

Automated method for the colorimetric determination of acyl esters in serum

ARNOLD ANTONIS, D. S. PLATT, and J. M. THORP

Medical Unit, St. George's Hospital Medical School, London, and Research Department, Pharmaceuticals Division, Imperial Chemical Industries, Ltd., Macclesfield, England

SUMMARY An automated procedure is described for the estimation of serum acyl esters. The method requires the preliminary extraction of serum with isopropanol. The lipid extract is then analyzed automatically by the colorimetric procedure based on the formation of ferric hydroxamates. The influence of a number of factors on the precision of the method is discussed.

KEY WORDS automatic · colorimetric determination serum · acyl esters · isopropanol extraction ferric hydroxamate

EPIDEMIOLOGICAL STUDIES of ischemic heart disease have created a need for rapid and reproducible methods for the routine investigation of serum lipid levels. While many excellent manual methods of analysis are available, these are usually time-consuming and require skilled technical assistance. Attention has therefore been directed to the development of new methods, or the modification of existing ones to suit the requirements of automated analytical equipment which has recently become available.

In the present paper a method for the automated colorimetric determination of acyl esters in serum lipid extracts is described. The estimation of ester groups is based on the alkaline hydroxylaminolysis of esters to form hydroxamic acids which produce highly colored Fe^{+++} chelate complexes with Fe^{+++} ions in acid solution. Since its original introduction as a spot test by Feigl et al. (1), this reaction has been applied by numerous investigators to the determination of serum acyl esters. The manual procedure of Antonis (2) has been readily adapted to automation, and has provided a precise and accurate method suitable for the analysis of 30 specimens of serum per hour.

The method requires the preliminary extraction of serum lipids with isopropanol and separation of the

precipitated proteins. The protein-free lipid extract is then fed into an autoanalyzer (Technicon Ltd., Surrey, England), where it reacts initially with an alkaline solution of hydroxylamine in methanol. After a time delay at room temperature, the mixture encounters an acid solution of ferric perchlorate in methanol, and finally passes through the flow-cell of a colorimeter with subsequent recording of the optical density at 520 $\text{m}\mu$.

REAGENTS AND STANDARDS

All reagents and solvents used were analytical reagent grade.

Alkaline Hydroxylamine Reagent

Stock: (a) 10% w/v NaOH solution in absolute methanol; (b) 10% w/v hydroxylamine hydrochloride solution in absolute methanol.

Working solution: equal parts by volume of (a) and (b) were mixed, allowed to stand for 10–15 min, and filtered. Daily requirement: about 300 ml.

Ferric Perchlorate Reagent

Stock: Colorless ferric perchlorate (B. Newton Maine, North Walsham, Norfolk, England, or G. Frederick Smith Chemical Co., Columbus, Ohio), 8.5 g, was dissolved in 20 ml of distilled water and made up to 250 ml with 72–73% perchloric acid.

Working solution: 45 ml of the stock ferric perchlorate solution was added to 755 ml absolute methanol. Daily requirement: about 800 ml.

Stock reagents are stable on the shelf. Working solutions are prepared just before use.

Standard Ester Solutions

Stock (25 meq/liter): 0.73783 g of triolein was dissolved in isopropanol and made up to 100 ml.

Working standards (0.5–2.5 meq/liter): 2 to 10 ml aliquots of the stock solution were diluted with approximately 80 ml of isopropanol, 10 ml of water was added, and each was made up to 100 ml with isopropanol. Standards correspond to serum total ester concentrations of 5–25 meq/liter when carried through the procedure.

PROCEDURE

Preparation of Serum Lipid Extract

One milliliter of serum was blown in a fine stream with swirling into 9 ml of isopropanol contained in a stoppered test tube, and the mixture was well shaken. Although extraction was probably instantaneous, the mixture was allowed to stand for 30 min with intermittent shaking. The fine protein precipitate was separated by centrifugation, and the clear supernatant lipid extract decanted either directly into a polystyrene cup for autoanalysis, or into a separate stoppered tube. Extracts may be stored at 4° until enough specimens (approximately 150) have been collected for analysis at a single session.

Automatic Analysis

The Technicon Autoanalyzer sampler II, proportioning pump, double-time delay coil (80 ft), colorimeter, and recorder are required, with the arrangement of flow lines as shown diagrammatically in Fig. 1.

A constant level source of blank solution (isopropanol–water 9:1, v/v) was provided by means of the reservoir fitted to the sampler, so as to maintain a constant flow of fluid through the system.

Lengths (5.5 in.) of silicone-rubber tubing [Esco (Rubber) Ltd., London, England] were used for all lines in the pump manifold. All tubing walls were 1 mm thick, and the internal diameters were as indicated in Fig. 1. It was essential to use silicone-rubber tubing since the plasticizers in standard Tygon and Solvaflex (Technicon Ltd.) were leached out by pure methanol and by the alkaline hydroxylamine reagent, with the production of a high reagent blank.

Pump tubing was replaced each week, or more often if necessary; the line for the hydroxylamine reagent was usually replaced at the start of a daily session.

The delivery line from the sampler to the pump was polythene tubing of 1 mm bore and 0.25 mm wall thickness (Lionel Andrews Ltd; London, England). Other delivery lines were polythene tubing of 1.5 mm bore and 0.60 mm wall thickness. After passing through the pump, the sample line was connected through glass h-pieces fitted with capillary side arms, and a glass debubbler T-tube as shown in Fig. 1. Tubes differing in diameter were connected by means of polythene nipples and silicone-rubber tubing. The time-delay coil was a double glass coil (80 ft) as normally supplied with a heating bath. This coil was placed in a water bath at

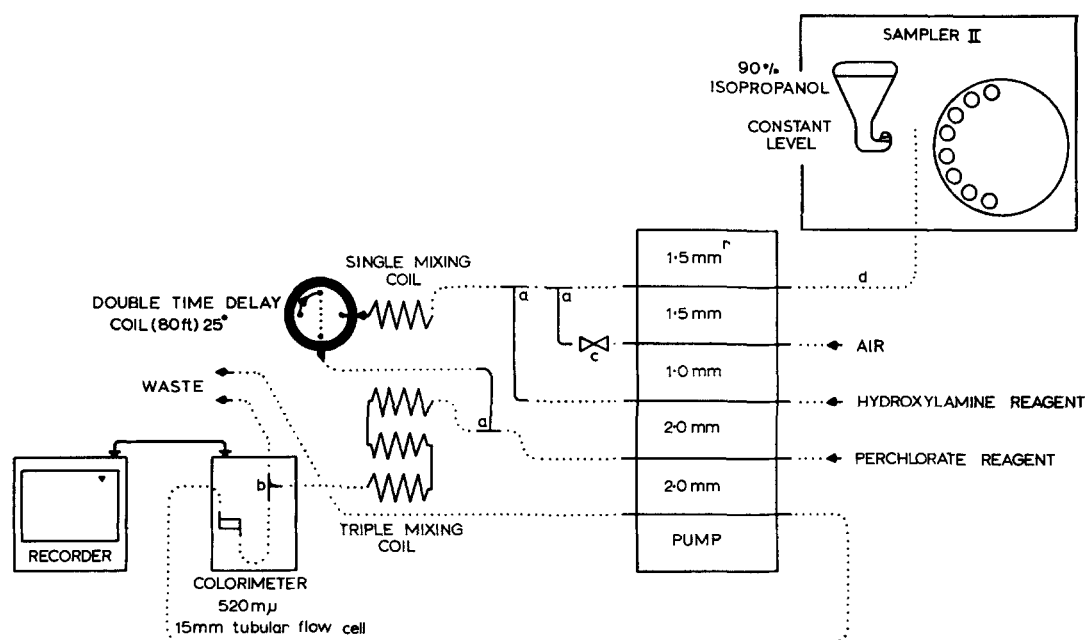


FIG. 1. Flow diagram for acyl ester determination. *r*, silicone-rubber pump tubing, bore as indicated, 1 mm wall throughout; *a*, glass h-piece, capillary side arm; *b*, glass debubbler T; *c*, 0.005 inch i.d. standard pump tubing pulse suppressor; *d*, polythene tubing, 1 mm i.d., 0.25 mm wall thickness. All other delivery tubing: polythene, 1.5 mm i.d., 0.60 mm wall thickness. All connections with silicone rubber tubing and polythene nipples.

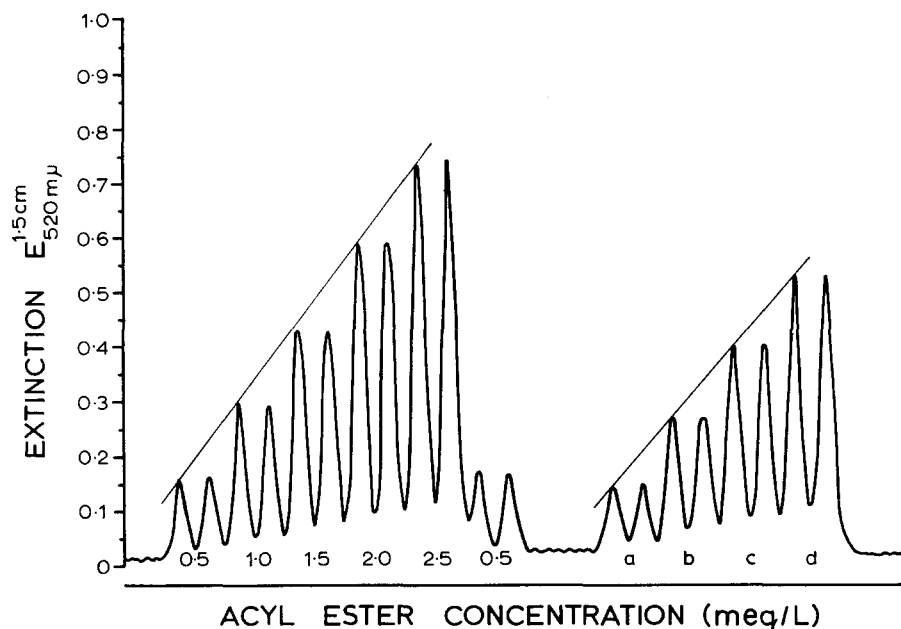


FIG. 2. Relationship between acyl ester concentration and extinction. Triolein standards in 90% aqueous isopropanol; range 0.5–2.5 meq/liter. *a-d*: serial dilutions of an isopropanol extract derived from a serum containing 23.26 meq of acyl esters per liter; 1–4 ml aliquots diluted to 5 ml with 90% aqueous isopropanol.

room temperature. Strict temperature control was not necessary, since temperature variation over the range 20–30° did not materially affect the analysis.

The sample plate was loaded with 40 specimens, covered with a cover plate, and run at the rate of 30 samples/hr with a 1:2 cam. Operating conditions therefore correspond to 40-sec sampling of specimen (approximately 0.9 ml of a 1:10 serum lipid extract) followed by 80 sec of solvent blank. By this procedure the sample line was washed between specimens, fluid flow remained constant, and evaporation of solvent from the sample cups was negligible.

The specimen drawn from the sampler (or alternately, blank solvent from the reservoir) and air were pumped, together with the alkaline hydroxylamine reagent, through a glass single mixing coil before entering the time delay coil. After leaving the latter (approximately 20 min later), the mixture encountered the perchlorate reagent and passed through a glass triple mixing coil followed by the glass debubbler before entering the 15 mm light-path tubular flow-cell of the colorimeter, the extinction being measured at 520 μ .

Although the baseline usually remained steady for runs of up to 8 hr, working standards were analyzed at the start of a session, and thereafter as every tenth specimen, in order to correct for drift which occasionally occurred.

With a suitable aperture in the colorimeter, the baseline was set to read 98–100% transmission with the blank (0–0.01 extinction with a linearized recorder). Under the conditions described above, a 2.5 meq triolein standard gave a reading of approximately 20% transmission (0.70 extinction).

At the end of a run the apparatus was washed through with methanol in the reagent lines, and dried by drawing air through the system, thus prolonging the life of the tubing.

RESULTS

The responses given by esters commonly found in serum lipids were almost identical. For lipid extracts containing 1 meq/liter, extinctions were as follows: triolein, 0.290; cholesteryl oleate, 0.292; dipalmitoyl lecithin, 0.288; and dipalmitoyl cephalin (Mann Laboratories, New York, N.Y.), 0.293. Sphingomyelin, with an amide fatty acid linkage, did not appear to react with the hydroxylamine reagent.

The relationship between extinction and concentration for a series of standards prepared from all the above compounds, and also with serial dilutions of serum lipid extracts, was linear for the range 0.5–2.5 meq/liter. This is clearly shown in Fig. 2, which is the tracing of a record obtained with triolein standards and with serial dilutions of a serum lipid extract.

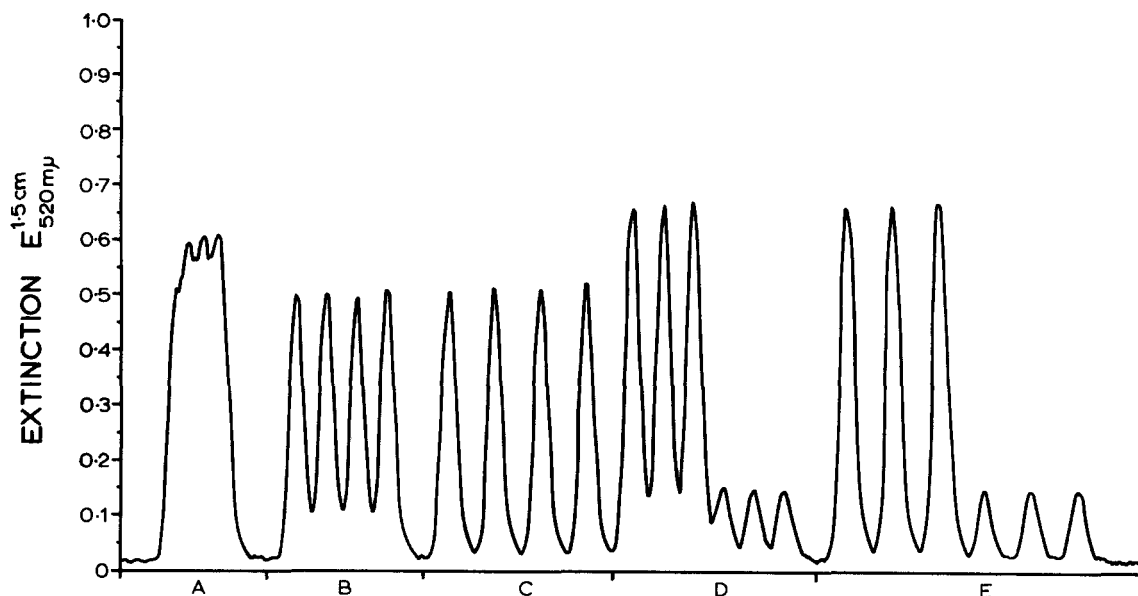


FIG. 3. Influence of blank wash between specimens. Constant volume of lipid extract sampled (approx. 0.9 ml over 40 sec). Sampling rates and ratios of specimens to blank were *A*, 60/hr, 2:1; *B* and *D*, 30/hr, 1:2; *C* and *E*, 20/hr, 1:3.5. In *D* and *E*, ratio of acyl ester concentrations was approximately 5:1.

The influence of the solvent blank (from the reservoir) interspersed between specimens is shown in Fig 3. The amount of specimen sampled (approximately 0.9 ml of serum lipid extract) was constant throughout, but the proportion of solvent blank was varied by the use of different cams as follows: *A*, with a sampling rate of 60 specimens/hr and a specimen to blank ratio of 2:1 (40-sec specimen and 20-sec blank); *B*, sampling rate 30 specimens/hr and ratio of 1:2 (40-sec specimen and 80-sec blank); *C*, sampling rate 20 specimens/hr and ratio of 1:3.5 (40-sec specimen and 140-sec blank). *D* and *E*, respectively, show the influence of the blank on specimens with high and low acyl ester concentrations. Proportions of specimen to blank were as in *B* and *C* and the ratio of concentrations was approximately 5:1. The extent of the effect produced by the blank is indicated by the decrease in optical density following each peak. Complete separation is indicated by exact return to the baseline.

The standard error of the method was 0.36 meq when determined on 20 pairs of serum specimens (acyl ester concentration 7–20 meq/liter of serum). Duplicate aliquots of extracts derived from 38 sera with acyl ester concentrations ranging from 12 to 20 meq/liter had an average difference of 0.39 meq with a standard error of a single estimation of 0.31 meq.

Fifty-four specimens of serum (acyl ester concentration 10.8–33.2 meq/liter) were analyzed by the automatic procedure (1 ml of serum + 9 ml of isopropanol) and by a manual method (2) (1 ml of serum made to 25 ml with alcohol-ether 3:1). Correlation was good, with an

average difference between the two methods of 0.24 meq, and a standard error of differences between paired items of 0.076 meq. This difference was well within the experimental errors of the two methods (0.36 and 0.34 meq, respectively); nevertheless the results with the automatic procedure were slightly but significantly ($P < 0.01$) higher than with the manual procedure.

The efficiency of isopropanol as a serum lipid extractant, indicated by the close correlation between results obtained with the automatic and manual procedures, has also been confirmed by the analysis of mixtures of, firstly, serum and isotonic saline, and secondly, two sera having low and high acyl ester concentrations, respectively. The results are shown in Table 1.

DISCUSSION

With the above manifold, reagents, and sampling rate (30 specimens/hr, 1:2 ratio), the relationship between extinction and concentration was linear for the range 0.5–2.5 meq/liter. Replication of results was good, and errors introduced by marked changes in concentration between consecutive specimens were small.

These desirable characteristics could not be maintained with a ratio of solvent blank to sample of less than 2:1. In Fig. 3, *A*, the wash of solvent blank between specimens was too small for efficient separation, and peaks overlapped. With a wash to specimen ratio of 2:1 as in *B* and *D* (Fig. 3) the optical density did not return to the baseline, but the influence of specimens on each other

was small, and the standard error between replicates (0.31 meq) was within the range normally acceptable for serum lipid analyses. In extreme cases, where the difference in acyl ester concentration of successive specimens was greater than 5:1, as in Fig. 3 (D), the error in the result obtained for the specimen having a low acyl ester concentration was of the order of 10%. When the record indicates that this sequence of specimens has occurred, it is necessary to repeat the analysis of the specimen having the low concentration. In this case the specimen should be placed following a sample of blank solvent.

In C and E (Fig. 3) the solvent wash between specimens resulted in the almost complete return of the optical density to the baseline between peak values, and the influence of successive specimens on each other was negligible. The precision of the method may therefore be considerably improved by increasing the solvent wash to specimen ratio, but in general the increase in precision does not outweigh the disadvantage of reducing the sampling rate to 20 specimens/hr.

Increasing the proportion of solvent wash to specimen while maintaining a sampling rate of 30 samples/hr may be achieved by the use of a cam with a 1:3 ratio. In this case, however, the amount of specimen sampled will be reduced to approximately 0.7 ml (30 sec) with a blank of 2.1 ml (90 sec), and the resulting extinction will be reduced proportionately. Although the relationship between extinction and concentration remains linear, the results with sera having low acyl ester concentrations would be subject to a greater proportional error. On the other hand, the range for analysis of sera having high acyl ester concentrations would be increased.

Clearly, the use of a particular cam influencing both the sampling rate and the amount of specimen sampled must depend on the expected range of acyl ester concentration of the sera, and on the numbers of sera required to be analyzed per day.

The influence of a number of factors on the final extinction has been investigated. The temperature at which hydroxylaminolysis takes place and the length of time of the reaction have a considerable effect on the yield of hydroxamic acid produced. With glyceryl esters such as triolein and lecithin, increasing the temperature from 25 to 50° with a time delay of 10 min resulted in a 20% lowering of the extinctions; however, with cholesteryl oleate the opposite effect (a rise of 15%) occurred. Increasing the time of the initial reaction at 25° from 10 to 20 min (by using an 80 ft instead of a 40 ft time-delay coil) had virtually no effect with triolein and lecithin, but increased the color yield for cholesteryl oleate so that for all three esters almost identical values were obtained. A minimum time is clearly necessary for quantitative hydroxamic acid formation at a particular

temperature. However, the use of elevated temperatures to improve the rate of formation of hydroxamic acids (especially from cholesteryl esters) is not advisable since this also tends to increase their rate of decomposition. The preferred procedure is therefore to carry out the reaction over a longer period at room temperature.

Within certain limits the presence of water in the initial reaction mixture did not appear to affect the rate of hydroxamic acid formation, as shown by the almost identical extinctions of equivalent solutions of different esters commonly found in serum lipids. With a manual procedure at room temperature, and using the reagents in the proportions recommended for the automated procedure (0.4 ml of hydroxylamine reagent, 0.9 ml of specimen, and 1.7 ml of ferric perchlorate reagent), extinctions have been compared on serum lipid extracts free from water or containing 10% water. In each case the final extinctions were almost identical. This is contrary to the experience of Skidmore and Entenman (3), who found a sharp decrease in extinction with increasing water content of the first reaction. On the other hand, Connerty et al. (4), whose results were in agreement with ours, found negligible effects due to water. In our procedure, the alkalinity of the first reaction is considerably greater than in the procedure recommended by Skidmore and Entenman, and this may account for the different findings.

The use of completely anhydrous conditions is not possible with serum isopropanol extracts unless they are taken to dryness and then reconstituted. Furthermore, this procedure is not recommended for the automatic method, since the presence of excess NaOH and NaCl in the hydroxylamine reagent causes precipitation of

TABLE 1 ACYL ESTER CONCENTRATIONS OF MIXTURES OF DIFFERENT SERA, OR OF SERUM AND SALINE

Mixture		Acyl Ester Concentration		Recovery
Serum	0.9% Saline	Found	Calculated	
ml		meq/liter		%
1.0	—	29.40
0.8	0.2	23.50	23.51	100.0
0.6	0.4	16.70	17.64	94.6
0.4	0.6	12.04	11.76	102.4
0.2	0.8	5.96	5.88	101.3
Mean				99.6
Serum A	Serum B	Found	Calculated	
1.0	—	7.63
0.8	0.2	15.00	15.36	97.7
0.6	0.4	22.96	23.10	99.4
0.4	0.6	31.10	30.83	100.9
—	1.0	46.30*		
Mean				99.3

* By analysis of mixture of 0.2 ml serum + 0.8 ml saline.

salts when the reagent is added to anhydrous isopropanol because of the lower solubility of these salts in the latter, and this would lead to a blockage in the time-delay coil.

Studies by Leffler (5) and Connerty et al. (4) have shown that isopropanol is an efficient extraction solvent for serum lipids, and values obtained for serum levels of cholesterol and its esters, glycerides, and phospholipids have agreed very closely with those obtained with other lipid extractants such as alcohol-ether 3:1 or chloroform-methanol 2:1. The above authors have recommended a 1:25 serum/solvent ratio with heating, but we have found that a 1:10 ratio at room temperature is equally effective, and that extraction of serum lipids is quantitative (as shown by comparison of the values obtained with the automatic and manual procedures). The results indicate that the isopropanol extraction technique was as efficient as the well established alcohol-ether procedure. This has also been confirmed by the results obtained from mixtures of sera with each other or with saline, as shown in Table 1. The 1:10 extraction procedure is applicable to sera with acyl ester concentrations not greater than 30 meq/liter. Most normal or slightly lipemic sera fall within this limit, but with highly lipemic sera (as in the example shown in the table) it is preferable to dilute the specimen with isotonic saline before extraction.

When 1:25 serum-solvent extracts are prepared, the volume of the precipitated protein relative to the final volume of the mixture is sufficiently small (approx-

mately 0.2%) to be ignored for calculation of serum lipid concentrations. With 1:10 serum-solvent extracts, the error due to the precipitated protein, or more correctly, due to the fact that 1 ml of serum of sp gr 1.025 at 20° actually contains 0.944 ml of water, is increased, since the volume of the extract would be 9.944 ml. There will also be a slight volume effect, due to the mutual solubility of water and isopropanol. The combined effect of these two errors would probably account for the slightly but significantly higher results obtained with the automated procedure as compared to the manual procedure. The error (approximately 1% for sera having acyl ester concentrations of 20 meq/liter) is sufficiently small to obviate the need for corrections in routine serum analyses. It could be eliminated, however, by increasing the volume of the isopropanol extractant to approximately 9.1 ml.

Our thanks are due to Professor A. C. Dornhorst and Dr. T. R. E. Pilkington for offering the facilities of their research laboratory, where this work was carried out.

Manuscript received September 21, 1964; accepted January 4, 1965.

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