99mTechnetium-HMPAO-labeled platelet scan in practice: Preparation, quality control, and biodistribution studies

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There is no biodistribution or imaging data on 99mtechnetium (Tc)-hexamethyl propylamine oxime (HMPAO)-labeled platelets in the literature. The current study aimed to present updated information about the clinical procedures for preparation and use of labeled platelets. Following two-step centrifugation at 1500 and 2500 rpm, the platelets were extracted from whole blood into platelet-rich plasma (PRP) above the buffy coat and then from PRP into a platelet pellet at the bottom of the tube. The 99mTc-HMPAO-labeled platelets were inspected for purity, viability, release of 99mTc from platelets, and sterility. Also, microscopic examination and thin layer chromatography (TLC) were performed. Biodistribution was assessed following necropsy in BALB/c mice and through imaging of New Zealand rabbits. The separation ratio was estimated at 98%, and radiochemical purity was measured to be 80%. The labeling efficiency was above 30% in more than half of the assays (range: 17-43%). The release of 99mTc from platelets was 9% per hour at 37ºC. After 24 hours, stability was estimated at 54% in the human serum. The target organs of mice included the spleen and liver. In rabbits, the imaging results indicated liver as the target organ. Thyroid uptake was negligible up to 90 minutes. Based on the findings, extraction of platelets and labeling them with 99mTc-HMPAO is a feasible and safe approach in routine practice.

Keywords: Platelet, 99mTc-HMPAO. Biodistribution. Labeling efficiency. Platelet extraction.
for clot detection may have clinical applications in pulmonary emboli, deep vein thrombosis, and cerebral venous sinus thrombosis, as discussed previously (Tafakhori et al., 2019). However, the biodistribution of 99mTc-HMPAO-labeled platelets has not been reported in the literature. In this study, we aimed to collect information on different methods for preparation of 99mTc-HMPAO-labeled platelets and to report quality control and biodistribution data in vivo.

There are some reports on the biodistribution of platelets, labeled with other tracers. In this study, we present further information beyond the existing knowledge (Goodwin et al., 1993, Knight et al., 2007), using one of HMPAO isomers. This study was necessary before proceeding to imaging clots in animals and humans to assess the diagnostic efficiency of labeling platelets, especially for clinical conditions, such as pseudotumor cerebri.

METHODS

The detailed information of instruments and tools in this study are summarized in Table I. The sterility of all materials and instruments, including syringes and tubes, was assured by examining the integrity of sealed packaging, according to the Good Laboratory Practice (GLP) principles. This study was approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran. All methods and procedures were performed in accordance with the international and regional ethical guidelines.

| Instruments | Specifications |
|-------------|----------------|
| Centrifuge  | Behdad and Labtron Company, Tehran, Iran (Digital Centrifuge, max: 6000 rpm) |
| Water bath  | Behdad and Labtron Company, Tehran, Iran |
| Dose calibrator | Capintec, Inc. (CRC-15R) |
| Universal hood | Labtron and Behdad Company, Tehran, Iran |

Extraction of platelets

Blood samples (51 mL) were drawn from healthy volunteers into 60 mL syringes, containing 9 mL of acid citrate dextrose (ACD) solution (Pars Isotope, Tehran, Iran), under standard skin disinfection conditions. The blood donors were adults, who were informed about the goals of the study and the possible hazards of blood donation. Informed consent was obtained from all participants. The blood samples were divided equally into two Falcon tubes (50 mL Conical Centrifuge Sterile Tubes, Maxwell, Wujiang, China) and mounted in the centrifuge. Platelet-rich plasma (PRP) was separated from whole blood at 1500 rpm (at 400 g with a shaft of 150 mm) for 15 minutes at room temperature (Rodrigues et al., 1999).

The supernatant (about 25 mL) was collected with a 2.5 mL syringe, followed by a needleless insulin syringe when necessary. It was then transferred to another Falcon tube. The pH was measured using a pH indicator paper and adjusted by adding anticoagulant citrate dextrose (ACD) in 10% increments of PRP volume to adjust pH between 6 and 6.5. Generally, pH adjustment is necessary, as platelets do not aggregate readily at pH of 6-6.5. The ACD solution had a pH of about 5-5.2 and was warmed to room temperature before use. Next, PRP was centrifuged again for 15 minutes at 2500 rpm (1050 g). The platelets formed sediments at the bottom of the tube as a platelet pellet (about 1.5-2 mL in volume). Next, the platelet-poor plasma (PPP) was removed using a syringe into a sterile Falcon tube and stored in a water bath at 37°C for later re injection.
The platelet pellet was re-suspended in 3 mL of ACD saline solution, which consisted of 2.8 mL of normal saline and 0.2 mL of ACD solution. The procedure was repeated a few times. After a few trials, the quality control parameters were examined acceptable, as described in the following sections of this paper. The causes of initial failures in the procedure included the low volume of blood samples; blood cell lysis; difficult collection of PRP after the first centrifugation due to contamination with RBCs and WBCs; lower or higher rpm or differences in centrifugation time; and variable amounts of stannous chloride in the HMPAO kit.

Pathology

The specimens for microscopic examination included direct smears and platelet suspensions. Direct smears were stained by the Wright-Giemsa staining method (Haugland, 1996). The platelet suspensions were cytocentrifuged for ten minutes at 500 g. Droplets of the platelet re-suspension in saline were smeared, and subsequently, dried and fixed in methanol; finally, they were stained via Wright-Giemsa staining for 30 minutes. The separation ratio was calculated as the ratio of platelet bulk to the total area occupied by the cells.

Preparation of 99mTc-HMPAO

For this purpose, 925 to 1110 MBq of Na\(^{99m}\)TcO\(_4\) in 2-3 mL of normal saline solution from a freshly eluted 99Mo/99mTc generator (Pars Isotope Co., Tehran, Iran) was calibrated by a dose calibrator and injected into the prepared HMPAO kit (Pars Isotope, Tehran, Iran). Radiation protection and aseptic standards were adopted, according to the manufacturer’s instructions. The HMPAO kit, which contained 0.5 mg of HMPAO, 7.6 µg of stannous chloride dihydrate, and 4.5 mg of sodium chloride, was kept in a nitrogen atmosphere. HMPAO was produced and distributed by Pars Isotope Co., Tehran, Iran (Mirahmadi et al., 2008). The solution was shaken for at least ten seconds before platelet labeling.

Labeling platelets with 99mTc-HMPAO

The suspension of platelets was injected into a vial, using a 2.5 mL syringe via dropwise addition and incubated in a water bath for 25 minutes at 37˚C. After 25 minutes of incubation to facilitate aspiration, 3 mL of autologous PPP was added. The suspension was centrifuged again for ten minutes at 1700 rpm (500 g). The labeled platelets accumulated in the pellet at the bottom of the tube (i.e., radio-labeled platelet pellet), and free 99mTc in the supernatant was removed by a syringe. The dried radiolabeled platelet pellet was re-suspended in 3 mL of autologous PPP (Avril et al., 2000). The quality control study of 99mTc-HMPAO, including thin layer chromatography (TLC), was conducted, as defined in the forthcoming section.

About 4-5 mL of the labeled platelet suspension, with an activity of 74-148 MBq, was immediately injected through an intravenous (IV) indwelling catheter in the marginal ear vein of the rabbits. To study the mice, about 0.2 mL of the final product, with an activity of 5.9 MBq, was directly injected into the lateral tail vein of mice.

Radiochemical purity of 99mTc-HMPAO

Combination of paper chromatography and TLC with silica gel (TLC-SG)

After preparing 99mTc-HMPAO, a droplet of about 5 µL was added to a TLC strip (aluminum-backed silica gel-60F\(_{254}\) plate; 10×1.5 cm) and Whatman paper (No. 1) at 1 cm above the bottom edge. After drying at room temperature, the strips and papers were placed in different solvents. The solvent movement was monitored starting at 1 cm above the bottom edge to the other end of the paper. Three types of mobile phase (solvents) and solid phases were used in this study: normal saline for running on TLC strips; a mixture of acetonitrile and normal saline (50:50) for running on Whatman paper No. 1; and 2-butanone (methyl ethyl ketone [MEK]) for running on TLC strips (Banerjee et al., 1999). Next, the papers were cut into two sections. The cutting points for normal saline, acetonitrile/normal saline, and MEK
were at 4.2, 0.8, and 1.7 cm above the origin, respectively (Pillai, 2008). The percentages of free pertechnetate (99mTcO4−), 99mTcO2, as well as primary and secondary 99mTc-HMPAO complexes, were calculated, using a well-type gamma counter.

**Quality control of platelets labeled with 99mTc-HMPAO**

**Visual examination**

The final product was inspected for visible aggregations, clots, and fibrin clumps in each step of the procedure; if detected, they were dissolved by gently rotating the suspension. Once the clot was dissolved, the product was used for re-injection, based on the WBC labeling guidelines (Erik et al., 2010).

**Labeling efficiency**

After the third round of centrifugation, the supernatant, including the free pertechnetate and unlabeled 99mTc-HMPAO, was removed by a syringe. The supernatant and the platelet pellet at the bottom of the Falcon tube were counted using a dose calibrator. The LE was calculated as the ratio of the platelet pellet activity to the sum of platelet pellet and supernatant activities. The obtained LE% was considered as the baseline LE%, and samples with LE% less than 30% were eliminated (Rodrigues et al., 1999; Martín-Comín et al., 2012).

**In vitro stability study in the human serum**

The labeled platelets with a volume of 1.5 mL were re-suspended in normal saline, and the total volume was adjusted to 5 mL. The obtained suspension was divided into five tubes. Next, 1 mL of PPP supernatant was added to each tube and incubated in a water bath at 37°C for one hour. The tube was centrifuged at 150 g for ten minutes after the first one hour (baseline) and also after the baseline (1, 2, 4, and 24 hours). The LE ratios in different tubes were calculated and compared with LE% at baseline (Shamshirian et al., 2016).

**Viability of platelets by eosin dye-exclusion test**

After extracting platelets re-suspended in normal saline, the viability of platelets was determined by the eosin dye-exclusion test. For this purpose, eosin dye entered the platelets with membrane damage. The platelet resuspension was diluted in saline by adding more normal saline (1:20 dilution). One drop of eosin (50 µL) was added to 200 µL of the platelet resuspension. The smear and platelet deposits over the plate were incubated for 30 minutes at 37°C. Next, microscopic examination was carried out for detecting clear and red platelets. This examination needs to be performed in the setup phase at any center (Erik et al., 2010; von Bruchhausen, Walter, 2012). Equation 1 was used to calculate the viability percentage:

\[
\text{Viability (\%)} = \frac{\text{Live cells}}{\text{total cells (live and dead cells)}} \times 100 \\
\text{(Equation 1)}
\]

**Release of 99mTc from platelets**

The release of 99mTc from platelets was assessed one hour after labeling. The platelet pellet was re-suspended in 3 mL of PPP (total volume up to 4 mL) and divided into four 10 mL tubes. Each tube contained 1 mL of the platelet suspension. Next, 2 mL of normal saline was added to two tubes, and 2 mL of plasma was added to the other two tubes. One tube of saline and one tube of plasma were incubated at 37°C for one hour, and one tube of saline and one tube of plasma were incubated at room temperature for one hour. After one hour, the suspensions were centrifuged at 1000 rpm (150 g) for ten minutes (Erik et al., 2010).

**Sterility and pyrogenicity tests**

Microbiological tests of sterility were conducted, according to the United States Pharmacopeia (USP) chapter 71 (USP XIX, 1975) in soybean digest medium and thioglycolate medium for 14 days at 30-35°C for bacteria and at 20-25°C for fungi. For the pyrogenicity assay, 99mTc-HMPAO was dissolved in 5 mL of normal saline. Next, 25 µL of solution was added to a Limulus
Amebocyte Lysate (LAL) kit and incubated for one hour at 37°C. Finally, the vial was inspected to assure that the final gel product was transparent.

**Biodistribution study**

The biodistribution experiments were carried out at the Nuclear Science and Technology Research Institute (NSTRI, Tehran, Iran), using eight male BALB/c mice, aged three months (weight: 20-25 g). They were kept in cool air-conditioned standard cages at 18-22°C (humidity: 30-70%) with free access to food and water. One of the mice died immediately after the injection, which might be due to the injection of an air bubble. The human platelet extract was transferred from Vali-Asr Hospital to NSTRI (maximum travel time of 20 minutes). The platelets were labeled using the same procedure described above.

A 0.2 mL suspension, consisting of 5.9 MBq of $^{99m}$Tc-HMPAO, was injected into mice via direct insulin syringe injection into the lateral tail vein. The mice were culled at 30, 60, and 120 minutes and at 24 hours post-injection by carbon dioxide asphyxiation. They were then dissected, and the organs of interest (e.g., thyroid gland, stomach, brain, liver, heart, blood, intestine, spleen, lung, kidney, bone, and muscle) were rinsed and weighed. Next, the radioactivity levels were measured using a gamma counter. The radiotracer accumulation was expressed as the percentage of injected dose per gram of tissue (ID/g%). Finally, the radio-pharmacokinetics of the tracer within the organs of interest were assessed (Mozaffari et al., 2015).

**Imaging studies**

In the final imaging stage of the study, two male rabbits, weighing 1 kg and 1.8 kg, respectively, were provided by the Animal Laboratory of the Faculty of Pharmacy (Tehran University of Medical Sciences, Tehran, Iran) and transferred to the imaging center (ten minutes of travel time). Initially, the rabbits were adapted to the environment by placing the cage under a cool surgical gown. Afterward, they were anesthetized by injection of ketamine (60 mg/kg) and xylazine (5 mg/kg) before inserting the 20F IV catheter gauge into the right marginal ear vein. The platelets (2 mL), labeled with $^{99m}$Tc-HMPAO (66.6 MBq of activity), were then injected. Next, the rabbits were transferred to the imaging room. After image acquisition, they received cefazolin sodium by intramuscular (IM) injection to prevent infection. They were then moved back to the Animal Laboratory of the Faculty of Pharmacy and monitored for ten days.

Imaging was performed by a gamma camera (AnyScan, Mediso, Budapest, Hungary) at the Department of Nuclear Medicine of Vali-Asr Hospital (Tehran University of Medical Sciences, Tehran, Iran). Five-hundred K-count static images were acquired, with the rabbits positioned in a supine position at 30, 60, and 90 minutes post-injection and also 16 hours after injection. The regions of interest were drawn over the right iliac artery, liver, spleen, heart, lungs, left kidney, and bladder. The uptake in these organs (count per pixel) was standardized for the target organ and illustrated as graphs, with the area under the ROC curve (AUC) indicating the accumulated dose within the organ.

**Statistical analysis**

Data were entered in Microsoft Excel (MS Office, 2016). Mean, standard deviation, and AUC for the accumulated doses were calculated, using Excel functions.

**RESULTS**

**Pathological examination**

Upon microscopic examination of both direct and cyt centrifuged smears, a large number of platelets were identified with a rare scattered population of lymphocytes in the background (3-4 lymphocytes per high power field), while no RBC was seen (Figure 1). More than 98% of the stained compounds composed of platelets (separation ratio >98%).
Quality control

TLC

The percentages of $^{99m}$Tc pertechnetate, $^{99m}$TcO$_2$\textsuperscript{2-}, secondary $^{99m}$Tc-HMPAO complexes (impurities), and primary complex (lipophilic complex) were 10.2%, 0.8%, 8.7%, and 80.3%, respectively. In another assay, the corresponding values were 5.5%, 1.9%, 8.8%, and 83.8%, respectively.

Visual inspection

Out of three final products, a visible clot was detected in one sample. We did not exclude this sample in our preclinical study; nevertheless, samples with visible clots should not be accepted for microscopic quality control and clinical use.

Labeling efficiency

After solving the third centrifuge platelet pellet in PPP, LE% was measured to be 56% after one hour of incubation in a water bath at baseline, which reduced to 55.7%, 55.5%, 55.3%, and 54.0% after 1, 2, 4, and 24 hours, respectively; the experiment was conducted on a sample.

Viability of platelets based on eosin dye-exclusion test

The percentages of viable platelets were 95% and 97% in two assays, respectively. The microscopic examination revealed no aggregation of platelets.

FIGURE 1 - The pathological examination of the final platelet suspension stained by the Wright-Giemsa method.
Release of $^{99m}$Tc from platelets

The leak ratios of $^{99m}$Tc from platelets in the plasma and normal saline media were 9% and 11% per hour at 37°C, respectively. These ratios for the plasma and normal saline media were 10% and 13% per hour at room temperature, respectively. For assessments, a portion of a single sample of labeled platelets was used.

Sterility and pyrogenicity tests

After 14 days, neither bacterial nor fungal growth was seen in the samples. The clear gel of LAL kit was inspected to assure the non-pyrogenicity of $^{99m}$Tc-HMPAO. Sterility and pyrogenicity tests, as described in this section, were not performed on the final product.

Biodistribution studies

The maximum activity per organ weight was detected in the liver after 30 minutes and in the spleen at 1 and 2 hours post-injection. The spleen and liver were the main target organs for the labeled platelets during the study (Table II and Figure 2).

| Organs   | 30 minutes | 1 hour | 2 hours | 24 hours |
|----------|------------|--------|---------|----------|
| Blood    | 3.70±0.13  | 3.70±0.56 | 0.87±0.01 | 0.26±0.01 |
| Heart    | 2.15±0.04  | 1.95±0.12 | 0.61±0.04 | 0.00±0.00 |
| Lung     | 2.60±0.08  | 6.20±0.28 | 1.57±0.10 | 0.26±0.06 |
| Liver    | 50.93±2.08 | 43.16±2.18 | 10.23±2.02 | 8.97±0.57 |
| Spleen   | 10.87±1.03 | 78.98±3.01 | 45.69±3.05 | 3.80±0.15 |
| Stomach  | 2.25±0.15  | 0.52±0.05 | 0.74±0.02 | 0.12±0.01 |
| Intestines | 1.89±0.05 | 3.98±0.67 | 1.95±0.28 | 0.45±0.01 |
| Kidneys  | 6.35±0.96  | 9.68±0.99 | 5.69±0.37 | 2.41±0.23 |
| Muscle   | 0.29±0.01  | 0.38±0.07 | 0.04±0.01 | 0.00±0.00 |
| Bone     | 0.42±0.02  | 0.95±0.12 | 0.93±0.05 | 0.13±0.02 |
| Thyroid  | 1.29±0.06  | 5.20±1.56 | 0.29±0.01 | 0.11±0.01 |
| Brain    | 0.08±0.01  | 0.14±0.05 | 0.01±0.00 | 0.00±0.00 |
A typical image of an injected rabbit after 90 minutes is presented in Figure 2. The uptake in the thyroid, salivary glands, and stomach was negligible, and the presence of free $^{99m}$Tc was found to be low. The liver was the target organ with the highest uptake, followed by the lungs, heart, spleen, and kidneys (Figure 3). Other organs and background activity were visualized negligibly. The rabbits appeared to do well during the ten-day follow-up.
FIGURE 3 - The activity of target organs at different intervals (30, 60, and 90 minutes and 16 hours) (a) and the accumulated activity over 16 hours after injection (b).

DISCUSSION

In the current study, a procedure was defined for labeling platelets with $^{99m}$Tc-HMPAO. Although there are some reports concerning the biodistribution of platelets, labeled by other tracers (Goodwin et al., 1993, Knight et al., 2007), in this study, we reported the biodistribution of $^{99m}$Tc-HMPAO-labeled platelets in mice and rabbits for the first time. Theoretically, many clinical applications are considered for platelet imaging. The sophisticated procedure of extracting platelets, quality control concerns, and clinical risk of using clotted solutions hinder the routine application of labeled platelet scans. Clot formation is generally a major concern, which can make the use of samples clinically impractical. The extraction of platelets and their separation from WBCs and RBCs are essential due to different physiological uptake sites of these cells (Danberry et al., 2015).
RBCs are denser than WBCs and platelets. After the first round of centrifugation, the buffy coat and PRP comprise WBCs and platelets, respectively, over the sedimented RBCs. With a higher centrifugation speed of PRP, the platelets were sedimented at the bottom of the tube, forming the platelet pellet. This platelet pellet was used for labeling and generating the injectable labeled platelet suspension (Becker et al., 1987). In this study, we assessed the feasibility of our proposed method, especially the centrifugation speed, gravity, and ratio of solutions used to label platelets efficiently with the $^{99m}$Tc-HMPAO complex. The efficiency of labeling, viability of platelets, their stability in the plasma up to 24 hours, and the release rate of $^{99m}$Tc from platelets were assessed, based on quality control standards.

The imaging and biodistribution studies revealed that the main target organs of labeled platelets were the liver and spleen. The low amounts of activity in the thyroid, salivary glands, and stomach indicated the low ratio of free pertechnetate. Regarding the trends of thyroid uptake, peak thyroid uptake at one hour corresponds to the general uptake pattern in different organs; therefore, it cannot be used as an indicator of either blood pool-related activity or the presence of free pertechnetate. Also, secretions from the kidney parenchyma and the bladder were acceptably low. The intestinal activity was even lower than the urinary activity, which indicates the low release of $^{99m}$Tc from the platelets.

The results of TLC revealed that the colloid, hydrophilic HMPAO, and free pertechnetate components of $^{99m}$Tc-HMPAO were below the standard thresholds. Since TLC was not applicable for the labeled platelets, the colloid content of the injected final product or the level of colloid formation after injection cannot be directly estimated. During this study, it was frequently observed that the labeling efficiency of platelets decreased periodically, which might be due to the use of older pre-prepared HMPAO kits during the study. The reduced stannous chloride was gradually oxidized. We tried to replace stannous chloride; however, the kit was highly prone to pH changes. Generally, the addition of stannous chloride reduces pH below the acceptable range (9-9.8); therefore, the kit would not be applicable anymore. For the purpose of platelet labeling, HMPAO should be produced and used freshly. The radiochemical purity of $^{99m}$Tc-HMPAO, according to a study by Decristoforo et al. (2000), should be above 80%. In our study, HMPAO was produced three times, with intervals of about three months, and the results indicated acceptable radiochemical purity (>80%) for $^{99m}$Tc-HMPAO.

Platelet imaging can be theoretically used in some important clinical settings, such as assessment of thrombotic events in the extremities and cerebral vasculature, besides detection of a migrating thrombosis, including pulmonary thromboembolism, from deep vein thrombosis (Vorne et al., 1993). The particular applications of platelet imaging can be related to pathologies with underlying vasculitis and clotting etiologies, including transplant rejections. We used this scan for the detection of cerebral venous sinus thrombosis as a differential etiology of pseudotumor cerebri (Tafakhori et al., 2019). We hope that labeled platelets can be integrated into routine nuclear medicine practice and that our results can facilitate the use of this method for the abovementioned indications. Also, sterility and clot formations are major concerns to be addressed. In future studies, the biodistribution data of labeled platelets with $^{18}$F-FDG products may provide additional information, including standard uptake value (SUV) data that we could not obtain.

**CONCLUSION**

In conclusion, extracting and labeling platelets with $^{99m}$Tc-HMPAO is a feasible and safe approach in routine medical practice. However, further studies are needed to evaluate this approach in different clinical settings.

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Not applicable.

**DECLARATION OF INTERESTS**

The authors declare no competing financial interests.
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