Am J Physiol Heart Circ Physiol
280: H108–H116, 2001.

Stable labeled microspheres to measure perfusion: validation of a neutron activation assay technique

CHRISTOPHER P. REINHARDT,[1,2] SETH DALHBERG,[1] MARK A. TRIES,[2]
ROBIN MARCEL,[1] AND JEFFERY A. LEPPO[1]
[1]*Myocardial Isotope Research Laboratory and Division of Cardiology, Department of Radiology and Medicine, University of Massachusetts Medical Center; and* [2]*BioPhysics Assay Laboratory (BioPAL) Incorporated, Worcester, Massachusetts 01606*

Received 10 April 2000; accepted in final form 17 August 2000

Reinhardt, Christopher, Seth Dalhberg, Mark A. Tries, Robin Marcel, and Jeffery A. Leppo. Stable labeled microspheres to measure perfusion: validation of a neutron activation assay technique. *Am J Physiol Heart Circ Physiol* 280: H108–H116, 2001.—Neutron activation is an accurate analytic method in which trace quantities of isotopes of interest in a sample are activated and the emitted radiation is measured with high-resolution detection equipment. This study demonstrates the application of neutron activation for the measurement of myocardial perfusion using stable isotopically labeled microspheres. Stable labeled and standard radiolabeled microspheres (15 μm) were coinjected in an in vivo rabbit model of myocardial ischemia and reperfusion. Radiolabeled microspheres were detected with a standard gamma-well counter, and stable labeled microspheres were detected with a high-resolution Ge detection after neutron activation of the myocardial and reference blood samples. Regional myocardial blood flow was calculated from the deposition of radiolabeled and stable labeled microspheres. Both sets of microspheres gave similar measurements of regional myocardial blood flow over a wide range of flow with a high linear correlation (r = 0.95–0.99). Neutron activation is capable of detecting a single microsphere in an intact myocardial sample while providing simultaneous quantitative measurements of multiple isotope labels. This high sensitivity and capability for measuring perfusion in intact tissue are advantages over other techniques, such as optical detection of microspheres. Neutron activation also can provide an effective method for reducing the production of low-level radioactive waste generated from biomedical research. Further applications of neutron activation offer the potential for measuring other stable labeled compounds, such as fatty acids and growth factors, in conjunction with microsphere measured flow, providing the capability for simultaneous measurement of regional metabolism and perfusion.

radioisotopes; stable isotopes; myocardium; ischemia; blood flow

SINCE ITS INTRODUCTION IN 1967, the radioactive microsphere technique has been the standard method for the measurement of regional tissue perfusion to organs (24). However, a major limitation of radiolabeled microspheres is the high disposal and administrative cost associated with generated low-level radioactive waste. The development of substitutes for radioactive materials has thus become an active field of research (18, 22, 23, 25, 28). Overwhelmingly, the focus of this research has been on optical-based detection technology. Because this detection technology cannot penetrate deeply into tissue, the digestion of the tissue matrix is required before the assay of optically labeled microspheres. This additional processing is expensive, time consuming, and may introduce additional experimental error. Therefore, optical detection is best suited for studies, such as tissue slide preparations, where the optical tracer can more easily be detected. Alternative nonradioactive methods that can directly measure tracer content in intact tissue and biological fluid samples with accuracy similar to radiolabeled microspheres would be desirable for the measurement of regional perfusion.

One potential approach is the use of stable isotopic tracers that can be assayed by neutron activation. Neutron activation is a well-known analytic technique whereby neutrons penetrate the sample and activate elements within the sample (1, 3, 7, 15, 20, 29, 31). After activation, elements of interest within the sample are rendered radioactive and can be quantified using high-resolution radiation counting equipment. This paper is the first to describe and validate the use of neutron activation for the assay of stable isotopically labeled microspheres for measuring regional myocardial perfusion. This approach was directly compared with the traditional radioactive microsphere method using an in vivo rabbit model of myocardial ischemia and reperfusion. Because only the samples of interest are activated, this assay can significantly reduce occupational exposure to radiation and reduce the amount of low-level radioactive waste generated. For example, the contamination of gloves and protective clothing, glass and plastic laboratory supplies, and waste products from animal housing and carcasses are avoided. In addition, this approach eliminates the digestion and

Address for reprint requests and other correspondence: C. P. Reinhardt, BioPAL, Inc., 10 New Bond St., Worcester, MA 01606 (E-mail: BioPAL@mx1.pair.com).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

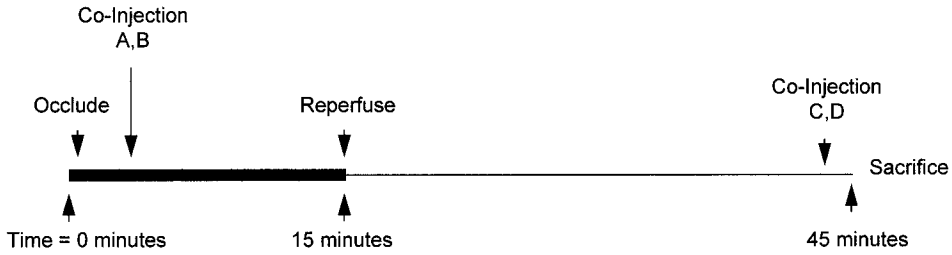

Fig. 1. Schematic of the experimental protocol.

Group 1 (n=5): A - ^{95}Nb, B - Sm, C - ^{103}Ru, D - La

Group 2 (n=5): A - ^{95}Nb, B - Sb, C - ^{103}Ru, D - Yb

Group 3 (n=5): A - ^{95}Nb, B - Re, C - ^{103}Ru, D - Eu

Group 4 (n=5): A - ^{95}Nb, B - Ir, C - ^{103}Ru, D - Au

Group 5 (n=5): A - Sm, B - La, C - Sb, D - Yb

Group 6 (n=5): A -Re, B - Eu, C - Ir, D - Au

Group 7 (n=5): A - Sm, B - La, C - Ir, D - Au

Group 8 (n=5): A - Re, B - Eu, C - Sb, D - Yb

microsphere recovery procedures required by optically based methods.

MATERIALS AND METHODS

Sensitivity measurements. To determine the linear range for elemental tracer detection by neutron activation, the following sensitivity study was undertaken using two of the stable isotope labels, samarium and lanthanum. Atomic absorption standard solutions of each element (Sigma Chemical, St. Louis, MO) were used to produce samples that contained 0.02–50 μg of each element. Each sample was placed in a vial containing 0.1 g of bovine liver tissue. A metallic monitor [20 μl; BioPhysics Assay Laboratory (BioPAL), Worcester, MA] was added to each vial to account for potential neutron flux density variations during activation analysis, as discussed in the APPENDIX. All vials were sent for analysis (BioPAL), where they were activated by exposure to a field of neutrons. Activated vials were stored for 48 h to allow short-lived activation products to decay. After this decay period, we performed spectrographic analysis to measure the concentration of the resultant radioactive nuclei in each vial. The general principles used to determine the elemental mass in each sample after neutron activation are outlined in the APPENDIX (3).

Microsphere preparation. Stable isotope-labeled microspheres (Sm, La, Sb, Yb, Eu, Re, Ir, and Au; 15 μm; BioPAL) were compared with standard radiolabeled microspheres (^{95}Nb and ^{103}Ru; 15 μm; NEN Research Products, North Billerica, MA). A coinjection consisted of a mixture of ~1 × 10^6 microspheres of each label, as described below in *Experimental Protocols* and shown in Fig. 1; therefore, the total injection contained 2 × 10^6 microspheres. The coinjection was suspended in 20% dextran with 0.01% polyoxyethylene-sorbitan monooleate (Tween 80). The microspheres were thoroughly mixed and vortexed before injection. Table 1 provides the radioactive half-lives and gamma-ray energies for each of the stable isotope-labeled microspheres after neutron activation.

It is important to note that the stable isotope label is cross-linked within the polystyrene microsphere matrix. In vitro testing over a range of temperature and pH variations has shown no isotope leakage from the microsphere. Therefore, stable isotope-labeled microspheres are assumed to be stable for the duration of the in vivo experiment.

Calculation of microsphere injection. The accuracy of any microsphere blood flow technique is dependent on having a sufficient number of microspheres present within the sample. Heymann et al. (10) states that a minimum of 400 microspheres are needed per sample for a 95% probability that the blood flow measurement will be within 10% of the true value. This microsphere estimate was later confirmed and refined by others (4, 16, 21). In this study, each microsphere label served as an independent flow marker. Assuming the heart receives 4% of the cardiac output, the initial 1 × 10^6 microsphere injection of each label would result in 40,000 microspheres uniformly distributed in the myocardium. If the myocardium is subdivided into 20 segments, each segment will contain 2,000 microspheres under normal conditions. However, because of the occlusion, we expect that some segments will, at minimum, have an 80% reduction in normal blood flow. As a result, 400 microspheres would be present in these low-flow segments, which is at the lower limit of acceptance.

Surgical preparation. The surgical procedures were conducted under the guidelines of the University of Massachusetts Medical Center (UMMC). The UMMC is accredited by the American Association of Laboratory Animal Care and meets all standards prescribed by the *Guide for the Care and Use of Laboratory Animals* [DHHS Publication No. (National Institutes of Health) 85-23, Revised 1985].

New Zealand White male rabbits (n = 40, weight 1–1.8 kg; Millbrook Farms, Amherst, MA) were sedated with intramuscular ketamine (35 mg/kg) and xylazine (5 mg/kg), intubated, and then anesthetized with 1–2% isoflurane administered through an anesthesia apparatus (Boyle model 50,

Table 1. *Stable isotope labels*

Element	Isotope	Half-Life	Gamma-Ray, keV
Samarium	^{153}Sm	1.929 days	103.2
Lanthanum	^{140}La	1.678 days	1,596.5
Antimony	^{122}Sb	2.70 days	564.1
Ytterbium	^{175}Yb	4.19 days	396.3
Europium	^{154}Eu	8.59 yr	123.1
Rhenium	^{186}Re	3.777 days	137.1
Iridium	^{192}Ir	73.83 days	316.5
Gold	^{198}Au	2.694 days	411.8

Harris-Lake, Cleveland, OH). The carotid artery was catheterized, and arterial pressure continuously monitored. Both femoral arteries were catheterized. One line was used to draw reference blood samples for microsphere determination of regional blood flow. The other line was used to measure arterial pH, P_{CO_2}, and P_{O_2}. Ventilatory parameters and supplemental oxygen were adjusted to maintain physiological arterial blood gases. The heart was exposed in a pericardial cradle through a left thoracotomy. A catheter was inserted into the left atrium for injection of microspheres to document regional flow patterns.

A major branch of the left circumflex artery was ligated for 15 min. This duration of coronary occlusion in rabbits has been shown to consistently produce regional ischemia with no infarction (6, 16). During the occlusion period, microspheres with two differing labels were coinjected through a left atrial catheter to measure hypoperfused myocardial blood flow. Simultaneously, a reference blood sample was drawn from a femoral arterial catheter using an withdrawal pump (model 94, Harvard Apparatus, Millis, MA) to calculate absolute myocardial blood flow. The withdrawal rate was set at 2 ml/min and collected over a 2-min interval, resulting in a 4-ml reference blood collection. After 15 min, the occluder was released, and the ischemic tissue was reperfused. After 30 min of reperfusion, a second coinjection of microspheres with two differing labels was given through the left atrial catheter to measure the extent of myocardial reperfusion. To eliminate potential sampling bias, animals were randomly placed into one of eight groups (Fig. 1).

Experimental protocols. Groups 1–4 were used to compare directly each of the eight different stable labeled microsphere sets to a coinjected set of radiolabeled microspheres. In *groups 5–8*, several combinations of four stable labeled microsphere sets were tested to characterize the sensitivity of measuring multiple labels and to demonstrate that there were no interfering reactions during analysis.

Tissue preparation. Hearts were harvested under increased anesthesia. The left ventricle was surgically isolated and cut into transmural slices, which were further subdivided into transmural segments. Each segment contained approximately equal concentrations of the endocardium and epicardium. Each segment was placed in a preweighed sample vial (BioPAL) and then reweighed. The average myocardial sample weighed ~0.154 ± 0.051 g. A metallic monitor (20 μl; BioPAL) was added to each vial to account for potential neutron flux density variations during neutron activation (3). The vials were then sealed.

In addition to the myocardium, representative samples from the brain, spleen, pancreas, stomach, small and large intestines, and right and left kidneys and lungs were collected from each subject. During sample dissection, careful consideration was given to organs having nonhomogeneous blood flow, such as the kidney, to ensure valid comparisons within each group. Like the myocardium segments, each sample was placed in a preweighed sample vial and then reweighed. A metallic monitor was also added to each vial and sealed. After the experimental protocol, livers were donated and therefore not available for sampling.

Absolute regional blood flow. Radiolabeled microsphere activity within each sealed vial was measured in a NaI (Tl) gamma-well counter (Auto-Gamma 5530, Packard Instruments, Downers Grove, IL). All samples were counted for 2 min and corrected for tracer decay during the counting period. The methodology used to obtain absolute blood flow (in $ml \cdot min^{-1} \cdot g^{-1}$) has been previously described (10). Briefly, the microsphere concentration of each segment [in disintegrations per minute (dpm)/g] was normalized to the microsphere concentration measured in the 2-min reference blood collection (in $dpm \cdot min \cdot ml^{-1}$).

Neutron irradiation and analysis. All tissue and blood samples were sent for analysis (BioPAL), where they were exposed to a field of neutrons. The vials were stored for 48 h to allow short-lived activation products to decay. After this decay period, spectroscopic analysis was performed on each sample, and corrections were made to account for interradionuclide crossover and tracer decay during the counting period (9, 13). In addition, instruments were calibrated to account for differences in sample volume and geometry. The results of the assay were reported as the number of disintegrations per minute measured for each stable labeled microsphere set, and the results were obtained within 4 days of sample submission. Absolute tissue blood flow measured by stable labeled microspheres was calculated as described above for the radiolabeled microspheres.

Although ultratrace amounts of rare earth elements, like samarium, may be present naturally in some tissue types, their concentration is very low, on the order of >0.001 μg/g of wet tissue (30). This amount represents <0.5% of the total mass of the label on just one microsphere. Therefore, this background concentration will clearly have no effect on the blood flow measurement and therefore was ignored.

Data analysis. All data are presented as means ± SD. A repeated measure ANOVA and Bonferroni *t*-test were used when multiple comparisons were made as a function of time. Statistical calculations were performed with the use of a commercially available computer program (SigmaStat, Jandel). Elemental mass measured by neutron activation was compared directly with the predetermined elemental mass using the analysis of Bland and Altman (2), where the difference in the measurement is plotted against the average elemental mass. Likewise, blood flow values measured by radiolabeled microspheres were directly compared with their corresponding values obtained from the coinjected stable labeled microspheres measurements. The data also were compared using the analysis of Bland and Altman (2), where the difference between the two methods are plotted against the average blood flow determined for each labeled microsphere.

RESULTS

Sensitivity curves. There was a strong linear relationship when the masses of samarium and lanthanum, determined by neutron activation analysis, were plotted as a function of their predetermined masses in dilutions of standard solutions (Fig. 2*A*). Neutron activation analysis accurately assessed trace quantities of both elements as low as 0.02 μg with good precision (Fig. 2*B*). The average mass of label per microsphere is ~0.030 ± 0.002 μg for each of the eight labels. The sensitivity study illustrates that neutron activation can accurately measure the presence of one microsphere in an intact tissue or blood sample.

Hemodynamics. Heart rate and peak systolic pressure data during baseline, occlusion, and reperfusion periods are contained in Table 2. All groups showed stability in heart rate during the experimental protocol. Blood pressure fell slightly during occlusion in all groups, although the difference did not reach statistical significance.

Absolute regional blood flow. Groups 1–4 directly compared each of the eight different stable labeled

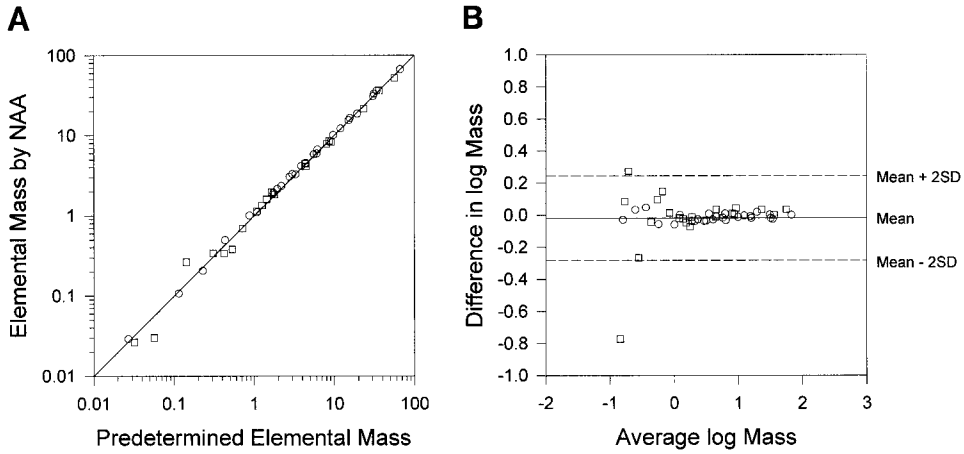

Fig. 2. ○, Samarium; □, lanthanum. *A*: predetermined elemental mass (in μg) is compared with neutron activation analysis (NAA) measurements (in μg) (Sm: $y = 0.02 + 1.00x$, $r = 1.0$; La: $y = 0.03 + 0.92x$, $r = 0.98$). *B*: difference against mean for neutron activation data.

microsphere sets to a coinjected set of radiolabeled microspheres. Fig. 3, *A* and *C*, illustrates a strong linear relationship when absolute blood flow measured with stable isotope-labeled microspheres was plotted as a function of blood flow measured by radiolabeled microspheres. Fig. 3, *B* and *D*, illustrates the precision of the blood flow measurement over a range of flows. Table 3 contains similar results for *groups 2–4*.

In *groups 5–8*, several combinations of four stable labeled microsphere sets were tested to characterize the sensitivity of measuring multiple labels and to demonstrate that there are no interfering reactions during analysis. For *group 5*, absolute blood flow measurements obtained from each stable labeled microsphere set plotted as a function of values obtained from its coinjected set of microspheres showed a strong linear relationship (Fig. 4, *A* and *C*) and good precision (Fig. 4, *B* and *D*). Table 3 contains similar results for *groups 6–8*. During spectrographic analyses, no major intraisotope interference was observed.

Absolute organ blood flow. Groups 1–4 directly compares each of the eight different stable isotope-labeled microsphere sets to a coinjected set of radiolabeled microspheres. For *group 1*, the blood flow measured by the stable isotope-labeled microsphere injection for each organ sample showed no statistical difference to its coinjected set of radioactive microspheres (Table 4). Similar results were also found for *groups 2–4* (data not shown). In *groups 5–8*, several combinations of four stable labeled microsphere sets were tested to characterize the sensitivity of measuring multiple labels. For *group 5*, the blood flow measured by the stable labeled microsphere injection for each organ sample showed no statistical difference to its coinjected set of stable labeled microspheres (Table 4). Like *groups 1–4*, similar results were also found for *groups 6–8* (data not shown).

DISCUSSION

The results of this study demonstrate the feasibility of using neutron activation to assay stable labeled microspheres in intact myocardial samples. The sensitivity of neutron activation analysis was shown to be linear over a wide detection range and, therefore, can theoretically measure the presence of one microsphere within a tissue segment. Data from this in vivo study show that stable labeled microspheres compare favorably with values obtained from radiolabeled microspheres. The turnover time for the assay was 4 days after submission of the samples, which is comparable to the 2- to 3-day delay associated with the digestion and recovery procedures of optically labeled microsphere techniques. Neutron activation analysis can also provide a cost-effective method for reducing the production of low-level radioactive waste generated from biomedical research.

Table 2. *Hemodynamic measurements*

	Heart Rate, beats/min			Peak Systolic Pressure, mmHg		
Group	Baseline	Occlusion	Reflow	Baseline	Occlusion	Reflow
1	155 ± 8	138 ± 36	150 ± 22	65 ± 5	54 ± 14	52 ± 9
2	164 ± 22	154 ± 20	151 ± 16	69 ± 7	58 ± 6	60 ± 14
3	124 ± 29	141 ± 24	124 ± 10	40 ± 27	37 ± 27	38 ± 34
4	175 ± 22	168 ± 11	156 ± 16	81 ± 14	70 ± 20	74 ± 14
5	109 ± 33	117 ± 40	118 ± 40	62 ± 14	58 ± 18	54 ± 19
6	161 ± 26	130 ± 24	131 ± 19	68 ± 10	63 ± 8	62 ± 10
7	112 ± 14	109 ± 29	126 ± 16	64 ± 24	55 ± 26	55 ± 24
8	128 ± 16	138 ± 25	137 ± 20	55 ± 1	54 ± 36	50 ± 15

Values are means ± SD. P = not significant (NS) vs. baseline; P = NS vs. groups for all values listed in Table 1.

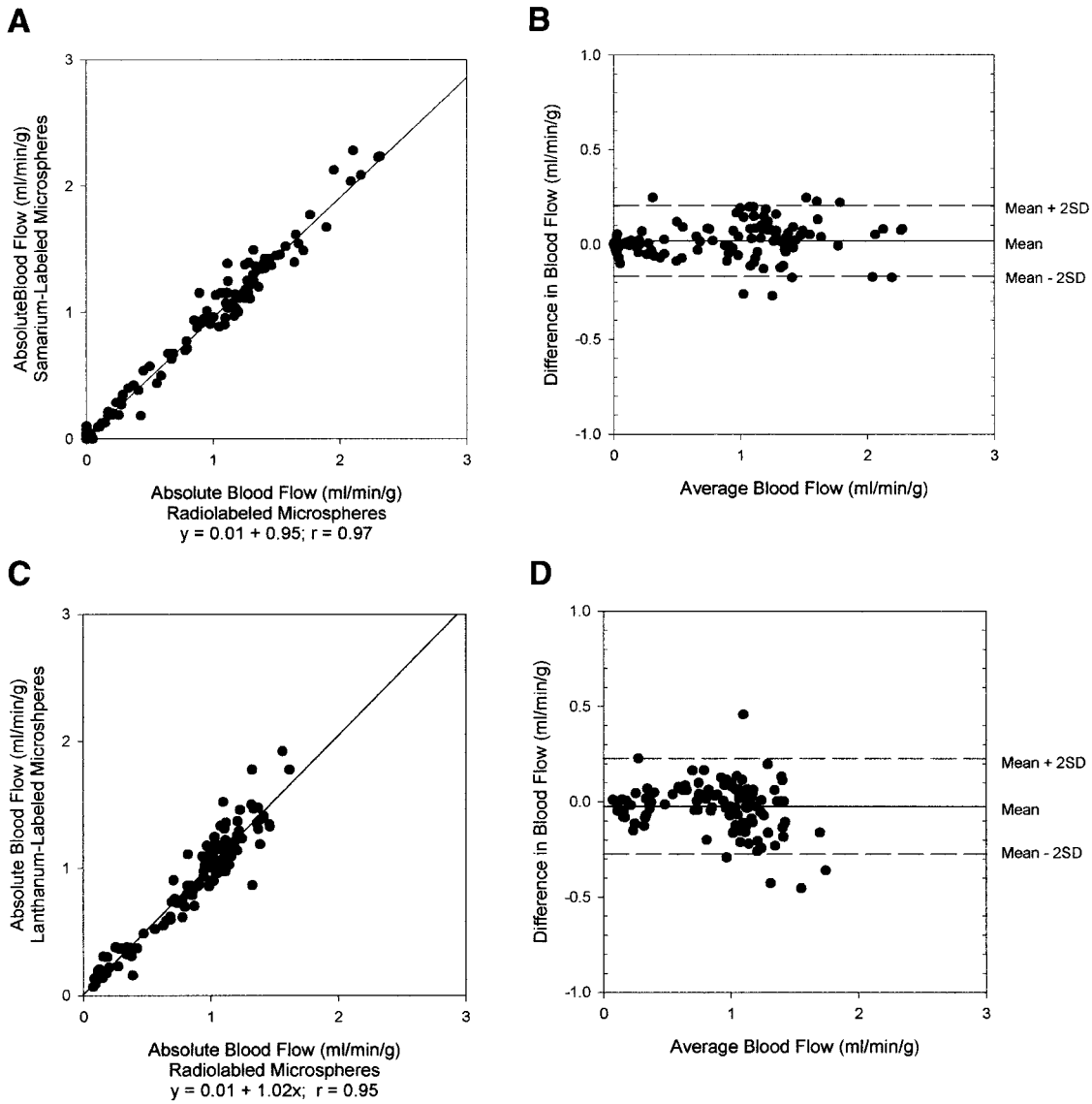

Fig. 3. Myocardial segments from *group 1*. *A*: absolute blood flow measured during occlusion by samarium-labeled microspheres is compared with values measured by radioactive microspheres (^{95}Nb). *B*: the difference between the 2 methods is plotted against the average blood flow determined for each labeled microsphere. *C*: absolute blood flow measured during reperfusion by lanthanum-labeled microspheres is compared with values measured by radioactive microspheres (^{103}Ru). *D*: the difference between the 2 methods is plotted against the average blood flow determined for each labeled microsphere.

Evaluation of fluorescent microsphere methods. The assay of stable labeled microspheres by neutron activation has several major advantages over current, nonradioactive fluorescent microsphere techniques. Fluorescent detection technology cannot penetrate deeply into tissue (22, 23). Therefore, the assay of fluorescent microspheres requires the digestion of the tissue matrix, thus destroying the sample in the process (18, 23, 25, 28). The digestion process frequently requires multiple separation and filtration steps that are expensive, time consuming, and may introduce sources of error. Therefore, a known concentration of "processing" microspheres are added to each sample to account for sample loss. Error estimates of 5–20% for chemical extraction techniques of this nature are not uncommon (22, 23). These tissue preparation procedures eliminate the possibility of tracking other fluorescently labeled compounds in conjunction with the microsphere assay. In addition, the digestion process requires the use of hazardous reagents. Like radioactivity, these reagents pose their own environmental and health risks.

In contrast, neutrons are highly penetrating (3). Therefore, neutron activation can be applied directly to the sample without additional processing. As a result, the potential for sample contamination and sample loss is eliminated. Activation of the sample results in a specific signal for each stable isotopically labeled microsphere. This signal is not compromised by the type of tissue being assayed, such as muscle versus fat, nor is the signal significantly affected by the presence of

Table 3. *Linear regression data*

Group	n	Occlussion				Reperfusion			
		Comparison	Linear regression	r	SD	Comparison	Linear regression	r	SD
1	119	^{95}Nb vs. Sm	$y = 0.01 + 0.95x$	0.97	0.09	^{103}Ru vs. La	$y = 0.01 + 1.02x$	0.95	0.13
2	101	^{95}Nb vs. Sb	$y = 0.001 + 0.99x$	0.99	0.14	^{103}Ru vs. Yb	$y = -0.08 + 1.06x$	0.96	0.14
3	90	^{95}Nb vs. Re	$y = -0.03 + 1.02x$	0.99	0.10	^{103}Ru vs. Eu	$y = 0.03 + 0.98x$	0.96	0.13
4	97	^{95}Nb vs. Ir	$y = -0.003 + 0.94x$	0.96	0.10	^{103}Ru vs. Au	$y = 0.05 + 0.92x$	0.95	0.08
5	90	Sm vs. La	$y = 0.01 + 0.97x$	0.99	0.01	Sb vs. Yb	$y = -0.002 + 1.00x$	0.97	0.13
6	90	Re vs. Eu	$y = 0.11 + 0.94x$	0.93	0.27	Ir vs. Au	$y = 0.05 + 1.02x$	0.97	0.11
7	91	Sm vs. La	$y = 0.05 + 0.97x$	0.95	0.11	Ir vs. Au	$y = 0.04 + 0.98x$	0.97	0.09
8	91	Re vs. Eu	$y = 0.01 + 0.97x$	0.99	0.08	Sb vs. Yb	$y = 0.04 + 0.97x$	0.97	0.15

histochemical stains and dyes, which are both problematic during fluorescent detection.

Neutron activation. Neutron activation analysis can provide researchers with capabilities not readily available with other assay technologies. Neutron activation is well known for its high sensitivity and elemental specificity for the simultaneous measurement of trace elements (1, 3, 7, 15, 20, 29, 31). This offers the poten-

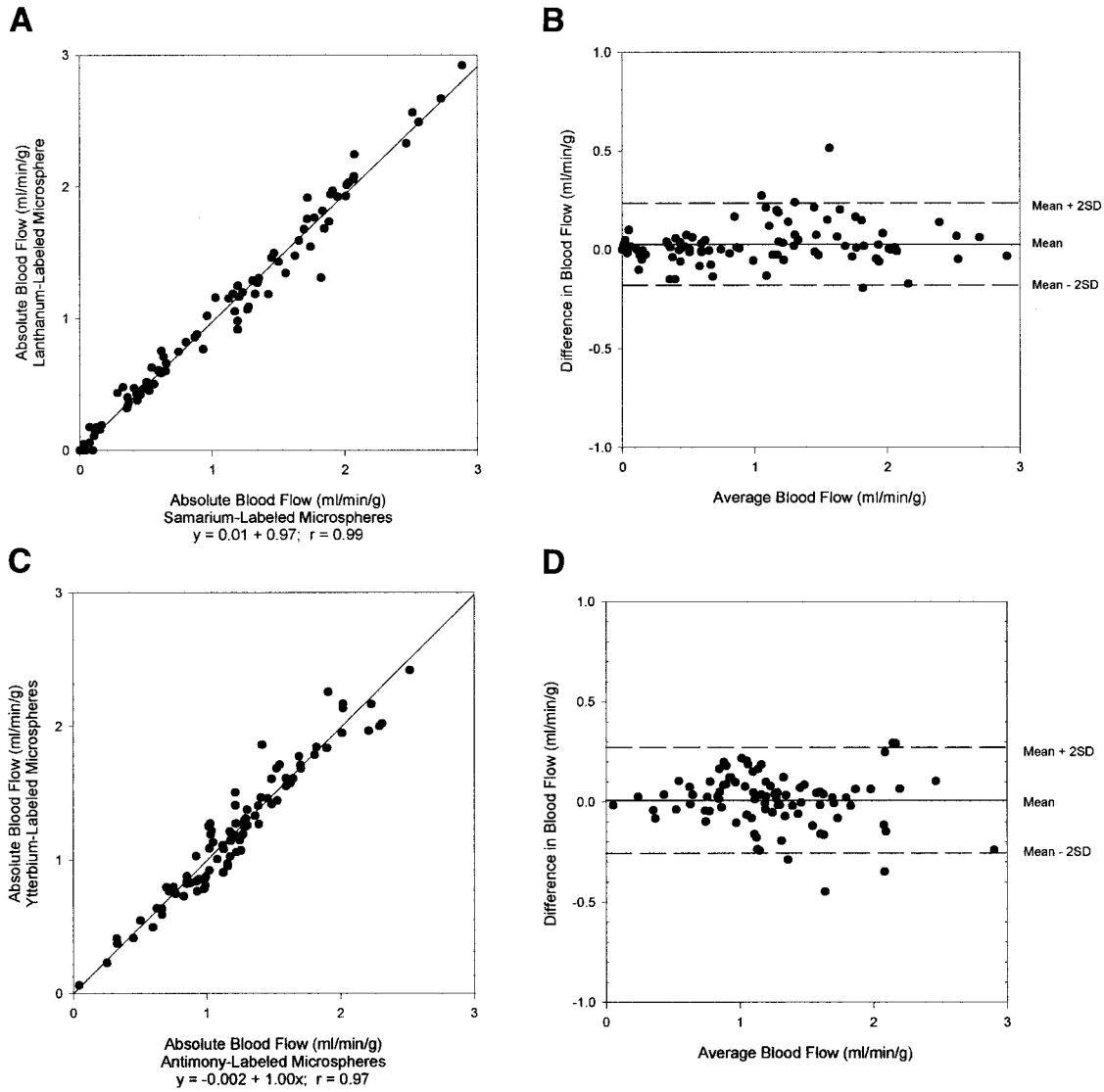

Fig. 4. Myocardial segments from *group 5*. *A*: absolute blood flow measured during occlusion by samarium-labeled microspheres is compared with values measured by lanthanum-labeled microspheres. *B*: the difference between the 2 methods is plotted against the average blood flow determined for each labeled microsphere. *C*: absolute blood flow measured during reperfusion by antimony-labeled microspheres is compared with values measured by ytterbium-labeled microspheres. *D*: the difference between the 2 methods is plotted against the average blood flow determined for each labeled microsphere.

Table 4. *Summary of blood flow values obtained from groups 1 and 5*

	Group 1				*Group 5*			
	Occlusion		Reperfusion		Occlusion		Reperfusion	
Organ	Sm	^{95}Nb	La	^{103}Ru	Sm	La	Sb	Yb
Brain	0.16 ± 0.05	0.18 ± 0.07	0.16 ± 0.11	0.14 ± 0.05	0.34 ± 0.12	0.32 ± 0.13	0.42 ± 0.24	0.48 ± 0.32
Spleen	0.64 ± 0.37	0.68 ± 0.44	0.61 ± 0.28	0.65 ± 0.37	0.44 ± 0.09	0.45 ± 0.08	0.85 ± 0.62	0.82 ± 0.66
Pancreas	0.66 ± 0.55	0.71 ± 0.56	0.56 ± 0.49	0.55 ± 0.40	0.40 ± 0.13	0.39 ± 0.12	0.53 ± 0.40	0.52 ± 0.36
Stomach	1.10 ± 0.55	1.10 ± 0.67	0.98 ± 0.38	0.74 ± 0.22	1.27 ± 0.26	1.24 ± 0.31	0.98 ± 0.43	1.02 ± 0.52
Small Intestine	0.33 ± 0.08	0.35 ± 0.12	0.29 ± 0.08	0.27 ± 0.07	0.50 ± 0.26	0.41 ± 0.16	0.59 ± 0.44	0.59 ± 0.45
Large Intestine	0.26 ± 0.11	0.26 ± 0.10	0.27 ± 0.09	0.23 ± 0.13	0.26 ± 0.12	0.27 ± 0.13	0.33 ± 0.15	0.30 ± 0.11
Right Kidney	2.65 ± 0.37	2.56 ± 0.42	1.49 ± 0.37	1.50 ± 0.35	1.53 ± 0.75	1.51 ± 0.71	1.47 ± 0.94	1.44 ± 1.01
Left Kidney	2.70 ± 0.64	2.75 ± 0.57	1.53 ± 0.48	1.81 ± 0.49	1.41 ± 0.73	1.51 ± 0.75	1.43 ± 1.05	1.33 ± 1.01
Right Lung	0.15 ± 0.05	0.16 ± 0.04	0.16 ± 0.13	0.13 ± 0.03	0.36 ± 0.32	0.38 ± 0.32	0.26 ± 0.17	0.23 ± 0.08
Left Lung	0.18 ± 0.09	0.18 ± 0.10	0.18 ± 0.15	0.15 ± 0.10	0.32 ± 0.24	0.24 ± 0.13	0.26 ± 0.09	0.28 ± 0.11

Values are means ± SD; $n = 5$ for each group. Blood flow values were measured as milliliters per minute per gram. P = NS for Sm vs. ^{95}Nb and La vs. ^{103}Ru for *group 1; P* = NS for Sm vs. La and La vs. Sb for *group 5.*

tial of being able to measure multiple isotopic tracers per assay. Unlike optical lasers, neutrons can penetrate solid tissue and opaque-liquid samples, thereby providing an assay that is completely self-contained and requires minimal sample preparation. Unlike other element detection methods, such as atomic absorption spectrophotometry, neutron activation is not chemically or physically destructive (3, 9). Therefore, samples can be archived, reassayed, and/or undergo additional analysis after neutron activation. Because only the samples of interest are activated, this assay can significantly reduce occupational exposure to radiation and reduce the amount of low-level radioactive waste generated. For example, the contamination of gloves and protective clothing, glass and plastic laboratory supplies, and waste products from research animal housing and carcasses are avoided. Stable isotopes do not undergo radioactive decay or cause radiokinistics (27), and, unlike optical probes, they do not lose their fluorescence over time. Therefore, stable isotope-labeled research products, such as microspheres, will have a significantly longer shelf life compared with alternative labeling methods.

Several researchers have proposed using neutron activation in conjunction with enriched or biologically rare isotopes as substitutes for radioactive isotopes in laboratory experiments (12, 26) and in clinical applications (5, 11, 19). Kitchin et al. (12), for example, successfully used neutron activation to measure iodine and bromine for the assay of 5-iodo-2′-deoxyuridine and 5-bromo-2′-deoxyuridine as DNA precursors for the determination of cell mitosis and death rates. Compared with both immunocytochemistry and fluorescent-activated cell sorting, they found that neutron activation-based studies of DNA synthesis were able to evaluate more cells per assay (up to 10^5 more) due to the elimination of signal quenching errors associated with both β-radiation and fluorescent counting, and the neutron activation method required less labor and in-house laboratory equipment. In addition, this technique offered improved accuracy and precision (coefficient of variation as low as 3–5%) compared with both immunocytochemistry and flow-activated cell sorting. Recently, Chou et al. (5) refined a neutron activation technique for the clinical assay of protein-bound iodine in blood serum samples. The measurement of protein-bound iodine, as well as the assay of tetra-iodothyronine (T4) and tri-iodothyronine (T3), is one of the conventional clinical tests of thyroid function. This refined neutron activation technique provided a simple and highly sensitive assay (down to a few parts per billion are theoretically achievable) while avoiding systematic and temperature-dependent errors associated with traditional clinical analytical techniques.

Despite the many advantages of neutron activation for biomedical research, this analytical method has not achieved widespread use due to a lack of familiarity within the biomedical community and the lack of readily available stable labeled materials specifically designed for biomedical research. This paper is the first to describe and validate stable labeled microspheres specifically developed for measurement by neutron activation analysis. Microspheres were developed with eight different isotopic labels, and the simultaneous measurement of four labels per sample was validated. The stable labeled microspheres were used in a similar manner as radioactive and optically based microspheres. However, only the samples of interest were sent for analysis of tracer(s) content, and the results of the assay were available within a few days. In addition to tissue measurements of blood flow, neutron activation analysis offers the potential of measuring other stable labeled compounds, such as fatty acids, antibodies, growth factors, etc., which may permit the comparison of regional metabolism and perfusion.

Economic comparisons of microsphere techniques. There are three principal technologies for measuring blood flow. Each has advantages and disadvantages, which can be express in an economic analysis. The cost of optically labeled microspheres and stable isotope-labeled microspheres are essentially identical (Triton Technologies, San Diego, CA). The cost of radiolabeled microspheres are 5 to 10 times more expensive per injection than microspheres for the other two methods. The experimental protocol from the injection of the subject animal to its death and tissue biopsy are iden-

tical. Cost differentials occur in the work up of the tissue. Radiolabeled microspheres produce large amounts of low-level radioactive waste disposal, which will mount rapidly in expense in the near term. Balancing this is the rapidity of obtaining an answer with little hands-on time for the technologist. Optically labeled microspheres require considerable technologist time (up to 3 days) to process samples (18, 23, 25, 28). In addition, training of the technologist before starting the first experiment may take up to several months before reliable results are obtained. Finally, this technology requires considerable capital expenditure in the purchase of fluorescent instrumentation and centrifuges (8, 14, 17). This method is thus suited to large laboratories with long and established programs in blood flow measurements. The workup using stable isotope labeling and neutron activation has no start-up costs, no requirement for training technologists, and no capital equipment requirements. The investigator need only mail samples to central processing laboratory (BioPAL) for quantitation. The technique is ideally suited for laboratories interested in using blood flow analysis for short periods of time and for small numbers of animals. The technique also avoids all associated expenses of chemical disposal found with optical methods and radioactive waste disposal. The current cost of this method is $10 per sample. However, as new applications for this technology are developed and additional assay service laboratories are formed, the cost of the assay may significantly reduce as it improves and gains popularity.

APPENDIX

The general principle underlying neutron activation is that an incident neutron is captured by an atomic nucleus forming a radioactive nucleus. An ideal radioactive nucleus would be short-lived and emit a gamma-ray during the decay process (9). The energy of the gamma-ray is discrete and distinct for each stable atom. Specialized, high-resolution detection equipment can than be used to identify and measure the emitted gamma-ray. The number of emitted gamma-rays is directly proportional to the total mass of the parent isotope. In practice, a sample is exposed to a flux of neutrons (φ, in m^2/s) for a given time (t). The specific activity (S, in s/kg) induced in any parent nuclide can be approximated from the formula (3)

$$S = 6.02 \times 10^{26}(\varphi)\,(\sigma)\,(f)\,(A^{-1})\,(0.5)^{t_1/t_{1/2}}[1 - (0.5)^{t/t_{1/2}}] \quad (1a)$$

where σ is the cross section for neutron interaction with the parent nuclide (in m^2), f is the fractional abundance of the parent nuclide, A is the atomic weight of the parent nuclide, t_1 is the time between activation and counting (in h), t is the activation period (in h), and $t_{1/2}$ is the half-life of the daughter nuclide (in h).

The sensitivity of any reaction can be estimated using *Eq. 1a*. For samarium, the potential reaction is $^{152}Sm(n,\gamma)^{153}Sm$; $t_{1/2}$ = 1.93 days. Therefore, using a flux of 2.0×10^{17} m^2/s and an irradiation time of 30 min followed by a decay period of 2 days, a specific activity of 1.5×10^{13} s/kg is obtained for ^{153}Sm. Conservatively, this technique can determine $<10^{-14}$ kg (0.01 ng) of samarium using standard counting equipment. It is important to recognize that increasing the neutron flux and/or increasing the duration of the neutron exposure will result in an increase in sensitivity of this assay. In addition, using an enriched isotope as the tracer will also increase sensitivity. For example, recalculating the specific activity for "enriched" samarium increases the estimated specific activity to 5.7×10^{13} s/kg (f = 1 rather than f = 0.26). *Equation 1a* also demonstrates that this assay only activates a minute population of the stable parent element present. Therefore, this assay can be repeated to retrieve results from expensive experiments that might otherwise be lost with competing technology.

The activation process will also activate other isotopes present in the sample, adding background noise to the signal, and thereby potentially decreasing the sensitivity of the assay. For biological samples, sodium and chloride pose the greatest concern. However, given their short activated half-lives (^{24}Na $t_{1/2}$ = 15 h; ^{38}Cl $t_{1/2}$ = 37 min) compared with isotopically labeled microspheres, this background activity will be low after the 2-day decay period.

If a standard containing a known mass (m_0) of the element of interest is exposed to the same flux of neutrons, then determination of the unknown mass (m) within the sample is given by

$$m = \frac{m_0 R}{R_0} \quad (2a)$$

where R and R_0 are the count rates of the resultant radioactive nucleus from the sample and standard, respectively. However, it is difficult to achieve a uniform neutron flux rate. Therefore, a monitor is used to gauge and correct for neutron flux nonuniformity. A monitor is an element that is not expected to be within the sample itself. A known concentration of this monitor is placed into both the standard and samples before neutron irradiation. Therefore, the flux rate can be "monitored" by measuring and then correcting differences in the generated specific activity of the monitor. As a result, the accuracy and precision of measuring the unknown mass is further enhanced, as follows

$$m = R\left(\frac{m_0}{R_0}\right)\left(\frac{R_{0,m}}{m_{0,m}}\right)\left(\frac{m_m}{R_m}\right) \quad (3a)$$

where R_m and $R_{0,m}$ are the count rates of the monitor in the sample and standard, respectively, and m_m and $m_{0,m}$ are their respective masses.

The authors thank Harriet Kay for editorial assistance.

This study was supported by a Phase I Small Business Innovation Research Grant from the National Heart, Lung, and Blood Institute (Grant 1 R43 HL-60603-01).

REFERENCES

1. **Advisory Group of the International Atomic Energy Agency.** Quality assurance in biomedical neutron activation analysis. *Anal Chim Acta* 165: 1–29, 1984.
2. **Bland JM and Altman DG.** Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1: 307–310, 1986.
3. **Bowen HJM.** Activation analysis. In: *Radiochemical Methods in Analysis*, edited by Coomber DI. New York: Plenum, 1975, p. 269–296.
4. **Buckberg GD, Luck JC, Payne B, Hoffman JIE, Archie JP, and Fixler DE.** Some sources of error in measuring regional blood flow with radioactive microspheres. *J Appl Physiol* 31: 598–604, 1971.
5. **Chou FI, Lin HD, Wei JC, Wang AY, and Lo JG.** Simplified measurement of protein-bound iodine with epithermal neutron activation analysis. *Nucl Med Biol* 20: 631–636, 1993.

6. **Connelly CM, Vogel WM, Hernandez YM, and Apstein CS.** Movement of the necrotic wavefront after coronary occlusion in the rabbit. *Am J Physiol Heart Circ Physiol* 243: H682–H690, 1982.
7. **Falley MP, Anderson DL, Zoller WH, Gordon GE, and Lindstrom RM.** Neutron-capture prompt gamma-ray activation analysis for multielement determination in complex samples. *Anal Chem* 51: 2209–2221, 1979.
8. **Hale SL, Alker KJ, and Kloner RA.** Evaluation of nonradioactive, colored microspheres for measurement of regional myocardial blood flow. *Circulation* 78: 428–434, 1988.
9. **Heydorn K.** *Neutron Activation Analysis for Clinical Trace Element Research*. Boca Raton, FL: CRC, 1984, p. 1–209.
10. **Heymann MA, Payne BD, Hoffman JIE, and Rudolph AM.** Blood flow measurement with radionuclide-labeled particles. *Prog Cardiovasc Dis* 20: 55–79, 1977.
11. **Kim HW, Yu YJ, and Greenburg AG.** Iron-58 and neutron activation analysis: a non-radioactive method for tracing hemoglobin iron. *Artif Cells Blood Substit Immobil Biotechnol* 22: 619–624, 1994.
12. **Kitchin KT and Brown JL.** Incorporation of 5-iodo-2′-deoxyuridine and 5-bromo-2′-deoxyuridine into rodent DNA as determined by neutron activation analysis. *Anal Biochem* 229: 180–187, 1995.
13. **Knoll GF.** General properties of radiation detectors. In: *Radiation Detection and Measurement*, edited by Ierardi LC. New York: Wiley, 1979, p. 79–103.
14. **Kowallik P, Schulz R, Guth BD, Schade A, Paffhausen W, Gross R, and Heusch G.** Measurement of myocardial blood flow with multiple colored microspheres. *Circulation* 83: 974–982, 1991.
15. **Landsberger S, Larson S, and Wu D.** Determination of airborne cadmium in environmental tobacco smoke by instrumental neutron activation analysis with a compton suppression system. *Anal Chem* 65: 1506–1509, 1993.
16. **Miura T, Downey JM, Ooiwa H, Ogawa S, Adachi T, Noto T, Shizukuda Y, and Iimura O.** Progression of myocardial infarction in a collateral flow deficient species. *Jpn Heart J* 30: 695–708, 1989.
17. **Mori H, Haruyama S, Shinozaki Y, Okino H, Iida A, Takanashi R, Sakuma I, Husseini WK, Payne BD, and Hoffman JIE.** New nonradioactive microspheres and more sensitive X-ray fluorescence to measure regional blood flow. *Am J Physiol Heart Circ Physiol* 263: H1946–H1957, 1992.
18. **Morita Y, Payne BD, Aldea GS, McWatters C, Husseini W, Mori H, Hoffman JIE, and Kaufman L.** Local blood flow measured by fluorescence excitation of nonradioactive microspheres. *Am J Physiol Heart Circ Physiol* 258: H1573–H1584, 1990.
19. **Neer R, Tully G, Schatz P, and Hnatowich DJ.** Use of stable ^{48}Ca in the clinical measurement of intestinal calcium absorption. *Calcif Tissue Res* 26: 5–11, 1978.
20. **Ng K-H, Bradley DA, Looi L-M, Mahmood CS, and Wood AK.** Differentiation of elemental composition of normal and malignant breast tissue by instrumental neutron activation analysis. *Appl Radiat Isot* 44: 511–516, 1993.
21. **Nose Y, Nakamura T, and Nakamura M.** The microsphere method facilitates statistical assessment of regional blood flow. *Basic Res Cardiol* 80: 417–429, 1985.
22. **Party E and Gershey EL.** A review of some available radioactive and non-radioactive substitutes for use in biomedical research. *Health Phys* 69: 1–5, 1995.
23. **Prinzen GW and Glenny RW.** Developments in non-radioactive microsphere techniques for blood flow measurement. *Cardiovasc Res* 28: 1467–1475, 1994.
24. **Rudolph AM and Heymann MA.** Circulation of fetus in utero: method for the studying distribution of blood flow, cardiac output and organ blood flow. *Circ Res* 21: 163–184, 1967.
25. **Sidi A and Rush W.** An alternative to radioactive microspheres for measuring regional myocardial blood flow. Part 1: colored microspheres. *J Cardiothorac Vasc Anesth* 10: 368–373, 1996.
26. **Snapka RM, Kwok K, Bernard JA, Harling OK, and Varshavsky A.** Post-separation detection of nucleic acids and proteins by neutron activation. *Proc Natl Acad Sci USA* 83: 8939–8942, 1986.
27. **Solary E, Bertrand R, Jenkins J, and Pommier Y.** Radiolabeling of DNA can induce its fragmentation in HL-60 human promyelocytic leukemic cells. *Exp Cell Res* 203: 495–498, 1992.
28. **Van Oosterhout MFM, Willigers HMM, Reneman RS, and Prinzen FW.** Fluorescent microspheres to measure organ perfusion: validation of a simplified sample processing technique. *Am J Physiol Heart Circ Physiol* 269: H725–H733, 1995.
29. **Versieck J and Vanballenberghe L.** Determination of tin in human blood serum by radiochemical neutron activation analysis. *Anal Chem* 63: 1143–1146, 1991.
30. **Wester PO.** Concentration of 24 trace elements in human heart tissue determined by neutron activation analysis. *Scand J Clin Lab Invest* 17: 357–370, 1965.
31. **Zecca L, Pietra R, Goj C, Mecacci C, Redice D, and Sabbioni E.** Iron and other metals in neuromelanin, substantia nigra, and putamen of human brain. *J Neurochem* 62: 1097–1101, 1994.