Measurement of organ blood flow with coloured microspheres in the rat

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Objective: The aim was to establish a method for measuring organ blood flow in rats using commercially available, coloured, dye extraction microspheres. **Methods:** A mixture of radiolabelled and dye extraction microspheres was infused into rats at rest (basal) and during intravenous administration of either angiotensin II (0.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$) or isoprenaline [12.5 $ng \cdot (g^{0.74})^{-1} \cdot min^{-1}$]. Tissues were removed and placed in test tubes, counted for radioactivity, then digested with 2N sodium hydroxide. Within the same tube, microspheres were isolated using centrifugation and the dye was extracted with dimethylformamide. The dye was quantified by spectrophotometry. **Results:** Recovery of microspheres averaged greater than 95% for all tissues studied; larger reagent volumes were required to achieve this level of recovery from white adipose tissue. Statistical analyses showed excellent correlations between blood flow values obtained by the dye extraction and radiolabelled microsphere techniques. Blood flow values obtained with the radioactive technique tended to be slightly higher. There were no differences in the results obtained with the two techniques when they were simultaneously used to measure changes in organ blood flow induced by angiotensin II or isoprenaline. **Conclusions:** The coloured, dye extraction microsphere technique accurately measures organ blood flow in rats. This technique is potentially useful for estimating blood flow in any animal, even if tissue sample size is limited.
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Coloured microspheres, which have been used to measure myocardial blood flow in dogs and pigs,[1 2] have obvious environmental and economic advantages compared to radiolabelled microspheres. However, a non-radioactive, coloured microsphere technique has not been reported for smaller species. Our goal was to establish a method for measuring organ blood flow in rats using commercially available, coloured, dye extraction microspheres.

The principles involving the use of radiolabelled microspheres,[3] including even mixing and distribution in the circulation, entrapment in tissues, and lack of haemodynamic effect, also apply to coloured microsphere techniques. With both radiolabelled and coloured microsphere techniques, the precision of the blood flow estimate is dependent on the number of microspheres in the sample.[4 5]

Coloured dye extraction and radiolabelled microsphere techniques differ in the way the number of microspheres is determined. Dye extraction microspheres must first be separated from tissues, and then quantified by using a spectrophotometer to measure the concentration of extracted dye. Radiolabelled microspheres do not have to be separated from tissue and are quantified with a gamma counter. These differences between techniques were addressed by Kowallik *et al*, who used dye extraction microspheres to measure myocardial blood flow in dogs and pigs.[2]

Variations in tissue composition and the small mass of most rat tissues present additional challenges when using dye extraction microspheres to measure organ blood flow in rats. The separation and recovery of microspheres from some tissues is more difficult than it is from myocardium. Also the number of microspheres per sample may be low due to the small mass and low flow rates of some tissues. Low numbers of dye extraction microspheres may lead to difficulty in detecting the absorbance produced by the extracted dye and contribute to a lack of precision in blood flow estimates.

We describe a coloured, dye extraction microsphere method of organ blood flow measurement in rats. The method includes a centrifugation technique to recover microspheres from hydroxide digested tissue and a way to determine percentage recovery of microspheres. We show how to calculate, based on the individual dye characteristics, the number of each colour of microsphere required in the infusion to produce a detectable signal in the tissue sample. We also discuss how to maximise the absorption signal produced by the dye extracted from the microspheres. Finally, simultaneous intraventricular infusions of radiolabelled and dye extraction microspheres were used for a direct comparison of the two techniques for measuring organ blood flow.

Methods

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Rat surgery

Overnight fasted male Sprague-Dawley rats of 300-400 g weight were anaesthetised with a mixture of sodium pentobarbitone, 30 $mg \cdot kg^{-1}$, chloral hydrate, 125 $mg \cdot kg^{-1}$, and magnesium sulphate, 63 $mg \cdot kg^{-1}$, given intraperitoneally. A catheter of PE50 tubing was positioned in the abdominal aorta by way of the femoral artery. A second catheter was inserted into the left ventricle via the right carotid artery. Proper positioning of the left ventricular catheter was determined by observing the characteristic left ventricular pressure waveform at surgery; verification of the position was made at necropsy. A third catheter, for drug infusion, was placed in the left jugular vein of rats that received angiotensin II or isoprenaline infusions. The catheters were filled with saline containing 20 $U \cdot ml^{-1}$ heparin, anchored with silk sutures, and exteriorised at the back of the neck. Microspheres were not infused until at least 3 h after completion of surgery, when the rats appeared alert and active.

Microspheres

Yellow, blue, and red 15 μm dye extraction Dye-Trak microspheres were obtained from Triton Technology. Radiolabelled 15 μm

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microspheres, labelled with ^{51}Cr, ^{46}Sc, or ^{113}Sn, were obtained from New England Nuclear. Reagents for the tissue digestion and microsphere isolation procedure were obtained from E-Z Trac.

Microsphere dilutions
To estimate accurately the numbers of dye extraction microspheres in the infusates and in the suspensions of microspheres used in recovery and dye content determinations, 5-10 identical dilutions of the commercial suspensions were prepared with 0.01% Tween 80 (Sigma). Samples of each of the dilutions were loaded into 3.2 μl Fuchs-Rosenthal haemacytometer chambers (C A Hauser and Son) and the microspheres were counted with an image analyser (Optomax) interfaced with a Nikon Optiphot microscope. The mean concentration of microspheres in these preparations was used as a basis for calculating numbers of microspheres for infusions and recovery determinations. To determine average dye content of the microspheres, 200 μl of the diluted commercial suspensions were taken with precalibrated pipettes within 3 s after slowly inverting the suspensions five times. These aliquots were placed in 1.5 ml centrifuge tubes and were centrifuged, dried, and extracted with dimethylformamide. The mean absorbance was determined with a spectrophotometer and divided by the average microsphere concentration to obtain an average dye concentration [absorbance units·(sphere·ml^{-1})$^{-1}$].

Microsphere infusions
The rats were placed in cylindrical acrylic restraint tubes, where they remained quietly for the duration of the experiment. The abdominal aorta and left ventricular catheters were connected to pressure transducers positioned at the level of the heart. Heart rate was measured using a biotachometer triggered by the arterial pressure waveform. Left ventricular pressure, arterial pressure, and heart rate were continuously recorded, except during infusion of microspheres and withdrawal of reference blood samples.

The infusates contained a mixture of radiolabelled and dye extraction microspheres; the two types of microsphere were therefore infused simultaneously and under the same conditions. Vials of commercial stock suspensions of both types of microsphere were sonicated for 3 to 5 min and inverted several times immediately before use. Microsphere infusates were diluted to the desired concentration with 0.9% sodium chloride containing 0.01% Tween 80. A 75 cm long piece of coiled PE-50 tubing[6] was then filled with 180 μl of the infusate. The coil was interposed between the left ventricular catheter and a 1 ml syringe. The syringe contained 0.5 ml of prewarmed (40°C) saline and was mounted in a variable speed infusion/withdrawal pump. With a second pump, reference blood samples were drawn from the abdominal aorta catheter at a rate of 0.84 ml·min^{-1} into preweighed 1 ml disposable syringes containing a drop of heparin saline in the tip. The reference blood withdrawal was started 10 s before the microsphere infusion was begun, and continued for 75 s. The microspheres and the saline flush were infused for 50 s at a rate of 0.6 ml·min^{-1}. The microsphere suspension (0.18 ml) was flushed from the infusion coil and into the left ventricle during the first 20 s of the infusion. The volume of blood drawn for the reference sample was replaced as saline during the coil flush and when the catheters were reconnected to the pressure transducers.

Simultaneous blood flow measurements with radiolabelled and dye extraction microspheres
In the first set of experiments the radioactive and dye extraction techniques were compared by infusing a mixture of 100 000 yellow dye extraction and 50 000 ^{51}Cr labelled radioactive microspheres into the left ventricle of six rats. These numbers represent the minimum number of dye extraction microspheres, as calculated for yellow (see table III), and the minimum number of radiolabelled microspheres, as suggested by published values.[7]

In the second set of experiments, radioactive and dye extraction techniques were compared using equal numbers of microspheres during intravenous administration of saline and again during intravenous administration of either angiotensin II or isoprenaline. In five rats, a mixture of 200 000 each of red dye extraction and ^{46}Sc labelled radioactive microspheres was infused 5 min after starting an intravenous administration of 0.9% sodium chloride (0.2 ml·min^{-1}). About 15 min after the first infusion of microspheres, the rats received an intravenous infusion of angiotensin II (Sigma). Five minutes after the start of the angiotensin II infusion, a mixture containing 200 000 each of yellow dye extraction and ^{113}Sn labelled radioactive microspheres was infused into the left ventricle. The dose of angiotensin II used, 0.5 μg·kg^{-1}·min^{-1}, was chosen because it consistently increased mean arterial pressure and decreased heart rate in preliminary experiments; a similar dose has previously been used in regional blood flow studies.[8] In the isoprenaline experiments, basal regional blood flows were determined by infusing a mixture of 200 000 each of red dye extraction and ^{46}Sc labelled radioactive microspheres into five rats which were being intravenously infused with ascorbate-saline vehicle (0.1 mg·ml^{-1} L-ascorbic acid in normal saline, pH 7.4, 0.2 ml·min^{-1}). The same rats were infused with 200 000 each of yellow dye extraction and ^{113}Sn labelled radioactive microspheres 30 min after the beginning of an intravenous administration of ±isoprenaline (Sigma) at a dose of 12.5 ng·(g$^{0.74}$)$^{-1}$·min^{-1}, as reported by Wickler *et al.*[9]

In an additional six rats, a second femoral catheter was implanted to monitor arterial pressure and heart rate during the microsphere infusion protocol. Two consecutive infusions of 440 000 dye extraction microspheres each had no effect on heart rate or arterial pressure.

Reference blood sample processing
The syringes containing the reference blood samples were weighed and the samples were emptied into conical 15 ml polypropylene tubes. The syringes were thoroughly rinsed with 4 ml of Blood Hemolysis Reagent (E-Z Trac No 108) which was added to the polypropylene tubes and mixed by inversion. Neither radiolabelled nor dye extraction microspheres were detectable in the reference blood syringes following this rinse. The tubes were then centrifuged for 30 min at 2000 *g*, and assayed for radioactivity in a gamma counter. The supernatants were drawn off and discarded, and the tubes were included in the tissue processing routine as described below.

Tissue digestion and processing
The procedure used for processing tissue and recovery of the microspheres was adapted from Hale *et al*[1] and is depicted in fig 1. All processing was carried out within the same tube; microspheres were not transferred from the tube into which the tissue samples were first placed.

In the first set of experiments, blood flow to the cardiac ventricles, kidneys, stomach, and proximal duodenum was determined. In the angiotensin and isoprenaline experiments, interscapular brown adipose tissue and samples of lung, liver, psoas muscle, and abdominal white adipose were also removed. Because lung tisssue was not consistently sampled to exclude the bronchial circulation, lung blood flow data were not analysed for drug effects.

The rats were killed by an overdose of pentobarbitone. The tissues were removed, blotted, placed into preweighed conical 15 ml polypropylene tubes, weighed, and assayed for radioactivity in a gamma counter. The raw counts were corrected for overlap by a computer program which was resident in the gamma counter and which used the method of simultaneous equations.[10] Tissue weights typically ranged between 0.5 and 1.5 g. Four millilitres of 2N sodium hydroxide were then added to each of the tubes containing tissues, and 2 ml of the hydroxide solution were added to the centrifuged reference blood samples. The tubes were capped and heated in a 90°C water bath, with vortexing every 10 to 15 min, until the tissue was dissolved (typically 45-60 min). Upon removal from the water bath, 10 ml (3 ml for reference blood samples) of Digest Reagent II (E-Z Trac No 106) were added to each tube. The tubes were mixed by inversion, and centrifuged at 2000 *g* for 30 min. The supernatants were drawn off, 10 ml (2 ml for reference blood) of Microsphere Counting Reagent (E-Z Trac No 107) were added to the tubes, and the tubes were vortexed. The tubes were reheated in the 90°C water bath, with

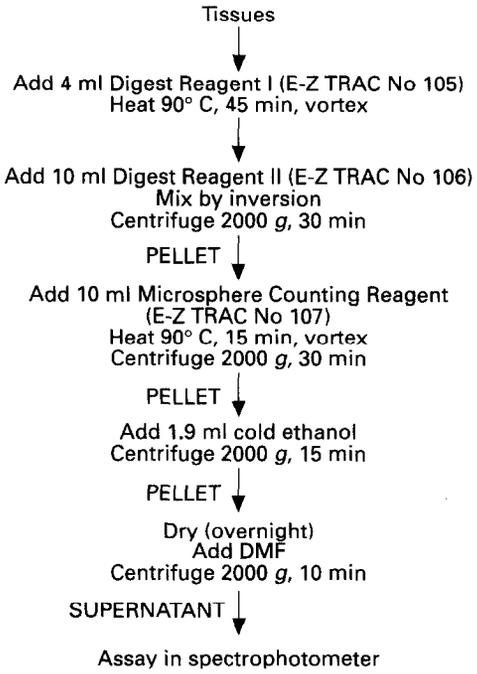

Figure 1 *Flow chart for sample digestion and microsphere isolation procedure.*

vortexing every 5 to 10 min, until any remaining tissue debris dissolved. The samples were then centrifuged at 2000 *g* for 30 min. The supernatants were aspirated, leaving about 100 μl in each tube, and 1.9 ml of ice cold absolute ethanol added. The tubes were vortexed and centrifuged at 2000 *g* and 4°C for 15 min. Following the ethanol wash, most of the ethanol was aspirated off; the remainder (about 100 μl) was allowed to evaporate overnight.

Dye extraction and measurement

To extract the dyes from the isolated, dried microspheres, 500 μl of dimethylformamide were added to each centrifuge tube, and the tube was briefly but vigorously vortexed. The samples were centrifuged (2000 *g*, 10 min) and the absorbance of the supernatant determined with a spectrophotometer using a 2 nm slit width. The absorption spectrum peaks for the yellow, red, and blue dyes are at 448 nm, 530 nm, and 672 nm, respectively. In the most recent experiments, dyes were extracted using 400 μl of dimethylformamide containing 0.5% Tween 80. These extracts (300 μl) were loaded into a 96-well quartz microplate and absorbances determined with a microplate reader. Ten namometre bandwidth filters, centered at 450 nm, 530 nm, and 670 nm, were used to quantify dye extracted from the yellow, red, and blue microspheres, respectively. With both methods of spectrophotometry, the absorbances at the red and yellow peak wavelengths were corrected for overlap from the blue and red dyes, respectively, using spectral stripping.[3] The minimum acceptable corrected absorbance was arbitrarily set at 0.010 absorbance units (AU). Several samples of psoas muscle yielded less than 0.010 AU of extracted dye. Data from these samples were excluded, and psoas muscle was not included in the pre-post drug comparisons.

Calculation of blood flow

The volume of reference blood collected was calculated by dividing the net weight of the reference sample by 1.05 $g \cdot ml^{-1}$, the specific gravity of blood. The flow rate of the reference sample, in $ml \cdot min^{-1}$, was calculated by dividing the reference blood volume by the time required for the withdrawal. For each infusion, the quotient of the reference blood flow rate divided by the activity of the reference sample was used as the basis for calculating tissue flow rates: $Q_s = A_s(Q_r/A_r)$. Q_r and Q_s represent flow in the reference blood and sample tissue, respectively, and A_r and A_s represent the activity, in $counts \cdot min^{-1}$ or AU, in the reference blood and sample tissue, respectively. Blood flow rates were divided by tissue weights to yield $ml \cdot min^{-1} \cdot g^{-1}$.

Determination of microsphere recovery

Before addition of the sodium hydroxide, 100 μl of a suspension of blue microspheres ($50\,000 \cdot ml^{-1}$) were added to each of the tubes containing the tissue and reference blood samples from the isoprenaline treated rats. The same amount was also added to each of 10 empty polypropylene tubes to serve as recovery standards. Tissue and blood samples were then processed as described above. The microspheres in the standards were washed with 100% ethanol and centrifuged at 2000 *g* for 15 min; the pellets were dried overnight and the blue dye extracted with dimethylformamide. The absorbances of the samples and standards at 670 nm were measured with a microplate reader. Percentage recovery was calculated for each tissue by dividing the mean absorbance of the tissue by the mean absorbance of the standards and multiplying by 100.

Statistical analyses

The correlations between basal flow rates measured by dye extraction and radiolabelling microsphere techniques were determined with least squares linear regression. The basal flows obtained with the two methods were also compared by the statistical method of Bland and Altman,[11] which describes the agreement between two methods based on the differences between individual measurements. Changes in regional blood flow due to administration of angiotensin II or isoprenaline and measured with the dye extraction technique were compared using one factor analysis of variance (ANOVA) to the changes in flow measured simultaneously with the radioactive technique. Dunnett's multiple comparison test was used to analyse microsphere recovery by comparing the absorbances (670 nm) of the tissue and blood samples with those of the standards. A P value of 0.05 was considered statistically significant. Unless otherwise stated, values are given as *mean*(SD).

Results

Recovery of microspheres

The heights of the columns in fig 2 indicate the recovery of blue microspheres from the standards and the tissue and reference blood samples. The mean absorbance of the extracts from the 10 recovery standards was 0.197(SD 0.007)

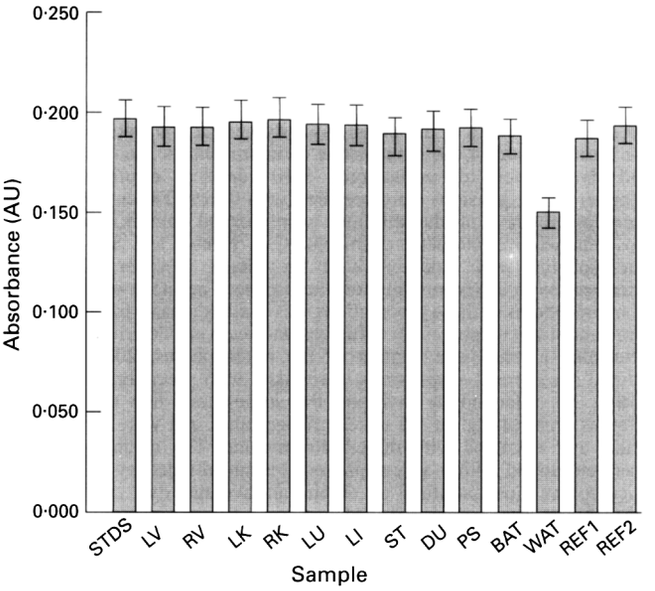

Figure 2 *Mean absorbance of dye extracted from blue microspheres isolated from tissues of five rats. Error bars = SD. STDS = recovery standards; LV and RV = left and right ventricles; LK and RK = left and right kidneys; LU = lung; LI = liver; ST = stomach; DU = duodenum; PS = psoas; BAT and WAT = brown and white adipose; REF1 and REF2 = reference blood samples. The mean absorbance of the standards, 0.197 AU, represents 100% recovery.*

AU (100% recovery). Processing of tissue samples resulted in a decrease in the absorbance only for white adipose tissue; the average recovery for every other sample type was greater than 95%. The mean absorbance of the white adipose tissue [0.150(0.016) AU, 76% recovery] was significantly lower than that of the standards. Because of the incomplete recovery of microspheres from this tissue, blood flow data for white adipose were omitted from further analyses.

In a subsequent experiment, we assessed the effect of larger volumes of processing reagents on the recovery of microspheres from white adipose tissue. By processing the tissue in 50 ml centrifuge tubes and increasing the volume of reagents threefold, the average recovery rose from 67.5% to 95.3%.

Comparison of radiolabelling and dye extraction microsphere techniques to measure basal blood flows

Correlations between blood flow values obtained with the radiolabelling and dye extraction microsphere methods were determined by linear regressions of the basal flow rates. The regression lines and plots of the residuals (dye extraction flow observed minus dye extraction flow predicted by regression) are shown in fig 3. The correlation coefficient for the yellow dye extraction *v* ^{51}Cr radioactive microsphere comparison is 0.983 (fig 3A), and for the red dye extraction *v* ^{46}Sc microsphere comparison, 0.982 (fig 3B). The slopes of the regression lines for these two comparisons are 0.90(0.02) and 0.93(0.03), and the intercepts are 0.08 and 0.05, respectively. For each regression, the residuals are uniformly distributed above and below the line $y = 0$ (fig 3C, 3D), supporting the use of linear regression analysis with these data.

In fig 4 the data are compared according to the statistical method of Bland and Altman.[11] For the yellow dye extraction *v* ^{51}Cr radioactive microsphere comparison, the mean difference in flows determined by the two micosphere methods (Q_{RA}-Q_{DE}) is 0.34 $ml \cdot min^{-1} \cdot g^{-1}$ (SD 0.46, fig 4A).

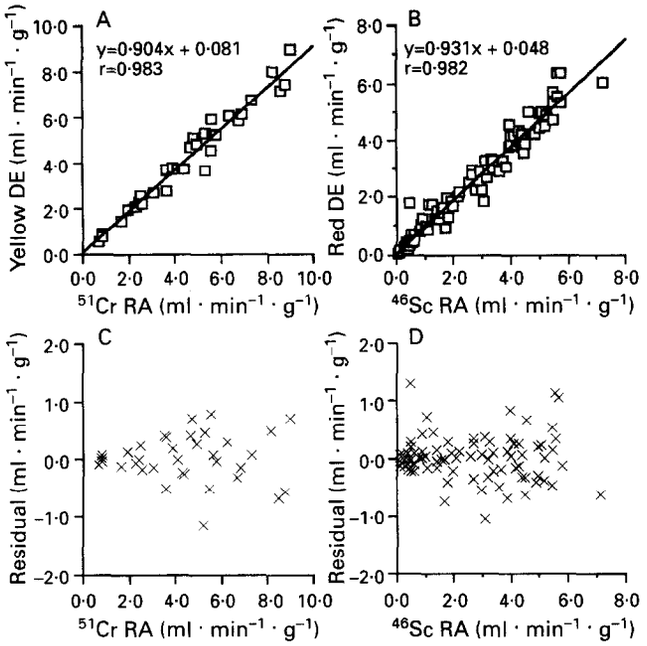

Figure 3 *Linear regressions of the basal flows measured simultaneously with ^{51}Cr radioactive (RA) and yellow dye extraction (DE) microspheres (A; 36 tissue samples from six rats) or ^{46}Sc RA and red DE microspheres (B; 92 samples from 10 rats). Plots of the residuals determined from the regression analyses of ^{51}Cr and yellow microspheres (C) or ^{46}Sc and red microspheres (D).*

Assuming a normal distribution, 95% of differences between the two techniques will lie within 2 SD of the mean, or between –0.58 and 1.26 ml·min^{-1}·g^{-1}. Similarly, the mean difference in flows for the red dye extraction *v* ^{46}Sc radioactive microsphere comparison (fig 4B) is 0.13 ml·min^{-1}·g^{-1} (SD 0.39), and the 95% interval ranges from –0.65 to 0.91 ml·min^{-1}·g^{-1}.

Comparison of radiolabelled and dye extraction microsphere techniques to measure changes in blood flow induced by angiotensin II and isoprenaline

Regional blood flow values, obtained by infusing mixtures of dye extraction and radiolabelled microspheres before and during intravenous administration of angiotensin II and isoprenaline, are shown in tables I and II, respectively. Angiotensin II (0.5 μg·kg^{-1}·min^{-1}) increased mean arterial pressure from an average of 122(14) to 153(18) mm Hg, and decreased heart rate from an average of 380(32) to 330(29) beats·min^{-1}. Angiotensin II-induced decreases in blood flow to the left ventricle, kidneys, stomach, duodenum, and brown adipose tissue were detected by both techniques.

The intravenous infusion of isoprenaline [12.5 ng·(g$^{0.74}$)$^{-1}$·min^{-1}] reduced mean arterial pressure from 126(13) to 96(9) mm Hg, and increased the average heart rate from 382(25) to 526(27) beats·min^{-1}. Both radiolabelling and dye extraction microsphere techniques detected a greater than twofold increase in blood flow to the ventricles, and a greater than eightfold increase in flow to the brown adipose tissue. Blood flow to the kidneys and stomach decreased in response to isoprenaline. These effects are similar to those previously reported in rats given the same dose of isoprenaline.[9]

The angiotensin II and isoprenaline induced changes in blood flow that were determined with dye extraction microspheres were not significantly different from the changes in flow measured with radiolabelled microspheres.

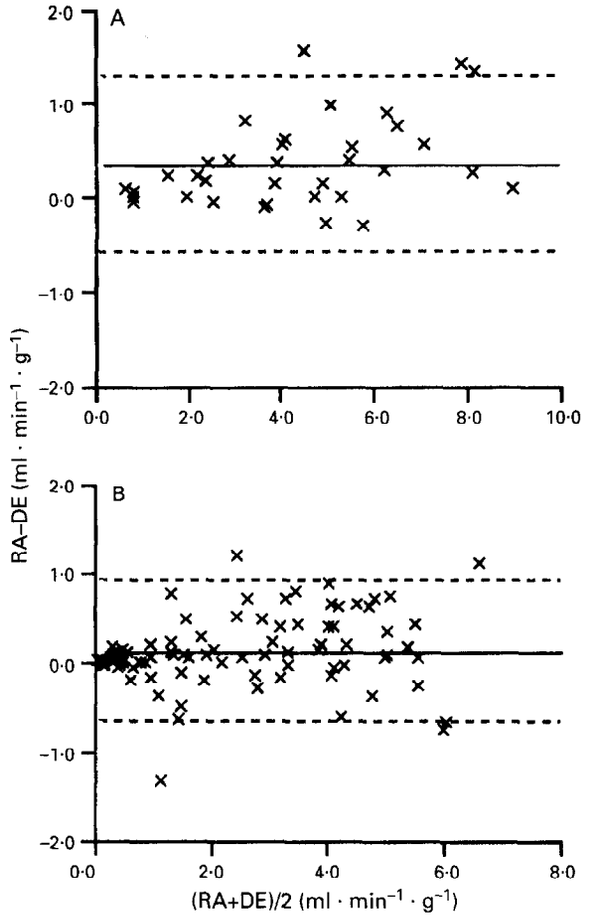

Figure 4 *Comparison by the method of Bland and Altman[11] of basal blood flows determined by ^{51}Cr radioactive (RA) and yellow dye extraction (DE) microspheres (A) or ^{46}Sc RA and red DE microspheres (B). The mean differences in flows (radioactive minus dye extraction), 0.34 ml·min^{-1}·g^{-1} (A), and 0.13 ml·min^{-1}·g^{-1} (B), are indicated by the solid lines. Dashed lines are 2 SD from the mean.*

Table I *Effect of angiotensin II (AII), 0.5 μg·kg^{-1}·min^{-1}, on regional blood flow (ml·min^{-1}·g^{-1}) in rats. Values are mean(SEM), n = 5 rats per group. Vehicle = 0.9% saline.*

Tissue		*Vehicle*	*AII*	*Change*	*P*
Left ventricle	RA	4.7(0.9)	3.9(1.0)	–0.8(1.6)	
	DE	4.2(1.1)	3.4(1.0)	–0.8(1.8)	0.985
Right ventricle	RA	4.4(1.8)	4.3(2.2)	–0.1(2.6)	
	DE	3.9(1.3)	3.9(1.8)	0.0(2.0)	0.969
Left kidney	RA	4.6(0.7)	1.2(0.3)	–3.3(0.6)	
	DE	4.3(0.7)	1.1(0.2)	–3.2(0.6)	0.670
Right kidney	RA	4.3(1.1)	1.1(0.3)	–3.2(0.8)	
	DE	3.9(0.9)	1.0(0.3)	–2.9(0.7)	0.622
Liver	RA	0.4(0.2)	0.4(0.2)	0.0(0.1)	
	DE	0.3(0.2)	0.4(0.1)	0.1(0.1)	0.052
Stomach	RA	1.0(0.3)	0.3(0.1)	–0.7(0.3)	
	DE	0.8(0.3)	0.3(0.1)	–0.5(0.3)	0.397
Duodenum	RA	2.3(1.0)	1.1(0.6)	–1.2(0.7)	
	DE	2.2(0.9)	1.0(0.5)	–1.1(0.7)	0.576
Brown adipose tissue	RA	4.3(3.3)	0.3(0.2)	–4.0(3.1)	
	DE	3.6(3.4)	0.5(0.4)	–3.1(3.0)	0.690

RA = radioactive; DE = dye extraction.
P = probability of no difference in change in flow as measured by dye extraction *v* radiolabelled microspheres, determined by ANOVA.

Table II *Effect of isoprenaline, 12.5 ng·(g$^{0.74}$)$^{-1}$·min^{-1}, on regional blood flow (ml min^{-1} g^{-1}) in rats. Values are mean(SEM), n = 5 rats per group. Vehicle = ascorbate-saline.*

Tissue		*Vehicle*	*Isoprenaline*	*Change*	
					P
Left ventricle	RA	4.6(0.8)	11.0(3.7)	7.3(2.3)	
	DE	4.6(1.1)	11.5(3.7)	6.8(4.4)	0.842
Right ventricle	RA	4.3(0.8)	11.1(3.7)	6.9(3.6)	
	DE	3.8(0.7)	11.1(4.7)	7.3(4.7)	0.883
Left kidney	RA	3.6(0.8)	2.6(0.4)	−1.1(1.5)	
	DE	3.8(0.9)	2.8(0.6)	−1.0(1.6)	0.936
Right kidney	RA	3.5(0.8)	2.5(1.0)	−1.1(1.1)	
	DE	3.8(0.7)	2.8(1.3)	−1.0(1.0)	0.907
Liver	RA	0.3(0.2)	0.3(0.2)	0.0(0.2)	
	DE	0.4(0.2)	0.3(0.2)	0.0(0.2)	0.860
Stomach	RA	0.5(0.2)	0.3(0.2)	−0.2(0.2)	
	DE	0.6(0.4)	0.3(0.1)	−0.3(0.2)	0.590
Duodenum	RA	2.0(0.6)	1.6(0.8)	−0.4(1.2)	
	DE	1.7(0.2)	1.8(0.7)	0.0(0.9)	0.518
Brown adipose tissue	RA	1.2(1.0)	10.3(3.4)	9.1(3.2)	
	DE	1.3(0.8)	10.5(4.3)	9.2(4.6)	0.963

RA = radioactive; DE = dye extraction.
P = probability of no difference in change in flow as measured by dye extraction *v* radiolabelled microspheres, determined by ANOVA.

Discussion

We have demonstrated a coloured, dye extraction microsphere technique which can be used for the accurate measurement of rat organ blood flow. The simultaneous left ventricular infusion of dye extraction and radiolabelled microspheres made it posible to compare directly blood flow data obtained with the two methods in rats. Regression analyses confirm linear relationships between basal blood flow values obtained with the two types of microspheres. Regression line slopes were slightly less than unity (0.90 and 0.93), suggesting a small difference in blood flow values obtained with the radiolabelling and dye extraction microsphere methods. A similar result (slope = 0.92) was obtained by Kowallik *et al*[2] when they compared blood flow values obtained with dye extraction and radiolabelled microspheres in dogs. Our studies do not provide any data which might help explain why the flows obtained with radiolabelled microspheres are slightly higher than those obtained with dye extraction microspheres. It is interesting to note, however, that the regressions reported here, which show the correlation in flows obtained with simultaneous infusion of dye extraction and radiolabelled microspheres, are not unlike those reported by Bassingthwaighte *et al*,[12] who compared the myocardial deposition of two types of radiolabelled microspheres simultaneously infused in rabbits.

The Bland and Altman[11] type of analysis of the basal blood flow data obtained with the two techniques showed good agreement. Consistent with the regression slopes, the flows determined by the radioactive method were slightly higher. The standard deviations of the differences between dye extraction and radioactive techniques (about 0.4 ml·min^{-1}·g^{-1}) are similar to those reported by Glenny *et al* in their comparison of radiolabelled and fluorescent microspheres,[13] but somewhat higher than that reported by Mori *et al*,[14] who compared radioactive and nonradioactive *x* ray fluorescence techniques of myocardial blood flow measurement in dogs. The reason underlying the wider range of differences in the dye extraction *v* radioactive comparison is unclear; the fact that we examined flows in a variety of tissues, while Mori *et al*[14] compared the radioactive and *x* ray fluorescence techniques only in canine myocardial tissue, may play a role.

Use of microsphere techniques to measure organ blood flow in the rat requires attention to conditions that are not present when measuring blood flow in dog or pig myocardium. With either radiolabelled or dye extraction microsphere techniques, some of the rat tissues of interest are small, and the number of microspheres trapped may be fewer than desired. About 400 microspheres should be present to maintain a 95% confidence level that the precision of the resulting blood flow estimate will be within 10%.[4 5] In larger animals, the solution to low sphere numbers in tissue samples is typically to increase the total number of microspheres infused. However, in small animals such as rats, increasing the number of microspheres infused is more likely to produce excessive embolisation of tissue and haemodynamic perturbations.[3] In small or large animals, increasing the number of experiments can help to offset the lower precision resulting from smaller numbers of spheres in individual flow estimates.[4] In the present study, when each tissue flow was determined in five rats, there were no significant differences between the radiolabelled and dye extraction microsphere techniques in the measurement of regional blood flow changes induced by intravenous angiotensin II or isoprenaline.

The dye extraction microsphere method is more limited than the radioactive method because more dye extraction microspheres are required to produce a minimum detectable signal. Counting accuracy can be improved with radiolabelled microspheres by counting the radioactive decay for longer periods of time. With dye extraction microspheres, the spectrophotometric signal produced by small numbers of microspheres may not be detectable over background. In the present study the number of microspheres in some psoas muscle samples was insufficient to yield the absorbance limit of 0.010 AU. This result underscores the need to maximise the signal obtained from dye extraction microspheres.

The signal produced by dye extraction microspheres can be maximised by using the colours of microspheres that produce the greatest absorption after dye extraction, and by using the smallest extraction volume possible. The minimum extraction volume depends on the spectrophotometry system. Of the dye extraction microspheres currently available, red has the highest "useful" dye content, averaging 2.59×10^{-5} AU·(sphere·ml^{-1})$^{-1}$. Dividing the lowest acceptable absorbance of 0.010 AU by the average dye content yields a concentration of 386 spheres·ml^{-1}. If the dye were extracted with 0.5 ml of dimethylformamide, 193 microspheres would be necessary to produce the acceptable absorbance.

Table III shows the number of each of the available colours of Dye-Trak dye extraction microspheres necessary to produce 0.010 AU with an extraction volume of 0.5 ml. Although we have quantified no more than three colours of

Table III *Numbers of dye extraction microspheres required in 0.5 ml to obtain minimum absorbance of 0.010 AU and to measure tissues perfused at 0.1 or 0.2 ml·min^{-1}, assuming a cardiac output of 100 ml·min^{-1}.*

Colour	*Dye content*[a]	*0.010 AU*	*Infusion required for*	
			0.1 ml·min^{-1}	*0.2 ml·min^{-1}*
Red	2.5905	193	193 000	96 500
Yellow	2.5740	194	194 000	97 000
White	1.7831	280	280 000	140 000
Orange	1.0378	482	482 000	241 000
Blue	1.0209	490	490 000	245 000
Eosin	0.8124	615	615 000	307 500
Violet	0.3166	1579	1 579 000	789 500

[a] $\frac{(AU)}{(spheres \cdot ml^{-1})} (\times 10^5)$

dye extraction microspheres simultaneously, Kowallik *et al*[2] quantified and separated five colours. Orange and eosin dye extraction microspheres have since become available. However, because eosin and red have nearly identical absorption peaks (~530 nm), they cannot be used in the same experiment.

Also shown in table III is the minimum number of each colour microsphere that would have to be infused into a rat with a cardiac output of 100 ml·min^{-1} to measure flow in a tissue perfused at either 0.1 ml·min^{-1} or 0.2 ml·min^{-1}. Microspheres having a low useful dye content are required in large numbers to measure blood flow, but the infusion of too many microspheres could produce haemodynamic effects in rats. Flaim *et al*[15] have reported that infusion of up to 8.5×10^5 microspheres does not cause haemodynamic effects in rats. In our preliminary studies, two consecutive infusions of 4.4×10^5 dye extraction microspheres had no effect on arterial pressure or heart rate in rats. Thus the total number of dye extraction microspheres to be infused must be carefully considered, and depends on the colours of the microspheres to be used, the number of infusions, and the estimated number of trapped microspheres in the tissues of interest having the lowest flows or the smallest mass or both.

Because the number of dye extraction microspheres infused is crucial in rat blood flow studies, it is important that microspheres do not become lost from the infusate by being trapped in syringes. Fortunately, the use of a coiled PE-50 tubing attached to an infusion pump, as described by Stanek *et al*,[6] allows for a smooth and virtually complete infusion of dye extraction microspheres into the left ventricle.

The accuracy of blood flow estimates obtained with the dye extraction method depends on the complete recovery of the microspheres following tissue digestion. The difficulty in isolating microspheres from tissues varies depending on the tissue composition. To date, microspheres have been recovered by either centrifugation[1] or filtration,[2] but the extent of recovery with either has not been reported. By modifying a centrifugation method which had been previously reported for coloured but not dye extraction microspheres,[1] we recovered virtually all (>95%) of the microspheres in all tissues except white adipose (76% recovery). We have since determined that 95% recovery of microspheres from white adipose tissue can be achieved by increasing threefold the volumes of reagents used for tissue processing. Larger (50 ml) centrifuge tubes were required for the larger volumes. A key feature of our isolation technique is that the entire procedure is carried out within the same tube; microsphere loss due to sample transfer is non-existent.

The advantages of using non-radioactive microspheres for regional blood flow measurement have been discussed previously.[1 2 13] A significant advantage of the modified dye extraction microsphere method presented here is the low cost of required equipment. Most laboratories have ready access to a spectrophotometer, centrifuge, and water bath, which are all that is necessary for this assay. We have considerably reduced the overall time required for these measurements by using a microplate reader, which can measure absorbances of 96 samples within seconds. However, the plate reader, which costs about $12 000, is not essential since many spectrophotometers can read sample volumes of 500 μl or less. Even with the microplate reader, the cost compares favourably with that of other non-radioactive measurement systems recently described by Mori *et al*,[14] by Abel *et al*,[16] and by Glenny *et al*.[13]

In summary, there is an excellent correlation and good agreement in absolute basal flow values obtained by using the radiolabelling and the modified dye extraction microsphere techniques in rats. There were no differences in the results obtained with the two techniques when they were simultaneously used to measure changes in organ blood flow induced by angiotensin II or isoprenaline. These results validate the dye extraction microsphere technique for measuring organ blood flow in rats. This technique is potentially useful for estimating blood flow in any animal, even if tissue sample size is limited.

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Key terms: Dye extraction microspheres; coloured microspheres; radioactive microspheres; organ blood flow; rat.

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