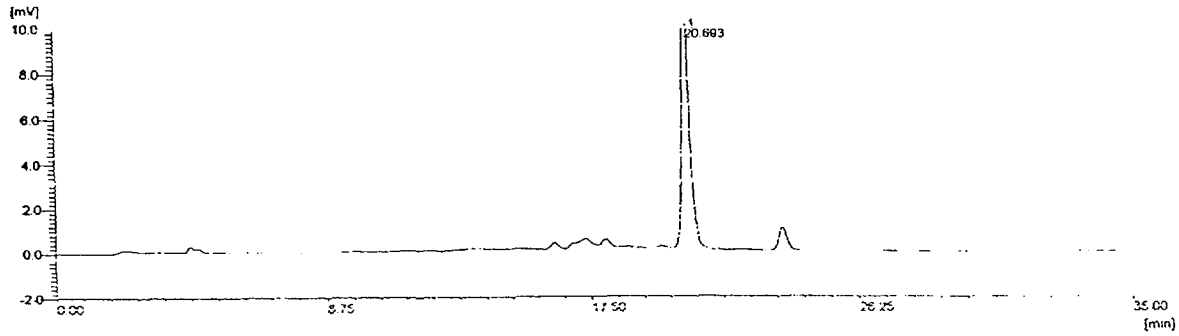

FIG. 1. HPLC profile of ^{125}I-RC-160 (about 80% radiolabelling yield).

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.

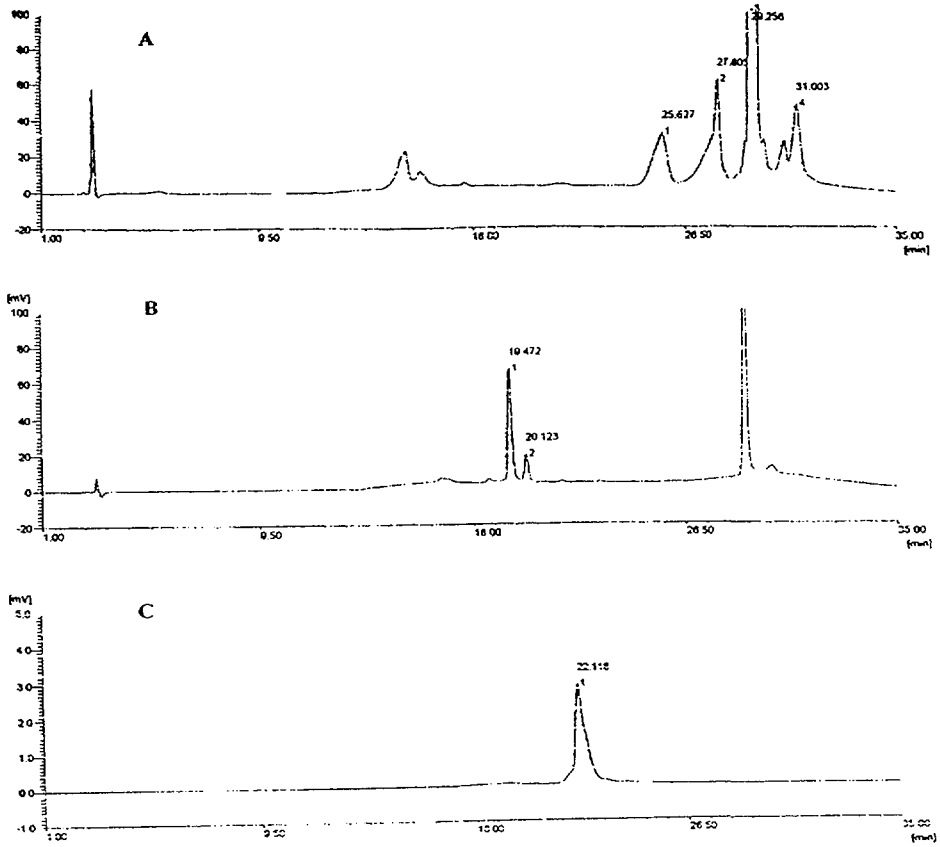

FIG. 2. HPLC profiles of the HYNIC-TOC: A reaction mixture of (Boc)HYNIC-(Boc)TOC; B-deprotected HYNIC-TOC conjugate; C–^{99m}Tc-HYNIC-TOC.

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.

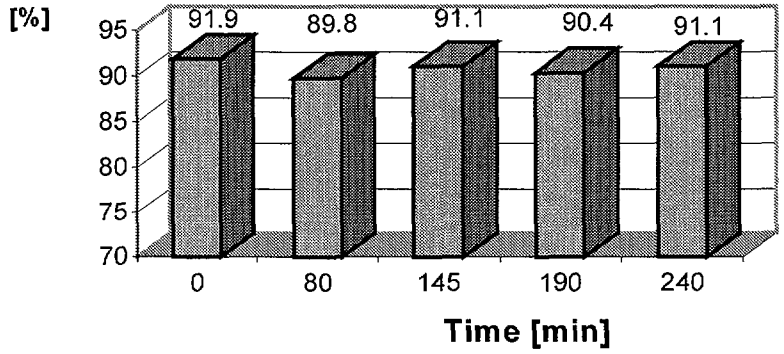

FIG. 3. Cysteine stability of ^{99m}Tc-tricine-HYNIC-TOC.

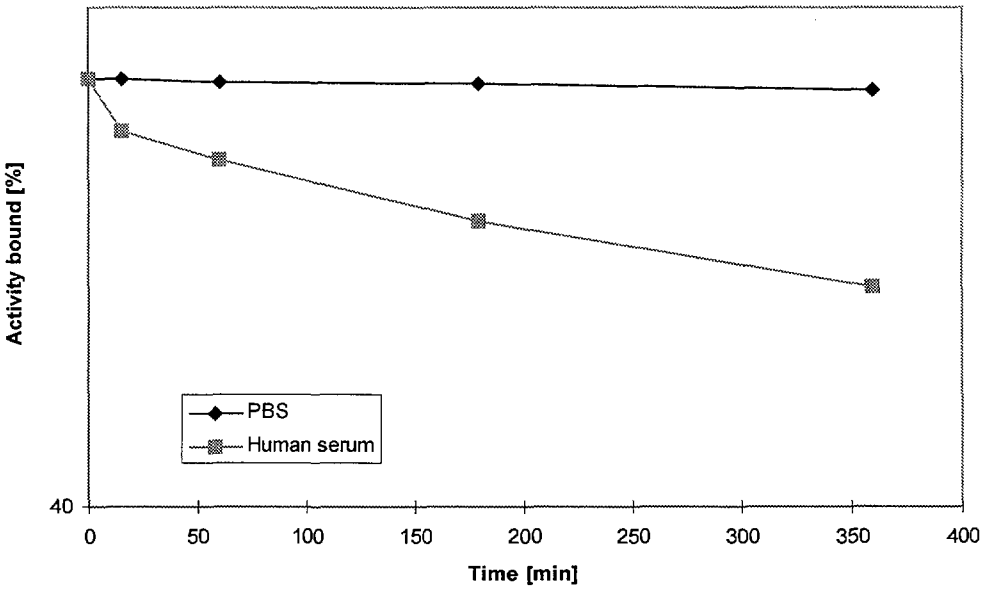

FIG. 4. Protein binding curve of ^{99m}Tc-tricine-nicotinic acid-HYNIC-TOC.

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.

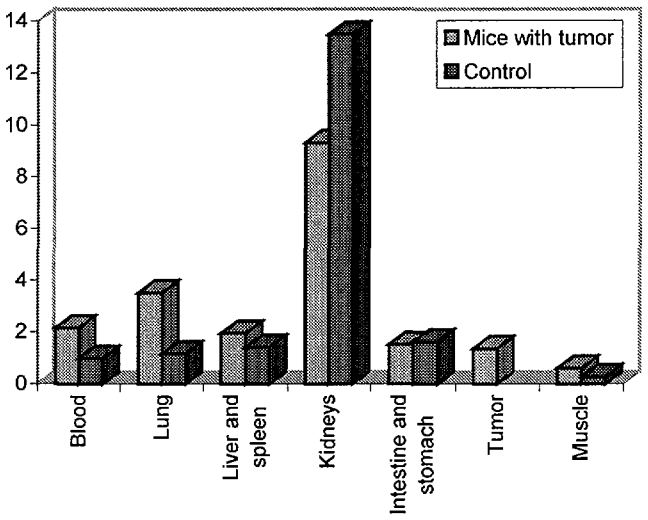

FIG. 5. Biodistribution of ^{99m}Tc-HYNIC-TOC in mice (tumour induced with breast cancer cells, activities measured in separated organs in 2 h pi).

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.

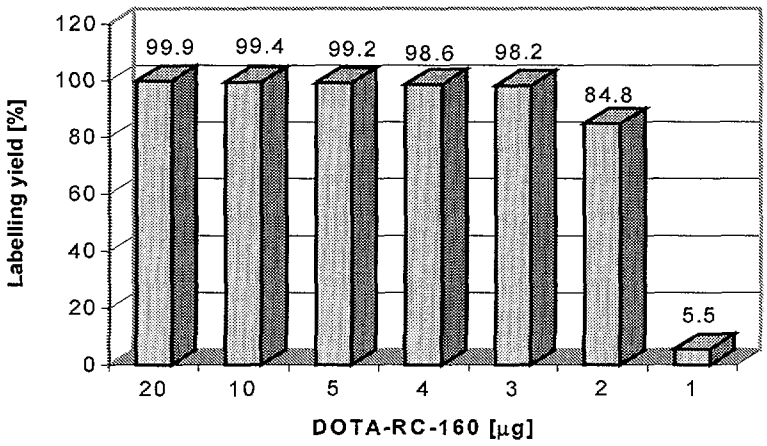

FIG. 6. ^{90}Y-DOTA-RC-60 labelling yields as function of amount of DOTA conjugate.

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.

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