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Synthesis and biological evaluation of receptor-based tumor imaging agent: ^{99m}Tc-folate-glucaric acid

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ABSTRACT

The aim of the present study was to prepare ^{99m}Tc-folate-glucaric acid and investigate the radiopharmaceutical potential for tumors imaging that over express folate receptor. Folate-glucaric acid was synthesized and the synthesized folate conjugate was confirmed with ^{1}H NMR and LC–MS/MS methods. Folate-glucaric acid was labeled with ^{99m}Tc, and its radiolabelling efficiency was found as 96 ± 2.0%. Biodistribution study of ^{99m}Tc-folate-glucaric acid was carried out *in vivo* using two groups of female Albino Wistar rats: folate receptor (FR) saturated and unsaturated. Biodistribution study showed that ^{99m}Tc-folate-glucaric acid indicated high uptake in folate receptor rich tissues such as breast, ovary and uterus. Therefore, ^{99m}Tc-folate-glucaric acid shows good radiolabeling and biodistribution in FR organs, the radiolabeled conjugate is a reason potentially useful radiopharmaceutical for detection of FR-positive tumors.

1. Introduction

Targeted drug delivery systems are well known to be critical for therapy and diagnosis of cancer. These systems have been successfully applied for the delivery of chemotherapeutic and immunotherapeutic agents, genes, liposomes, proteins and radioimaging agents into tumors (Wang et al., 1997; Sudimack and Lee, 2000; Guo et al., 1999). Generally targeting agents with low molecular weight are combined with chelating agents, and labeled with suitable radionuclide (^{99m}Tc, ^{111}In, $^{66/67/68}$Ga, ^{64}Cu) for nuclear medicine applications (Okarvi and Jammaz, 2006; Guo et al., 1999; Ke et al., 2004; Jurisson and Lydon, 1999; Mindt et al., 2008). One of the most widely used targeting agent is folic acid (FA), which is a low molecular weight (441.4 Da) vitamin needed for DNA synthesis and one-carbon metabolism by eukaryotic cells.

Radiolabeled folate-chelate conjugates with low molecular weight are widely used in receptor-targeted radiopharmaceutical due to FA has a high-affinity for folate receptors (FR) ($K_d \sim 10^{-10}$ mol/L). FA binds to the folate receptors at cell surfaces via its γ-carboxyl group and folate conjugates internalize into the cell by receptor mediated endocytosis (Zhang et al., 2010; Guo et al., 1999; Okarvi and Jammaz, 2006; Sudimack and Lee, 2000; Kim et al., 2007; Ke et al., 2003). The folate-based radiopharmaceuticals have important advantages in imaging of wide variety of tumor types, such as ovary, endometrial, kidney, colorectal and breast. These are, increase of target/non-target tissue ratio and drug toxicity, can be prevented at very low concentrations (Okarvi and Jammaz, 2006; Sudimack and Lee, 2000). Recently many folate-based radiopharmaceuticals have been synthesized and evaluated for tumor imaging, such as ^{99m}Tc-citro-folate, ^{111}In-DTPA-folate, ^{99m}Tc-DTPA-CKK$_2$-PEG-folate and ^{67}Ga-deferoxamine-folate (Altiparmak et al., 2010; Wang et al., 1997; Gu et al., 2010; Ke et al., 2004).

In this study a new folate conjugate, folate-glucaric acid, was synthesized and labeled with ^{99m}Tc. The potential of radiolabeled conjugate with low molecular weight was evaluated in normal female rats for FR positive tumor imaging agent in nuclear medicine.

2. Materials and methods

2.1. Materials

Folic acid, dicyclohexylcarbodiimide, hydrazine hydrate and glucaric acid were purchased from a supplier (Sigma–Aldrich Chemical Co., Steinheim am Albuch, Germany). N-hydroxysuccinimide was purchased from another supplier (Merck Co., Darmstadt, Germany).

2.2. Synthesis of folate-hydrazide

In the first step, N-hydroxysuccinimide (NHS), which is an ester of folic acid, was synthesized (Guo et al., 1999; Okarvi and Jammaz, 2006). For this synthesis, 1 g (2.3 mmol) folic acid was dissolved in 50 mL dimethylsulfoxide (DMSO). Then, NHS and dicyclohexyl

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carbodiimide (DCC) (1:1 ratio molar excess) were added to the solution. The reaction was allowed to proceed for 16 h at room temperature under stirring. The solution must be protected from light through all reaction time. The by-product dicyclohexylurea was removed by filtration. The NHS-folate in DMSO solution was stored at −20 °C. In the second step, synthesizing folate-hydrazide was done. NHS-folate solution was slowly added to hydrazine hydrate (2 mL) under stirring at room temperature. The product folate-hydrazide was converted into hydrochloride salt by adding HCl and then precipitated with acetonitrile/diethylether (1:1 v/v). The precipitate was centrifuged and then reprecipitated with ethanol. The final precipitate was washed with solvent mixtures of ethanol:diethyl ether (the ratio in mixture 1: 50/50, mixture 2: 80/20 and 100% ethanol) and the obtained precipitate was finally lyophilized under vacuum. Then, obtained folate-hydrazide, yellow powder, was stored at −20 °C.

2.3. Synthesis of folate-glucaric acid

Folate-glucaric acid was synthesized as follows: glucaric acid of 24.8 mg (0.1 mmol) was dissolved in DMSO and then 4.5 mg (0.01 mmol) folate-hydrazide was added to glucaric acid solution. Finally, 0.01 g (0.089 mmol) $CaCl_2$ was added to the final solution and stirred for approximately 1 h until the reaction was complete (Altiparmak et al., 2010). At the end of the reaction, solution was obtained by centrifugation. The precipitate was washed in a small volume of water and then reprecipiated with ethanol. The pellet was lyophilized and stored at −20 °C.

The molecular structures of NHS-folate, folate-hydrazide and folate-glucaric acid were confirmed by proton nuclear magnetic resonance spectroscopy (^{1}H NMR: Bruker 400 MHz spectrometer, Berlin, Germany). LC–MS/MS analyses of NHS-folate, folate-hydrazide and folate-glucaric acid were performed on an Agilent 6460 series system (Agilent ZORBAX Eclipse Plus C-18 narrow bore column, 3:7 Acetonitrile:20 mM Potassium Phosphate, pH: 6.2, isocratic, ambient, 0.3 ml/min).

2.4. Radiosynthesis of ^{99m}Tc-glucarate

In order to prepare radiolabeled glucarate; one mg (4 μmol) glucaric acid was firstly dissolved in 500 μL water in a glass vial. Secondly, 100 μL $SnCl_2{\cdot}2H_2O$ aq. solution (1 mg $SnCl_2{\cdot}2H_2O$/1 mL H_2O) was added to the solution and then mixed. Finally, ^{99m}Tc-sodium pertechnetate (37 MBq) was added and incubated at room temperature for 15 min. The labeling efficiency of ^{99m}Tc-glucarate was determined by thin layer radio chromatography (TLRC), using ready plates of ITLC-SG. After developing in three different solvent systems [0.9% sodium chloride solution, methanol/water (20/80) solution, and ethanol/ammonia/water [(2/1/5)], the strips were scanned on the TLC scanner (Bioscan AR-2000, Washington DC, USA). For the RHPLC analysis, the procedures described by Altiparmak et al. were followed (Altiparmak et al., 2010). We used a low pressure gradient HPLC system with LC-10ATvp quaternary pump, UV detector (Shimadzu SPD-10ATvp, Macherey-Nagel, EC 250/4.6 Nucleodur 100-5 C18 column) and 20 μL loop and equipped with a Cd(Te) detector equipped with a RAD-501 single channel analyzer. The HPLC process was run using 0.1% TFA/acetonitrile, 0.1% TFA/water at a flow rate of 1 mL/min. The flow rate was set at 1 mL/min and the UV detector at 240 nm.

2.5. Radiosynthesis of ^{99m}Tc-folate-glucaric acid

Synthesized folate-glucaric acid [0.5 mg (0.77 μmol)] was dissolved in 200 μL DMSO. 100 μL of the solution by taking from the solution was diluted with 200 μL water. Then the following solutions were added one by one into a glass tube: 300 μL folat-glucaric acid, 100 μL tin chloride (1 mg $SnCl_2{\cdot}2H_2O$/1 mL H_2O), 37 MBq $Na^{99m}TcO_4$. The glass tube was incubated for 15 min at room temperature. After the incubation, radiochemical product was analyzed by RHPLC and TLRC. The sample (5 μL) was spotted at the origin of three ITLC-SG strips (1.5 cm × 10 cm). The strips were developed in different solvent systems [0.9% sodium chloride solution, methanol/water (20/80) and ethanol/ammonia/water (2/1/5)] and then scanned using the TLC scanner.

2.6. Radiochromatography

This process was conducted with a Gelman electrophoresis chamber. Application points on cellulose acetate strips for cathode and anode poles were marked and moistened with 0.9% NaCl solution. Each compound was set on the strip and placed into the chamber. Applied voltage and standing time were set at 250 V and 120 min, respectively. The strips were then counted by using the TLC scanner.

2.7. Stability in human serum

The serum stability of ^{99m}Tc-folate-glucaric acid was determined by incubating 200 μL of the labeled compound with 500 μL of human serum at 37 °C. The sample was analyzed in the time intervals of 30, 60, 120, 180 and 1440 min by TLRC and radioelectrophoresis.

2.8. Lipophilicity

The lipophilicity (log P) of the radiolabeled compound was determined by using 300 μL radiolabeled conjugate, ^{99m}Tc-folate-glucaric acid, added to a pre-mixed suspension of 2 mL n-octanol in 2 mL water. The tube was vortexed for 1 h at room temperature, and then centrifuged at 3000 rpm for 5 min. Aliquots (0.5 mL) from each phase were taken for counting. The radioactivity was counted using a Cd(Te) detector. The partition coefficient was fixed as partition coefficient = $\log_{10}$ (counts in n-octanol layer/counts in aqueous layer). The experiments were repeated in three times and the results were averaged.

2.9. Biodistribution studies in organ tissues with saturated and unsaturated FRs

All animal experiments were approved by the Animal Ethics Committee of Ege University, and performed in accordance with the published guidelines. The biodistributions of the radiolabeled conjugate in organs with saturated and unsaturated FRs were investigated using 18 female Albino Wistar rats with weights ranging between 130 g and 180 g. The saturated study was designed to establish reference measurements for comparison of the radioactivity obtained from the unsaturated study. Three animals were used for each time interval and the biodistribution data from the different organs of each rat were recorded and averaged.

FR saturation study; each animal was administered folic acid [0.10 mg (0.23 μmol)] via intravenously (iv). After 30 min from folic acid injected, ^{99m}Tc-folate-glucaric acid (10 μg, 2.5 GBq/μmol) was injected through the same route. The uptake efficiency of the radiolabeled conjugate was evaluated at 60, 120 and 180 min after injection. The percent of injected dose per gram of tissue weight (% ID/g) was then calculated considering all the measurements.

In the receptor unsaturated study, ^{99m}Tc-folate-glucaric acid conjugate (0.015 μmol, 2.5 GBq/μmol) was injected only via iv route to the rats. The animal was sacrificed at ether atmosphere and the organ tissues were removed and counted to establish whether the uptake of the conjugate was specific in the receptor express-

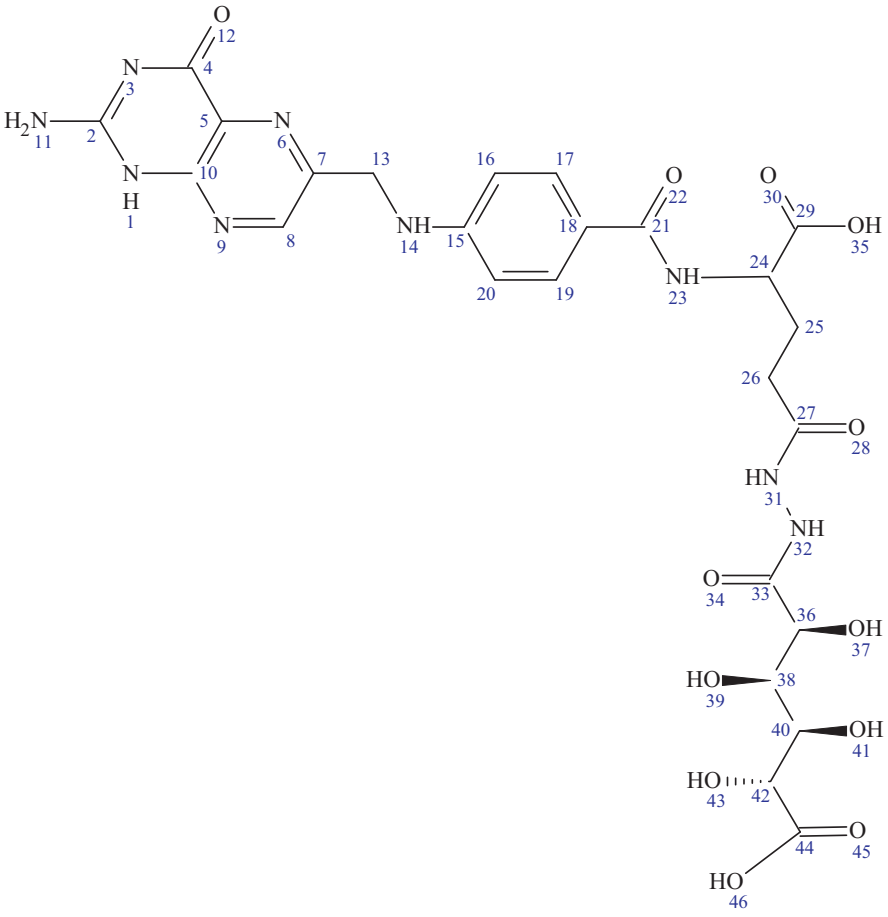

Fig. 1. Structure of folate-glucaric acid.

ing target tissue. The tissues were weighed and counted for their radioactivities.

2.10. Statistical analysis

The data obtained from the biodistribution studies were evaluated by a statistics program (Univariate Variance Analyses and Pearson Correlation). Probability values < 0.05 (SPSS Version 10) were considered to be significant. A Pearson Correlation between the organs of saturated and unsaturated animals tested for ^{99m}Tc-folate-glucaric acid was obtained.

3. Result and discussion

3.1. Characterization of folate-glucaric acid

The elementary and intermediate compounds of synthesis of folate-glucaric acid were confirmed at each step by TLC using 2-propanol:chloroform (7/3) mobile phase solution and then UV lamb was used determining of R_f values. According to the R_f values, folate-glucaric acid remained at the bottom of TLC strip (R_f = 0.0) but other compounds were distinctly separated as listed in Table 1.

NMR and LC–MS/MS spectroscopy were used to characterize and confirm the synthesized folate-glucaric acid. ^{1}H NMR (DMSO): δ 1.84–1.96 (m, 1H, $CHCH_2CH_2$, 25), 2.03–2.10 (m, 1H, CH_2CH_2C, 26), 2.65–2.67 (m, 1H, H, CHCHCH, 40), 3.84–3.87 (m, 1H, CHCHC, 42), 4.28–4.33 (q, 1H, CHCHCH, 38), 4.11–4.21 (m, 1H, $NHCHCH_2$, 24), 4.47 (d, J = 2.4 Hz, 1H, CCHCH, 36), 6.88 (t, J = 6 Hz, H, CCH_2NH, 13), 6.63 (d, J = 4 Hz, 2H, CCHCH, 16–20), 7.65 (d, J = 3.6 Hz, 2H, CHCHC, 17–19), 8.64 (s, CCHN, 1H, 8). Proton NMR spectra were measured by a Brunker 400 MHz spectrometer. The reported chemical shifts are against TMS (tetramethylsilane). The ^{1}H NMR spectroscopy proved to be the most valuable tool for determining the linkage property of azide groups. Since, the azide group can coordinate to the main structure in two possibilities, γ or α. The NMR spectrum of the compound (Folat-azid) in aromatic and aliphatic regions shows 9 resonance peaks. Those 9 well-resolved resonance peaks correspond to five different aromatic ring protons; and remaining aliphatic peaks can be attributed to–CH and –NH groups in the chain. The position of azidation (γ or α) can be identified by the assistance of CH proton at the position of 24 (Fig. 1).

In the case of azidation at α position, the corresponding peak for 24, should shift to up field (around 5.50 ppm), comparing to NMR of folic acid. In fact, the NMR spectrum of folat-azid, there was no remarkable change for the peak at 4.47 ppm comparing to folic acid, which means the azidation at γ position. NHS-folate, folate-hydrazide and folate-glucaric acid were identified by LC–MS/MS and their molecular masses were determined as 577, 455 and 643, respectively.

Table 1
R_f values of folic acid, NHS-folate, folate-hydrazide, glucaric acid and folate-glucaric acid in 2-propanol:chloroform (7/3) solution.

Compounds	R_f value
Folic acid	0.2
NHS-folate	0.6
Folate-hydrazide	0.2
Glucaric acid	1.0
Folate-glucaric acid	0.0

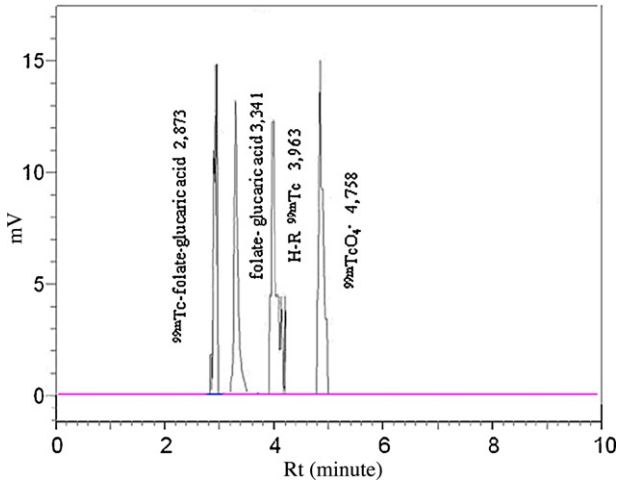

Fig. 2. RHPLC of folat-glucaric acid, radiolabeled compund, reduced ^{99m}Tc and $^{99m}TcO_4^-$.

3.2. Quality control of ^{99m}Tc-folate-glucaric acid

The radiolabeling efficiency of ^{99m}Tc-folate-glucaric acid was confirmed using TLRC and RHPLC methods. R_f values of ^{99m}Tc-glucaric acid, ^{99m}Tc-folate-glucaric acid, $^{99m}TcO_4^-$ and reduced ^{99m}Tc were determined as 0.77, 0.15, 1.00 and 0.09 by TLRC, respectively, when mobile phase 1 (ethanol/NH_3/water 2/1/5 v/v) was used. For mobile phase 2 (methanol/water 20/80 v/v), R_f values of ^{99m}Tc-glucaric acid, ^{99m}Tc-folate-glucaric acid, $^{99m}TcO_4^-$ and reduced ^{99m}Tc were 1.00, 0.20, 1.00 and 0.12, respectively. On the other hand, when 0.9% NaCl was used as mobile phase 3, the R_f values were measured to be 0.62, 0.10, 1.00 and 0.15, respectively. From the results of RHPLC analysis, R_t values of ^{99m}Tc-folate-glucaric acid, $^{99m}TcO_4^-$ and reduced ^{99m}Tc were found as 2.8, 4.7 and 3.9 min, respectively (Fig. 2). These results indicate that the folate-glucaric acid was successfully labeled with a high yield ($96.0 \pm 2.0\%$).

The serum stability result showed that ^{99m}Tc-folate-glucaric acid remained stable during incubation at 37 °C up to 3 h in human serum. Lipophilicity was determined by counting radiolabeled folate congujate between n-octanol and water. Partition coefficient (Log P) was calculated as -1.13 ± 0.02 for ^{99m}Tc-folate-glucaric acid. The result present that this compound has a water solubility property, which is very important for *in vivo* distribution studies. According to many studies, hydrophilic conjugates have showed high affinity for folate receptors.

3.3. Biodistribution studies in Albino Wistar rats

The biodistribution evaluation of ^{99m}Tc-folate-glucaric acid in receptor unsaturated and saturated rat groups is plotted in Figs. 3 and 4. As seen in Fig. 3, the uptake of labeled folate-glucaric acid in lung (0.85%ID/g), liver (6.0%ID/g, $p < 0.05$), small intestine (0.97%ID/g) and spleen (0.43%ID/g) reached the highest values at 60 min after post injection (pi). Then the uptake decreased gradually in the organs with time. The corresponding values (%ID/g) in FR including organs such as ovary, breast and uterus were determined as 0.09 ($p < 0.05$), 0.07 ($p < 0.05$) and 0.31($p < 0.05$), respectively.

The results of receptor saturated experiments are presented Fig. 4. The data indicated that its uptake was high in lung (%ID/g: 0.15, $p < 0.05$), liver (%ID/g: 0.20, $p < 0.05$) and kidney (%ID/g: 0.34) at 120 min and then decreased with time. According to the data, the uptake of ^{99m}Tc-folate-glucaric acid reached maxima in folate receptor rich tissues such as ovary (0.04%ID/g, $p < 0.05$), breast

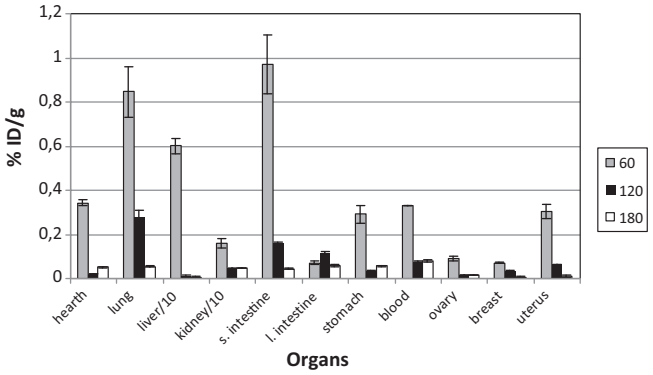

Fig. 3. Biodistribution of ^{99m}Tc-folate-glucaric acid in unsaturated Albino Wistar rats as a function of time.

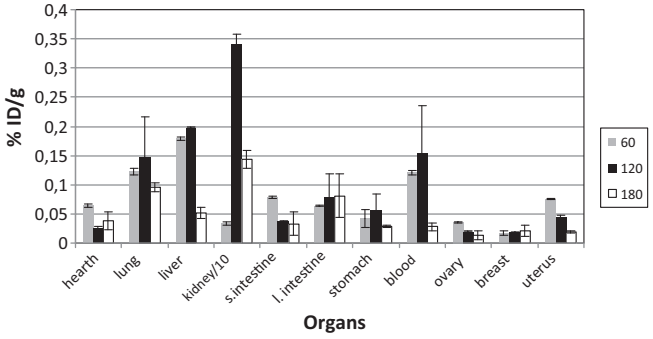

Fig. 4. Biodistribution of ^{99m}Tc-folate-glucaric acid in saturated Albino Wistar rats as a function of time.

(0.02%ID/g, $p < 0.05$) and uterus (0.08, %ID/g, $p < 0.05$) at 60 min after injection.

The uptake of ^{99m}Tc-folate-glucaric acid for ovary, breast and uterus from the saturated and unsaturated receptor biodisturibition data are shown all together for comparison from Fig. 5. As seen in Fig. 5, the uptake decreased in ovary, when the receptor was saturated. The decrease was 60% at 60 and 18% at 180 min. Data in Fig. 5 shows that the %ID/g values for uterus also decreased, when the receptor was saturated such as 76% at 60 and 30% at 120 min. The uptake in breast decreased, when the receptor was saturated. The decrease for this organ was 76% at 60 and 44% at 120 min. When the receptor saturated, the uptake in the organs decreased as reported by Altiparmak. The percent uptake of radiolabeled compound in FR positive organs showed a significant decrease when compared with Altiparmak's data (Altiparmak et al., 2010). Therefore, folate receptor was blocked by unlabeled folic acid and the uptakes in ovary, uterus and breast were determined to decrease as suggested by Altiparmak et al. (2010). As known FRs exhibit limited expression

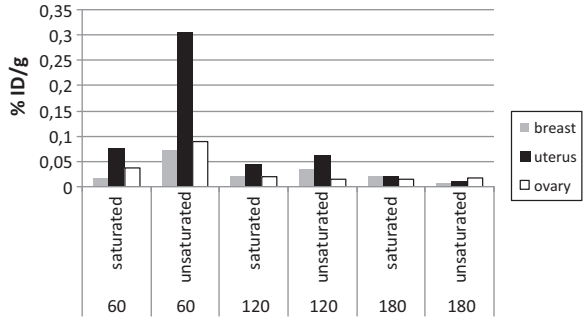

Fig. 5. Receptor saturated and unsaturated biodistrubition studies of ^{99m}Tc-folate-glucaric acid in FR positive organs.

on healthy cells, but are often present in large numbers on cancer cells. For example, FRs are over expressed on epithelial cancers of the ovary, mammary gland, colon, lung, prostate, nose, throat, and brain. So the use of folate ligand as targeting ligand can be potential strategy for treatment and/or monitoring of FR positive tumors (Hilgenbrink and Low, 2005). Consequently, ^{99m}Tc-folate-glucaric acid might be specific for ovary, uterus and breast. In the other studies, many folate-based radiopharmaceuticals such as ^{99m}Tc-citro-folate, ^{99m}Tc-MAG$_3$-FA, ^{99m}Tc-MAG$_2$-FA, ^{99m}Tc-TEPA-folate, ^{99m}Tc-DTPA-folate and ^{99m}Tc-HYNIC-folate have showed an efficient uptake in the tissues including folate receptor (Altiparmak et al., 2010; Okarvi and Jammaz, 2006; Panwar et al., 2009; Mathias et al., 2000; Guo et al., 1999).

The results together demonstrated that ^{99m}Tc-folate-glucaric acid showed a higher uptake in liver and small intestine compared to the other folat-free organs. Folic acid and its derivatives taken by the small intestine are metabolized in the liver (Mindt et al., 2008; Trump et al., 2002). Therefore, these organs showed apparent uptake.

Our results demonstrated that the elimination of ^{99m}Tc-folate-glucaric acid occurred via urinary system from blood. Kidneys are the main clearance organ due to folate conjugate. Since folate receptors are usually located on proximal tubules. Additionally, hydrophilic conjugates, such as ^{99m}Tc-folate-glucaric acid show a fast clearance from the blood via kidneys (Trump et al., 2002; Kim et al., 2007; Zhang et al., 2010; Gu et al., 2010; Altiparmak et al., 2010).

In summary, ^{99m}Tc-folate-glucaric acid showed good localization in FR rich organs such as uterus, ovary and breast and rapid clearance from blood via urinary system.

4. Conclusions

The folate-glucaric acid was successfully labeled with ^{99m}Tc at high radiolabelling yield ($96 \pm 2.0\%$) and radiolabeled folate conjugate remained stable in human serum up to 3 h. Furthermore, the conjugate was found to be neutral and hydrophilic. As a result of the biodistribution study conducted in the rats showed that the ^{99m}Tc-folate-glucaric acid has significant uptake in folate receptor rich tissues such as breast, ovary and uterus. In conclusion, the radiolabeled conjugate shows good radiolabeling and biodistribution in FR organs, ^{99m}Tc-folate-glucaric acid might be potentially useful in the field of receptor-based targeted radiopharmaceutical.

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