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Comparison of some physico-chemical parameters and biological behaviour of fullerenol labelled with technetium -99m

T. MAKSIN, D. DJOKIĆ, D. JANKOVIĆ, A. DJORDJEVIC[a], O. NEŠKOVIĆ*
The Institute of Nuclear Sciences "Vinča", POB 522, 11001 Belgrade, Serbia
[a]University of Novi Sad, Faculty of Natural Sciences and Mathematics,
Trg Dositeja Obradovica 3, Novi Sad, Serbia

The biological behavior of fullerene derivatives shows their considerable potential for medical application. Polyhidroxilated, water soluble fullerenol $C_{60}(OH)_{22}$, used in these studies, was synthesized by procedure complete substitution of bromine atoms from polybromine derivatives $C_{60}Br_{24}$ in alkaline media. In order to investigate the pharmacological behaviour and metabolism of the fullerenol, quickly and conveniently, we present the way and possibilities for its labelling with ^{99m}Tc ($T_{1/2}$=6.02h, E =141keV), which bind to the outside, leaving the cage intact. Two different labelling approaches with technetium-99m were performed: direct labelling by tin (II) chloride method and labelling with ^{99m}Tc(I) using $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor. The radiochemical studies results have shown that content of free $^{99m}TcO_4^-$ in the samples was depend on fullerenol-stannous chloride ratio and increased if ratio was higher. HPLC quality control results of $^{99m}Tc(CO)_3$-$[C_{60}(OH)_{22}]$ have shown that the labelling yield, in alkaly media, was 95.35%. Protein binding to HA was species-dependent process. The biological behaviour of labelled fullerenol were different in dependence of labelling method and the time of sacrificed of animals. From recent investigations it can be concluded that oxidation state in technetium coordination complexes has a great impact on in vitro and in vivo behaviour of the these complexes.

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1. Introduction

Previous work suggested that fullerenes have successfully reacted with organic molecules which bond to the outside, leaving the cage intact[1]. Also, the diameter from 0.71nm (C_{60}) to about 0.84nm (C_{84}) is large enough to be able to contain a variety of atoms of different sizes. Such characteristics would be especially useful in biomedical applications where radiolabelling of biological molecules and pharmaceuticals have been used extensively for medical imaging and therapy[2].

Fullerenes derivatives have some potential effects, e.g. specific cleavage of DNA, antiviral activity, inhibition of HIV protease and photodynamic therapy[3,4] so it has become a challenging research field at present. Fullerenols, water-soluble polihydroksylated fullerenes (C_{60}(OH)n n=10-26), are very important kinds of fullerene derivatives because it is suitable for biological study. The biological behavior of fullerenol derivatives shows their considerable potential for medical application.

Technetium-99m is still the radionuclide of choice because of his ideal physical properties ($T_{1/2}$=6.02h, Eγ=141keV) for many applications in nuclear medicine. For radiopharmaceuticals preparation it was often used like technetium pertechnetate (TcO_4^-), which have to be reduced in lower oxidation state. Besides this labelling approach, it is possible to use hydrophilic organometallic $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor to forming Tc (I) radiopharmaceuticals based on the tricarbonyltechnetium (I) core[5-9]. Three molecules of water are labile coordinated to Tc(I) and could be readily exchanged.

In this paper the results of the conditions and possibilities investigation of water soluble fullerenol $C_{60}(OH)_{22}$ labelling with ($^{99m}TcO_4^-$) using by stannous chloride method and $[^{99m}Tc(CO)_3(H_2O)_3]^+$ as precursor for ^{99m} Tc(I) were presented. The radiochemical purity, pharmacokinetics and biodistribution of labelled compounds were investigated.

2. Experimental

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

The starting polybrominated derivative $C_{60}Br_{24}$ was prepared by a previously described procedure [10]. To 40 mg of $C_{60}Br_{24}$ was added 5 ml of 30% NaOH at the temperature of 22°C. After 15 minutes, the heterogenous system turned into clear, dark-brown solution. The total duration of the reaction of substitution of polybrominated derivative was 60 minutes. The addition of ethanol yielded a brown precipitate of fullerenol. The heterogeneous system was stirred for 10 min and the precipitate separated from the etanolic solution of NaOH and NaBr. Separation procedure was repeated until achieving pH 7 of the aquesous solution of fullerenol. Residual amounts of NaOH and NaBr were removed from the aqueous solution by mixing it with 100 mg of combined ion exchange resin for 30 min at stirring. Atomic absorption spectrometry

showed the presence of only traces of sodium (2 ppm) in the fullerenol solution. The reaction yield of fullerenol was 85%.

Apparatures used for physico-chemical characterisation of fullerenol $C_{60}(OH)_{22}$ were: FTIR Thermo-nicolet, nexus 670, derivatographc Paulik-Erdey MOM-1000, TG, DTG, DTA, ^{13}C NMR (750 MHz). MALDI TOF (AB Applied Biosystems, Voyager-DE PRO, Framingham, USA).

Fullerenol was labelled with ^{99m}Tc using by tin (II)-reduction method. Three samples with different molar ration of Sn(II) and fullerenol were prepared, using 1.40×10^{-3} M aqueous solutions 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 of technetiumpertechnetate ($^{99m}TcO_4^-$) in saline from ^{99m}Tc-generator (Vinča), the mixtures were heated for 40 min in boiling water bath.

$[^{99m}Tc(CO)_3(H_2O)_3]^+$ ion was prepared by addition of 1 ml of ^{99m}Tc-pertechnetate (0.925 – 3.7 GBq $^{99m}TcO_4^-$ eluted in saline from ^{99m}Tc-generator, Vinča Institute) to a penicillin vial with lyophilised form of 7.15 mg sodium carbonate, 4.5 mg sodium boranocarbonate, 2.85 mg sodium tetraborate and 8.5 mg sodium tartrate (IsoLink™ ,Mallinckrodt Medical B.V., The Netherlands). After heating for 30 min in boiling water bath and cooling, pH of solutions were adjusted to ~7.5 (using indicator paper for control pH) with 1 M HCl .

The samples of fullerenol were prepared by dissolving in water appropriate amount of substances for obtaining solution with 1.5 mg fullerenol / ml. The pH of solutions was adjusted at 9.0. ^{99m}Tc-carbonyl fullerenol complexes were prepared by 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 boiling water bath.

Radiochemical purity - For 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 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 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 mobile phase (flow rate 0.7 ml / min) at room temperature.

Protein binding and lipophilicity measurements - *T*CA 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 37^0C for different time intervals) was used[11]. All lipophilicity measurements for ^{99m}Tc- and ^{99m}Tc(I)-labelled fullerenol were carried out by solvent extraction method with n-octanol equilibrated with 0.15 mol/dm^{-3} phosphate buffers (pH=3.5÷7.5)[12]. The measurements were performed at room temperature.

Animal biodistribution - The biodistributions studies of ^{99m}Tc-labelled compounds were carried out on health white Wistar rats (four weeks old). The animals (n=6 animals for each interval) were sacrificed at different time after application of 0.1 ml of ^{99m}Tc-labelled compound (~74kBq). The radioactivity per whole organ of interest (or gram) was measured in a NaI (Tl) □-detector and the percentage of radioactivity related to administrated dose was determined.

3. Results and discussion

Physico-chemical characterisation of fullerenol $C_{60}(OH)_{22}$

The results of synthesis of fullerenol $C_{60}(OH)_{22}$ were presented as: FTIR spectrum of fullerenol $C_{60}(OH)_{22}$: 3427, 1627, 1419, 1080 cm^{-1}. ^{13}NMR (D_2O) spectrum of alkaline reaction mixture of fullerenol: singlet peak at δ=77.7 ppm (C-OH) and multiplet at δ=140 ppm. ^{13}C NMR (D_2O) spectrum of fullerenol: a singlet peak at δ=169.47 ppm and a multiplet peak at 160-110 ppm (158, 157, 155, 153, 149, 145, 143, 141, 140, 139, 138, 136, 132, 129, 126, 125, 121, 120, 118, 116, 112, 110 ppm). MALDI (matrix 2,5-dihydroxybezoic acid) (*m/z*): MALDI (matrix α-cyano-4-hydroxycinnamic acid) (*m/z*): 720 (C_{60}^+), 721 ($C_{60}H^+$), 722 ($C_{60}H_2^+$), 737 ($C_{60}(OH)^+$), 788 ($C_{60}(OH)_4^+$), 805 ($C_{60}(OH)_5^+$), 839 ($C_{60}(OH)_7^+$), 856 ($C_{60}(OH)_8^+$), 1009 ($C_{60}(OH)_{17}^+$), 1043 ($C_{60}(OH)_{19}^+$), 1060($C_{60}(OH)_{20}^+$), 1094 ($C_{60}(OH)_{22}^+$). Molecular peak of $C_{60}(OH)_{24}$ was not detected. DTG, DTA, TG reveal two thermal changes in the range of 120–395 ^0C, corresponding to the loss of mass of 34 % (22 OH groups) and at 430 ^0C, loss of mass was 66 % (temperature of sublimation of C_{60}).

Radiochemical purity –The results of radiochemical purity investigations of ^{99m}Tc-labelled fullerenol by Sn(II) reduced method (three mixtures with different molar ratio fullerenol : Sn(II)), have shown in Table 1, as mean values from at least three Whatman 1 and ITLC sheets and mobile phases. The radiochemical studies results have shown that content of free $^{99m}TcO_4^-$ was depend on fullerenol-stannous chloride ratio and increased if the ratio was higher (10:1). From the comparison of these data it could be seen that amount of stannous chloride in the preparation had a great influence on the labelling yield. But these applied methods could not separate labelled fullerenol from $^{99m}TcO_2$, which is usually impurity in the process of labelling with pertechnetate.

The same chromatographic methods for investigation of $^{99m}Tc(CO)_3$- $[C_{60}(OH)_{22}]$ labelling yields were used. The obtained results have shown that applied chromatographic methods could not separate labelled fullerenol from radiochemical impurities like as $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor or free $^{99m}TcO_4^-$.

HPLC analysis - The quality control results of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor, as well as ^{99m}Tc-carbonyl tagged fullerenol, obtained with heatting of the samples in

boiling water for 30 min, were presented as HPLC chromatograms, in Fig. 1 and Fig. 2.

The retention time values (R_t) for $[^{99m}Tc(CO)_3(H_2O)_3]^+$ and $^{99m}TcO_4^-$, obtained by gradient HPLC with 0.05 M triethylammonium phosphate (TEAP), pH = 2.25 and methanol as mobile phase (flow rate 0.7 ml/min, room temperature) were 4.736 min and 12.624 min respectively. HPLC quality control results of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor have shown that radiochemical purity was higher than 95 %. The HPLC results performed by isocratic HPLC with 90% TEAP, 9 % H_2O and 1% CH_3OH as mobile phase, with flow 0.7 ml/min, confirmed that hydrophilic organometallic $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor allows forming of Tc(I) complexes with fullerenol. The retention time value (R_t) for $^{99m}Tc(CO)_3$-fullerenol (pH around 9.0, carbonyl:fullerenol=1:1v/v), was 15.923. The labelling yield was 95.35%, with 4.65% of free, not bound $[^{99m}Tc(CO)_3]^+$ as radiochemical impurities. If the labelling was performed at pH around 5.5, better results were obtained for samples with carbonyl:fullerenol = 3:1v/v then 9:1v/v. If the ratio was 9:1v/v, almost there was no labelling (more than 90% is $[^{99m}Tc(CO)_3]^+$ precursor), but if the ratio was 3:1v/v the labelling yield was about 60 %, with 40% of free, not bound $[^{99m}Tc(CO)_3]^+$.

Protein binding and lipophilicity measurements

The results of $^{99m}TcO_4^-$, $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor, ^{99m}Tc-carbonyl tagged fullerenol, as well as ^{99m}Tc labelled fullerenol, bound to 12 % HA, determined by TCA precipitation method, are presented in Table 2. It could be seen that binding to HA was species-dependent process. Protein bound values obtained for ^{99m}Tc-fullerenol were much higher (~76%) than the obtained values for pertechnetate-99m in saline solution (~3.50 %). The values of protein binding for ^{99m}Tc-carbonyl tagged fullerenol were about 40%, that was much lower than the some one for $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor (~85%).

Lipophilicity also influence on the in vivo behaviour of imaging agents and make the basis of anticipation of their accumulation and the route of elimination. The results of lipophilicity measurements for $^{99m}TcO_4^-$, ^{99m}Tc-fullerenol, $[^{99m}Tc(CO)_3(H_2O)_3]$ and $^{99m}Tc(CO)_3$-fullerenol were presented at Figure 3, as a dependence of distribution coefficient of given labelled compounds on pH. The obtained l-octanol-buffer distribution pH profiles have shown that the distribution coefficients for fullerenol labelled with $^{99m}TcO_4^-$ was very high than the same one for free $^{99m}TcO_4^-$ that pointed at on its very hidrofobic character. The results of distribution of complexes between inorganic and organic solvents indicated that all $^{99m}Tc(CO)_3$-$[C_{60}(OH)_{22}]$ radioactivity remained in aqueous phase, thus the distribution coefficient was around zero. No change in extractability with pH was observed. These values for distribution coefficient was lower then the same one for precursor $[^{99m}Tc(CO)_3]^+$.

Animal biodistribution The biodistribution results of ^{99m}Tc-$[C_{60}(OH)_{22}]$, $[^{99m}Tc(CO)_3(OH)_2]$ and $^{99m}Tc(CO)_3$-$[C_{60}(OH)_{22}]$ are presented in Table.3, Table.4 and Table.5 respectively, as a percentage of administrated doses per organ of animal, determined by comparison of tissue radioactivity with a radioactivity of suitable aliquots (standards) of prepared preparations. These data showed that the biological behaviour of labelled fullerenol were different in dependence of the labelling method and the time of sacrificed of animals. The biodistribution results for fullerenol labelled with $^{99m}TcO_4^-$ by direct tin (II) method were in agreement with radiochemical purity results: for the sample with molar ratio fullerenol:Sn (II)=5:1, a lot of radioactivity in liver (>70 %) may suggest that in this condition of labelling in samples the content of hydrolysed reduced ^{99m}Tc ($^{99m}TcO_2$) as radiochemical impurity was high or that this samples showed higher affinity for liver due to its hidrophobic character. Fullerenol coordinated to $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor gave quite different biodistribution results in comparison with the results for ^{99m}Tc-$[C_{60}(OH)_{22}]$. The percentages of radioactivity in liver was significantly lower (from 20.1 to 14.1% depend on time), but the acumulation in kidneys was higher as well as the acumulation in the intestine.

Table 1. The radiochemical purity results of ^{99m}Tc-Fullerenol, thirty minutes after labelling (Mean values ± SD)

Labelled Compound Time (min)		^{99m}Tc –Fullerenol $(I)^+$	^{99m}Tc –Fullerenol $(II)^+$	^{99m}Tc –Fullerenol $(III)^+$
SG / saline	a	96.5± 0.7	83.8± 1.0	88.4± 5.8
	b	3.5± 0.2	16.2± 0.3	11.6± 0.5
SG/ Acetone	a	96.7± 0.2	92.9± 1.8	60.0± 1.9
	b	3.3± 0.1	7.1± 0.3	40.0± 0.3
Whatman No1/ saline	a	94.8± 0.1	85.4± 0.7	55.8± 0.6
	b	5.2± 0.1	14.6 ± 0.3	44.2± 0.1
Whatman No1/ acetone	a	95.5± 0.1	92.0± 0.2	81.7± 0.1
	b	4.5± 0.1	8.0 ± 0.2	18.3± 0.2

a - ^{99m}Tc-fullerenol and $^{99m}TcO_2$, R_f=0.0 – 0.2

b – $^{99m}TcO_4^-$, R_f=0.7 – 0.8

*Molar ration fullerenol:Sn(II)= 2:1 (I); 5:1 (II); 10:1 (III).

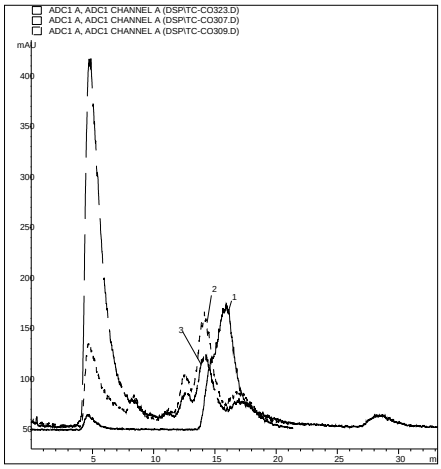

Fig 1. HPLC chromatogram of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor.

Table 2. The percentage of protein binding results for $^{99m}Tc(CO)_3$-Fullerenol and ^{99m}Tc- Fullerenol (12 % HA, TCA-method, 37 ^{0} C).

Tissue	Time (min)			
	20	120	240	24(h)
Blood	0.94±0.16	0.49±0.07	0.29±0.02	0.096±0.006
Lung	5.47±1.37	1.02±0.08	0.85±0.05	0.84±0.05
Liver	70.33±0.3.92	72.35±1.21	76.6±2.6	72.42±0.40
Kidney	0.86±0.31	1.30±0.18	1.58±0.11	2.25±0.13
Muscle$^+$	0.017±0.007	0.007±0.002	0.00	0.00
Bone$^+$	0.24±0.10	0.39±0.14	0.69±0.046	0.34±0.05
Thyroid	0.021±0.009	0.046±0.023	0.022±0.013	0.00±
Spleen	0.65±0.13	1.03±0.10	1.45±0.08	1.57±0.39
Intenstine	1.04±0.21	2.39±1.2	4.18±1.05	2.10±0.10

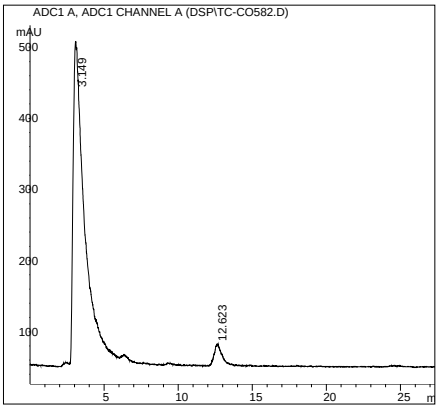

Fig 2. HPLC chromatogram: fullerenol: Tc(I)= 1:1v/v (1); 1:3 (2) and 1:9 (3).

Table 3. Organ distribution data in Wistar rats for ^{99m}Tc-[$C_{60}(OH)_{22}$] (% ID/organ, mean value ± SD).

Time (min) / Compounds	20	60
$^{99m}TcO_4^-$	3.5 ± 0.4	3.1 ± 0.4
^{99m}Tc -Fullerenol	76.8 ± 0.2	76.2 ± 0.2
[$^{99m}Tc(CO)_3(H_2O)_3$]	84.9 ± 1.1	83.1 ± 0.8
$^{99m}Tc(CO)_3$ -Fullerenol	40.9 ± 0.9	40.6 ± 0.9

*Mean±SD - The percentage of protein binding is the mean value from three measurements
+ - % ID/g

Table 4. Organ distribution data in Wistar rats for $^{99m}Tc(CO)_3(OH)_2$] precursor (% ID/organ, mean value ± SD).

	T(min)			
	5	20	60	120
Blood	7.5±1.2	5.9±1.0	5.0±0.4	2.25±0.95
Lung	3.4±0.8	2.5±0.6	2.1±0.6	1.5±0.2
Liver	11.6±0.3	14.1±0.5	14.3±0.2	14.4±0.3
Kidney	7.2±1.1	7.0±0.2	6.6±0.5	5.1±0.1
Mussle+	0.19±0.09	0.17±0.08	0.17±0.06	0.14±0.05
Bone+	0.28±0.08	0.34±0.11	0.31±0.10	0.27±0.09
Tireoidea	0.91±0.05	0.753±0.0053	0.58±0.02	0.25±0.11
Spleen	0.65±0.05	0.75±0.04	0.58±0.18	0.92±0.22

+ - % ID/g

Table 5 . Organ distribution data in Wistar rats for $^{99m}Tc(CO)_3$-[$C_{60}(OH)_{22}$] (% ID/organ, mean value ± SD).

Tissue	T i m e (h)		
	2	4	24
Blood	2.4±0.1	0.5±0.1	0.1±0.05
Lung	2.1±0.5	1.45±0.05	0.85±0.05
Liver	20.1±0.5	18.3±0.6	14.1±1.1
Kidney	5.3±0.6	4.3±0.3	2.33±0.15
Muscle+	0.16±0.01	0.15±0.03	0.15±0.09
Bone+	0.59±0.04	0.59±0.05	1.56±0.04
Thyroid	0.2±0.01	0.11±0.01	0.066±0.053
Spleen	0.66±0.03	0.48±0.04	0.35±0.08
Intenstine	16±6	32±9	5.1±3

+ - % ID/g

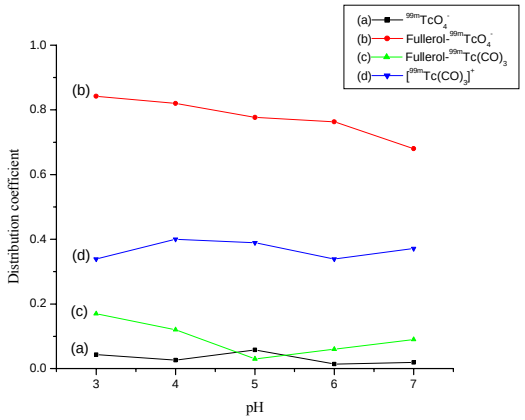

Fig. 3. l-octanol-buffer distribution coefficients (The results are the mean value from six measurements)

4. Conclusions

Polyhidroxilated, water soluble fullerenol $C_{60}(OH)_{22,}$ used in these studies, was synthesized by procedure complete substitution of bromine atoms from lybromine derivatives $C_{60}Br_{24}$ in alkaline media. Fullerenol is dark brown powder substance soluble in water.

From recent investigations it can be concluded that oxidation state in technetium coordination complexes has a great impact on in vitro and in vivo behaviour of the these complexes. The results presented in this paper confirmed the fact that complexion between the investigated compound, fullerenol, and reduced ^{99m}Tc was highly dependent on molar ratio fullerenol : Sn(II)-ion. But in all samples, the labelling yield for direct ^{99m}Tc-labelling of fullerenol was poor. The used methods could not separate labelled fullerenol from $^{99m}TcO_2$, which is usually unpurity in the proceses of labelling with pertechnetate. However, the used chromatographic methods was not also able to separate radiochemical impurities from labelled ^{99m}Tc-carbonyl labelled fullerenol.

HPLC quality control results for $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor, as well as the quality control results of ^{99m}Tc-carbonyl complexes of fullerenol presented as HPLC chromatograms show that the radiochemical purity of precursor was higher than 95 % and the labelling yield was more then 94% in samples with carbonyl:fullerenol = 1:1 v/v.

It could be seen that there is also a big difference between protein bound values obtained for ^{99m}Tc-fullerenol and ^{99m}Tc-carbonyl tagged fullerenol. While the stabile, no-reactive $^{99m}TcO_4^-$ showed only a weak binding to human albumin, the most reactive radiolabelled tricarbonyl precursor itself bound to that protein more effectively. There were the big differences between values of distribution coefficients for fullerenol labelled by $^{99m}TcO_4^-$ and labelled by $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor. The fullerenol labelled with $^{99m}TcO_4^-$ possessed hydrophobic character while labelled by $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor gave compound with very hydrophilic character.

The obtained results have shown that the biological behaviour of labelled fullerenol were different in dependence of labelling method.

Those experiments, pointed at the fact that fullerenol labelled with technetium-99m ($^{99m}TcO_4^-$ and $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor) might be useful. The future investigations on the animals with some kind of tumors would confirm, whether fullerenol labelled with ^{99m}Tc, could be used in diagnostic purpose.

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$[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor (IsoLink^{TM}) was obtained owing to amiability of Tyco Healthcare, Mallinckrodt Medical B.V., The Netherlands, free of charge.

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*Corresponding author: oliveran@vin.bg.ac.yu