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

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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.

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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 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 $Na_2^{99m}TcO_4^-$ 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 200C° 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].

Patients' selection

Patients' data are shown in Table 1. Four female patients with breast cancer at different stages were selected for this study at Gujranwala Institute of Nuclear Medicine and Radiotherapy (GINUM). The mean age of the patients was 35y ranging from 28y-52y. All patients were under chemotherapy except patient No. 1 who had been scanned before the start and also after chemotherapy. Patients had no history of allergy. Each patient gave her written consent after being fully informed about the whole procedure. The protocol of this study was accepted by the Ethical Review Committee of GINUM, according to the legislation of Pakistan.

Study protocol: Before starting imaging studies besides clinical examination, routine blood and biochemical lab tests of all patients were examined like complete blood count (CBC), liver function tests (LFT), serum urea, and creatinine, blood pressure, blood sugar level, urine chemical and microscopic examination and also tumor receptors ER, PR and Her-2-Neu. A dose of 555MBq of ^{99m}Tc-MTX was then administered intravenously (i.v.) in 30sec to acquire dynamic images of both breasts. Scintigraphic results were co-evaluated with breast ultrasonography (USG) and X-rays mammography. Diagnosis was verified by biopsy.

Imaging protocol: The dynamic acquisition comprised of 10 frames of 60sec each. Anterior and posterior whole body images were acquired at 30, 60, and 120min, post injection (p.i.). To obtain clear visualization of the tumor, more static images were acquired at various additional positions, e.g., anterior, posterior, left lateral or right lateral. Images were recorded by using two dual head gamma cameras: ECAM by Siemens, Germany along with INFINIA® gamma camera by General Electric GE®, USA, both equipped with low-energy, all-purpose collimators. Data processing was done on ECAM workstation using ESOFT software, SYNGO™, Siemens, Germany.

Biodistribution and semi-quantitative analysis

The radiopharmaceutical drug was injected under single photon emission tomography (SPET) dual head gamma cameras INFINIA or ECAM. On the whole body anterior and

Table 1. *Patients' history*

Patient ID	History
Patient 1	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.
Patient 2	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 metasteses.
Patient 3	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.
Patient 4	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.

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 placement of the ROI around the area of increased accumulation of the tracer was followed by a mirroring ROI over the contralateral site. Percentage of the injected dose at these time intervals was calculated using the following formula: Percentage injected dose in an organ=100X (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 calculated. 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}Tc- some other chemical components were formed like, reduced ^{99m}Tc-MTX, free pertechnetate ($^{99m}TcO_4^-$) and hydrolyzed $^{99m}TcO_2$, which were separated by PC and ITLC using acetone and saline as the 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, in this case the ^{99m}Tc-MTX complex and the free $^{99m}TcO_4^-$ appeared at *Rf* = 0.9-1.0, and the $^{99m}TcO_2$ was detected at *Rf*=0.00-0.01. The overall labeling yield of the ^{99m}Tc-MTX complex was more than 95.0% as shown in Figures 1 and 2.

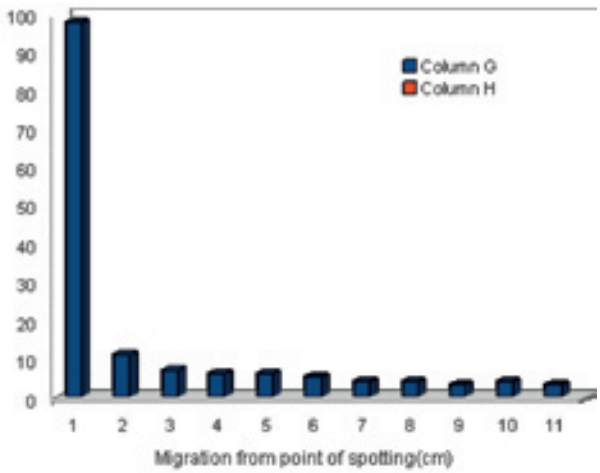

Figure 1. Paper chromatography pattern of ^{99m}Tc-MTX. Free pertechnetate moves towards the solvent front while labeled ^{99m}Tc-MTX remained at the origin of the paper.

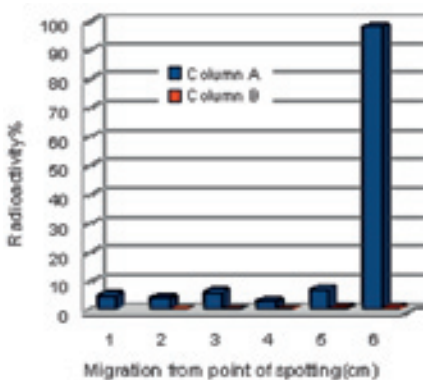

Figure 2. Instant thin layer chromatography pattern of ^{99m}Tc-MTX.The hydrolyzed form remained at the origin of ITLC and the labeled ^{99m}Tc moved towards the solvent front.

Safety in humans

All patients remained well with no adverse reactions after the i.v. injection of ^{99m}Tc-MTX. Patients' blood pressure, heart, respiratory rate and body temperature were not altered before and at 4h post injection of ^{99m}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 various time intervals are shown in Figure 3. Percentages 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}Tc-MTX in the primary breast tumor was increased from 0h: 1.90%±0.53% to a maximum 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 (Table 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 visulization time, target to non target ratios were also calcu-

Table 2. *Biodistribution data of ^{99m}Tc-MTX in the 4 breast cancer patients (consequent data), expressed as percentage of the injected dose*

	Uptake of ^{99m}Tc-MTX in kcts					Percentage of injected dose in an organ			
Time	WBS	RB	LB	RK	LK	RB	LB	RK	LK
0h	1984.0	34.1	43.7	87.7	91.1	1.7	2.2	4.4	4.6
1h	1364.0	35.0	59.3	258.1	227.3	2.6	4.3	18.9	16.7
2h	1062.0	33.0	41.0	160.0	123.0	3.1	3.9	15.1	11.6
0h	2734.0	36.0	26.0	84.0	86.0	1.3	1.0	3.1	3.1
1h	2224.1	46.0	36.0	126.0	119.0	2.1	1.6	5.7	5.4
2h	1425.3	32.0	24.0	94.9	90.2	2.2	1.7	6.7	6.3
0h	2773.0	33.0	45.0	187.0	125.0	1.2	1.6	6.7	4.5
1h	1889.2	26.4	14.1	50.4	84.7	1.4	0.7	2.7	4.5
2h	1332.6	17.1	9.0	32.4	69.1	1.3	0.7	2.4	5.2
0h	3356.5	33.6	33.6	73.3	87.6	1.0	1.0	2.2	2.6
1h	2087.3	33.7	31.0	76.9	95.2	1.6	1.5	3.7	4.6
2h	1513.4	24.6	22.0	68.2	93.9	1.6	1.5	4.5	6.2

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

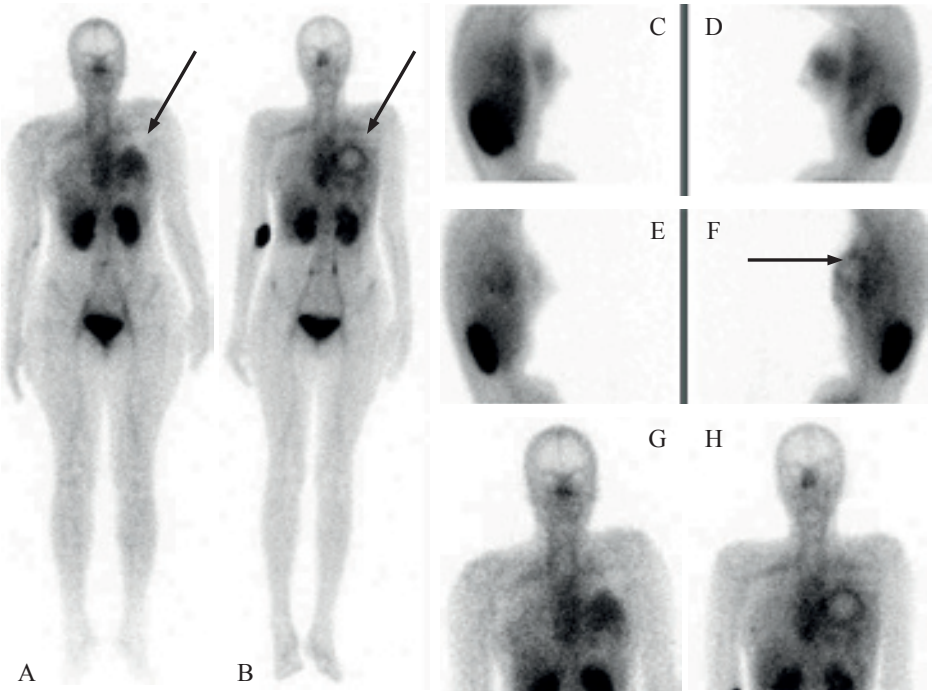

Figure 3. Biodistribution of ^{99m}Tc-MTX. Static views in patient 1. A and B. are baseline and after 1h p.i. whole body scans, C. and D. show right and left lateral views of right and left breasts, respectively of the baseline study, E. and F. show the right and left lateral views of right and left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

lated. 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*

	Mean % values of the injected dose		
	0h	1h	2h
Tumor	1.90±0.53	3.13±1.33	3.00±1.16
RK	5.46±1.95	10.33±7.54	9.56±5.57
LK	4.93±1.00	10.40±5.95	9.76±2.89

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

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

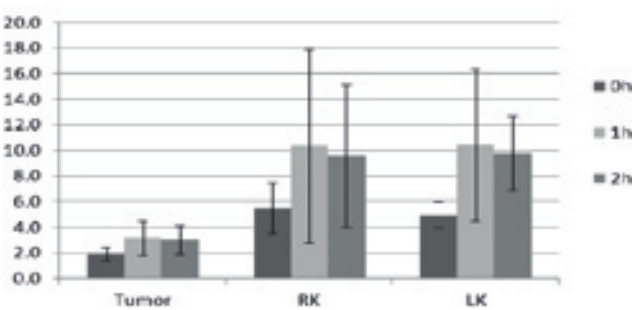

Figure 4. Scintigraphic biodistribution of ^{99m}Tc-MTX. The values are mean percentages of the injected dose for the right and left kidney of the 4 patients at 0h, 1h and 2h, taking whole body counts as 100% of the injected dose.

Table 4. *T/NT ratio at 0h, 1h and 2h*

Patient	T/NT ratio		
	0h	1h	2h
1	1.28	1.69	1.24
2	1.38	1.28	1.33
3	0.73	1.87	1.91
4	1.00	1.09	1.12
Mean±SD	1.10±0.29	1.48±0.36	1.40±0.35

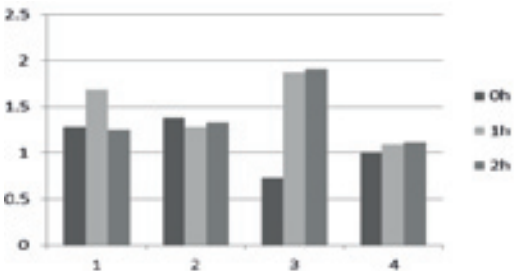

Figure 5. T/NT at 0h, 1h and 2h in the 4 patients using the uptake in the breast tumor as targeted data and while uptake in the normal breast as non-targeted data.

the effect of imaging by ^{99m}Tc-MTX. Additionally, ^{99m}Tc-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 ^{99m}Tc-MIBI are needed.

In conclusion, the present study indicates the ability of ^{99m}Tc-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.

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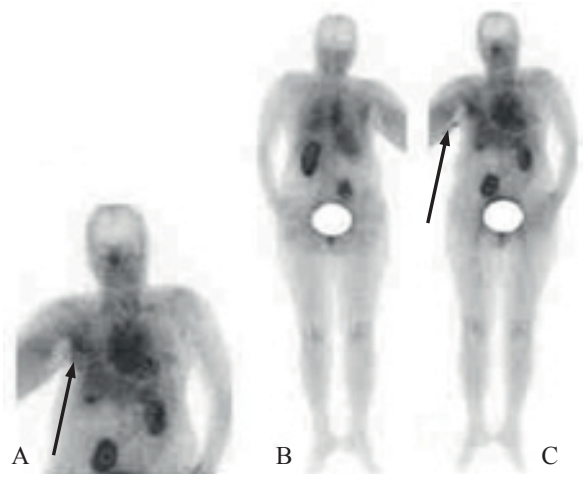

Figure 6. Biodistribution of ^{99m}Tc-MTX in patient 3 with uptake in the skin of the involved right arm surgical scar (arrows). A. and C. anterior and B. posterior whole body scans.

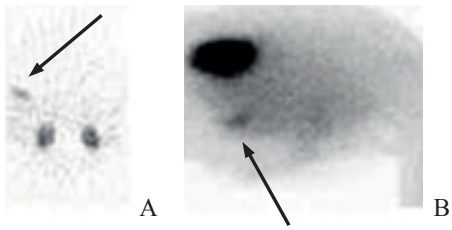

Figure 7. Patient 2 with IDC of the right breast, stage IV after surgery. Ultrasound showed liver metastases. A. SPET slices of the liver at the level of kidneys showing tracer uptake (arrow) in the metastatic focus interiorly and normal uptake in the kidneys posteriorly. B. Right lateral prone image of the liver showing focal metastatic uptake anterior to the right kidney.

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