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Evaluation of an I^{123}-Radiolabeled Peptide as a Targeting and Imaging Agent for Cell Membrane Receptors

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ABSTRACT

During the last years investigators have focused on the importance of labeled peptides in multiple areas in nuclear medicine. The development, optimization and applications of methodologies for the labeling of peptides with ^{18}F and ^{123}I are very important goals in both benign and malignant disorders. The objective of current study focused on the optimization of synthesis, quality control, *in vitro* and *in vivo* evaluation of ^{123}I radiopharmaceuticals based on peptides. The selective chemotactic peptide was N-formyl-Met-Leu-Phe (N-For-MLF). This peptide is a bacterial product that possibly via cell membrane receptors binds to polymorph nuclear leukocytes and mononuclear macrophages and causes leukocyte chemo taxis. The using of labeling procedures prosthetic groups was applied. After labeling, the fate of the labeled FMLF, stability *in vivo*, bio distribution and pharmacokinetics were investigated in rodents and white blood cell. The results showed that peptides such as FMLF do not lend themselves for direct labeling method using chloramine-T. Therefore, their labeling must be considered by in directed method. Also, with increasing in pH, yield of labeled FMLF decreased with increasing in pH perhaps because of interaction OH to carboxyl group of SIB resulting iodobenzoicacid. Foremore, biological studies of labeled FMLF showed a low uptake in thyroid but a high at urine and bladder. Perhaps because of FMLF molecular weight is low in comparison to other peptides; accordingly this molecule could pass from blood to urine.

Key words: FMLF, radiolabeled peptide, radio iodination, membrane receptors

INTRODUCTION

Nuclear medicine techniques are increasingly being used in the detecting and therapy of disease. The most sensitive imaging methods are those using nuclear probes for Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET). Specific kind of incarnate probes have been expansion for tumor receptor imaging and therapy. Since receptors of these agents have over expression on the tumor cells and so their pharmacokinetics, these peptides are very application in nuclear medicine (Okarvi, 2004; Reubi, 2003; Langer and Beck-Sickinger, 2001; Van Den Bossche and Van de Wiele, 2004; Reubi *et al.* 2005; Mariani *et al.*, 2006). Because of the importance of labeled peptides in multiple areas in nuclear

medicine the development and optimization of methodologies for the labeling of peptides with ^{18}F and ^{123}I is a very important goal. During the last three decade searchers have focused on monoclonal antibodies (MAbs) that are potentially ideal agents for a variety of applications in both benign and malignant disorders. But Experience over the past decade clarifies the advantages and limitations of MAbs (Blok *et al*., 1999; Dimitroulopoulos *et al*., 2003; Virgolini *et al*., 1995, 1996; Vaidyanathan and Zalutsky, 1996, Fischman *et al*., 1993).

In recent years the search for agents with specific targeting has led to a variety of molecules such as fragments, chimeric and humanized antibodies, immune adhesions and single chain antigen binding proteins and variable domain peptide molecules. Since the discovery of peptide receptors and the synthesis of small, biologically active peptides, it has been recognized that these molecules can provide new approaches for radiopharmaceutical development. In many cases of cancers an over expression of receptors is observed which makes such receptors an attractive target for tumors imaging. Many other peptides and receptor systems have been investigated in experimental animals and *in vitro* studies and have been suggested as imaging agents (Okarvi, 2004; Reubi *et al*., 2005; Blok *et al*., 1999).

One of the output bacterial is N-Formyl-Met-Leu-Phe (N-For-MLF) that by the cell membrane receptors attach to a lot of immune cells in body. Binding of this peptide or of many synthetic analogues initiates leukocyte chemo taxis (Zalutsky and Narula, 1987; Wilbur *et al*., 1989; Bakker *et al*., 1991; Lister-James *et al*., 1996; Babich *et al*., 1993; Greenwood *et al*., 1963; Salacinski *et al*., 1981).

Foremore, studies developed by Zalutsky and Narula (1987) and Wilbur *et al*. (1989) demonstrated that antibodies can be radio iodinated using the synthesized precursor N-succinimidyl-(tri-n-butylstannyl) benzoate intermediate (ATE), that is needed for making labeled SIB ((N-succinimidyliodobenzoate). The use of SIB reagent for protein labels significantly thyroid uptake of radioiodine. Objective of current study focused on the optimization of synthesis, quality control, *in vitro* and *in vivo* evaluation of ^{123}I radiopharmaceuticals based on peptides and selective labeling procedures using prosthetic groups were applied. Additionally the investigation includes on the fate of the label, stability *in vivo*, bio distribution and pharmacokinetics, rodents and also cell were made (Salacinski *et al*., 1981; Angelberger *et al*., 1995; Wilbur *et al*., 1989; Bolton and Hunter, 1973; Wood *et al*., 1975).

MATERIALS AND METHODS

Radio-iodination of FMLF: Labeling studies were done both by direct method using chloramine-T according to Khawli and Kassis (1989) and indirect method using [^{125}I and ^{131}I] SIB according to Zalutsky and Narula (1987). Since FMLF does not lend itself for direct radio-iodination, it was seen that, after labeling, the most unreacted radio-iodine was isolated from the reaction mixture by a column packed with Sephadex G-50 eluted with 0.01M PBS, pH 7.4. Briefly, radio-ioidination of FMLF via [^{125}I and [^{131}I] SIB was performed.

Biological studies: According to Vaidyanathan *et al*. (1995) and Zalutsky and Narula (1987) both in normal mice and the ones bearing 50 μL turpentine for 24 h, promoted inflammation in right leg (twelve normal mice and twelve per injected turpentine ones weighting 20-28 g). Each animal was received 6-12 μCi [^{125}I] FMLF intravenously via tail vein (three mice per time point), sacrificed

0.5, 2, 4 and 24 h post injection and dissected. The tissues of interest were discarded, washed with saline, dried, weighed and counted by a gamma counter to obtain bio distribution. The tissue bio distribution results were expressed as the percentage of the injected dose, localized per gram of tissue. Presence of radioactivity in urine and bladder encouraged to that make the SPECT scan on the mice which were received [^{131}I] iodide and [^{131}I] FMLF. These scans confirmed the difference radioactivity localized in different organs, especially in thyroid.

Biological evaluation of the chemotactic-SIB conjugate, *in vitro* studies: The ability of the labeled peptide conjugate to bind to human Polymorph Nuclear Leukocytes (PMN) was determined using *in vitro* assays described in the literature (Vaidyanathan *et al*., 1995; Vaidyanathan and Zalutsky, 1997) with some modification taking into account this laboratory facilities. In order to determine the effect of derivatization with SIB on the potency of the peptide and in the biological activity, competitive binding to PMN and superoxide production were made.

Cell preparation: Human PMNs were isolated using a density gradient centrifugation method as described in the literature (Vaidyanathan *et al*., 2005).

Isolation procedure:

- **Blood collect:** As 30-40 mL of blood from normal voluntary was used in each experiment used sodium citrate 3.8% as anticoagulant in relation blood/anticoagulant 9:1. The initial count of total leukocytes present was made in a Neuberger camera making a dilution 1/20 of a sample of the blood with 3% acetic acid
- **Buffy-coat:** In a 15 mL tube with 5% Dextran (PM 100, 000-200, 000) in phosphate-buffered saline pH 7.4 (PBS) added the blood in relation blood/dextran 1:5. Is important to add the blood slowly drop by drop in the center of the tube. The cells were allowed to settle 45 min at room temperature. In this step normally 30% of the cells are lost. The supernatant was washed in PBS or culture media (RPMI 1640, Sigma R1383), pH 7.4 and centrifuged twice at 400×g for 10 min. The cellular pellet was re-suspended in culture media
- **Gradient separation:** Used per colldensity 1.130±0.005 g mL^{-1} (Sigma P1644) and PBS to prepare two solutions of different densities (1.119 and 1.077 g mL^{-1}). The cells re-suspended in culture media (4 mL) from step (2) was layered over percoll gradients consisting of 3 mL of each of two densities (1.119 and 1.077 g mL^{-1}) in 10 mL conical tubes. PMNs were harvested from the interface between the two solutions following centrifugation at 1000×g for 25 min at 2°C. The cells were washed in culture media (RPMI 1640, Sigma R1383), pH 7.4 and centrifuged twice at 400×g for 10 min. The cells were then re-suspended in a small volume of incubation buffer (1.7 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 117.0 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM PMSF, pH 7.4)
- **Cell count and determination of cell viability:** A sample of isolated cells from (3) was diluted1:2 with 0.4% Trypan Blue and after 4 min they were count in a Neubauer camera. Viable cells counted from death cells (stained cells). Finally cell suspension was diluted at a

concentration of 1×107 cells mL^{-1}. Cell preparation contained more than 95% PMNs as assessed by light microscopy of a sample of Giemsa-stained specimens. Cell yields of 35×106-50×06 cell from 40 mL of blood were obtained

RESULTS AND DISCUSSION

Synthesis of following intermediate products was performed:

- Tri-n-butyl stannyl-3 and 4-(tri-n-buthylstannyl) benzoate in >30% yield
- N-succinimidyl-3 and 4-(tri-n-buthylstannyl) benzoate, ATE in 70% yield
- N-succinimidyl-3 and 4-iodobenzoate, SIB in 80% yield

Radio iodination of SIB with radioiodine 125 and 131 was performed satisfactorily in radiochemical yield more than 40-70% (Fig. 1). Comparative HPLC analysis of IK, NCS, ATE, SIB (cold) and the elution profile of radioiodinated SIB shows a high radio chemical purity in [^{125}I and ^{131}I] SIB, 99%.

FMLF labeling was done both by direct method using chloramine-T and indirect method via radio iodinated SIB, showed that FMLF does not lent itself for direct labeling using chloramine-T (Fig. 2).

FMLF labeling via radioiodine SIB was performed at different pH 8.5, 9.0, 9.5 and 10.0, disclose that radiochemical yields decrease with increasing pH (Fig. 3) and the best yield was found at pH 8.5.

Biological behavior of labeled FMLF was studied in normal mice and 24 h after the animal received 6-12 μCi [^{125}I] SIB-FMLf (Fig. 4, 5). These results showed an accumulation in the inflamed leg. In Fig. 5 could be see that in the inflamed mice, in almost all the tissues, the uptake is lower than in the normal mice. The reason could be the progressive accumulation of activity by the inflammation.

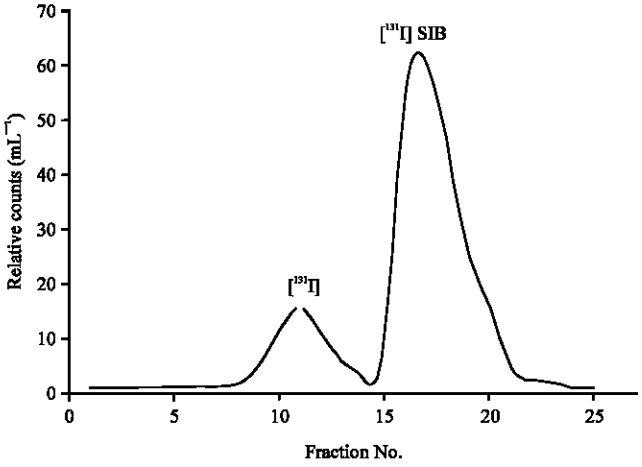

Fig. 1: Relative activity mL^{-1} of each fraction to calculate labeling yield. Radio iodination of SIB with radioiodine 125 and 131 was performed satisfactorily in radiochemical yield more than 40-70%

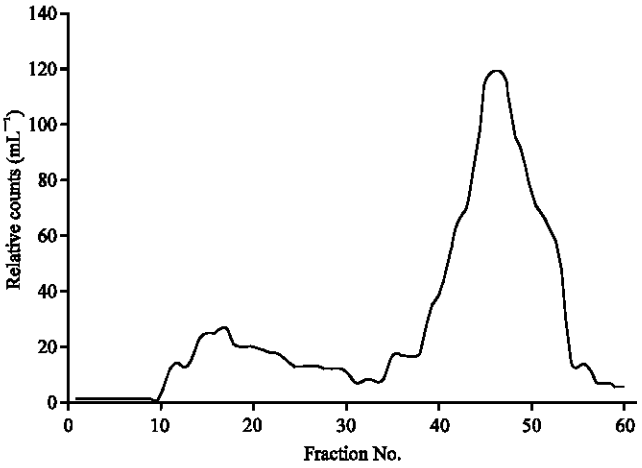

Fig. 2: Labelling studies were done both by direct method using chloramine-T and indirect method using [^{125}I and ^{131}I]SIB. Since FMLF does not lend itself for direct radioiodination, it was seen that, after labelling, the most unreacted radioiodine was isolated from the reaction mixture by a column packed with Sephadex G-50 eluted with 0.01 M PBS, pH 7.4. The fractions between 20-22 mL were labelled FMLF. 3B: radiochemical yields decrease with increasing pH

A comparative SPECT scan was performed with normal mice 2 h after the animals received about 30 μCi [^{131}I] iodide and [^{131}I] SIB-FMLF to distinguish why high labeled FMLP has been localized in bladder and urine (Fig. 6). The results of biodistribution showed a very low thyroid and stomach uptake of radioiodine. These results indicate that the labelling of proteins using the ATE intermediate produced labelled proteins with a decreased rate of dehalogenation *in vivo*. This result is similar to that obtained by Zalutsky and Narula (1987).

The procedure for ATE synthesis and its intermediate products must be reconsidered due to low yield of intermediate products.

Protein labeling by SIB which is itself radiolabelled by "^{125}I" and "^{131}I" at pH = 8.5 was done with high yield. As pH increased, pattern of marks of FLMF decreases due to the reaction of carboxyl SIB and "OH" which causes the formation of mono Iodobenzoic acid. Chemical peptides labeled with radioactive element like ^{131}I, ^{125}I, ^{3}H, ^{18}F, ^{99m}TC, ^{111}In is preferable method for identifying and determining infection.

While the result our current experiments was 66% within 5 min while zalatsky obtained 72% in the same experiment. There are several factors which cause this difference. These are solubility of FMLF derivatives in solution compare to buffer which was used for monoclonal antibody, also apart from the positive effect of solubility of solution heterogeneous system products is better than homogenous system. In addition, since the ^{125}I SIB is an active ester comparative hydrolysis under aqueous condition will reduce the access for conjugating of antibody. At last the reaction is dependent on the pH and the yield increased, respectively.

The small size of this protein causes passing through the glomerular of kidney, so high activity was observed in urine and bladder. In this study the highest activity observed in urine and bladder. This is not surprising since the protein small and can pass through the glomerular of kidney. Foremore, not due to labeled incorrectly, because it was found that during the color scan, Na^{131}I, ^{125}I FMLF was injected to one of the mice, the highest uptake observed. Concomitantly, in the

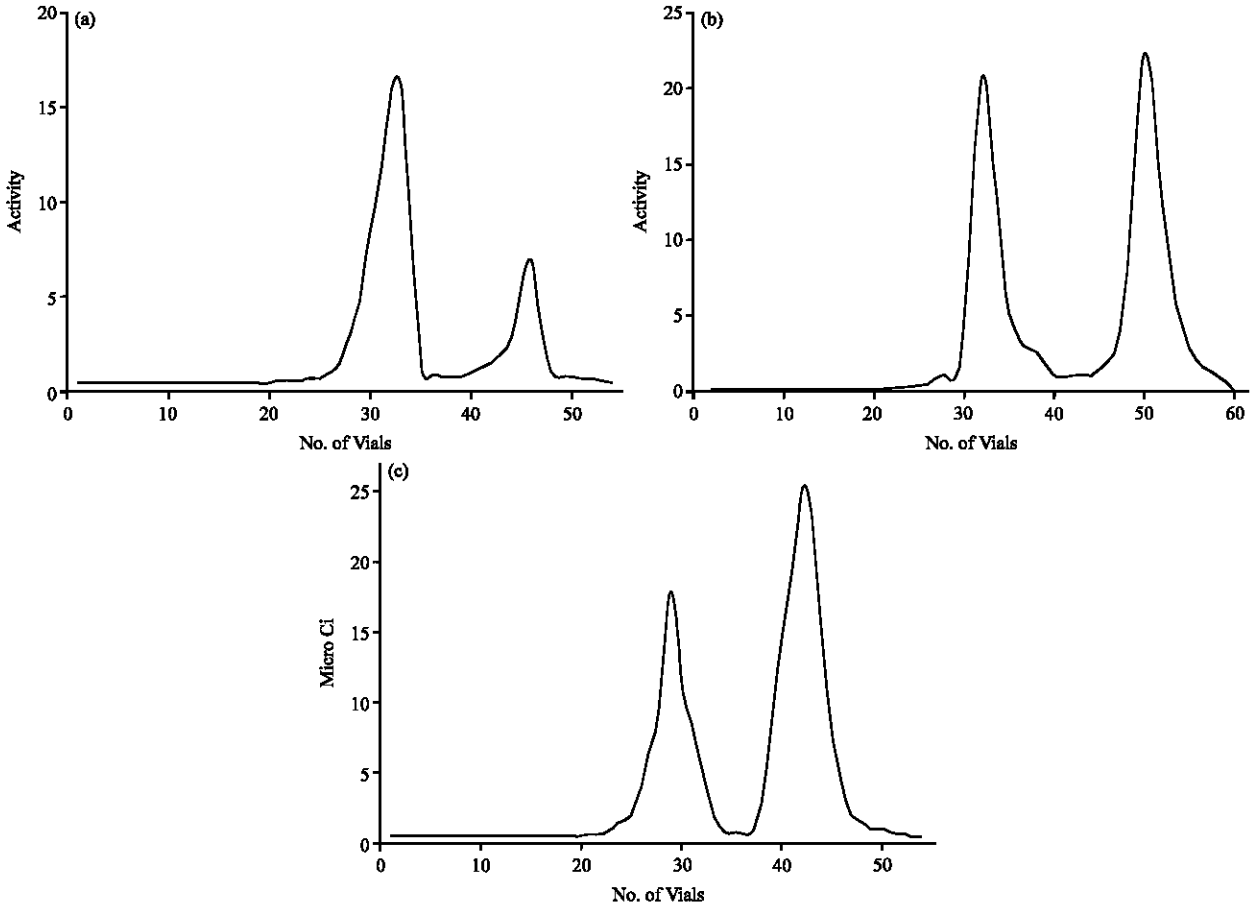

Fig. 3(a-c): FMLF was added to the labeled IB (200 μL FMLF/50 μL 0.1 M borate buffer). FMLF labeling via radioiodine SIB was performed at different pH 8.5 (3A), 9.0 (3B), 9.5 and 10.0 (3C), disclose that radiochemical yields decrease with increasing pH and the best yield was found at pH 8.5 (3A), (a) Best yield was found at pH 8.5, (b) Radiochemical yields decrease with increasing pH and (c) Radiochemical yields decrease with increasing pH

thyroid and the abdominal region showed low activity. If $Na^{131}I$, ^{125}I FMLF were injected to the same mice after two hours the highest radio activity were observed in abdominal cavity and low uptake in thyroid. The highest absorption of FMLF was seen in kidney, liver, stomach and gut. Initial activity was high in stomach, kidney and gastrointestinal tract. Color scan showed, after injection of labeled peptides to mice, thyroid has low due to free iodine (unlabeled) is absorbed in thyroid. Then, we was concluded that the amount of unlabeled iodine is low but labeling performed correctly, Fishman *et al.* (1987) report this difference were 8 times. Factor such as lipophilicity, catabolism of labeled peptide must be considered in labeling of protein. Higher uptake and slow rise absorption was height in the injected right leg with turpentine compare to other organ as indicates the infection and increase influx of blood in that area.

The amount of absorption in turpentine muscle, due to inflammation, more than the amount in a normal muscle. This peptide was conjugated to PMN specifically and maximum activity was 66%. It is possible that severe neutropenia created by this peptide, so there is a need for more

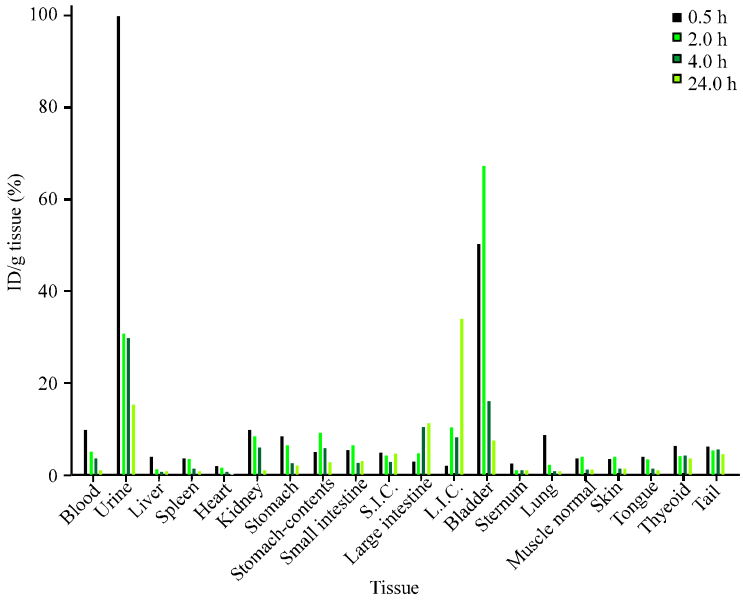

Fig. 4: Results of study of biological behavior of labelled FMLF (N-formyl-Met-Leu-Phe) in normal mice. The small size of this protein causes passing through the glomerular of kidney, so high activity was observed in urine and bladder. The highest absorption of FMLF was seen in kidney, liver, stomach and gut. Initial activity was high in stomach, kidney and gastrointestinal tract

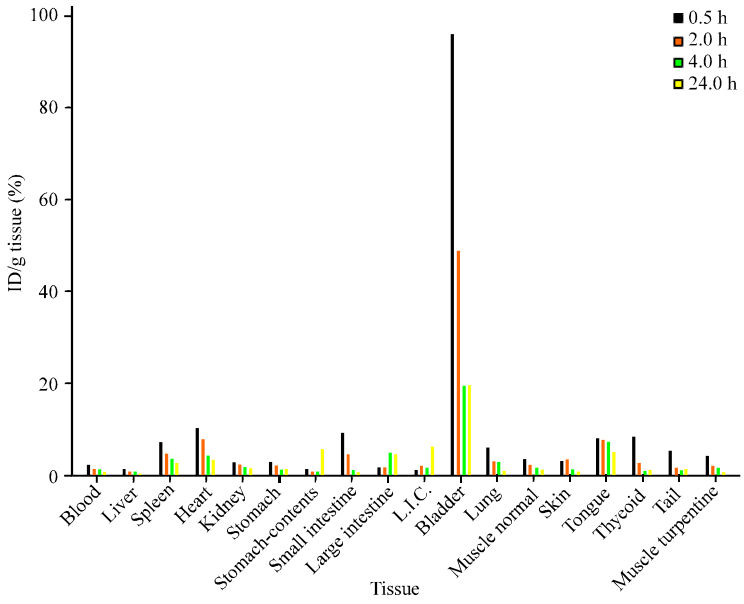

Fig. 5: Results of study of biological behavior of labelled FMLF (N-formyl-Met-Leu-Phe) in inflamed mice. Can see that in the inflamed mice, in almost all the tissues, the uptake is lower than in the normal mice. The reason could be the progressive accumulation of activity by the inflammation

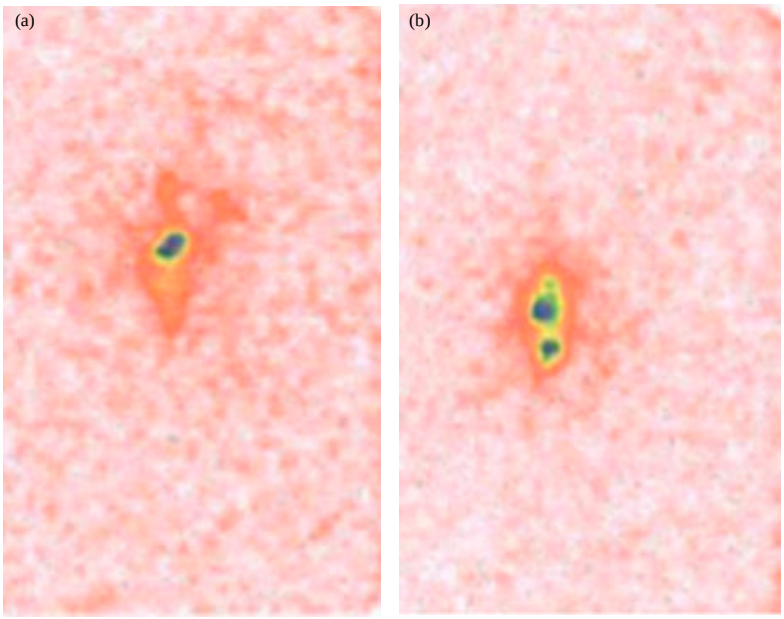

Fig. 6(a-b): Comparative SPECT (single photon emission computed tomography scan) performed with normal mice 2 h after the animals received about 30 μCi [^{131}I] SIB-FMLFand [^{131}I] iodide. The results of bio distribution showed a very low thyroid and stomach uptake of radioiodine. These results indicate that the labeling of proteins using the ATE intermediate produced labeled proteins with a decreased rate of dehalogenation *in vivo*, (a) Scan has taken 35 min after injection with [^{131}I] SIB-FMLF, Normal Mouse Source activity 6.1 micro Cori Accumulated Radio-isotope in the thyroid: o micro Cori Accumulated Radio-isotope in the abdomen: 1.8 micro ci and (b) Scan has taken 35 min after injection with [^{131}I] NaI Normal Mouse Source activity 5.4 micro Cori accumulated Radio-isotope in the thyroid: 0.62 micro Cori Accumulated Radio-isotope in the abdomen: 0.9 micro Cori

preparation for clinical research. This can only be done by using of ^{125}I SIB with the highest activity which is achieved by using of ^{125}I with height specific activity. Using of small size protein is more preferential than antibody due to the clear more rapidly from blood pool and normal organ. The proportion of absorbed activity of labeled peptides in organs, especially gastro intestinal tract to blood is more than this proportion for the peptides which are labeled by technetium (Okarvi, 2004; Reubi *et al*., 2005; Blok *et al*., 1999). This can be the result of more lipidophily of peptides to iodine.

CONCLUSION

Biological studies of labeled FMLF showed a low uptake in thyroid but a high at urine and bladder. Perhaps because of FMLF molecular weight is low in comparison to other peptides; accordingly this molecule could pass from blood to urine. Foremore, Tumor cell express significantly height amount of receptor as compared to normal tissue with referring to previous studies small peptides had more advantage compare with antibody due to easy penetration to tumor, easy clearance from blood.

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