notes on methodology

Use of a rapid ultrafiltration technique in the determination of plasma glycerol

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SUMMARY The use of plasma ultrafiltrates in the enzymatic analysis of glycerol has been compared with filtrates prepared by using metaphosphoric acid and heat denaturation. The results which were obtained with these three methods of deproteinization are comparable; however, the membrane ultrafiltration technique is the simplest of the procedures and yields an undiluted filtrate which is free of foreign ions.

SUPPLEMENTARY KEY WORDS: enzymatic · plasma ultrafiltrates

THE ESTIMATION of plasma glycerol levels is frequently a cumbersome procedure, primarily because of the difficulty in obtaining a clear protein-free filtrate. Deproteinization is usually accomplished with metaphosphoric or perchloric acids; this procedure necessitates repeated centrifugations and introduces foreign ions which, in high concentrations, can interfere with the analysis. In addition, plasma glycerol concentrations are normally quite low, and the use of precipitating agents markedly dilutes the glycerol concentration in the ultrafiltrate. Accordingly, we have investigated various methods for preparing protein-free filtrates of plasma which would circumvent these problems. In this paper we have compared the results for the enzymatic determination of glycerol from filtrates prepared by using metaphosphoric acid, heat denaturation, and ultrafiltration techniques.

Deproteinization Procedures. Aliquots of human plasma were deproteinized by the following procedures.

(*a*) *Metaphosphoric Acid.* To 1.0 ml of plasma there was added 1.5 ml of distilled water, and then 0.5 ml of 10% metaphosphoric acid. This solution was thoroughly mixed with a test tube vibrator, and allowed to stand for 15 min prior to centrifugation at 2000 rpm (1000 *g*) for 20 min. The resulting supernatant was decanted and recentrifuged for 10 min in order to obtain a clear protein-free filtrate.

(*b*) *Heat Denaturation.* 1.0 ml of plasma was mixed thoroughly with 1.5 ml of 0.2 M acetate buffer, pH 4.4 (1), and incubated at 80°C for 30 min (2). The sample then was cooled to room temperature prior to centrifugation at 2000 rpm for 30 min. The resulting supernatant was decanted and, if required, was centrifuged for an additional 10 min.

(*c*) *Membrane Ultrafiltration.* Approximately 3.0 ml of plasma was placed into a Centriflo[1] membrane cone and centrifuged at 2000 rpm (1000 *g*) for approximately 30 min. To assure a constant flow rate these cones were initially filled with distilled water and placed in their supports overnight. Prior to use they were centrifuged at 1000 rpm (250 *g*) for 10 min in order to remove all the residual water.

Procedure. The enzymatic procedure of Spinella and Mager (3) for glycerol was utilized for the analyses of all the protein-free filtrates. The method was further simplified by preparing, just prior to use, a combined reagent, thus decreasing the number of individual pipettings.

Briefly, the procedure consisted of adding the following to a cuvette (10 mm light path): 1.0 ml of the protein-free fitrate, 1.5 ml of $MgCl_2$–hydrazine buffer (1 mg/ml of $MgCl_2$ in 1 M hydrazine–hydrazine dihydrochloride buffer, pH 9.6), and 0.2 ml of the combined reagent which contained 1.0 ml each of 0.064 M ATP, 0.038 M NAD, 0.38 M cysteine hydrochloride, and 0.2 ml of a solution of glycerokinase (1.0 mg/ml). The contents were mixed thoroughly by inversion. After incubation at 30°C for approximately 10 min the absorbance (OD_1) at 340 nm was measured in a Zeiss spectrophotometer. Then 0.01 ml of a solution of α-glycerophosphate dehydrogenase (10 mg/ml) was added, and the test solution was again mixed by gently inverting the cuvette. The absorbance of this solution was noted at approximate 1 min intervals until the reaction had reached completion (OD_2). The total increase in absorbance (OD_2–OD_1) is directly proportional to the glycerol concentration in the filtrate. 1.0 ml of a solution of glycerol (0.01 μM/ml) was used to monitor the assay.

Results. To compare the heat denaturation method with the metaphosphoric acid procedure, 10 different samples of human plasma were deproteinized by these two techniques and then assayed in duplicate. The mean glycerol concentration (μM/ml) ± SEM for these analyses were 0.092 ± 0.014 and 0.089 ± 0.014, respectively. These two groups are not statistically different from one another.

In another series, 20 different samples of human plasma were simultaneously processed by the metaphosphoric acid and the membrane ultrafiltration techniques, and they were analyzed in duplicate for glycerol. The mean glycerol levels ± SEM obtained for these two pro-

[1] Amicon Corp., Lexington, Mass.

TABLE 1 MEAN GLYCEROL ANALYSES OF ULTRAFILTRATES OF THREE DIFFERENT PLASMA SAMPLES PROCESSED SIMULTANEOUSLY IN SEPARATE CONES

	Plasma No. 1	Plasma No. 2	Plasma No. 3
		μM/ml	
	0.043	0.291	0.099
	0.044	0.296	0.100
	0.043	0.287	0.103
	0.043	0.301	0.103
	0.044	0.296	0.100
	0.043		
	0.043		
	0.042		
Mean	0.043	0.294	0.101
±SEM	0.0002	0.002	0.0008

cedures were identical, 0.066 ± 0.007 μM/ml, with a range of 0.038–0.138 μM/ml for the metaphosphoric acid filtrates, and 0.031–0.137 μM/ml for the ultrafiltrates. To evaluate the precision of the individual analyses, the standard error of the duplicates for both procedures was calculated. A value of 0.002 μM/ml was obtained for each method.

To test the reproducibility of the ultrafiltrates obtained from different Centriflo cones, aliquots of three plasma samples were placed in separate cones, and after centrifugation the glycerol in the resultant ultrafiltrates was analyzed. The mean and the SEM for these analyses are tabulated in Table 1. To determine the recovery of added glycerol when using this ultrafiltration technique, equal volumes of plasma and solutions of glycerol (0.05 or 0.1 μM/ml) were mixed. A mean recovery of 98% (range 94–104%) was obtained from the analyses of 18 different samples prepared in this manner.

Discussion. It is apparent from the data that the glycerol analyses of plasma filtrates prepared with metaphosphoric acid are in excellent agreement with those obtained from membrane ultrafiltrates and from solutions deproteinized by heat denaturation. In addition, glycerol levels of ultrafiltrates from single plasma samples which were prepared from several different cones are quite similar; thus, the reproducibility of the procedure using membrane ultrafiltrates is comparable to that of the older methods.

A marked advantage of using ultrafiltrates in this analysis is that the glycerol level, normally very low in human plasma, is not diluted; consequently, the change in absorbance is severalfold greater than that in methods utilizing metaphosphoric acid or heat denaturation. Moreover, no foreign ions are introduced into the test solution, thus minimizing any possible interference with the analytical procedure.

Because of these important advantages, as well as the relative simplicity of preparing the ultrafiltrates, we are now using this membrane ultrafiltration process in all determinations of plasma glycerol. We have also successfully demonstrated the application of these ultrafiltrates for the determination of bound and free calcium (4), as well as the analyses of plasma glucose, urea nitrogen, uric acid, and creatinine (5). It is suggested that this preparative method could be profitably applied to the analysis of plasma lactate and pyruvate, since unit concentrations of these constituents are usually low and deproteinization is quite cumbersome.

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REFERENCES

1. Dawson, R. M. C., D. C. Elliot, W. H. Elliot, and K. M. Jones. 1959. Data For Biochemical Research. Clarendon Press, Oxford, England. 197.
2. Gibbs, R. J. 1954. *Arch. Biochem. Biophys.* **52:** 340.
3. Spinella, C. J., and M. Mager. 1966. *J. Lipid Res.* **7:** 167.
4. Farese, G., M. Mager, and W. F. Blatt. *Clin. Chem.* In press.
5. Farese, G., and M. Mager. *Clin. Chem.* In press.