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Research Article

A New ^{99m}Tc Labeled Peptide: ^{99m}Tc β-Casomorphin 6, Biodistribution and Imaging Studies on Rats [1]

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Abstract

Peptide radiopharmaceuticals have an increasing significance in nuclear medicine practice. β-casomorphin is a digestive peptide with 6 amino acids (Tyr-Pro-Phe-Pro-Gly-Pro). N terminal amino acid chain mainly tyr-pro-phe-pro structured exogen opioid peptid type beta cosomorphin are μ-receptor agonistic activity with priority. Animal studies show that β-casomorphins can act as opioid receptor agonists. The aim of this study was to label β-casomorphin with ^{99m} Tc and to examine its usefulness as an opioid receptor binding radiopharmaceutical in *Albino Wistar rats* and cancer cells. β-casomorphin was labeled with ^{99m} Tc radionuclide using bifunctional chelating agent. Quality control studies were done by Instant Thin layer chromatography (ITLC) and High performance liquid chromatography (HPLC) methods. Binding efficiency of the compound was more than 99%. It was observed as stable for at least 3 h at room temperature. Lipophilicity was also performed for labeled molecule. Imaging studies for ^{99m} Tc labeled molecule was done in rats by using gamma camera. For biodistribution studies; ^{99m} Tc labeled molecule was injected to the rats from tail vein and radioactivity per gr weight of each organ was measured as count per second (cps). Receptor specificity was evaluated in imaging and biodistribution studies in experimental animals. Cell labeling studies were also performed on *breast* and ovarien cancer cells. In terms of evaluating the biodistribution of ^{99m} Tc-β-casomorphin molecule in rats, *uterus* and *ovary* displayed high involvement. It was also confirmed by cell labeling studies. If the radiopharmaceutical is radiolabeled with therapeutic radionuclides it would be useful for therapy for *uterus, ovary* and *breast* tumors.

Keywords: *β-casomorphin, Cancer cell , Peptide, ^{99m}Tc, receptor*

^{99m}Tc Bağlı Yeni Bir Peptid: Ratlarda ^{99m} Tc β-Kazomorfin 6 Molekülünün Biyodağılım ve Görüntüleme Çalışması

Özet

Peptit radyofarmasötikleri nükleer tıp pratiğinde giderek artan bir öneme sahiptir. β- kazomorfin 6 amino asit (Tyr-Pro-Phe-Pro-Gly-Pro) ile bir sindirim peptitidir. Tyr-Pro-Phe-Pro aminoasit zinciri ile başlayan ekzojen opioid peptid türevi β-kazomorfinler öncelikle μ-resptör agonistik aktiviteye sahiptir. Hayvan çalışmaları β-kazomorfin türevlerinin bir opioid reseptör agonisti olduğunu göstermiştir. Bu çalışmanın amacı, β-kazomorfin'i ^{99m} Tc ile işaretleyerek opioid reseptörler radyofarmasötiği olarak kullanabilirliğini Albino Wistar ratlarda ve kanser hücrelerinde incelemektir. β-kazomorphin bifonksiyonel molekül kullanılarak ^{99m} Tc radyonüklidi ile işaretlendi. Kalite kontrol çalışmaları, ince tabaka kromatografisi (ITLC) ve yüksek performanslı sıvı kromatografisi (HPLC) yöntemleri ile yapıldı. Bileşiğin işaretlenme verimi %99'dan fazla idi. Oda sıcaklığında, en az 3 saat boyunca stabil kaldığı tespit edildi. İşaretli molekülün lipofilite çalışması da gerçekleştirildi. ^{99m}Tc ile işaretli molekülün görüntüleme çalışmaları sıçanlarda gamma kamera kullanılarak gerçekleştirildi. Biyolojik dağılım çalışmaları için ^{99m}Tc işaretli molekül kuyruk veninden sıçanlara enjekte edildi ve her organın gr ağırlığı başına radyoaktivitesi sayım/saniye olarak ölçüldü. Molekülün reseptöre bağlanma özelliği deney hayvanlarında görüntüleme ve biyolojik dağılım çalışmaları ile değerlendirildi. Hücre işaretleme çalışmaları meme ve yumurtalık kanser hücreleri için yapıldı. ^{99m} Tc β-kazomorfin molekülünün biyolojik dağılımı sıçanlarda değerlendirildiğinde, rahim ve yumurtalıkta yüksek tutulum gösterdiği görüldü. Molekülün reseptör etkinliği hücre işaretleme çalışmalarıyla da doğrulandı. Molekül tedavi radyonüklitleri ile işaretlendiğinde ise rahim ve yumurtalık tümörleri tedavisinde yararlı olabilecektir.

Anahtar sözcükler: *β-kazomorfin, Kanser hücresi , Peptid, ^{99m}Tc, reseptör*

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INTRODUCTION

Peptides are receptor specific molecules. They have an important role not only in diagnosis but also in treatment modalities. Peptide radiopharmaceuticals are developed for this purpose became important in nuclear medicine practice. Use of receptor-specific peptide compounds has an increasing significance not only in imaging, but also in treatment modalities [1-6].

Imaging of receptors on tumor cell surface using radiolabeled regulatory peptides becoming ingradually important strategy in oncology research [7]. High affinity of radiolabeled peptides for receptors indicates molecular targets for diagnosis and treatment of many cancer types.

Several clinically relevant radionuclides have been used for labeling bioactive peptides either for diagnostic imaging (^{99m}Tc, ^{111}In, $^{68/66}$Ga, ^{18}F, ^{123}I, ^{64}Cu) or for therapy (^{111}In, $^{64/67}$Cu, ^{90}Y, ^{177}Lu, ^{213}Bi) [2-4,6].

Small-sized peptides (compounds having short chain with less than 30 amino acids) manifest rapid pharmacokinetics. They easily penetrate into extravasculary tissue space, are excreted rapidly and their clearance from the blood is fast [7-10].

Rapid pharmacokinetics are ideal for labeling peptides with a radioisotope that has a short half-life, such as ^{99m}Tc. Among all the radioisotopes used in nuclear medicine ^{99m}Tc is still the most widely applied for diagnostic purpose, mainly because of the ready availability, low cost, excellent imaging properties, favorable dosimetry and high specific activity [11,12]. ^{99m}Tc-labeled peptides, particularly those of a lipophilic nature, are often excreted through the hepatobiliary system, and the subsequent accumulation in the intestine may obscure receptor-mediated uptake in tumor sites in the pelvis [13,14]. In our previous studies CCK8 (Asp-Tyr(SO3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) was labeled with ^{99m}Tc using bifunctional chelates [Glucoheptonate (GH) and Diethylene-Triamine-Pentaacetate (DTPA)] and it was shown that this could have a high potential for practice in tissues containing CCK receptors [15]. We also studied on ^{99m}Tc exorphin C peptide molecule for biodistribution and imaging on tumor bearing rats [16].

β-casomorphin is an exogenous bioactive opioid peptide derivative from enzymatic digests of bovine casein [17]. β-casomorphin is a β-casein derive peptide sequence present in the milk protein. β-casomorphin release from parent protein molecule during *gastrointestinal* digestion. It was reported that *uterus* and *ovary* express opioid receptors [17,18].

Animal studies show that β-casomorphins can act as opioid receptor agonists are derived-casein.

Animal studies clearly show that β-casomorphins derived from β-casein are opioid-like peptides [19,20].

N terminal amino acid chain mainly tyr-pro-phe- pro structured exogen opioid peptid type beta casomorphin are receptör agonistic activity with priority [21,22].

In this study β-casomorphin has 6 aminoacid (Tyr-Pro-Phe-Pro-Gly-Pro) sequenced peptide molecule was labeled with ^{99m}Tc and investigated its radiopharmaceutical potential by imaging and biodistribution studies in rats.

MATERIAL and METHODS

All animals were treated in accordance with protocols approved by the Animal Care and Use Committee of Ege University.

HPLC equipment: is an equipment which has high pressure-four gradient LCD display screen microprocessor controlled pump, variable wave length UV/VIS detector, sample automatic injection and control unit (Perkin Elmer Series, 200 USA). There is a fraction collector on the equipment to collect the carrier phase (Foxy 200).

Gamma camera: Philips Forte Gamma Camera (for scintigraphy).

In this study, linear peptide molecule was designed with 6 amino acid sequences (Tyr-Pro-Phe-Pro-Gly-Pro) and it was synthesized by PepMetric Technologies commercially. All other chemicals were supplied from Sigma-Aldrich Co.

UV absorption spectral analysis was performed by spectrophotometer. Purity of synthesized molecule was checked in HPLC equipment at 214 nm wave length by using C-18 RP as column and as eluents 0.1% TFA and water as solution. Purity of synthesized molecules was 97%.

Labeling and Quality Control Studies with ^{99m}Tc

- Preparation of Solutions

Preparation of peptide solution: Peptide was dissolved in pure water. Peptide solutions were fractioned and kept in tapped tubes at -20°C to be used 1 tube (250 µg/mL) at each time. Out of 250 µg/mL peptide solution, 0.1 mL was used for labeling process.

Labeling of peptides with ^{99m}Tc: In labeling procedures, firstly direct labeling method was used, but since it could not reveal sufficient labeling, desired result was obtained similar with our previous study [15,16,23].

GH molecule was used as bifunctional agent in indirect labeling method. Ten mg of GH (Sigma Chemical Co.) was dissolved in 1 ml distilled water, 300 µg (in 0.5 ml water) stannous chloride and 259 MBq of ^{99m}Tc (in 0.5 mL), 25 µg peptide (in 0.1 mL), were added to the solution and then the mixture was heated for 10 min at 90°C. Firstly reduction

of technetium was (^{99m}Tc) was accomplished by tin and then reduced ^{99m}Tc was bound to peptide molecules using bifunctional agent GH at pH value of 5 *(Fig. 1)*.

After cooling to room temperature, quality control assessments of ^{99m}Tc-β-casomorphin, was done by ITLC and HPLC methods. Rf values of labeled compounds for 0.9% NaCl and acetone were determined using ITLC-SG (1x10) strips *(Table 1, Fig. 2, Fig. 3)*.

Conditions for HPLC: The wavelength of labeled compound was measured as 195 nanometers in the spectrophotometer. Solutions of 0.1% TFA and acetonitrile at ratio of 20/80 with a flow velocity of 1 mL/min was set up, wavelength was set up 195 nanometer for UV detector *(Fig. 4)*.

- Determination of Lipophilicity

Lipophilicity values are important in understanding biologic transport characteristics of the peptide molecule. Distribution of a single molecule between two different phases can be defined as distribution factor.

Table 1. *Rf values of relevant complexes*
Tablo 1. *İlgili komplekslerin Rf değerleri*

Compound	Physiological Serum (*Rf*)	Acetone (*Rf*)
Na^{99m}TcO4	1	1
^{99m}Tc O2	0	0
^{99m}Tc GH	0.4	0
^{99m}Tc-β-casomorphin	1	0

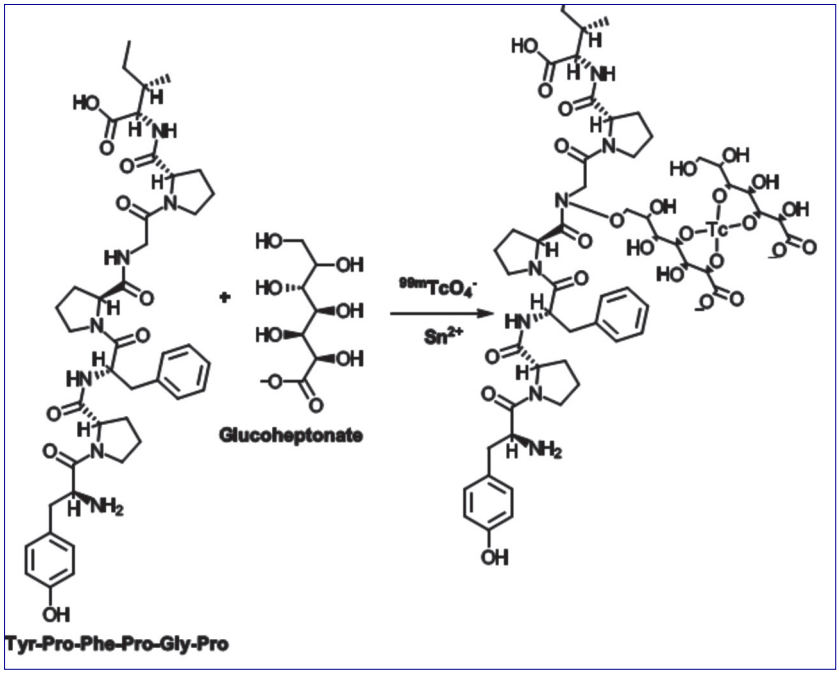

Fig 1. β-casomorphin + GH + ^{99m}Tc O4

Şekil 1. β-kasomorphin + GH + ^{99m}Tc O4

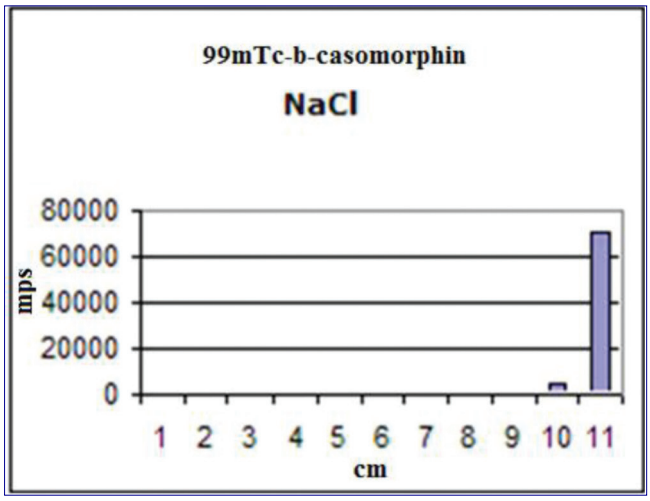

Fig 2. ITLC-SG diagram of ^{99m}Tc-β-casomorphin in NaCl 0.9%

Şekil 2. %0.9 NaCl'de ^{99m}Tc-β-kazomorphin'in ITLC-SG diyagramı

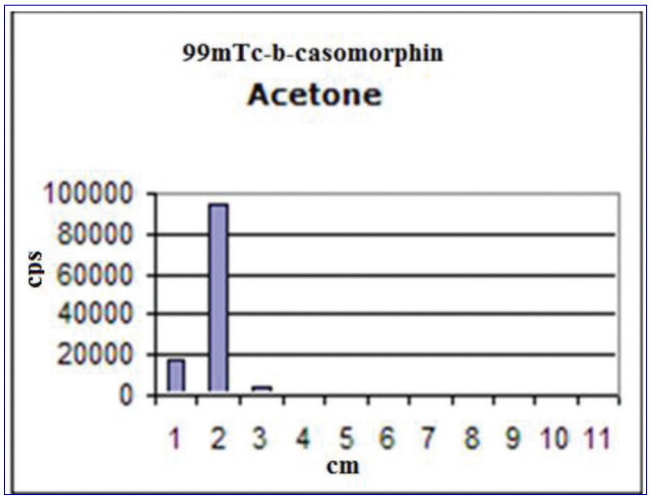

Fig 3. ^{99m}Tc-β-casomorphin ITLC-SG in acetone

Şekil 3. Aseton'da ^{99m}Tc-β-kazomorphin ITLC-SG diyagramı

Experimental lipophilicity values of labeled compounds were determined by extraction method carried out in octanol for two different solutions 0.9% NaCl and H_2O. For lipophilicity, activities in octanol and water phase and those in octanol and 0.9% NaCl phase were counted in counter equipment as cps. Lipophilicity values were obtained by rationing the counts in both phases and calculating their logarithm. After mixing 500 µL octanol, 500 µL 0.9% NaCl or H_2O and 10 microcurie ^{99m}Tc labeled peptide in a vortex mixer for 1 min, they were centrifuged at 4.000 rpm. Low phase and upper phase radioactivity were counted in dose calibrator and phases were rationed and logarithm was calculated [22,24,25]. The lipophilicity was calculated using the formula

logP = A0/Aw

Besides, theoretical lipophilicity values were compared with experimental values using ACD chemsketch computer program *(Table 2)*.

Experimental Animal Studies

Albino wistar female rats weighing 150-200 g were used for animal experiments. Animals were housed under constant conditions of temperature (20-22°C) and lighting (12 h light-12 h dark). All animals had access to regular rat chow and water *ad libitum* except when scheduled for testing and for sacrifice.

- Scintigraphic Studies

Labeling of peptide molecule with ^{99m}Tc was performed and after reaching a sufficient binding level, scintigraphy and biodistribution experiments were done on experimental animals.

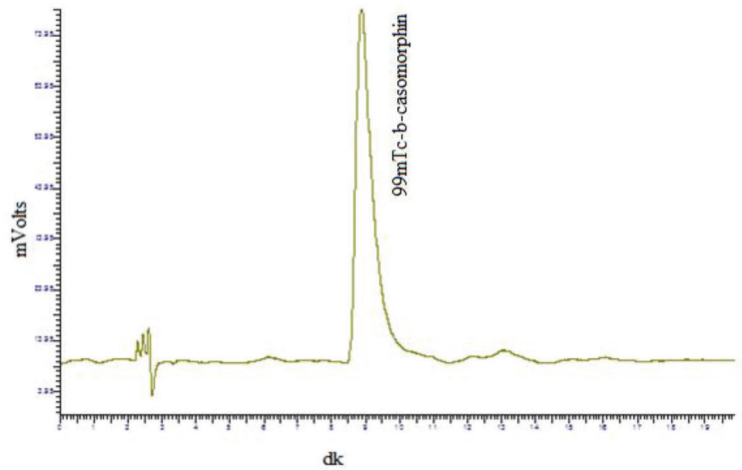

Fig 4. HPLC diagram of ^{99m}Tc-β-casomorphin

Şekil 4. ^{99m}Tc-β-kazomorfin'in HPLC diyagramı

Fig 5. ^{99m}Tc-β-casomorphin biodistribution studies on rats

Şekil 5. Ratlarda ^{99m}Tc-β-kazomorfin biyodağılım çalışması

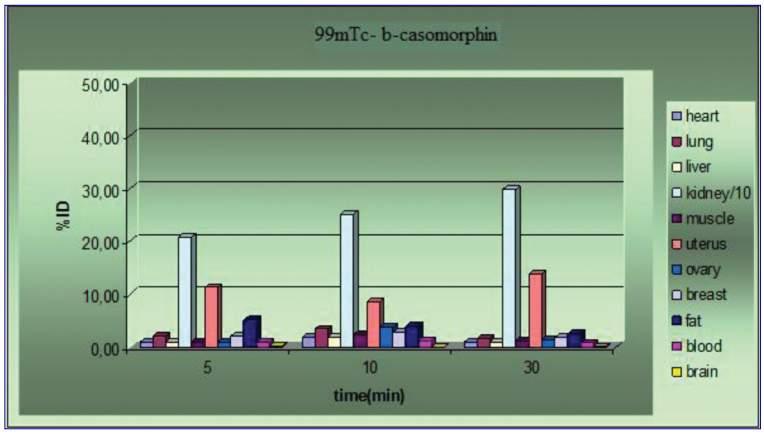

Table 2. *Therotical lypophilicity value of unlabeled β-casomorphin was similar with ^{99m}Tc-β-casomorphin*

Tablo 2. *Bağlanmamış β-kazomorfin'in kuramsal lipofilitesi ^{99m}Tc-β-kazomorphin ile benzerdir*

Peptide Molecule	Lypophilicity Values (logP)		
	Water	%NaCl	Theoretical
^{99m}Tc-β-casomorphin	1.8±0.1	1.27±0	1.42±0.95

Quality control studies demonstrated that labeled compounds can be administered to living beings. Experimental animals were used as three rats per group. Rats were given general anesthesia, 100 microcurie ^{99m}Tc-β-casomorphin was injected from tail vein and scintigraphy was performed using angle gamma camera. Distribution in the body was followed up by obtaining static images in (256x256) matrix at 5, 10, 15, 20, 30,

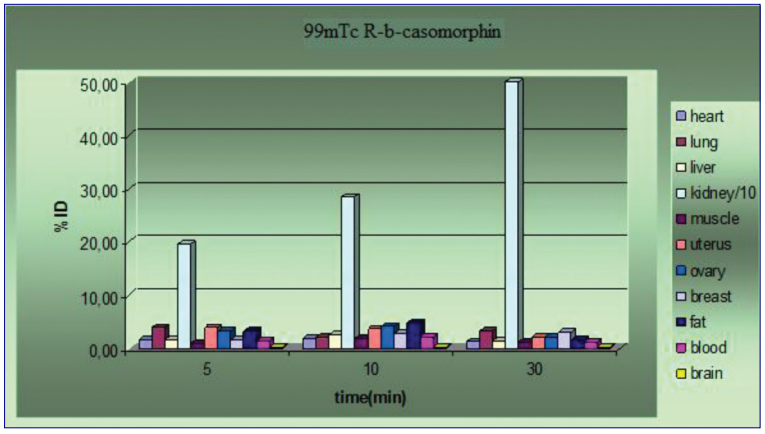

Fig 6. ^{99m}Tc-β-casomorphin reseptor saturated biodistribution studies on rats

Şekil 6. Ratlarda satüre ^{99m}Tc-β-kazomorfin reseptör biyodağılım çalışması

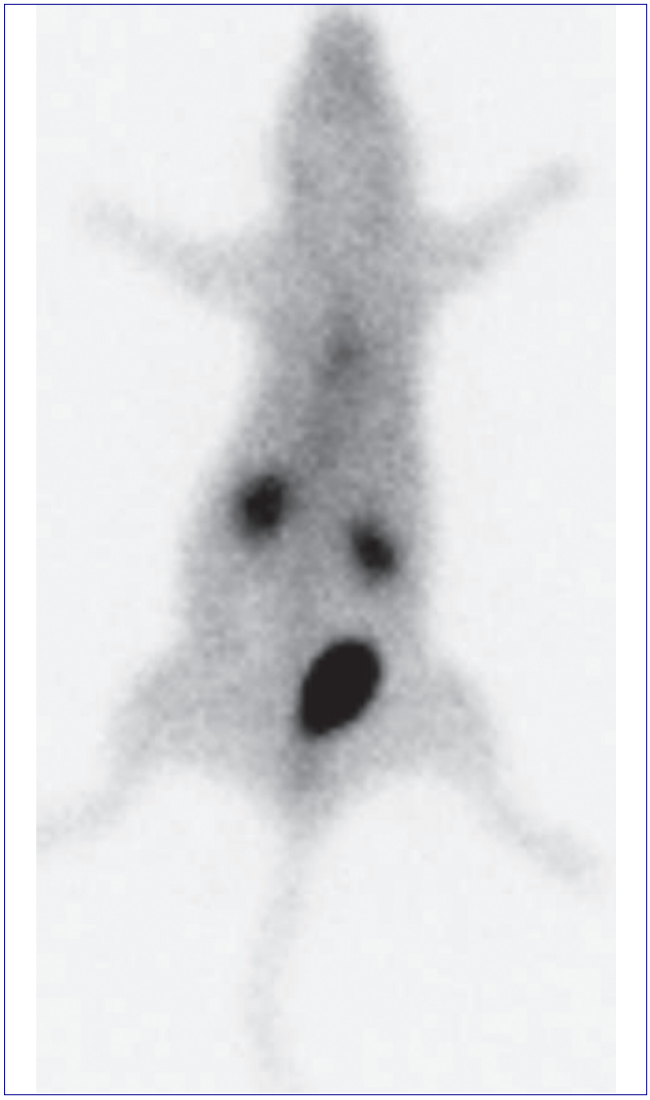

Fig 7. ^{99m}Tc-β-casomorphin scintigraphic imaging on rats
Şekil 7. Ratlarda ^{99m}Tc-β-kazomorfin sintigrafik görüntüleme

60, 120 min and 24 h. For receptor saturation studies, pure β-casomorphin was given prior to radioactive substance and scintigraphic imaging was done under the same conditions *(Fig. 5)*.

Table 3. *Uptake rate of peptide molecule for target organs*
Tablo 3. *Hedef organlarda peptid molekülünün tutulum oranı*

99mTc-β-Casomorphin (Organs)	Unsaturated Receptor		Saturated Receptor	
	%ID/g	Organ/Bg	%ID/g	Organ/Bg
uterus	7.02	7.97	0.69	1.23
ovary	5.61	6.7	0.41	0.70
breast	5.3	6.02	0.72	0.48
muscle=bg	0.88	1	0.88	1

Table 4. *The labeling efficiency of peptide molecule in accordance with cell types*
Tablo 4. *Hücre tiplerinde peptide molekülünün bağlanma etkinliği*

Tc-99m Labeled Molecule	Cell Cultures		
	MCF7	MDAH2774	WL38(control)
Tc-99m-β-Casomorphin	90.71±9%	80.32±10%	31±8%

For each organ, a graphic was drawn corresponding with time intervals using the counts obtained from images cps.

- Biodistribution Studies

Biodistribution studies were also done on rats. Rats given 100 microcurie labeled molecule was sacrificed at 5th, 10th and 30th min, being three rats from each group. Each organ was extracted and activity was measured in gamma counter. Percentage of activity per gram corresponding cps was calculated. Receptor saturation studies were carried out under the same conditions. Just before radioactive substance injections at 5th, 10th and 30th min, rats were injected 10 µg/0.5 mL pure peptide molecule for receptor saturation. Biodistribution graphic was created with percentage of injected dose amount (ID/g%) for counts per grams corresponding time interval *(Fig. 6, Fig. 7, Table 3)*.

Cell Culture Studies

The radiopharmaceutical potentials of peptide radiopharmaceuticals in cancerous cell and normal cell were examined in the cell culture studies of peptide radio-

pharmaceutical. The cell culture process took place at the Department of Medical Biology of Faculty of Medicine in Ege University. All cell lines were supplied from American Type Culture Collection (ATCC, UK). BIOAMF-1 (Biological Industries, Israel), BIOAMF-1 supplement for the reproduction of WL-38 (Lung Fibroblast) cell lines in culture medium and RPMI 1640 (Biological Industries, Israel) for the reproduction of MDAH-2774 (Ovarian endometriotid adenocarcinoma) and MCF-7 (breast cancer) cell lines were used by adding 2 mM L-glutamin, 1% Penicillin Streptomycin to the medium. The cells were incubated in the incubator (Thermo, US) maintaining at 95% relative humidity and with 5% CO_2 until the adequate reproduction was obtained from the cells. The proliferation, passaging (by Trypsinization method) and follow-up of cells were monitored through the inverted microscope (Olympus, Japan). Production and passaging processes were continued until the number of cells in each cell line reached 107 per mL.

Cell Labeling Studies

The cells were examined under the microscope at 10x, 40x and 100x magnifications. It was observed that the cells were healthy and enough in number. The cell numbers were studied to be 10^7 cell/mL. The cells were put into falcon tubes and 2 mL medium (RPMI 1640) was added. They were centrifuged at 2.500 rpm for 5 min. The supernatant was removed. 5 mCi/mL Tc-99m-β-casomorphin was added on the cells (MCF7, MDAH2774 and WL38) After the incubation at room temperature for 10 min, 2 mL RPMI-1640 was added. They were centrifuged at 2.500 rpm for 5 min. The upper layer was taken and the labeling efficiency of cells was calculated through the measurement of activities in the lower layer and upper layer *(Table 4)*.

RESULTS

In this study, peptide molecule was labeled with ^{99m}Tc and its usability as opioid receptor pharmaceutical was analyzed. Peptides were labeled with ^{99m}Tc using GH as bifunctional agent. Binding efficiency of the labeled compound was more than 99%. It was observed to be stable for 3 h at room temperature.

Imaging and biodistribution studies on rats were also evaluated for crucial organs. Since *heart* and *lungs* are crucial organs, long term involvement of radiopharmaceutical in these organs is not desired. Our studies on imaging and biodistribution favor these results. It was observed that the radiopharmaceutical was excreted by the *kidneys*. ^{99m}Tc-β-casomorphin molecule, which is slightly lipophilic, was also excreted through *hepatobiliary* route beside *kidneys*.

Uterus, ovaries and *breast* are considered as target organs in studies about biodistribution of ^{99m}Tc-β-casomorphin molecules on rats. Especially in receptor saturation studies, activity decreased during first minutes in *uterus ovaries* and *breast* of the rats while it increased in *stomach* and *small intestines*. This suggests that *uterus ovaries* and *breast* of the female rats constitute targets. Radioactivity in all other tissues was unremarkable. Each of the six amino acids in the sequence Tyr-Pro-Phe-Pro-Gly-Pro appears to be integral to the site recognized by cancer cells through the cell-surface receptors. This conclusion is based on the complete lack of activity that was observed with synthetic peptides in which substitutions were made by using the most closely related amino acid. Since these conservative substitutions give inactive peptides, it seems reasonable to conclude that other, less closely related amino acids [21].

The partition coefficient is defined for dilute solutions as the molar concentration ratio between octanol and water phases at equilibrium: P=A0/Aw [21]. The lipophilicity for ^{99m}Tc- β-casomorphin was given as -2.21±1.44 while 1.42±0.95 for β-casomorphin and -4.07±0.90 for GH by the ACD programmer and found to be 1.8±0.1 for Tc-99m β-casomorphin, experimentally (n=5). Theoretical lipophilicities of ^{99m}Tc complexes cannot be predicted by ACD. It seems that ^{99m}Tc complexing is increasing the lipophilicity. On the other hand, lipophilicity of β-casomorphin was reduced by conjugation with GH, since β-casomorphin has more lipophilicity than GH. Similar lipophilicity value for another ^{99m}Tc labeled seven amino acid sequenced ^{99m}Tc-YGGSLAK (Tyr-Gly-Gly-Ser-Leu-Ala-Lys) lipophilicity value was 1.46±0.13 while theoretical lipophilicity was 1.73. They reported extraction way as hepatobiliary however the target organs were not *ovaries, uterus* or *breast* [13]. In receptor saturation studies, target organs activity decreased with blocking opioid receptors while excretion rate increased. This means that opioid receptor expressing organs uptake labeled molecule, in receptor blocking situation, molecule can not be uptaken by receptor and it is excreted by *kidney*.

In the cell labeling studies, MCF7, MDAH2774 (breast and ovarien cancer) and WL 38 (normal tissue) cells were labeled in vitro with Tc-99m-labeled peptides. It was observed that Tc-99m-β-casomorphin bound to MCF7 and MDAH2774 cancer cell with a high efficiency such as 90.71±9 %80.32±10% respectively.

DISCUSSION

Renal excretion was also observed as expected from small peptides [9]. Moreover, the radiopeptide is rapidly cleared via the *urinary system*, showing much lower *liver* uptake. After saturating receptors, there was increased *renal* and *gastrointestinal* excretion. Increased activity was seen in *intestine, kidney* and *bladder* after receptor saturation study.

Animal studies clearly show that β-casomorphins

opioid-like peptides are derived from β-casein [19-26]. β-Casomorphins are exogenous opioid peptides derivatives containing the common N-terminal amino acid sequence Tyr-Pro-Phe-Pro and have preferential μ-receptor agonistic activity [25].

In terms of evaluating the biodistribution of ^{99m}Tc-β-casomorphin molecule in rats, *uterus* , ovary and breast displayed high involvement at 5th and 10th min in normal biodistribution, it decreased at 30th min. On the contrary, if the receptors were saturated, low involvement was observed in first minutes and increased activity occurred at 30th min. A reverse receptor-peptide reaction is suggested by binding of the peptide molecule at first and then decreasing of the binding as time passes. After saturation of the receptors, this gradual increase in activity that was low at first shows that the radiopharmaceutical is specific to *uterus, ovaries* and *breast* receptors.

In ^{99m}Tc-β-casomorphin molecule, however when the receptor is compared to saturated biodistribution, increase of activity in *kidneys* and *urinary bladder* indicate that labeled molecule is excreted from the body more rapidly. When the radiopharmaceutical is evaluated in terms of *uterus, ovaries* and *breast,* the molecule seems to be more specific for these organs.

In biodistribution studies on rats, the facts that the compound had a low *hepatobiliary* excretion, higher *renal excretion* and rapid pharmacokinetics in the organs correspond to expected characteristics. In cell labeling studies, when breast, ovarien and uterus cancer are compared with control cells results show that labeled molecule can be used for both diagnosing and treatment these type of cancer cells.

Analysis of variance (ANOVA) was applied to the results and data were analyzed statistically. It is suggested that the radiopharmaceutical can be used in diagnosis of especially *uterus ovaries* and *breast* cancer and when labeled with therapeutic radionucleotide, treatment radiopharmaceutical potential would be attributed to it. For *uterus, ovaries* and *breast* and for the tumors related to these organs, it is demonstrated that a convenient radiopharmaceutical can exist.

Radionuclide labeled molecule ^{99m}Tc-β-casomorphin is useful for *uterus ovaries* and *breast* cancer cells. The peptide molecule must have appropriate chemical design to the receptor in order to be able to have affinity for tumor tissue at cellular level. The affinity towards cancer cells can be increased through causing change in the amino acid sequence of peptide molecule. The fact that radiolabeled peptides have high affinity towards cancer cell receptor shows the molecular targets for the diagnosis and treatment for *uterus ovaries* and *breast* cancer. The use of peptide for peptide receptor radionuclide therapy when it is labeled with the therapy radionuclides such as ^{177}Lu, whose amino acid sequence is Tyr-Pro-Phe-Pro-Gly-Pro can be convenient in conformity with *in vivo* applications.

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