Variables in the Technicon-N-24 Method for the Determination of Serum Cholesterol

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Abstract

A modification of the Technicon-N-24 method for serum cholesterol is described. It is possible to obtain precise results which agree on the average with those obtained with the reference Abell-Kendall method. The level of cholesterol found by the AutoAnalyzer method depends primarily on the ratio of the rates of flow of the lipid extract to that of the premixed color reagent and the time of heating of the color reaction at 95C. In this modification, the flow rate of isopropyl alcohol extract of sample or standard is kept at 0.9 ml/min and the ratio of flow of extract to that of premixed color reagent is maintained between 1:10 and 1:12 while the time of heating of the color reaction is maintained between 95 and 120 sec. Other conditions necessary for precise results are discussed. The precision obtained on the average in several experiments ranged from 2.2 to 2.7 mg% as standard deviation between duplicates.

Introduction

In recent years several procedures have been proposed for the automated determination of serum cholesterol using the AutoAnalyzer. These methods have been based on the reaction of cholesterol with *p*-toluenesulfonic acid (1,2) or ferric chloride (3–6). The AutoAnalyzer-N-24 method (4) which is based on the ferric chloride reaction, is one of the more widely used procedures.

This report covers a study of the precision and accuracy obtainable with the AutoAnalyzer-N-24 method. Manual ferric chloride methods similar in principle to the AutoAnalyzer-N-24 method have been generally found to give high results when compared to the Abell-Kendall method (7) which is considered a reference method by most authorities (8–10). In the present investigation, however, the ratio of rates of flow of premixed color reagent to rate of flow of sample extract, and the time of heating of the color reaction, have been adjusted so that the results obtained with the AutoAnalyzer-N-24 method are not high but on the average agree quite well with the values found with the Abell-Kendall procedure. In addition, several other points of technique are described which were not included in the original procedure but which are necessary for good precision and reproducible results.

Experimental

The Technicon AutoAnalyzer-N-24 method was carried out according to the procedure described by the manufacturer except for the following modifications. The sulfuric acid concentration was lowered 2 volume percent; i.e., 420 ml instead of 430 ml per liter of color reagent. The level of the premixed color reagent in the reagent reservior was kept between 20 and 22 cm above the bench top on which the AutoAnalyzer was placed. In all experiments reported, except those using the continuous filter, 60 sample cups were run per hour with an isopropyl alcohol wash cup between each determination for a total of 30 determinations per hour. Analyses were run at the rate of 40 determinations per hour when the continuous filter was used.

The rate of flow of sample or standard extract was maintained at 0.9 ml/min and ratio of the rate of flow of sample or standard extract to that of the premixed color reagent was held between 1:10 and 1:12 except for the experiments listed in Tables I and II. The initial rate of flow of the premixed color reagent was adjusted to 10.7 ml/min with new tubing. The premixed color reagent was allowed to run through the new tubing for 2 hr during which time the flow rate diminished about 2.5% and then stabilized. Ratios of rate of sample extract flow to rate of flow of premixed reagent were then maintained between 1:10 and 1:12. The tubing for the premixed color reagent was changed when the flow rate dropped below 10 ml/min.

TABLE I

Effect of Time of Heating at 95C on the Cholesterol Level of Serum

Experiment number	Time of heating at 95C	Sample No. 1		Sample No. 2	
		Cholesterol level AA[a]	AA-AK[a] level	Cholesterol level AA[a]	AA-AK level
	Seconds	Mg %	Mg %	Mg %	Mg %
1	78	139	−8	216	−37
2	93	141	−6	243	−10
3	107	144	−3	250	−3
4	112	145	−2	251	−2
5	120	149	+2	250	−3
6	130	150	+3	259	+6
7	136	155	+8	263	+10

[a] AA = AutoAnalyzer; AK = Abell-Kendall method.
All experiments were done in duplicate and standards were run under each condition. The rate of flow of color reagent was 9.9 ml/min and that of the sample extract was 0.7 ml/min except for experiments 1 and 2 in which the rate of flow of sample extract was 1.1 and 0.8 ml/min, respectively.
The cholesterol levels of sera 1 and 2 were 147 and 253 mg%, respectively, as determined by the Abell-Kendall method.

TABLE II

Effects of Ratio of Flow of Sample Extract and Color Reagent on Cholesterol Level of Serum

Sample extract flow	Ratio of flow of sample extract/color reagent	Sample No. 1		Sample No. 2	
		Cholesterol level AA[a]	AA-AK[a]	Cholesterol level AA[a]	AA-AK level
Ml/min		Mg %	Mg %	Mg %	Mg %
1.1	1:9.0	184	+4	268	+1
0.78	1:9.2	171	−9	242	−25
1.0	1:11.0	187	+7	279	+12
0.9	1:12.0	179	−1	260	−7
0.8	1:12.4	183	+3	268	+1
0.66	1:12.7	173	−7	255	−11
0.36	1:16.7	165	−15	249	−18
0.60	1:18.1	167	−13	251	−16

[a] AA = AutoAnalyzer; AK = Abell-Kendall method.
In this experiment the time of heating was kept between 105 and 118 sec. All determinations were in duplicate and standards were run under each condition. The results are given as the means of the duplicates and were calculated with aid of the appropriate standards, as described in the experimental section. Other conditions are as described in the experimental section. Cholesterol levels of sera 1 and 2 were 180 and 267 mg%, respectively as determined by the Abell-Kendall method.

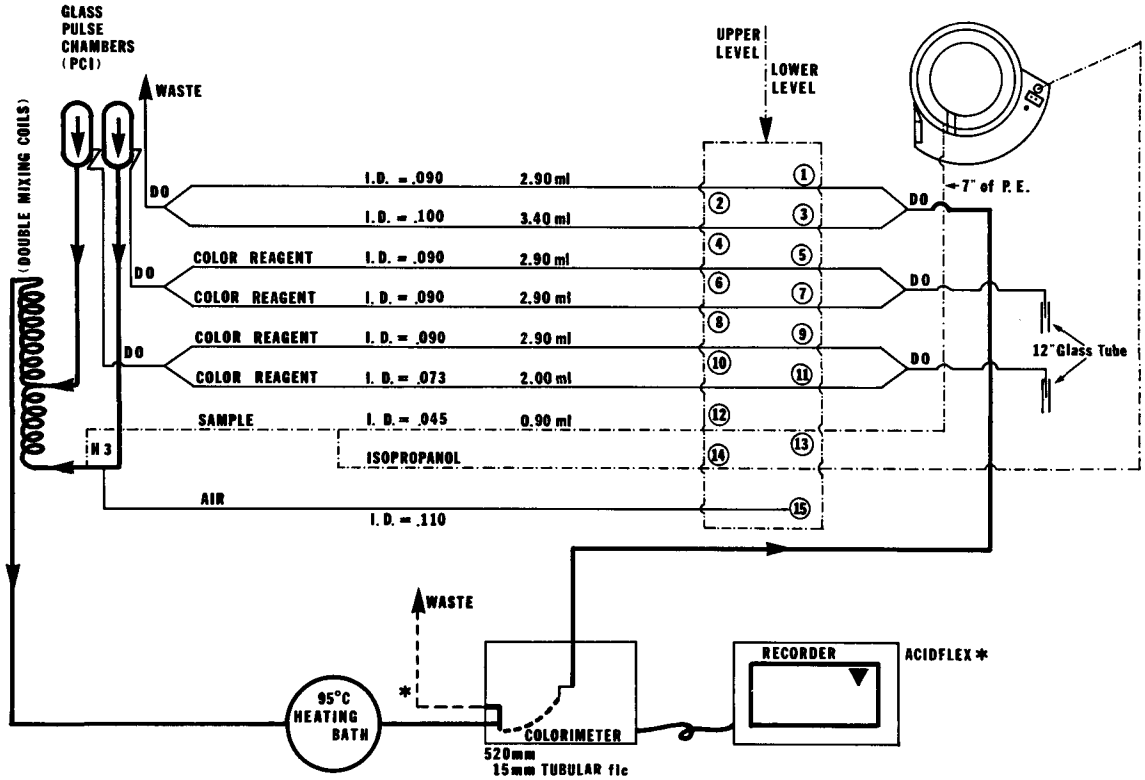

FIG. 1. Manifold diagram for modified AutoAnalyzer-N-24 method.

The time of heating in the bath at 95C was kept between 95 and 120 sec by use of a glass coil containing a volume of 24 to 28 ml, and by adjustment of the rate of flow of air.

A flow diagram illustrating the manifold of the modified AutoAnalyzer-N-24 method is given in Figure 1. The flow rate for the premixed color reagent is the initial rate with new tubing.

Solvaflex tubing was used for the sample extract and the isopropyl alcohol wash. Acidflex tubing was used for all the experiments reported in this paper with the exception of those reported in Tables I and II, in which Tygon tubing was employed. Tygon tubing has proved to be more resistant to the acid premixed color reagent and is now used for this reagent. Acidflex tubing is used to connect the heating coil to the flow cell of the colorimeter. This section of the tubing was changed each day. Unless otherwise specified, the position on the sample turntable of all replicate standards and samples was randomized. The results were recorded as percent transmittance which was read to the nearest 0.1%. These data were converted to absorbance, and the results calculated from the absorbancies of four cholesterol standard solutions equivalent to 100, 200, 300, and 400 mg%. These standard solutions were analyzed in duplicate with each experiment.

The Abell-Kendall method (7) was used as the reference procedure. Cholesterol was purified according to the method of Fieser (11).

Results

Effect of Time of Heating at 95C and the Rate of Flow

The level of cholesterol determined in serum was found to be influenced by the rates of flow of the sample extract and the premixed color reagent, and the time of heating of the reaction mixture at 95C. As can be seen in Table I, the values obtained increased from 9 to 18% as the time of heating was increased from 78 to 136 sec. Also, as illustrated in Table II, the apparent cholesterol content of serum cholesterol can be altered simply by varying either the rate of flow of the sample extract or the ratio of the rate of flow of sample extract to rate of flow of mixed color reagent. The fact that standard solutions of cholesterol were treated in the same manner as the sample does not compensate for changes in rates of flow of extract or premixed reagent, or in time of heating at 95C. Apparently the mixture of cholesterol esters and cholesterol in the serum reacts at different rates with these parameters than does cholesterol in the standard solutions.

Comparison of N-24 Method and Abell-Kendall Method

Tables I and II illustrate that it is possible to select rates of flow of color reagent and sample extract and times of heating of the reaction mixture so that the results obtained with the AutoAnalyzer-N-24 method agree with those obtained by the Abell-Kendall procedure. Table III shows experiments illustrating the close agreement between cholesterol levels found with the Abell-Kendall method and those obtained with the AutoAnalyzer-N-24 procedure under the conditions of reagent and extract flow and time of heating of color reaction given in the experimental section.

TABLE III

Comparison of Cholesterol Levels Obtained with AutoAnalyzer-N-24 Method and Those Obtained with the Abell-Kendall Procedure

Experiment number	Method	No. of samples	Range of values	Mean	Precision SD[a]
			Mg %	Mg %	Mg %
1	AutoAnalyzer-N-24	42	133–392	205	2.2
	Abell-Kendall	42	132–393	209	5.3
2	AutoAnalyzer-N-24	20	107–368	225	2.3
	Abell-Kendall	20	107–359	223	4.0

[a] Standard deviation based on differences between duplicates.

All samples were run in duplicate by each method. For Experiment 1 the duplicates were randomized over a 6-day period. The correlation coefficient r was +0.991 and +0.998 for experiments 1 and 2, respectively.

TABLE IV

Analysis of Serum Samples by the Autoanalyzer-N-24 Technique Compared to the Same Method Using the Continuous Filter Technique

Method	Range	Mean	Precision (standard deviation)
	Mg%	Mg%	Mg%
AutoAnalyzer-N-24	149–306	220	2.7
AutoAnalyzer-N-24 with continuous filter	150–310	223	2.4
Abell-Kendall method	149–309	222	

Each of the 8 samples was analyzed in duplicate on each of 4 days by both AutoAnalyzer methods and on one day in duplicate by the Abell-Kendall procedure. Four reference sera containing 155, 200, 250, and 280 mg % cholesterol as determined by the Abell-Kendall method, were analyzed in duplicate each day. An average factor relating milligrams per cent in reference samples to absorbance was calculated each day. This factor was used to calculate the results for that day. The correlation coefficient r was +0.996, +0.996, and +0.997 for results obtained with the AutoAnalyzer manual method and the Abell-Kendall method, with the continuous filter and the Abell-Kendall method, and with the continuous filter and the manual N-24 procedures, respectively.

Analysis Using Continuous Filter

The continuous filter is a device with which it is possible to automate the process of preparing an isopropyl alcohol extract of serum. In this laboratory, isopropyl alcohol solutions of cholesterol could not be used as primary standards. However, as showin in Table IV, excellent agreement was obtained among results using the AutoAnalyzer-N-24 method as described in the experimental section, the same method with the continuous filter device, and the Abell-Kendall procedure. Levene and Zak (5) also report that similar results are obtained with either the continuous filter or the usual manual extraction but did not show comparison with another method. In the present experiments consistent results were not obtained with a conversion factor calculated from the cholesterol content of a single pooled reference serum as previously recommended (3). In order to obtain reproducible results with the continuous filter, it was necessary to calculate an average daily conversion factor from the daily analyses of at least four reference sera whose cholesterol content covered the usual range of values in serum for this lipid.

Precision of Modified N-24 Method

The precision which can be obtained in the modified AutoAnalyzer-N-24 method is illustrated in Tables III and IV. It can be seen that highly precise results with standard deviation as low as 2.2 mg% can be obtained for duplicate analyses of samples whose duplicates are randomized over a period of 6 days.

Discussion

Under the conditions outlined in the experimental section, the results obtained with the AutoAnalyzer-N-24 method show a high degree of precision, and unlike those found in most ferric chloride methods, check very closely with the results found by a generally recognized reference procedure, the Abell-Kendall method. Rates of sample extract and color reagent flow, and time of heating at 95C are interdependent parameters which influence the apparent cholesterol level. None of these variables can be changed without redetermination of optimal conditions for the other two.

The automated preparation of isopropyl extracts is possible with the continuous filter. The results obtained also agree with those found by the modified N-24 procedure and the Abell-Kendall method. However, the method employing continuous filtration has the inherent disadvantages of procedures in which secondary instead of primary standards are used.

Careful adherence to the conditions for the determination as outlined in the present publication is essential for accurate and reproducible results. For example, the sulfuric acid concentration of the original premixed color reagent was lowered two volume percent from the level previously recommended (4) since the viscosity of the original reagent was too high to permit optimal pumping and hence adequate mixing of the color reagent with the lipid extract. In addition, it was necessary that the level of the premixed color reagent in the reagent reservoir be maintained within specified limits or the base line of the recording of absorbance would gradually shift upward as the level of the reagent dropped. Thus a drop of 2 cm in the specified level of the reagent caused a shift of 0.0066 absorbance units in the base line.

Careful attention must also be given to other conditions which are given in the original method. It is of particular importance to prevent evaporation of extracts not only during filtration but also when the sample extracts are placed on the sample turntable. However, the most difficult variable to control in this procedure is the rate of pumping of the mixed color reagent. This variable in turn makes it difficult to control the ratio of flow of sample extract to flow of color reagent and the time of heating of the reaction mixture at 95C. The Acidflex or Tygon tubing used for this reagent is readily attacked by the premixed color reagent. Since under these conditions the walls of the tubing swell with subsequent decrease in rate of flow of color reagent, maintaining the rate of flow of the color reagent at a constant value is one of the most troublesome aspects of the AutoAnalyzer-N-24 method. It is necessary to check the flow rates at the beginning and end of a run and with new tubing to make allowances for the decrease in flow rate which occurs after one or two hours. Since there is considerable variation from one batch of tubing to another, it is necessary to test each new batch.

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