Fluorescent microspheres are superior to radioactive microspheres in chronic blood flow measurements

MATTHIJ F. M. VAN OOSTERHOUT, FRITS W. PRINZEN, S. SAKURADA, ROBB W. GLENNY, AND J. ROBERT S. HALES

Fluorescent microspheres are superior to radioactive microspheres in chronic blood flow measurements. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H110–H115, 1998.—The accuracy of the fluorescent (FM) and radioactive microsphere (RM) techniques is similar in acute experiments but has not been established in chronic experiments. In the present study various combinations at least pairs of FM and/or RM labels were injected simultaneously between 2 mo and 5 min before each animal was killed. Blood flow was determined in many organs. Intramethod mean difference and variation did not change over time for FM but increased significantly for RM (from 1.8 ± 1.4 to 25.6 ± 21.8% and from 4.4 ± 3.2 to 32.4 ± 23.0% at 5 min and 2 mo, respectively). Also the FM-RM (from 1.8 to 6.5) techniques is similar in acute experiments but has not been validated to a limited extent and because FM may offer the various benefits mentioned above, we compared the validity of the FM and RM techniques in chronic experiments. Two of the factors determining the accuracy of the microsphere method (5, 10, 13) deserve special emphasis in chronic experiments, i.e., the absence of both leaching of the label from the microspheres and disappearance of microspheres from the site of entrapment (10). In a study on leaching of radioactive microspheres from the heart, Consigny et al. (3) showed that microspheres appearing in the venous circulation are almost exclusively smaller than 12 µm in diameter. A similar conclusion was reached by a study of Medvedev et al. (12), who determined the content of 46Sc-labeled spheres with a diameter of 15 µm in donor hearts and in lungs of the recipients. The latter study also indicated some leaching of 46Sc from the beads. Losses of 125I (7) and 113Sn have also been reported (9).

The present experiments were performed in rabbits. Various combinations at least pairs of FM and RM labels were injected simultaneously at 2 mo, 1 mo, and 5 min before the animal was killed. The accuracy of the RM and FM methods was evaluated by quantification of the variation and mean difference of blood flow values between RM and FM and between different labels of RM as well as of FM in a large number of organs. Because RM lost label within 1 mo, a second set of animals was studied to compare the various isotopes in experiments lasting 1, 7, and 14 days.

THE FLUORESCENT MICROSPHERES (FM) technique has been demonstrated to be an excellent alternative to the radioactive microspheres (RM) technique in acute experiments (2, 4, 14). The accuracy of the FM technique is comparable to that of its radioactive counterpart, and although the FM method is more time consuming, the lack of radiation for workers and the environment makes the fluorescent method safer and presents fewer legislative problems. The high costs of storage and disposal of waste are also avoided.

Nonradioactive microspheres may offer even greater advantages in chronic experiments. In the case of isotopes with short half-life, high specific activities are required at the time of injection. This causes a larger radiation load for animals and their environment during the early phase of the experiment. The release of isotopes with excreta from animals, although usually low, is also a concern.

Because the use of RM in chronic experiments has only been validated to a limited extent and because FM may offer the various benefits mentioned above, we compared the validity of the FM and RM techniques in chronic experiments. Two of the factors determining the accuracy of the microsphere method (5, 10, 13) deserve special emphasis in chronic experiments, i.e., the absence of both leaching of the label from the microspheres and disappearance of microspheres from the site of entrapment (10). In a study on leaching of radioactive microspheres from the heart, Consigny et al. (3) showed that microspheres appearing in the venous circulation are almost exclusively smaller than 12 µm in diameter. A similar conclusion was reached by a study of Medvedev et al. (12), who determined the content of 46Sc-labeled spheres with a diameter of 15 µm in donor hearts and in lungs of the recipients. The latter study also indicated some leaching of 46Sc from the beads. Losses of 125I (7) and 113Sn have also been reported (9).

The present experiments were performed in rabbits. Various combinations at least pairs of FM and RM labels were injected simultaneously at 2 mo, 1 mo, and 5 min before the animal was killed. The accuracy of the RM and FM methods was evaluated by quantification of the variation and mean difference of blood flow values between RM and FM and between different labels of RM as well as of FM in a large number of organs. Because RM lost label within 1 mo, a second set of animals was studied to compare the various isotopes in experiments lasting 1, 7, and 14 days.

METHODS

In vitro test on leaching. To test potential leaching of fluorescent labels from FM, we took 20 samples of 1 ml each from a stock solution containing a mixture of all FM used in the present study; the spheres were suspended in Hemaccel. Samples were stored in the dark for 3 days and for 2 mo, either at 37°C (the temperature at which microspheres stay while the animal is still alive) or at −20°C (the temperature at which blood samples are stored while the animal is still alive in the chronic situation and at which all samples were stored between gamma counting and processing for fluorometry). At the end of the storage period the samples were processed like the tissue and blood samples, and the fluorescence was determined (see Sample processing and blood flow determination). Because the product information from the supplier states that decay of the fluorescent labels is small (~1% after 6 mo of storage in the dark), any decrease in the dye content during the 2 mo of storage was assumed to be due to leaching of the dye from the beads during the storage.

Experimental protocol. The experiments were performed according to the "Guiding Principles in the Care and Use of Animals" approved by the Council of The American Physiological Society. New Zealand White rabbits (1.5–4 kg) were used...
for this study. At each of the injection times the rabbits were
anesthetized with pentobarbital sodium (30 mg/kg body wt)
injected via a marginal ear vein. Also, for each injection
catheters were placed in a femoral artery via the saphenous
artery for withdrawal of a reference sample and in the left
ventricle via a carotid artery for injection of the microspheres.

Arterial reference samples were withdrawn using a syringe-
pump, and the sample speed (3–5 ml/min) was calculated by
weighing the blood samples afterward. Approximately 5 min
after microsphere injection either the rabbit was killed by an
overdose of pentobarbital or the catheters were removed and
the animal was allowed to recover. In the latter situation, the
saphenous artery was ligated and the carotid artery was left
patent by removing the catheter and then suturing the
punctured wall.

The following microspheres (diameter of each: 15 ± 1 µm)
were used. The RM (NEN) were 142Ce, 51Cr, 89Sr, 113Sn, 103Ru,
95Nb, and 46Sc: the specific activities (mCi/g) of the nuclides
were 3–5 for Ce, 30–45 for Cr, 7–11 for Sr, 5–10 for Sn, 7–11
for Ru, 10–15 for Nb, and 5–10 for Sc. The FM (Molecular
Probes) were blue, blue-green, yellow-green, orange, red,
and crimson.

In seven animals pairs of RM and/or pairs of FM were
simultaneously injected at 5 min, 1 mo, and 2 mo before
animals were killed. In four animals all RM or all FM were
injected simultaneously at either 5 min or 2 mo before death.

To determine the time course of the change in accuracy of the
RM method (see RESULTS) in 12 experiments various combina-
tions of RM were injected at 1, 7, or 14 days before death.
Each of the RM and FM was used at least once at each of the
sample times.

All microspheres (all labels of FM and/or RM) were mixed
together in one syringe before injection and flushed in after
ultrasonic mixture with 5 ml of warm saline. Two hundred
fifty thousand microspheres per kilogram body weight of each
ultrasonic mixture with 5 ml of warm saline. Two hundred
fifty thousand microspheres per kilogram body weight of each
were punctured wall.

Overdose of pentobarbital or the catheters were removed and
after microsphere injection either the rabbit was killed by an
weighing the blood samples afterward. Approximately 5 min
after microsphere injection either the rabbit was killed by an
overdose of pentobarbital or the catheters were removed and
the animal was allowed to recover. In the latter situation, the
saphenous artery was ligated and the carotid artery was left
patent by removing the catheter and then suturing the
punctured wall.

The following microspheres (diameter of each: 15 ± 1 µm)
were used. The RM (NEN) were 142Ce, 51Cr, 89Sr, 113Sn, 103Ru,
95Nb, and 46Sc: the specific activities (mCi/g) of the nuclides
were 3–5 for Ce, 30–45 for Cr, 7–11 for Sr, 5–10 for Sn, 7–11
for Ru, 10–15 for Nb, and 5–10 for Sc. The FM (Molecular
Probes) were blue, blue-green, yellow-green, orange, red,
and crimson.

In seven animals pairs of RM and/or pairs of FM were
simultaneously injected at 5 min, 1 mo, and 2 mo before
animals were killed. In four animals all RM or all FM were
injected simultaneously at either 5 min or 2 mo before death.

To determine the time course of the change in accuracy of the
RM method (see RESULTS) in 12 experiments various combina-
tions of RM were injected at 1, 7, or 14 days before death.
Each of the RM and FM was used at least once at each of the
sample times.

All microspheres (all labels of FM and/or RM) were mixed
together in one syringe before injection and flushed in after
ultrasonic mixture with 5 ml of warm saline. Two hundred
fifty thousand microspheres per kilogram body weight of each
ultrasonic mixture with 5 ml of warm saline. Two hundred
fifty thousand microspheres per kilogram body weight of each

Statistics. Intermethod variability (mean difference and
variation) was determined by comparing the mean of the
blood flow values obtained with the two simultaneously
injected RM (QRM) to the mean values obtained with the two
simultaneously injected FM (QFM) for each individual sample.
The Q values were used rather than values from individual
labels to obtain the most precise blood flow value. Intra-
method variability was determined by comparing individual
values of each label to the Q value of the same kind (RM or
FM). Per-experiment intramethod mean difference and varia-
tion were calculated as the mean value and SD of QRM – QFM,
or QFM – QRM, so a positive mean difference indicates lower
values for RM than for FM. Data were also compared by
means of the analysis of Bland and Altman (1), where the
difference between the two methods (QFM – QRM for inter-
method comparison and QRM – QFM for intramethod compara-
tion) was plotted against QRM or QFM, respectively. Changes in mean difference and variation over
time were evaluated for significance using one-way ANOVA
and subsequent post hoc testing, with P < 0.05 considered
significant.

RESULTS

The in vitro studies, in which FM were stored for 2
mo at either −20 or 37°C, showed no significant
leaching of fluorescent label, except for a 10% loss of
fluorescence at 37°C and a 13% loss of red at
−20°C. Red also tended to lose label at 37°C (Table 1).

In Fig. 1 the results obtained in one typical experi-
ment are presented and in Table 2 the pooled data for
all experiments are shown. In the acute measurements
blood flow values obtained from all microsphere labels
correlated very well with each other. Consequently,
inter- and intramethod errors were small. In Fig. 1, A
and B, it can be seen that the intramethod variability
(mean difference and variation) for the FM increased

Table 1. Effect of storage of fluorescent microspheres at 37 and −20°C on loss of label

<table>
<thead>
<tr>
<th>Label</th>
<th>Blue</th>
<th>Blue-green</th>
<th>Yellow-green</th>
<th>Orange</th>
<th>Red</th>
<th>Crimson</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage at 37°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>124.40 ± 2.41</td>
<td>120.80 ± 6.40</td>
<td>460.41 ± 27.93</td>
<td>127.84 ± 13.18</td>
<td>51.41 ± 4.68</td>
<td>40.81 ± 0.93</td>
</tr>
<tr>
<td>1 mo</td>
<td>122.04 ± 5.01</td>
<td>109.89 ± 4.28*</td>
<td>452.47 ± 36.31</td>
<td>125.75 ± 7.32</td>
<td>47.21 ± 4.36</td>
<td>36.50 ± 2.53*</td>
</tr>
<tr>
<td>Average loss, %</td>
<td>1.9</td>
<td>0.0</td>
<td>1.7</td>
<td>1.6</td>
<td>8.2</td>
<td>10.5</td>
</tr>
<tr>
<td>Storage at −20°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>122.51 ± 9.08</td>
<td>113.60 ± 2.28</td>
<td>504.70 ± 18.02</td>
<td>135.41 ± 5.65</td>
<td>50.24 ± 1.24</td>
<td>39.08 ± 2.05</td>
</tr>
<tr>
<td>2 mo</td>
<td>121.55 ± 7.14</td>
<td>114.79 ± 6.22</td>
<td>504.50 ± 16.77</td>
<td>132.60 ± 5.73</td>
<td>44.15 ± 3.33*</td>
<td>38.59 ± 0.25</td>
</tr>
<tr>
<td>Average loss, %</td>
<td>0.8</td>
<td>−0.1</td>
<td>0.0</td>
<td>2.1</td>
<td>12.1</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Data at 3 days and 2 mo of storage are means ± SD of fluorescence values in arbitrary units from 5 samples at each time point. *P < 0.05 vs. 3-day value by t-test.
slightly over time, predominantly due to relatively low values for blue and blue-green in samples from heart and lungs in this particular experiment. Nevertheless, slope and intercepts of the regression equations for FM intramethod comparison were not significantly different from unity and zero, respectively

\[y = 1.03x + 0.01\] (r = 0.96, standard error of estimate (SEE) = 0.06) after 5 min, \(y = 0.89x - 0.07\) (r = 0.90, SEE = 0.20) after 1 mo, and \(y = 0.91x + 0.06\) (r = 0.88, SEE = 0.34) after 2 mo. For the whole group of experiments the FM-FM intramethod variation did not change significantly over time (Table 2). Also, there was no systematic mean difference for any of the FM labels at any time point.

Figure 1, E and F, shows an increase in the intramethod variability for RM over time. Regression equations for RM intramethod comparison were \(y = 1.05x + 0.01\) (r = 0.97, SEE = 0.08) after 5 min, \(y = 0.72x + 0.13\) (r = 0.91, SEE = 0.19) after 1 mo, and \(y = 0.84x - 0.02\) (r = 0.93, SEE = 0.21) after 2 mo. After 1 mo the slope was significantly lower than unity. For the whole group the increase in RM-RM intramethod variation was statistically significant at 2 mo (Table 2).

In the experiment shown in Fig. 1, the intermethod mean difference increased at 1 and 2 mo (Fig. 1, C and D). This was due to a decrease of the slope of the regression equation for the FM-RM comparisons from 0.86 and 0.92 at 5 min [not significantly different from 1] to 0.81 and 0.57 after 1 mo and 0.49 and 0.57 after 2 mo (all significantly lower than unity). Intercepts were not significantly different from zero. For all experiments only the increase in mean difference at 2 mo reached the level of significance. Because the intermethod mean difference is defined as \(\sigma_{FM} - \sigma_{RM}\) (see METHODS), the positive intermethod mean difference indicates lower blood flow estimations by RM than by...
FM. The increase in intramethod mean difference at 1 mo was not significant because of higher RM blood flow estimates in one experiment. In this particular experiment Cr proved to result in higher flow values than the other RM and FM labels for several organs. Intermethod variation significantly increased at 1 and 2 mo (Table 2).

After 2 mo blood flow values from RM were on average 40% lower than those obtained with FM (Table 2). This difference was smallest for Sn, Ce, and Ru (Fig. 2). In the case of Ce and Ru, however, this was due to disproportionately high values in brain (Ru and Ce) and liver (Ce). In the latter organ Ce blood flow was more than twice the FM values (Fig. 2). Blood flow values obtained with Cr and Nb were ~50% lower than those obtained with FM. This difference was similar in all organs for Nb, whereas brain flow values obtained with Cr were closer to the FM values (Fig. 2). These interisotope and interorgan differences in RM blood flow estimations explain the high FM-RM intermethod variability (Table 2). At the time of death significant amounts of Ce, Cr, Ru, and Nb were found in the urine and feces of some animals. Blood flow estimations from the various FM labels were similar in all organs (data not shown).

When RM were injected 1 day before the animal was killed, all labels except Cr gave similar blood flow values (Fig. 3). Injection of RM 7 days before the animal was killed resulted in 15–30% underestimation of blood flow by Cr, Sr, and Nb compared with Sc. During the experiments lasting 14 days a slightly more pronounced underestimation was observed for Sr, Cr, and Ce (Fig. 3).

**DISCUSSION**

This study demonstrates that in chronic experiments of up to 2 mo blood flow estimation by FM is reproducible, whereas RM lead to variable results and usually lower values than those obtained with FM. Therefore, FM are to be preferred for blood flow measurements in experiments lasting longer than 1 day.

The mean difference and variation between any two fluorescent labels do not significantly change within 2 mo. This indicates that FM perform virtually as well in chronic as in acute studies and that FM are suitable to follow changes in absolute blood flow over time, at least for 2 mo. The reliability of FM is probably due to stability of labels. This idea is supported by the in vitro studies.

The present study cannot exclude dislodgment of spheres over time. Loss of spheres from a tissue is likely to be reflected in a relatively high lung blood flow value as the spheres are trapped by the lungs (6, 11). In the present study, however, comparison of absolute lung flows between various injection times is hampered by variable conditions between injections, such as ambient temperature, which may influence shunt flow. Although the absence of dislodgment cannot be proven
in the present study, three previous studies virtually exclude the likelihood of dislodgment of microspheres in chronic studies. Hales and Cliff (8) reported that losses from the rabbit ear or thoracic tissue had stopped within a few minutes after injection and were not detectable for up to 8 wk. Consigny et al. (3) demonstrated that over a period of 5 wk part of the microspheres with diameters <12 µm disappear from the myocardium but hardly any dislodging of microspheres with diameters ≥15 µm occurs. Medvedev et al. (12) counted activity of hearts from donor rats that had previously been injected with Sc-labeled RM. The hearts were retrogradely perfused by attaching the ascending aorta to the abdominal aorta of the recipient animals. Within 4 wk these investigators did not find accumulation of activity in the lungs of the recipient animal (12).

The leaching of blue-green and crimson at 37°C in vitro may, in theory, lead to underestimate of tissue blood flows determined with these labels because FM in the tissues remain at this temperature while the related reference blood samples were stored at −20°C. Figure 1, A and B, shows some examples of increased intramethod variability. However, in vitro leaching was relatively small (10% in 2 mo) compared with the errors observed with the RM method, and in vivo the increase in FM intramethod variability over time was not statistically significant (Table 2). Red appeared to be leached from the spheres at a similar rate at −20 and 37°C. Therefore, this leaching would not result in a decline of blood flow, calculated with the reference method.

The generally limited extent of dislodgment of 15-µm-diameter microspheres in general in combination with the minimal leaching of label from RM indicate that absolute blood flows can be determined accurately with FM in chronic experiments. The generally lower and more variable blood flow estimations by RM in chronic experiments, therefore, indicate poor performance of RM under these circumstances. While on average RM underestimate blood flow by up to 40% compared with FM, considerable differences are found between isotopes and between organs. This can be observed from the intermethod variation at 2 mo, which is considerably larger than both intramethod variations. Cr, Sr, and Nb underestimated blood flow most (Cr already within 1 day; Fig. 3). Although underestimation by Ce and Ru appeared to be less compared with other isotopes, this observation is, in part, due to selective overestimation of blood flow in organs like the brain and the liver.

This variability in blood flow estimation by RM is most likely not caused by dislodgment of the spheres, because in that case blood flow to the lungs should be higher for RM than for FM. Instead, lung flows were underestimated by RM as much as other organs like the heart. Therefore, the most likely explanation is the loss of radioactive label from the beads. While loss of activity from the beads in most organs can explain the underestimate of tissue blood flow, selective binding of isotopes in some organs can explain overestimation, as, for example, by Ce in the liver and Ce and Ru in the brain.

The interorgan variation in leaching of the isotopes suggests that leaching is dependent on the environment. This environment is different in the various organs, because microspheres move to the interstitium within 1–3 wk (3, 8), thus becoming subject to the chemical environment of each particular organ. In the present study leaching of label is also suggested by the presence of Ce, Cr, Ru, and Nb in urine and feces at the time some animals were killed. The data of the present study also indicate that the leaching process is different between isotopes. Hales et al. (9) attributed falsely high blood flow values for baboon liver obtained with Sn (manufactured by 3M) to minute losses of that label from tissues in general and uptake in the liver. Similarly, 125I appeared to be lost from spheres entrapped in the kidney, gastrointestinal tract, and bone and taken up by thyroid and fat in sheep (7). These observations suggest that coating and/or chemical binding to the resin of the bead is of variable efficiency.

The present results are not in contradiction with those of Consigny et al. (3), who studied the loss of microspheres from the myocardium. These investigators determined the chronic loss of microspheres from the myocardium from the radioactivity counted within and outside the heart rather than counting microspheres. In this approach loss of label from spheres will not be observed if loss is equal within and outside the heart.

The large variability in blood flow estimations between the various radioactive labels, as well as the increasing underestimate of flow with time, starting within a week after injection of the spheres, makes blood flow data from RM in chronic experiments unreliable. Although the interisotope variation was limited to 30% in organs like heart, lungs, and kidneys, variation in the brain amounted to as much as a factor three.

This poor performance of RM stands in contrast to the good correlation between RM and FM blood flow estimation in acute experiments (<1 day), as has also been demonstrated in previous studies (4, 14). Actually, the accuracy of the RM was slightly better than that of the FM, although in previous studies the accuracies of both methods were found to be similar. This may be due to the fact that in the present study determination of radioactivity was performed soon after the animal was killed, whereas the fluorescence measurements were performed after storage of the samples for up to 1 yr. The in vitro studies show that long-term storage of samples at −20°C may cause some leaching or destruction of spheres.

We conclude that in chronic animal experiments the FM method is superior to the RM method, presumably because after periods of 1 day and longer several isotopes are leached from spheres in the tissue and may accumulate in other tissues. The accuracy of organ blood flow determination with FM does not deteriorate within 2 mo. This advantage in the accuracy of blood flow measurements comes in addition to other advantages of FM, like lack of radiation and minimal decay of the label over time.
The authors are indebted to Gari Gazibarich for assistance with the animal experiments, Anmita Rousseau for performing the fluorescent microsphere assays, and Robert S. Reneman for critically reviewing the manuscript.

Present address of J. R. S. Hales: Dept. of Veterinary Clinical Sciences, Univ. of Sydney, Camden, New South Wales, Australia.

Address for reprint requests: F. W. Prinzen, Dept. of Physiology, Univ. of Maastricht, PO Box 616, 6200 MD Maastricht, The Netherlands.

Received 20 October 1997; accepted in final form 16 March 1998.

REFERENCES


