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TECHNICAL NOTE

Phthalaldehyde microprotein method: Usefulness and potential errors

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Two major technical advances introduced by Brenner et al [1, 2] have been responsible for recent breakthroughs in understanding the process of glomerular ultrafiltration at the single nephron level. These are the development of a method for directly measuring glomerular capillary pressure [1] and the microadaptation of the colorimetric protein method of Lowry et al [2, 3] to estimate changes in colloid osmotic pressure between the afferent and efferent ends of the glomerular vasculature [4]. The good correlation between colloid osmotic pressure as calculated from the arterial protein concentrations and direct measurement of the colloid osmotic pressure has established the usefulness of the protein method to accurately measure intravascular oncotic forces [5]. Because the colorimetric Lowry microprotein method is both time consuming and necessitates the use of a frequently unstable, relatively high intensity colorimetric scale for sample reading, attempts have been made to use other microprotein techniques as substitutes for the Lowry method [6–9]. Viets et al [7] have compared the usefulness of two fluorescent microprotein methods in micropuncture studies. Of these, the phthalaldehyde fluorescent microprotein method was preferable and appeared to be equally as useful as the Lowry method, with certain minor limitations. Our laboratory has spent the last 2 years evaluating the applicability of the phthalaldehyde technique to microprotein analysis in micropuncture studies. Although we have found the method to have the advantage of being less time consuming than the Lowry technique, we have noted certain limitations and potential errors that could artificially lower the estimate of protein content in plasma samples. Spuriously low protein concentrations could result in underestimations of afferent and efferent arteriolar oncotic pressures and overestimates of single nephron filtration fractions. This report is designed to describe the limitations of phthalaldehyde fluorescent protein technique and point out its potential errors in measuring protein concentration while comparing its usefulness to that of the colorimetric micro-Lowry method.

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Methods and results

Two techniques of plasma protein measurement were used: (*a*) the standard Lowry protein method as modified for micro samples [2, 3] and (*b*) a fluorometric method that relies on the fluorescence produced by a mixture of primary amines with *o*-phthalaldehyde [6, 8, 9].

Reagents for the fluorometric method included *o*-phthalaldehyde (Eastman Kodak, Rochester, New York) dissolved in 95% ethanol (800 mg in 10 ml), which was added to 1 liter of 0.4 M boric acid solution titrated to a pH of 9.7 with potassium hydroxide containing 2 ml of 2-mercaptoethanol. Brij (1.0 g/liter) (Pierce Chemical Co., Rockford, Illinois) was added to the filtered reagent to enhance fluorescence. This reagent mixture was covered and heated to 38° C while being stirred. Cuvettes were prepared from 100-μl capacity, 116-mm-long Microcaps (Drummond Scientific Co., Broomall, Pennsylvania). Each Microcap was divided into two 58-mm-long segments, and one end of each was sealed with a microflame (Water Welder, Henes Mfg. Co., Phoenix, Arizona). At least 20 nl of sample was added to each cuvette, containing 6 μl of the reagent described above. If smaller quantities of sample were used, then fluorescence was influenced by sample size. This relationship is shown in

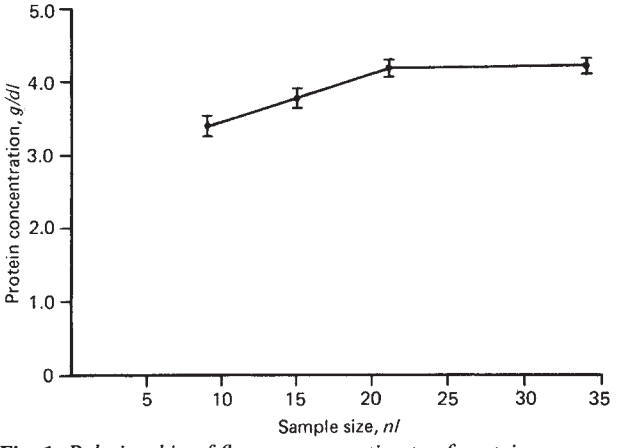

Fig. 1. *Relationship of fluorescence estimate of protein concentration to size of plasma sample using the o-phthalaldehyde technique.* Below a sample size of 20 nl, the estimated protein concentration is influenced by quantity of sample. Above 20 nl, protein concentration estimate is not altered by sample size. Each data point represents the mean ± SD of ten measurements.

Fig. 1. (Sample size down to 8 nl did not influence the colorimetric reading with the micro-Lowry technique.) The mixture of sample and *o*-phthalaldehyde was then centrifuged for 3 min and the open ends of the cuvettes sealed with the microflame. Centrifugation was carried out two additional times for 3 min after inverting the cuvettes. Samples were either read immediately in a darkened area or could be left several hours in a darkened area at room temperature before reading without significant decline in fluorescence.[1] Samples were read with an Aminco solid state fluorocolorimeter with a microcuvette holder. A mercury vapor lamp assembly (GE 85-watt) was used as the excitation source. Filters consisted of one of optimum excitation at 340 nm and one at optimum emission of 455 nm.

Standards consisted of bovine serum albumin (Sigma Chemical Co., Chicago, Illinois) at measured concentrations of 1.0, 2.0, 2.5, 5.0, 7.5, and 10 g/dl and rat serum. The latter was ultrafiltered with an Amicon ultrafiltration cell (Amicon Corp., Lexington, Massachusetts) through a Diaflo UM20 membrane (Amicon Corp., Lexington, Massachusetts) to half its original volume and diluted with ultrafiltrate of rat serum to protein concentrations approximating those of the bovine serum albumin standards as described by Viets et al [7].

[1] In the study of Viets et al [7], there was a decrease in fluorescence with time when the samples were not protected from normal daylight intensity. In the present study, it was found that the fluorescence reading was stable over several hours if the sample was read and stored in a dark room. This was similar to the finding of Weidekamm, Wallach, and Fluckiger [9].

Figure 2 shows the slopes of a series of ten measurements at each concentration of the relationship of protein content by using bovine serum albumin standards to fluorometric and colorimetric readings with the *o*-phthalaldehyde and micro-Lowry methods, respectively. Both slopes were linear through the range of 1.0 to 10.0 g/dl. Figure 3 shows the slopes of a series of ten measurements at each concentration, as described above, when rat serum standards were substituted for bovine serum albumin. The colorimetric readings with the micro-Lowry technique had the same linear relationship to protein concentration as they did with bovine serum albumin. Although the fluorometric readings also were related linearly to protein content between 2.0 and 10.0 g/dl, the straight-line relationship was lost below the former value. To determine the cause of nonlinearity, we added rat serum ultrafiltrate to lyophilized human plasma protein (Monibiol 1, Scientific Products, McGraw Park, Illinois) at concentrations of 1.0, 1.5, 2.0, 2.5, 5.0, 7.5, and 10 g/dl. When ten determinations at each concentration were made, a fluorometric reading-protein concentration curve was found to be identical to that when rat serum standards diluted with rat serum ultrafiltrate was used. Rat serum ultrafiltrate alone produced a small amount of fluorescence, which was equivalent to 0.4 to 0.5 g/dl protein content. These findings indicate that the "fluorescent noise" seen at protein concentrations less than 2 g/dl was due to

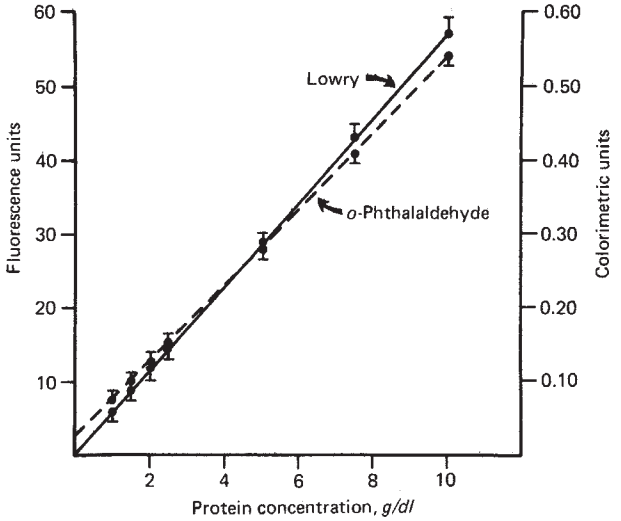

Fig. 2. *Relationship of protein concentration to fluorescent and colorimetric readings with the o-phthalaldehyde and Lowry methods, respectively, when standard solutions are prepared with bovine serum albumin.* Both techniques have linear relationships over the ranges examined. Each data point represents the mean ± SD of ten measurements.

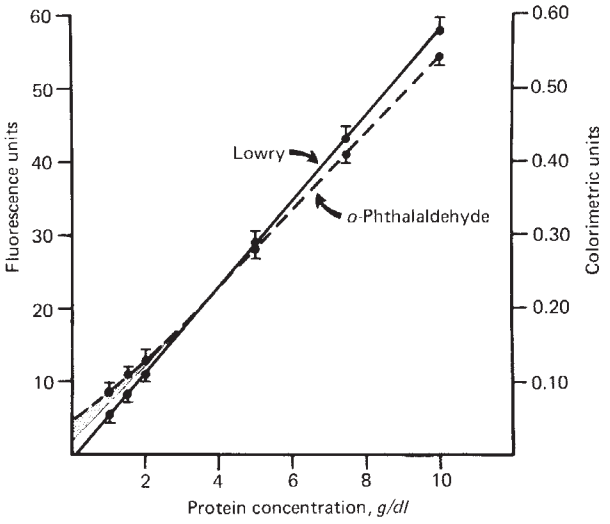

Fig. 3. *Relationship of protein concentration to fluorescent and colorimetric readings with the o-phthalaldehyde and Lowry methods, respectively.* When standards are prepared from rat serum, the fluorometric readings are not linearly related to protein concentration below 2.0 g/dl due to fluorescence of small-molecular-weight, nonprotein substances. The *hatched area* shows the region of nonlinearity. The background fluorescence noted at protein concentrations less than 2.0 g/dl was not additive to protein fluorescence above this level. The Lowry method showed the same linearity as when bovine serum albumin standards were used. Each data point represents the means ± SD of ten measurements.

factors present in the rat ultrafiltrate. The critical observation, however—as evidenced by the rat serum fluorescent standard curve—was that the small amounts of fluorescence produced by the rat serum ultrafiltrate was not additive to the fluorescence produced by protein above 2.0 g/dl.

When appropriate volumes of standard and sample solution were used (that is, greater than 20 nl), it was found that bovine serum albumin standards produced slightly but significantly lower results with the *o*-phthalaldehyde method when measuring rat serum samples. Determinations of protein concentrations were carried out on rat serum that had been ultrafiltered to one half of its original volume and diluted 1:1 and 3:1 with the ultrafiltrate. Nine determinations at each concentration or dilution gave the following results: micro-Lowry, 2.55 ± 0.07, 5.20 ± 0.05, and 10.20 ± 0.23 g/dl; *o*-phthalaldehyde, 2.43 ± 0.10, 4.92 ± 0.06, and 9.8 ± 0.21 g/dl (all $P < 0.01$).

With the completion of these standardization experiments, micropuncture studies were carried out in Munich Wistar rats to determine the usefulness of the fluorescent method compared with the micro-Lowry technique in measuring protein concentration in systemic arterial and efferent arteriolar plasma. Eight rats weighing between 150 and 250 g that had been fasted for 10 hours were prepared for micropuncture studies as previously described [10]. A maintenance infusion of Ringer's solution was given via the external jugular vein at a rate of 1.5 ml/hr. Star vessels representing efferent arterioles were identified on the surface of the kidney, and a minimum of three and up to five samples of blood were drawn with pipettes (14 μ O.D.) which had been pretreated with a 1:10 (vol) solution of Dow Corning 1107 fluid (Dow Corning Corp., Midland, Michigan) and trichlorethylene. Femoral artery blood samples were collected prior to and following the series of efferent arteriolar blood collections. The micro-Lowry colorimetric and *o*-phthalaldehyde fluorescent protein methods were used to measure protein content of the femoral artery and efferent arteriolar plasma samples after they had been separated from red cells by centrifugation. In this series of determinations, rat serum standards were used. These measurements and the calculated single nephron filtration fractions are shown in Table 1. There were no significant differences in protein concentration estimates of either femoral artery or efferent arteriolar plasma samples with the two techniques. The single nephron filtration fractions were the same as well.

Discussion

The results of this study indicate that the *o*-phthalaldehyde fluorescent method is equally as accurate as the micro-Lowry colorimetric technique in estimating protein concentration in nanoliter quantities of plasma, confirming the results of Viets et al [7]. The measurement of plasma protein concent in femoral artery and efferent arteriolar blood samples in a series of ten Munich Wistar rats showed identical results with the two methods. The advantages of the fluorescent method is its rapid performance and stability of the fluorescent reading. The procedure can be carried out in approximately one-half the time that the micro-Lowry method requires.

On the other hand, there are certain technical considerations and limitations with the fluorescent technique that need to be taken into account to make it equally as accurate as the colorimetric method. First, it was found that the fluorescence of a plasma sample was correlated with sample size below a critical volume of 20 nl. At 20 nl and above, sample size did not affect the fluorescence of the

Table 1. Systemic and efferent arteriolar plasma protein concentrations[a]

	Micro-Lowry method			*o*-Phthalaldehyde method		
Rat no.	C_{AA}	*g/dl*	C_{EA}	C_{AA}	*g/dl*	C_{EA}
1	4.6		6.0	4.3		6.0
			6.1			6.0
			6.1			6.0
	4.5		6.2	4.5		5.9
2	4.7		7.1	4.5		7.3
			7.1			7.2
			7.0			7.3
	4.5		6.9	4.5		7.0
3	5.4		7.5	5.3		7.6
			7.4			7.6
	5.0		7.4	5.1		7.6
4	4.9		7.0	4.9		6.8
			6.7			7.0
	5.0		6.7	4.6		6.7
5	4.6		7.7	4.8		7.6
			7.6			7.8
			7.6			7.9
	5.0		7.6	5.0		7.8
6	4.9		7.9	4.8		7.6
			7.7			7.5
	—		7.8	—		7.6
7	5.5		8.2	5.6		8.3
			8.2			8.2
			8.6			8.4
	5.6		8.5	5.6		8.3
8	—		8.2	—		8.1
			8.3			8.2
	5.5		8.2	5.4		8.1
9	5.1		7.8	4.9		7.8
			7.9			8.0
	4.7		7.7	4.7		7.7
10	5.2		7.9	5.2		8.0
			7.9			8.0
			7.8			7.9
			8.2			8.2
	5.4		8.2	5.2		8.3
Mean	5.0		7.5	4.9		7.5
± SD	±0.36		±0.70	±0.39		±0.71
N	18		36	18		36

[a] Abbreviations are: C_{AA}, afferent arteriolar plasma protein concentrations (considered equal to femoral artery plasma protein concentration); and C_{EA}, efferent arteriolar plasma protein concentrations. Data shown are the individual results of all samples from each rat.

sample and reagent mixture. The volume requirement for the *o*-phthalaldehyde method in part may explain the previously noted differences in results of 25% from the micro-Lowry technique when using bovine serum albumin standards [7]. Viets et al [7] used standard and plasma sample volumes between 3 and 5 nl. In the present study, when at least 20 nl of standard or sample was added to the fluorescent reagent, there was a 10% difference in an estimate of protein concentration when bovine serum albumin standards were used. Thus, it appears that both adequate volume and the use of rat serum standards are required for optimal accuracy with the fluorescent method.[2]

The second technical consideration discovered in this study was that there is a small amount of fluorescence present in an ultrafiltrate of rat plasma. The fluorescence produced would be equivalent to a protein measurement of approximately 0.4 g/dl. It was found that if ultrafiltrate was added to lyophylized human plasma protein at low concentrations of protein, the ultrafiltrate fluorescence affected the estimate of protein concentration when the latter was < 2 g/dl. Above 2 g/dl, fluorescence was not affected by the ultrafiltrate. Thus, at very low protein concentrations, the fluorescence of ultrafiltrate distorted the estimate of protein concentration, but above 2 g/dl this ultrafiltrate factor was not additive and protein concentration estimates were linearly related to fluorescence between 2 and 10 g/dl. Although it is known that *o*-phthalaldehyde reacts with all primary amines, this fluorescent "noise" seen with ultrafiltrate could not be due solely to amino acids and filterable peptides because their total concentration would be less than 35 mg/dl [11]. According to Rubin [12], the unmasked fluorescence of ultrafiltrate at very low protein concentrations would disappear at higher protein concentrations because of the quenching effect of increasing concentrations of fluorescing substance. This fluorescence phenomenon would explain the nonadditive nature of the fluorescence seen in ultrafiltrates and the effect on protein concentration estimate at low levels of protein below 2 g/dl. This small amount of fluorescence that was seen with ultrafiltrate but not with saline was noted by Viets et al [7], but he found no apparent difference between ultrafiltrate and isotonic saline as diluent in the preparation of rat serum standards following rat serum ultrafiltration. It is unclear whether the small amount of fluorescence was additive to the actual protein fluorescence up to 10 g/dl or whether the fluorescence was sufficiently low that it did not affect the linearity of the rat serum standard curve.

This study demonstrates that, although the *o*-phthalaldehyde fluorescent protein technique has the advantage of being simple and rapid, it has potential errors if certain precautions are not taken. Both the effect of sample volume on fluorescence and the presence of detectable fluorescence in

[2] It should be pointed out that an effect of volume of sample on fluorescence was not found by another worker, in that results with a micro-*o*-phthalaldehyde technique using 3 to 5 nl of sample and a macro technique gave similar results (Deen, personal communication).

serum ultrafiltrate could result in an underestimation of protein concentration and calculated afferent and efferent arteriolar oncotic pressure. A sample size less than 20 nl would cause the error in the former situation, and an investigator's decision to subtract ultrafiltrate fluorescence from sample fluorescence would cause the falsely low estimate of protein concentration in the latter situation. An investigator might choose to subtract ultrafiltrate fluorescence if he were not aware of the nonlinearity of the fluorescence-protein relationship at low concentrations of the latter. As pointed out, falsely low estimates of intravascular oncotic pressure can result in erroneous conclusions concerning the conditions of filtration equilibrium or disequilibrium in glomerular dynamics [13, 14]. Errors of this kind can cause unnecessary controversy in the study of glomerular dynamics.

Acknowledgments

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