notes on methodology

Modified enzymatic procedure for the routine determination of glycerol and triglycerides in plasma

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SUMMARY A modification of Wieland's enzymatic procedure for glycerol analysis is presented. It is simple and precise, and readily applicable to the routine analysis of plasma and tissue glycerol and triglycerides. Optimal precision is obtained in samples containing 0.003–0.400 μmole of glycerol per ml, which in this method is equivalent to plasma levels of 0.011 to 1.40 μmoles/ml.

KEY WORDS determination · glycerol · triglycerides · plasma · enzymatic · modification · metaphosphoric acid · glycerokinase

THE RECENT INTRODUCTION of plasma glycerol levels as a dynamic index of lipolytic events has heightened the desirability of a simple, specific, and precise analytical technique. Glycerol levels in plasma or tissue may be determined enzymatically (1–7), colorimetrically (8), fluorimetrically (9), or chromatographically (10). Since the enzymatic techniques are specific, and require no elaborate instrumentation, an attempt was made to modify the various enzymatic procedures and to develop a technique that would be applicable to the routine analysis of plasma both for glycerol and triglycerides.

Reagents. 0.5 N and 1 N NaOH; 10% HPO_3 (w/v); 0.1 M ATP[1] in 0.2 M phosphate buffer pH 7.4; 0.5 M cysteine-HCl; 0.0225 N NAD[1] in phosphate buffer pH 7.4; 0.1 M $MgCl_2$; 1 M hydrazine–hydrazine dihydrochloride buffer, pH 9.4 (prepared by adding a M solution of the salt to 1 mole of the hydrazine base); glycerokinase;[1] α-glycerophosphate dehydrogenase[1] (α-GPD); 0.100 mM glycerol standard prepared weekly from a refrigerated 20.0 mM stock solution.

Glycerol Analysis. Into a 12 ml centrifuge tube, add 1.0 ml of plasma, 2.0 ml of water, and 0.5 ml of 10% HPO_3. Mix well with a test tube vibrator. Centrifuge at 3000 rpm for 10–20 min, decant the supernatant liquid into another test tube, and centrifuge again.

Pipette the following into a 3 ml cuvette (10 mm light path): 0.26 ml 0.5 N NaOH; 1.00 ml of supernatant solution (a blank is prepared by pipetting 1.26 ml of H_2O in a cuvette; the standard contains 1.00 ml of 0.1 mM glycerol solution plus 0.26 ml of H_2O); 1.50 ml of hydrazine buffer; 0.06 ml of cysteine-HCl; 0.03 ml of ATP; 0.03 ml of $MgCl_2$; 0.10 ml of NAD; and 0.01 ml of glycerokinase solution. Mix the contents thoroughly by inversion.

Incubate the above mixture at 30° for 10 min in a water bath or in the thermoregulated cell compartment of the Beckman Spectrophotometer. Read optical density (OD) at 340 mμ.

Add 0.01 ml of α-GPD to each cuvette, mix thoroughly and determine the OD at 340 mμ of each solution at intervals of about 1 min until a constant value is obtained (generally 5–10 min for normal plasma levels).

Calculations. During the enzymatic conversion of glycerol, 1 mole of NADH is formed for each mole of glycerol. Since the extinction coefficient, at 340 mμ, of 0.01 μmole of NADH is 0.062, the following equation is applicable:

$$\frac{\Delta\ OD_{sample} - \Delta\ OD_{blank}}{0.062}\ (0.01)(3.0)(3.5) =$$

μmoles of glycerol per ml of plasma,

where Δ OD = the change in absorbance. Since the dilution in the deproteinization is 1 to 3.5 and the final cuvette volume is 3.0 ml, these factors are included in the above calculations.

Triglyceride Analysis. The procedure for the enzymatic determination of glycerol can be applied to triglyceride analysis, by prior conversion of the triglyceride to fatty acids and glycerol. Plasma samples were extracted, chromatographed on silicic acid columns, and saponified essentially as outlined in the colorimetric method of Carlson and Wadström (11). Two drops of 2.5% KOH and a 1 hr incubation period were found necessary for complete saponification, after which the samples were evaporated to dryness, boiled in 10 ml of hexane, and extracted with 1.0 ml of 0.67 M H_2SO_4 (11). A 0.5 ml aliquot of the sulfuric acid extract was pipetted into a cuvette followed by 0.26 ml of water and 0.5 ml of N NaOH, which adjusts the solution to approximately pH 7.4. The enzymatic glycerol assay was then performed as outlined above.[2]

Circulating plasma glycerol and triglyceride levels were determined on 18 subjects. The proposed method for triglyceride analysis was compared with an estab-

[1] Sigma Chemical Company, St. Louis, Mo. or California Biochemical Corporation, Los Angeles, Calif.

[2] The equation given above for calculating plasma glycerol levels is applicable for estimating plasma triglyceride levels (in mg/100 ml) by substituting 1402 for the dilution factor 3.5. This factor is based on an assumed average molecular weight of 876 for plasma triglycerides, and compensates for the 8-fold dilution of the saponification mixture before enzymatic assay of 0.5 ml.

TABLE 1 MEAN PLASMA GLYCEROL LEVELS

Author	n	Mean	SEM
		μ mole/ml	
Present method	18	0.049 (0.033–0.079)	0.003
Garland and Randle (6)	5	0.048	0.004
Carlson and Orö (4)	14	0.070	0.004
Shafrir and Gorin (5)	28	0.080	0.006*
Jellum and Björnstad (10)	9	0.085*	0.009*

* Calculated from authors' data.

lished colorimetric method (11), by assaying in duplicate 17 of these plasma samples. The plasma assayed was from normal army volunteers (ages 20–25). After an overnight fast of approximately 12 hr, the men reported to the laboratory and rested quietly for an additional 2–3 hr prior to venipuncture. The blood samples were introduced into chilled heparinized centrifuge tubes and the plasma was immediately separated by centrifugation at 4°.

Results. Standard solutions containing from 0.002 to 1.00 mM glycerol were added undiluted into cuvettes and enzymatically assayed as described above. A linear spectrophotometric response was obtained up to 0.400 mM. Although glycerol levels as low as 0.002 mM can be readily detected and measured, optimal precision is in the range 0.003–0.400 mM (equivalent to 0.011–1.40 μmoles of glycerol per ml of plasma). The daily reproducibility of the procedure was determined by repeatedly analyzing a 0.100 mM glycerol solution to give a mean value of 0.102 mM, SEM 0.00087 (n = 38).

Recovery studies were performed by the addition of various amounts of glycerol standard (0.040–0.100 μmole) to plasma samples. The mean value for six recoveries, assayed in duplicate following deproteinization, was 97.4%.

The mean fasting plasma glycerol level of the 18 normal men of this study was 0.049 mM (Table 1). The precision[3] of duplicate analyses of each plasma supernatant was 0.003 mM. Mean values from the literature for the enzymatic determination of plasma glycerol are also listed in Table 1. The mean plasma glycerol level obtained in this study is similar to that reported by Garland and Randle (6), but is lower than the values of other authors. Since basal plasma gycerol levels are affected by previous nutritional status, hours of fasting, per cent body fat, and emotional state, the differences between the levels reported by various investigators (Table 1) may be reflections of some or all of these factors.

For verification of the triglyceride analysis, purified samples of trilaurin, triolein, and tristearin (Hormel Institute, Austin, Minn.) were dissolved and diluted in chloroform, and analyzed colorimetrically and enzymatically. For these comparisons, solutions containing varying known amounts of triglyceride (36–100 mg/100 ml) were assayed on 10 different occasions. The average difference between the enzymatic assay and theoretical levels was equal to 0.3%, while the enzymatic and colorimetric analyses differed by 1.3%.

TABLE 2 PLASMA TRIGLYCERIDE ANALYSIS BY ENZYMATIC AND COLORIMETRIC METHODS

Method	n	Mean	Range	SEM	Precision of a Single Determination*
		mg/100 ml			
Enzymatic (present)	17	98.8	37–215	10.4	7.42
Colorimetric (11)	17	101	32–193	10.0	7.43

* $SD_d = (\Sigma d^2/2N)^{1/2}$.

The comparative data for 17 enzymatic and colorimetric plasma triglycerides analyses are shown in Table 2. Statistical analysis by the paired "*t*" test demonstrated no significant difference between the two methods ($SE_{Diff.} = 3.22$, $t = 0.713$, $P > 0.40$).

Discussion. The described procedure, which is basically a modification of Wieland's technique (1), introduces certain new features: first, the use of metaphosphoric acid for protein precipitation, which permits simpler and more precise sampling of the protein-free filtrate; second, the introduction of glycerokinase before adding α-GPD, which seems to produce a more stable system by bringing all the substrate to a uniform level of reactivity prior to the reduction of NAD. Under such circumstances, we have found that the reaction goes rapidly to completion, and precise monitoring of timed intervals is not mandatory. Thus, the change in absorbance is obtained after the reaction is completed and without the need for any extrapolation.

The observed maximum spectrophotometric linearity was up to 0.400 μmole of glycerol per ml of cuvette sample, which is greater than the amount normally present in aliquots of plasma utilized for glycerol and triglyceride analysis (i.e., 1.40 μmoles of glycerol per ml of plasma and 273 mg of triglyceride per 100 ml of plasma). The precision noted in the triglyceride analyses is in agreement with what has been reported by others for this determination (12), and the variability is probably the result of sample manipulation through extraction, chromatographic separation, and saponification processes rather than errors in the enzymatic determination.

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[3] Precision is defined as the estimate of the standard deviation of the difference (*d*) between duplicate determinations of *N* specimens: $SD_d = (\Sigma d^2/2N)^{1/2}$.

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