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An AutoAnalyzer method for estimating serum glyceride glycerol using a glycerokinase procedure

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SYNOPSIS The glyceride glycerol analysis depends, after saponification of triglycerides, on a linked enzymatic procedure using glycerokinase, pyruvate kinase, and lactate dehydrogenase: the final conversion of NADH to NAD^+ is followed fluorimetrically. Twenty analyses can be performed per hour on the AutoAnalyzer; recoveries of added triglycerides ranged between 90 and 104%. In a mixed male and female group the normal range for glyceride glycerol was 2·5 to 15·5 mg/100 ml (0·2-1·4 mmol/l) fasting, and 2·5 to 18·0 mg/100 ml (0·2-1·6 mmol/l) postprandially using fresh serum. There was a significant rise postprandially in older men.

There are increasing requests for the estimation of serum triglycerides, particularly since the introduction of the drug clofibrate (Atromid-S).

Triglycerides are normally measured as glyceride glycerol and there are a number of different types of method available, eg, manual colorimetric (Carlson and Wadström, 1959), manual enzymatic (Wieland, 1957; Eggstein and Kreutz, 1966), automatic fluorimetric (Kessler and Lederer, 1965), and automatic colorimetric (Lofland, 1964). Colorimetric and fluorimetric methods require preliminary treatment of the sample to remove interfering substances and impart specificity, and we felt, therefore, that automation of a specific enzymatic method would be useful. The automated method described in this paper is based on the enzymatic method of Eggstein and Kreutz (1966) and depends on the following reactions:

$$\text{glyceride glycerol} \xrightarrow[\text{KOH}]{\text{ethanolic}} \text{glycerol} + \text{fatty acids}$$

$$\text{glycerol} + \text{ATP} \xrightarrow[(Mg^{++})]{\text{glycerokinase}} \text{glycerophosphate} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow[(Mg^{++})]{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADPH} + H^+ \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + NAD^+$$

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Materials and Methods

APPARATUS

The Technicon AutoAnalyzer was used attached to a Locarte fluorimeter (model LFM/5) with a flow-through cell and a Rikadenki recorder. An LF.1 filter was used on the primary (excitation) side, and a wavelength of 470 nm with a cut-out filter, LF.6, on the secondary (emission) side. The LF.1 filter transmits between 254 and 400 nm. The LF.6 filter transmits light only of wavelengths greater than 470 nm.

REAGENTS

Ethanolic KOH (0·5N)
Dissolve 6 g glycerol-free KOH (Griffin and George) in 200 ml absolute ethanol. The normality of this solution should be checked by titration and appropriately diluted with absolute ethanol to bring it to 0·5N.

$MgSO_4$ (0·1M)
Dissolve 5·0 g $MgSO_4\ 7H_2O$ A.R. in 200 ml distilled water.

Triethanolamine buffer (0·1M, pH 7·6) containing 0·004M $MgSO_4$ (solution A)
Dissolve 37·2 g triethanolamine hydrochloride

A.R. and 1·972 g $MgSO_4$ $7H_2O$ A.R. in 1,500 ml distilled water, bring the *p*H to 7·6 with N NaOH (about 80 ml), and dilute to 2 litres with distilled water. Stable at 4°C.

Phosphoenolypyruvate (0·011M) and ATP (0·033M) (solution B)
Dissolve 0·363 g ATP (Boehringer) and 0·045 g phosphoenolpyruvate (Boehringer) in 20 ml of the triethanolamine buffer (solution A). Stable for at least two weeks at 4°C.

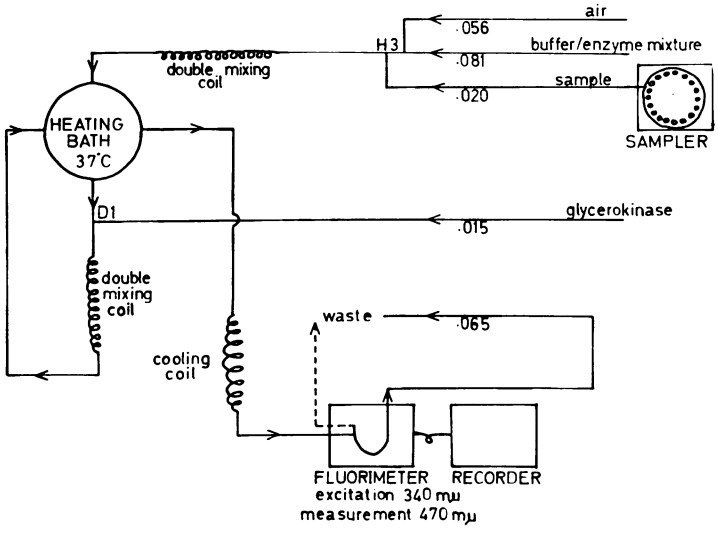

Fig. 1 *Diagram of AutoAnalyzer manifold for determination of serum glyceride glycerol.*

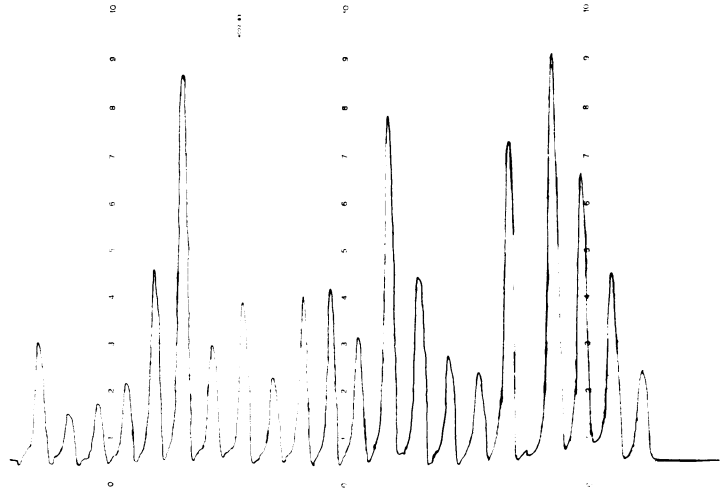

Fig. 2 *Calibration curve for standard glycerol solutions on the right (first four samples), followed to the left by typical AutoAnalyzer tracing for glyceride glycerol determination.*

Lactate dehydrogenase (2 mg/ml) pyruvate kinase (1 mg/ml) (solution C)
Mix 4 ml lactate dehydrogenase solution (5 mg/ml, specific activity about 360 u/mg, Boehringer) with 1 ml pyruvate kinase (10 mg/ml, specific activity about 150 u/mg, Boehringer) and 5 ml normal saline. Stable for at least two weeks at 4°C.

NADH (0·018M) (solution D)
Dissolve 24 mg NADH (Sigma) in 2 ml aqueous sodium chloride solution (0·15 M: isotonic saline). Prepare freshly for each batch of analyses.

Buffer-enzyme mixture
Mix 500 ml solution A with 10 ml solution B and 2·4 ml solution C. Add 2 ml solution D. This is sufficient reagent for the standards and about 24 tests and blanks, and should be prepared freshly for each day's run.

Glycerokinase (0·05 mg/ml)
Dilute 0·1 ml glycerokinase (5 mg/ml specific activity about 85 u/mg Boehringer) to 10 ml with normal saline. Prepare freshly for each batch of analyses.

Stock standard glycerol (10 mg/100 ml)
Dissolve 10 mg glycerol A.R. (BDH) in distilled water to a volume of 100 ml.

Working glycerol standards
Dilute 10, 20, 30, and 40 ml stock glycerol solution each to 100 ml with distilled water to give working standards of 1, 2, 3, and 4 mg/100 ml respectively.

METHOD

Saponification
Pipette 0·2 ml fresh serum and 0·5 ml ethanolic KOH (0·5N) into a centrifuge tube. Stopper, mix, and saponify in a water bath at 70°C for 30 minutes. Cool, add 1·5 ml $MgSO_4$ solution, shake well and centrifuge for 10 minutes. The clear supernatant is used for assay of the total glyceride glycerol. The supernatant can if necessary be left at room temperature overnight before analysis.

Operating procedure
A diagram of the AutoAnalyzer manifold is shown in Figure 1. A 2:1 cam is used with a Technicon sampler 2. The fluorimeter should be switched on at least 30 minutes before use to allow it to stabilize. The photomultiplier and 'shorted direct' switches should be switched on about 10 minutes before the start of the run. Place the buffer-enzyme mixture in an ice bath. Aspirate the buffer-enzyme mixture for 10 minutes, with the glycerokinase tube pumping buffer. After about 10 minutes' aspiration, sample the standards and tests at a speed of 40 per hour alternatively with water, ie, 20

samples per hour. After about 12 minutes' aspiration, ie, a further two minutes, place the glycerokinase tube in the glycerokinase mixture: this procedure avoids undue waste of the glycerokinase solution. After about 15 minutes' aspiration (a further three minutes) the buffer-enzyme mixture will reach the fluorimeter, when the base line should be adjusted. About 12 minutes after the last test has been aspirated the glycerokinase can be stopped and the blanks run. The blanks are run in the same way as the tests except buffer is pumped through the glycerokinase line continuously. The times given are approximate and depend on the details of construction of the manifold.

Calculation

A calibration curve is drawn for the standards and mg glycerol/100 ml read off for the tests and blanks. Then:

(test-blank) $\times$ 11 = mg/glyceride glycerol per 100 ml serum

or

(test-blank) $\times$ 11 $\times$ 10/92 = mmol glyceride glycerol per litre.

Results

CALIBRATION CURVE

A typical calibration curve is shown in Figure 2. The curve is linear up to and including 4 mg glycerol/100 ml. Any serum with a glycerol concentration higher than this should be appropriately diluted with isotonic saline and reestimated.

FREE GLYCEROL

In preliminary experiments, by omitting the saponification procedure, the free glycerol in the serum before saponification was also estimated. As this was always less than 1 mg/100 ml, even in very lipaemic sera, a parallel free glycerol estimation was omitted as a routine procedure.

BLANK ESTIMATIONS

It is necessary to carry out a blank determination on every test. Omission of the blank results in a 10-30% overestimation of the serum triglyceride level.

REPRODUCIBILITY

The within-batch reproducibility of the method was checked by assaying a pooled serum 20 times. The mean value was 34·2 mg/100 ml with a SD of 0·737 and a coefficient of variation of 2·2%. It was not possible to do a between-batch reproducibility because of the instability of serum glyceride glycerol which necessitated samples being analysed within 24 hr of collection.

RECOVERY

Recovery experiments were carried out using tributyrin, triolein, and tripalmitin. The tributyrin was dissolved in ethanolic KOH (0·5 N), and the triolein and tripalmitin in chloroform, before addition to the serum and saponification. The average recovery of tributyrin was 97·8% with a range of 93·3 to 104·4%, of triolein 97% with a range of 90·5 to 104·5%, and of tripalmitin 94·2% with a range of 90·3 to 97·2%.

CARRY-OVER

The carry-over from one specimen to the next was checked by running 4 mg/100 ml standards followed by water and then by 1 mg/100 ml standards as in the procedure described. The carry-over was found to be nil.

NORMAL RANGE

The normal range (95% limits) for glyceride glycerol in serum was established by estimating the fasting (morning) levels and the postprandial levels (one to two hours after lunch) in 24 male and female members of the hospital staff whose ages ranged between 22 and 62 years. The normal fasting range was 2·5 to 15·5 mg/100 ml and the normal range postprandially was 2·5 to 18 mg/100 ml. The fasting values showed no significant difference either between males and females ($t = 1{\cdot}54$, $P > 0{\cdot}1$) or between under and over 30 years age groups ($t = 0{\cdot}93$, $P > 0{\cdot}2$). When the postprandial values were divided into two groups, under 30 years and over 30 years of age, the values in the younger group were found to be significantly lower than those in the older age group ($t = 3{\cdot}34$, $P < 0{\cdot}005$). When the postprandial values were divided according to sex the males were found to have significantly higher triglyceride levels than the females ($t = 2{\cdot}84$, $P < 0{\cdot}01$; Fig. 3). Eighteen samples were tested both fasting and postprandially and 12 of these showed a rise in glyceride glycerol in the postprandial state. These results are shown graphically in Figure 4. It is interesting to note that the range of normal fasting values is apparently the same whatever the age and sex of the subject, whereas there is a significantly higher postprandial value in older subjects and in males. This finding is to be investigated on a larger series, and if confirmed, may be important in relation to the problems of serum lipids and disease.

SAPONIFICATION

In preliminary experiments saponification of the triglycerides was carried out with alcoholic potassium hydroxide and the *p*H value adjusted with perchloric acid before analysis to be within the range 6 to 8. This method was time consuming, and trouble was encountered due to precipitation of fatty acids during analysis causing turbidities. For this reason the saponification method of Schmidt and Dahl (1968), where the fatty acids are precipitated by magnesium before

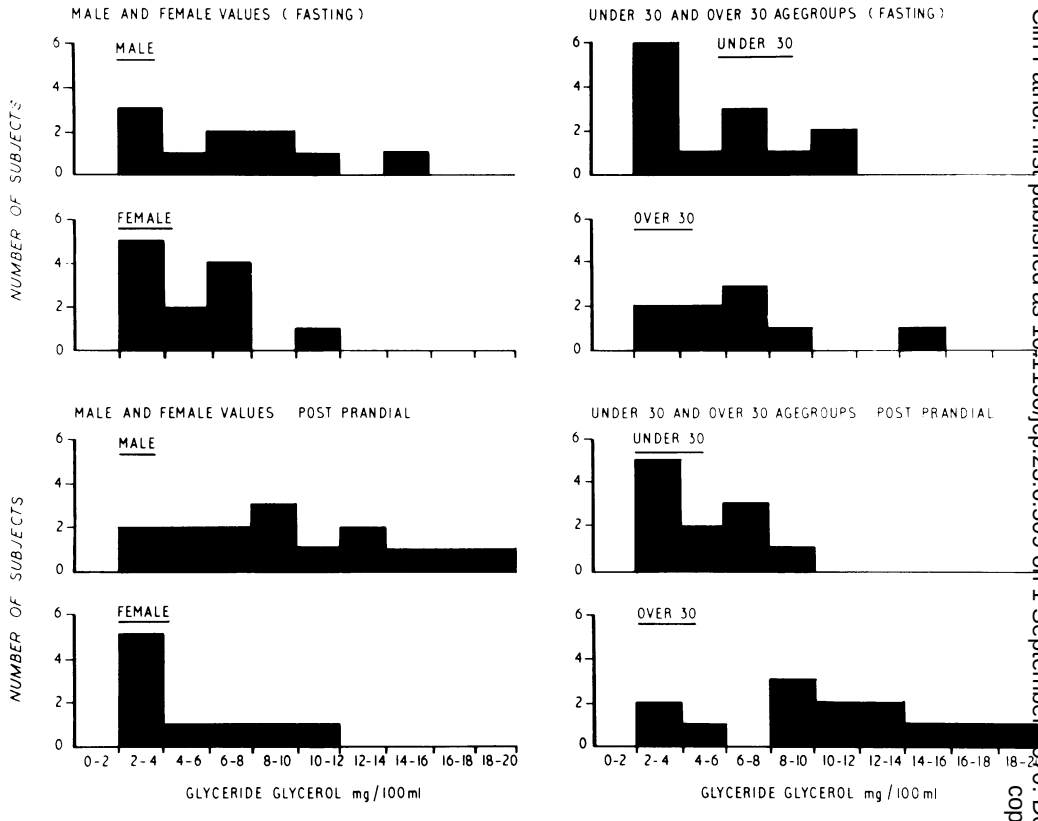

Fig. 3 *Histograms of normal glyceride glycerol values according to age, sex, and absorptive state.*

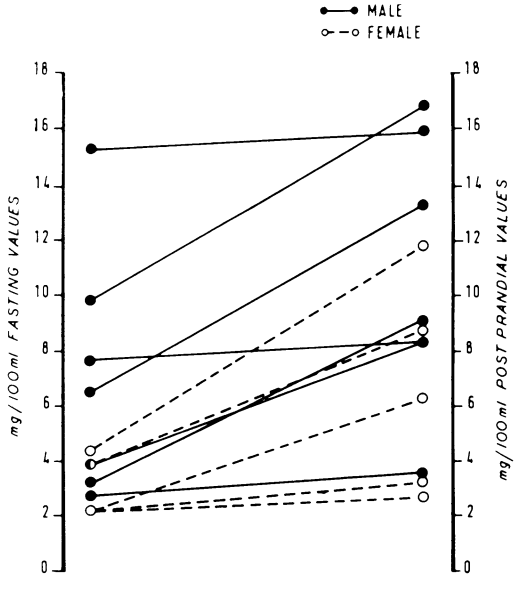

Fig. 4 *Comparison of fasting and postprandial glyceride glycerol levels.*

analysis of glycerol, was adopted and found to be satisfactory for analysis of serum. The methods of Sperry (1955) and Folch, Lees, and Stanley (1957) were also tried but although these were also satisfactory for saponification the direct method described was preferred because of its greater simplicity.

KEEPING PROPERTIES OF GLYCERIDE GLYCEROL

The keeping properties of glyceride glycerol were investigated both in the serum and in the saponified extract. The specimens were kept in a series of covered tubes at −20 °C, 4 °C, and 25 °C. The value found for glyceride glycerol in the serum and for the glycerol in the saponified extracts was stable for 24 hr, then increased on keeping at all temperatures, reaching a plateau at about three days: it is therefore necessary to use fresh serum for this analysis. This phenomenon requires further study.

COMPARISON BETWEEN AUTOMATIC AND MANUAL PROCEDURES

The Table shows a comparison between the present automatic procedure and the manual enzymatic method of Eggstein and Kreutz (1966). The free glycerol concentration was consistently higher with the manual method than with the automated

Sample	Free Glycerol (mg/100 ml)		Glyceride Glycerol (free glycerol not subtracted) (mg/100 ml)	
	AutoAnalyzer	Manual	AutoAnalyzer	Manual
1	0·8	1·4	23·3	24·0
2	0·8	1·6	25·3	24·0
3	0·6	1·1	20·4	19·8
4	0·9	1·5	19·5	19·8
5	0·6	1·6	13·2	14·0
6	0·5	1·3	15·4	13·0
7	0·6	2·2	13·2	13·0
8	0·9	1·1	14·3	14·0

Table *Present method and the manual enzymatic method compared*

method. The values for total glyceride glycerol showed good agreement.

EFFECT OF GLUCOSE

Solutions of glucose (50 mg/100 ml and 100 mg/100 ml in water) were run through the standard procedure in the same way as the glycerol solutions. The peaks obtained were read as 'glyceride-glycerol'. At both concentrations values equivalent to about 0·8 mg glyceride glycerol/100 ml were obtained.

EFFECT OF PHOSPHOLIPIDS

Concentrations of α-lecithin between 50 and 250 mg/100 ml (6 to 30 mg lecithin glycerol) were added to a serum containing 10 mg glyceride glycerol/100 ml and the samples run through the procedure. Lecithin is not measured by this procedure, shown by zero recovery of the added lecithin glycerol.

Discussion

The normal range found by this method is similar to that of the standard colorimetric procedure of Carlson and Wadström (1959), who found a normal range of 3·8 to 14 mg/100 ml in the postabsorptive state in young men. Using this enzymatic procedure, Eggstein and Kreutz (1966) found a significantly wider range of 2 to 22 mg/100 ml (mean ± 2 SD). The reason for this discrepancy is not clear.

The value for free serum glycerol was consistently lower by the AutoAnalyzer method than by the manual method. This may be due to the fact that in the AutoAnalyzer procedure a continuous running blank is used and subtracted from the test result, whereas in the manual procedure all the reagents except the glycerokinase are mixed and the optical density is allowed to reach a stable level before the addition of glycerokinase and analysis of glycerol. It was practically always impossible to stabilize the optical density before the addition of glycerokinase in the manual method even with a 'blank' period of one hour or more. The free glycerol in serum estimated by the present procedure was between 0·5 and 1·0 mg/100 ml in the normal group tested: and similar results have been reported by Laurell and Tibbling (1966). It would appear that a considerable part, if not all, of the measured glycerol may be an artefact due to traces of hexokinase in the glycerokinase used. This hexokinase will convert any glucose present to glucose-6-phosphate with the production of ADP from the ATP present and this ADP will continue through the enzymatic reactions used for estimating glycerol. This does not occur in total glyceride glycerol estimations as any glucose present is destroyed by the saponification procedure. Solutions of glucose (50 mg/100 ml and 100 mg/100 ml), when run by the standard glyceride glycerol procedure, both gave peaks equivalent to about 0·8 mg glyceride glycerol/100 ml which is approximately equal to the level of free glycerol estimated by this method in serum. A concentration of glucose of 50 mg/100 ml thus appears to be sufficient to saturate the interfering enzymes.

This automated procedure for serum glyceride glycerol estimation has now been in use in this laboratory for six months and has proved to be simple to use and reproducible. The cost of reagents for these estimations is approximately 4 shillings (£0.2) for each serum analysed.

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