APPENDIX-II, 4.

IATROSCAN INSTRUMENT APPLICATION

TLC/FID

No. 14

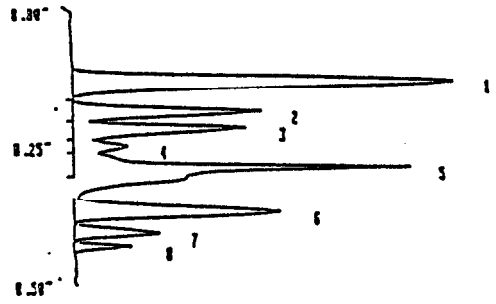
Analysis of serum lipids by the Iatroscan

Analysis of serum lipid by the Iatroscan

By means of the Iatroscan lipid in serum, namely cholesterol ester (E. Chol.), triglyceride (TG), fatty acid (FA), free cholesterol (F. Chol.), phosphatidyl choline (PC), phsphatidyl sphingomyelin (SM), lysophosphatidyl choline (Lyso-PC) can be separated and assayed simultaneously (Fig. 1).

Fig. 1 - Normal human serum lipids

SAMPLE 10 15:26 MAY 28 1987



CAL. METHOD 91

	SF	PA	PB
	.100000E+01	.100000E+01	.100000E+01

ISTD PEAK 0.197 3902 M

NO.	NAME	RT	A OR H	MK	CONC
1	CHO.E	0.142	11103	M	2.8432
2	I.S	0.197	3902	M	1.0000
3	TG	0.229	3762	M	0.9642
4	FA	0.259	1095	M	0.2808
5	CHO	0.298	6646	M	1.7032
6	PC	0.391	5295		1.3569
7	SM	0.419	1479		0.3790
8	LPC	0.441	616		0.1579
9					
	TOTAL		33902		8.6873

Compdnents:

1. Cholesterol ester
2. Internal standard (Cholestreol acetate)
3. Triglyceride
4. Fatty acid
5. Cholesterol
6. Phosphatidyl choline
7. Sphingomyelin
8. Lysophosphatidyl choline

Conditions:

Stationary phase: CHROMAROD-SIII

Mobile phase:

1st.Chloroform:Methanol:Water 40:20:2.5 4cm
2nd.Chloroform:Methanol:Water 40:20:2.5 4cm
3rd.Chloroform:Methanol:Water 40:20:2.5 4cm
4th.Hexane:Ether:Formic acid 60:5:0.5 11cm

Gas flow: H2 160mL/min, Air 2 L/min

Scanning speed: 30 sec/scan

Integrator attenuation: 16

1. Method

Quantitative analysis of serum lipids shall be made according to the following four steps:

(1) Preparation of the calibration curve

(2) Extraction of serum lipids

(3) Analysis of lipids extracted

(4) Quantitation based on the calibration curve

Details for the above are explained respectively as follows:

1.1 Preparation of the calibration curve

Flame ionization detector of the IATROSCAN varies in its sensitivity depending on substances to be detected. Therefore, it is required to provide the respective calibration curve ready from the measured peak areas and amounts of the components.

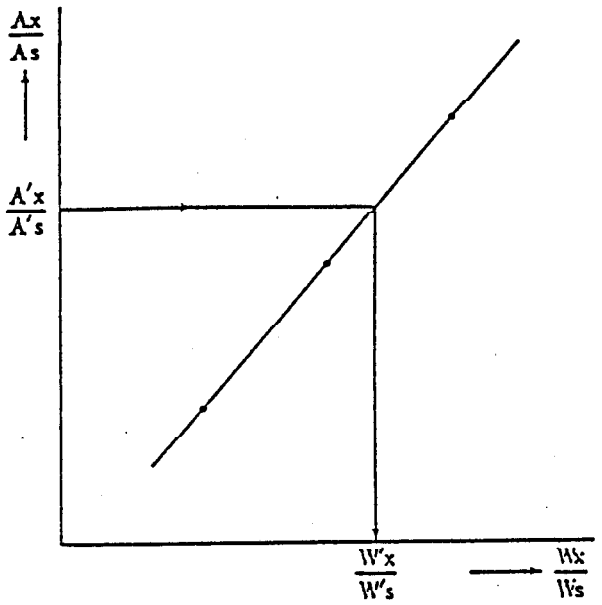
There are absolute and internal standard calibration curves, but herein serum lipid analysis is applied with the internal standard calibration curve which may be more appropriate in this particular application.

[Analysis with the internal standard calibration curve]

Record a chromatogram of the mixed sample, in which a known amount of the internal standard is added to a known amount of the pure components to be detected, and measure the respective peak area.

Take the ratio of the sample component and the internal standard in weight (Wx/Ws) along the abscissa (x-axis), and the ratio of the sample component and the internal standard in peak area (Ax/As) along the ordinate (y-axis) respectively, and prepare a calibration curve as showin in Fig. 2.

Fig. 2 - Calibration curve of the internal standard



Ax: Peak area of the component X

As: Peak area of the inner standard

Wx: Weight of the component X

Ws: Weight of the inner standard

Add a known amount of the internal standard (n) to a known amount of the sample (W) appropriately to have them within the range of the calibration curve and mix to be homogenous, then develop a chromatogram adjusting a spot to have a peak of the internal standard nearly the same height as that of the calibration curve made ready under the same conditions.

From thus made chromatogram, obtain a ratio in the peak area (A'X/A's) of the test component (A'x) and the internal standard (A's) as well as a ratio in weight (W'x/W's) of the test component (W'x) and the internal standard (W's) from the calibration curve respectively. Thus, according to the following equation the content (X) can be calculated.

$$X = \frac{(W'x/W's) \times n}{W}$$

Here is explanation to be made on the calibration curves of E. Chol., TG, F. Chol , PC, and SM prepared by applying Cholesterol Acetate (Chol. A) to the internal standard.

1.1.1. Preparation of the standard mixtures

Here is explanation to be made for preparation of the standard mixture with sample solution used in this analysis.

(1) Preparation of the internal standard component with solution.

Weight as acculately as 50mg of Cho. A by means of chemical balance, and take it by measuring pipette with Folch's solution (Chloroform : Methanol, 2 : 1) in a measuring flask of 50mℓ to make it to the internal standard component.

(2) Preparation of the original solution of standard mixtures.

Acculately weight 25mg of Cho. E, 100mg of TG, 8mg of Cho., 15mg of PC, 4mg of SM and 2.5mg of LPC by means of chemical balance, and take these by measuring pipette with Folch's solution in a measuring flask of 50mℓ. This is the standard mixture sample solution.

(3) Preparation of the standard mixture solution No. 2

Take 4mℓ of the original solution of standard mixture to which add 3mℓ of the internal standard component solution. This is the standard mixture No. 2.

(4) Preparation of the standard mixture solution No. 3

Take 1mℓ of the standard mixture solution No. 2 to which add 1mℓ of the internal standard mixture. This is the standard mixture No. 3.

The following standard lipids were used.

Code	Component	Manufacturer
Cho. E	Cholesterol palmitate	Tokyo Kasei Kogyo
Cho. A	Cholesterol acetate	Tokyo Kasei Kogyo
TG	Trilaurin	P-L Biochemicals, Inc.
Cho.	Cholesterol	Nu-Chek-Prep. Inc.
PC	L-a-phospatidyle choline (egg yolk)	Sigma
SM	Sphingomyelin (egg)	Avanti-Polar-Lipids, Inc.
LPC	Lysolecithin (egg)	Avanti-Polar-Lipids, Inc.

Note: It is recommended that each component concentration in the standard mixture solution needs to be appropriate prepared for the particular analysis.

1.1.2. Development of the standard mixtures

Analyze the standard mixtures to obtain the respective data for preparation of the calibration curves conforming to the tetramerous development method.

(1) Preparation of the developing solvents

Prepare the following two types of solvent.

Solvent A:

Chloroform : methanol : water 40 : 20 : 2.5

Solvent B:

Hexane : ether : formic acid 60 : 5 : 0.5

(2) Chromarod

Use Chromarod-SIII on the stationary phase

(3) Development

1) Chromarod-SIII blank scanning

2) Sample spot : Spot 1μℓ of the standard mixture

3) First development : Develop with solvent A to 4 cm.

4) Drying : Dry for 1 - 2 minutes by a hair dryer with cool air.

5) Second development : Develop with solvent A to 4 cm.

6) Drying : Dry for 1 - 2 minutes by a hair dryer with cool air.

7) Third development : Develop with solvent A to 4 cm.

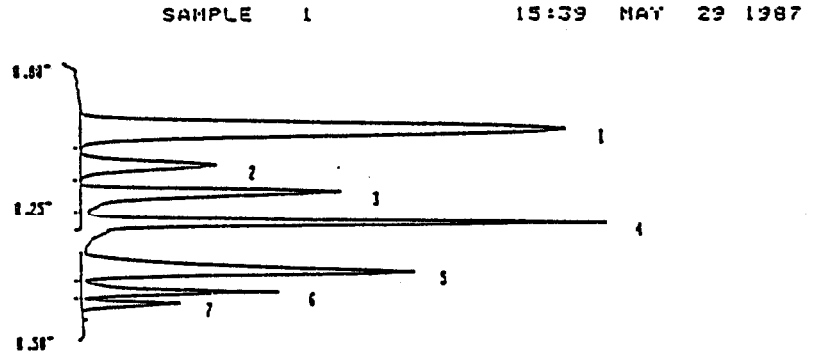
8) Drying : Dry for 1 - 2 minutes by a hair dryer with cool air.

9) Fourth development : Develop with solvent B to 11 cm.

10) Drying : Dry for 1 minute in a drying chamber at 100°C.

11) Scan to obtain chromatogram.

Fig. 3 - Analytical example by the standard mixtures



CAL. METHOD 01

		SF	FA	FB
		.100000E+01	.100000E+01	.100000E+01

ISTD	PEAK	0.133	2979	M	
NO.	NAME	RT	A OR H	MK	CONC
1	CHO.E	0.125	15973	M	5.3696
2	I.S	0.133	2979	M	1.0000
3	TG	0.233	5531	M	1.8564
4	CHO	0.293	7552	M	2.5344
5	PC	0.384	7675	M	2.5760
6	SM	0.420	2559	M	0.8557
7	LPC	0.439	1157		0.3883
	TOTAL		43420		14.5718

Components:	Conditions:
1. Cho.E	Stationary phase: CHROMAROD-SIII
2. I.S. (Cho.A)	Mobile phase:
3. TG	1st. Chloroform:Methanol:Water 40:20:2.5 4cm
4. Cho	2nd. Chloroform:Methanol:Water 40:20:2.5 4cm
5. PC	3rd. Chloroform:Methanol:Water 40:20:2.5 4cm
6. SM	4th. Hexane:Ether:Formic acid 60:5:0.5 11cm
7. LPC	Gas flow: H2 160 mL/min. Air 2 L/min
	Scanning speed: 30 sec/scan
	Integrater attenuation: 16

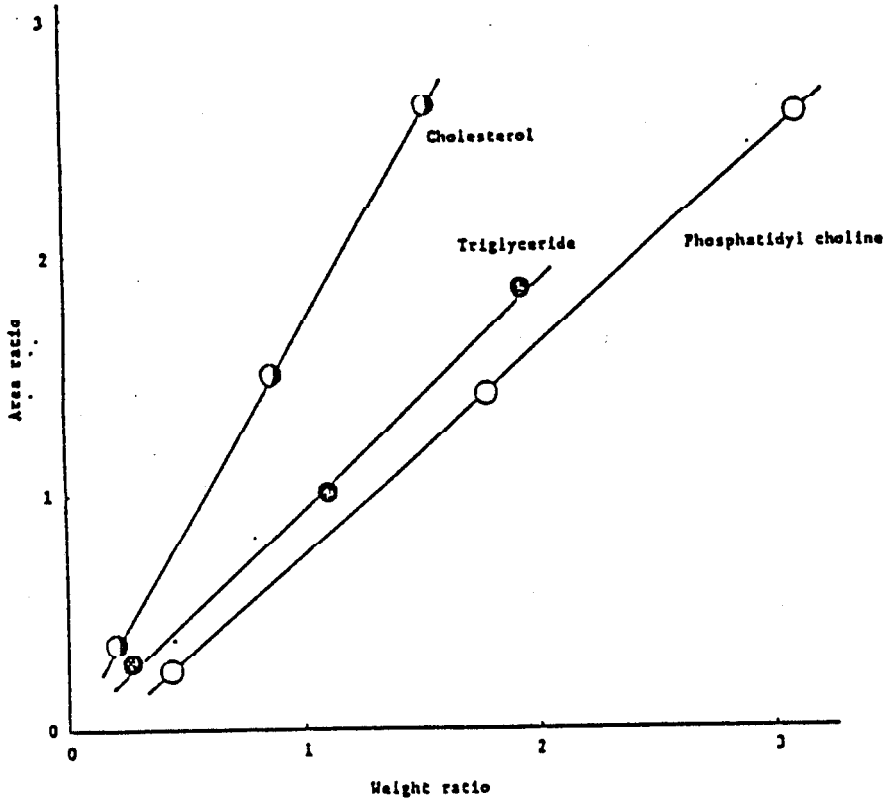
By means of this tetramerous development, polar lipids are separated by the first to third developments, and netural lipids pushed yp the solvent front is separated by the fourth development, therefore, total components can be analyzed at a time. To have the better result, all analycal conditions must be maintained particularly, amount of developing solvent, distance of development and sample amount are always kept in constant.

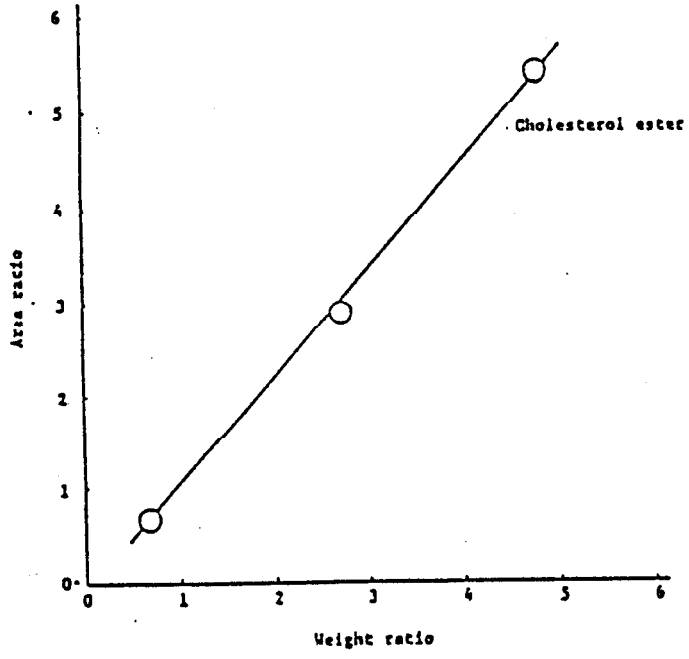
1.3 Calibration curve

From the chromatogram obtained, acquire the respective peak area ratio (Ax/As) against lipid standards and the internal standard.

On a logarithmic graph paper, draw the calibration curves having the weight ratio (Wx/Ws) of the lipid standard and the internal standard on the abscissa (x-axis) and peak area ratio (Ax/As) on the ordinate (y-axis).

Fig. 4 - Internal standard calibration curves





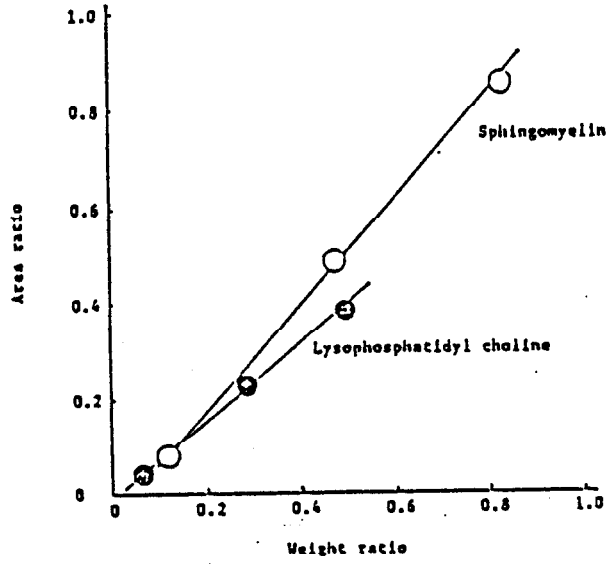


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Table 1 - Internal standard calibration data

	Standard mixture	Cho.E	TG	Cho	PC	SM	LPC
Weight ratio Ws/Ws	Original	4.78	1.94	1.54	3.12	0.83	0.49
	2	2.74	1.11	0.88	1.79	0.47	0.29
	3	0.68	0.28	0.22	0.44	0.12	0.07
Peak area raito Ax/As	Original	5.44	1.86	2.63	2.59	0.86	0.39
	2	2.91	1.01	1.50	1.42	0.49	0.23
	3	0.67	0.28	0.38	0.25	0.08	0.04

2. Extraction of serum lipids

As lipids present in serum combined with protein, sacharide, etc. as a rule and in order to analyze lipids, it is required to extract beforehand.

2.1 Extraction

The following procedures may be taken to extract serum lipids.

(1) Collect serum 0.5mℓ into a test tube with cap provided.

(2) Add Folch solution 10mℓ in the serum collected and shake it for 30 seconds by an agitator.

(3) Then filter and put the solution filtered into a test tube with cap provided.

(4) Add pure water 2mℓ in the solution filtered and shake.

(5) Then centrifuge for 10 minutes at 2,000 rpm.

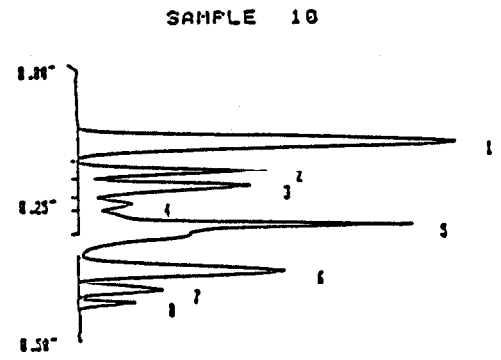
(6) Remove supernatant and evaporate the lower layer substances under reduced pressure.

(7) Dissolve the evaporated substances again with the international standard solution, 0.25mℓ.

3. Analysis of extracted lipids

Extracted lipids are analyzed completely in the same manner as that of the standard mixtures for calibration. Spot 1μℓ of extracted lipid on Chromarod-SIII, and manipulate in the same manner as that of the standard mixture. From the chromatogram thus made, a peak area ratio of each component and the internal standard is obtained.

Fig. 5 - Analytical results of normal human lipids in serum



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4th.Hexane:Ether:Formic acid 60:5:0.5 11cm

Gas flow: H2 160mL/min, Air 2 L/min

Scanning speed: 30 sec/scan

Integrator attenuation: 16

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4. *Quantitation by Calibration*

Serum lipids are separated as shown in Fig. 5, and peak area ratio (Ax/As) of each component and the internal standard is obtained as shown in the Table below.

	Cho.E	TG	Cho	PC	SM	LPC
Peak area ratio Ax/As	2.85	0.96	1.70	1.36	0.38	0.16

From the calibration curves, the weight ratio can be converted as follows:-

	Cho.E	TG	Cho	PC	SM	LPC
Weight ratio Wx/Ws	2.55	1.03	1.00	1.71	0.38	0.21

From values in the above, lipid contents in serum are calculated as follows:

[Calculation of lipid contents in serum 1dℓ.]

Content of a component in serum is with the following equation.

$$\text{Content (mg/dℓ)} = \frac{(Wx/Ws) \times \text{mass of the internal standard (mg)}}{\text{Amount of serum collected (dℓ)}}$$

In case of E. Chol,

Wx/Ws = 2.55

mass of the internal standard: 1.03mg/mℓ, 0.25mℓ,

from this 1.03 x 0.25 = 0.2575 (mg)

amount of serum: 0.5mℓ = 0.005dℓ

substitute these values into the equation,

content = 2.55 x 0.2575/0.005 = 131 (mg/dℓ)

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Content of other components can be obtained in the same manner as shown in the Table below.

(unit: mg/dl)

Cho.E	TG	Cho	PC	SM	LPC
131	53	52	88	20	11

As stated above, from the internal standard calibration method, lipids in serum can be quantitated.

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