Recent Advances in Automated Lipid Analysis

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Abstract

This paper reviews some of the recent developments in automated instrumentation which apply to the determination of chemically significant blood lipids such as cholesterol, triglycerides and phospholipids. Many complex lipid determinations can be performed on the continuous flow system of Skeggs following simple preliminary extraction.

This system, commercially available as the AutoAnalyzer is composed of modules which are capable of extraction, digestion and hydrolysis. Analytical endpoints can be determined by colorimetry or fluorometry with automatic recording of results.

Introduction

THIS PAPER WILL REVIEW some of the recent developments in automated instrumentation which apply to the determination of clinically significant blood lipids such as cholesterol, phospholipids and triglycerides. The continuous flow analytical system, described by Skeggs in 1957 for the determination of blood urea nitrogen (1), has been adapted to a wide variety of chemical determinations which can be accomplished using the principles of proportional pumping of samples and reagents, with subsequent colorimetry or fluorometry and automatic recording.

The introduction of new equipment to perform specific analytical functions such as extraction, digestion, and hydrolysis has gradually expanded the capabilities of this system.

The combination of simple preliminary manual purification, followed by automated processing for the remainder of the analytical procedure, permits application to complex lipid determinations.

Cholesterol

The first efforts to analyze lipids on the AutoAnalyzer (Technicon Instruments Co., Ardsley, N.Y.) were directed at the determination of serum cholesterol. The method of Zak et al. (2) was chosen as the analytical procedure because of its greater sensitivity as compared to the Liebermann-Burchard chromophore and near equivalence of color yield for cholesterol in its free and esterified form. This relationship is strongly dependent on the temperature of the reaction. In fact, Girard and Assous (3) were able to differentiate between free cholesterol and its esters by reacting a serum extract at 20C for the free form and 65C for total cholesterol. In the manual Zak procedure, an exothermic reaction is used to develop color. This is produced by carefully layering the sulfuric acid over a mixture of sample extract and ferric chloride in acetic acid. Then the mixture must be rapidly mixed to develop sufficient temperature. The success or failure of the method is mostly dependent on this step.

A characteristic of the AutoAnalyzer continuous flow system is constant proportioning and mixing of reagents. This proved most useful in automating the Zak procedure where this step is of critical importance. The initial AutoAnalyzer method was described by Kessler in 1959 (4). A particular problem in his work was the effect of organic solvents and strong acids on the Tygon tubing normally used to pump reagents. Silastic tubing was found to withstand glacial acetic acid and the isopropanolic extract of serum. Since no tubing was then available to pump concentrated mineral acids, a bag-bottle displacement device was used to deliver concentrated sulfuric acid into the system.

The sample extract was first diluted with an air-segmented stream of glacial acetic acid containing ferric chloride. Following the addition of a sulfuric acid, the flowing stream passed through a small chamber containing a magnetic mixing bar to insure complete homogeneity. The mixture then passed through a series of glass coils inside a hot air bath where a constant temperature of 80C was maintained. At this temperature all forms of cholesterol reacted equally.

Shortly after its introduction, the automated procedure was improved by replacing the cumbersome bag-bottle displacement device with Acidflex pump tubes. Not only does this material withstand concentrated mineral acids, but in addition, it pumps organic alcohols, chloroform and a variety of other solvents. The displacement device remains the last resort for reagents which cannot otherwise be pumped. The availability of Acidflex pump tubes allowed for faster flow rates, increasing the usable rate of analysis to 40 samples per hour. It was found that the 80C air bath, a special module, was not necessary when the ratio of sulfuric to acetic acid pumping rates was between 0.55 and 0.80. The heat generated by the mixing of the two acid streams was sufficient for complete color development.

The next step in the development of the AutoAnalyzer cholesterol method was the use of a combined, stable, color reagent. Although combining the two acid streams in situ resulted in an exothermic reaction, the manifold required daily calibration to ensure the ratio of delivery rates stayed within the range needed for maximum color development. A reagent composed of 350 mg $FeCl_3 \cdot 6H_2O$ per liter of a 3.5% water, 30% sulfuric acid, 66.5% acetic acid mixture was developed by Levine and Zak (5) for use with the Technicon 15-mm tubular flow cell. The combined reagent was added to the sample extract in two steps and thoroughly mixed prior to passage through a standard AutoAnalyzer 95C heating bath. Color was developed in the bath and then read at 520 mμ in the colorimenter.

Ordinarily, serum components are separated from protein in the AutoAnalyzer by use of continuous flow dialysis. This approach was not feasible for cholesterol which must first be freed from its protein bonding and then extracted into isopropanol to remove chromogenic interferences. As the demand for cholesterol determinations increased, a means of preparing serum extracts automatically was devised—the Technicon continuous filter (Fig. 1). Serum enters the mixing block (*D*) through nipple (*B*) and is mixed by a paddle (*C*), attached to a stirring motor with a stream of isopropanol entering at (*A*). Serum protein immediately precipitates as it comes

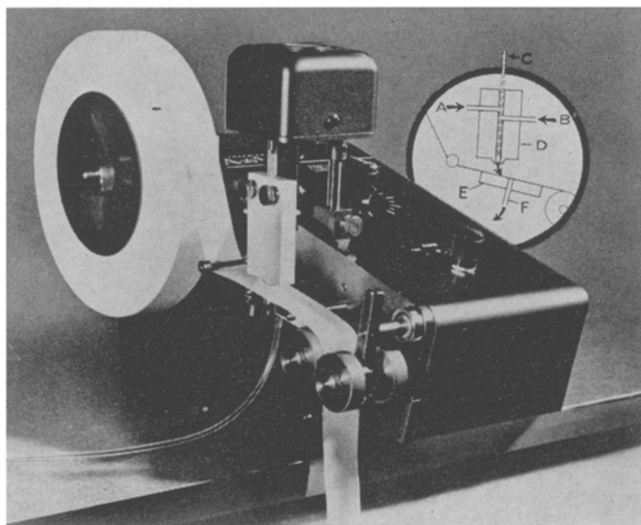

FIG. 1. Technicon AutoAnalyzer Continuous Filter.

in contact with the solvent and is deposited onto a moving strip of filter paper passing over a platen (*E*). Isopropanol containing the extracted cholesterol can then be aspirated into the analytical system through nipple (*F*). The precipitate and remaining solvent pass on to a waste container. By combining serum extraction on the continuous filter with the analytical procedure of Levine and Zak it was possible to sample serum directly at the rate of 20 per hour with a minimum of technician effort.

The original cholesterol method for the tubular flow cell (N-24) described above was found to be precise and correlate well with the Abell-Kendall method (6) when properly controlled. However, some laboratories found the procedure to be imprecise. Investigation revealed that extended dwell time in the 95C heating bath was the major cause of the difficulty. During the heating step the reaction mixture was going from room temperature to 95C, a considerable temperature gradient.

Block et al. (7) devised a new manifold (N-24A) using an anhydrous color reagent containing 275 mg of $FeCl_3$ per liter of a solution containing one part sulfuric acid to two parts acetic acid. The flow diagram is shown in Fig. 2. A 1:20 extract of serum in isopropanol may be prepared by hand or automatically on the continuous filter. The color reagent is preheated to 95C in the heating bath, segmented with air, and the sample added to it before there is any significant drop in temperature. Color is developed in three vacuum-jacketed single mixing coils and read at 550 mμ. Instead of a rapidly increasing temperature gradient, the color reaction now occurs at a high, relatively constant temperature. The preheated color reagent is quite limpid compared to its viscous state at room temperature. This enables serum extracts to be determined at the rate of 40 samples per hour and a sampling rate of 30 per hour when the continuous filter is used. Table I compares the day-to-day precision of the AutoAnalyzer method with the Abell-Kendall.

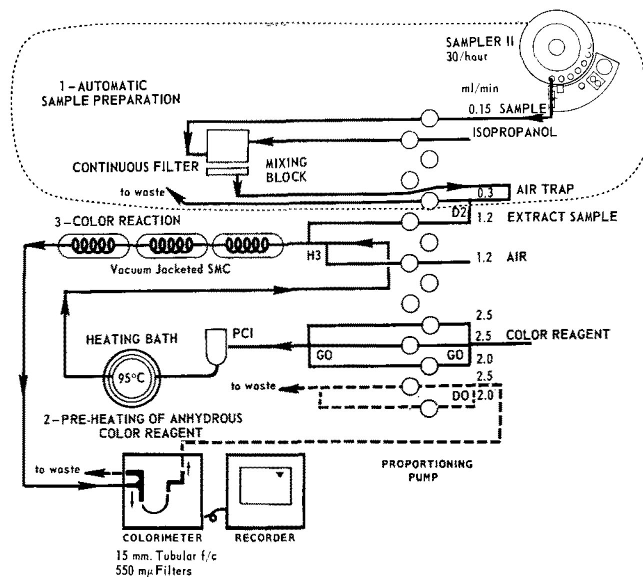

FIG. 2. Flow diagram for total cholesterol method of Block, Jarret and Levine.

Triglycerides

As data has accumulated on the possible relationship of serum cholesterol to heart disease, it has become apparent that other biochemical parameters are involved. Triglycerides appear to be an important factor but their analysis is difficult and time-consuming. Lofland in 1964 (8) and Kessler and Lederer in 1965 (9) have presented methods which make use of an isopropanol extract which can also be used for determining cholesterol.

Lofland's procedure is based on the removal of phospholipids from the extract by the use of Doucil, a zeolite. An aliquot of the phospholipid-free extract is then mixed with alcoholic KOH and saponified at 70C for 20 min. The solvent is then evaporated off and the samples are reconstituted with 0.2 N H_2SO_4 and transferred to sample cups.

The glycerol obtained by saponification of the triglycerides is then analyzed using the flow diagram in Fig. 3 at the rate of 40 samples per hour. The samples are mixed with periodate to oxidize the glycerol to formaldehyde. Excess periodate is then destroyed by the addition of sodium arsenite and the formaldehyde is reacted with chromotropic acid in a double coil 95C heating bath to produce a colored product measured at 570 mμ. Up to a 100 samples can be processed in a day when batch operations are used.

TABLE I

Determination of Serum Total Cholesterol
Serum total cholesterol (mg/100 ml)

Serum Pool	Day	Analytical method	
		Block et al. AutoAnalyzer	Abell et al.
J	1	217 ± 4.4 (2.0)[a]	226 ± 1.4 (0.6)
	2	216 ± 6.9 (3.2)	220 ± 0.0 (0.0)
	3	216 ± 0.0 (0.0)	223 ± 1.4 (0.6)
K	1	291 ± 1.7 (0.6)	300 ± 0.0 (0.0)
	2	285 ± 4.7 (1.6)	291 ± 0.0 (0.0)
	3	285 ± 4.7 (1.6)	298 ± 1.4 (0.5)
L	1	366 ± 2.2 (0.6)	351 ± 4.1 (1.2)
	2	365 ± 6.2 (1.2)	345 ± 2.2 (0.6)
	3	367 ± 9.3 (2.5)	348 ± 2.2 (0.6)
M	1	192 ± 0.0 (0.0)	203 ± 1.2 (0.6)
	2	194 ± 4.0 (2.1)	200 ± 0.0 (0.0)
	3	192 ± 0.0 (0.0)	191 ± 3.4 (1.8)
N	1	199 ± 5.0 (2.5)	205 ± 2.4 (1.2)
	2	187 ± 6.0 (3.2)	202 ± 0.2 (0.1)
	3	191 ± 2.0 (1.0)	192 ± 5.2 (2.7)
P	1	224 ± 0.0 (0.0)	233 ± 0.0 (0.0)
	2	226 ± 2.0 (0.9)	233 ± 1.0 (0.4)
	3	226 ± 1.0 (0.4)	228 ± 2.8 (1.2)

[a] Mean ± S.D. of 4 replicate samples, and coefficient of variation expressed as per cent.

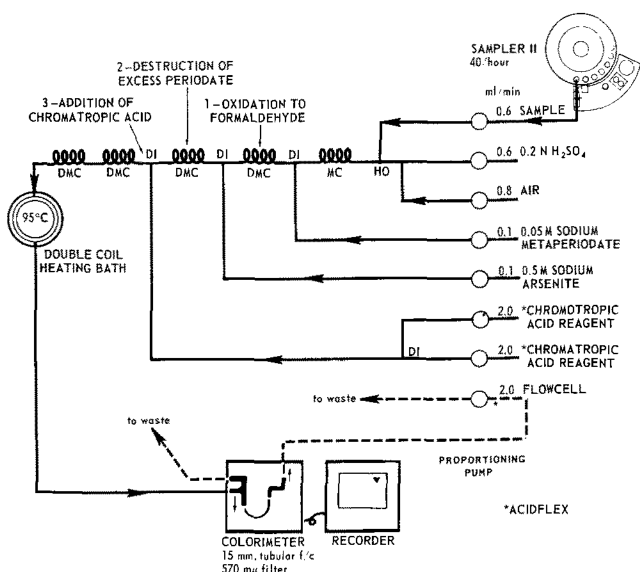

FIG. 3. Flow diagram for triglycerides method of Lofland.

The determination has been simplified by automation of the saponification step by Kessler and Lederer in the development of a new fluorometric procedure for triglycerides. Their work is based on the Hantzsch condensation reaction between an amine, beta diketone, and an aldehyde. They prepare a phospholipid-free extract by adding zeolite to the isopropanol extract. In addition, Lloyd's reagent and a copper-lime mixture are added to remove chromagens and glucose interferences, respectively. The extract may be used for the simultaneous determination of cholesterol and triglycerides by combining the two manifolds with a stream splitter.

The flow diagram for the procedure is shown in Fig. 4. The extract is mixed with 0.5% KOH in 1 part water and 3 parts isopropanol and then passed through a 50C heating bath coil for approximately 5 min. During this time complete saponification of the triglycerides occurs and the stream is then mixed with periodate and acetylacetone reagent. During the mixture's passage through the second 50C heating bath coil, the glycerol is oxidized to formaldehyde by the periodate and then condensed with the acetylacetone reagent to produce a fluorescent product, 3,5-diacetyl-1,4-dihydrolutidine. This is measured in a Technicon Fluorometer using a 405 mμ interference filter for the primary and a 485 mμ sharp-cut as the secondary. A blank determination is carried out on the extract by eliminating the saponification step.

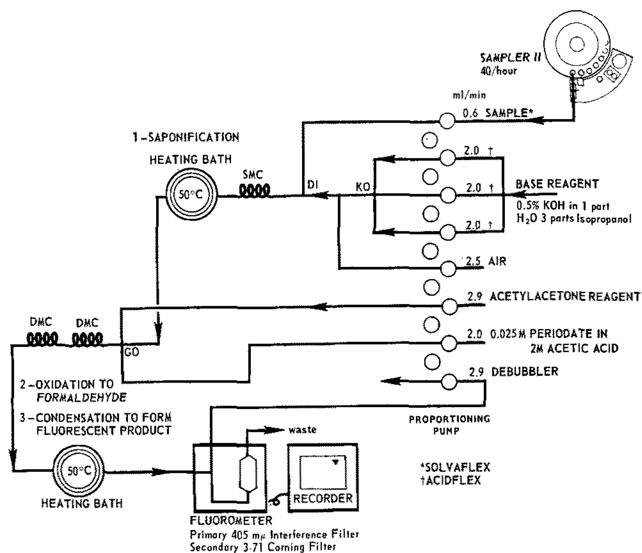

FIG. 4. Flow diagram for fluorometric triglycerides method of Kessler and Lederer.

TABLE II

Reproducibility of Control Pool, mg triglyceride/100 ml

Day	Value	Day	Value
1	125	11	123
2	121	12	120
3	118	13	123
4	115	14	124
5	119	15	118
6	118	16	116
7	117	17	116
8	118	18	119
9	117	19	121
10	122	20	120
	Average	119.5	
	Standard Deviation	±2.9	

Both assay and blank determinations are sampled at the rate of 40 per hour giving a net rate of 20 per hour. Table II indicates the reproducibility of the method for a serum pool over 20 days. Table III compares the automated procedure with a modified Carlson-Wadström technique.

Phospholipids

The automation of serum phospholipids presented an even more difficult problem in sample pretreatment than triglycerides. In the widely used method of Zilversmit and Davis (10), the protein-phospholipid complex is first precipitated with 10% TCA. This is followed by centrifugation and decantation of the supernatant which contains the inorganic phosphate. The precipitated complex is then digested with perchloric acid and the digest is assayed by a colorimetric molybdate method for phosphorus. The most time-consuming operation in the procedure is the digestion, which requires careful attention to ensure complete oxidation of the organic material.

Whitley and Alburn (11) approached the problem by using the Technicon continuous flow digestor which had been developed for use in Kjeldahl nitrogen digestion. This module is essentially a rotating glass helix which carries a mixture of sample and oxidizing solution over a series of high temperature heating elements. They found that vanadium pentoxide was an excellent catalyst when used with a mixture of perchloric and sulfuric acids. Complete oxidation was accomplished in less than 2 min. This module

TABLE III

Triglyceride Comparisons, mg per 100 ml

Specimen	Manual[a]	Kessler and Lederer AutoAnalyzer
P7	135	138
S6	355	350
W83	53	57
M38	106	107
M44	289	282
M87	98	97
F23	53	37
M39	222	229
S15	269	275
Th81	212	227
M43	210	209
M42	2090	2020
W5	2215	2330
F38	64	66
Th94	136	115
S12	1615	1710

[a] Modified Carlson-Wadström technique.

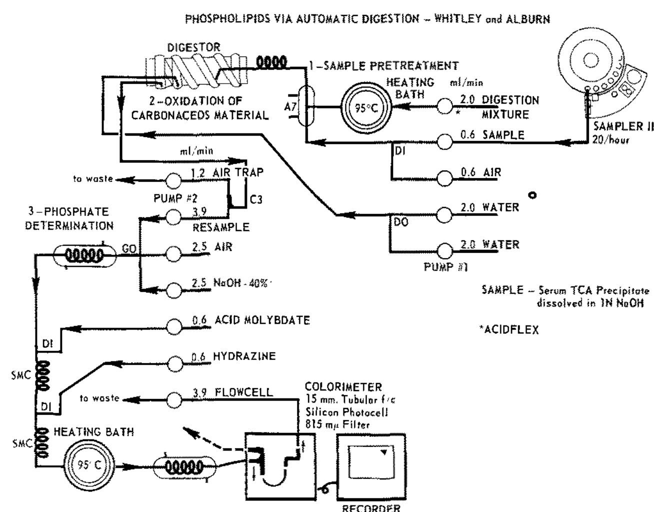

FIG. 5. Flow diagram for Whitley and Alburn's method for phospholipids incorporating automatic digestion.

has also had wide application in oxidizing protein in the determination of protein-bound iodine.

The analytical procedure is outlined in the flow diagram in Fig. 5. Samples are run at the rate of 20 per hour. The precipitate is reconstituted with IN NaOH and mixed with hot oxidizing solution and then passed through the digestor. An aliquot is then resampled and partially neutralized with 40% NaOH. Molybdate and hydrazine are then added and the mixture passes through a large bore heating bath. Final color is read at 815 mμ in a colorimeter equipped with silicon photocells.

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