Cholesterol Determinations in Serum

A RAPID DIRECT METHOD

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SUMMARY

When cholesterol, dissolved in acetic acid, is mixed with premixed ferric chloride reagent and heated at 50°C for 15 minutes, a bright pink colour forms which is stable for 1 hour. The reagent contains acetic acid, sulphuric acid, ferric chloride and phosphoric acid, and is stable for years. Sterols extracted from saponified serum by petroleum ether produce an absorbance directly proportional to the cholesterol present. When whole serum is dissolved in acetic acid and treated with the reagent, the absorbance of the solution is equal to the sum of the absorbance due to cholesterol plus an amount due to other serum components which is constant in healthy persons. By using two reference sera to set up the linear standard curve of absorbance versus cholesterol concentration, a rapid precise method for serum cholesterol is obtained, applicable to healthy persons. The results of this direct method were well in agreement with those from established methods.

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The need for an accurate, rapid method for determining total cholesterol has existed for many years. Direct methods in use[1-3] have several disadvantages, e.g. accurate temperature control of the colour-developing process is impossible; most procedures are very time-consuming; the colour reagent is unstable and must be freshly prepared prior to each batch of determinations.

In most of the direct methods, the elevated temperature required to drive the colour formation is produced by adding sulphuric acid to a tube containing acetic acid. This procedure of increasing the temperature is extremely poor because it cannot be precisely controlled.

In the present investigation the ferric chloride reagent, introduced by Zlatkis *et al.*[1] was modified to overcome the above disadvantages. The new colour reagent, consisting of sulphuric acid, phosphoric acid and ferric chloride, is premixed with acetic acid, thus avoiding the increase in temperature when the reagent is mixed with the serum sample dissolved in acetic acid. The colour development is produced by placing the tube, after all the ingredients have been thoroughly mixed, in a thermostatically controlled waterbath for a predetermined time.

The direct method of determining cholesterol in serum consists of mixing 20 μL of serum with 1 ml of acetic acid, adding 5 ml of colour reagent, heating at 50°C for 15 minutes and measuring the absorbance. Part of the absorbance in this method is due to substances other than cholesterol. Correction for this extra absorbance is made by using serum samples of known cholesterol content as standards. A second method is described where the serum is first saponified. The unsaponifiable fraction of serum lipids is subsequently isolated, dissolved in acetic acid and subjected to the same colour-developing process. With this procedure the colour is caused by cholesterol alone and its accuracy is similar to that of the K5 modification[4] of Abell *et al.*'s method.[5] Compared to methods[4-6] using Liebermann-Burchard reagent, our procedures have the advantage that the colour reagent is stable for years, the final colour intensity is constant for at least an hour, the sensitivity is much higher and the volume of serum required much smaller.

MATERIALS AND METHODS

Reagents

All chemicals used were of the highest quality.

Ferric chloride solution: Two and a half grams of $FeCl_3.6H_2O$ were dissolved in 85% phosphoric acid and diluted to 100 ml with phosphoric acid.

Premixed ferric chloride colour reagent: Acetic acid, 25 volumes, and sulphuric acid, 25 volumes, were mixed and cooled to about 50°C. Two volumes of the ferric chloride solution in phosphoric acid were added with mixing. For convenience and safety the technique used was as follows: A 200-ml portion of glacial acetic was placed in a 500-ml polyethylene flask equipped with a stopper. A 200-ml portion of concentrated sulphuric acid was added. The flask was stoppered and mixed by inverting a few times. When the temperature began to rise the pressure was released by loosening the stopper momentarily and the flask was cooled in cold water. Holding the stopper firmly, the flask was shaken for 10 seconds and cooled, the process being repeated a few times until the temperature had fallen below 50°C. The mixture was transferred to a glass-stoppered bottle, mixed thoroughly with 16 ml of ferric chloride solution and allowed to stand until the air bubbles had escaped.

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Reference standards: Two reference sera are used for standardisation of the direct method, one with a relatively low cholesterol content, and the other high in cholesterol. Portions of serum are distributed into 4-ml screw-cap vials and stored at −20°C.

In this condition serum was found to give consistent values for more than 2 years. Individual vials are taken from the freezer when needed and the serum used for as long as 1 week if stored at 4°C. When the volume of serum in a vial was reduced to about one-third, the remainder was discarded because of the danger of a change in concentration due to evaporation.

The cholesterol concentrations of the 2 reference sera were repeatedly determined by the saponification procedure described below or by some other reliable method which includes saponification.[4-6]

Cholesterol standard: The stock cholesterol standard is made by dissolving 240 mg of pure crystalline cholesterol in 1 000 ml of redistilled absolute ethanol. To prepare the working standard, a selected volume of stock standard is diluted with exactly 4 volumes of ethanol to give a concentration of 48 μg of cholesterol/ml.

Apparatus

A type MDD-20 self-adjusting pipette was used (Microchemical Specialties Co., Berkeley, California, USA). Two syringe pipettes were adjusted to deliver 1 and 5 ml, respectively. The volumes delivered by the pipette must be reproducible within about 0,010 ml, but the exact capacities are not important. A motor-driven test tube shaker or buzzer was used. The most convenient shaker used was the Vortex Junior, made by Scientific Industries Inc., Springfield, Massachusetts, USA. Very vigorous shaking by hand will do if the number of samples is small.

A colorimeter or spectrophotometer with matched tubes (the procedure described is for volumes of 6 ml in tubes about 20 mm in diameter) and a waterbath with a rack holding about 60 tubes, were used.

Serum Samples

Blood samples were collected either by venepuncture or from the ear lobe or finger-tip into centrifuge tubes or capillaries (75,0 × 2,0 mm).

After the blood had clotted the tubes were centrifuged. When capillary tubes are used they are sealed at one end before centrifuging, and after centrifuging they are broken above the sedimented cells, the tip of the capillary is brought into contact with the tip of the self-adjusting pipette which fills automatically.

Direct Procedure

The samples of serum and reference standards (duplicate), were measured into colorimeter tubes using a single 0,020-ml pipette, which delivered precisely reproducible volumes. Two empty tubes were used to which the reagents were added to serve as blanks. One millilitre of acetic acid was added to each tube with the small syringe pipette. The tube was immediately shaken by the buzzer until the precipitate which first formed was dissolved. Five millilitres of colour reagent was then added to each tube with a syringe pipette. It was again shaken vigorously for 30 seconds and transferred to the waterbath rack. The rack was placed in the waterbath at 50°C for 15 minutes to develop the colour. Fifteen minutes after removal from the waterbath, the absorbance was read in a colorimeter at 550 nm wavelength, after the zero of the instrument had been set with a reagent blank.

A standard curve was prepared by plotting the absorbance values of the two reference serum samples against their cholesterol concentration (in mg/100 ml) and drawing a straight line through these points. From the absorbance of each unknown, using this standard curve, the cholesterol concentration was read. Alternatively the concentration may be calculated as follows: Let A be the absorbance of a sample and C the concentration of cholesterol in the corresponding serum. Let subscripts h and l indicate the high reference serum and low reference serum, respectively. The slope of the calibration curve obtained from the difference in absorbance of the two reference samples was:

$$s = \frac{C_h - C_l}{A_h - A_l}$$

From the absorbance of a reference serum (either A_h or A_l) the intercept of the calibration curve was:

$$i = C_l - sA_l$$

For each unknown serum the real cholesterol concentration was: $C = sA + i$. Starting with separated serum, one person can analyse 25 samples in duplicate in 2½ hours by this direct method.

Standardisation Procedure Including Saponification

To obtain the cholesterol concentration of the reference sera, 0,020-ml quantities (measured from a pipette calibrated by weighing the serum delivered) were saponified and the cholesterol was extracted with petroleum ether according to the modification of the Abell method described below.[5] This modification is a half scale version of the K5 method of Anderson *et al.*[4] with minor alterations. To the 0,020-ml serum sample in a glass-stoppered tube 1 ml of ethanol is added. For standards 1-ml portions of working cholesterol standard solution containing 48 μg of cholesterol per ml were dispensed into similar tubes using syringe pipettes calibrated by weighing water. For blanks, 1-ml portions of ethano. were used. To each tube was added 0,10 ml of 33% aqueous KOH. The tubes are stoppered, swirled gently, and heated in a 40°C airbath for 90 minutes. After removal from the airbath 0,50 ml of water and 3 ml of petroleum ether (boiling range about 60° to 80°C) were added to each tube, the stopper wetted with water and inserted firmly, and the tube shaken vigorously for 60 seconds. After the layers had separated, most of the upper layer was removed

by a capillary pipette and transferred without loss to a tube which was one of a matched set fitting the colorimeter. It is permissible to leave as much as 0,20 ml of the upper layer behind but it is not permissible to transfer any of the lower layer. After the transfer the pipette was rinsed inside and outside with petroleum ether adding the rinsings to the main portion. Three millilitres more of petroleum ether were added and the extraction repeated. A third similar extraction was made to insure complete recovery of cholesterol. The petroleum ether was evaporated by a vigorous stream of air or nitrogen blowing out of a small tube inserted into the neck of the colorimeter tube. Heating the tube by placing an electric light bulb near it will speed up the process, but this is not necessary.

When the petroleum ether was completely evaporated the cholesterol was dissolved in 1 ml of acetic acid by shaking vigorously for about 1 minute with the buzzer, allowing it to stand for 15 minutes and shaking again in the same way. All of the surface which was wet by petroleum ether should be washed by acetic acid. Colour development and reading were done as described above for the direct procedure. The true cholesterol concentrations of the reference sera were calculated on the basis that the cholesterol present is directly proportional to the absorbance (after subtracting the blank value).

RESULTS

Absorbance Curves

The absorption spectrum of the coloured product formed by cholesterol with the colour reagent was measured and is shown in Fig. 1. Measured quantities of cholesterol standard solution were placed in colorimeter tubes, the solvent was evaporated, the cholesterol was dissolved in 1 ml of acetic acid and the colour was developed as described. The absorbance was found to be strictly proportional to the amount of cholesterol up to 120 μg per tube which corresponds to a serum concentration of 600 mg/100 ml.

The absorbance curves for the coloured products formed with 0,020 ml of a serum sample containing 36,60 μg of cholesterol and with an equal amount of pure cholesterol are shown in Fig. 1. The solutions were rose-red in colour and free from turbidity. The wave length of maximum absorbance was 550 nm for both curves. The absorbance for this particular serum with these reagents at 550 nm was greater than that for the pure cholesterol by an amount equivalent to 12,30 μg of cholesterol.

Ratio of Acetic Acid to Sulphuric Acid

Equal portions, 0,020 ml each, of a serum standard were treated by the direct method, except that the volume

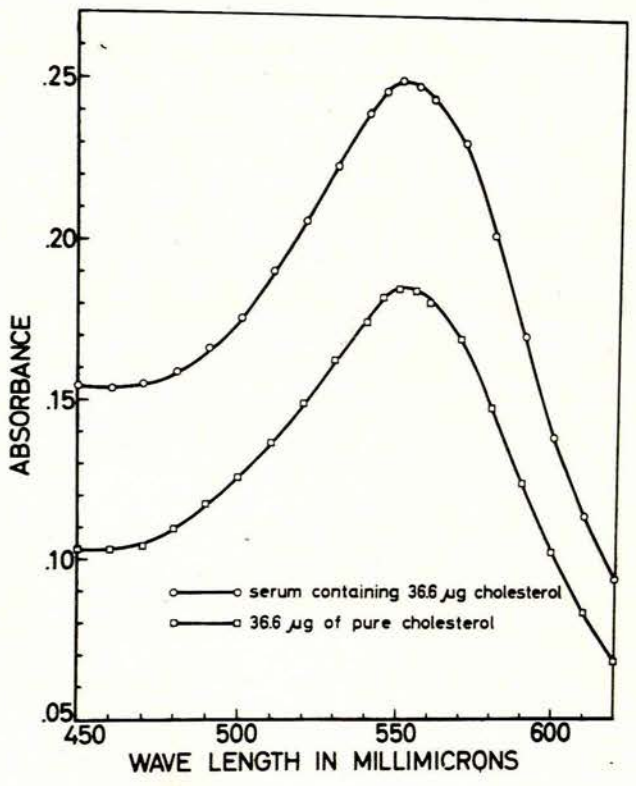

Fig. 1. Absorbance curves for the colour formed by the direct method with serum and with an amount of pure cholesterol equal to that in the serum. The measurements were made in matched 20-mm tubes with a Coleman Junior spectrophotometer.

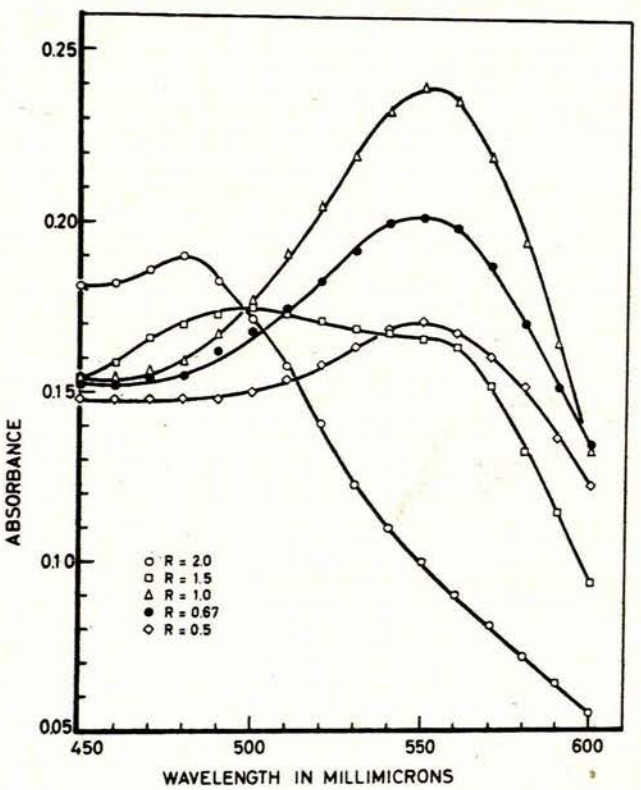

Fig. 2. Absorbance curves of serum treated by the direct method using colour reagents with various values of the ratio (R) of acetic acid to sulphuric acid. The normal value of R is 1.

ratio (R) of acetic acid to sulphuric acid in the colour reagent was changed systematically. Absorbance curves of these samples are given in Fig. 2. The normal ratio (R = 1) gave a higher absorbance peak at 550 nm than any other ratio. When the ratio was increased to 1,5 or 2,0 a second absorbance peak appeared at about 480 nm. The colour of this solution resembled the salmon-pink colour obtained in the method of Searcy *et al.*[2] for which the ratio (R) is 3. With ratios of one or smaller, the colours produced gave maximum absorbance peaks at 550 nm.

When the phosphoric acid concentration in the colour reagent was gradually increased (Fig. 3) while the $FeCl_3.6H_2O$ concentration was kept constant at 100 mg/100 ml, the colour intensity increased with increasing phosphoric acid concentration reaching a maximum at about 2 to 3 ml of phosphoric acid per 100 ml of reagent. With higher concentrations of phosphoric acid the colour intensity decreased. With a simultaneous increase in the phosphoric and iron concentrations, the colour intensity increased, reaching a maximum at 100 mg of $FeCl_3.H_2O$ and 4 ml of phosphoric acid per 100 ml of reagent. This relationship is shown in two different ways in Figs 3 and 4.

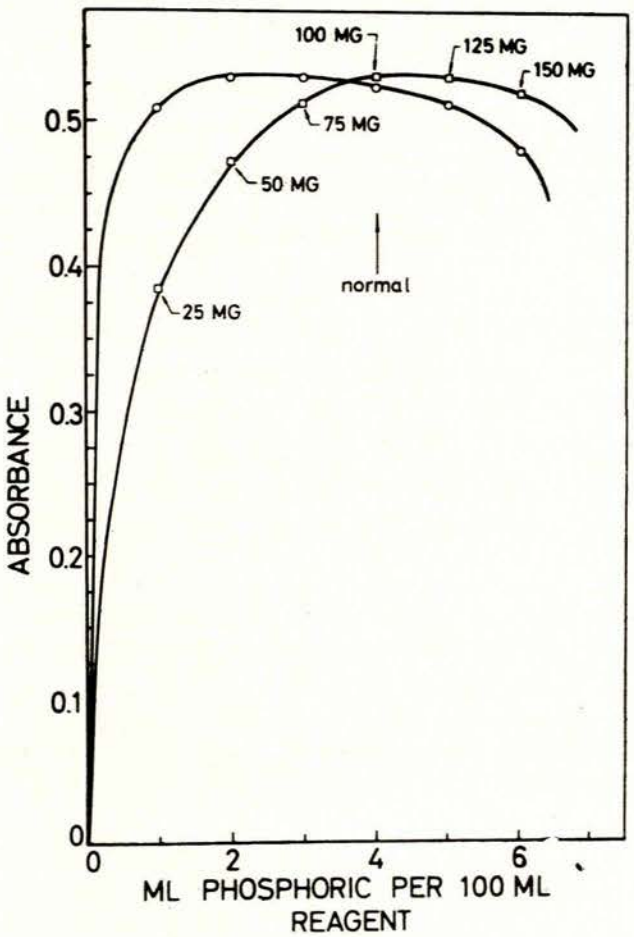

Fig. 3. The effect of changing phosphoric acid with and without a simultaneous change in ferric chloride concentration in the colour reagent. ○ = $FeCl_3.6H_2O$ constant (100 mg/ml); □ = changing concentration of $FeCl_3.6H_2O$. The amount of the iron salt added to 100 ml of reagent is given for each point.

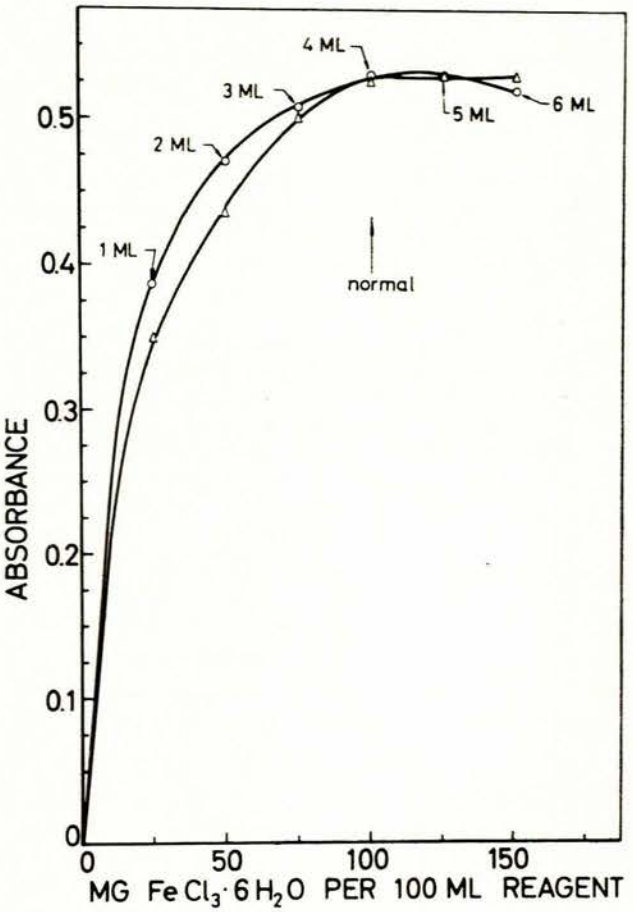

Fig. 4. The effect of changing the ferric chloride concentration in the colour reagent with and without a simultaneous change in phosphoric acid. △ = phosphoric acid constant (4 ml/100 ml reagent); ○ = changing phosphoric acid. The volume of phosphoric added to 100 ml of reagent is given for each point.

Influence of Changing Phosphoric Acid and Iron Concentrations on Colour Produced

When only the $FeCl_3.6H_2O$ concentration was increased (phosphoric acid constant at 4 ml/100 ml) the colour intensity increased, as shown in Fig. 4, to a maximum with 100 mg of ferric chloride per 100 ml of reagent. Higher concentrations of $FeCl_3.6H_2O$ within the concentrations tested had no further influence. These tests show that the concentration of phosphoric acid in the normal colour reagent (about 4 ml/100 ml) and the concentration of iron (about 100 mg $FeCl_3.6H_2O$ per 100 ml) give a colour intensity close to the maximum.

Effect of Water on Colour Development

The normal reagent was compared with the same reagent to which either 2,5% or 5,0% of water had been added in the analyses of 7 serum samples and of pure cholesterol. The results are given in Fig. 5. Each 2,5% of water decreased the absorbance due to cholesterol by about 9%. When still more water was added to the reagent, the colour intensity was further decreased and the time required to reach maximum intensity at 50°C was prolonged beyond 15 minutes.

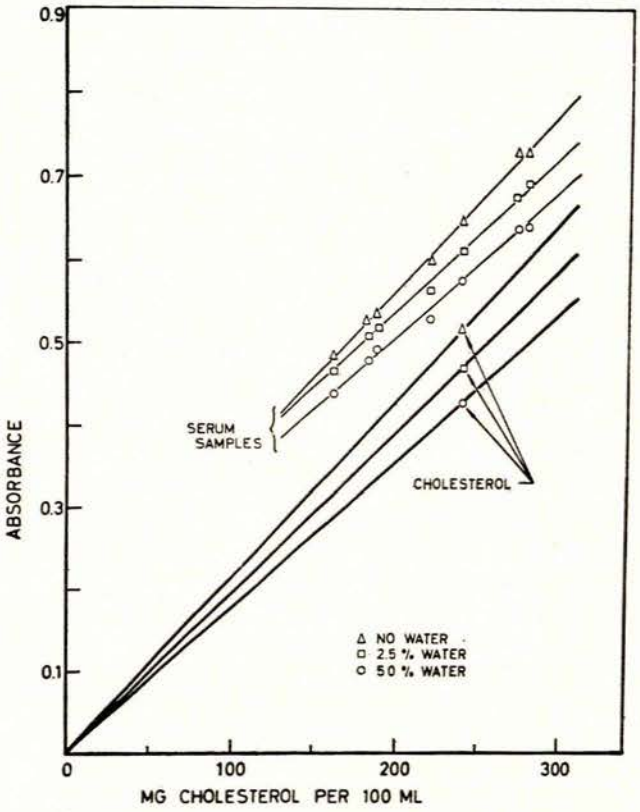

Fig. 5. The effect of water content of the colour reagent on the standard curve (least square lines for 7 analysed serum samples and lines for pure cholesterol).

Effect of Time and Temperature on Colour Development

The effects of time and temperature on colour development were studied using tubes prepared with reference plasma to which acetic acid and colour reagent were added as usual. The tubes were heated at selected temperatures (40° - 80°C) for selected lengths of time (5 - 30 minutes), and cooled in air for 3 minutes before reading the absorbance. At 40°C the time required to reach maximum absorbance was at least 20 minutes. At 50°C the absorbance reached a maximum in 15 minutes and remained unchanged for at least 15 minutes longer at this temperature. At 60°C the maximum absorbance was reached in 10 minutes and was 0,70% higher than the maximum reached at 50°C. Continued heating at 60°C resulted in a uniform decrease in absorbance which amounted to 0,70% in 15 minutes. At 80°C the absorbance reached a maximum in about 5 minutes, which was 1,0% higher than the 50°C maximum. Continuing at 80°C caused a rapid decrease in absorbance of 14% in 25 minutes. The most useful combination of time and temperature seems to be that described in the procedure, 15 minutes at 50°C. Once the colour is formed, the tubes can be kept at room temperature for at least an hour without detectable change in absorbance.

Reproducibility of the Direct Technique

Eight serum samples which had been carefully and repeatedly analysed by the K5 modification of the Abell method, were also analysed on 6 different occasions with 3 different batches of colour reagent by the direct procedure. Using 2 of the 8 serum samples as reference standards, the cholesterol content of the other 6 were calculated. The results are shown in Table I.

TABLE I. RESULTS OF 6 REPEATED DUPLICATE DETERMINATIONS OF CHOLESTEROL IN 6 NORMAL SERA BY THE DIRECT METHOD. THE STANDARDS WERE TWO OTHER NORMAL SERA WHICH BY THE K5 METHOD CONTAINED CHOLESTEROL 183 AND 274 mg/100 ml

	Direct values (mg/100 ml)							Mean K5 value
Sample	1	2	3	4	5	6	Mean	(mg/100 ml)
1	159	158	159	158	152	159	158	152
2	179	177	177	179	176	176	177	182
3	186	190	185	187	188	189	188	187
4	216	216	221	221	212	212	216	222
5	231	232	234	230	232	229	231	240
6	280	277	283	267	276	281	278	280

The mean values agree reasonably well with the K5 mean values. The error of the method, calculated as the pooled standard deviation between occasions, was 3,3 mg/100 ml.

Reliability of the Direct and Saponification Procedures

To test the reliability of the new procedures, the cholesterol concentrations of 30 serum samples which had been analysed by the K5 method, were carefully determined by the direct and by the saponification method. The means of the duplicate determinations are given in Table II. If the K5 value is accepted as the correct cholesterol value for each sample, the direct method gave values which on the average were higher by 0,3 mg/100 ml and the standard deviation of the differences was 5,5 mg/100 ml. Compared in the same way, the saponifi-

cation method gave results lower on the average by 1,7 mg/100 ml, and the standard deviation of the differences was 3,9 mg/100 ml.

TABLE II. COMPARISON OF CHOLESTEROL VALUES, MEANS OF DUPLICATE (mg/100 ml) OF 30 SERUM SAMPLES BY THREE METHODS

Sample No.	Direct method	Saponification method	K5 method
1	236	244	242
2	140	139	140
3	232	229	237
4	179	176	177
5	156	154	149
6	268	270	272
7	82	86	90
8	120	122	126
9	86	92	95
10	134	128	135
11	183	177	178
12	164	160	161
13	147	138	140
14	140	135	135
15	237	234	244
16	157	152	158
17	127	117	120
18	158	149	155
19	69	80	77
20	123	125	120
21	96	96	94
22	116	115	113
23	178	172	168
24	147	140	140
25	123	124	123
26	118	112	113
27	224	222	229
28	165	164	170
29	109	107	110
30	121	116	116
Mean	151,2	149,2	150,9

Further evidence about the reliability of the direct method was obtained using 167 serum samples, ranging from 0 to 332 mg/100 ml in cholesterol concentration and analysed by both the direct and the K5 methods. The mean of the differences between the 2 methods was 0,54 mg/100 ml and the standard deviation of the differences was 6,10 mg. The greatest difference was 15 mg.

Effect of Fatty Acids

Rhodes[2] reported that esters and glycerides of unsaturated fatty acids give a colour with a ferric chloride reagent. The possibility that part of the colour in the direct method might be caused by fatty acids was therefore tested. Quantities of oil similar to the amount of triglycerides in a typical serum sample were added to 1 ml of acetic acid and treated by the usual colour-development procedure. Corn oil, coconut oil, olive oil, and menhaden oil were tested and all the tubes gave more than 99% transmission of light. It is concluded that under the conditions of the method, fatty acids do not contribute to the colour.

DISCUSSION

The method described for forming a coloured derivative of cholesterol is very similar to the modification of Rosenthal *et al.*[3] of the method described by Zlatkis *et al.*[1] These authors make use of the heat liberated when sulphuric acid is mixed with acetic acid to raise the temperature of the reaction mixture and bring the colour development to completion. We suspected that this method of colour development was liable to cause erratic results because the temperature was not well controlled. Room temperature, speed of adding and mixing reagents, and size and shape of containers[3] are among the factors which affect the course of the temperature and consequently the progress of colour development. Experiments showed that a mixture of acetic acid, sulphuric acid, phosphoric acid and ferric chloride can be prepared which when mixed with cholesterol (dissolved in acetic acid) produces an intense pink colour if the temperature is properly adjusted. The final mixing of reagents causes no release of heat and the temperature is easily controlled by use of a waterbath.

The intensity of the colour formed from a given amount of cholesterol was found to depend mainly on the ratio of acetic to sulphuric acid. The greatest sensitivity is at a volume ratio of 1:1 in the colour reagent, or 3,4 ml to 2,4 ml in the final mixture of 6 ml total. The addition of phosphoric acid (0,2 ml per 6 ml final volume) further increase the intensity of the colour. It also results in a colour reagent which remains perfectly clear even after standing for a long time. This premixed ferric chloride colour reagent gives colour intensities suitable for photometric measurement, with amounts of cholesterol ranging from 5 to 120 μg when the final volume is 6 ml in a tube of about 20 mm diameter.

The colour developed is completely stable for 60 minutes at room temperature and for at least 30 minutes at 60°C when the procedure is strictly applied. After this the colour intensity falls off at a rate of about 1,5% per hour if the tubes are exposed to room light from fluorescent lamps.

Some people object to using samples as small as 0,020 ml because they fear that the error of pipetting will be excessive. It has been the practice in this laboratory to buy self-adjusting pipettes calibrated by the manufacturer to contain 0,020 ml. The analyst is required to standardise the pipette by weighing it (1) after rinsing with dichromate cleaning solution, water and acetone and drying by aspiration of air; (2) filled with distilled water at a known temperature; (3) filled with serum at the same temperature; and (4) after blowing out the serum as completely as possible and removing all the droplets from the tip by touching it repeatedly to a clean glass surface. After steps (3) and (4) have been repeated enough times to give results consistent to 0,10 mg there is no longer any

reservation about the accuracy. The volume contained at 20°C is calculated from the weight and temperature of the water contained as described in textbooks of quantitative analysis.[9] The fraction of serum delivered is calculated from the weights. This fraction multiplied by the volume contained gives the volume of serum delivered at 20°C. For routine analytical work, in which batches of 50 or more samples are analysed day after day, reference sera offer advantages over a cholesterol standard. The practice in this laboratory is to make a pool of 200 - 300 ml of serum, distribute it into 3-ml portions in 4-ml screw-cap vials and store them at −20°C. One vial is opened each Monday and it is usually enough to serve as reference for a week, during which time it is stored at 4°C.

It is recommended that the low and high serum standards should have cholesterol concentrations of approximately 100 and 300 mg/100 ml, respectively. The greater the difference in cholesterol concentration of the reference sera the less will be the change in the slope of the standard curve introduced by small errors in readings. When the direct technique is used for cholesterol determinations in sera of species other than man, the serum of that particular species should be used for reference standards.

The possibility of error in the direct method due to colour formation by unsaturated fatty acids or their esters seems to be ruled out by the trials with added oils. The observed results are not inconsistent with the observation of Rhodes[7] that glycerides of unsaturated fatty acids when treated with a similar colour reagent gave weak absorbance at wavelengths between 440 and 490 nm.

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