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Fluorescent vs. radioactive microsphere measurement of regional myocardial blood flow

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Abstract

Objectives: This study compared simultaneous regional myocardial blood flow (RMBF) measurements using fluorescent microspheres (FM) and radiolabeled microspheres (RM). The utility of an internal standard during processing was also examined. **Methods:** Paired FM and RM were injected into the left atrium of 9 anaesthetised rabbits. RMBF was altered by use of either regional ischaemia or (−)-N^6-(2-phenylisopropyl)-adenosine. Radioactivity of blood reference and tissue samples was quantitated using standard methods. Samples were then digested with potassium hydroxide and microspheres recovered by vacuum filtration, with an additional label of FM as the internal standard. FM labels were extracted using Carbitol® acetate and quantitated using fluorescence spectroscopy. Agreement between the fluorescent and radioactive methods was assessed using both orthogonal regression and difference-against-mean analyses. **Results:** Using recovery-*un*corrected data, the slope of the orthogonal regression of RM and FM-determined RMBF was not statistically different from 1, but the intercept was statistically different from 0 [−0.03(0.01), $P = 0.005$] and the mean RMBF by each method differed from one another [1.24(0.08) vs. 1.17(0.08) ml · min^{-1} · g^{-1}, $P = 0.0002$]. The mean ± 2 s.d. of the differences of RMBF (RM minus FM) was +0.07 ± 0.30 ml · min^{-1} · g^{-1}. Although recovery of FM from tissue averaged 97.6(1.2)%, use of the internal standard to correct for losses substantially improved the agreement between RM and FM-determined RMBF: the orthogonal regression slope was not statistically different from 1, the intercept was not statistically different from 0, and the means of the flows were not different. The mean ± 2 s.d. of the differences of RMBF was −0.01 ± 0.22 ml · min^{-1} · g^{-1}. The internal standard also improved RMBF estimates from samples with simulated large spillage during processing. **Conclusion:** Fluorescent microspheres are an equivalent alternative to radiolabeled microspheres for the estimation of RMBF. Although the overall recovery of microspheres using this technique was high, use of an internal standard is recommended for correction of random losses.

Keywords: Microspheres, fluorescent; Microspheres, radioactive; Coronary blood flow; Rabbit, atria

1. Introduction

The use of radiolabeled microspheres for determination of regional myocardial blood flow (RMBF) has become standard [1]. As the costs of using radiolabels have increased, alternative non-radioactive microsphere methods of RMBF determination have received increasing attention. These have included labor-intensive manual counting of colored [2] or fluorescent [3] microspheres, an expensive X-ray fluorescence method [4], and more recently, tissue digestion with dye-extraction and spectroscopy of either custom-dyed colored microspheres [5] or commercially available fluorescent microspheres [6-8]. In the latter studies, fluorescent and radiolabeled microspheres were compared for the determination of RMBF in pigs [6] and dogs [7,8]. The recovery of microspheres during filtration has not been reported previously, and the recovery of microspheres using centrifugation with a sucrose cushion was only 72.1(1.4)% [3]. The present study was designed to compare RMBF measured by fluorescent and radiolabeled microsphere methods in rabbits using an internal standard to test and correct for microsphere losses. Also in contrast to previous studies [6,7], the use of the less volatile solvent 2-(2-ethoxyethoxy)ethanol acetate (Carbitol® acetate) was evaluated. Statistical analyses utilised in the present study were specific to assessing agreement, rather than correlation, between the fluorescent and radiolabeled methods. These modifications resulted in an improved method and excellent agreement between recovery-corrected fluorescent and radiolabeled microsphere-determined RMBF.

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2. Methods

Animals used in this study were allowed access to food and water ad libitum until induction of anaesthesia. With local Institutional Animal Care and Use Committee approval, all animals received humane treatment in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institute for Health (NIH publication No. 85-23, revised 1985).

2.1. Surgical preparation

Nine male New Zealand white rabbits (2.8–3.3 kg) were anaesthetised with 30 mg · kg^{-1} sodium pentobarbitone via a marginal ear vein. Anaesthesia was maintained with 10 mg supplements of intravenous pentobarbitone as needed. After tracheostomy, rabbits were mechanically ventilated (MDI ventilator, Mobile, AL). Polyethylene catheters were inserted into the right jugular vein for drug administration, right carotid artery for measurement of blood pressure, and thoracic aorta via the right femoral artery for blood reference sampling. A left thoracotomy was performed in the fourth intercostal space and the heart was suspended in a pericardial cradle. The left atrium was cannulated with a short length of polyvinyl chloride tubing (with stopcock, dead space < 0.5 ml) for injection of microspheres. A surface electrocardiogram was monitored throughout the surgery.

2.2. Experimental protocols

Microsphere injection

Radiolabeled microspheres (RM, diameter 15.5 ± 0.1 μm, density 1.364 g · cm^{-3}, suspended in 0.9% NaCl with 0.01% Tween-80) were obtained from DuPont/NEN Research Products (Boston, MA), and fluorescent-labeled microspheres (FM, diameter 15.5 ± 0.3 μm, density 1.055 g · cm^{-3}, suspended in 0.9% NaCl with 0.02% Tween-20) were obtained from Molecular Probes (Eugene, OR). Randomly paired RM (cerium-141 or niobium-95) and FM (green or orange) were injected under one of the following conditions: regional myocardial ischaemia or (−)-N^6-(2-phenylisopropyl)-adenosine (*R*-PIA) infusion. To achieve ischaemia, a 4-0 silk ligature was placed around a proximal left coronary artery and passed through a short segment of soft polyvinyl chloride tubing to form a snare. The snare was tightened with a micrometer until visual evidence of cyanosis, wall motion abnormalities, and electrocardiographic changes were observed. To achieve stable hyperaemia, the adenosine agonist *R*-PIA was administered intravenously in a dose of 200 μg · min^{-1} in NS, starting at least 2 min before microsphere injection and continuing throughout the reference withdrawal. During *R*-PIA administration, heart rate was maintained at either 200 or 250 beats · min^{-1} with atrial pacing (Model S9D, Grass Instruments, Quincy, MA).

Six rabbits received 2 pair of RM and FM, and 3 received 1 pair. Approximately equal numbers of RM and FM varying from 0.3 to 2 million each (dose inversely proportional to anticipated RMBF) were sonicated, vortexed, combined into a single syringe, and injected immediately into the left atrium over 30–50 s followed by a 3-ml normal saline flush. Five to 10 s before microsphere injection, a 120-s heparinised blood reference collection was started at a rate of ≈ 2 ml · min^{-1} (Model #G-74900-20 syringe pump, Cole-Parmer, Niles, IL). Reference blood flow was calculated as the difference between glass syringe weights pre- and post-withdrawal, corrected for blood density (1.05 g · ml^{-1}), divided by collection time. No rabbit received more than a total of 4 million microspheres.

Routine tissue and blood processing

Hearts from the animals having regional ischaemia were excised and transferred to a modified Langendorff apparatus. The coronary snare was subsequently re-tightened to fully occlude the coronary artery, and zinc-cadmium-sulfide particles (ZnCdS, 1-10 μm diameter, Duke Scientific Corp, Palo Alto, CA) were infused to delineate the ischaemic area. These particles fluoresce under ultraviolet light but only minimally affect the fluorescence intensity at the excitation (Ex) and emission (Em) wavelengths used to quantitate microsphere dyes (see "Results"); potentially ischaemic tissue was therefore delineated as a negative image. Hearts were then trimmed of great vessels, atria, and epicardial fat, separated into right and left ventricles, and further sectioned into ZnCdS-negative and -positive pieces of ≈ 1 g each. In the animals receiving *R*-PIA, hearts were excised, trimmed, and sectioned into ≈ 1 g right and left ventricular pieces. Each heart yielded 4–5 tissue samples. All tissue samples were weighed and placed into glass test tubes. Blood syringes were emptied into glass test tubes and each syringe was then rinsed 3 times with 2% Tween-80 into its corresponding test tube. Preliminary experiments demonstrated that this procedure recovered more than 99% of microspheres in the syringes.

The radioactivities of all samples were determined using a Germanium crystal gamma counter (Micrad, Inc, Knoxville, TN) using 145 keV (^{141}Ce) and 766 keV (^{95}Nb) windows and a 10-min counting period.

Tissue samples were subsequently digested overnight by adding freshly prepared warm 4N KOH (5 ml per g of tissue) and Tween-80 (final concentration 2%). Blood samples were digested using 16N KOH (0.4 ml per ml of blood-and-rinse solution) and Tween-80 (final concentration 2%). A standardised aliquot of sonicated, vortexed, and continuously stirred blue FM ($\approx$ 1500 microspheres/30 μl 2% Tween-80) was pipetted into each tissue and blood sample (Eppendorf #4810, Brinkman Instruments Inc., Westbury, NY, with #21-375D pipette tips, Fisher Scientific, Pittsburgh, PA), to be used as an internal standard for recovery calculations. Each digested sample was vacuum-filtered to recover the microspheres on a 10-μm pore polycarbonate membrane filter (Poretics Corp, Livermore, CA). The sample container and filtration apparatus were promptly rinsed 3 times each with 2% Tween-80 over the same filter. The filter and filtered material were punched into a polypropylene cryovial using a short length of polypropylene (PE-190) tubing which was then left in the vial. After all tissue and blood samples were filtered in this manner, 2 ml of solvent [2-(2-ethoxyethoxy)ethanol acetate (Carbitol® acetate), Aldrich Chemical, Milwaukee,

WI] were pipetted into each cryovial, and all vials were capped.

Thirty to 180 min after addition of solvent, each vial was shaken and a portion of the solution was transferred into 0.7-ml (10 mm path length) glass cuvette. The fluorescence intensities of a solvent blank and each sample were then determined (Hitachi F-2000 with R372 photomultiplier tube, Tokyo, Japan; 10 nm Ex and Em slit widths). The fluorescence intensities of $\geqslant 3$ standardised aliquots of blue FM (similar to those added to the tissue and blood samples), unfiltered but allowed to air-dry overnight and then dissolved into 2 ml solvent, were also measured. These were subsequently averaged to become the standard of 100% recovery (F_{100}). The Ex/Em wavelengths used were: 449/497 (green), 528/553 (orange), and 354/418 (blue).

Tissue and blood processing for simulated spillage

To test the ability of the internal standard to correct for large losses during sample processing, two additional rabbits underwent surgical preparation and injection of 1 pair of RM and FM, with insertion of bilateral femoral artery cannulae for simultaneous withdrawal of 2 blood reference samples. The tissue and blood samples were processed as previously described, except that the digested samples were split over 2 separate filter membranes to mimic large "spillage". One sample each of tissue and blood were processed without splitting to allow independent assessment of the ability of the internal standard to correct for loss of tissue sample or loss of reference sample.

2.3. *In vitro studies*

The precision of the fluorometer and linearity of the signal were tested by 3 repeated measurements of the fluorescence intensity of 7 different serial dilutions of the 3 labels of FM, bracketing the range of fluorescence intensities measured in the study.

The potential interference of ZnCdS particles in the measurement of microsphere dye fluorescence was also examined. When excited with ultraviolet wavelengths, ZnCdS emits a yellow wavelength and might therefore add to the fluorescence intensity of FM dyes measured in the fluorometer. However, the presence of particulate matter in the fluorometer path may decrease the strength of both the excitation signal entering and the emission signal leaving the sample. In order to test these possibilities, ZnCdS particles were incubated overnight in 4N KOH. Twenty-seven aliquots, approximating the amount delivered to the average tissue sample, were pipetted from this ZnCdS-plus-KOH suspension during constant stirring, then filtered in the same manner as tissue samples. Green, orange, and blue FM were dissolved into solvent in numbers approximating those found in the average tissue sample ("low") or 10 times that ("high"). Two milliliters of solvent containing these "low" or "high" concentrations of microsphere dyes, or 2 ml of pure solvent, were then pipetted into each of 9 filter-containing cryovials and 9 cryovials without filters. The fluorescence intensity of each was measured.

To assess the error associated with the addition of the internal standard as well as the overall recovery of microspheres during filtration, 10 standardised aliquots of blue FM were pipetted into cryovials, air-dried, and dissolved into 2 ml solvent, and 10 additional standardised aliquots were individually filtered and then dissolved into 2 ml solvent. Fluorescence intensity was measured as above.

The number of RM in each sample was estimated as follows: aliquots of each isotope of RM were streaked onto graph paper and counted under a microscope. Squares with known numbers of RM were then analysed in the gamma counter. The isotopes' half-lives were then used in the standard decay formula to calculate a daily radioactivity/sphere ratio. The number of RM in a sample was then calculated by dividing the radioactivity of the sample by the corresponding radioactivity/sphere ratio.

The number of FM in each sample was estimated as follows: aliquots of each lot of each label of FM were streaked onto a coverslip overlying a hemocytometer and counted under a microscope. Coverslips with known numbers of FM were then immersed in 2 ml solvent and analysed in the fluorometer. (Coverslips were used because graph paper ink dissolves in solvent and might have interfered with the fluorescence measurements.) A fluorescence intensity/sphere ratio was thus generated. The number of FM in a sample was then calculated by dividing the fluorescence intensity of the sample by the appropriate F/sphere ratio.

2.4. *Calculation of myocardial blood flow*

Regional myocardial blood flow (RMBF) was calculated as:

$$\text{RMBF} = Q_b \cdot S_t / S_b$$

where Q_b was the reference blood flow ($\text{ml} \cdot \text{min}^{-1}$), S_t was the radioactive or fluorescence signal of the tissue sample normalised per gram wet weight (g^{-1}), and S_b was the radioactive or fluorescence signal of the blood reference.

For the fluorescent method, all fluorescence signals were corrected using the appropriate blank. Recovery of microspheres from tissue or blood was calculated as:

$$\%\text{Recovery} = 100 \cdot F_s / F_{100}$$

where F_s was blue fluorescence of the sample (tissue or blood) and F_{100} was the average blue fluorescence of the recovery standard. FM-determined RMBF was calculated with ("corrected") and without ("uncorrected") a normalisation for recovery.

2.5. *Data analysis*

Data were analysed using Crunch Statistical Package software (version 4.07, Crunch Software Corporation, Oakland, CA). The coefficient of variation was used to describe repeated measurements. Simple linear regression was used to describe the correlation between fluorescence intensity and serial dilutions of FM. Analysis of variance was used to describe the effect of ZnCdS on fluorescence intensity.

Orthogonal regression was used to compare the two methods of RMBF estimation, which have the same units and are measured with error. Since we were not interested in predicting one method of RMBF measurement from another (i.e., one method is not dependent on the other as in a y-on-x regression), the orthogonal regression was appropriate as it minimises the perpendicular distance between the data values and regression line, thus assuming equal weighting of the random error in x and y. The hypothesis that the slope of the resulting regression line is one, and the intercept is zero, was then tested. A paired groups t-test was used to evaluate if the means of the RMBF measurements by each method were different. The Bland-Altman difference-against-mean method [9] was further utilised to graphically demonstrate the agreement between the two methods of RMBF estimation. Significance was assumed for P-values < 0.05. Data are expressed below as mean (s.e.m.).

3. Results

3.1. In vitro studies

There was no fluorescence overlap between green, orange, and blue fluorescent dyes using the solvent and fluorometer described. The averages of the coefficients of variation generated from each of the 7 dilutions read thrice were 0.39(0.08), 0.67(0.30), and 0.56(0.11)% for green, orange, and blue, respectively. Linear regression of fluorescence intensity vs. dilutions of FM yielded strong correlations for each dye (green, $r = 0.9996$; orange, $r = 0.9997$; blue, $r = 0.9997$). The range of fluorescence intensities used corresponded to approximately 0–17000 green FM, 0–22000 orange FM and 0–12000 blue FM per 2 ml solvent.

There was a small amount of "intrinsic" fluorescence intensity of KOH-exposed, filter-captured ZnCdS particles in pure solvent: 0.16(0.01), 0.14(0.01), and 1.04(0.18), equalling 0.15, 0.09, and 1.24% of the tissue samples' average fluorescence for green, orange, and blue, respectively. Overall, however, the presence of ZnCdS particles reduced the fluorescence signal of low and high concentrations of each microsphere dye by 0.75–3.48% (compared to dye-containing samples without ZnCdS particles, $P < 0.025$), excepting orange in "high" concentration ($P = 0.20$).

The fluorescence intensity of 10 standardised aliquots of a suspension of blue FM, unfiltered, air-dried, and dissolved into solvent, was 130.0(1.9). The fluorescence intensity of 10 standardised aliquots of blue FM, filtered before being dissolved into solvent, was 122.9(1.7). Recovery of the blue FM during filtration averaged 94.5(1.3)% in this test.

3.2. Regional myocardial blood flow: routine processing

Thirty-nine physical samples were analysed for RMBF: 27 at 2 different flow conditions plus 12 at 1 flow condition (severe ischaemia), totalling 66 paired RMBF measurements. Recovery of blue FM from tissue averaged

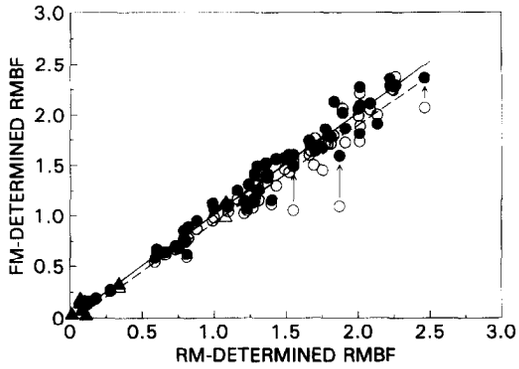

Fig. 1. Relationship between fluorescent microsphere (FM) and radiolabeled microsphere (RM)-determined regional myocardial blood flow (RMBF): orthogonal regression. Symbols represent individual tissue pieces which were analysed both with (closed symbols) and without (open symbols) a correction for recovery of microspheres during filtration. Circular symbols denote tissue samples containing ≥ 400 microspheres of any label; triangular symbols denote samples containing < 400 microspheres of any label. Note that there are several apparent underestimations of RMBF by the fluorescent technique in samples processed without difficulty, which could be identified and corrected by use of the internal standard (arrows). For regression statistics, please refer to the text.

97.6(1.2)%. The plot and orthogonal regression of RM vs. recovery-uncorrected FM-determined RMBF are shown in Fig. 1 (open symbols and dashed line). The slope of the regression was 0.97(0.15), not statistically different from 1 ($P = 0.28$), but the intercept of $-0.03(0.01)$ was statistically different from 0 ($P = 0.005$). A paired groups t-test further showed that the means of the RM and uncorrected FM-determined RMBF were different [1.24(0.08) vs. 1.17(0.08) $ml \cdot min^{-1} \cdot g^{-1}$, respectively; $P = 0.0002$]. When analysed by the method of Bland and Altman (Fig. 2, open symbols and dashed lines), the mean of the

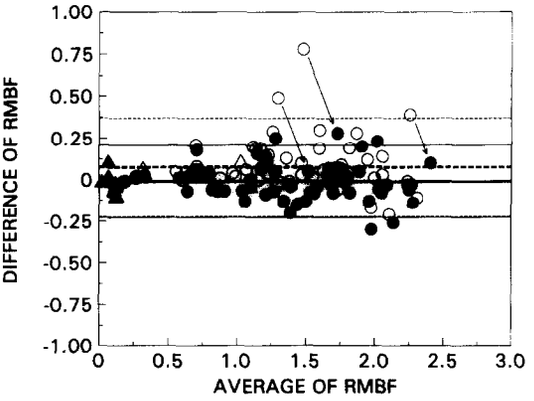

Fig. 2. Relationship between fluorescent microsphere (FM) and radiolabeled microsphere (RM)-determined regional myocardial blood flow (RMBF): Bland-Altman analysis. The difference of RMBF determined by the two methods, calculated as RM-determined RMBF minus FM-determined RMBF ($ml \cdot min^{-1} \cdot g^{-1}$), is plotted vs. the average of RMBF by both methods. Symbols represent individual tissue pieces which were analysed both with (closed symbols, solid lines) and without (open symbols, dashed lines) a correction for recovery of microspheres during filtration. Circular symbols denote tissue samples containing ≥ 400 microspheres of any label; triangular symbols denote samples containing < 400 microspheres of any label. The means of the differences of RMBF are depicted with thick lines; mean ± 2 s.d. are depicted with thin lines. Note that there are several apparent underestimations of RMBF by the fluorescent technique in samples processed without difficulty, which could be identified and corrected by use of the internal standard (arrows).

(normally-distributed) differences between RM and uncorrected FM-determined RMBF was $+0.07$ ml $\cdot$ min^{-1} $\cdot$ g^{-1}, and the mean $\pm$ 2 s.d. was $+0.37$ and -0.23 ml $\cdot$ min^{-1} $\cdot$ g^{-1}, respectively.

The plot and orthogonal regression between RM and recovery-corrected FM-determined RMBF are also illustrated in Fig. 1 (closed symbols and solid lines). Using the corrected FM data, the orthogonal regression yielded a slope of 1.01(0.11), which was not statistically different from 1 ($P = 0.57$), and an intercept of -0.00(0.01), which was not statistically different from 0 ($P = 0.96$). The means of the RM and corrected FM-determined flows were not different by paired groups t-testing [1.24(0.08) vs. 1.25(0.08) ml $\cdot$ min^{-1} $\cdot$ g^{-1}, respectively; $P = 0.31$]. When analysed by the method of Bland and Altman (Fig. 2, closed symbols and solid lines), the mean of these differences between RM and corrected FM-determined RMBF was -0.01 ml $\cdot$ min^{-1} $\cdot$ g^{-1}, and the mean $\pm$ 2 s.d. was $+0.21$ and -0.23 ml $\cdot$ min^{-1} $\cdot$ g^{-1}, respectively.

3.3. Influence of number of microspheres / sample

The average number of FM/tissue sample was 3264(462) and 4268(696) for green and orange, respectively. However, 9 of the 66 samples were estimated to have < 400 microspheres of any label (radioactive or fluorescent) per sample. If the 9 samples with < 400 microspheres of a label were excluded from the statistical analyses, orthogonal regression between RM and corrected FM-determined RMBF yielded a slope of 1.02(0.14), which was not statistically different from 1 ($P = 0.82$), and an intercept of -0.02(0.01), which was not statistically different from 0 ($P = 0.07$). The means of the RM and FM-determined flows were not different by paired groups t-testing [1.36(0.08) vs. 1.37(0.08) ml $\cdot$ min^{-1} $\cdot$ g^{-1}, respectively; $P = 0.42$]. Likewise, excluding the 9 samples with < 400 microspheres of a label from the Bland-Altman difference-against-mean analysis yielded a mean difference between RM and corrected FM-determined RMBF of -0.01 ml $\cdot$ min^{-1} $\cdot$ g^{-1}, and the mean $\pm$ 2 s.d. was $+0.22$ and -0.25 ml $\cdot$ min^{-1} $\cdot$ g^{-1}, respectively.

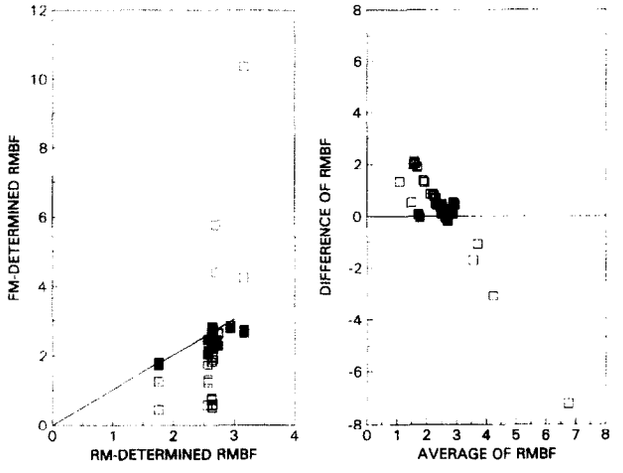

Fig. 3. Ability of the internal standard to improve data from samples with simulated spillage during microsphere isolation. FM-determined RMBF was calculated using "spilled" tissue and "unspilled" blood samples or vice versa. Open squares denote recovery-uncorrected data; closed squares denote recovery-corrected data. *Left panel:* Orthogonal regression. The regression line of the recovery-corrected, routinely processed data is depicted for reference. Recovery-uncorrected tissue sample loss results in underestimations of RMBF by FM (open squares below the line), while blood sample loss results in overestimations (open squares above the line). *Right panel:* Bland-Altman analysis. The mean difference of the recovery-corrected, routinely processed data is depicted for reference.

3.4. Regional myocardial blood flow: simulated spillage

The 2 additional animals yielded 10 tissue samples and 4 blood samples. Eight of the 10 tissue samples were split during processing (after gamma counting and after addition of the standardised aliquot of blue FM), yielding 16 "spilled" and 2 "unspilled" tissue samples. Two of the 4 blood samples were split, yielding 4 "spilled" and 2 "unspilled" blood samples. In the split samples, recovery of blue FM ranged from 20.8–82.1% in tissue, and from 26.9–67.6% in blood. Orthogonal regression and difference-against-mean plots of RM vs. FM-determined RMBF with and without recovery-correction are shown in Fig. 3. The regression line and mean difference for the routinely processed, recovery-corrected data are included for reference in their respective panels.

4. Discussion

The present data demonstrate excellent agreement of FM and RM-determined RMBF over a range of 0.01–2.46 ml $\cdot$ min^{-1} $\cdot$ g^{-1} (by RM data) in rabbits. Because predicting FM-determined RMBF from RM-determined RMBF was not as important as testing that the two measures are equally good, orthogonal regression was performed. Using recovery-corrected data, the slope of the orthogonal regression was not statistically different from one, nor was the intercept different from zero. In combination with the demonstration that the means of the blood flow measurements were not different, we conclude that the FM and RM methods are no different in their relative RMBF determinations. This conclusion was corroborated by the Bland-Altman analysis, which graphically demonstrated a zero mean difference between the two methods.

4.1. Assessment of the agreement between two methods

When assessing agreement between two methods, the variability of each method is important: when an "old" method has variability, a "new" method with no variability will not agree with it perfectly [9]. Therefore, the agreement between RM and FM-determined RMBF cannot be expected to be better than the amount of error intrinsic to the RM method. RMBF by RM determination has been reported to be within 20% of timed volumetric coronary sinus flow, approximately 90% of the time [1,10]. Further, the duplicate variability of RMBF estimated by two labels of RM injected simultaneously (defined as 100 times the absolute value of the difference of RMBF as determined by the two labels, divided by the arithmetic mean of the pair) has been calculated to be 20% when > 385 RM are present in both blood reference and tissue samples and 10% when > 2000 RM are present in the blood reference

and > 475 RM are present in the tissue samples, with 95% confidence [11]. Subsequently, duplicate variability of two labels of RM used to determine RMBF by the blood reference method has been measured to be between 5–9% [2,12].

Any sources of error in the estimation of regional blood flow by entrapment of microspheres in tissue should apply equally to the radioactive and fluorescent techniques. This includes the issues of microsphere size, mixing (sonication, vortexing, site of injection), and distribution (number injected) [13]. In this study, FM and RM of comparable size were used in equal quantities and mixed similarly. There were 8077(2085) microspheres of any label in the blood reference samples. Excluding tissue samples with < 400 microspheres, Bland-Altman analysis demonstrated that approximately 95% of the differences between RM and corrected FM-determined RMBF lay in the range of +0.22 to −0.25 $ml \cdot min^{-1} \cdot g^{-1}$, with a variability between the methods of 7.2(0.8)%. Since the variability between the FM and RM-determined RMBF is of the same magnitude as that reported for two labels of RM, we conclude that the fluorescent technique for estimation of RMBF does not add to the error traditionally accepted with the radiolabeled approach.

4.2. Microsphere recovery techniques

FM dyes can be extracted directly from myocardial sample-plus-KOH solutions by the addition of an organic solvent, followed by centrifugation and transfer of the upper layer of solvent into a cuvette [7]. This technique has the advantage of minimisation of potential microsphere losses during transfer and filtration of solutions. However, Abel et al. compared myocardial flow distributions of RM and FM, not absolute flow volume using a blood reference sample, and the resulting correlation coefficient ($r = 0.77$) was not as high as reported in comparable studies utilising other microsphere recovery methods ($r > 0.97$) [3,5,6]. In the present data, the corresponding correlation was: $r = 0.99$, $FM = 1.00 \cdot RM + 0.02$, standard error of the estimate = 0.11.

Reported methods for isolation of microspheres from enzymatic-or KOH-digested myocardium and blood have included separation by differential centrifugation [2,3] or filtration [5,6]. With a centrifugation/sucrose cushion method, the recovery of FM from myocardial tissue was reported to be 72.1(1.4)%; recovery was assessed by loss of radioactivity from RM which had been injected concomitantly into the animal [3]. The percentage recovery of microspheres has not been reported previously with a KOH digestion and filtration technique. In the current study, the average 97.6% recovery was quite high; nonetheless, the agreement between FM and RM-determined RMBF was improved by use of the (non-radioactive) internal standard.

4.3. Utility of the internal standard

Use of the internal standard to correct for variable filtration losses in estimating RMBF by FM was very helpful. Visual inspection of both Figs. 1 and 2 demonstrates 3 obvious outliers (more than 2 s.d. from mean). RMBF data from these routinely-processed samples with otherwise unrecognised processing losses [3 of 66 RMBF determinations (5%) or 2 of 39 physical samples (5%)] were clearly improved by application of a recovery correction. The recovery of FM for these points was as low as 76.2%. Fig. 3 further suggests that the internal standard can substantially improve RMBF estimates calculated using tissue samples with even greater losses, as well as blood reference samples with large losses.

4.4. Technical aspects of the fluorescent dye quantification

A variety of different solvents have been used for FM dye extraction, including Cellosolve®, xylenes, and ethyl acetate. Cellosolve® acetate (2-ethoxyethanol acetate) dissolves microspheres readily, the fluorescent dyes are stable in it, and it is less volatile than xylenes so that there is less evaporation before dye concentrations are determined [6]. Ethyl acetate, used in the organic extraction technique [7], is a flammable solvent with a boiling point near room temperature (77°C). Additionally, ethyl acetate absorbs water (up to 3.3% w/w), a characteristic that could introduce volume-related errors [14]. The solvent used in the current study, Carbitol® acetate, dissolves microspheres readily and is even less volatile (boiling point 218.5°C) than Cellosolve® acetate (boiling point 156°C) [14]. Fluorescence intensities were read between 30 and 180 min after solvent was added to the microspheres to minimise/standardise potential evaporative losses of solvent before reading.

Carbitol® did have some significant intrinsic fluorescence at blue wavelengths, so a daily solvent blank was subtracted from all fluorescence intensity readings. The fluorescence intensity of KOH-digested, filtered tissue and blood samples containing no microspheres was no different from solvent at the orange Ex/Em wavelengths used, but was minimally higher than solvent at the green wavelengths used ($P = 0.03$ by ANOVA analysis of solvent, blood, and tissue "blanks"). However, the difference of green fluorescence intensities between blood or tissue and solvent was 0.063 and 0.055, equalling 0.02 and 0.05% of the blood and tissue samples' average fluorescence intensities, respectively. As with Cellosolve® acetate, Carbitol® acetate degrades plastic cuvettes so that glass cuvettes must be used.

The choice of fluorometer excitation and emission slit widths is also important. Glenny et al. used Ex and Em slit widths of 4 nm but noted that use of slit widths in the range of 6–8 nm should result in a larger fluorescent signal with little change in spillover, allowing > 1.25 ml of solvent to be used for each sample and thus reducing the error for unequal volumes [6]. Indeed, Abel et al. achieved excellent separation of peaks using 10-nm slit widths and 3 ml solvent. In the present study, 10-nm slit widths were used with no significant spillover, and signal strength was adequate with 2 ml solvent. In addition to the possible reduction of error due to unequal solvent volumes, use of larger volumes of solvent reduces the problem of "quenching", which occurs at high dye concentrations. This is supported by the finding of linearity of fluores-

cence intensity over a greater range of estimated microsphere numbers: over the range of 25–16000 microspheres/1.25 ml solvent, Glenny et al. reported a correlation of $r > 0.99$. The correlation was improved ($r > 0.999$) by narrowing the range to 50–2000 microspheres/1.25 ml solvent. The present study shows correlation coefficients of $r = 0.9996$–0.9997, over the approximate range of 0–17000 green spheres, 0–22000 orange spheres and 0–12000 blue spheres in 2 ml of solvent.

The precision of the fluorescent signal as assessed by the coefficient of variation for repeated measurements of the same sample has been reported to be 0.2–1.7% [6]. In the present study, a similar precision was found (0.39–0.67%) using a less expensive fluorometer. The costs of several microsphere quantification methods have been summarised recently [6]. To this summary we would add that the < $16000 fluorometer used in the present study is quite satisfactory, albeit not automated.

4.5. Number of labels injected

Although several are available from Molecular Probes, only two fluorescent labels were injected in this study. This was done in order to avoid significant alteration of blood flow due to embolisation of large numbers of microspheres, given that each type of FM was paired with a RM. Omitting the RM, smaller numbers of more labels of FM could certainly be injected at more time points, keeping in mind that as the anticipated flow is lowered, the number of microspheres injected and/or the volume of the tissue and blood reference samples must be increased to maintain precision of the measurement. Despite left atrial injections of up to 2 million microspheres (of any label) in rabbits, we could not reliably obtain ≥ 400 microspheres per ≈ 1 g tissue sample in myocardium receiving < 0.2 ml $\cdot$ min$^{-1} \cdot$ g^{-1}. In these circumstances, we may accurately conclude that RMBF was low, but the estimation is made with less precision due to a greater impact of the distribution variability of (any types of) microspheres.

The green and orange labels were chosen because of the increased efficiency of our photomultiplier tube at low visible Ex and Em wavelengths; a preliminary study in our laboratory showed that the signal detected for crimson (Ex/Em = 600/635 nm) was significantly weaker than for the blue, green, yellow-green, orange, or red colors. Given that photomultiplier tubes with wider detection ranges are available, and that the total numbers of microspheres needing to be injected at one time could be reduced, the number of labels could easily be increased using fluorescent microspheres alone to estimate RMBF. As noted by Glenny et al., however, as the number of fluorescent labels in increased, correction for spillover may become necessary [6].

4.6. Possible influence of technique of ischaemic area delineation on RMBF determinations

In this study, ZnCdS particles were used to delineate ischaemic area. These particles were nominally 1–10 μm in diameter and tissue was filtered using a 10-μm pore size. Nevertheless, some particles (possibly larger or clumped) remained on the filters. Although minimally fluorescent themselves at the wavelengths used to quantitate microsphere dyes, the presence of ZnCdS particles in solutions containing microsphere dyes reduced the fluorescent signal in the range of 2–3%. This is presumably because the particles themselves blocked the amount of exciting light entering the solution, and/or absorbed emitted light from the microsphere dyes in the same solution, more than they added "intrinsic" fluorescence. As a result, a small reduction of fluorescent-microsphere-estimated RMBF probably occurred in those tissue samples containing particles (non-ischaemic areas of hearts with snares). In the future, if particles are to be used to delineate ischaemic tissue, the use of small syringe filters for transfers of the samples into the cuvettes may minimise any particulate interference. If a dye is to be used to delineate ischaemic tissue, prior testing of possible interference of fluorescent microsphere dye quantification is recommended, as the tissue dye could possibly stain the microspheres and thus be present during fluorescence measurements.

4.7. Conclusion

Fluorescent microspheres can be used as an alternative to radiolabeled microspheres in the determination of regional myocardial blood flow in rabbits. The overall recovery of microspheres using a KOH digestion and filtration technique was high. Nevertheless, use of an internal standard is recommended for correction of filtration losses.

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