Preparation of $^{99m}$Tc-labeled methotrexate by a direct labeling technique as a potential diagnostic agent for breast cancer and preliminary clinical results

Abstract
Methotrexate (MTX) is being used in clinical oncology for the treatment of a wide variety of cancers. The aim of the present study was to label directly MTX with $^{99m}$Tc by using Sn/pyrophosphate as reducing agent and to use this labeled compound as a potential anticancer radiopharmaceutical for breast cancer imaging. We studied the labeling efficiency of the $^{99m}$Tc-MTX compound by paper chromatography and instant thin layer chromatography (ITLC) in acetone and saline and found it to be more than 95%. In vitro stability of labeled MTX in serum was studied up to 5h. Partition coefficient in n-octanol and saline indicated that the labeled radiopharmaceutical was hydrophilic. We then tested $^{99m}$Tc-MTX in 5 breast cancer female patients. Protein bound $^{99m}$Tc-MTX showed rapid clearance from blood. The biodistibution data suggested that $^{99m}$Tc-MTX was cleared by the kidneys and the liver. Patients’ data also showed highly significant uptake of $^{99m}$Tc-MTX in breast cancer. In conclusion, this study indicated that $^{99m}$Tc-MTX may be used as a potential diagnostic agent for breast cancer patients imaging and may show treatment efficiency in case MTX is to be used for treatment.

Introduction
Methotrexate (MTX) is a chemotherapeutic agent for breast cancer, head and neck cancer, leukemia, lymphoma, lung cancer, osteosarcoma, urine bladder cancer etc. [1]. In breast cancer the drug is being used for adjuvant chemotherapy [2]. Methotrexate allosterically inhibits dihydrofolate reductase (DHFR), an enzyme that participates in tetrahydrofolate synthesis. The affinity of methotrexate for DHFR is about one thousand-fold that of folate. Dihydrofolate reductase catalyses the conversion of dihydrofolate to active tetrahydrofolate. Folic acid is needed for the de novo synthesis of the nucleoside thymidine, required for DNA synthesis. Also, folate is needed for purine base synthesis, so purine synthesis will be inhibited. Methotrexate, therefore, inhibits the synthesis of DNA, RNA, thymidylates, and proteins having a greater toxic effect on rapidly dividing cells, such as malignant cells and myeloid cells [3].

We thought that MTX labeled with a radionuclide could be taken up by cancer cells more than by normal cells and could thus be applied to diagnose breast cancer. In a previous study we labeled MTX with technetium $^{99m}$Tc and studied its uptake in the animals’ tumors [4]. In this study we did the same labeling procedure and additionally, we also report our first potential clinical results in diagnosing breast cancer patients by $^{99m}$Tc-MTX. No similar study we were able to find in the medical literature.

Materials and methods
All chemicals used for this research were analytically derived from the following sources: Methotrexate, stannous chloride, ascorbic acid and sodium citrate were purchased from Aldrich, USA, Technetium-$^{99m}$ generator was purchased from Pakistan Institute of Nuclear Science and Technology (PINSTECH), Pakistan and saline from Ostuka, Pakistan.

Radiopharmaceutical kit; formulation of the compound
Formulation of the MTX kit was carried out by modifying the method previously published by our team [4]. Twenty mg of MTX in 18mL of double distilled water were dissolved by few drops of 1N NaOH. Then 30mg of ascorbic acid and 20mg of sodium citrate were added in the stirred solution. Two mL of stannous tartrate (5mg/mL) and (2mL) of pyrophosphate (5mg/mL) were then added with constant stirring, after pH was adjusted to pH 8.0-8.5 and a fraction of 1mL of the whole solution was dispensed in a 10mL serum vial after pass-
ing through 0.22 micrometer membrane filter. A dose of 925MBq of Na,$^{99m}$TcO$_4$ was eluted from Pakgen generator from PINSTECH was added in the vial and incubated for 15min at room temperature. No MAG3 was used.

In vitro stability of the radiopharmaceutic complex: In vitro stability of the $^{99m}$Tc-MTX complex was estimated for various intervals of time up to 5h at room temperature. Aliquots at different time intervals were applied on chromatography paper (PC) and instant thin layer chromatography (ITLC- Silica Gel) 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 of significant loss of metal-complex stability, it was not advisable to use the radiopharmaceutical for clinical applications. Free pertechnetate in the radiometal complex was calculated using PC up to 6h and was found to be about 0.258% at any time tested, which was within acceptable limits.

Safety of $^{99m}$Tc-MTX
The radiopharmaceutical kit was synthesized under sterile conditions. Laminar flow hood was sterilized with absolute alcohol 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 i.v. 100μg/kg of the $^{99m}$Tc-MTX complex every day. 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 the Institute of Nuclear Medicine and Oncology, Lahore (INMOL) Hospital, Pakistan Animal study rules, which generally follow the international rules. The $^{99m}$Tc-MTX complex was also tested in animal models using Swiss mice as mentioned before [4] and showed significant uptake in the naturally developed tumor (moderately differentiated adenocarcinoma in the lower abdomen) as compared to normal tissues, indicating that MTX was more specific in the above mentioned tumors than in normal tissues [4].

Table 1. Patients’ history

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>History</th>
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<tr>
<td>Patient 1</td>
<td>A 30y old young unmarried female with left breast, invasive ductal carcinoma (IDC) grade-III, stage II-B, tumor size of 6x7cm, was on neo-adjuvant chemotherapy. No distant metastases were observed. The patient showed mild to moderate response to FAC: 5FU, adriamycin (doxorubicin) and cyclophosphamide and additionally received neo-adjuvant radiotherapy with also mild response.</td>
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<tr>
<td>Patient 2</td>
<td>A 28y old female with right breast cancer, IDC-grade-II, tumor size was 3x2.2cm and stage IV with liver metastases under palliative treatment of chemotherapy (FACx6) also responded well to treatment with resolution of liver metastases.</td>
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<tr>
<td>Patient 3</td>
<td>A 52y old female with right breast cancer, IDC stage III-C. The patient had completed treatment in 2011 with taxotere (docetaxel), adriamycin (doxorubicin), and cyclophosphamide (TAC) chemotherapy. The patient showed recurrence at the surgical scar of mastectomy and the adjacent skin of the right arm and moderate right arm lymphedema.</td>
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<tr>
<td>Patient 4</td>
<td>A 31y old female with right breast cancer, grade III-IDC with skin involvement received five cycles of neo adjuvant chemotherapy. The breast lump of 4.1x2.2cm regressed significantly in size and surgery was planned.</td>
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posterior views, a region of interest (ROI) was drawn around
the tumor and/or metastases and the geometric mean of
these counts was considered as 100% of the injected dose
at that particular time. Regions of interest were also drawn
around the tumors of the involved breast, the kidneys, the
heart and urine bladder. Scans with positive findings were
analyzed semi-quantitatively by calculating T/NT counts of
various ROI of the 0h, 1h and 2h images (Fig. 5). Exact place-
ment of the ROI around the area of increased accumulation
of the tracer was followed by a mirroring ROI over the con-
tralateral site. Percentage of the injected dose at these time
intervals was calculated using the following formula: Per-
centage injected dose in an organ = 100× (organ counts at a
particular time)/ (total-body counts at that time).

Statistical analysis
Due to the small number of our patients no statistical results,
such as sensitivity, specificity and accuracy could be calcu-
lated. Nevertheless, correlation with the diagnostic results
from radiology and pathology was shown to be useful.

Results
Quality control
During the labeling process of MTX with \(^{99m}\text{Tc}\) some other
chemical components were formed like, reduced \(^{99m}\text{Tc-MTX},\)
free pertechnetate \((^{99m}\text{TcO}_4^-)\) and hydrolyzed \(^{99m}\text{TcO}_2\) which
were separated by PC and ITLC using acetone and saline as
the mobile phase. In PC, \(^{99m}\text{TcO}_4^-\) had an Rf of 0.8-0.9, while
the \(^{99m}\text{Tc-MTX}\) and the hydrolyzed \(^{99m}\text{TcO}_2\) appeared at
Rf=0.00-0.01. The hydrolyzed fraction was separated from
the other two fractions by using saline, in this case the
\(^{99m}\text{Tc-MTX}\) complex and the free \(^{99m}\text{TcO}_4^-\) appeared at
Rf= 0.9-1.0, and the \(^{99m}\text{TcO}_2\) was detected at Rf=0.00-0.01. The overall la-
beling yield of the \(^{99m}\text{Tc-MTX}\) complex was more than 95.0%
as shown in Figures 1 and 2.

Safety in humans
All patients remained well with no adverse
reactions after the i.v. injection of \(^{99m}\text{Tc-MTX}.\)
Patients’ blood pressure, heart, respiratory
rate and body temperature were not altered
before and at 4h post injection of \(^{99m}\text{Tc-MTX.}\)
Continuous follow-up of up to two weeks
showed no abnormal change in the clinical
status of the patients.

Dynamic and delayed images taken at vari-
ous time intervals are shown in Figure 3. Per-
centages of the injected dose in each organ
are given in Tables 2 and 3 and in Figure 4.

The above data show that the uptake of
\(^{99m}\text{Tc-MTX}\) in the primary breast tumor was
increased from 0h: 1.90%±0.53% to a maxi-
mum at 1h, of 3.13%±1.33% and decreased at
2h (3.00%±1.16%). A similar pattern of uptake
was observed in the left and right kidneys (Ta-
ble 2 and Fig. 4). Excretion of the radiolabeled
drug through kidneys and urine bladder was
noticed. Due to technicalities, measurements
were not possible at 10min intervals.

To evaluate the optimum visualization time,
target to non target ratios were also calcu-

| Table 2. Biodistribution data of \(^{99m}\text{Tc-MTX}\) in the 4 breast cancer pa-
tients (consequent data), expressed as percentage of the injected dose |
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<tbody>
<tr>
<td></td>
<td>Uptake of (^{99m}\text{Tc-MTX}) in kcts</td>
<td>Percentage of injected dose in an organ</td>
<td></td>
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<tr>
<td>Time</td>
<td>WBS</td>
<td>RB</td>
<td>LB</td>
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<td>----------------</td>
<td>----------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>0h</td>
<td>1984.0</td>
<td>34.1</td>
<td>43.7</td>
</tr>
<tr>
<td>1h</td>
<td>1364.0</td>
<td>35.0</td>
<td>59.3</td>
</tr>
<tr>
<td>2h</td>
<td>1062.0</td>
<td>33.0</td>
<td>41.0</td>
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<td>----------------</td>
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<td>----------------</td>
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<tr>
<td>0h</td>
<td>2734.0</td>
<td>36.0</td>
<td>26.0</td>
</tr>
<tr>
<td>1h</td>
<td>2224.1</td>
<td>46.0</td>
<td>36.0</td>
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<tr>
<td>2h</td>
<td>1425.3</td>
<td>32.0</td>
<td>24.0</td>
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</tr>
<tr>
<td>0h</td>
<td>2773.0</td>
<td>33.0</td>
<td>45.0</td>
</tr>
<tr>
<td>1h</td>
<td>1889.2</td>
<td>26.4</td>
<td>14.1</td>
</tr>
<tr>
<td>2h</td>
<td>1332.6</td>
<td>17.1</td>
<td>9.0</td>
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<tr>
<td>1h</td>
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<td>31.0</td>
</tr>
<tr>
<td>2h</td>
<td>1513.4</td>
<td>24.6</td>
<td>22.0</td>
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</table>

Horizontal lines separate patients 1-4. WBS: Whole body scan, RB: Right breast, LB: Left breast, RK: Right kidney, LK: Left kidney

Figure 1. Paper chromatography pattern of \(^{99m}\text{Tc-MTX}.\) Free pertechnetate moves towards the solvent front while labeled \(^{99m}\text{Tc-MTX}\) remained at the origin of the paper.

Figure 2. Instant thin layer chromatography pattern of \(^{99m}\text{Tc-MTX.}\) The hydro-
yzed form remained at the origin of ITLC and the labeled \(^{99m}\text{Tc}\) moved towards the solvent front.
Other researchers studied the uptake of MTX in animals' by using $^{18}$F-Fluoro-deoxyglucose ($^{18}$F-FDG)-MTX PET/CT and showed significant uptake in solid type of cancers [6]. Other researchers [7] have shown uptake of $^{99m}$Tc-MTX complex in animal models in breast cancer cells and excretion of the tracer through the kidneys.

Our method for $^{99m}$Tc-MTX is simpler and seems to be less expensive [7, 8]. Our data of high T/NT ratio for optimal visualization of the breast tumor at 1h, slightly differ from those of a previous study of ours using $^{99m}$Tc-5-fluorouracil ($^{99m}$Tc-5-FU), which was reported to be at 2h [9]. Imaging at 1h is more convenient in practice although it is obvious from the actual means ±standard deviations that there is no statistical difference between the values at 1h and at 2h. More measurements every 10min after emptying urine bladder are needed.

Our method was able to show a skin-scar recurrence and liver metastasis as well (Fig. 6, 7).

A follow-up $^{99m}$Tc-MTX scan performed in patient 1 who had received 6 cycles of neo-adjuvant chemotherapy and radiotherapy, two weeks before the scan showed significantly reduction in the size of breast tumor, appearing as a large centrally photopenic area in the left breast (Fig. 3 B, F and H).

It was also worth mentioning that all our patients were studied during chemotherapy, which did not seem to alter counted. The counts in T/NT tissues, i.e. in the breast tumor to the no tumor having breasts, of the 4 patients were used for the T/NT ratio. These data indicated that the mean T/NT ratio was maximum at 1h, i.e., 1.48±0.36% (Table 4 and Fig. 5).

Discussion

Our study provides original clinical evidence for $^{99m}$Tc-MTX prepared by a direct labeling method, as a possible breast cancer imaging agent. We have at present simplified the radiolabeling procedure previously used by us in an animal study [4].

Safety clinical trial tests are essential for any drug before it is widely used and our study was initially approved from the Ethical Committee of GINUM, according to related rules in Pakistan. The percentage uptake of the injected dose both by the breast tumor and the kidneys was maximum at 1h after injection of $^{99m}$Tc-MTX. $^{99m}$Tc-MTX uptake was not well detected at any other body site as shown in the whole body images except in urine bladder and some other organs like the lungs, the heart and the liver where uptake of the radiopharmaceutical was diffuse and very poor. Earlier studies with MTX also showed a preferable uptake in animals’ breast tumor cells [4, 5].

Table 3. Mean % values of the injected dose of the 4 patients in the breast tumor and the kidneys

<table>
<thead>
<tr>
<th></th>
<th>Mean % values of the injected dose</th>
<th>0h</th>
<th>1h</th>
<th>2h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td></td>
<td>1.90±0.53</td>
<td>3.13±1.33</td>
<td>3.00±1.16</td>
</tr>
<tr>
<td>RK</td>
<td></td>
<td>5.46±1.95</td>
<td>10.33±7.54</td>
<td>9.56±5.57</td>
</tr>
<tr>
<td>LK</td>
<td></td>
<td>4.93±1.00</td>
<td>10.40±5.95</td>
<td>9.76±2.89</td>
</tr>
</tbody>
</table>

RK: Right kidney, LK: Left kidney, ID: injected dose (555MBq)

Figure 3. Biodistribution of $^{99m}$Tc-MTX. Static views in patient 1. A and B. are baseline and after 1h p.i. whole body scans, C. and D. show right and left lateral views of right and left breasts, respectively of the baseline study, E. and F. show the right and left lateral views of right and left breasts, respectively at 1h p.i. and G. and H. are anterior views of the chest showing both breasts in the baseline and the 1h follow-up study.

Figure 4. Scintigraphic biodistribution of $^{99m}$Tc-MTX. The values are mean percentages of the injected dose for the right and left kidney of the 4 patients at 0h, 1h and 2h, taking whole body counts as 100% of the injected dose.
the effect of imaging by ⁹⁹mTc-MTX. Additionally, ⁹⁹mTc-MTX being a chemotherapeutic agent itself may be applied as to indicate the effect of chemotherapy.

This study is in progress in order to eliminate drawbacks such as measurements at shorter periods of time, to include a larger number of patients and do more precise measurements at various sites of the human body.

Cost effectiveness, the radiation burden of this technique and more studies comparing other techniques used for detecting breast cancer and their metastases, like PET/CT and ⁹⁹mTc-MIBI are needed.

In conclusion, the present study indicates the ability of ⁹⁹mTc-MTX as a radiopharmaceutical to diagnose not only primaries but also small metastatic lesions of the skin and the liver of patients with breast carcinoma even during chemotherapy.

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The authors declare that they have no conflicts of interest.

Bibliography