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.

3.1. 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 4°C, 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 4°C 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 –80°C. 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 –20°C. The reconstituted product was determined for peptide concentration by UV-spectrophotometer at 280 nm and the solution was also stored at –20°C.

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.

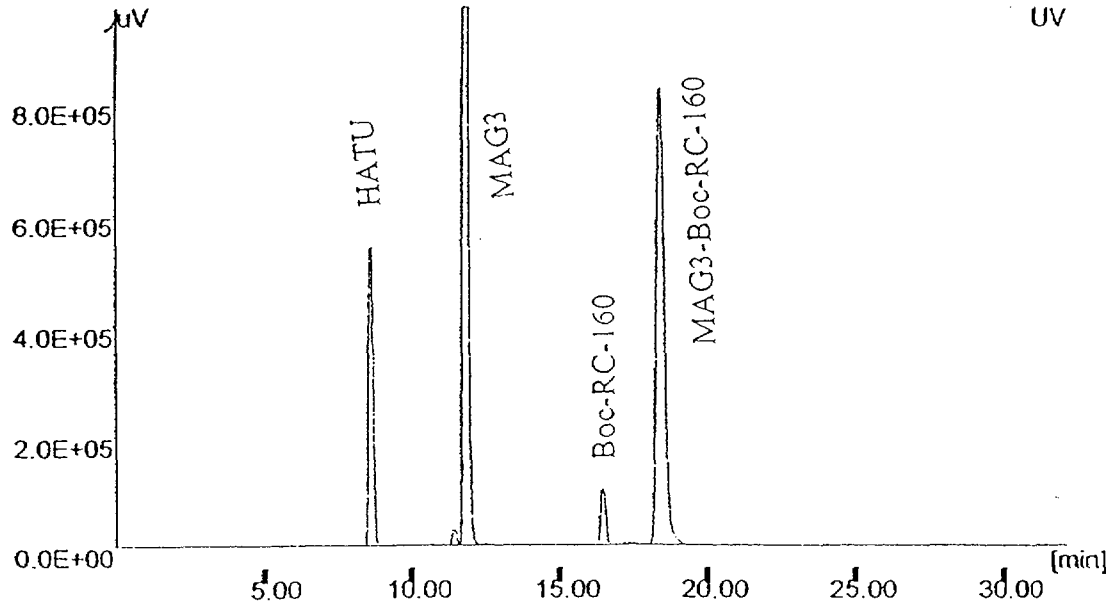

FIG. 1. HPLC chromatogram of reaction mixture of Boc-MAG-3-RC160 and Boc-HYNIC-RC160 (Crest Pak C-18, 5 μm , 4.6 x 150 mm).

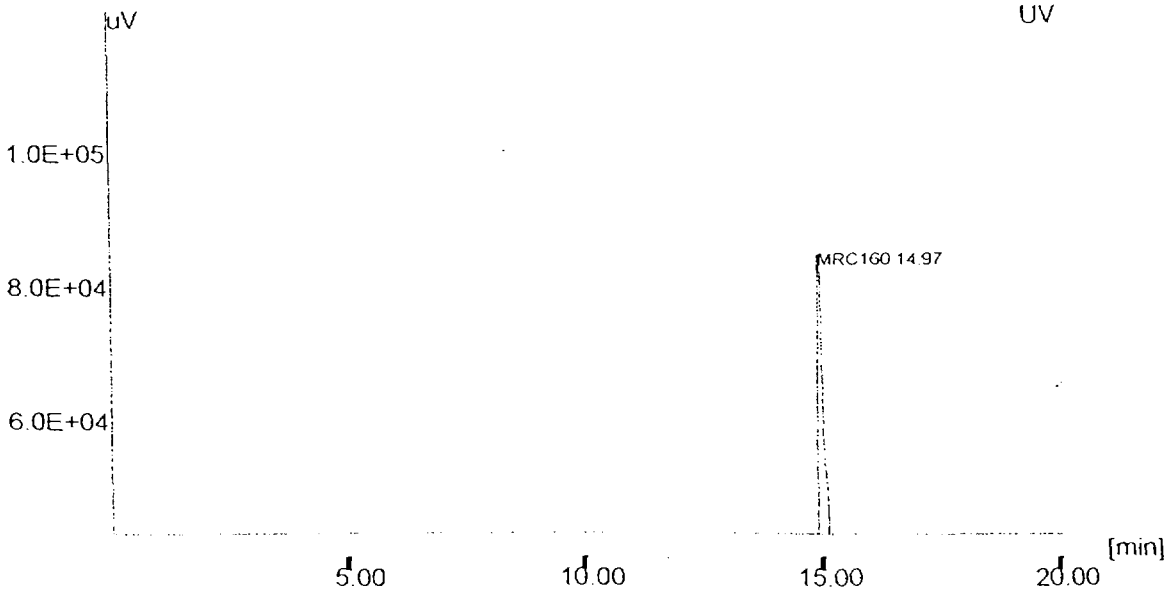

FIG. 2. HPLC chromatogram of deprotection MAG-3-RC160 (Crest Pak C-18, 5 μm , 4.6 × 150 mm).

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	-	-

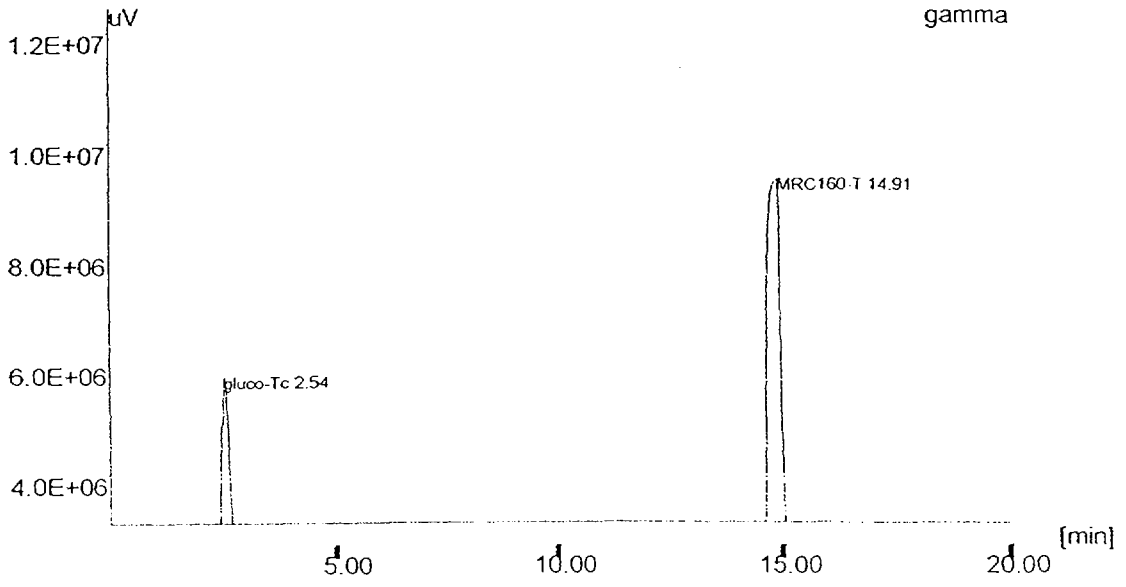

FIG. 3. Radiochromatogram of ^{99m}Tc-MAG-3-RC-160 (Crest Pak C-18, 5 μm, 4.6 × 150 mm).

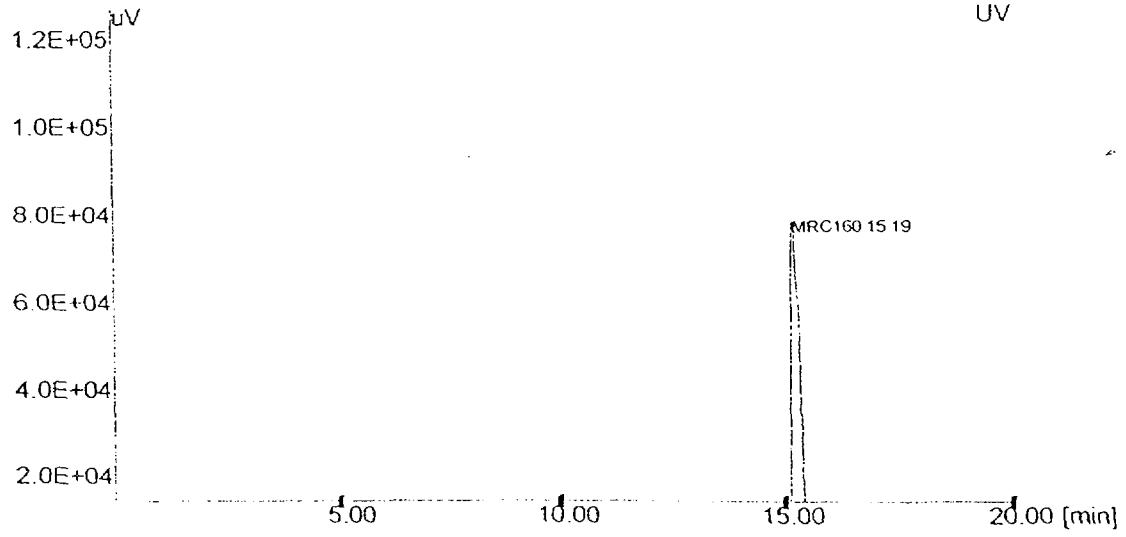

FIG. 4. UV-chromatogram of ^{99m}Tc-MAG-3-RC-160 (Crest Pak C-18, 5 μm, 4.6 × 150mm).

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.

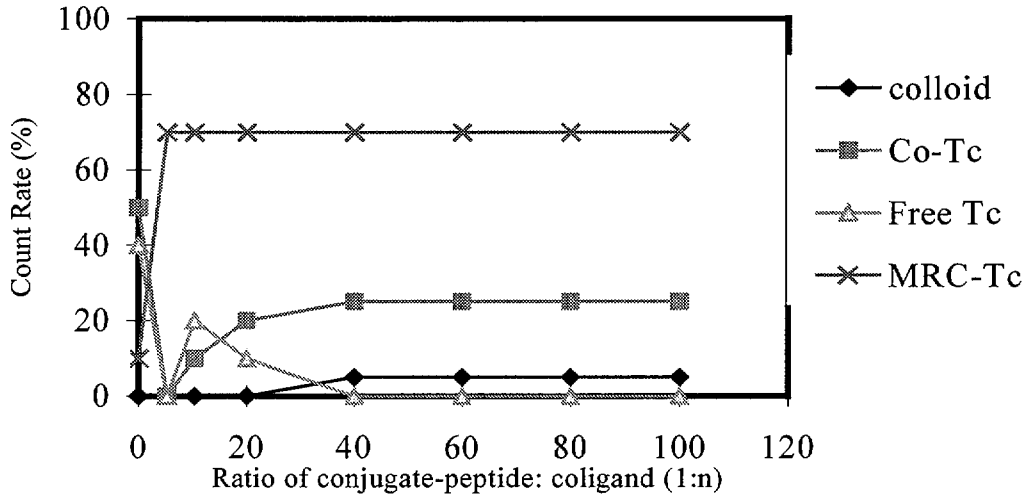

FIG. 5. Effect of mole ratio of MAG-3-RC-160 to glucoheptonate to the labelling yield.

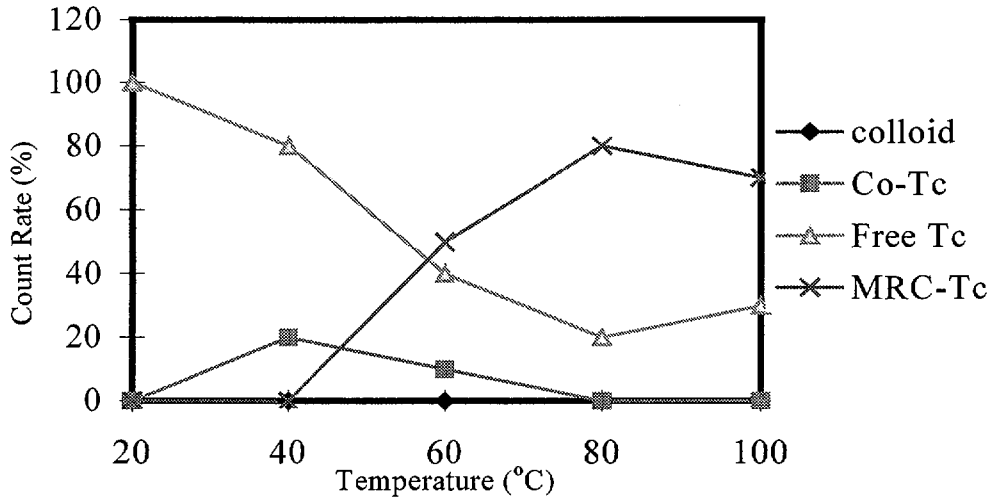

FIG. 6. Effect of temperature in labelling of MAG-3-RC-160 with ^{99m}Tc.

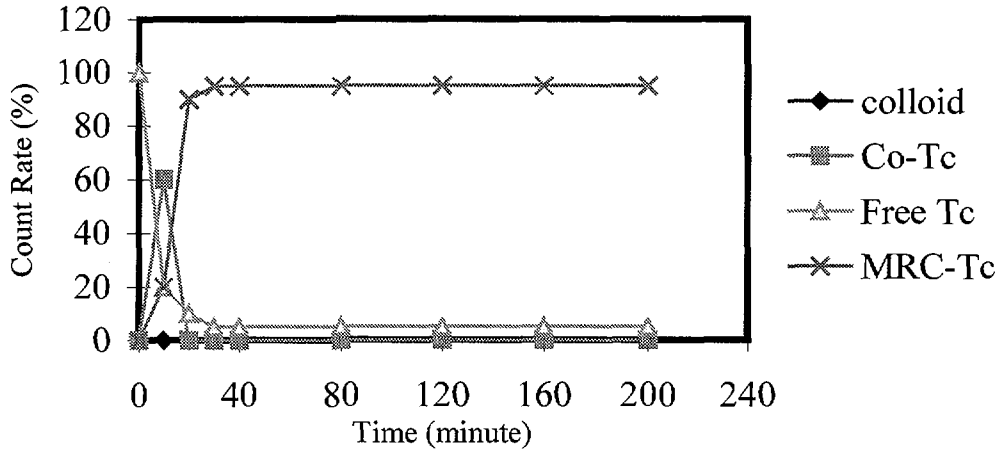

FIG. 7. Effect of reacting time in labelling of MAG-3-RC-160 with ^{99m}Tc.

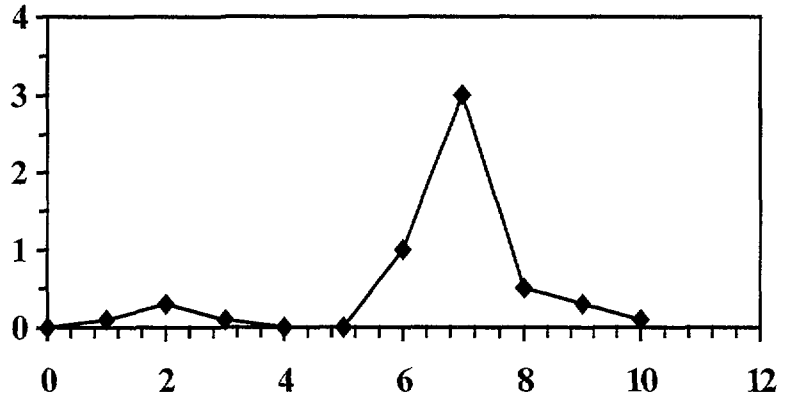

FIG. 8. Elution profile of purification by SEPPAK; AA = acetic acid and CAN = 50% acetonitrile in water.

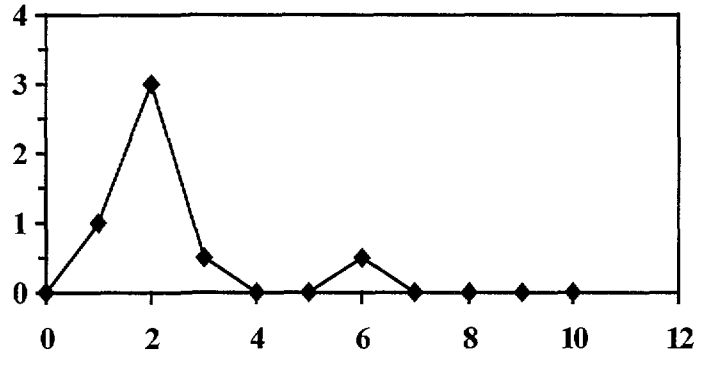

FIG. 9. Elution profile of the purification by Sephadex G-25.

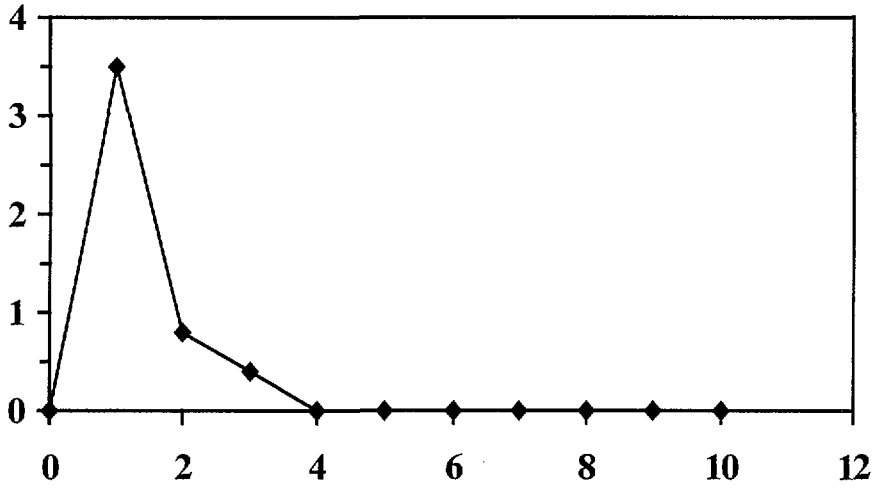

FIG. 10. Elution profile of the purification by Dowex.

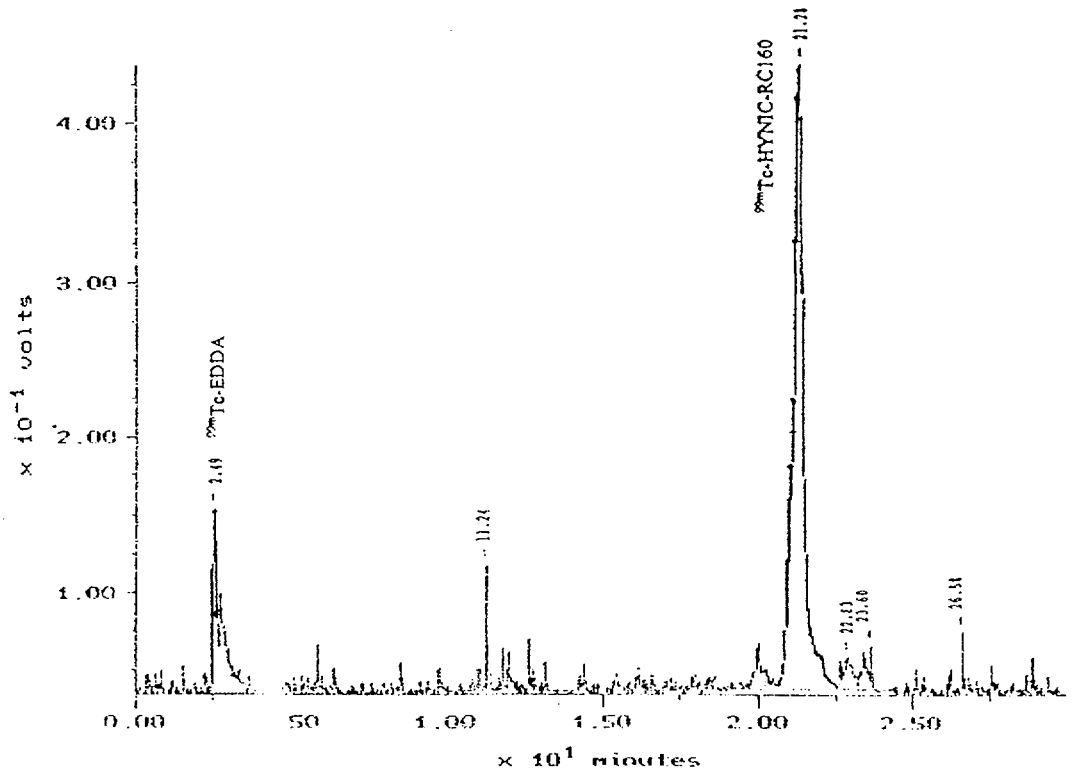

FIG. 11. Radiochromatogram of the HPLC purification of ^{99m}Tc-HYNIC-RC-160 (Ultremex C-18, 4 × 250 mm).

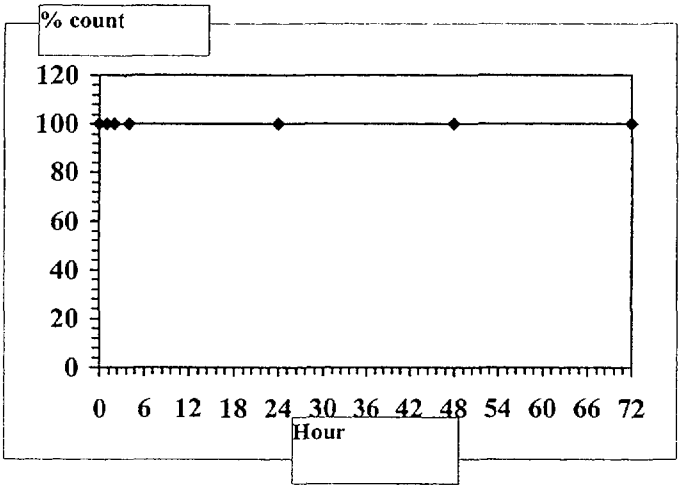

FIG. 12. Stability of radioligand were determined by HPLC.

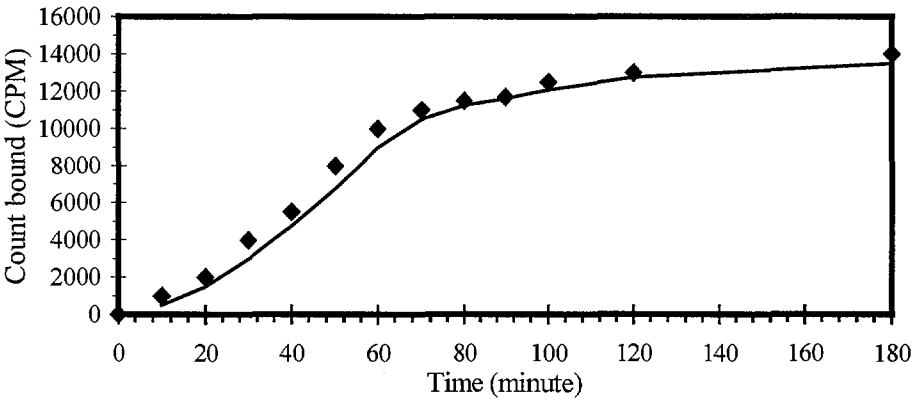

FIG. 13. Time course of specific binding of ^{99m}Tc-MAG-3-RC-160.

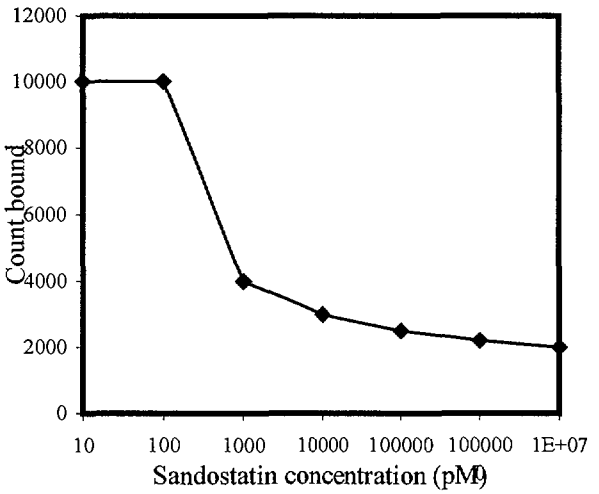

FIG. 14. Competitive binding of ^{99m}Tc-MAG-3-RC-160 in the presence of Sandostatin.

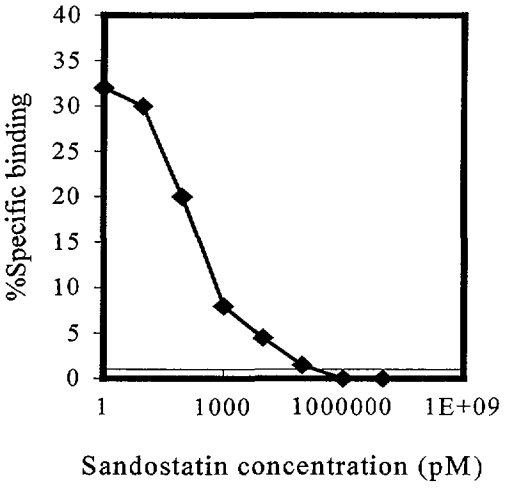

FIG. 15. Per cent specific binding of ^{99m}Tc-MAG-3-RC-160 in the presence of Sandostatin.

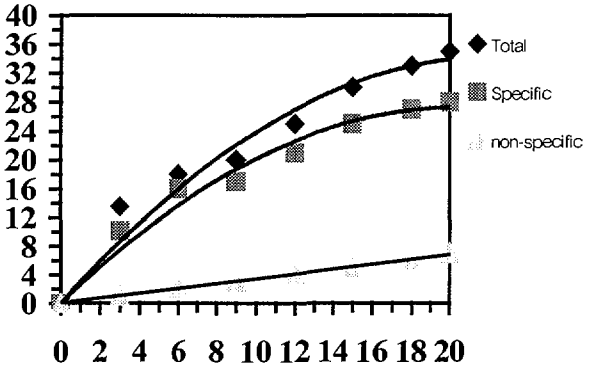

FIG. 16. Saturation curve of ^{99m}Tc-MAG-3-RC-160.

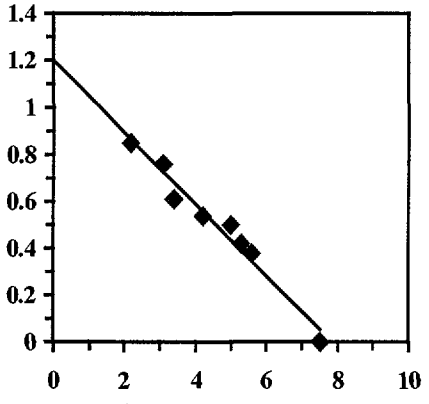

FIG. 17. Scatchard plot of data in to cortical membrane. incubated at room temperature 60 min in the presence of Sandostatin.

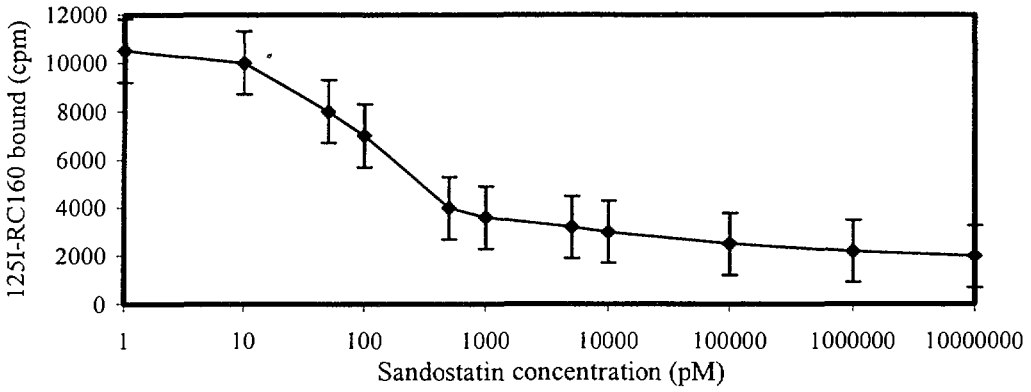

FIG. 18. Competition binding curve of 125I-RC160 to rat cortex membrane in the presence of Sandostatin.

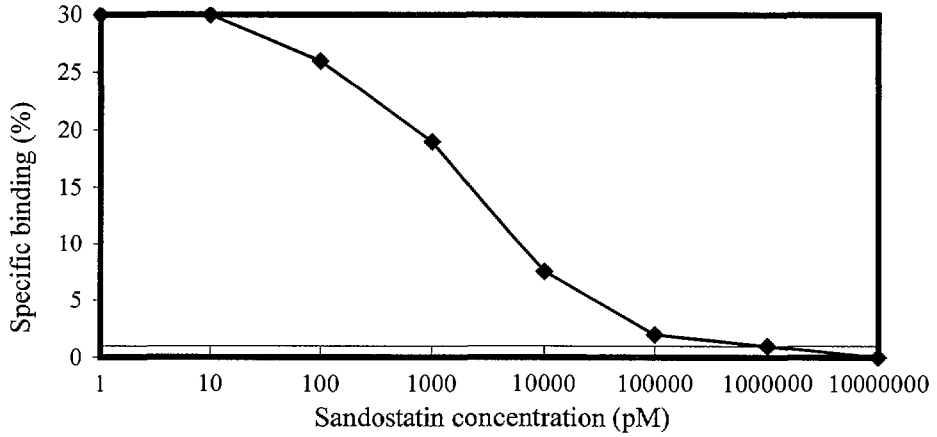

FIG. 19. %SRB of ^{125}I-RC-160 to rat cortex membrane in the presence of Sandostatin.

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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