

Preparation and biodistribution in mice of a new radiopharmaceutical –technetium-99m labeled methotrexate, as a tumor diagnostic agent

Ume Kalsoom Dar^{1,2} BS,
Irfan Ullah Khan¹ PhD,
Muhammad Javed³ MSc,
Fayyaz Ahmad¹ MSc,
Muhammad Ali⁴ MSc,
Syed Waqar Hyder¹ MSc

1. Radiopharmacy and PET Radiochemistry Division, Institute of Nuclear Medicine & Oncology (INMOL), New Campus Road, PO Box 10068, Lahore- 54600, Pakistan

2. Institute of Chemistry, University of the Punjab, Quaid-e-Azam Campus, Lahore- 54590, Pakistan

3. Gujranwala Institute of Nuclear Medicine (GINUM), Gujranwala, Pakistan

4. School of Biological Sciences (SBS), University of the Punjab, Quaid-e-Azam Campus, Lahore- 54590, Pakistan

Keywords: Radiolabeling
- ^{99m}Tc-methotrexate
- Tumor - Biodistribution

Correspondence address:

Irfan Ullah Khan PhD
Radiopharmacy and PET Radiochemistry Division, Institute of Nuclear Medicine & Oncology (INMOL), New Campus Road, PO Box 10068, Lahore- 54600, Pakistan
E-mail: drirfankhan69@gmail.com

Received:

9 April 2012

Accepted revised:

2 July 2012

Abstract

Our aim was to develop the procedure for radiolabeling of an anticancer drug e.g., methotrexate with ^{99m}Tc for tumors diagnosis. *The study included* the radiolabeling of methotrexate, *in vitro* stability of radiolabeled drug, *in vitro* binding of radiolabeled drug with plasma protein, partition coefficient and biodistribution of radiolabeled drug in mice. *Results showed* 98.2±0.5% radiolabeling of methotrexate with technetium-99m (^{99m}Tc). *In vitro* stability was studied for 5h and 79.3±5% of the drug was bound with plasma proteins. Partition coefficient of the labeled drug showed that it was highly hydrophilic. Biodistribution study in tumor bearing mice exhibited high uptake in tumor cells which were further investigated by histopathological studies. *In conclusion*, our study indicates that technetium-99m labeled methotrexate is a potentially strong tumor diagnostic agent with low uptake in normal tissues.

Hell J Nucl Med 2012; 15(2): 120-124

Published on line: 27 July 2012

Introduction

Receptor targeted radiopharmaceuticals highly improve the sensitivity and specificity of nuclear imaging procedures [1]. The ligands which are capable of concentrating at pathological sites have been derivatised with chelator radionuclide complexes and they are used as non-invasive probes for diagnostic purposes [1-4]. Folic acid and its analogs have been used as ligands to localize radionuclides to tumors. Biologically active molecules that can selectively interact with specific type of cells are the attractive vehicles for the delivery of radioactivity to the desired tissue or cancerous cells [5]. Small bioactive molecules are now widely in use for the development of radiopharmaceuticals because of their favorable pharmacokinetic characteristics such as low toxicity, rapid blood clearance, low uptake in non targeted areas and high uptake in targeted tissue [6].

The folic acid and its analogs have high affinity for the folate receptor positive tissues and show uptake in tumor cells by folate receptor mediated endocytosis [1]. Folate receptors are over expressed in various human tumors that include breast, cervical, renal, ovarian and colorectal malignancies but are present in very limited amount in normal tissues making folate and its analogs potential molecular targets for tumoral imaging [2, 3].

Methotrexate (MTX) is an anticancer agent commonly known as amethopterin; it is an antimetabolite of folic acid analog. Methotrexate inhibits the synthesis, repair and cellular replication of DNA. This inhibition occurs because of irreversible binding of methotrexate with dihydrofolate reductase, which prevents reduction of dihydrofolate to the active tetrahydrofolate. It is widely used for the treatment of cancer, for ectopic pregnancy, for autoimmune diseases, for the induction of medical abortions and higher doses of MTX can produce a therapeutic response in patients with bone metastases [7, 8]. Specifically, MTX is used for the treatment of neck, esophageal, gastric, pancreatic, lung, ovarian, bladder, cervical, breast, head, colorectal and penile carcinomas [9].

We designed MTX for the diagnosis of various types of cancers by labeling it with technetium-99m (^{99m}Tc) and tried to deliver high doses of ^{99m}Tc-MTX to selected malignant sites in targeted tumors or tissues in mice, while minimizing the radiation burden to the surrounding tissues. Various parameters were studied which referred to *in vitro* stability of the radiolabeled drug: protein binding, partition coefficient, biodistribution in animal models, and reported in this original article.

Materials and methods

All chemicals used for this research were of analytical grade. Methotrexate and stannous tartrate were purchased from Sigma Aldrich, USA. Hydrochloric acid and acetone were

purchased from Merck, Germany. Trichloroacetic acid was purchased from BDH laboratory reagents, England. Technetium-99m generator was purchased from Pakistan Institute of Nuclear Science and Technology (PINSTECH), and phosphate bottles were purchased from Ostuka, Pakistan. Mice were obtained from the animal house at School of Biological Sciences, University of the Punjab, Lahore, Pakistan.

Radiolabeling and radiochemical purity analysis

Twenty mg MTX were dissolved in 10mL of distilled water with continuous stirring. Two drops of 5N NaOH was added during stirring to clear the solution. Afterwards, 10mg ascorbic acid and 500µg stannous tartrate were added. Furthermore, pH was maintained at 8.2-8.5 with 1N HCl and 0.1N HCl. The resultant solution was passed through 0.22µm membrane filter. One mL/kit of resultant solution was dispensed in sterilized serum vials. Five hundred and fifty five MBq Na^{99m}TcO₄ eluted from ⁹⁹Mo-⁹⁹Tc generator were added to the kit and incubated at room temperature for 5min. Radiochemical purity of the ^{99m}Tc-MTX was determined by using two simple chromatographic techniques, e.g., instant thin layer chromatography (ITLC) and paper chromatography using a 3MM Whatman paper, employed to find out the percentage of hydrolyzed, reduced radioisotope bound to ligand and of free pertechnetate. Acetone was used as a mobile phase for paper chromatography and saline was used for ITLC. Small aliquots from the reconstituted kit were spotted on the respective strips. The strips, after elution, were cut in fractions of 1cm and counted for radioactivity in a well type scintillation counter.

In vitro stability of radiocomplex

In vitro stability of the ^{99m}Tc-MTX complex was estimated by incubating ^{99m}Tc-MTX at room temperature for various intervals of time up to 5h. To assess the dissociation/ degradation of the labeled complex at room temperature, aliquots at different time intervals were applied on PC and ITLC-SG strips. The PC strips were developed in 100% acetone and the ITLC-SG strips in saline. The percentage dissociation of the complex at a particular time interval was detected by the percentage of free pertechnetate (%) at that time. In case of significant loss of metal-complex stability, it was not advisable to use the radiopharmaceutical for experimental or clinical applications.

Protein binding and lipophilicity

In vitro protein binding of ^{99m}Tc-MTX was carried out in human blood by protein precipitation. To 3mL fresh human plasma, 1mL of the labeled complex was mixed and incubated for 1h at 37°C. Contents of the tube were centrifuged at 3000rpm for 10min for separation of serum and blood cells. After mixing approximately equal volume of 10% trichloroacetic acid (TCA), the mixture was centrifuged at 3000rpm for 10min. Residue was separated from supernatant and both layers were counted for radioactivity in a well type gamma counter. Protein binding of the complex was expressed as the fraction of radioactivity bound to protein, in percentage of the total radioactivity.

The lipophilicity was determined by measuring the partition coefficient between organic and aqueous layers. An aliquot of 100µL of ^{99m}Tc-MTX was mixed with 200µL of phosphate buffers of pH 6.6, 7.0 and 7.6 in three separate vials, followed by addition of 200µL of n-octanol in each vial. The

contents in all vials were shaken and the two layers allowed to separate by leaving the contents undisturbed for 10min at room temperature. Both the fractions were collected separately and the radioactivity was measured in known aliquots of each fraction. Same procedure was repeated by replacing phosphate buffers with saline.

Safety of ^{99m}Tc-MTX

The radiopharmaceutical kit was synthesized under sterilized conditions. Laminar flow hood was sterilized with spirit under UV light exposure for 24h. Apparatus used for the kit formulation was sterilized in a preheated oven at 200°C for 2h. The dose-related toxicity was investigated in a group of three rabbits for five consecutive days by injecting 100µg/kg of ^{99m}Tc complex. No signs of toxicity were observed till 72h after the last i.v. injection. The animal toxicity study was performed in accordance with the current rules of INMOL Hospital, Lahore, Pakistan.

Biodistribution

All animal experiments were carried out with the approval of the Ethics Committee of INMOL. Six female Swiss Webster mice of age 24±4 days having weight 18±2g were studied. All mice had naturally developed tumor in lower abdomen and upper right thorax region. Tumoral uptake was studied in Swiss Webster mice (26g-28g) after administering approximately 37MBq of the ^{99m}Tc-MTX into the tail vein (Fig. 1). The mice were sacrificed by cervical dislocation at 30, 60 and 90min post administration of the drug. Organs were collected, weighed and counted for radioactivity. The radioactivity was measured in various organs including tumor, which was expressed as percent injected dose per gram and tumor was further investigated by histopathological studies.

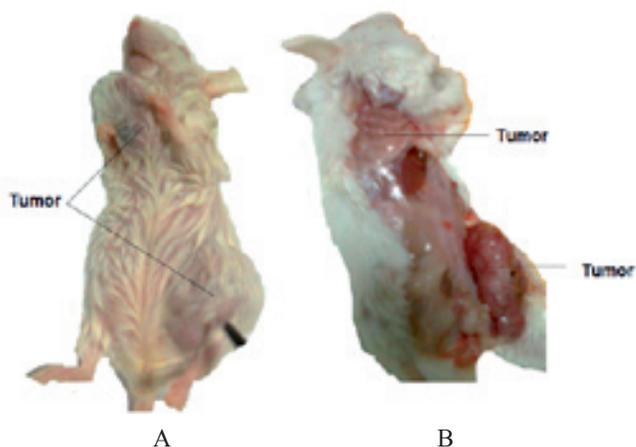


Figure 1. Swiss Webster mouse with a carcinoma in the thorax and the left side of lower abdomen injected with ^{99m}Tc-MTX. **A:** Before dissection, **B:** After dissection.

Histopathology of tumor

Specimens of 2x2cm diameter with smooth and reddish appearance were bisected and placed on tissue cassettes. The samples were processed in the processor for 14-15h. Specimens were embedded, blocks prepared with wax and frozen. After cutting slides by the microtome to the size of 3microns, samples were prepared for staining and reporting. The blocks were cut; slides were dewaxed on a hot plate, and dipped in xylene. The process was repeated

three times, and the slides were then placed in 95% ethanol for 10-20min followed by 75% ethanol for 5-7min. This process was repeated three times and then slides were placed in haematoxyline for 15min and in running tab water for 10min. Decolorization was developed by placing the slides for 1-2sec in standard acid decolorizer. The rinsing was carried out in standard ammonia solution containing 2ml NH₃ in 8mL of water, followed by rinsing in water and placing in eosin for 5min. The slides were further rinsed through ethanol by using increasing concentrations of 75%, 80% and 95%. Slides were finally placed in a xylene container, cleaned, dipped in mounting media and covered with slide caps.

Results

Radiochemical purity and in vitro stability

During labeling of MTX with ^{99m}Tc, three products namely ^{99m}Tc-MTX complex, free pertechnetate (^{99m}TcO₄⁻) and reduced or hydrolyzed ^{99m}TcO₂, were formed, and were separated by PC and ITLC. In PC, ^{99m}TcO₄⁻ had an Rf of 0.8-0.9, while the ^{99m}Tc-MTX and the hydrolyzed ^{99m}TcO₂ appeared at Rf=0.00-0.01. The hydrolyzed fraction was separated from the other two fractions by using saline, so that the ^{99m}Tc-MTX complex and the free ^{99m}TcO₄⁻ appeared at Rf=0.9-1.0, and the ^{99m}TcO₂ was at Rf=0.00-0.01. The overall labeling yield of the ^{99m}Tc-MTX complex, as calculated by the above methods, was 98.2±0.5%. This complex was sufficiently stable. The *in vitro* stability analysis at room temperature up to 5h showed a degradation of only 1%-1.7%. This high labeling efficacy shows the validity of the labeling technique. Graphical presentation is given in the (Fig. 2).

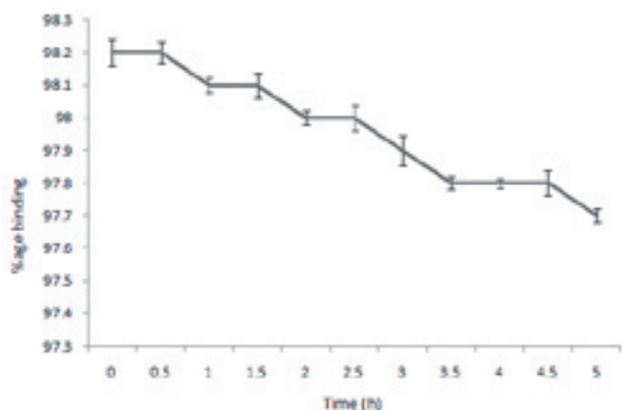


Figure 2. In vitro stability of ^{99m}Tc-MTX up to 5h at room temperature.

Protein binding and lipophilicity

Before studying the uptake of the drug in mice, we studied its *in vitro* binding with plasma proteins. The protein binding of ^{99m}Tc-MTX assessed in human serum was found to be 79.3±5%. Lipophilicity of ^{99m}Tc-MTX was also measured. Data indicated that ^{99m}Tc-MTX had maximum binding at the hydrophilic layer in phosphate buffers of pH 6.6, 7.0 and 7.6 in saline, and negligible amount of activity observed in lipophilic media (octanol), thus suggesting that radiolabeled drug was hydrophilic in nature. Data are shown in Table 1.

Biodistribution

Drug was injected under single photon emission tomography (SPET) gamma camera to study the biodistribution of the drug especially in the tumor. Mice were dissected at 30, 60 and 90min post injection, intervals. Various organs (mentioned in Table 2) were collected and counted for radioactivity in a gamma scintillation counter. Percentage of injected dose per gram (%ID/g) determined from various organs is shown in Table 2.

Histopathology of the tumors

After haematoxyline-eosin staining, histopathology specimens of the tumors showed small granular cancer cells with hyperchromatic nuclei and a few granular cells containing secretions in lumina. Abnormal mitotic figures were also seen. These morphological features, as shown in Figure 3 are suggestive of moderately differentiated adenocarcinoma, originated in abdomen and thorax.

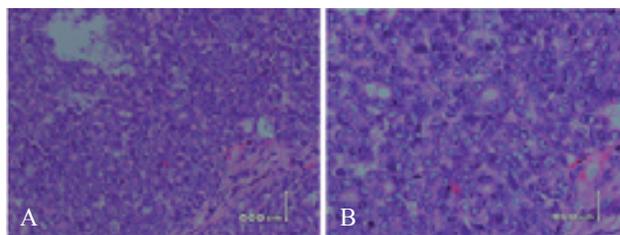


Figure 3. Immunohistochemistry data of tumor tissue samples obtained after dissection from mice. A: Tumor of the lower abdomen after staining with hematoxyline eosin (HE). B: Tumor of thorax after staining with HE.

Discussion

The high accumulation of ^{99m}Tc-MTX in tumor tissues of mice allows to deliver the high doses of the radiopharmaceutical to selected malignant tumors, while minimizing the radiation doses to normal tissues.

Table 1. Percentage of ^{99m}Tc-MTX in the hydrophilic and lipophilic areas

^{99m} Tc-MTX % detected in the:		Hydrophilic media (%)	Lipophilic media (%)
Hydrophilic Media	Lipophilic Media		
Phosphate buffer pH=6.6	Octanol	99.3±0.3	0.3±0.03
» » pH=7.0	»	99.1±0.5	0.9±0.05
» » pH=7.6	»	99.6±0.2	0.4±0.05
Saline	»	99.8±0.1	0.2±0.05

Table 2. Percentage of ID/g of MTX in various organs of mice

Tissue	%ID/g at 30min	%ID/g at 60min	%ID/g at 90min
Tumor 1 (lower abdomen region)	5.87±0.12	6.22±0.29	6.32±0.46
Tumor 2 (upper right thorax region)	2.84±0.23	3.76±0.22	3.77±0.17
Blood	0.65±0.01	0.76±0.04	0.69±0.09
Liver	4.19±0.46	3.11±0.55	1.44±0.98
Heart	0.31±0.02	0.17±0.03	0.15±0.06
Muscle	0.21±0.09	0.45±0.1	0.32±0.07
Spleen	0.37±0.04	0.31±0.02	0.15±0.04
Lungs	0.76±0.10	0.51±0.99	0.34±1.1
Stomach	0.002±0.001	0.002±0.001	0.002±0.001
Bladder	2.84±0.23	3.95±0.32	4.12±0.54
Kidneys	3.47±0.93	4.77±1.22	5.98±1.16
Tail (injection site)	32.38±1.43	30.09±2.99	27.69±2.54

A rather simple method was used for labeling of MTX with ^{99m}Tc .

Drugs bind to plasma proteins or many other biological materials like glycoprotein, lipoprotein, albumin, erythrocytes, and α , β -, and γ -globulins. Albumin is an important plasma protein with a concentration of 3.5 to 5g/dL in serum [10]. Radiolabeled drug and plasma protein interactions affect the pharmacokinetic parameters such as metabolism, volume of distribution, and excretion of the drug, and accordingly its dosage [11]. Radiolabeled drug binding with plasma proteins is an essential parameter for measuring the effectiveness of the chelating moiety to coordinate the radiometal. The transchelation is involved in the process in which radiometal in labeled drug transchelates to blood proteins, particularly, albumin. So it is important to study the *in vitro* blood protein binding with radiolabeled drug before it is applied to any organism. The binding of drug with blood protein decreases the concentration of drug in plasma. The free or unbound drug is responsible for the side effects and pharmacological activities in the body [12-14]. Plasma proteins provide a depot for drugs by maintaining buffered free drug levels and assist its distribution [15]. As mentioned earlier, ^{99m}Tc -MTX showed 79.3±5% binding with blood proteins.

Partition coefficient is an essential parameter to be studied for any drug. The lipophilic characteristics of drug affect its binding to the receptor targets [16, 17]. Lipophilic drugs tend to be toxic because of their longer retention and wider distribution in body, while, hydrophilic drugs show rapid clearance from the body. It is highly recommended to use drugs with high hydrophilic properties [18]. Our data indicate that ^{99m}Tc -MTX showed significant hydrophilic characteristics. It rapidly reached the target area and was rapidly cleared.

Previously reported studies showed that MTX, e.g., a folic acid analog showed receptor binding uptake in tumoral cells with very limited uptake in normal cells [7-9]. On the basis of these findings, we performed the biodistribution study of ^{99m}Tc -MTX in tumor bearing mice. Prominent uptake was observed in the liver and the tumor specimens. These findings support the argument that uptake of a radiotracer is de-

pendent on various factors, like the nature of the complex, pH, blood flow, plasma concentration, etc. The retention of the radiolabeled drug in the liver might be due to MTX metabolism in the liver. The high uptake of ^{99m}Tc -MTX in the tumors showed the possibility of its application in cancer diagnosis. The most probable route of excretion of ^{99m}Tc -MTX and its metabolites was through the kidneys [19, 20]. We did not acquire more images in the mice because of technical reasons. We currently continue this research.

In conclusion, a sample technique for labeling MTX with ^{99m}Tc was described and furthermore, the biological-chemical and the biodistribution of ^{99m}Tc -MTX in mice. All the above data indicate that ^{99m}Tc -MTX can be a useful means for tumor diagnosis.

The authors declare that they have no conflicts of interest.

Bibliography

- Guo W, Hinkle GH, Lee RJ. Tc-HYNIC-folate: A novel receptor-based targeted radiopharmaceutical for tumor imaging. *J Nucl Med* 1999; 40: 1563-9.
- Mathias CJ, Wang S, Waters DJ et al. Indium-111-DTPA as potential folate receptor targeted radiopharmaceutical. *J Nucl Med* 1998; 39: 1579-85.
- Ilgan S, Yang DJ, Higuchi T et al. ^{99m}Tc -ethylenedicysteine-folate: a new tumor imaging agent. Synthesis, labeling and evaluation in animals. *Cancer Biother Radiopharm* 1998; 13: 427-35.
- Linder KE, Wedeking P, Ramalingam K. *In vitro* and *in vivo* studies with α - and γ -isomers of ^{99m}Tc -OXA-folate show uptake of both isomers in folate-receptor (+) KB cell lines. *Soc Nucl Med Proc 47th Annual Meeting* 2000; 41: 119P.
- Okarvi SM. Peptide-based radiopharmaceuticals: Future tools for diagnostics imaging of cancers and other diseases. *Med Res Rev* 2004; 24: 357-97.
- Okarvi SM, Jammaz IA. Preparation and *in vitro* and *in vivo* evaluation of technetium-99m labeled folate and methotrexate conjugates as tumor imaging agents. *Cancer Biother Radiopharmac* 2006; 21: 49-60.

7. Methotrexate: The American Society of Health-System Pharmacists. <http://www.drugs.com/monograph/methotrexate.html>. Retrieved 3 April 2011.
8. Jaffe N. Recent advances in the chemotherapy of metastatic osteogenic sarcoma. *Cancer* 1972; 30: 1627-30.
9. Devita VT, Hellman S, Rosenberg SA. *Cancer: Principles and Practice of Oncology*. 5th edn. Philadelphia: Lippincott-Raven 1997; 1180-1.
10. Reza M. Role of Protein Binding in Pharmacokinetics. *Amer J Pharm Educ* 2005; 69: Article 103: 1-8.
11. Rolan PE. Plasma protein binding displacement interactions. Why they are still regarded as clinically important? *Br J Clin Pharmacol* 1994; 37: 125-8.
12. Nix DE, Matthias KR, Ferguson EC. Effect of ertapenem protein binding on killing of bacteria. *Antimicrob Agents Chemother* 2004; 48: 3419-24.
13. Scaglione F, Demartini G, Arcidiacono MM et al. Influence of protein binding on the pharmacodynamics of ceftazidime or ceftriaxone against gram-positive and gram-negative bacteria in an *in vitro* infection model. *Chemother* 1998; 10: 29-34.
14. Zeitlinger MA, Sauermann R, Traummüller F et al. Impact of plasma protein binding on antimicrobial activity using time-killing curves. *J Antimicrob Chemother* 2004; 54: 876-80.
15. Talbert AM, George ET, Elaine H, Peter LF. Determination of Drug-Plasma Protein Binding Kinetics and Equilibria by Chromatographic Profiling: Exemplification of the Method Using L-Tryptophan and Albumin. *Anal Chem* 2002; 74: 446-52.
16. Eisenberg D, McLachlan AD. Solvation energy in protein folding and binding. *Nature* 1986; 319: 199-203.
17. Miyamoto S, Kollman PA. What determines the strength of noncovalent association of ligands to proteins in aqueous solution? *Proc Natl Acad Sci USA* 1993; 90: 8402-6.
18. Pliska V, Testa B, Waterbeemd H. *Lipophilicity in Drug Action and Toxicology*. John Wiley & Sons Ltd New York 1996; 439.
19. Henderson GB, Montague WB. Irreversible inhibitors of methotrexate in L1210 cells characteristics of inhibition by an N-hydroxysuccinimide ester of methotrexate. *Biochim Biophys Acta* 1983; 735: 123-30.
20. Huang WT, Yao HH, Lo JM. Preparation and biolocalization of technetium-99m complexes with N₂O₂ schiff-base compounds. *Ann Nucl Med Sci* 2004; 17: 13-9.



Louis-Leopold Boilly, 1825: *The blind musicians*. Handmade lithography