Assay of Triglycerides Using the Perkin Elmer C-4 Automatic Analyzer

By R. J. M. VAN OERS, R. J. H. SCHOLTIS, N. A. SCHMIDT, J. F. VAN DE CALSEYDE and A. M. J. KUYPERS

Department of Clinical Chemistry, Ignatius Hospital, Breda, The Netherlands

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EGGSTAIN's method for the enzymatic assay of triglycerides was adapted to the Perkin Elmer C-4 Automatic Analyzer and the repeatability, recovery, linearity and drift within a series were checked. The assay of triglycerides with the Perkin Elmer C-4 Automatic Analyzer was found to be highly reliable and rapid. With a few exceptions, it was found to be not necessary to estimate the free glycerol in addition to the total glycerol, which considerably reduces the length of this procedure.

Die Methode von EGGSTEIN zur enzymatischen Bestimmung von Triglyceriden wurde an den automatischen Analysator Perkin Elmer C-4 angepaßt. Wiederholbarkeit, Wiederfindung, Linearität und Drift innerhalb der Serie wurden untersucht. Die Bestimmung der Triglyceride mit diesem Gerät ist sehr zuverlässig und schnell. Abgesehen von einigen Ausnahmen ist die Bestimmung des freien Glycerins neben dem Gesamtglycerin nicht erforderlich, wodurch der Zeitaufwand wesentlich verringert wird.

The demand for the automation of triglyceride assays is increasing with the demand for this procedure. So far only a semi-automated assay on a manually prepared isopropanol extract of the serum, based on the oxidation of glycerol to formaldehyde and the formation of a fluorescent product, has been described (1). The present authors believe that the Perkin Elmer C-4 Automatic Analyzer provides a suitable instrument for the enzymatic assay of triglycerides by EGGSTEIN's method (2, 3, 4).

This enzymatic assay was adjusted to the Perkin Elmer C-4 Automatic Analyzer, and the repeatability, recovery, linearity and drift within a series were checked.

In order to determine whether the concentration of free glycerol should also be estimated in routine triglyceride assays, the free glycerol concentrations found in well over 1,000 assays were compared with the total glycerol concentrations.

The Perkin Elmer C-4 Automatic Analyzer (Fig. 1) is a discrete sampling automatic analyzer by which up to four different assays may be carried out at the same time. The rate of the apparatus is 120, 60, 40 or 30

Fig. 1
Perkin Elmer C-4 Automatic Analyzer

Fig. 2
Rotary table with two reagent additions in channel II and channel IV. Sample cups are visible in the outer channel

samples per hour, varying with the number of assay channels (1, 2, 3 or 4 channels respectively).

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Material and Methods

Principle of the reaction

Glycerol is enzymatically estimated in serum before (free glycerol) and after (total glycerol) alkaline saponification. The amount of NADH utilized, which is determined at 340 nm, is a measure of the amount of glycerol.

- a. Glycerokinase
- b. Pyruvate kinase
- c. Lactate dehydrogenase.

Reagents

Ethanol potassium hydroxide 0.5N: Dissolve 3.3 g of potassium hydroxide (Merck 5033, $\approx$ 86% KOH) in 10 ml of twice distilled water, allow to cool and make up to 100 ml with 96 per cent ethanol.

This remains stable for one month at approximately 4°.

Magnesium sulphate: Dissolve 37 g of $\text{MgSO}_4 \cdot 7 \text{ aq.}$ (Merck 5886) and make up to 1,000 ml with distilled water.

0.9 Per cent sodium chloride: Dissolve 9 g of NaCl (Merck 6404) in one litre of distilled water.

Triethanolamine buffer 0.1M, pH 7.6: Dissolve 14 g of triethanolamine (Merck 8379) and 1 g of $\text{MgSO}_4 \cdot 7 \text{ aq.}$ in 900 ml of distilled water. Adjust to pH 7.6 with HCl 6N and make up to 1,000 ml with distilled water.

NADH-Phosphoenolpyruvate-ATP solution: Make up 20 mg of NADH di-sodium salt (Boehringer 15142 CNAB) 120 mg of ATP di-sodium salt (Boehringer 15028 NAAH) and 14 mg of phosphoenolpyruvate-mono-potassium salt (Boehringer 15179 SPAB) to 120 ml with triethanolamine buffer.

Prepare fresh solution in each case.

The concentration of NADH has been chosen in such a way that the reaction will be linear up to 6.0 mm.

Lactate dehydrogenase-Pyruvatekinase solution: Make up 1 ml of lactate dehydrogenase suspension (10 mg/2 ml, Boehringer 15371 ELAC) and 0.25 ml of pyruvate kinase suspension (10 mg/ml, Boehringer 15744 EPAU) to 50 ml with triethanolamine buffer, pH 7.6.

Prepare fresh solution in each case.

Glycerokinase solution: Make up 0.25 ml of glycerokinase suspension (5 mg/ml, Boehringer 15746 EGAP) to 25 ml with triethanolamine buffer, pH 7.6.

Prepare fresh solution in each case.

Glycerol standard: Accurately weigh out approximately 1,000 mg of glycerol (Merck 4094) and make up to 1,000 ml with glycerol free water.

This remains stable for approximately two months at about 4°. Make up 5 ml of this solution to 100 ml with distilled water.

Molarity of this standard solution = $\text{weight (mg)} \cdot \frac{87}{92} \cdot \frac{1}{2000} = \text{mm.}$

Triolein standard: Transfer contents of a triolein ampoule (Sigma T-7502) to 50 ml volumetric flask with a small quantity of ether. Make up to 50 ml with 96 per cent ethanol. Molarity of this standard solution $\approx$ 2 mm.

Method of estimation

When EGGSTEIN's enzyme triglyceride assay (2) is used, the sample to be analysed is added to a triethanolamine buffer, pH 7.6, containing ATP, phosphoenolpyruvate, pyruvate kinase, NADH and lactate dehydrogenase. The extinction is monitored for 5–10 minutes (Fig. 3). Glycerokinase having been added, extinction is followed again for 5–10 minutes.

The difference in extinction before and after addition of the glycerokinase solution, extrapolated to the time at which glycerokinase is added, provides a measure of the amount of glycerol in the sample to be analysed.

Fig. 3

Determination of glycerol. Extinction before and after addition of glycerokinase

- I. total glycerol II. free glycerol
- a) addition of sample b) addition of glycerokinase
- non-specific reactions

Enzyme assays of the free glycerol in the serum are accompanied by non-specific reactions (Fig. 3), resulting in reduced extinction. These non-specific reactions do not occur when glycerol is assayed in saponified samples, which have been correctly neutralized. The following method was adopted by the present authors in performing this assay with the Perkin Elmer C-4 (Fig. 4).

With the exception of glycerokinase, the reagents and the sample are introduced into both channel I and channel II. Within ten minutes after adding the sample, the glycerokinase solution is introduced into channel I and an equal volume of triethanolamine buffer is introduced into channel II. The actual reaction therefore occurs in channel I, whereas changes in extinction resulting from dilution and nonspecific reactions, if any, can only occur in channel II. Within eleven minutes after glycerokinase has been added to channel I, the extinction of channel I is determined in comparison

Fig. 4

Determination of glycerol by comparison of the extinction of channel I (Δ) with that of channel II ($\bullet$)

- a) addition of sample b) addition of glycerokinase to channel I

with that of channel II (Fig. 4), which corresponds with ΔE of Figure 3 as the extinctions in channels I and II run parallel to one another following the addition of glycerokinase. This assay using the Perkin Elmer C-4, accordingly requires two channels, resulting in a maximum number of 60 samples per hour.

Adjustment of Perkin Elmer C-4

Channel selector: 1 + 2; 25°; 5 Reagent pumps sampling pump, total quantity of sample 400 μ l

Channel	I	II	III	IV
Wave-length, nm	340	340	—	—
Sample volume, μ l	200	200	—	—
1. NADH — phosphoenolpyruvate — ATP μ l	1,000	1,000	—	—
2. Lactate dehydrogenase — pyruvate kinase μ l	200	200	—	—
3. Glycerokinase μ l	200	—	—	—
4. Triethanolamine Buffer μ l	—	200	—	—
5. Triethanolamine Buffer μ l	1,000	1,000	—	—

Method of saponification

The following materials are pipetted into a centrifuge tube:

ethanolic KOH 0.5N 0.5 ml
serum 0.2 ml.

The tubes are well sealed and saponified for thirty minutes at 70°. After cooling, the following solution is added:

MgSO₄ solution 0.15M 1.0 ml

This is well mixed and then centrifuged again for five minutes at 3,000 r.p.m. The supernatant fluid is stored at 4°. In lieu of serum, 0.2 ml of 0.9 per cent NaCl should be used as a saponification blank in the above saponifying procedure. 0.2 ml of the triolein standard are also saponified and estimated.

Saponified sample factor: 17.7

Free glycerol factor: 2.08

$$\text{Saponified sample factor} = \frac{2.6 \cdot 8.5}{6.23 \cdot 0.2} \text{ and free glycerol factor} = \frac{2.6}{6.23 \cdot 0.2}$$

in which 2.6: total volume

8.5: dilution factor of saponified sample

6.23 · 10³: millimolar coefficient of extinction of NADH at 340 nm

0.2: volume of sample.

As shown in Figure 5, solutions 1 and 2 are initially transferred to the reaction vessel, followed by the sample. This will start the secondary reaction. Ten minutes later, the glycerokinase solution is introduced into channel I, triethanolamine buffer being introduced into channel II in lieu of this solution.

Fig. 5

Adjustment of the Perkin Elmer C-4 Automatic Analyzer

In order to bring the extinction within a measurable range, triethanolamine buffer is introduced into the two channels at position 1. The reaction will therefore occur in channel I. Within eleven minutes after the glycerokinase solution has been added, the Perkin Elmer C-4 transfers the contents of channel I to flow cuvette 3 and that of channel II to flow cuvette 4. The transmission of cuvette 3 is automatically adjusted to one hundred per cent by the width of the slit, and cuvette 4 is measured against cuvette 3.

Results

Table 1 shows the ratio of free glycerol to total glycerol in over 1,000 serum samples, each in mMoles per litre. Of the free glycerol, the average, standard deviation and number of free glycerol assays in each area are stated.

It is apparent from Table 1 that when the total glycerol increases to 6.0 mM, the free glycerol will only increase by a minute amount to approximately 0.16 mM. When the normal total glycerol levels of the 1,000 assays are estimated by the methods as suggested by NEUMAN (5) and HOFFMAN (6), these levels are found to vary from 0.6 to 2.2 mM.

When routine triglyceride assays are carried out, it is not necessary to estimate the concentrations of free glycerol. If the lipid pattern is found to be normal when the concentration of triglycerides shows an increase (in which case the lipid pattern is invariably determined), the free glycerol concentration of the sample may still be determined so that the triglyceride level can be adjusted.

Repeatability

Duplicate determinations are made of the total glycerol concentration, i. e. the serum sample is saponified and assayed on two consecutive days.

Table 2 provides an example of a series of duplicate determinations.

Tab. 2

Results of duplicate (triplicate) triglyceride assays (mMoles/l glycerol) in brackets: outliers

Day:											
1	2	3	1	2	1	2	1	2	3	1	2
1.1	1.3		3.1	3.1	3.0	2.9	1.4	1.3		0.9	0.9
1.4	1.5		1.8	1.8	1.0	1.1	(0.5)	1.9	2.0	2.5	2.4
1.5	1.6		1.6	1.5	1.2	1.3	0.8	0.9		1.6	1.5
0.7	0.7		1.8	1.7	1.0	1.1	0.8	0.9		1.9	1.7
0.9	1.0		0.5	0.6	1.6	1.6	3.6	4.1		1.4	1.3
(3.3)	2.3	2.2	2.4	2.5	1.3	1.3	4.5	4.9		1.3	1.2
1.2	1.3		2.0	2.0	2.3	2.3	(4.4)	1.8	1.6	2.0	2.0
0.9	0.9		2.1	2.2	4.6	4.7	1.9	1.9		1.2	1.2
1.5	1.6		1.7	1.8	2.1	3.0	3.2	3.4		3.1	3.2
3.5	3.3		1.7	1.8	1.5	1.5	0.7	1.0		2.1	1.9

The above table shows that, with a few exceptions, the maximum difference between two duplicate levels is 0.2 mMoles per litre. Glycerol assays using the Perkin Elmer C-4 are readily repeatable but duplicate estima-

Tab. 1
Comparison of free glycerol and total glycerol (mMoles/l)

Total glycerol	0.0—0.5	0.5—1.0	1.0—1.5	1.5—2.0	2.0—2.5	2.5—3.0	3.0—3.5	3.5—4.0	4.0—4.5	4.5—5.0	5.0—5.5	5.5—6.0
Free glycerol $\bar{x}$	0.07	0.07	0.09	0.10	0.11	0.12	0.14	0.13	0.15	0.16	0.13	0.13
S. D.	0.06	0.05	0.06	0.06	0.08	0.08	0.09	0.09	0.12	0.15	0.08	0.17
Number of probands	21	209	273	231	115	63	34	27	15	13	6	8

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Tab. 3

Stability of saponified samples of triglycerides (mMoles/l glycerol)

Serum						
1	0.7	1.0	0.7	0.7	0.8	0.7
2	0.9	0.9	0.9	0.9	0.8	0.8
3	1.9	2.1	2.1	2.2	2.0	2.1
Saponified day	1	2	3	3	3	3
Assayed day	2	3	4	5	6	9

tions are essential because of the occasional outliers which are due to the saponification procedure.

Efforts were made to determine whether saponified samples would remain stable.

For this purpose, samples were saponified and assayed on different days. Table 3 shows that saponified samples stored at approximately 4°, continue to display a similar activity a few days later.

Drift and recovery of the Perkin Elmer C-4

In order to determine the drift within a series of assays as well as the recovery of the Perkin Elmer C-4, a glycerol standard was introduced whenever ten samples had been assayed, and estimated with an adjusted theoretical factor based on the millimolar coefficient of extinction of NADH at 340 nm. Table 4 shows that a

Tab. 4

Drift and recovery of a glycerol standard within a series (mMoles/l)

3.72 = 99%	3.67 = 98%	3.73 = 99%
3.78 = 101%	3.50 = 93%	3.67 = 98%
3.70 = 99%	3.61 = 96%	3.80 = 101%
3.75 = 100%	3.71 = 99%	3.79 = 101%
3.71 = 99%	3.67 = 98%	3.94 = 105%
3.91 = 104%	3.59 = 96%	3.83 = 102%
3.77 = 101%	3.57 = 95%	3.90 = 104%
1st series	2nd series	3rd series

significant drift is absent in a series of assays of approximately seventy samples, and that recovery of the glycerol standard is almost one hundred per cent.

Linearity of assays

The area for which linearity holds good, will vary with the concentration of NADH. Estimates made at 340 nm, are more sensitive than those made at 360 nm. Thus if 340 nm is used the maximum amount of NADH which can be dissolved in solution 1 is 20 mg per 120 ml, in order to make the automatic one hundred per cent adjustment of transmission by the width of the slit practicable.

Fig. 6

Linearity of the triglyceride assay

Fig. 7

Order of introduction of samples

As shown by Figure 6, the reaction continues to be linear up to 6.0 mMoles per litre when the concentration of NADH is 20 mg/120 ml.

The Figure 7 shows the order in which samples were introduced, in which adequate determination of quality was practicable.

- Sites 1—4: Reagent blank, i. e., instead of a sample, 0.2 ml of 0.9 per cent NaCl are introduced into channels 1 and 11, and cuvette 4 is measured against cuvette 3 which is set at zero.
- 5—6: Glycerol standard.
- 7: Saponification blank; this must be ≤ 0.2 , and its value is deduced from the total glycerol level. This may be done by zeroing cuvette 4 against cuvette 3.
- 8: Triolein standard, saponified. This serves to check saponification.
- 9—18: Serum samples or serum hydrolysates.
- 19—20: Reagent blank and glycerol standard respectively.
- 31—32: Identical to 19—20.
- Another reagent blank and a triolein standard are used again at the end of the series.

Conclusion

EGGSTEIN's enzyme triglyceride assay, adapted to the Perkin Elmer C-4 automatic analyzer by the present authors, was found to be highly reliable and rapid. With a few exceptions, it was found to be not necessary to estimate the free glycerol in addition to the total glycerol, which considerably reduces the length of this procedure.

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Drs. R. J. M. van Oers
Stichting Ignatius-Ziekenhuis
Ignatiusstraat 11
Breda, The Netherlands