M. Akınlı et al. / Hacettepe J. Biol. & Chem., 2021, 49 (3), 243-252

Research Article

Hacettepe Journal of Biology and Chemistry

journal homepage: www.hjbc.hacettepe.edu.tr

Biodistribution of Whey Protein Labeled with Tc-99m on Experimental Animals

Tc-99m ile İşaretli Whey Proteinin Deney Hayvanlarındaki Biyodağılımı

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ABSTRACT

In this study, whey protein was radiolabeled with Tc-^{99m} using the SnCl2 reduction method. The paper electrophoresis technique was done in the radiochromatography studies. SF (saline solution) was used as a suitable mobile phase. At the same time, the labeling yield of ^{99m}Tc-WHEY was found to be about 95%. Then, lipophilicity and stability studies were carried out, respectively. Finally, imaging and biodistribution studies were completed using Albino Wistar Rats. In vivo studies showed that ^{99m}Tc-WHEY was accumulated in the breast, the ovaria and the pancreas. In conclusion, all results showed that ^{99m}Tc-WHEY may be helpful in the diagnosis of the breast, the ovaria and the pancreas in the future.

Key Words

Whey protein, biodistribution, ^{99m}Tc, scintigraphy, protein powder supplement.

ÖZ

Bu çalışmada Whey proteini kalay klorür indirgenme metodu kullanılarak Tc-^{99m} ile işaretlenmiştir. Radyokromatografi çalışmalarında kağıt elektroforezi tekniği kullanılmıştır. Uygun hareketli faz olarak SF (serum fizyolojik) kullanılmıştır. Aynı zamanda Tc-^{99m} ile işaretli Whey protein'in bağlanma verimi yaklaşık %95 olarak bulunmuştur. Daha sonra, sırasıyla lipofilite ve stabilite çalışmaları gerçekleştirilmiştir. Son olarak Albino Wistar sıçanlarda görüntüleme ve biyodağılım çalışmaları yapılmıştır. Sonuç olarak, bütün elde edilen sonuçlar Tc-^{99m} ile işaretli Whey protein'in gelecekte meme, yumurtalık ve pankreas kanserlerinin teşhisinde kullanılabileceğini göstermiştir.

Anahtar Kelimeler

Whey proteini, biyodağılım, ^{99m}Tc, sintigrafi, protein tozu suplemanı.

Article History: Received: Dec 19, 2019; Revised: Sep 20, 2020; Accepted: Oct 8, 2020; Available Online: May 6, 2021.

DOI: https://doi.org/10.15671/hjbc.656021

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INTRODUCTION

Supplements are commonly used to improve athletic performance. The consumption of these supplements by both professional and amateur athletes has become popular in recent years [1,2].

The use of the supplements by athletes has been published in many sources and literatures [3]. According to Burke and Cato, supplements consumed by athletes may be appropriate and practical [4]. The supplements must contain enough quantity to overcome the athlete's nutritional shortage. As a result, convenience of the supplements should be scientifically confirmed.

Protein powders are prevalently used by athletes as nutritional supplements [5,6]. Most of them contains whey proteins (WH), but many of them have different proteins, such as milk and meat hydrolysates as ingredient.

WH consists of higher amounts of essential amino acids than the amounts contained in sources of protein such as egg and soy [7].

WH produced from cheese was considered a waste product in the past. Its disposal in the environment raised some important environmental problems [8]. The discovery of the benefits of WH as a supplement raised its importance in the cheese production industry. The composition of WH varies according to the type of cheese and milk used. WH contains some proteins, such as αlactalbumin, βgalactoglobulin, serum albumin, immunoglobulins, lactoferrin, and galactoperoxidase. They have various chemical, physical, and functional properties [9-11]. WH has also exhibited important antioxidant activity, probably due to cysteines. It can be used for the treatment of oxidative stressassociated diseases with this aspect.

The aim of this study is to assess the biodistribution of the ^{99m}Tc-WH on the rats. Firstly, radiolabeling studies were completed. The radiolabeling yield of ^{99m}Tc-WH was found to be 95%. After that, lipophilicity and stability studies were done, respectively. The serum stability experiments showed that about 85% of WH labeled with ^{99m}Tc existed in the rat serum within 2 h. Finally, biodistribution and imaging studies were carried out. ^{99m}Tc-WH was observed to have accumulated in the different organs such as the breast, the ovaria and the pancreas in the biodistribution studies.

Consequently, ^{99m}Tc-WH will be a good radiopharmaceutical for the diagnosis of diseases in organs such as the breast, the ovaria and the pancreas in the future.

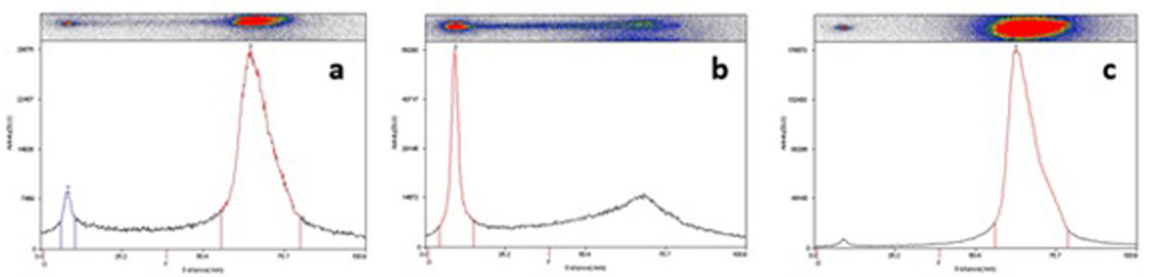

Figure 1. TLRC chromatograms of 99mTc-WH (a), Reduced ^{99m}Tc (b) and Na99mTcO4 (c).

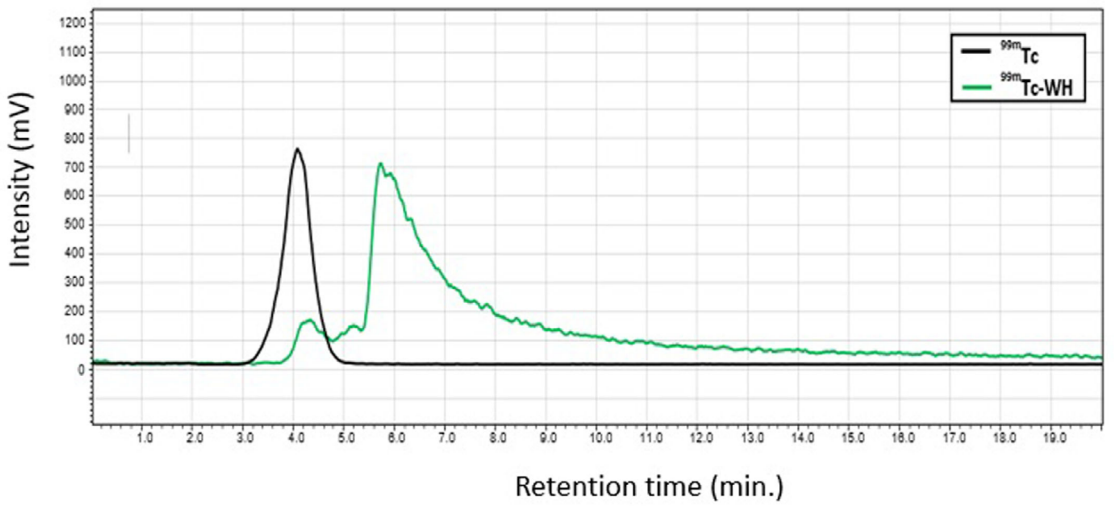

Figure 2. HPLRC chromatogram of ^{99m}Tc-WH (pH=12).

Table 1. . R_f values and radiolabeling yields of ^{99m}Tc-WH in pyridine/acetic acid/water (3:5:1.5).

Sample	R_f (cm)	Yield (%)
^{99m}Tc-WH	0.794	95.4
Reduced ^{99m}Tc	0.111	100
^{99m}Tc	0.788	100

MATERIALS and METHODS

Na $^{99m}TcO_4$ was provided by Medicine Faculty of Manisa Celal Bayar University. All chemicals were purchased from Merck and Sigma. A Bioscan AR 2000 and Cyclone Plus Storage Phosphor System and a RAD 501 single-channel analyzer were used in the radiochromatography studies. High performance liquid radio chromatography (HPLRC) and paper electrophoresis studies were carried out by using HPLC instrument (LC-10ATvp quaternary pump and SPD-10A/V UV detector and a syringe injector equipped with a 1-mL loop and NUCLEODUR 7-µm reversed-phase (RP)-C-18 column 250x21 mm I.D., Macherey–Nagel) and Gelman Instrument in Ege University Institute of Nuclear Sciences, respectively.

Radiolabeling studies of WH with ^{99m}Tc

WH was dissolved in distilled water. $SnCl_2$ solution (1 mg/1 mL) was prepared. In the labeling of WH with ^{99m}Tc; 250 µg of WH and 100 µL of $SnCl_2$ were added into a vial. The $Na^{99m}TcO_4$ (1 mCi, 37 MBq) eluted from ^{99}Mo/^{99m}Tc generator was added to the vial. All components (WH, $SnCl_2$ and $Na^{99m}TcO_4$) were mixed and pH of the mixture was set as 12 with 1 M NaOH then the mixture was shaken up and was incubated at 25°C for 30 min. $^{99m}TcO_2$ was applied in the similar conditions without WH.

Quality control studies

Thin layer radiochromatography (TLRC), HPLRC and paper electrophoresis techniques were used for quality control studies of the ^{99m}Tc-WH.

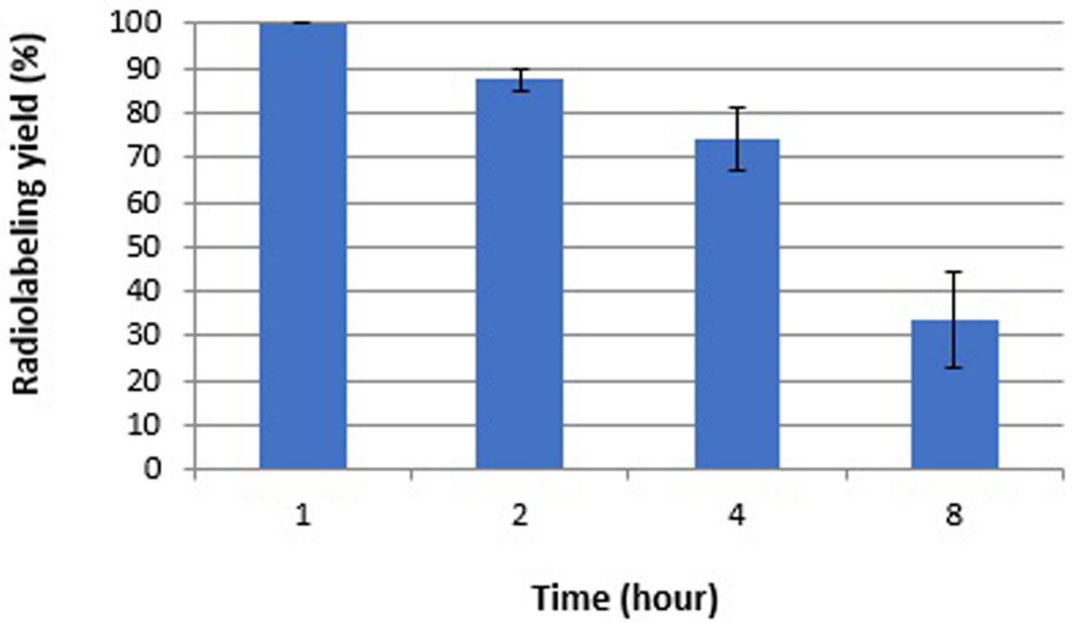

Figure 3. Stability graphic of ^{99m}Tc-WH.

TLRC studies

R_f values of ^{99m}Tc-WH, $Na^{99m}TcO_4$ and $^{99m}TcO_2$ were obtained using TLRC. A small amount of the substance to be analyzed (analyte) was placed on a strip of paper (the stationary phase) above the level of the solvent (mobile phase). Different mobile systems were studied. Plates were counted by Cyclone Plus Storage Phosphor System.

Paper electrophoresis studies

Paper electrophoresis studies was done using physiological saline as an electrolyte solution and a Gelman electrophoresis chamber supply (Gelman Instrument Company, Ann Arbor, MI). 10 µL of solutions of the $Na^{99m}TcO_4$ and ^{99m}Tc-WH was performed to the middle of Whatman 3 Chr paper, which were wetted with the electrolyte solution. Left side of the point of the application on paper strip was selected as anode and other side of it was selected as cathode. 300 V potential was enforced for 90 min. At the end of this time, the migration of radioactivity was measured with Bioscan AR2000.

HPLRC studies

^{99m}Tc-WH, $Na^{99m}TcO_4$ and $^{99m}TcO_2$ were assayed with HPLRC then samples were eluted under same conditions of HPLC analysis. Samples were analyzed by a scintillation detector.

Stability of ^{99m}Tc-WH in the rat blood serum

The stability of ^{99m}Tc-WH in the rat blood serum was measured. For this, 100 µg/100 µL of the ^{99m}Tc-WH and 300 µL of rat blood serum were incubated at 37°C. The aliquots were investigated in the different times (1, 2, 4 and 8 hours) by TLRC technique after radiolabeling at 25°C.

The partition coefficient (logP) of ^{99m}Tc-WH

300 µL of n-octanol and 300 µL of phosphate buffer (pH 7) were added into a tube and then 100 µL of ^{99m}Tc-WH sample was added to this solution. This solution was shaken at 25°C for 1 min. The mixture was then centrifuged at 2,500 rpm for 30 min. 100 µL of n-octanol and 100 µL of buffer layers were transferred into other tubes. Finally, radioactivities of the layers were measured with a RAD 501. Measurements were replicated three times. The logP was calculated with following equation given below:

Table 2. Radiolabeling yield of ^{99m}Tc-WH (pH=12) according to HPLRC analysis.

Sample	Retention time (min.)	Area	Height	Peak start	Peak end	Area %
^{99m}Tc	4.346	64201585	126304	3.758	4.800	5.79
^{99m}Tc-WH	5.738	51552354	74865567	4.800	11.958	94.21

logP = log($A_{n\text{-}octanol}/A_{buffer}$) (A: Radioactivity in cps: counts per second).

Biodistribution studies on female Albino Wistar rats

Biodistribution studies were done with the approval of the Relevant Institutional Animal Review Committee of Manisa Celal Bayar University, (Number: 22/05/2018/71,637.435-) Manisa, Turkey.

The results are given as percentage of injected radioactivity per gram of tissue (% ID/g) for some selected organs. The mean values are expressed for three rats. Female Albino Wistar rats, which are approximately 150–200 g were used in these studies. The WH labeled with ^{99m}Tc was sterilized by using membrane filter (0.45 µm). After that 0.10 mL of the ^{99m}Tc-WH solution [(1 µg/0.50 mCi) /rat] was injected via tail vein of the animals. The rats were sacrificed in the different times (30, 60 and 90 min) using ketamine and xylazine mixture as an anesthetic. The organs taken and blood were excised, weighed. Then, radioactivities of them were measured by using Cd(Te) detector equipped with a RAD 501. % ID/g values of organs were calculated. The data obtained from % ID/g of ^{99m}Tc-WH versus time graphs were plotted using Excel program.

Scintigraphy studies

In the biodistribution of ^{99m}Tc-WH, scintigraphy studies were carried out by using healthy female Albino Wistar rats. Scintigraphy studies were done in Manisa Celal Bayar University, Department of Nuclear Medicine. GE Infinia Dual Headed Gamma Camera (Hacarmal, Tirat, Israel) system was utilized to display the radioactivity of labeled compound, uptake and scintigraphic imaging of ^{99m}Tc-WH in rat metabolism. This camera can detect γ-irradiations of ^{99m}Tc.

For this, 11.1 MBq (300 µCi), 64.8 µg (360 µL) of ^{99m}Tc-WH was injected to rats and then scintigraphic images of ^{99m}Tc-WH were taken in the different times given above. Rats were anesthetized by xylazine and ketamine mixtures to obtain the static images.

Statistical analysis

Statistical evaluations were done with univariate Variance Analysis and Pearson correlation using the SPSS 15 program (SPSS, Inc., Chicago, IL). Probability values (P) below 0.05 were considered as statistically significant.

Table 3. Paper electrophoresis Rf values of ^{99m}Tc-WH, Red. ^{99m}Tc and $Na^{99m}TcO_4^-$.

Electrophoresis R_f	Electrical charge	
Sample	Positive (+)	Negative (-)
^{99}mTc-WH		0.17
Reduced ^{99m}Tc	0.02	0.02
Na^{99m}Tc		0.30

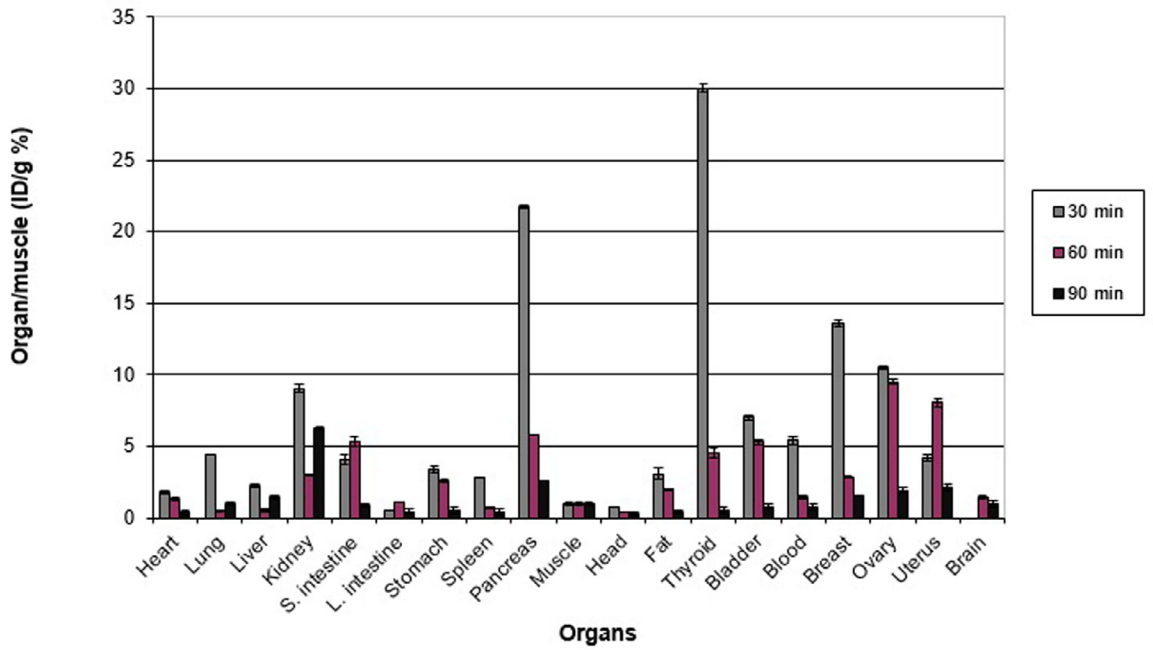

Figure 4. Biodistribution graphic of ^{99m}Tc-WH (organ/muscle ID/g %).

RESULTS and DISCUSSION

WH was radiolabeled with ^{99m}Tc in the quality control studies. ^{99m}Tc is a suitable radionuclide for diagnosis and it can be got easily from ^{99}Mo/^{99m}Tc generator eluate in pertechnetate form ($Na^{99m}TcO_4$). Radiolabeling reaction was done by using $^{99m}TcO_4^-$ to go on with complexing in forming ^{99m}Tc-WH. $^{99m}TcO_2$ was applied individually to prove that $^{99m}TcO_4^-$ was reduced successfully. The radiolabeling yield of ^{99m}Tc-WH was calculated by means of TLRC, HPLRC and paper electrophoresis techniques, respectively. In the TLRC studies pyridine/acetic acid/water (3:5:1.5) were used as a mobile phase. ^{99m}Tc-WH moved upwards (R_f = 0.8), when $^{99m}TcO_2$ remained at the origin as seen in Figure 1. The radiochemical purity (RCP) was stated by subtracting the sum of the percent of reduced hydrolyzed technetium and free pertechnetate from 100%. The radiochemical yield was found to be the mean value of three experiments. The yield was obtained about 95±2%. Additionally, R_f values and radiolabeling yields of ^{99m}Tc-WH in pyridine/acetic acid/water (3:5:1.5) were given in Table 1. Paper electrophoresis was carried out to determine the charge of ^{99m}Tc-WH. As seen Table 2, $Na^{99m}TcO_4$ remained at the application point when ^{99m}Tc-WH was moved toward to anode.

Also, radiochemical purity of ^{99m}Tc-WH was investigated by HPLRC as seen in Figure 2. Retention times of TcO_4^- and ^{99m}Tc-WH, were found to be 4.348±0.256 and 5.738±0.102 (min), respectively. HPLRC studies were also given in Table 3.

The results of the serum stability experiments showed that about 100 and 85 % of ^{99m}Tc-WH existed as an intact complex within 1 and 2 h, respectively. Figure 3 shows the graph of radiolabeling yield of ^{99m}Tc-WH versus time. High stability behavior of radiolabeled WH shows that ^{99m}Tc-WH can stay in live metabolism for a long time. In this period ^{99m}Tc-WH will not lose its stability in the metabolism.

In addition to this, the logP of ^{99m}Tc-WH was detected, and the logP was obtained as 0.13±0.01 (n = 3).

The results of biodistribution of ^{99m}Tc-WH in rat tissues were given in Figure 4. Figure 4 represents the biodistribution results of ^{99m}Tc-WH obtained in 30, 60, and 90 min after its administration to rats. The results of biodistribution study were given as % ID/g values given in Table 4. Researching the results, we can see that ^{99m}Tc-WH has partially high involvement values in the bladder and the kidneys of the female rats. It was found that

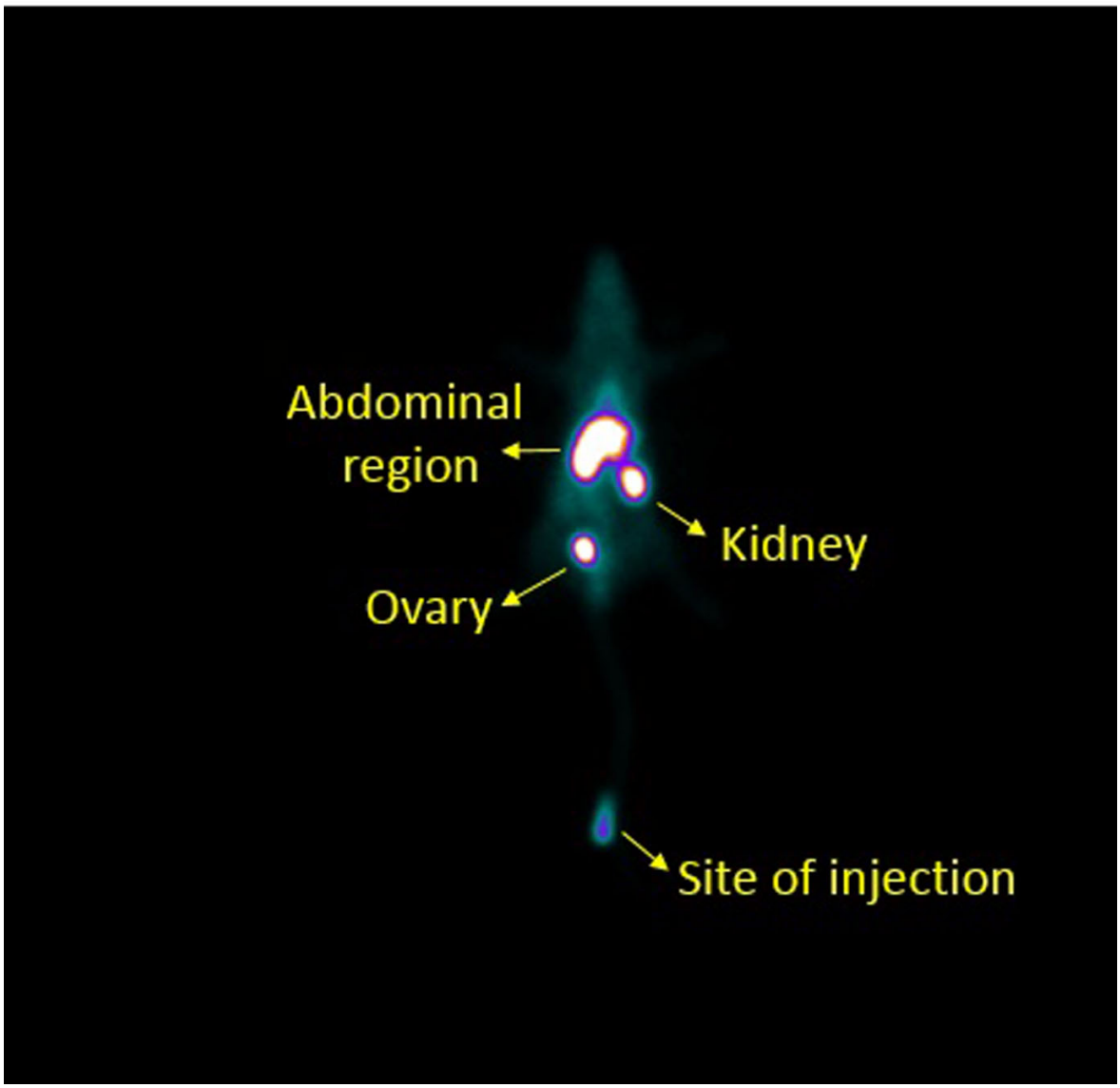

Figure 5. Scintigraphy image of ^{99m}Tc-WH.

^{99m}Tc-WH was metabolized by way of the urinary and intestinal tract on the female rats. Thorough, clearance of this compound is by way of the hepatobiliary duct. As seen in Figure 4 it is seen that the uptakes in the breast and the pancreas decreased with time. It was also observed that ^{99m}Tc-WH remained in the metabolism (about 90 min) and was not cleared rapidly. Additively, high uptake of ^{99m}Tc-WH was observed in the different organs such as the breast, the ovary, and the pancreas within 30 min. The %ID/g values of the ^{99m}Tc-WH in the breast, the ovary, and the pancreas were found to be 1.212±0.212, 0.936±0.091, and 1.935±0.013 at 60 min, respectively.

After the ^{99m}Tc-WH was injected to rats, static images were obtained by using a dual head gamma camera. Static image of ^{99m}Tc-WH in female rats demonstrated that significantly high radio activities existed in the ovary, the kidney, and the abdominal region in 30 min as seen in Figure 5. We also observed that ^{99m}Tc activity

Table 4. %ID values of biodistribution study of ^{99m}Tc-WH.

	30 min.	60 min.	90 min.
Heart	0.159±0.110	0.140±0.084	0.183±0.153
Lung	0.395±0.022	0.049±0.069	0.480±0.153
Liver	0.201±0.018	0.059±0.016	0.700±0.090
Kidney	0.805±0.286	0.316±0.079	3.095±0.042
S. Intestine	0.367±0.354	0.555±0.346	0.429±0.118
L. Intestine	0.049±0.,020	0.113±0.007	0.225±0.063
Stomach	0.299±0.211	0.270±0.066	0.259±0,132
Spleen	0.250±0.051	0.076±0.060	0.221±0.200
Pancreas	1.935±0.013	0.610±0.015	1.287±0.223
Muscle	0.089±0.126	0.105±0.002	0.494±0.013
Head	0.068±0.040	0.045±0.008	0.156±0.116
Fat	0.275±0.388	0.205±0.079	0.190±0.105
Thyroid	2.680±0.315	0.478±0.3	0.256±0.243
Bladder	0.628±0.159	0.555±0.202	0.391±0.159
Blood	0.482±0.335	0.148±0.105	0.382±0.211
Breast	1.212±0.212	0.301±0.009	0.754±0.012
Ovary	0.936±0.091	0.999±0.193	0.953±0.293
Uterus	0.285±0.262	0.359±0.202	0.327±0.262
Brain	0	0.291±0.007	0.180±0.227

increased appropriately with time in the chest region and the abdominal region. It can be explicated that radioactivity in the chest region is possibly due to the activity in the lungs with reference to ROI values given in Table 5. In addition to this, the clear ^{99m}Tc uptake in the abdominal region is probably due to the high activities in the stomach, the liver and the intestines. Values of ROI indicated that the highest ^{99m}Tc activity was obtained from the stomach in all time intervals, thus the radioactivity detected in the abdominal region was probably due to the stomach. This can be explained by the ^{99m}Tc eliminated from ^{99m}Tc-WH that showed a high uptake in this organ [12-18].

There are limited studies about milk proteins labeled with a radionuclide in the literature. One of them is about combining of radiolabeled whey proteins into casein micelles by heat processing published by Noh and Richardson [19-29]. In this study, WH was labeled with ^{14}C then radioactivities of the WH and radioactivities of the washed casein pellets from renneted skim milk were measured. It was found that the radiolabeling technique was very sensitive and useful for tracing low levels of interaction between whey proteins and casein in heated milk systems.

In a different study carried out by Rehner et al, it was investigated that influence of proteins on availability of zinc I. gastrointestinal transit time of casein and whey protein and zinc absorption in weaned rats [22]. The availability of zinc from isolated casein was compared with that from whey protein in 23-25 day old rats. The study was planned to show the course of the gastrointestinal transit time of either chyme radiolabeled by ^{141}Ce or ^{65}Zn in groups of 9 to 12 animals each. From this study, it was finalized that short-term experiments were not suitable for comparison of zinc availability from diets containing different proteins. Furthermore, it could be supposed that preabsorptive processes in the stomach were vital for the availability of zinc.

Table 5. . ROI values of ^{99m}Tc-WH.

	Thyroid	Heart	Righ Lung	Lef Lung	Stomach	Liver	Right Kidney	Left Kidney	Uterus
30 min.	2.17	5.4	3.07	2.73	22.7	18.73	24.63	30.1	22
60 min.	1.89	4.6	2.52	2.3	21.35	14.29	30	39.9	27.42
90 min.	2.3	5.81	3.36	3	30.98	16.45	33.47	46.03	38.26
120 min.	2.32	5.06	2.6	2.52	29.32	18.76	33.68	45	36.8
150 min.	2.11	5.47	3.06	2.9	27.81	20.29	46.68	35.98	36.08
180 min.	2.29	5.88	3.7	2.6	32.95	13.27	39.16	49.34	42
210 min.	2.54	6.31	3.3	3.36	3.36	12.03	46.16	59.97	51.77

Altosaar et al investigated proteins labeled with ^{14}C to follow biodistribution of ingested proteins [20]. For this, it was investigated by screening the biodistribution of ^{14}C in rats that were fed ^{14}C-labeled CD14 protein, a receptor for bacterial lipopolysaccharide. In addition to this bovine serum albumin (BSA) and casein were also labeled with ^{14}C and used for comparative analysis. It has been reported that the accumulation of ^{14}C label in the organs of ^{14}C-CD14-fed rats was tentatively significant from ^{14}C-BSA and ^{14}C-casein-fed rats.

CONCLUSIONS

In conclusion, ^{99m}Tc-WH has significant impact on the radiopharmacy. ^{99m}Tc-WH radiotracer evaluated in Albino Wistar rats showed high initial uptakes with the retention time in the different organs such as the breast, the ovary, and the pancreas. High initial uptakes of these target organs will help to diagnose some diseases in patients, who suffer from diseases related with the target organs.

CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ACKNOWLEDGEMENTS

We thank to Buket Ateş for the technical assistance during the animal experiments. The authors thank to Mustafa Üzümcü for providing Whey protein. Finally, we also thank Norma Merce Yagcier for reviewing our study as a native English speaker.

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