An evaluation of cholesterol determinations in serum and serum lipoprotein fractions by a semi-automated fluorimetric method

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SYNOPSIS A sensitive automated fluorimetric method for the estimation of cholesterol is described which is suitable for the routine determination of both serum and serum lipoprotein cholesterol. Analyses can be performed at 60/hour in the serum range of 5 mg to 400 mg/100 ml. The method shows a high degree of analytical precision and good yields from recovery experiments.

We describe a cholesterol method which involves the measurement of the stable fluorescent product of a Liebermann-Burchard reaction: the cholesterol extract mixed with trichloroethane, acetic anhydride, and concentrated sulphuric acid is excited at 546 m μ and fluoresces at 590 m μ . Originally described by Albers and Lowry (1955), the method was modified by McDougall and Farmer (1957) and Carpenter, Gotsis, and Hegsted (1957). Carpenter's method was subsequently automated by Antonis (1964). The automated technique does not appear to have been evaluated either for measuring serum or the lipoprotein fraction cholesterol.

This paper describes modifications of the automated method and evaluates the measurement of cholesterol in serum and serum lipoprotein fractions. Cholesterol concentrations in the range 5 mg-150 mg/100 ml in lipoprotein fractions and up to 400 mg/100 ml in serum may be measured under similar analytical conditions, at a sampling rate of 60 per hour.

Materials and Method

REAGENT

The reagent is made immediately before use by mixing 400 ml of redistilled 1, 1, 2-trichloroethane¹ and 80 ml of analytical reagent grade

¹Kodak, Ltd, Kirkby, Liverpool.

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acetic anhydride, cooling the mixture to -15°C and then adding 20 ml of concentrated sulphuric acid.

STANDARDS

Working cholesterol standards in the range of 20 mg/100 ml to 400 mg/100 ml are prepared in analytical grade chloroform and extracted as described for serum according to Bragdon (1960).

SAMPLE PREPARATION

Serum

One millilitre of serum is extracted into 24 ml of a 2:1 chloroform-methanol mixture (Bragdon, 1960). The extraction mixture is washed with 0.1N sulphuric acid. The lower chloroform layer of the resultant two-phase system contains all the lipid in 16 ml and is used for analysis.

Separation of lipoprotein fractions

Very low density (Sf 12-400), low density (Sf 0-12), and high density lipoproteins are separated sequentially from chylomicron-free serum in a Beckman preparative ultracentrifuge in graded-density salt solutions, by a method essentially that of Havel, Eder, and Bragdon (1955).

Three millilitres of each fraction is extracted into chloroform-methanol as already described.

Fig. 1 Manifold and flow diagram.

Automated procedure

The automated system consists of a Technicon AutoAnalyzer sampler II and proportioning pump; a 25°C heating bath containing a 40 ft delay coil, a Locarte filter fluorimeter fitted with a flow cell, and, a 10 mV potentiometric recorder (Fig. 1). The primary filters activate at 546 m μ (Corning filters 4010 and 5120) and emission is measured at 590 m μ (Corning filter 2424). Glass sample cups are used. Results are interpreted from a standard curve which is linear up to 400 mg/100 ml and corrections are made for the dilutions involved. Commercially prepared control sera^{1,2} are included in each batch of analyses.

Results

A straight-line relationship is obtained with standard cholesterol in the range 20-400 mg/100 ml; a chart record showing a duplicated standard curve and typical sample peaks is shown in Figure 2.

This method shows a high degree of reproducibility and precision and a satisfactory degree of correlation was obtained in a comparison with the manual method previously used in this laboratory (Henley, 1957); 116 serum samples were analysed and compared with results obtained on the same samples by the manual method of Henley (1957). The distribution and correlation coefficient (r) are shown in Fig. 3, where $r = 0.980$ ($p < 0.001$). The respective mean values obtained by the manual and automated methods were 186 mg and 182 mg/100 ml. There was no significant difference between the results from the two methods at any level. The within-method variance was the

¹Hyland-Baxter Ltd, Thetford, Norfolk.

²William R. Warner Ltd, Eastleigh, Hants.

Fig. 2 Recording of (a) standard curve, (b) duplicate standards and (c) replicate samples.

Fig. 3 Correlation between manual and automated method.

same as the variance between them as calculated by the F test. The standard error of the mean of replicated samples was 0.6. Recovery of added cholesterol from serum averaged 97% (93 to 99%) over the range 40 to 370 mg/100 ml.

Total cholesterol and lipoprotein fraction cholesterol were measured on 29 serum samples. The results in mg/100 ml and the percentage recoveries (Table) show good agreement between total serum cholesterol and the sum of the individual fractions.

Subject ¹	Date	Serum Cholesterol (mg/100 ml)				Sum of Fractions	Recovery (%)
		Total	VLD	LD	HD		
W.R.	15.5. 1968	164	27	87	50	164	100
D.C.	16.5	208	26	111	38	175	84
K.D.	16.5	184	12	110	43	165	90
	17.5	162	9	102	39	150	93
A.M.	16.5	130	16	87	30	133	102
	17.5	124	17	73	29	119	96
W.D.	30.5	191	11	118	41	170	89
	31.5	174	8	114	42	164	94
J.P.	30.5	172	67	73	25	165	96
	31.5	168	65	70	23	158	94
B.O'K.	27.6	162	22	78	50	150	93
	28.6	144	18	78	43	139	97
K.F.	22.8	140	16	90	42	148	106
	23.8	148	16	88	41	145	98
W.L.	30.9	632	540	60	14	614	97
	3.10	532	464	60	27	551	104
J.A.	7.10	124	13	77	40	130	105
	8.10	138	21	75	37	133	96
F.D.	7.10	154	68	38	14	150	98
C. de H.	7.10	182	20	95	69	184	101
D.P.	7.10	142	9	88	50	147	104
M.T.	7.10	424	47	328	45	420	99
	8.10	430	43	340	44	427	99
E.T.	7.10	224	21	167	45	233	104
	8.10	236	25	174	48	247	105
I.E.	9.10	154	19	73	59	151	98
	10.10	154	17	71	59	147	96
D.S.	9.10	287	83	167	61	311	108
Mean recovery							98.1%

Table Serum cholesterol and serum lipoprotein fractions

¹VLD, LD, HD = very low, low and high density lipoprotein respectively

Discussion

Current interest in atherosclerosis and the lipaemias has heightened the demand for precise measurement not only of serum cholesterol, but also for measurement of the cholesterol, triglyceride, and phospholipid fractions that comprise the lipid moiety of the lipoproteins.

This cholesterol method provides a high degree of sensitivity essential for accurate measurement of low cholesterol concentrations and is fairly specific. For, although under the conditions of this assay a mixture of products is formed, evidence for which is shown by the intense emission of blue fluorescence when excited with 365 m μ light, in fact, activation at 546 m μ and measurement of the red-orange fluorescence (590 m μ) gives high specificity for cholesterol (Albers and Lowry, 1955). With the automated system described and using a sampler II, a back reservoir is not required, and there is an effective gain in sampling rate, 60 an hour compared with 30 an hour. The 1 : 2 sample-to-wash ratio provides a good wash pattern without carry-over, which increases the sensitivity in the lipoprotein fraction assays and also diminishes the sample volume required.

Chloroform is chosen as the extracting medium for several reasons. The sample extract can be used directly for analysis without prior need to dry extracts and reconstitute the residues in

trichloroethane. It was found, in fact, that results were more reproducible when using chloroform extracts in the reaction than when trichloroethane extracts were prepared, though care must be taken to prevent evaporation from a considerably lower boiling-point solvent.

The bromide ions in the mixed salt solutions in which the lipoprotein fractions are suspended cause considerable interference in the automated colorimetric procedure (Technicon Instrument Co., Methodology sheet no. 24). In that procedure a polar solvent extract (iso-propanol) is used from which both the lipids and the salt ions are extracted. The fluorimetric method described is particularly advantageous in being an almost totally non-aqueous system, and halide ions are eliminated at the initial extraction step.

It is important to extract the standard solutions in a similar manner to the serum samples, since the very small mutual solubility of chloroform and water alters the slope of the standard curve by about 10%.

Inherent advantages of this method are the simplified manifold and the increased rate of analysis. Our results confirm that the method is suitable for the routine analysis of serum cholesterol and the cholesterol of serum lipoprotein fractions. It matches in sensitivity the semi-automated method used for measuring glyceride-glycerol described previously from this laboratory (Cramp and Robertson, 1968).

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