Modification of Lofland's colorimetric semiautomated serum triglyceride determination, assessed by an enzymatic glycerol determination

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ABSTRACT Erroneously high values for serum triglyceride levels obtained with the semiautomated method of Lofland were found to be due to contamination of the isopropanol extracts with glucose or other carbohydrate. Treatment of the isopropanol extracts with a mixture of copper sulfate and calcium hydroxide removed the contaminating glucose. Analysis of the glucose-free extracts by either the semiautomated or manual colorimetric method gave values in good agreement with each other and with those obtained by a new specific enzymatic method. The simple modification described in this paper obviates the necessity for the more expensive automated fluorometric apparatus.

SUPPLEMENTARY KEY WORDS interference by carbohydrate · removal · Garland and Randle

THE DESIRABILITY of an automated or semiautomated method for the determination of triglycerides in large numbers of serum samples has been recognized for some time. Van Handel and Zilversmit (1) described a manual colorimetric method, which is reliable, but not suitable for the routine analysis of very large numbers of samples. A semiautomated method, based on the colorimetric method of Van Handel and Zilversmit was developed by Lofland (2) for use with the AutoAnalyzer (Technicon Instruments Corporation, Ardsley, N.Y.). However, in our laboratory, Lofland's semiautomated method yielded results that were consistently higher than those obtained by the Van Handel and Zilversmit procedure. Lofland made one notable modification of the Van Handel and Zilversmit method, in using isopropanol instead of chloroform to extract serum triglycerides. Since both methods basically depended on the colorimetric estimation of formaldehyde formed by periodate oxidation of triglyceride glycerol, the reason for the discrepancies most probably lay in the different methods used for extraction of the triglycerides.

In order to determine what factors were responsible for the discrepancies, we decided to develop a method for serum triglyceride determination which was independent of periodate oxidation of glyceride glycerol. This method would then be used to establish the triglyceride level of serum samples, so that Lofland's procedure could be modified to give more reliable results. In choosing such a method, it seemed that an enzymatic determination of glyceride glycerol would offer the advantages of both specificity and freedom from the necessity for periodate oxidation. This paper describes the enzymatic method developed and the use of this method in identifying the source of error in the semiautomated method of Lofland (2).

METHODS

Enzymatic Determination of Glycerol

Spinella and Mager (3) described the adaptation of Wieland's enzymatic glycerol determination (4) to the determination of plasma triglycerides. The method as described depended upon the determination of glycerol with glycerokinase and α-glycerophosphate dehydrogenase and spectrophotometric measurement of the conversion of NAD to NADH. The treatment of plasma samples, separation of phospholipids on silicic acid chromatographic columns, and saponification procedure that these authors recommend prior to the enzymatic glycerol analysis were quite protracted, as was the final enzymatic determination of glycerol which took up to 10 min. Accordingly, we sought a less elaborate but equally reliable procedure for extraction and saponification of triglycerides, and a more rapid enzymatic assay.

Garland and Randle (5) described a rapid enzymatic assay of glycerol that depended on the phosphorylation of glycerol with ATP and glycerokinase and the enzymatic determination of the ADP formed by reaction with phosphoenolpyruvate in the presence of pyruvate kinase. The pyruvate formed was reduced by NADH under the influence of lactate dehydrogenase, and oxidation of NADH was determined spectrophotometrically. For our purposes, the method worked well with some minor modifications.

Garland and Randle (5) employed a triethanolamine buffer, pH 7.6, in their glycerol determination. By substituting potassium glycylglycine buffer (0.5 M, pH 7.4), which was found to be satisfactory, we avoided the use of triethanolamine which cannot always be obtained in pure form, and is volatile and troublesome to handle. The method was also scaled down from that described by Garland and Randle, so that the final reaction was conducted in a total volume of 0.8 ml in microcuvettes with a 1 cm light path. A considerable saving in the more expensive reagents and enzymes was effected this way, and at the same time much smaller quantities of glyceride could be measured reliably.

Reagents

The reaction mixture for the determination of glycerol was prepared in two steps. Solution A, which could be stored in the frozen state for at least 4 wk, consisted of 44 mg of $MgSO_4 \cdot 7H_2O$ and 98 mg of phosphoenolpyruvate (as the tricyclohexylammonium salt) dissolved in 10 ml of 0.5 M potassium glycylglycine buffer, pH 7.4. Solution B, the "final reaction mixture," was prepared freshly as required by addition of the following to 5.0 ml of Solution A: 0.4 ml of 0.1 M ATP (potassium salt); 0.4 ml of pyruvate kinase (rabbit muscle; 2 mg/ml. Boehringer Mannheim Corp., New York); 0.2 ml of lactate dehydrogenase (bovine heart; crystalline suspension in 55% saturated ammonium sulfate. Worthington Biochemical Corporation, Freehold, N.J.); and 0.5 ml of 9 mM NADH (64 mg of NADH sodium dissolved in 10 ml of H_2O, frozen in 1.2 ml samples and thawed as required). The "final reaction mixture" was kept in ice until required.

Glycerokinase

A 1:10 dilution of glycerokinase (Boehringer Mannheim Corporation) was made in 0.05 M potassium glycylglycine buffer, pH 7.4. This dilution was performed each day and the solution was kept in ice until needed.

Procedure for Glycerol Determination

0.2 ml of solution B was placed in each test cuvette (1 ml capacity, 1 cm light path) of a Beckman DU spectrophotometer. The blank cuvette was filled with water. Aliquots of a standard glycerol solution (30 μg/ml in water) were added to the cuvettes, together with enough water to bring the total volume to 0.78 ml. A reagent blank was run in which all reagents were present except glycerol. After the solutions had been mixed with narrow-gauge glass rods, the initial OD was read at 340 mμ with the blank set at zero OD (100% transmission). 0.02 ml of the diluted glycerokinase preparation was then added, the contents of the cuvette were mixed, and the OD was read until no further decrease occurred, when it was assumed that the reaction had proceeded to completion. This usually took less than 5 min; 12 test cuvettes could be used simultaneously if the glycerokinase was added sequentially to the cells at fixed time intervals.

The glycerol content of the sample could be calculated from the final OD of the sample minus the OD of the blank, using the molar extinction coefficient for NADH. Values thus obtained were found to range from 90 to 107% of the amount of glycerol added (Table 1). In our hands, direct proportionality between glycerol concentration and OD change could not be obtained with more than 6.0 μg of glycerol in 0.8 ml of the final mixture in the cuvette. Aliquots taken were chosen so that the amount of glycerol in the final mixture was always below 6.0 μg.

Application of Enzymatic Procedure to Determination of Triglycerides

The serum triglyceride determinations were carried out in three stages.

Stage I: Isopropanol Extraction. Preliminary experiments were performed with Mazola corn oil (Best Foods Division, Corn Products Co., New York) or purified triolein (Applied Science Laboratories Inc., State College, Pa.) as the source of triglycerides. Standard triglyceride solutions were prepared in redistilled chloroform in concentrations of 25, 50, or 100 mg/100 ml, so that they were similar to the concentrations expected

TABLE 1 RECOVERY OF GLYCEROL ADDED TO CUVETTES: ENZYMATIC METHOD

Expt.	Glycerol		
	Added to Cuvette	Found*	Recovery
	μg		%
I	1.5	1.6	106.5
	3.0	3.0	100.0
	6.0	5.9	98.4
II	1.5	1.5	100.0
	3.0	2.7	90.0
III	1.5	1.4	93.2
	3.0	2.8	93.4

* Determined by calculation from observed OD change and molar extinction coefficient for NADH (see Methods).

to be present in the serum. For the removal of phospholipids, 1.0 ml aliquots of the standard triglyceride solutions or of rat serum were added to 1.0 g samples of zeolite (W.A. Taylor & Company, Baltimore, Md.) that had been ground, sieved, and activated as described by Lofland (2) in 20-ml glass-stoppered centrifuge tubes. After the suspension had been mixed on a Vortex Junior mixer, 9.0 ml of redistilled isopropanol was added, the tubes were stoppered, and the contents were thoroughly mixed. At this stage, the tubes could be conveniently left overnight, without shaking, which resulted in complete removal of the phospholipids; alternatively, removal of the phospholipids could be completed by mechanical shaking for 1 hr at room temperature. The isopropanol extracts were cleared by centrifugation at 2000 rpm for 10 min at room temperature.

Stage II: Saponification and Extraction of Glycerol. The following procedure was used to achieve complete saponification, as checked by thin-layer chromatography by the method of Wood, Imaichi, Knowles, Michaels, and Kinsell (6). Unsaponified samples were used as blanks. 1 ml aliquots of the isopropanol extracts were pipetted into 12-ml conical stoppered centrifuge tubes. The isopropanol was removed by evaporation under a stream of nitrogen at 60°C. 1 ml of Skellysolve F (petroleum ether, bp 30–60°C, Skelly Oil Co., Kansas City, Mo.) was added to each tube. 0.1 ml of 90% ethanol was added to the blanks, and 0.1 ml of 10% KOH in 90% ethanol to the samples to be saponified. The tubes were stoppered and incubated with gentle agitation at 40°C for 30 min. 0.2 ml of 2 N H_2SO_4 was added to the saponified samples and mixed. To the blank (i.e., unsaponified) samples 0.2 ml of distilled water was added and mixed. 4 ml of Skellysolve F was then added to all tubes and the fatty acids were extracted by shaking the tubes for 10 sec. After the phases had separated, most of the petroleum ether phase was removed by aspiration, and residual Skellysolve F was removed by a gentle stream of nitrogen at 60°C. The saponified samples were then neutralized by the addition of a predetermined volume of 2 N aqueous KOH (usually about 0.15 ml) and diluted equally by the addition of distilled water to give a final volume of 1.2 ml. The blanks were diluted to an equal volume with distilled water.

Stage III: Enzymatic Glycerol Determination. The glycerol content of the aqueous solutions so prepared was then determined enzymatically as described above. A typical arrangement of the cuvettes for such a determination is shown in Table 2. The triglyceride content of the unknown sample was calculated from the standard curve relating the OD change to the triglyceride content of the standards, shown in Fig. 1.

With the triolein standards, it was possible to calculate recovery by using the extinction coefficient for NADH and the known glycerol content of triolein. Recoveries of triolein so calculated were 94–102%. Recoveries of triolein equivalent to 97% or better were obtained in experiments where serum and standard triolein solutions were added together to zeolite and extracted by the above procedure.

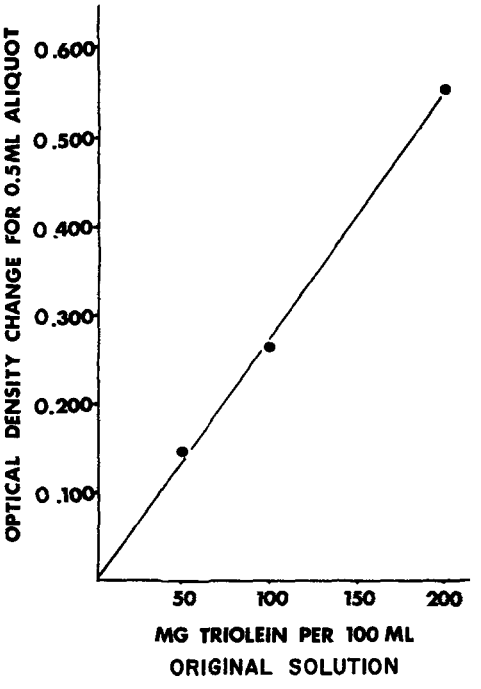

FIG. 1. Typical standard curve for the enzymatic determination of triolein glycerol. The OD changes were determined with no more than 6.0 μg of glycerol in the cuvettes; with the higher triolein concentrations, it was necessary to use smaller aliquots of the final aqueous glycerol solutions. For purposes of constructing this diagram, the OD changes for these samples were mathematically adjusted to what would have been seen with a 0.5 ml aliquot of the final aqueous glycerol sample.

OTHER METHODS FOR SERUM TRIGLYCERIDE ESTIMATION

The manual method of Van Handel and Zilversmit (1) was used, as modified by Newman, Liu, and Zilversmit (7).

The semiautomated colorimetric method of Lofland (2) was used initially, and modified as described in this paper.

The saponification of samples for the modified semiautomated colorimetric procedure was as described in the addendum to Lofland's method (1965 *Anal. Biochem.* **10:** 178) except that the samples were not evaporated after saponification. Instead the samples were acidified with 2.5 ml of 0.2 N H_2SO_4 and fatty acids were extracted with two 5 ml washes of Skellysolve F and were run on the AutoAnalyzer as described by Lofland (2).

TABLE 2 TYPICAL EXPERIMENTAL ARRANGEMENT OF REAGENTS IN CUVETTES FOR THE ENZYMATIC METHOD

	Cuvette No.			
	1	2	3	4
Water	0.8*	0.58	0.08	0.08
Reaction mixture (solution B)	—	0.20	0.20	0.20
Saponified sample	—	—	0.5	—
Nonsaponified sample	—	—	—	0.5
Glycerokinase	—	0.02	0.02	0.02

* Figures are volumes in ml added to the cuvettes.

OTHER DETERMINATIONS

Glucose was determined by the Glucostat method (Worthington Biochemical Corporation) or the hexokinase–glucose-6-phosphate dehydrogenase method of Slein, Cori, and Cori (8). Chloride was determined by the Whitehorn method (9).

Phospholipid phosphorus was determined on the isopropanol extracts by evaporation to dryness, extraction, and purification of the residues by the method of Folch, Lees, and Sloane Stanley (10), and phosphorus determined by the method of Bartlett (11).

RESULTS AND DISCUSSION

Validity of the Enzymatic Method

The validity of the enzymatic method for determination of glyceride glycerol described above was established by comparison of results obtained with this method and those obtained on duplicate samples of the same rat sera by the method of Van Handel and Zilversmit (1). Table 3 shows good agreement between the results.

Comparison of Extraction Procedures of Lofland (2) and of Kessler and Lederer (12)

Isopropanol and zeolite are used in both the Lofland (2) and the Kessler and Lederer (12) extraction procedures, and both procedures determine formaldehyde generated from glyceride glycerol by periodate oxidation, one by a colorimetric, the other by a fluorometric method. However, the isopropanol extracts prepared by the Kessler–Lederer method are freed from glucose by treatment with copper–lime reagent, and from bilirubin by treatment with hydrated aluminum silicate (Lloyd's reagent). Since these treatments might well remove those materials yielding erroneously high results in Lofland's procedure, the following experiment was performed. 15 male Wistar rats (Royal Hart Breeders, Middletown, N.Y.) were killed under hexobarbital anesthesia (10 mg/kg, i.p.) and blood was obtained from the jugular veins. The 15 samples of serum were each divided into two aliquots, one of which was extracted by the Lofland procedure (zeolite alone) and the other by the Kessler–Lederer procedure (zeolite, copper–lime, Lloyd's reagent). Each of the 30 isopropanol extracts so obtained was then analyzed for its triglyceride content by both the enzymatic method and the Lofland AutoAnalyzer colorimetric procedure. The results, given in Table 4, show that the enzymatic method gave essentially the same result for any given serum sample with either method of extraction. On the other hand, the

TABLE 3 MANUAL VAN HANDEL–ZILVERSMIT TRIGLYCERIDE METHOD COMPARED TO ENZYMATIC METHOD

Rat No.	Serum Triglyceride	
	Van Handel–Zilversmit	Enzymatic
	mg/100 ml	
1	70	69
2	70	59
3	52	52
4	67	61
5	68	68
6	80	65
7	65	62
8	73	85
9	57	54
10	79	80
Mean ± SEM	68.1 ± 2.8	65.5 ± 3.3

TABLE 4 ENZYMATIC AND SEMIAUTOMATED DETERMINATION OF SERUM TRIGLYCERIDES, EXTRACTED ACCORDING TO LOFLAND AND ALSO BY THE METHOD OF KESSLER AND LEDERER

Rat No.	Serum Triglycerides			
	Lofland Extract		Kessler–Lederer Extract	
	Manual Enzymatic Method	Lofland's AutoAnalyzer Method	Manual Enzymatic Method	Lofland's AutoAnalyzer Method
	mg/100 ml			
1	56	84	53	60
2	43	77	46	41
3	44	77	48	52
4	42	76	40	40
5	76	109	75	84
6	43	79	34	44
7	54	83	52	61
8	65	97	64	72
9	43	76	48	53
10	56	91	59	56
11	63	93	59	62
12	47	80	46	52
13	80	109	74	80
14	55	84	51	51
15	56	108	72	79
Mean ± SEM	55.5 ± 3.2	88.2 ± 3.2	54.7 ± 3.2	59.5 ± 3.8

There is no significant difference between the first, third, and fourth methods; the second method is significantly different, $P < 0.005$.

Lofland procedure gave significantly higher results when the isopropanol extracts were treated with zeolite alone than when they were treated with zeolite, copper–lime, and Lloyd's reagent.

Identification of the Interfering Material

We concluded that isopropanol extracts of serum, when treated with zeolite alone contain some material which gives a positive formaldehyde reaction when it is oxidized with periodate. Treatment of the isopropanol extracts with the mixture recommended by Kessler and Lederer (12) apparently removes this material. The interfering material is not likely to be phospholipid because, in both Lofland's (2) experiments and our own, it was not possible to detect phospholipid phosphorus in the isopropanol extracts treated with zeolite. In addition, glycerol arising from contaminant phospholipids would presumably be detected by the specific enzymatic method which gave the same values with either method of extraction.

It seemed possible that the interfering material might be carbohydrate (12); accordingly, efforts were made to assess the amount of glucose in the isopropanol extracts. We observed that when isopropanol extracts that had been treated with zeolite alone were evaporated to dryness under nitrogen, the resulting residue was almost insoluble in chloroform, but it was soluble in water. This material was found to give a positive reaction when tested by the colorimetric procedure for glyceride glycerol, but it contained no glycerol when examined by the enzymatic method. We considered it possible that the interfering material could be separated from the glycerol-containing lipids by extraction of serum with isopropanol and zeolite followed by evaporation of the isopropanol and extraction of the residue with chloroform. Accordingly, 270 ml of pooled isopropanol extracts from 27 ml of rat serum was treated with 54 g of zeolite. After centrifugation, the clear supernate was evaporated to dryness, and the residue was extracted twice with 100 ml of chloroform. The residue was analyzed for glucose by both the coupled hexokinase–glucose-6-phosphate dehydrogenase method (8) and the glucose oxidase (Glucostat) method. The results are shown in Table 5. The residue contained between 173 and 187 μg of glucose per mg of residue. The remainder of the residue appeared to be largely composed of chloride, based on titration of an aqueous solution of the residue for inorganic chloride content and calculation of the results as sodium chloride.

The concentration of glucose in the isopropanol extract treated with zeolite alone, calculated from these figures, was 16 μg/ml. When solutions of glucose in isopropanol of graded concentration were taken through the Lofland AutoAnalyzer procedure for triglyceride, the values shown in Table 6 were obtained. The results were read off a standard curve for triolein. 16 μg glucose per ml gave a color equivalent to approximately 50 mg/100 ml of triolein. It would appear, therefore, that the amount of glucose remaining in isopropanol extracts of rat serum treated with zeolite alone can account for the discrepancies between the triglyceride values obtained with the Lofland and the Kessler–Lederer methods of extraction. It should be pointed out, however, that the glucose that contaminates the isopropanol extracts cannot have been completely oxidized by the periodate treatment: according to calculations 16 μg of glucose per ml should be equivalent to approximately 80 mg/100 ml of triglyceride, whereas a value of only 50 mg/100 ml was obtained in practice. It is possible that a further source of variation in the original Lofland method is that the contaminating glucose is oxidized to an extent which varies from sample to sample, and that part of the variation is due to the variable loss of glucose during alkaline saponification.

TABLE 5 ANALYSIS OF CHLOROFORM-INSOLUBLE MATERIAL IN 270 ML OF ISOPROPANOL EXTRACT (1:10) OF RAT SERUM ACCORDING TO LOFLAND

Dry Residue after Evaporation and Chloroform Washes	24.6 mg
	μg/mg residue
Glucose determinations	
Slein, Cori, and Cori (8)	187
Glucostat	173
Chloride as NaCl	610

TABLE 6 ERRORS OF TRIGLYCERIDE ANALYSIS DUE TO GLUCOSE

Concn of Glucose in Isopropanol*	Triglyceride Equivalents by AutoAnalyzer Method
μg/ml	*mg/100 ml*
10	31
20	58
40	92

* Concentration of glucose calculated to be in isopropanol extracts treated with zeolite alone was 16 μg/ml.

The Modified Lofland Procedure

These results suggested that if the original Lofland semiautomated method (2) were modified by substituting the Kessler–Lederer (12) extraction mixture for the plain zeolite recommended by Lofland, the colorimetric procedure might yield reliable triglyceride values. To test this possibility, we analyzed 20 samples of rat serum for their triglyceride content by three methods: the manual Van Handel–Zilversmit method (1); the original Lofland

semiautomated method (2); and the Lofland procedure modified by treating the isopropanol extracts with the Kessler–Lederer (12) mixture to remove contaminant carbohydrates and bilirubin. Table 7 shows the values obtained by the three methods on all 20 serum samples. It is clear that there is good agreement between the Van Handel–Zilversmit and the modified Lofland procedures. Once again, the original Lofland procedure gave erroneously high results compared with the other methods.

To measure the variability of the Lofland procedure, modified as recommended, a large pool of serum was prepared from 30 rats. The triglyceride content of the pool was measured by 40 repeated enzymatic determinations and by 40 determinations with our modification of the Lofland semiautomated procedure. Table 8 shows that the methods gave mean values which were in excellent agreement. Furthermore, as judged by the standard errors, both methods appeared to have the same variability on repetition.

It seems therefore, that substitution of the Kessler–Lederer (12) mixture for the plain zeolite recommended by Lofland (2) permits reliable determinations of triglycerides by the semiautomated colorimetric procedure that Lofland describes. This simple modification obviates the necessity for the more expensive fluorometric apparatus. With the modified method reliable measurements of triglyceride levels up to 250 mg/100 ml can be made. Beyond that level, it is necessary to make a more dilute isopropanol extract and to use suitably diluted standards.

TABLE 7 COMPARISON OF MANUAL VAN HANDEL–ZILVERSMIT METHOD WITH AUTOMATED PROCEDURE OF LOFLAND AND MODIFIED PROCEDURE USING EXTRACT OF KESSLER AND LEDERER

Rat No.	Serum Triglyceride		
		AutoAnalyzer	
	Van Handel–Zilversmit	Zeolite (Lofland)	Zeolite + Copper–Lime + Lloyd's (Kessler–Lederer)
	mg/100 ml		
1	58	76	54
2	52	78	50
3	35	63	35
4	47	78	48
5	51	75	56
6	62	85	60
7	47	78	50
8	61	86	51
9	57	86	57
10	38	72	39
11	75	96	71
12	55	75	52
13	52	73	43
14	44	72	38
15	38	69	41
16	46	82	50
17	54	85	43
18	46	90	43
19	45	72	48
20	49	86	50
Mean ± SEM	50.6 ± 2.1	78.9 ± 1.8	49.0 ± 1.9

TABLE 8 REPEATED SERUM TRIGLYCERIDE DETERMINATION ON THE SAME RAT SERUM POOL

Serum Triglycerides	
Enzymatic	AutoAnalyzer
mg/100 ml	
54.4 ± 2.58 (SD)	55.2 ± 2.48 (SD)
SEM ± 0.41	SEM ± 0.40

40 determinations were made by the enzymatic method and the semiautomated colorimetric method of Lofland (2), modified as described in the text.

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