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Research Article

Red blood cells labelling with ^{99m}Tc-d,l-HMPAO: an alternative method for specific cases

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Summary

The effectiveness of the classic methods for red blood cells (RBCs) labelling with ^{99m}Tc has been demonstrated in nuclear medicine. However, nuclear physicians have found, in certain circumstances, this diagnostic technique fails and poor quality images are obtained. In this work we report on an alternative method that is of useful in these occasions, for *in vitro* labelling RBCs with ^{99m}Tc-d,l-HMPAO complex. The study shows a high and reproducible labelling efficiency (94.14 ± 0.38), using low amount of tin. The RBCs were isolated from plasma and other interfering blood cells before adding ^{99m}Tc-d,l-HMPAO. The tracer was retained and the elution rate from RBCs was low (less than 6% after 120 min). The preclinical results indicate that this new method could be a good alternative to the standard classic methods for specific cases.

Key Words: red blood cells labelling; ^{99m}Tc-d,l-HMPAO RBC; ^{99m}Tc lipophilic complex; ^{99m}Tc-RBC and blood pool imaging

Introduction

Among the different classic methods of labelling red blood cells (RBCs), pretinning is the most commonly used. Labelling may be done *in vitro*,

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in vivo, or by a combined *in vivo*/*in vitro* (*in vivitro*) technique.[1–3] These classic techniques have proven to be effective and useful for over 20 years. The main aim of these techniques is to obtain images which are adequate for clinical purpose with minimum potential risk to the patient. However, nuclear physicians have argued and found that, in certain circumstances, these diagnostic techniques fail and values of lower quality have been obtained. Some drawbacks are directly related to the technique as well as other factors which can limit their applications.[4–6] In *in vivo* and *in vivitro* methods, the patient is given a large quantity of tin salt intravenously. The tin ion is considered toxic and adverse reactions have been noted. In gated blood-pool studies, carried out routinely by labelling *in vivo* and *in vivitro*, it is frequent to find labelling efficiency as low as 40–60%. The variability and lower labelling efficiency can be attributed to the interaction with certain drugs . This implies poor quantification and lower quality of images. In the classic methods, RBCs are labelled randomly and other cells are labelled, such as leukocytes, that can cause a decrease in sensitivity and specificity in studies of digestive hemorrhages of low flow.

Taking into consideration the above-mentioned disadvantages, Martti Vorne and Risto Laitinen[7] proposed a new method for labelling RBCs *in vitro* with the lipophilic ^{99m}Tc-d,l-HMPAO complex (HMPAO: Exametazime, Ceretec^{TM}) with clinical applications in humans to detect acute gastrointestinal bleeding. This technique did not present satisfactory results in labelling efficiency, variability and elution rate.

In this article, we propose a new method of labelling RBCs, using the same ^{99m}Tc-d,l-HMPAO complex. Preliminary data suggest that it could be a good alternative to the classic methods for specific clinical cases.

Materials and methods

Formulation of ^{99m}Tc-d,l-HMPAO complex

Fresh pertechnetate eluates were obtained from a ^{99}Mo/^{99m}Tc generator (Ultra-Technekow FM) to ensure high labelling of HMPAO ligand.[5] The ^{99m}Tc-d,l-HMPAO complex was prepared from a commercial Ceretec Kit using two methods. (1) ''Standard method'' (Ceretec 0 h): Four vials were labelled according to prospectus norms. The interval of

activity of ^{99m}Tc-HMPAO added to RBCs was 277.5–555 MBq (7.5–15 mCi), contained in a volume of 4.5 ml. (2) 'Aliquots method': The Ceretec vials were reconstituted previously with 2 ml of physiological saline solution. No stabilizer agent has been added to the vials. One milliliter of this solution was withdrawn and deposited in a vacuum vial used to elute the generator. Four aliquots (1 ml) were labelled after 1 h (Ceretec 1 h) with the desired activity of pertechnetate in 4 ml of saline isotonic solution. The range of activity of ^{99m}Tc-d,l-HMPAO used for the labelling of RBCs was 555–722 Mbq (15–19 mCi) in 4.5 ml of the radioactive solution. Another 5 aliquots (1 ml) were labelled after 2 h (Ceretec 2 h) and the interval of activity used to label the red cellular suspension was 500–566.1 MBq (13.5–15.3 mCi) of ^{99m}Tc-d,l-HMPAO in 4.5 ml. The radiochemical purity was determined, according to Neirinckx *et al.*[8]

RBC labelling method with ^{99m}Tc-d,l-HMPAO

This labelling method was carried out on 13 healthy volunteers as follows: 10 ml of venous whole blood was drawn from each volunteer by a syringe with 2 ml of acid-citrate-dextrose (ACD). The first wash was carried out by adding 5 ml of physiological saline solution to the tube which was gently swirled and then centrifuged for 6 min at 100 *g*. The content was separated into 3 layers: the top or supernatant (TL-1), the intermediate (IL-1) and the bottom layer (BL-1) with the pellet of RBCs. The TL-1 and IL-1 were extracted and reserved at 37°C. A second wash, under the same conditions, was carried out on the BL-1. The TL-2 and IL-2 layers were also extracted and reserved at 37°C. Labelling was carried out on the remaining RBCs, BL-2. Red cells were incubated with ^{99m}Tc-d,l-HMPAO for 10 min at 37°C. During this period, the tube was gently swirled various times. ^{99m}Tc-d,l-HMPAO was prepared as described previously (see section on formulation). In four volunteers, Ceretec 0 h was used, in another four, Ceretec 1 h and in the remaining five, Ceretec 2 h. Afterwards, the tube was centrifuged for 6 min at 500 *g*, and the RBCs-bound radioactivity and free radioactivity were determined.

Hematologic analysis

The test was carried out on a healthy patient three times in a period of two weeks. The blood, with ACD, before subjecting it to any

manipulation was the control. A blood test was done during the different steps of the procedure until the injectable formula was ready. Information such as population, size, HGB, HCT, MCV, was obtained with an automated hematologic instrument. All of the pellets were observed by an optical microscope.

Retention of ^{99m}Tc-d,l-HMPAO in red cells after consecutive washes with saline

The ability to hold ^{99m}Tc-d,l-HMPAO was tested in four labelled pellets with activities of 221.6 (Ceretec 0 h), 481 (Ceretec 1 h), 342.9 (Ceretec 2 h) and 358.5 MBq (Ceretec 2 h). Cells were resuspended with 10 ml of saline solution and gently swirled for 1 min. The tubes were then centrifuged for 6 min at 625 *g*, and the cells-associated radioactivity fraction and free radioactivity in the saline medium were determined in a dose calibrator. This washing procedure was repeated at 30, 90 and 120 min. The cells were incubated during the trial at 37°C.

Elution of ^{99m}Tc-d,l-HMPAO red cells in a mixture of plasma and saline solution

Two labelled pellets of RBCs with Ceretec 1 h (599.4 and 603.1 MBq) were reconstituted with their corresponding plasma and saline solution which were obtained from the first and second wash (TL-1 and TL-2), previously incubated at 37°C. The cell suspension was placed in a 37°C incubator for 23 h and 35 min and was occasionally swirled gently. One-milliliter samples were collected at different time intervals and deposited into a test tube. The tube was centrifuged for 6 min at 626 *g*. Cell-bound radioactivity and activity in the supernatant medium were then determined in a dose calibrator.

Elution of ^{99m}Tc-d,l-HMPAO red cells in plasma

Twenty milliliters of venous whole blood, from two normal volunteers, was drawn with a syringe (4 ml ACD). Half of the blood sample (10 ml) was labelled with Ceretec 2 h. Cell-free plasma, from the unlabelled half of the blood sample (10 ml), was separated by centrifugation for 6 min at 500 *g* and placed in a 37°C incubator and reserved. The labelled autologous red cells, with activities of 481 and 488.4 MB, were resuspended in this cell-free plasma and incubated at 37°C for 24 h.

One-milliliter samples were collected at different time intervals and the same steps as above were followed.

Distribution and images of radiolabel pellet treated by a leukocytes isolator

Three pellets of red cells were labelled with Ceretec 0 h, 1 h and 2 h, and the activities were 617.9, 617.9 and 573.5 MB, respectively. An isolator agent of leukocytes, 7 ml of 6% hydroxyethyl starch (Hes Grifols), was added to the labelled pellet. The suspension of cells was swirled gently for 1 min and it was left to settle for 3 h. Once this time period transpired, scintigraphy images were taken from the tube. As soon as the image was acquired, 300 µl of a hemolytic solution of acetic acid was added to the tube and it was then centrifuged at 156 *g* for 6 min. Afterwards, a new image of the tube with the cells hemolysated was acquired.

Results

Quality control of ^{99m}Tc-d,l-HMPAO

The percentage of purity of the primary complex was as follows: 93.5% in Ceretec 0 h ($n=4$), 92% in Ceretec 1 h ($n=4$) and 93% in Ceretec 2 h ($n=5$).

Labelling efficiency of red blood cells

The efficiency of red blood cells (ERBCs) mean of the 13 trials was high and reproducible, 94.33 ± 0.46 (mean $\pm$ s.d.). When the standard method to labell Ceretec ($n=4$) was used, the quantity of Cl_2Sn added to the cells was 5.8 µg and a labelling yield of 94.74 ± 0.36 was obtained. When the aliquots of the Ceretec solutions method ($n=9$) were employed, the quantity of Cl_2Sn was decreased to half the amount (2.8 µg). An efficiency of 94.14 ± 0.38 was obtained by this method, despite the low quantity of tin. With respect to the lapse of time, from the reconstitution of the Ceretec until it was radiolabelled with ^{99m}Tc (0, 1 and 2 h), there were not any significant differences in the ERBCs.

Hematology

The data in Figures 1 and 2 represent the analytical mean values from the hematologic report of the patient who underwent the test three times in a period of two weeks.

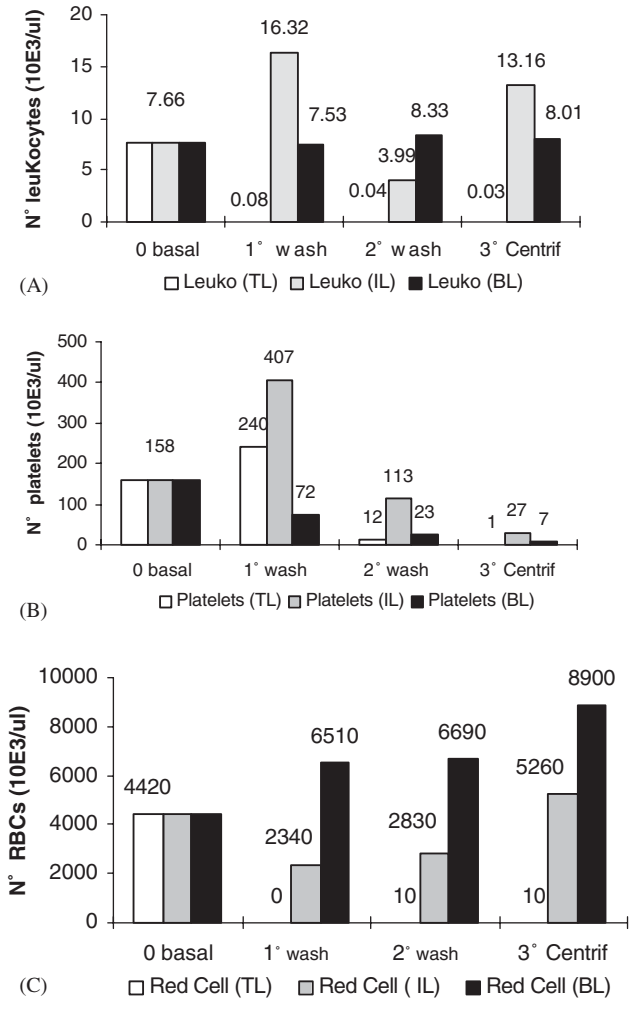

Figure 1. Number of leukocytes (A), platelets (B) and red cells (C) found per microliter in the different layers formed during the manipulation of the blood until the pellet of red cells is obtained. 0 basal: whole blood with ACD before manipulations, TL: top layer, IL: intermediate layer and BL: bottom layer

The main process of elimination of leukocytes was done in the intermediate layer (Figure 1A). The rate of washing of the intermediate layer was higher than the supernatant by 204, 99 and 438 times in the first wash, second wash and in the final centrifugation, respectively. During the process of washing, platelets were also

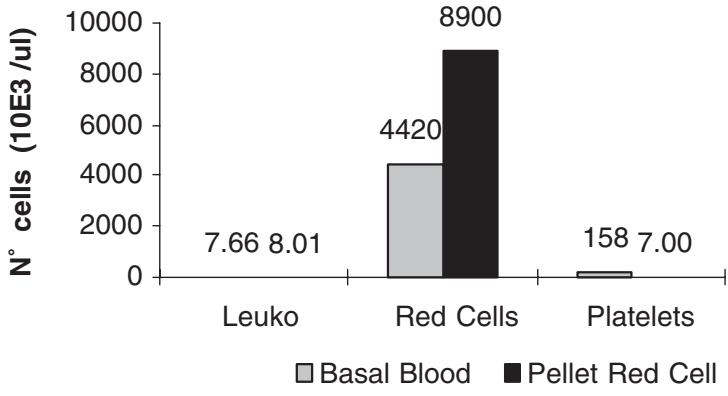

Figure 2. Type and number of cells per microliter in basal blood (With ACD) and in the pellet of red cells without reconstituting

eliminated (Figure 1B). The process occurred in the intermediate and supernatant layers and TL/IL in the first, second and third wash were, respectively, 1.99, 9.4 and 27.

Figure 2 shows that there existed a gain of red cells in the pellet with respect to leukocytes and platelets 1.92 and 45.6, respectively. When the pellet of red cells, with a volume of 4 ml, was reconstituted with physiological saline solution to a final volume of 10 ml for its reinjection, the cellular concentration per microliter of resuspended injection was 2.5 times lower than that of the hematologic report. As a result, the percentage of the different types of cells remained as follows: 80.5% for red cells, 41.8% for leukocytes and only 1.8% for platelets.

The percentage of leukocytes was modified during the washing process. In basal blood, the percentage was 50.8% neutrophils, 38.5% lymphocytes and 5.4% monocytes and the corresponding percentage in the pellet were 80.4%, 13.1% and 2.9%. The relation of neutrophils/lymphocytes increased in the pellet from 1.3 to 6.1. Plasma was eliminated during the procedure with two washes.

The results obtained, with an automated hematologic instrument and visual inspection, suggested that the labelling operation did not damage the RBCs significantly.

Distribution and images of radioactivity in the pellet

Figure 3 shows the radiodistribution of a labelled pellet of RBCs which was resuspended with an isolator agent of cells and allowed to deposit

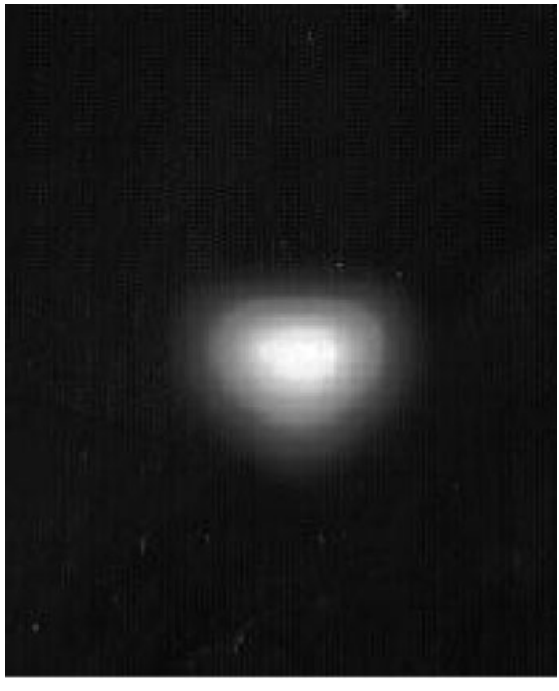

Figure 3. Image of a pellet of RBC (342.9 MBq) labelled with reconstituted Ceretec 2 h and allowing for sedimentation for 3 h with an isolator and differentiator of cells

for 3 h. All of the radioactivity was bound to red cells pelleted to the bottom; however, the image did not show other cell-band radioactivities in the supernatant medium. On the other hand, there was no release of ^{99m}Tc-d,l-HMPAO from red cells to the medium; therefore, the binding of complex radioactivity with red cells was irreversible, even after a prolonged period. Our protocol, of labelling, isolated red cells from other types of cells and did not cause hemolysis.

To prove that the radioactivity was bound to undamaged red cells, a hemolytic agent was added to the above tube. Figure 4 shows that hemolysis had occurred and the radioactivity was freed in all mediums of the tube.

Washing trial in saline solution

The results, obtained in the washing trial with saline ($n = 4$), demonstrated that 95.3–98.6% of ^{99m}Tc-d,l-HMPAO in the initial partition was irreversibly retained.

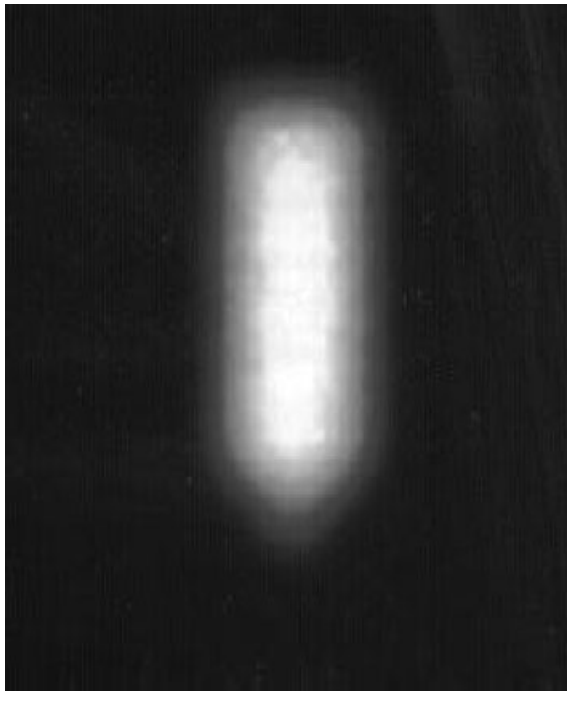

Figure 4. Gammagraphic image obtained after adding the hemolytic reactive to the tube of Figure 3

Elution in a saline solution and plasma mixture. Elution in plasma

To know what medium is better to resuspend the cells in the radiolabelled pellet was resuspended in saline solution–plasma mixture and with pure plasma. The elution rate, in the initial 10–60 min, was lower than 5% in the medium mixture and about 3% in pure plasma. The percentage of ^{99m}Tc-d,l-HMPAO, in the plasma-saline mixture and in the plasma fraction, was about 4–5% at 90 min of incubation and approximately 10–12% at 180 min (Figure 5).

Discussion

Choice of anticoagulant

The choice of the anticoagulant, used for collecting the blood samples, was important because it could interfere in the labelling process. Experiments showed[9–12] that the use of (ACD) provided more efficient

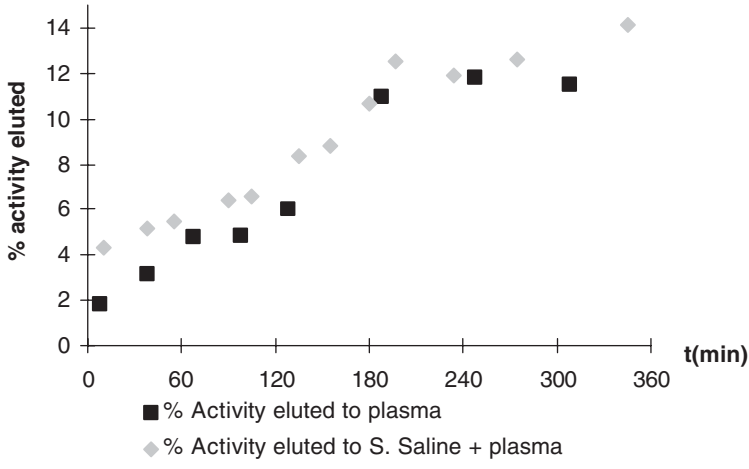

Figure 5. Percentage of eluted activity from RBCs to the different studied extrahematic mediums

labelling than the use of heparin. It was also found that ^{99m}Tc could form a complex with heparin in the presence of tin and this complex undergoes renal clearance and raises urinary bladder activity. We selected, ACD instead of heparin to avoid this drawback.

Remove plasma before adding ^{99m}Tc-d,l-HMPAO

The percentage of the primary complex, ^{99m}Tc-d,l-HMPAO, was higher than 90%, immediately after complex formation, throughout our experiments. It is well known that the primary complex presents a high kinetic of conversion to a secondary less lipophilic complex with a lower capacity of penetration through the cellular membranes. It was necessary to preserve, in our protocol, the primary complex. It was demonstrated[13] that the primary complex converted to the secondary one very rapidly in the presence of plasma and that the plasmatic fraction retained between 40 and 80% of the complex with respect to the red cells. A similar result was obtained by Vorne and Laitinen.[7] Considering the above, we believe that the removal of plasma, before adding ^{99m}Tc-d,l-HMPAO, improves the results, given that: (a) it guarantees greater stability of the primary complex and (b) the activity of ^{99m}Tc-d,l-HMPAO bound to proteins is lower.

The procedure reported by Vorne and Laitinen,[7] who have radiolabelled RBC in a plasma medium, gave poor labelling efficiency

(47%) and was irreproducible (32–64%). In contrast, the data from our study shows higher labelling yields (94%) and is reproducible (93.6–94.9%). These results suggest that the labelling operation without plasma gives better labelling yields and a lower degree of variability. The explanation may be that the undesirable 'plasma effect' is avoided. Furthermore, many commonly used medications have been shown to alter RBC tagging.[14–18] If we consider that, the main biological carrier of drugs are plasmatic proteins, it is conceivable to conclude that radiolabelled RBC without plasma could decrease these possible interferences.

It is not necessary to pretreat the RBC with tin

The tin ion is considered toxic and has been proven to cause adverse reactions in patients. The toxicity depends upon the doses. Another fact is that tin remains in the organism for a long time, more than 3 months.[19,20] Therefore, if the patient has to undergo an ulterior exploration with pertechnetate, during this time period, the tracer could suffer an anomalous intravascular retention and an abnormal biodistribution. In both cases, it is necessary to minimize the quantity of tin to be administered to the patient.

The pertechnetate ion freely diffuses in and out of the erythrocytes. So, all the classic methods need to use a pretreatment technique that involves incubation of either whole blood in the patient or previously separated RBC with a reducing agent containing tin before labelling with pertechnetate.[21–23] The reaction is done in blood.

In *in vivo* and *in vitro* methods, the dose injected into the patient (depending upon the patient's weight) can be between 750–2200 μg of stannous salt.[24,25,5] The net Kit method, for the labelling of RBCs in whole blood *in vitro*, contains between 2 and 50 μg of tin. In order to obtain a high tagging efficiency it is necessary to use a chelating agent, such as EDTA, and an oxidant agent, such as, NaClO.[6,3,23]

Blood contains competitive elements for the tracer as well as for tin. However, we previously formulated the neutral lipophilic complex, ^{99m}Tc-d,l-HMPAO, and not in the blood. These subtle differences in the procedure and the use of a neutral compound, that can pass easily through the cells, permit the administration of smaller quantities of tin, between 2.8 and 5.8 μg ,and produce a high ERBC. We believe that the amount of tin administered could be further decreased.

Hematological considerations

The data from previous work,[26,7] showed that the affinity of ^{99m}Tc-d, l-HMPAO to whole blood was 40% for plasma and platelets, 8% for mononuclear cells and 5% for granulocytes. If these were reduced or eliminated, prior to the addition of ^{99m}Tc-d,l-HMPAO, more activity would remain for the target red cells. This also has implications from a clinical point of view. In the conventional methods, the red cell labelling is done in whole blood and is random; therefore, leukocytes are also labelled, among other cells, which tend to be uptaken by bone marrow.[26–28] The accumulation of these cells can cause a false positive-negative diagnostics in suspected gastrointestinal bleeding.

In our new method, almost all platelets and more than half of the leukocytes, from whole blood, were eliminated by a simple washing procedure with saline solutions without having to add foreign substances, like polypred, which can cause adverse reactions.[29] The probability of making a diagnostic error in gastrointestinal hemorrhages would be lower.

Elution trial

In general, the RBC radiopharmaceutical labelled with ^{99m}Tc is considered ideal for diagnostic nuclear medicine applications. When a high ERBC is obtained, the radionuclide is tagged firmly and is not released from the cells.

The method proposed by Vorne and Laitinen[7] gave a high rate of elution. At 30 min, it was 7–8%, at 60 min, 10% and between 2.5 and 3 h, about 20%. The same data was reported by Danpure *et al.*[30] who obtained a value of 15% after 120 min of incubation. In our protocol, the retention of ^{99m}Tc-d,l-HMPAO in red cells was of 95.3–98.6%, indicating that the complex was irreversibly fixed to red cells. Similar results were obtained by Nakamura *et al.*[13] Furthermore, the results obtained from the elution rate test, in both mediums used in the present study, represented half of the values found by the above authors. At 30 min, it was 3–4%, at 60 min, 5%, and between 2.5–3 hours, lower than 11%.

Theoretically, a technique is preferable if it provides higher ERBC and less elution of the tracer from the cells. Given the ability of the image/quantitative measurement to demonstrate disease and to delineate anatomical structures, detection, differential diagnosis and

localization will be improved. Thus, the preclinical data suggest that this protocol has fewer limitations. However, it must be evaluated *in vivo* in order to prove its clinical usefulness.

Conclusion

From a technical point of view, the preclinical results obtained, with the new method of RBCs labelling with the ^{99m}Tc-d,l-HMPAO complex, are optimal. A high ERBC was obtained, a lower amount of tin was employed, plasma and other interfering cells were eliminated, irreversibility in the labelling and a low elution rate were achieved.

These technical parameters indicate that the new method could serve as a useful tool for digestive hemorrhages or other blood pool studies in which a high-quality diagnosis is needed. It may be useful when pharmaceutical interferences or sensitivity to the tin ion exists.

The method is not adequate in routine practice considering its elevated cost. But, for specific cases, as mentioned in this study, it could be a good alternative to the classic methods given that its benefits override cost considerations.

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