special communication

Application of HPLC to counting of colored microspheres in determination of regional blood flow

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Mazoit, Jean Xavier, Régine Le Guen, Anne Decaux, Pierre Albaladejo, and Kamran Samii. Application of HPLC to counting of colored microspheres in determination of regional blood flow. *Am. J. Physiol.* 274 (*Heart Circ. Physiol.* 43): H1041–H1047, 1998.—Colored microspheres have become popular compared with radioactive microspheres because they do not use radioactivity. However, they suffer from a much greater variability in their determination. We have developed a new method for assaying the dye using high-performance liquid chromatography (HPLC) with internal standard. This technique permits accurate determination of ≤400 spheres in rat blood, heart, kidney, liver, and brain with a relative error [coefficient of variation (CV)] <10%. To date, only three colors (white, yellow, and red) may be used because, of the five colors tested, one (violet) served as internal standard and another (blue) exhibited marked degradation during extraction. Compared with the classical spectrophotometric technique, HPLC allows a three to five times improvement in reproducibility with a relative error significantly lower ($P < 0.01$) than with direct spectrophotometry. Although this new technique appears to be more time consuming than the classical method, its use seems to be preferable because of the improvement in measurement sensitivity.

high-performance liquid chromatography; internal standard

THE MICROSPHERE TECHNIQUE is still considered the "gold standard" for measuring regional blood flow, either as an absolute value or relative to other organ flow (4, 13). Until recently, only radioactive spheres were used, but the emergence of colored microspheres provides the opportunity for a simple and safe technique, particularly by avoiding the use of radioactivity (9, 12). Briefly, the technique is as follows. A known number of microspheres is injected in a mixing chamber such as the left atria or the left ventricle, and a reference arterial sample is drawn at a known withdrawal rate. After animals are euthanized, the number of spheres is measured both in the reference sample and in the sample organ of interest. Regional blood flow is then calculated relative to the reference withdrawal rate. Thus errors in both measurements will participate in the error in regional blood flow calculation. In fact, measurements of regional blood flow by microspheres suffer from lack of precision due to distributional variation governed by the Poisson distribution and due to assay imprecision (2, 5, 6). The assay error is most often neglected in case of radioactive microspheres because increasing counting time meets the required precision (2). However, in the case of colored microspheres, the assay error may be the primary cause of imprecision (see RESULTS). One may thus be tempted to increase the number of microspheres injected. However, increasing the number of spheres in both tissue and reference samples will not decrease the error because the assay generally has a constant coefficient of variation (CV). Moreover, if one increases the number of spheres injected per measurement in the rat, serious hemodynamic disturbances are expected to occur (11). It is, then, of utmost importance to reduce the assay error when one uses colored microspheres. In that respect, the usual direct spectrophotometric method derived from radioactivity measurement lacks precision (see RESULTS). If some studies have addressed the question of recovery of dye (8), none (in our knowledge) has studied the problem of reproducibility of the assay.

We now propose *1*) the use of an internal standard technique and *2*) a new assay using high-performance liquid chromatography (HPLC), which we compare with the direct spectrophotometric assay in terms of reproducibility, i.e., relative error.

METHODS

Materials and reagents. Colored (white, yellow, violet, red, and blue) microspheres (DyeTrak Triton 15.5 μm) were purchased from Bioseb (Paris, France). Inasmuch as the whole study is based on the principle of the reference sample, we used the particle concentration recorded on the manufacturer's chart (3,000,000 spheres/ml except for the violet spheres, whose concentration was recorded to be 3,300,000 spheres/ml) with no further verification. *N*,*N*,-dimethylformamide (DMF) (HPLC grade) and polyoxyethylenesorbitan monooleate (Tween 80) were purchased from Sigma (St. Quentin Fallavier, France). Acetonitrile (HPLC grade), ethanol (low grade with 4% methanol; a preliminary experiment has shown that the quality of extraction and background noise were not modified whether HPLC-grade or low-grade ethanol was used), NaCl, NaH_2PO_4, and H_3PO_4 were purchased from Prolabo (Paris, France).

Chromatography. Chromatographic analysis was performed under isocratic conditions at room temperature. The HPLC system consisted of a Shimadzu LC9A pump (Touzart et Matignon, Les Ulis, France), a variable-wavelength ultraviolet (UV)-visible detector (model 1050, Hewlett-Packard, Les Ulis, France), and a Linseis L 6512B pen recorder

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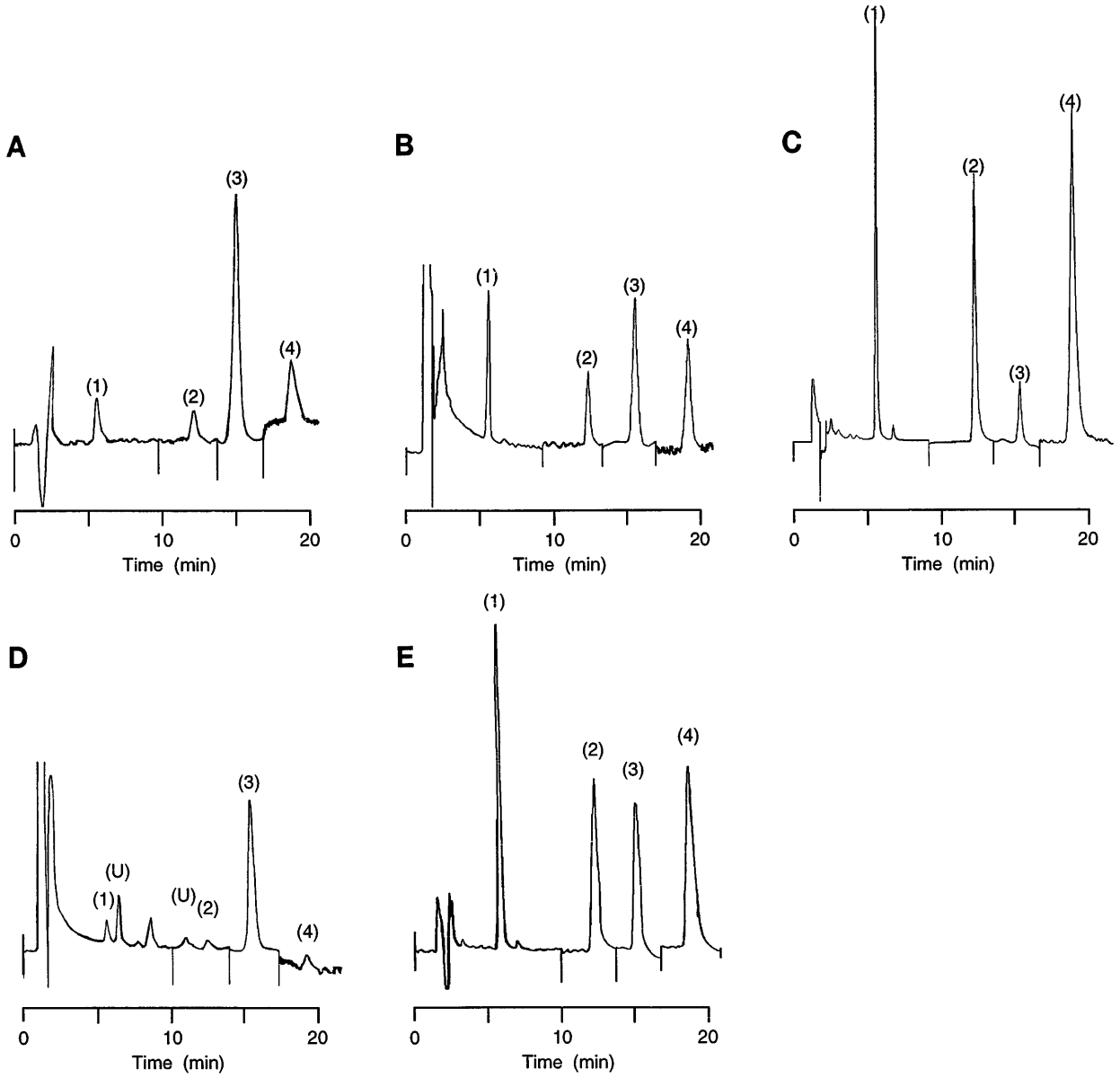

Fig. 1. *Top*: chromatograms obtained after extraction of 0.5 ml blood containing 25 yellow (2), 50 white (1), and 50 red spheres (4) with 1,500 violet spheres (3) as internal standard (IS) (*A*); 125 yellow (2), 250 white (1), and 250 red spheres (4) with 1,500 violet spheres (3) as IS (*B*); or 1,000 yellow (2), 2,000 white (1), and 2,000 red spheres (4) with 1,500 violet spheres (3) as IS (*C*). *Bottom*: chromatograms obtained after extraction of rat brain samples with the following measured content: 27 white (1), 11 yellow (2), and 20 red spheres (4) with 1,500 violet spheres (3) as IS (*D*) and 451 white (1), 445 yellow (2), and 335 red (4) spheres with 1,500 violet spheres (3) as IS (*E*). In *D*, U is unknown peak.

(Bioblock, Illkirch, France). The separation was performed on a Waters Resolve 5-μm spherical C_{18}, 3.9 × 300 mm column (Waters, St. Quentin en Yvelines, France). The mobile phase consisted of 35% 0.01 M NaH_2PO_4 buffer containing 0.001 M sodium dodecyl sulfate and 65% acetonitrile (vol/vol) and was set at a flow rate of 1.2 ml/min. Twenty-five microliters of the final extract were injected in the system. Detection was made at wavelengths of 370, 448, 268, and 530 nm, respectively, for the white, yellow, violet, and red dye. The blue spheres were not used because a preliminary experiment showed that a marked degradation of the dye occurred during extraction with the appearance of multiple peaks after chromatographic separation.

Spectrophotometric procedure. Sample spectra were recorded using the same variable-wavelength UV-visible detector as used for HPLC, set in the scanning mode. One hundred to one hundred twenty microliters of the extract were injected using a direct injection port with minimal dead volume (Rheodyne, Touzart et Matignon, Les Ulis, France). Absorption was measured at 335, 375, 405, 435, 475, 505, 535, 565, and 595 nm. Dye concentration was determined using a modification of the procedure described by Schosser et al. (15). This modification allows the use of a number of wavelength measurements greater than the number of unknown dye concentrations by using linear least-squares regression using the Faddeev-Leverrier algorithm (10) rather than a simple matrix inversion procedure. Because background noise in biological samples is greater in the lower wavelength spectrum (typically at wavelengths between 190 and 400 nm), we compared the results obtained using the set of nine

wavelengths to the results obtained using a reduced set of seven wavelengths (from 405 to 595 nm) and to the results obtained with the classical four-point technique [number of measurements equal to number of colors (405, 475, 535, and 595 nm)].

Extraction. The extraction procedure was always performed using 1% Tween 80. The spheres were gently vortex-mixed for 30–60 s (at low speed to be sure that no foam was formed), then put in an ultrasonic bath and hand-mixed just before sampling with a micropipette. Direct extraction of dye from the spheres was performed using DMF and was considered complete. Extraction from biological material was performed as follows. Heparinized blood (500 µl) or 200- to 300-mg tissue samples (heart, kidney, liver, or brain) obtained from Sprague-Dawley rats were spiked with known amounts of spheres in 5-ml polypropylene tubes. The spheres were introduced into the tubes by pipetting known numbers of spheres (in 50 µl saline with 1% Tween 80). In spiked samples to which a known number of violet spheres were added, this number served as an internal standard reference. For the lower concentrations tested (50 yellow, 100 white, and 100 red spheres) and for extraction after in vivo injection, 1,500 violet spheres were added to the sample as internal standard. Two milliliters of distilled water with 1% Tween 80 were added, and the tubes were hand-shaken. One milliliter of KOH (12 M) was added, and the tubes were put in a water bath shaker at 70°C for 2 h 30 min. After being cooled, the tubes were centrifuged at 2,500–3,000 revolutions/min for 20 min. The lower part was carefully aspired with a thin needle and discarded, leaving the spheres in the upper layer (0.5–0.75 ml). Four milliliters of ethanol containing 1% Tween 80 and 100 µl H_3PO_4 (6 M) were added, and the tubes were vortex-mixed for 30–40 s and ultrasonicated for 4–5 min. After centrifugation at 2,500–3,000 revolutions/min for 10 min, the supernatant was discarded and the procedure was repeated. The residue was then evaporated to dryness under vacuum at 35°C for 40 min. The dry residue was extracted with 200 µl DMF, vortex-mixed for 30–40 s, and ultrasonicated for 4–5 min. After centrifugation for 10 min at 4,000 revolutions/min, 25 µl of the supernatant were injected into the HPLC system or 100–120 µl were directly injected in the spectroscopic cell for measurement by the traditional technique.

Protocol design. Experiments were conducted using five replicates. First, we assessed the pipetting variability *1*) by pipetting 50 µl of solutions containing 4,000, 400, and 40 white microspheres (these concentrations were obtained by serial dilutions) into test tubes and *2*) by pipetting 150,000 white microspheres (50 µl) in a polyethylene tube (PE-50, Guerbet Louvres, France) using a model 44 Harvard pump (Harvard, Les Ulis, France) and rinsing the tube with 2 ml water containing 1% Tween 80. Because each dye did not display the same absorbance intensity, we normalized the mixtures of spheres according to their respective absorbance, i.e., to the intensity of their respective signal. Recovery and reproducibility of extraction and detection in biological samples were tested using three different mixtures: *1*) 50 yellow, 100 white, 100 red, and 200 violet spheres (violet spheres were not added at this time when an internal standard procedure was used); *2*) 400 yellow, 800 white, 800 red, and 1,600 violet spheres; and *3*) 1,500 yellow, 3,000 white, 3,000 red, and 6,000 violet spheres.

In vivo experiments. Two Sprague-Dawley rats weighing 380–400 g were anesthetized with pentobarbital (50 mg/kg ip). The left femoral artery was cannulated using a 22-gauge Teflon short catheter (Insyte, Beckon-Dickinson, Dun Laoghaire, UK), and a polyethylene catheter (PE-50, Guerbet Louvres, France) was advanced in the left ventricle via the right carotid artery for dye injection (8). *Rat 1* received a mixture of 300,000 white, 300,000 yellow, and 300,000 red spheres over a 40-s period using a model 22 Harvard pump (Harvard, Les Ulis, France), and *rat 2* received a mixture of 100,000 white, 100,000 yellow, and 100,000 red spheres. The animals were euthanized with an overdose of pentobarbital, and the organs (heart, brain, and right and left kidneys) were dissected and cut into five samples weighing 150–300 mg each (200–400 mg for brain tissue). After extraction as described above and elution with 200 µl DMF, 25 µl were injected into the HPLC system and then 100–120 µl were injected directly into the spectrophotometer.

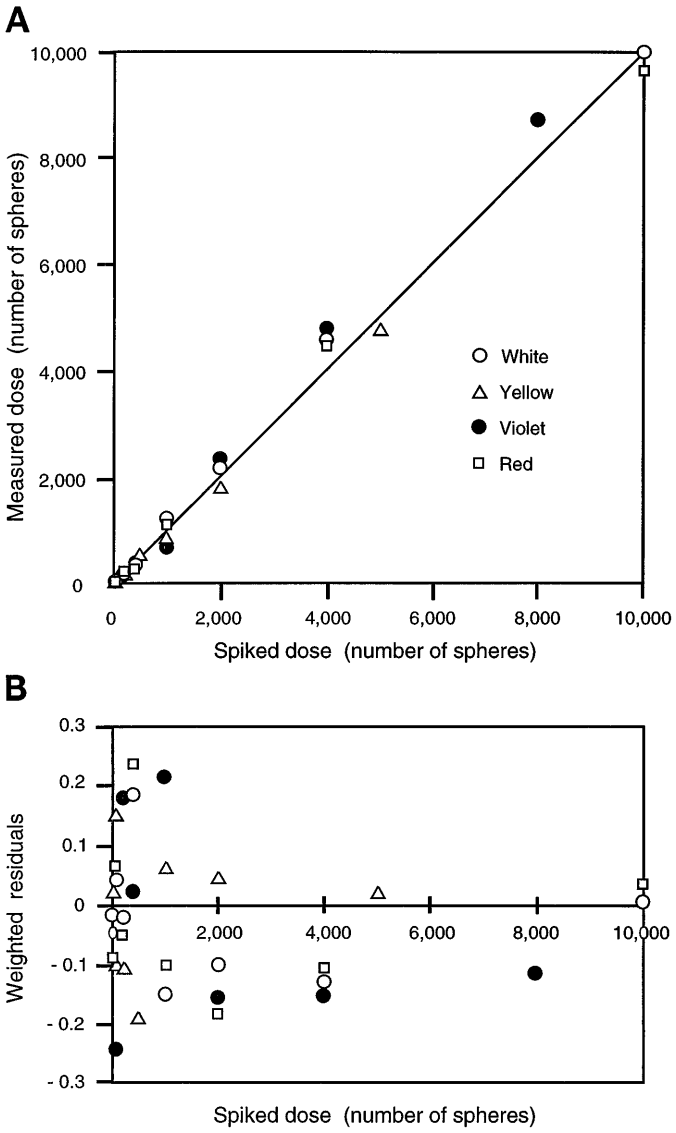

Fig. 2. Measured vs. spiked dye concentration obtained after fitting an 8-point standard curve (7 points for violet) (*A*), and the corresponding weighted residuals (*B*). Weighting as the inverse of concentration squared led to small bias.

Data are presented as means of replicate experiments, and CVs (relative standard deviation or relative error) are expressed as percentages. Comparisons between CVs used the Fisher-Snedecor *F* test (1).

RESULTS

HPLC. The separation was optimal with mobile phase containing 65% acetonitrile (vol/vol) (Fig. 1). Under the above-described conditions, retention times

Table 1. *Recovery and intraday reproducibility of HPLC*

No. of Spheres	Blood			Heart			Kidney			Liver			Brain		
	Recovery	CV_1	CV_{IS}	Recovery	CV_1	CV_{IS}	Recovery	CV_1	CV_{IS}	Recovery	CV_1	CV_{IS}	Recovery	CV_1	CV_{IS}
White															
100	78	37	11	83	12	6.8	85	19	10	73	22	5.4	80	17	13
800	78	29	10	81	13	5.7	94	22	6.8	77	24	5.2	82	13	3.5
3,000	80	13	3.4	95	17	4.0	94	8	3.4	80	12	2.3	97	10	3.2
Yellow															
50	70	21	18	84	21	16	88	20	4.1	69	34	12	76	17	10
400	82	18	9.0	86	14	6.5	92	13	3.8	78	17	3.0	72	13	1.4
1,500	76	13	1.8	79	10	4.5	89	9	4.8	78	13	2.3	95	11	3.9
Violet															
200	75	38		86	16		81	14		66	25		92	14	
1,600	74	18		85	17		89	16		81	15		79	13	
6,000	74	12		89	19		87	12		75	25		93	9	
Red															
100	87	37	11	90	23	11	90	30	13	69	35	18	82	14	8.8
800	84	26	8.9	84	14	6.5	88	22	5.8	79	14	5.1	75	14	3.7
3,000	77	13	2.7	94	13	5.1	89	9	4.2	79	14	1.9	88	13	4.2

Values are in percent; $n = 5$ replicate experiments. Three concentrations (no. of spheres in 0.5 ml blood or 200–300 mg tissue) have been used for each color according to their relative absorbance. CV_1, coefficient of variation (relative standard deviation) of measured concentration without internal standard (IS); CV_{IS}, coefficient of variation of calculated concentration when 1,500 violet spheres were added as IS. HPLC, high-performance liquid chromatography.

were as follows: white spheres, 5.1 min (CV 1.3%); yellow spheres, 10.6 min (CV 1.0%); violet spheres, 12.6 min (CV 0.7%); and red spheres, 13.2 min (CV 0.7%). The lower limits of detection of the dye (directly extracted from the spheres) at four times the size of the basal noise were 2.5 spheres (white), <1 sphere (yellow), 12.5 spheres (violet), and 2.5 spheres (red). The CV within the same extracted sample at four times the lower limit of detection were 0.6% (white spheres), 1.0% (yellow spheres), 1.7% (violet spheres), and 3% (red spheres). The errors in pipetting were, respectively, 4, 7, and 15% for the solutions containing 4,000, 400, and 40 spheres. The error in pipetting 50 µl of spheres in PE tubing was 4%. An eight-point calibration curve was constructed in blood (7 points for violet spheres). The curves were found to be linear from 20 to 5,000 spheres (yellow, $r = 0.998$), from 40 to 10,000 spheres (white, $r = 0.996$; red, $r = 0.995$), and from 80 to 10,000 spheres (violet, $r = 0.993$) (Fig. 2). However, recovery of dye was incomplete even at sphere concentrations ≥400 per sample (Table 1). Reproducibility was poor, but when violet spheres were used as internal standard, reproducibility markedly increased with a CV between 1.8 and 5.1% at the higher concentration and between 4.1 and 18% at the lower concentration, depending on the type of spheres and type of tissue (Table 1).

Direct spectrophotometry. In contrast to the above results concerning HPLC, the classical direct spectrophotometric method showed very poor reproducibility, and the difference between classical spectrophotometric and HPLC methods using internal standards was highly significant (Table 2). After scanning the samples, the calculations used linear least squares with a nine-point method (from 335 to 595 nm), a seven-point

Table 2. *Intraday reproducibility of traditional spectrophotometric method with and without internal standard compared with new HPLC technique*

No. of Spheres	Blood			Heart			Kidney			Liver			Brain		
	CV_1	CV_{IS}	CV_{HPLC}	CV_1	CV_{IS}	CV_{HPLC}	CV_1	CV_{IS}	CV_{HPLC}	CV_1	CV_{IS}	CV_{HPLC}	CV_1	CV_{IS}	CV_{HPLC}
White															
800	46	52	10†	42	26	5.7†	39	33	6.8†	65	50	5.2†	54	37	3.5†
3,000	21	31	3.4†	21	14	4.0*	18	15	3.4†	14	10	2.3†	22	19	3.2†
Yellow															
400	45	41	9.0†	44	21	6.5*	38	32	3.8†	50	51	3.0†	47	29	1.4†
1,500	25	25	1.8†	34	21	4.5†	22	19	4.8†	45	20	2.3†	21	24	3.9†
Violet															
1,600	65			40			48			54			42		
6,000	23			34			25			27			38		
Red															
800	33	39	9.6†	31	36	6.5†	34	36	5.8†	49	37	5.1†	24	25	3.7†
3,000	26	20	2.7†	21	14	5.1*	21	12	4.2*	15	16	1.9†	12	7.2	4.2^{NS}

Values are in percent; $n = 5$ replicate experiments. Results obtained with 7 wavelengths measurements are displayed. Three concentrations have been used for each color as in Table 1, but lower concentration for each color is not represented because it was considered under the limit of detection (mean bias or CV > 100%, or both mean bias and CV > 60%). CV_{IS} and CV_1, CV of traditional spectrophotometric method with and without IS, respectively; CV_{HPLC}, CV obtained with new HPLC technique (with IS). $*P < 0.05$, $†P < 0.01$, CV_{IS} vs. CV_{HPLC}. ^{NS} Nonsignificant difference.

method (obtained by deleting the first 2 points, at 335 and 375 nm, because the high background noise observed at these wavelengths was supposed to decrease reproducibility), or the classical four-point method (number of measurements equal to number of unknowns). We observed an improvement in the quality of results when the reduced seven-point method was used compared with the nine-point technique. In contrast, the four-point technique always showed an impairment in the quality of results (except in the case of white spheres, for which the 3 techniques gave similar results). It was not possible to measure dye content at sphere concentrations less than 200 (yellow), 400 (white and red), or 800 spheres per milliliter (violet): CVs and bias both always exceeded 60% at these concentrations.

Because the HPLC technique showed that recovery of dye after extraction was incomplete, we estimated the bias obtained with the direct spectrophotometric technique by comparing the results with those obtained by direct extraction of spheres in saline. With HPLC, mean bias ($n = 5$) was in the range of -4.7 ± 12, -4.1 ± 5.6, and -3.3 ± 2.1% for the low, medium, and high concentrations, respectively, whereas with direct spectrophotometry (7 points with internal standard), mean bias was in the range of -17 ± 19 and -6.8 ± 12% for the medium and high concentrations, respectively.

In vivo comparison of the two techniques. The results of the in vivo experiment are summarized in Table 3. With the exception of brain measurements in *rat 2* (which received only 100,000 spheres), the two methods gave similar mean results, but HPLC always showed lower CVs than the traditional spectrophotometric technique using internal standard. However, the variability within an organ was still greater than the in vitro variability.

Table 3. *Comparison between HPLC and traditional spectrophotometry with internal standard*

	HPLC		Spectrophotometric	
	Mean no. of spheres	CV_{HPLC} %	Mean no. of spheres	CV_{IS} %
	Rat 1			
Heart (169 ± 49 mg)				
White	4,855	12	5,285	42*
Yellow	4,091	20	4,787	52*
Red	4,245	14	5,469	36*
Brain (333 ± 90 mg)				
White	721	20	794	38^{NS}
Yellow	698	27	791	42^{NS}
Red	743	18	993	70*
Right kidney (227 ± 39 mg)				
White	2,799	6.7	3,041	22*
Yellow	2,495	9.4	3,225	32*
Red	2,533	18	3,531	43^{NS}
Left kidney (220 ± 42 mg)				
White	3,116	7.9	3,522	42†
Yellow	2,911	21	2,874	36^{NS}
Red	2,561	10	2,231	55*
	Rat 2			
Heart (190 ± 54 mg)				
White	1,230	17	1,224	58*
Yellow	1,050	19	1,324	62*
Red	1,402	19	1,681	63*
Brain (279 ± 138)				
White	181	22	455	47^{NS}
Yellow	209	19	387	58*
Red	204	25	498	85*
Right kidney (207 ± 33 mg)				
White	1,007	17	909	39^{NS}
Yellow	888	19	783	34^{NS}
Red	1,040	20	1,043	40^{NS}
Left kidney (223 ± 35 mg)				
White	1,053	21	884	55*
Yellow	903	11	954	43*
Red	1,053	16	900	61*

Values in parentheses are tissue sample weights (mean ± SD) for each organ; $n = 5$ replicate experiments. For each technique, 1,500 violet spheres/sample were used as internal standard. *Rat 1* received a mixture of 300,000 white, 300,000 yellow, and 300,000 red spheres. *Rat 2* received a mixture of 100,000 white, 100,000 yellow, and 100,000 red spheres. The number of spheres recovered is normalized for 200 mg tissue. *$P < 0.05$, †$P < 0.01$ CV_{IS} vs. CV_{HPLC}. ^{NS}Nonsignificant difference.

DISCUSSION

The present study shows that assaying dye content of colored microspheres by HPLC with internal standard is a major improvement compared with the direct spectrophotometric assay. The extraction step needs to be carefully performed. In fact, use of surfactant is critical. Some authors use filtration in the washing-extraction process (12). This may improve the sample washing procedure but with the risk of losing spheres. We made the choice of working in a unique tube. This procedure is assumed to lose fewer spheres but may result in incomplete clean-up procedure. In fact, recovery was incomplete at all particle concentrations, especially in blood and in the liver, as already described by Hakkinen et al. (8). However, these authors used a much larger number of spheres in their samples (5,000 spheres) compared with the range of spheres used in our experiment.

The lower limit of detection of the HPLC technique was far below the number of spheres usually encountered in tissue or blood samples. When there are ≥400 microspheres in the sample, the relative error of the assay is then between 2 and 10% with the yellow, white, and red microspheres. To date, only three different measurements are made possible because, of the five available colors, one served as internal standard (violet) and another (blue) exhibited marked degradation during the extraction or separation procedure, leading to the unexpected observation of three different peaks. We chose violet as standard because its absorbance is much lower than the absorbance observed with the other colors, thus leading to a greater CV at the critical value of 400 spheres/sample: ~1,500 spheres appears then to be the adequate number to be added as internal standard (see Fig. 1). The use of the internal standard markedly improved the precision of the technique (Table 1). This is not surprising because recovery appears incomplete even at high particle concentrations. It should also be noted that we only measured recovery relative to dye directly extracted from spheres in DMF and not relative to the pure dye molecule. The technique of isocratic HPLC with UV-visible spectropho-

tometric detection is simple and easy to implement. However, it is certainly more time consuming than the direct spectrophotometric method; the use of an autosampler is highly recommended.

At the critical value of 400 spheres/sample, the direct spectrophotometric method cannot be recommended because of unacceptable error in flow measurement due to lack of precision of the assay. The high background noise associated with the direct spectroscopic measurement method is responsible for this imprecision. In contrast, chromatography adds a separation step that markedly increases the signal-to-noise ratio and permits a single determination of the compound of interest.

In vivo injection of spheres in rats confirms these findings. The mean number of spheres measured in samples by the two methods is not markedly different. This confirms the results of the in vitro experiments showing that bias was (on average) not very important. Nevertheless, when the number of spheres in the samples was low (<300 in brain of *rat 2*), a systematic difference in the number of spheres measured by the two techniques became visible. The average intraorgan CV was three times greater with traditional spectrophotometry than with HPLC (Table 3). In fact, physiological variability in sphere deposition in organs is well documented. For example, Bassingthwaighte et al. (3) showed that relative dispersion of flows measured in baboon, sheep, and rabbits using spheres or soluble markers was 23–30% (mean of 6–11 animals). Our results of single-organ variability in sphere content (measured with HPLC) is consistent with these findings. Nevertheless, the combined error made in measuring reference blood and tissue contents is expected to increase the resulting CV of regional blood flow.

Cost and time requirements. The extraction procedure is the same for the two techniques. However, these two techniques differ in equipment, in time needed for spectrophotometry (traditional or after chromatographic separation), and in data processing requirements. The classical spectrophotometric technique requires the use of a spectrophotometer with an automatic scanning mode, leading either to a total spectrum or to the measurement of absorbance at predetermined wavelengths, usually coupled with a computerized recording system, whereas HPLC needs a simpler spectrophotometer (with the same precision and sensitivity). After the spectroscopic procedure, a computer program is needed for inverting the matrix of absorbance. On the other hand, HPLC requires the use of other equipments (pump, injection port, and column) and also the use of solvents. Moreover, HPLC is time consuming, and the use of an automatic sampler is highly recommended. The counting unit and computer software used for traditional spectrophotometry cost approximately $18,000, whereas an HPLC system costs approximately $17,000–$20,500 (isocratic pump $6,000, injection port and tubing $1,500, UV-visible detector $8,000, and pen recorder $1,500 or integrator $5,000). The replacement of the column ($500 for ~1,000 samples, i.e., $0.50 per sample assayed) and the extra cost of solvents ($30 for ~100 samples, considering mobile-phase recycling) have also to be taken into account. Also, there is an extra cost due to the time required for chromatographic separation. This cost is about $6–$8 per sample, considering $25 per hour for personnel costs. The addition of an automatic sampler to the HPLC system is highly recommended. Its cost ($6,000–$8,000) is then paid off after ~1,000 samples are assayed. Finally, the use of HPLC adds an extra cost of about $5,000–$8,000 for equipment and $0.80 per sample assayed compared with the traditional spectrophotometric technique. However, many laboratories already possess an HPLC system that can be used for different purposes.

In conclusion, HPLC with internal standard appears to give much less variability due to assay errors in the measurement of regional blood flow with colored microspheres. Moreover, this new technique allows the accurate detection of as low as 50–100 spheres, whereas the traditional technique, even when corrected with the use of an internal standard, cannot permit the detection of fewer than 200–400 spheres. Compared with other nonradioactive microsphere methods that have recently emerged [e.g., fluorescence (7) and X-ray fluorescence (14)], the colored microsphere technique has the advantage of simplicity of use associated with moderate cost. The lower limit of detection of colored spheres using HPLC favorably compares with the limit of detection obtained with fluorescent microsphere techniques (7), but the number of available different spheres is much lower when using colored rather than fluorescent spheres. The benefit of using HPLC with internal standard, then, is to increase the precision of the method and to allow the injection of a limited number of spheres, thereby decreasing the hemodynamic consequences of sphere embolization (11).

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