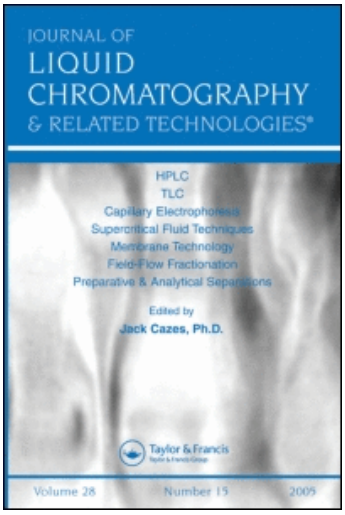


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# Enzymatic Determination of Triglycerides in Conjunction with High Performance Liquid Chromatography

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# ENZYMATIC DETERMINATION OF TRIGLYCERIDES IN CONJUNCTION WITH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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## ABSTRACT

The glycerol kinase (GK) catalyzed reaction involving the conversion of glycerol and adenosine triphosphate (ATP) to glycerol-3-phosphate and adenosine diphosphate (ADP) has been used in conjunction with HPLC for the determination of triglycerides. After alkaline hydrolysis of the triglycerides to glycerol, the enzyme reaction was carried out. The ADP formed and the remaining ATP were then separated by HPLC and the ADP peak area correlated to the concentration of triglycerides originally present in the sample. Linearity of the method was established from 28-180 mg/dl with a reproducibility of 6.5% RSD. A comparison between the HPLC method and the standard coupled enzyme system for triglycerides in real serum indicated a correlation coefficient of 0.977.

## INTRODUCTION

Triglycerides (esters of glycerol and fatty acids) make up a general type of lipid found in plasma. They combine with proteins in the form of water soluble complexes termed lipoproteins. Abnormalities in lipid metabolism are characterized by an excess of one or more of the lipoproteins. These can be detected by plasma cholesterol and triglyceride levels. Elevated triglyceride levels are likely to be found in diabetes mellitus, liver disease and atherosclerosis (1).

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Several methods for the determination of glycerol or glycerol derived from serum triglycerides have been reported. A two enzyme system involving glycerokinase (GK) and glycerophosphate dehydrogenase (GPD) has been employed for the determination of glycerol, however the equilibrium constant for the GPD-catalyzed reaction was unfavorable requiring the use of the trapping agent hydrazine (2). Another coupled enzyme system for glycerol, involving GPD and diaphorase, indicated good selectivity but the procedure was somewhat lengthy (3). Hercules and Sheehan (4) coupled the chemiluminescent luciferase reaction to the GK enzyme reaction and related the production of light due to the residual ATP to the initial glycerol present in the sample.

Probably the most common method for the assay of serum triglycerides is that basically reported by Bucolo and David (5). It is based on four coupled enzyme reactions.

$$\text{Triglycerides} \xrightarrow{\text{lipase}} \text{Glycerol} + \text{Fatty Acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-P} + \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}.$$

where ATP is adenosine triphosphate, ADP is adenosine diphosphate, PK is pyruvate kinase, PEP is phosphoenolpyruvate, LDH is lactate dehydrogenase, and NADH and $NAD^+$ are the reduced and oxidized forms, respectively, of nicotinamide adenine dinucleotide. The amount of NADH oxidized is represented by a decrease in absorbance at 340 nm.

Previously, we have used high performance liquid chromatography (HPLC) to facilitate the assay of kinase enzymes such as creatine kinase (6). To avoid the necessity of a coupled enzyme system for the assay of glycerol, we have used HPLC to separate and quantitate the ADP produced in the GK reaction. The amount of ADP can be related to the glycerol formed after alkaline hydrolysis of the triglycerides present in the sample. Besides avoiding the instability problems of NADH, the expense of enzymes and substrates required in the coupled systems is eliminated.

## MATERIALS

### Apparatus

The liquid chromatograph consisted of an Altex Model 110-A high pressure pump (Altex, Berkely, CA), a Rheodyne Model 7125 injector ( Rheodyne, Berkely, CA) and an Altex Model 153 UV detector (254 nm). A 5 cm x 4.1 mm I.D. precolumn packed with octadecyl (C-18) derivatized pellicular silica and a 25 cm x 4.1 mm I.D. R-Sil C-18 working column (Alltech Associates, Deerfield, Illinois) were used for the separations. Peaks were recorded by an Omniscribe Model B-5000 recorder (Houston Instruments, Austin, TX.) Peak areas were calculated using a HP 3000 minicomputer equipped with a HP-7221A plotter (Hewlett-Packard, Palo Alto, CA).



### Chemicals

All water used was distilled and deionized. Tetrabutylammonium hydrogen sulfate was supplied by either Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO). Trizma buffer [tris(hydroxymethyl)aminomethane], ATP (sodium salt), GK (lyophilized from E. coli), control serum (type 1A normal), mercapto-ethanol, and triolein were all obtained from Sigma. All substrates and enyzmes were stored refrigerated at 0-5°C or frozen in a desiccator as recommended. Real serum samples were obtained from Mercy Hospital, Hamilton, OH.

## METHODS

The triolein standard solutions, ranging from about 25-280 mg/dl, were prepared by dissolving the appropriate amount in isopropanol and then diluting to 25 ml with absolute ethanol. The glycerokinase was reconstituted to a concentration of 8 units/ml with 0.02 M, pH 7.0, sodium phosphate buffer containing 0.001 M 2-mercaptoethanol. Stock ATP and magnesium acetate solutions of 0.033 M and 2.5 mM, respectively, were prepared using 0.1M Tris buffer, pH = 7.0 . An aliquot no greater than 3 ml of the triglyceride

standard was added to 0.5 ml of ethanolic KOH and diluted to 3.5 ml with ethanol. Saponification of this solution was allowed to occur for 30 minutes at 70°C. The sample tubes were covered with Parafilm to decrease evaporation. After cooling, 1 ml of 0.15 M $MgSO_4$ was added to precipitate the free fatty acids and the sample tubes were then centrifuged. Volumes of 0.5 ml were taken of either the control serum or the real serum samples for saponification and treated as described previously for the triolein standards.

The assay mixture consisted of 3.0 ml buffer/$Mg^{+2}$ solution, 0.1 ml ATP solution, and 0.5 ml hydrolyzate supernatant. For the serum samples the hydrolyzate volume was either 0.5 ml or 1.0 ml. To initiate the reaction, 0.3 ml of the GK solution was pipetted into the test tube and the mixture was swirled. The test tubes were immediately placed in a 25°C bath for 10 minutes.



The ADP formed in the assay was separated from the excess ATP by the use of HPLC. The mobile phase consisted of 88% 0.1 M $KH_2PO_4$, 0.025 M tetrabutylammonium hydrogen sulfate and 12% methanol, adjusted to a pH 7.1 with NaOH and was pumped at a flow rate of 1.5 ml/min. The sample injection volume was 20 µl. The detector was generally set at 0.04 absorbance units full scale (a.u.f.s.), however occasionally 0.08 a.u.f.s. was required. The temperature was ambient and the chart speed was 0.25 cm/min.

The Sigma Triglyceride Diagnostic Kit (No.335-UV) based on the 4 enzyme system was employed as a reference triglyceride technique and was used as written. The Sigma assays were carried out using a Hewlett-Packard 8450A UV-VIS spectrophotometer.

## RESULTS

The glycerokinase used for the assay contained several minor enzyme impurities, two of which were hexokinase and myokinase. The enzyme hexokinase can catalyze the reaction shown below.

$$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose-6-P} + \text{ADP}$$

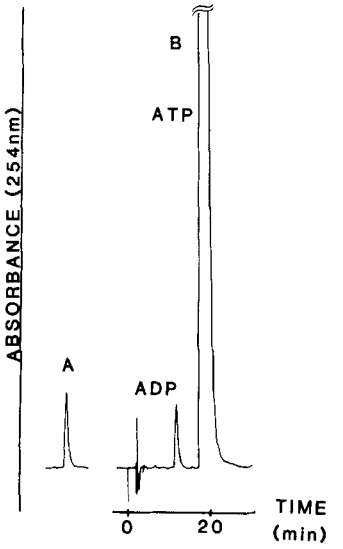


FIGURE 1. HPLC check for possible interferences by hexokinase. (A)-Blank assay containing buffer/$Mg^{+2}$ solution, ATP and GK as described in METHODS section. (B)-Blank assay as in A with glucose (200 mg/dl). Both enzyme reactions were incubated for 10 minutes and then quenched by immersing the test tube in boiling water for 2 minutes. This quenching procedure was determined not to be necessary and in fact increased the blank ADP peak due to ATP decomposition.



In this case the amount of ADP formed would be larger than the true value. Figure 1 shows the comparison of a blank sample with and without the presence of 200 mg/dl of glucose. This level of glucose is higher than that typically formed in serum. It can be concluded that the presence of hexokinase would not be expected to interfere with the glycerokinase catalyzed reaction. Myokinase can catalyze the following reaction.

$$2\ ADP \longrightarrow AMP + ATP$$

Since ADP was the product monitored in the assay, the presence of myokinase could contribute a negative error. Since it has been shown that the presence of AMP will inhibit myokinase (7), assays were performed comparing the amount of ADP formed with and without the presence of AMP in the assay mixture. As

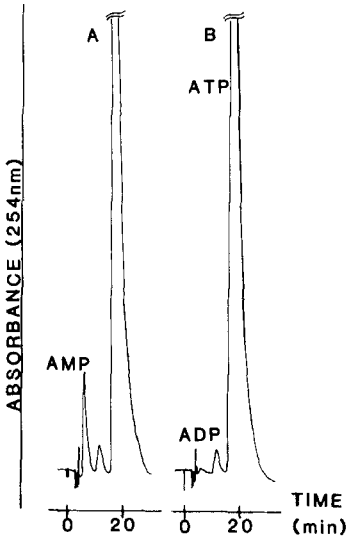


FIGURE 2. HPLC check for possible interference by myokinase. (A)-Blank assay as in Figure 1A. (B)-Blank assay as in A with AMP (2 mM). Both enzyme reactions were incubated 30 minutes and no quenching was carried out.



shown in Figure 2, any possible myokinase impurity did not appear to interfere with the assay of glycerol.

A typical separation of ADP and ATP obtained for a 28 mg/dl range of triolein is given in Figure 3. Other constituents present either in the standards or the serum samples were either nonabsorbing or retained on the precolumn and did not interfere with the separation. The time for complete separation of ADP and ATP was about 20 minutes. Assuming the detection limit is twice the response of the blank ADP peak, a value of about 10 mg/dl can be calculated. A plot of triolein concentration versus the ADP peak area given in Figure 4 demonstrates the linearity of the assay. A slope of 0.27 $cm^2/10$ mg/dl with an intercept of -0.64 and a correlation coefficient of 0.9944 were calculated. The relative average standard deviation for 3 determinations was found to be 6.5%.

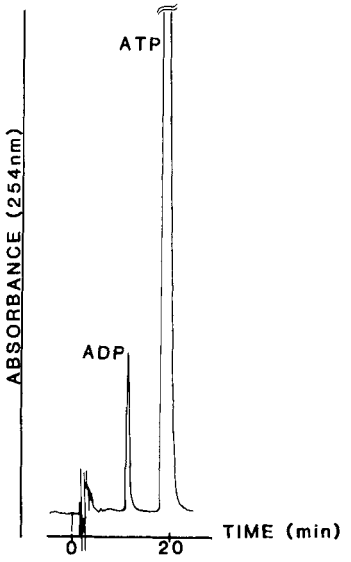


FIGURE 3. Chromatogram obtained for a sample originally 28 mg/dl in triolein.

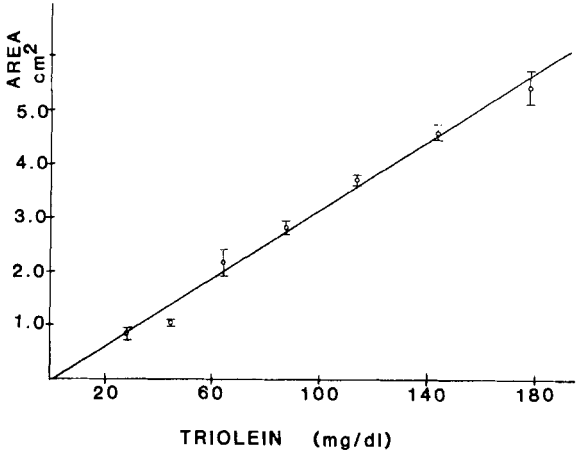


FIGURE 4. ADP peak area as a function of mg/dl of triolein. Error bars represent the standard deviation of 3 determinations.

TABLE 1. RECOVERY OF TRIGLYCERIDES ADDED TO SERUM

| Triglycerides Added mg/dl | Total Triglycerides Found | Recovery % |
|---|---|---|
| 0 | 78 ± 10 | --- |
| 94 | 153 ± 2.0 | 88 |
| 136 | 230 ± 9.8 | 107 |
| 176 | 256 ± 0.1 | 100 |
| 214 | 308 ± 0.5 | 105 |
| 250 | 376 ± 7.5 | 114 |
| | Avg. | 103% |



TABLE 2. COMPARISON OF THE HPLC METHOD WITH THE SIGMA ENZYMATIC METHOD

| Sigma* | Range* | n | HPLC* | Range* | n |
|---|---|---|---|---|---|
| 71.6 | 2.6 | 2 | 79.7 | 10.8 | 2 |
| 121.0 | 2.6 | 2 | 136.6 | 20.7 | 3 |
| 213.9 | 4.3 | 2 | 249.5 | 1.2 | 2 |
| 85.9 | --- | 1 | 111.7 | 0.4 | 2 |
| 151.9 | 19.1 | 2 | 158.6 | 25.7 | 3 |
| 69.0 | 13.0 | 2 | 78.9 | 0.7 | 2 |
| 79.9 | --- | 1 | 73.9 | 16.8 | 3 |
| 60.8 | --- | 1 | 99.0 | 4.4 | 2 |
| 151.0 | --- | 1 | 164.7 | 1.1 | 2 |

*All values expressed in mg/dl of triglycerides.

A study of the percent recovery of triglycerides added to control serum is summarized in Table 1. The % recovery ranged from 88 to 114% with an average value of 103%. The average standard deviation in the total triglycerides found was about ± 5 mg/dl.

Results of the comparison of the HPLC method with the Sigma Reference assay for triglycerides is given in Table 2. The average range for both methods was about 8 mg/dl. A plot of the HPLC values versus the Sigma results gave a correlation coefficient of 0.9770.

## DISCUSSION

The linearity of the system essentially covered the normal range for triglycerides in serum. This range is 40-160 mg/dl for males and 35-135 mg/dl for females (8). However, samples up to 380 mg/dl could be determined with good accuracy (Table 1) if diluted first or by reducing the sensitivity of the HPLC detector.

The triglyceride determinations using the HPLC method tended to be slightly higher than those found by the reference method. This may be due to a volume displacement error that occurs when proteins are precipitated because the serum volume containing the triglycerides decreases by the volume of proteins precipitated (8). Since larger sample aliquots were used in the HPLC method than the reference method, a consistent discrepency could result. Smaller sample aliquots could probably be used particularily if the injection volume and/or the detector sensitivity were increased. Total analysis time for the HPLC method was about 25 minutes discounting the time required for saponification. The sample throughput could probably be increased substantially by using a computer-controlled HPLC that could switch a valve between the injector and column to divert the ATP band to waste instead of the column before subsequent automatic injection of the next sample.

Application of the assay for other samples such as antifungal creams containing the triglyceride triacetin should be straightforward. It is also

expected this general method would be applicable to the determination of other important clinical substrates that are involved in kinase enyzme systems such as the glucose-hexokinase catalyzed reaction.

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