 

ORIGINAL ARTICLE

# Study of normal biodistribution and uptake patterns of novel anticancer radiopharmaceutical complex $^{99m}$Tc-Methotrexate

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## Abstract

*Objective* Methotrexate (MTx) is an anticancer agent used in the treatment of various cancers. The objective of this study was to document the biodistribution of $^{99m}$Tc-labelled Mtx ($^{99m}$Tc-MTx) in normal subjects and patients with breast cancers.

*Methods* We prepared the $^{99m}$Tc-MTx kit by a direct labelling method and studied its biodistribution in volunteer subjects and patients with breast carcinoma breast. This clinical study was preceded by animal trials [1].

*Results* The normal biodistribution pattern in humans was characterized by nonspecific uptake in the body with the $^{99m}$Tc-MTx behaving like a blood pool agent with no evidence of specific organ uptake. The kidneys were seen to be the main route of excretion. Biodistribution data of patients with carcinoma breast showed excellent tracer uptake in the tumour and showed no other nonspecific tracer uptake.

*Conclusion* This initial clinical trial showed that $^{99m}$Tc-labelled anticancer drug can be successfully used for tumour scintigraphy, which appears to be a major breakthrough as this method of labelling and scanning may be useful in future tumour staging, calculating the sensitivity of tumours to certain anticancer agent and response evaluation during chemotherapy.

***Key words***: *$^{99m}$Tc-methotrexate, biodistribution, breast cancer*

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## Introduction

The drug methotrexate (MTx) is a chemo therapeutic agent used in the treatment of breast cancers, head and neck cancers, leukaemia, lymphoma, lung cancers, osteo sarcomas, bladder cancers, etc. [2]. In breast cancer, the drug is being used as an adjuvant and neoadjuvant chemotherapeutic agent [3]. Cancer causes abnormal growth of cells by multiple changes in gene expression leading to deregulation in the balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites causing significant morbidity and, if untreated, death of the host [4].

Methotrexate has been previously labelled with $^{99m}$Tc using a conjugate mercaptoacetyl triglycine (MAG3) and its gamma scintigraphic properties have been studied in animal models of tumour [1, 5, 6]. Recently published animal studies also used $^{99m}$Tc-MAG3-MTx complex as a tumour imaging agent in animals [6]. However, the use of conjugate makes the kit costly and more difficult to prepare due to the complexity of the labelling procedure and heating of the compound for proper labelling. It is therefore desirable to explore new methods for direct and simple labelling of the anticancer agents along with preservation of the chemical characteristics of the agent without increasing its *in vivo* toxicity. This study aimed at direct labelling of $^{99m}$Tc with Methotrexate and studying the biodistribution in normal human subjects and patients with breast cancer following an animal model study [1].

## Materials and Methods

Several chemicals used in this research were purchased from Aldrich, USA (Methotrexate, stannous chloride ascorbic acid and sodium citrate), with normal saline procured from Ostuka, Pakistan. The technetium-99m generator was purchased from Pakistan Institute of Nuclear Science and Technology (PINSTECH).

MTx kit formulation was carried out by modified method published by our team [1]. 20 mg of MTx was dissolved in 18 ml of D/D (double distilled) water by using few drops of 1N NaOH. Ascorbic acid (30 mg) and sodium citrate (20 mg) were added in the stirred solution. Next, 2 ml of stannous tartrate (5 mg/ml) in pyrophosphate (5 mg/ml) solution were added with constant stirring and pH adjusted to 8.0-8.5 and a fraction of 1 ml was dispensed in a 10 ml serum vial after passing through 0.22 µm membrane filter. Sodium pertechnetate (925 MBq) eluted from Pakgen® generator from PINSTECH was added into the vial and incubated for 15 min at room temperature.

*In vitro* stability of the $^{99m}$Tc-MTx radiometal complex was estimated for various intervals of time up to 5h. To assess the dissociation of the labelled complex at room temperature, aliquots at different time intervals were applied on 3 mm chromatography paper (PC) and ITLC-SG strips. The PC strips were developed in 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 there was a significant loss of metal-complex stability, it was advised to not use the radiopharmaceutical for clinical applications. Free pertechnetate in radiometal complex was calculated using PC up to 5 hours and it was found to be 0.258% at any time, which was within acceptable limits.

The radiopharmaceutical kit was synthesized under sterile conditions. Laminar flow hood was sterilized with methylated spirit under UV light exposure for 24h. Apparatus used for the kit formulation was sterilized in a preheated oven at 200$^{o}$C for 2h. The dose-related toxicity was investigated in a group of three rabbits for five consecutive days by injecting 100 mg/kg of $^{99m}$Tc complex. No signs of toxicity were observed until 72h after the last i.v. injection. The animal toxicity study was performed in accordance with the current rules of INMOL Hospital, Lahore, Pakistan. $^{99m}$Tc-MTx complex has been tested in the animal models using mice by Dar *et al.* who demonstrated significant tumour uptake as compared to the normal organs confirming that MTx is more specific and therefore effective at tumour level

rather than in the normal tissues [1].

The study was approved by the Ethical Review Board of the Gujranwala Institute of Nuclear Medicine and Radiotherapy (GINUM). Three female patients with proven breast cancer and one female normal volunteer were studied. The subjects were fully informed of the procedure and written consent obtained.

Before starting imaging studies, routine blood and biochemical lab tests of all subjects including a complete blood count (CBC), liver function tests (LFT), urea and creatinine were determined. Besides these clinical investiga tions, blood pressure and blood sugar of all subjects were also monitored along with ER, PR, Her-2-Neu status of tumour receptors. Urine samples were collected for routine chemical and microscopic examination. All these investigations were considered as baseline. A dose of 555 MBq of $^{99m}$Tc-MTx was given i.v. in 30-s to acquire dynamic images of both breasts. During the study, vital signs were monitored for any significant change from baseline. Scintigraphic results were co-evaluated with ultrasonography (USG) of the breasts, mammography and the diagnosis was verified by biopsy of the cancerous specimen.

Imaging protocol comprised of a dynamic acquisition of ten 60-sec frames. Anterior and posterior whole-body images were acquired at 30, 60, and 120 min postinjection. To obtain clear visualization of the tumour, static images were additionally acquired in various positions (anterior, posterior, left lateral, right lateral). Images were recorded by a large field-of-view (LFOV) dual-headed gamma camera (ECAM® by Siemens™, Germany), equipped with a low-energy, all-purpose collimator for acquisition. Data processing was done on ECAM® work station using ESOFT® by SYNGO™.

Pharmacokinetics and biodistribution was studied by region-of-interest (ROI) analyses. An ROI was drawn around the whole-body on anterior and posterior views, and counts with geometric mean method were considered 100% of the injected dose at that particular time. ROIs were also drawn around other important organs including the tumour, the kidneys, the heart and the bladder. The background regions were placed close to the primary ROIs for background correction. Scans were also analysed qualitatively. The study population was much too small to allow for statistical analyses (e.g. sensitivity, specificity and accuracy); however, correlation with the diagnostic results from radiology and pathology was crucially undertaken and proved to be accurate.

Protein binding of $^{99m}$Tc-MTX was investigated by taking a 1.6 ml blood sample and centrifuging it at 3000rpm for 10 minutes to obtain a layer of blood cells and a layer of serum. The blood cell layer was wasted and 2 ml labelled kit was added to 0.3 ml of serum and incubated for one hour at 37°C. Next, an equal volume of trichloroacetic acid (10%) was added to the tube and shaken for ten minutes. The tube was again centrifuged at 3000rpm for 10 minutes for obtain supernatant solution and the residue. The two layers were separated and their activities measured. The percentage of supernatant and residue were calculated from the formula:

$$\% \text{ supernatant} = \frac{\text{supernatant counts}}{\text{supernatant + residue counts}} \times 100$$

$$\% \text{ residue} = \frac{\text{residue counts}}{\text{supernatant + residue counts}} \times 100$$

*In vitro* stability in blood was determined by adding 0.2 ml of the labelled kit to a 0.4 ml aliquot of blood and incubating at 37°C for 30 min and hourly QC was performed.

*In vitro* stability in serum was determined by centrifuging 5 ml aliquot of blood for 10 minutes at 3000rpm. The serum was separated in a sample vial and blood cell layer was wasted. Labelled kit (0.2 ml) was added to the serum (0.2 ml) and incubated at 37°C for 30 min and hourly QC performed.

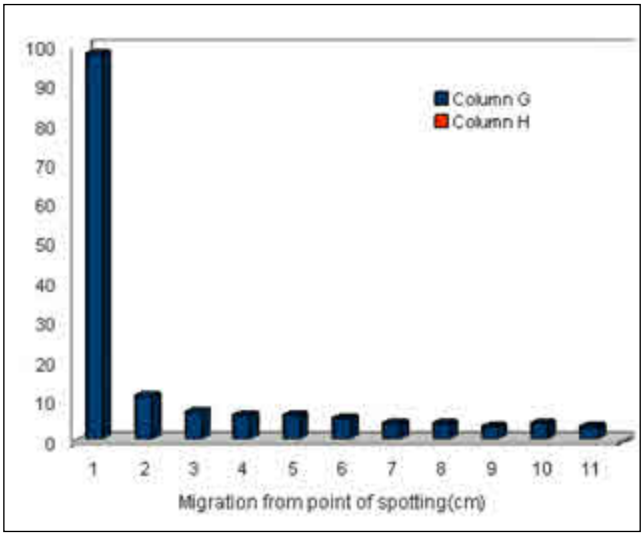


***Figure 1*** *Paper chromatography pattern of $^{99m}$Tc-methotrexate. Free pertechnetate migrated toward the solvent front while labelled $^{99m}$Tc remained at the origin of paper*

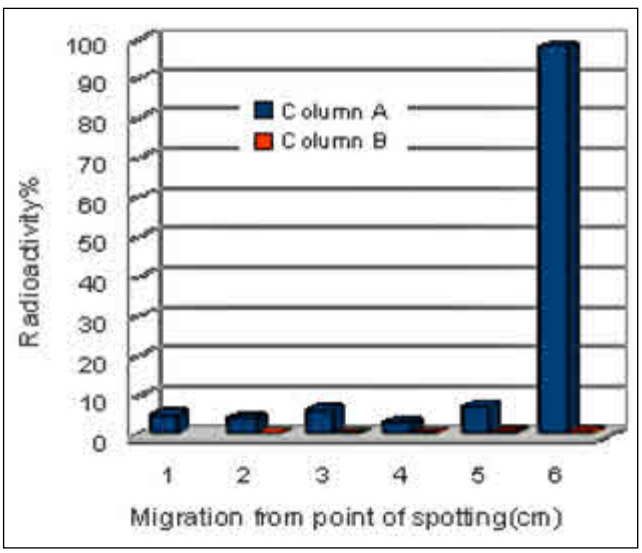


***Figure 2*** *ITLC-SG pattern of $^{99m}$Tc-methotrexate. The hydrolyzed component remained at the origin of paper and labelled $^{99m}$Tc moved towards the solvent front*

## Results

During labelling process of MTx with $^{99m}$Tc different species were formed including bound reduced $^{99m}$Tc with drug, free pertechnetate ($^{99m}TcO_{4-}$) and hydrolyzed $^{99m}TcO_2$, which were separated by PC and ITLC using acetone and saline as mobile phase. In PC, $^{99m}TcO_{4-}$ had an *Rf* of 0.8-0.9, while the $^{99m}$Tc-MTx and the hydrolyzed $^{99m}TcO_2$ appeared at *Rf*=0.00-0.01. The hydrolyzed fraction was separated from the other two fractions by using saline as mobile phase by ITLC Silica Gel (SG). In this case the $^{99m}$Tc-MTx complex appeared at *Rf* = 0.9-1.0, and the $^{99m}TcO_2$ was detected at *Rf*=0.00-0.01. The overall labelling yield of the $^{99m}$Tc-MTx complex was more than 95.0±1.5% as shown in Figures 1 and 2.

The three patients as well as the normal control remained well and no adverse reactions were reported following i.v. injection of $^{99m}$Tc-MTx. Subject's blood pressure, heart rate, respiratory rate and body temperature were recorded before injection and at 4h after injection of $^{99m}$Tc-MTx. WBC and RBC counts and ER, PR, Her2 Neu were also examined. USG of the breasts, mammography and biopsy of the cancerous specimen were supporting diagnostic tools. All tests including both the clinical and the laboratory investigations after the $^{99m}$Tc-MTx matched well with the baseline tests data. The subjects' vital signs, ECG and blood tests of were monitored during and after the injections and no signs of toxicity were noticed up to three days.

The pharmacokinetics in the single subject with breast cancer was studied. The agent was injected with the patient lying on the imaging table positioned under the SPECT gamma camera. The whole-body and static scan images of the subject showing normal biodistribution of radiotracer are shown in Figure 3.

## Discussion

Our study provides the first clinical evidence of the normal biodistribution of $^{99m}$Tc-MTx in humans as a possible breast cancer imaging agent. We have modified the radiolabelling procedure previously used in animals [1]. The quality control of $^{99m}$Tc-MTx was performed by

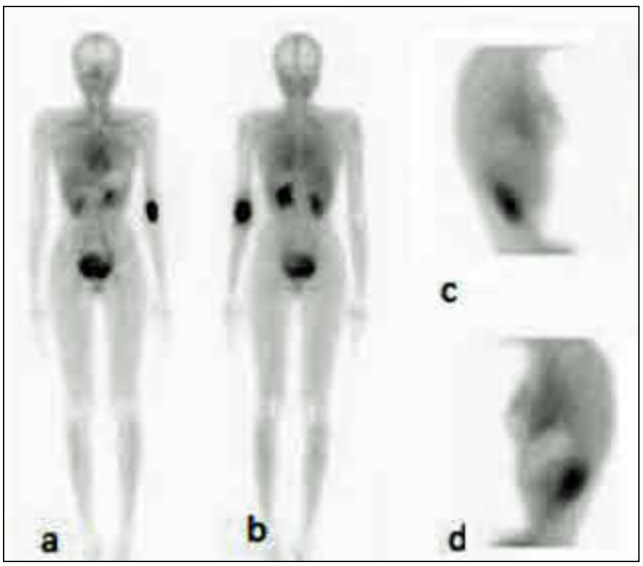


***Figure 3*** *Normal biodistribution of $^{99m}$Tc-MTx in the control subject: whole-body scans at 1-hour in the anterior (a) and posterior (b) projections followed by static trunk views in the right lateral (c) and the left lateral (d) projections showing the right and the left breasts with the patient lying prone*

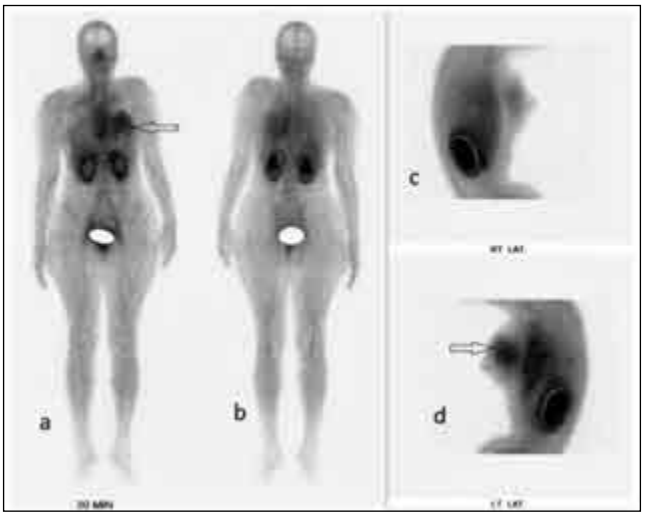


***Figure 4*** *Biodistribution of $^{99m}$Tc-MTx in a patient with left breast cancer (arrows): whole-body scans at 1-hour in the anterior (a) and posterior (b) projections followed by static trunk views in the right lateral (c) and the left lateral (d) projections showing the right and the left breasts with the patient lying prone*

using paper and instant thin layer chroma tography (ITLC). Results showed that 95%±1.5% of the drug was radiolabelled. *In vitro* stability of $^{99m}$Tc-MTx was studied up to 24 hours in fresh sample of blood and serum. No significant dissociation of $^{99m}$Tc-MTx was observed. The scintigraphic procedure was used to evaluate the biodistribution and biokinetics of the radiopharmaceutical.

Clinical safety trial tests are essential for any drug before it is widely used. The project was approved by the Ethical Review Board and all relevant safety consideration were taken into account including the preparation of the radiopharmaceutical under sterile conditions and animal studies with appropriate monitoring for 72h. On completion of the animal studies and following publication of the findings of the study [1], we proceeded with the human studies as outlined in this paper. Normal biodistribution study in the control subject showed prompt distribution of the tracer in the blood and excretion from the kidneys and urinary bladder (Figure 3). Normal blood pool is seen in the heart and mild uptake in the liver and spleen is also appreciable but this is apparently blood pool activity only. No other nonspecific uptake was noted, which is reassuring and was the expected finding. Main route of excretion are the kidneys. Scintigraphy in the patient with breast cancer showed prompt uptake of radiotracer by the primary tumour (Figure 4) with gradual increase in the concentration of radiotracer in the tumour with maximum tumour uptake seen at 1 hour postinjection. The maximum uptake of injected dose was in the kidneys and the urinary bladder and was seen to increase with time. An important feature was the lack of non-specific uptake of $^{99m}$Tc-MTx at any other body site. The main routes of excretion of tracer were from kidneys though there is non-specific blood pool activity in the liver and spleen (see Figure 4). Our study also showed marked tumour uptake immediately after injection which increased with time. This specific uptake of $^{99m}$Tc-Mtx was also previously evidenced and reported in animals [1].

Our work is well supported by previously published studies [5-6], which demonstrated good uptake of the $^{99m}$Tc-labelled MAG3-MTx complex in breast cancer in animal models together with excretion of the radio pharmaceutical by the the kidneys. Our method appears to have a distinct advantage over some other reported animal studies where $^{99m}$Tc labelled MAG3-MTx was seen to be mainly excreted through faeces via liver [7]. The direct labelling of MTx has the advantage of predominant renal excretion, which is clinically important because non-specific hepatic uptake can cause difficulty in evaluation of liver lesions. The other advantage of the direct labelling method is the low cost of the kit and the simplified formulation.

When compared to other studies, $^{99m}$Tc-MTx has three distinct advantages including: 1) it is a tumour-specific agent which can be used for diagnostic imaging and as well as therapy and follow-up; 2) the target-to- background ratios are high with $^{99m}$Tc-MTx as the liver activity is almost near background when compared to $^{99m}$Tc-MIBI; and c) $^{99m}$Tc-MTx can also be used in monitoring response to neo-adjuvant chemotherapy in breast cancer.

$^{99m}$Tc-MTx can potentially be used not only for diagnostic imaging and tumour staging but also for follow-up studies performed for monitoring the response of chemotherapy in breast cancer in an analogous fashion to FDG-PET in lymphomas and other tumours. It is quite plausible that $^{99m}$Tc-MTx may be the first ever SPECT anti-cancer radio pharmaceutical that may be introduced in oncology for staging of breast carcinoma.

## Conclusion

We have demonstrated that the direct labelling methodology of $^{99m}$Tc-MTx is a cost effective, simple-to-perform and a time efficient method. The patient data shows sufficient uptake in tumour cells with very low uptake in normal tissues, which indicates its potential for use as an imaging agent in breast carcinoma. The subjective data from our study indicates that $^{99m}$Tc-MTX in the absence of a breast tumour behaves like blood pool agent. Renal excretion is the normal dominant route of excretion. Remarkable uptake in breast cancer tissue showed that this technetium labelled anticancer agent can be used for tumour scintigraphy. The initial results of the our study in humans with the new direct labelling method has underscored the potential of labelling and imaging of anticancer drugs with technetium for tumour imaging and monitoring the response to chemotheray. However, more work is needed to explore the full potential of this new method.

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