SCINTIGRAPHY OF THE DOMESTIC DOG USING [^{99m}Tc(CO)$_3$(H$_2$O)$_3$]-C$_{60}$(OH)$_{22-24}$

A. DJORDJEVIĆ*, B. AJDINOVIĆ[a], M. DOPUDJA[a], S. TRAJKOVIĆ[a], Z. MILOVANOVIĆ[a], T. MAKSIN[b], O. NEŠKOVIĆ[b], V. BOGDANOVIĆ[c], Đ. TRPKOV[b], J. CVETIĆANIN[b]

University of Novi Sad, Faculty of Sciences, Department of Chemistry, Trg Dositeja Obradovića 3, 21000 Novi Sad, Serbia

[a]*Military Medical Academy, Crnotravska 11, 11000, Belgrade, Serbia*

[b]*The Institute of Nuclear Sciences "Vinča", POB 522, University of Belgrade, 11000 Belgrade, Serbia*

[c]*Institute of Oncology Sremska Kamenica, Institutski put 4, 21204 Sremska Kamenica, Serbia*

In this study we performed the dynamic and static scintigraphy of the domestic dog, using the newly synthesized radiopharmaceutical, [^{99m}Tc(CO)$_3$(H$_2$O)$_3$]-C$_{60}$(OH)$_{22-24}$. In the current study, an advanced one-step method for the functionalization of fullerenol by ^{99m}Tc is described. Optical properties of as-prepared samples and the mechanism responsible for the functionalization were investigated using UV–VIS and FTIR spectroscopy, respectively. Also, the presence of the Tc complex on fullerenol was confirmed by using the energy dispersive X-ray spectroscopy, HPLC and MALDI TOF techniques.This simple and effective method of producing a new radiopharmaceutical is of interest not only for its application in various areas of technology and biology, but also for investigating its potential use in radiation technology for nanoengineering of materials. With dynamic scintigraphy, performed during 30 minutes (120 frames, 15 sec per frame), we obtained ratios of heart, liver and spleen counts: 222/249/168; 178/320/217; 120/348/239 respectively. By static scintigraphy after 1 hour, we detected the activity in heart, liver, spleen and intestines. After 4 hours, the radiopharmaceutical activity was detected in salivary glands. The detection after 21 hours showed the activity in kidneys and urinary bladder, while the activity in intestines was absent. After 24 hours, we detected the activity in liver, spleen, kidneys and urinary bladder. Pharmacokinetic investigations performed in this study are of key interest for the further fullerenol *in vivo* research.

(Received December 2, 2010; accepted December 22, 2010)

Keywords: Biodistribution, Fullerenol, [^{99m}Tc(CO)$_3$(H$_2$O)$_3$]-C$_{60}$(OH)$_{22-24}$, dynamic, Static, Scintigraphy, *In vivo*, Dog

1. Introduction

Studies on the biological properties of fullerene C_{60} and its derivatives started a decade ago as curiosity-driven studies and are now flourishing as an area of transdisciplinary research [1-3]. The water-soluble fullerene derivative, fullerenol $C_{60}(OH)_{22-24,}$ demonstrates important biological activity, such as: free radical scavenging activities in chemical and biological systems *in vitro* and *in vivo* [4-9], cytotoxicity against human tumor cell lines [10], cardioprotective effects in doxorubicin-induced cardiotoxicity [11], cardio-, hepato- and nephro-protective effects in rats with mammary carcinomas [12–14]. Pharmacokinetic investigations of fullerenol are of key

*Corresponding author: dvadj@nadlanu.com

interest, from the aspects of planning and potential application of fullerenol in *in vivo* models, recognizing the fullerenol virtue as a cardioprotective agent, during doxorubicin and radio-induced toxicity. In the studies of Q. N. Li and Y. G. Li [15-18], radiolabelled fullerenol $C_{60}(OH)_x$ (x=22-26) derivatives with ^{67}Ga, ^{127}I and ^{99m}Tc were investigated. The first and preliminary experiments (performed on New Zealand rabbits), using ^{99m}Tc-$C_{60}(OH)_x$, by single photon emissions computed tomography, radiopharmaceuticals were detected in *reticuloendothelial* organs, but not in the brain [18]. The preliminary investigations on Wistar rats using $[^{99m}Tc(CO)_3(H_2O)_3]$-$C_{60}(OH)_{22-24}$, detected the highest activity in liver, at three time periods (2, 4 and 24 hours) [19]. The activity in intestines increased between 2 and 4 hours, decreasing at 24 hours. The kidney activity decrease was time-dependent [19, 20].

2. Experimental

Synthesis of fullerenol $C_{60}(OH)_{22-24}$

Our results are very similar to those previously published [21-23].

Fullerenol $C_{60}(OH)_{22-24}$ was synthesized in alkaline media by complete substitution of bromine atoms from $C_{60}Br_{24}$. Briefly, the polybromine derivative $C_{60}Br_{24}$ was synthesized through catalytical ($FeBr_3$) reaction of C_{60} in Br_2[20]. Fifty milligrams of $C_{60}Br_{24}$ was mixed in 5mL of NaOH pH= 10 for 2 h at room temperature.After the reaction was completed, the solvent was evaporated at 40 $^{\circ}$C, and the mixture was rinsed five times with 10 mL portions of 80% ethanol. The aqueous solution of fullerenol (20 mL) with residual amounts of NaOH and NaBr was applied on the top of the combined ion-exchange resin DOWEX MB50 QC121815 R1 (20 g) and eluted with demineralized water until discoloration. The solution of fullerenol (pH =7) was evaporated under low pressure; a dark brown powder substance remaining.

Synthesis of $[^{99m}Tc(CO)_3(H_2O)_3]$-$C_{60}(OH)_{22-24}$

Fullerenol was labelled with ^{99m}Tc using the tin (II)- reduction method. Three samples with different molar ratios of Sn(II) and fullerenol were prepared, using 1.40×10^{-3} M aqueous solutions of fullerenol and 9.75×10^{-3} M Sn(II) as $SnCl_2\times2H_2O$; fullerenol : Sn(II) = 2:1, 5:1 and 10:1. pH of the solutions adjusted at 5.5. After adding technetiumpertechnetate ($^{99m}TcO_4^-$) in saline from the ^{99m}Tc generator, the mixtures were heated for 40 min in a boiling water bath.

The $[^{99m}Tc(CO)_3(H_2O)_3]^+$ ion was prepared by the addition of 1 ml of ^{99m}Tc -pertechnetate (0.925 – 3.7 GBq $^{99m}TcO_4^-$ eluted in saline from ^{99m}Tc -generator) to a penicillin vial containing in lyophilized form:7.15 mg sodium carbonate, 4.5 mg sodium boranocarbonate, 2.85 mg sodium tetraborate and 8.5 mg sodium tartarate (IsoLink™ Mallinckrodt Medical B.V., The Netherlands). After heating for 30 min in the boiling water bath and cooling, the pH of solutions were adjusted to approximately 7.5 with 1 M HCl (using indicator paper for pH control). The samples of fullerenol were prepared by dissolving in water the appropriate amount of substances for obtaining the solution with 1.5 mg fullerenol / ml. The pH of solutions was adjusted to 9.0. ^{99m}Tc-carbonyl fullerenol complexes were prepared by the addition of 0.1 ml of fullerenol solutions to 0.5 ml of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor with appropriate pH values. The vials were heated for 30 min in the boiling water bath.

Instrumentations

Apparatuses used for the physico-chemical characterisation of synthesized fullerenol were: FTIR Thermo-nicolet, nexus 670, derivatograph Paulik-Erdey MOM-1000, TG, DTG, DTA, ^{13}C NMR (750 MHz), MALDI TOF (AB Applied Biosystems, Voyager-DE PRO, Framingham, USA) and particle size distribution was determined using a Zeta-sizer Nano ZS with 633 nm He–Ne laser (Malvern, UK).

Radiochemical purity - For the determination of radiochemical purity of all ^{99m}Tc-labelled compounds, the standard paper (Whatman No1) and instant thin layer chromatography (ITLC-SG) with two solvents (acetone and saline) was used.

HPLC analysis - The quality control of the obtained $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor (pH = 10 -11) was performed by gradient HPLC (Liquid Chromatograph, Hewlett Packard 1050, S/N with UV and Raytest gamma flow detector) on the RP C18 column (250 x 4.6 x 5 mm). The solutions of 0.05 M triethylammonium phosphate (TEAP), pH = 2.25 and methanol were used as the mobile phase. The labelling efficiency for ^{99m}Tc-carbonyl tagged fullerenol was determined in isocratic HPLC with 90 % TEAP: 9 % H_2O:1% CH_3OH, pH = 2.25 as the mobile phase (flow rate 0.7 ml / min) at room temperature and saline was used.

Protein binding and lipophilicity measurements - TCA precipitation method for determining the percentage of ^{99m}Tc- and ^{99m}Tc(I)-labelled fullerenol bound to protein (12% human albumin - National Blood Transfusion Institute, Belgrade, incubation at 37 ^{0}C for different time intervals) was applied. All lipophilicity measurements for ^{99m}Tc and ^{99m}Tc(I)-labelled fullerenol were carried out by the solvent extraction method with n-octanol equilibrated with 0.15 M phosphate buffers (pH=3.5-7.5). The measurements were performed at room temperature.

Scintigraphy

Dynamic scintigraphy of the dog was performed during 30 minutes (120 frames, 15 sec. per frame, in the matrix 128x128), after the administration of 2.085 mCi ^{99m}Tc-F, using the gamma camera LFOV Searle, which was situated above the dog's head, thorax and abdomen. Static scintigraphy was performed in the matrix of 256x256 in time intervals of 1 and 4 hours after the dose administration (during 3 minutes or 1000 000 counts), as well as, 21 and 24 hours after the dose administration (during 5 minutes).

Animal preparation

The adult male dog belonging to the German Shepherd breed (aged:3 years, body mass:28 kilos) was under veterinary care and sedation (in time intervals of 1, 4, 21 and 24 hours) with premedication: 0,003 mg/kg (i.v.) kombelen-a (acepromazin), 15 min later 0,03 mg/kg (i.m.) ketamine-chloride (5% solution) and 0,1 mg/kg atropin (s.c.). The animal was safely immobilized and TcF was injected intravenously. After the experiment, the dog was examined by the veterinarian and transported back to the military breeding unit. Experiments were approved by the National Animal Ethical Committee of Republic of Serbia and were conducted in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

3. Results and discussion

Analysis of synthesized materials

Analysis of fullerenol: FTIR $C_{60}(OH)_{24}$: 3427, 1627, 1419, 1080cm^{-1}; ^{13}C NMR(D_2O) reaction mixture: singlet peaks at δ = 77.7 ppm and multiplet at δ=140 ppm, ^{13}C NMR (D_2O) of $C_{60}(OH)_{24}$: singlet peaks δ=169.47 ppm and multiplet peak at 160–110 ppm; TG, DTA,

TG: reveals two thermal changes in the temperature interval of 120–395°C, with corresponding mass loss of 35.7% (23.7 OH groups) and at the temperature of 430°C, mass loss was 64.3% (this was the temperature of C_{60} sublimation). Elementary analysis of fullerenol provided the following: C, 63.0%; H, 2.0% calc.: C, 63.83%; H, 2.13%. Dimensions of fullerenol nano particles (FNP) (pH=5.1) were; 50.3, 60.0, 71.5 and 85.4 nm, with average dimension 61.1 nm. FNP (pH=5.1) had two values for ζ-potential, -51.4 mV and -26.4 mV. The average value of ζ-potential was -47.2 mV. The negative ion MALDI-TOF mass spectrum of fullerenols showed a well-defined pattern of ion fragmentations with a nearly constant, consecutive weight increase of

17 mass units compared to C_{60}. Results of MALDI TOF analysis of $C_{60}(OH)_n$ are given in Figure 1. Detection of a molecular ion at *m/z* 1094-1128 was indicative for the composition of fullerenols as containing 22-24 hydroxy addenda per C_{60} cage. It was found that the molecular stability largely depends on the quantity of impure groups, especially the highly oxygenated carbons in fullerenols, but less so on the hydroxyl number.

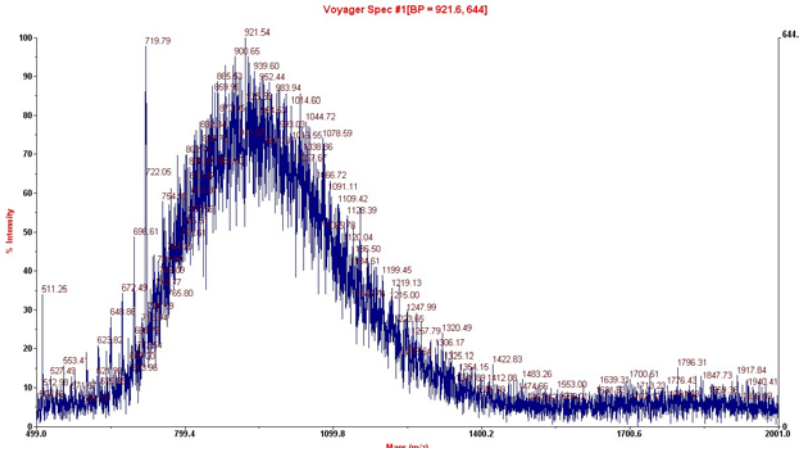

Fig. 1. MALDI TOF mass spectrum of fullerenol $C_{60}(OH)_{22-24}$.

Analysis of [^{99m}Tc(CO)$_3$(H$_2$O)$_3$]-$C_{60}(OH)_{22-24}$:

The chromatograms of $^{99m}TcO_4$-tagged fullerenol, as well as fullerenol alone, were obtained after heating the samples in boiling water for 30 min and are presented as HPLC chromatograms in Fig. 2 and Fig. 3 respectively.

The retention time values (Rt) for $^{99m}TcO_4$- tagged fullerenol and fullerenol alone, obtained by HPLC, using 90% methanol and 10% water as mobile phase (flow rate 0.5 ml/min, temperature 25°C) were 4.736 and 12.624 min respectively. According to the HPLC chromatograms, it can be deduced that complexes of $^{99m}TcO_4$-fullerenol were formed.

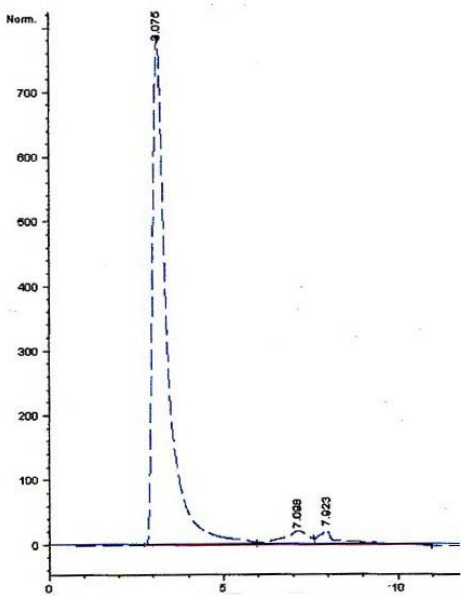

Fig. 2. HPLC chromatogram of fullerenol.

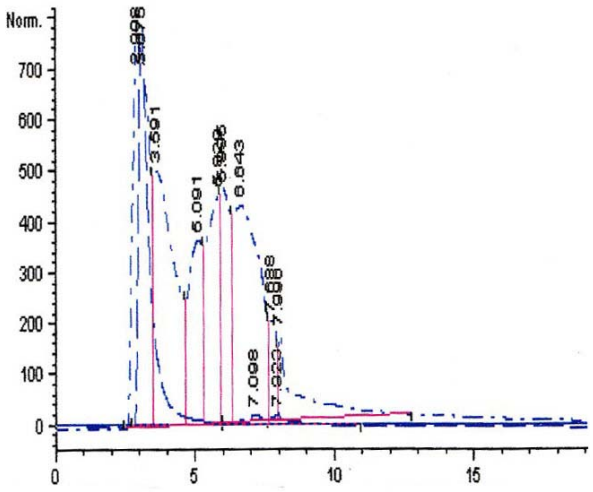

Fig. 3. HPLC chromatogram of fullerenol labeled with TcO_4^-

MALDI TOF spectrum of labelled fullerenol is presented in Figure 4.

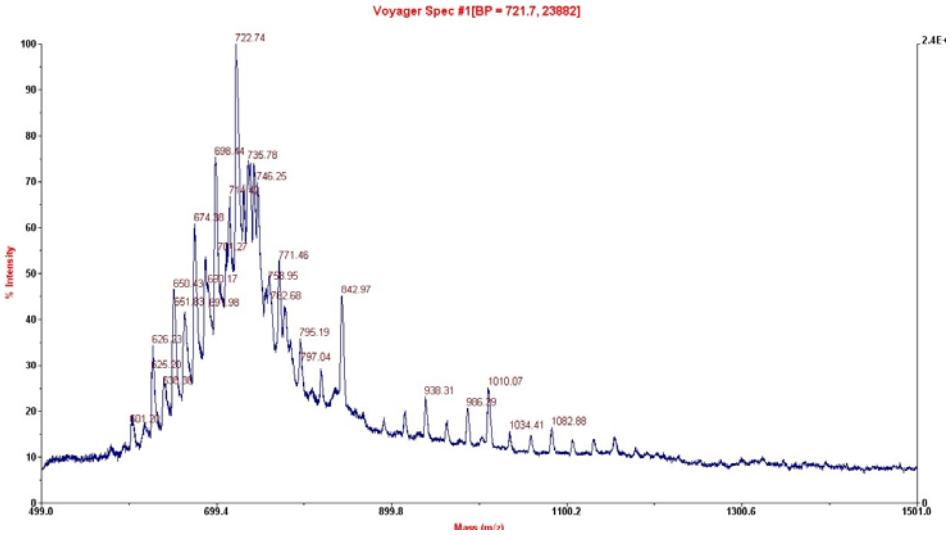

Fig. 4. MALDI TOF spectrum of $[^{99m}Tc(CO)_3(H_2O)_3]$-$C_{60}(OH)_{22-24}$:

Fig. 4 represents the MALDI TOF mass spectrum of $[^{99m}Tc(CO)_3(H_2O)_3]$-$C_{60}(OH)_{22-24}$:. Observation from larger to smaller masses, reveals the first peak with an m/z value of 1082.88 which corresponds to fullerenol with 22 OH groups ($C_{60}(OH)_{22}^+$). Peaks at m/z values of 1010.07, 842.97 and 771.46, correspond to $C_{60}(OH)_{17}^+$, $C_{60}(OH)_7^+$ and $C_{60}(OH)_3^+$ respectively.

It is clearly seen how the loss of OH groups of fullerenol takes place under the influence of laser radiation. The peak at 722.74 corresponds to fullerene C_{60}^+. By fullerene C_{60}^+ fragmentation, which means C_2 loss, the fragments C_{60-2n}^+ are obtained. In the mass spectrum (Figure 4) these fragments correspond to m/z values of: 698.44, 674.38, 650.43, 626.23 and 601.20.

Dynamic scintigraphy

Dynamic scintigraphy analysis showed the presence of ^{99m}Tc-F in heart, showing its decrease until the 12th minute, followed by further increase until the 22nd minute, and by final decrease until the end of detection. The activity observed in liver was increased until the 450th second, this level being permanent until the end of detection. The spleen activity time-course

curve is of the similar shape as in the case of liver, however, the activity in liver is higher. The kidney activity time-course curve is of similar shape as in the case of spleen, but with a moderate decrease observed until the end of detection (Figure 5). Dynamic scintigraphy analysis did not detect radiopharmaceutical activity in urinary bladder.

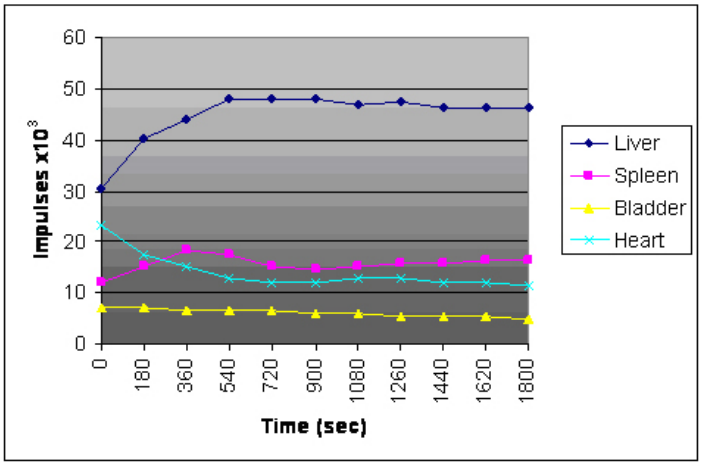

Fig. 5. Dynamic scintigraphy analysis of dog.

The number of counts in dynamic scintigraphy analysis per pixel in heart, liver and spleen (in time intervals of 1, 5 and 15 minutes) is shown in Table 1.

Table 1. The number of counts in dynamic scintigraphy analysis in the dog, per pixel in heart, liver and spleen with [$^{99m}Tc(CO)_3(H_2O)_3$]-$C_{60}(OH)_{22}$.

Time interval → Organ ↓	1 minute	5 minutes	15 minutes
Heart	222	178	120
Liver	249	320	348
Spleen	168	217	239

Static scintigraphy

After 1 hour: the detection above the dog's abdomen showed one million counts within 141 seconds (10^6 counts/141 seconds), demonstrating the strong presence of ^{99m}Tc-F in heart, liver and spleen. The pelvic region scintigram (10^6 counts/172 seconds) showed intensive accumulation of ^{99m}Tc-F, the area of accumulation was of irregular shape, which may correspond to intestinal activity. This image was also present in the scintigram after 4 hours, and it was absent after 21 and 24 hours.

After 4 hours: static scintigraphy analysis of the dog's head (870760 counts /180 seconds) showed the accumulation of ^{99m}Tc-F in salivary glands. The pelvic scintigram showed the activity which may correspond to the intestinal activity. The scintigraphic analysis of abdomen showed the accumulation of ^{99m}Tc-F in heart, liver and spleen. These results did not alter significantly between 4 and 21 hours.

After 21 hours: static scintigraphy analysis of the dog showed the following results: head (42025 counts /300 seconds), abdomen (172141 counts / 300 seconds), pelvis (179803 counts /300

seconds). We detected the ^{99m}Tc-F accumulation in spleen, liver, salivary glands, kidneys and urinary bladder. The intestinal activity is absent at this time-point.

After 24 hours: static scintigraphy analysis showed the following results: abdomen (121165 i counts in/ 300 seconds) and pelvis (129424 counts/ 300 seconds). We detected the ^{99m}Tc-F activity in liver, spleen, kidneys and urinary bladder. The number of i counts obtained by static scintigraphy analysis in heart, liver and spleen is shown in Table 2.

Table 2. The number of i counts obtained by static scintigraphy analysis in heart, liver and spleen with $[^{99m}Tc(CO)_3(H_2O)_3]$-$C_{60}(OH)_{22-24}$.

Time interval→ Organ ↓	1 hour	4 hours	21 hours	24 hours
Heart	46	37	6	4
Liver	124	123	20	15
Spleen	97	84	14	12

Figure 6. shows images obtained by the static scintigraphy analysis of dog: 1. heart activity after 1 hour; 2. liver activity after 1 hour; 3. kidney and urinary bladder activities after 24 hours; 4. kidney and urinary bladder activities after 21 hours; 5. liver, kidneys and urinary bladder activities after 24h; 6. salivary glands activity after 24 hours; 7. heart activity after 24 hours.

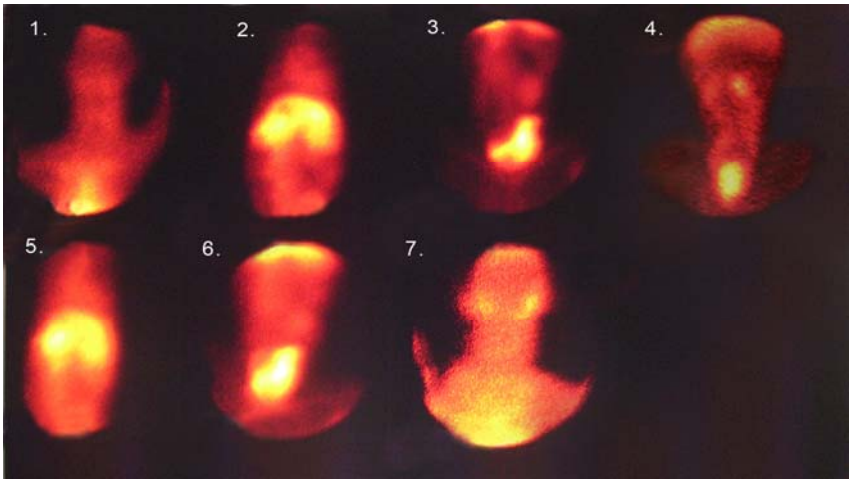

Fig. 6. Static scintigraphy analysis of dog

4. Conclusions

With dynamic scintigraphy of the dog, we detected the radiopharmaceutical $[^{99m}Tc(CO)_3(H_2O)_3]$-$C_{60}(OH)_{22}$ in the heart, liver and spleen. The activity in heart progressively decreased. The activity in liver and spleen has increased during the first nine minutes, finally the activity in all three organs being stabilized until the 30th minute. The static scintigraphy, after 1 hour detected activity in heart, liver, spleen and intestine, while after 4 hours, radiopharmaceutical activity was detected in salivary glands. After 21 hours, the activity was detected in kidneys, urinary bladder and urinary tract, while intestinal activity was absent. After 24 hours, the activity was detected in liver, spleen, kidneys and urinary bladder. The accumulation of the radiopharmaceutical $[^{99m}Tc(CO)_3(H_2O)_3]$-$C_{60}(OH)_{22-24}$ was detected in heart, by both dynamic and static scintigraphy. According to our results, we may conclude that scintigraphic analysis shows

liver and urinary elimination of the radiopharmaceutical [$^{99m}Tc(CO)_3(H_2O)_3$]-$C_{60}(OH)_{22-24}$ in the dog.

Acknowledgment

This research was supported by the Ministry of Science and Technological Development, Republic of Serbia, Grant No. III45005. Credit is given to Prof. Karmen Stankov for critical reading of the manuscript and useful suggestions concerning medicine. We thank Prof. Nadežda Petranović for donating the C_{60}.

References

[1] W.A. Jensen, R.S. Wilson, I.D. Schuster, Bioorg. Med. Chem. **4**, 767-779 (1996).
[2] S. Bosi, T. Da Ros, G. Spalluto, M. Prato, Eur. J. Med. Chem. **38,** 913-923 (2003)**.**
[3] A. Djordjević, G. Bogdanović, S. Dobrić, Journal of BUON review article **11,** 391-404. (2006).
[4] G. Bogdanović, V. Kojić, A. Djordjević, J. Čanadanovic-Brunet, M. Vojinović-Miloradov, V. Baltić, Toxicol. in vitro **18,** 629-637 **(**2004)
[5] S. Mirkov, A. Djordjević, N. Andrić, S. Andrić, T. Kostić, G. Bogdanović, M. Vojinović-Miloradov and R. Kovačević, Nitric Oxide - Biol. Ch. **11**, 201-207 (2004).
[6] A. Djordjević, J. Čanadanovic-Brunet, M. Vojinović-Miloradov, G. Bogdanović, Oxid. Commun. **27,** 806-812 (2005).
[7] V. Bogdanović, K. Stankov, I. Ičević, D. Žikić, A. Nikolić, S. Šolajić, A. Djordjević, G. Bogdanović, J. Radiat. Res. **49,** 321-327 (2008).
[8] S. Trajković, S. Dobrić, V. Jaćević, V. Dragojević-Simić, Z. Milovanović, A. Djordjević, Colloid Surface B **58,** 39-43 (2007).
[9] S. Trajković, S. Dobrić, A. Djordjević, A. Dragojević-Simić, Z. Milovanović, Mater. Sci. Forum **494,** 549-554 (2005).
[10] V. Kojić, D. Jakimov, G. Bogdanović, A. Djordjević, Mater. Sci. Forum **1,** 543-548 (2005).
[11] R. Injac, M. Perse, M. Boskovic, V. Djordjevic-Milić, A. Djordjevic, A. Hvala, A. Cerar, B. Strukelj, Technol. Cancer Res. T. **7**, 15-25 (2008).
[12] R. Injac, M. Perse, N. Obermajer, V. Djordjevic-Milic, M. Prijatelj, A. Djordjevic, A. Cerar, B. Strukelj, Biomaterials **29**, 3451-3460 (2008).
[13] R. Injac, M. Perse, M. Cerne, N. Potocnik, N. Radic, B. Govedarica, A. Djordjevic, A. Cerar, B. Strukelj, Biomaterials **30,** 1184-1196 (2009).
[14] R. Injac, M. Boskovic, M. Perse, E. Koprivec-Furlan, A. Cerar, A. Djordjevic, B. Strukelj, Pharmacol. Rep. **60,** 742-749 (2008).
[15] Y. G. Li, Q. N. Li, Y. P. Zhang, X. Huang, W. Li, J. Nucl Radio. **24**, 161-163 (2002).
[16] Y. G. Li, X.D. Zhang, Q.N. Li, W. Li, J.Radioanal. Nucl. Ch. **250,** 363-364 (2001).
[17] Q. N. Li, Y. Xiu, R. Liu, Q. Qu, X. Shun, Chin. Sci. Bull. **46,** 1615-1617 (2001).
[18] Q. N. Li, Y. Xiu, X. D. Zhang, R. Lui, Q. Du, X. Shuan, Nucl. Med. Biol. **29**, 707-710 (2002).
[19] T. Maksin, D. Djokić, D. Janković, A. Djordjević, O. Nešković, J. Optoelectron. Adv. Mat. **9**, 2571-2577 (2007).
[20] A. Djordjević, M. Vojinović-Miloradov, N. Petranović, A. Devečerski, D. Lazar, B. Ribar, Fullerene Sci. Techn. **6**, 689-694 (1998).
[21] Y. C. Chaing, Y. W. Swirtczewski, S. Chang,. S. K. Hsu, S. Cameron, K. Creegan, J. Chem. Soc. Chem. Commun. **24,** 1791-1792 (1992).
[22] J. Li, A. Takeuchi, M. Ozawa, X. Li, K. Saigo, K. Kitazawa, J. Chem. Soc. Chem. Commun. **23**,1784-1785 (1993).
[23] Y. Chen, R. F. Cai, S. Chen, Z. E. Huang, J. Phys. Chem. Sol. **62**, 99-101 (2001).