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Quantitative Thin-Layer Chromatography with a Flame Ionization Detector for Serum Lipid Measurements in the Routine Clinical Laboratory

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Flame ionization detectors (FID) are widely used in gasliquid chromatography and are now utilized in other types of chromatography. Padly (1969) reported a method using a thin quartz rod covered with silica gel or alumina passed through the flame of a FID. In Japan, analytical procedures using a TLC-FID combination have been developed. This paper describes a method for rapid separation and identification of lipids such as cholesterol esters, free cholesterol, triglyceride and phospholipids by the TLC-FID method in view of the future development of a screening method for serum lipids, and introduces an internal standard method for quantitative analysis. In addition, the serum lipid fraction of normal male subjects with different mean ages were determined by TLC-FID.

(Key Words: Serum Lipid, Thin-Layer Chromatography, Flame Ionization Detector, Iatroscan)

INTRODUCTION

Recent interest in the variation of lipid concentrations in serum has promoted the development of rapid, accurate methods for measuring lipid fractions such as cholesterol esters, free cholesterol, triglyceride and phospholipids. There are many reports (1) (5) on the determination of serum lipid fractions, but little work (8) has been done using the IATROSCAN (Iatron, JAPAN).

Thin-layer chromatography (TLC) has many advantages over other chromatographic techniques for the determination of lipids, but quantitative evaluation of TLC had been a difficult task. Tedious analytical procedures and less precise data are the disadvantage of TLC analysis.

Cotgreave (2) and Szakasits (7) described a technique of quantitative determination in which a traveling hydrogen flame stage passes over a TLC plate, and Padley (4) reported a method using a thin quartz rod covered

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with adsorbent and passing through the hydrogen jet flame of a FID.

The idea of Padley (4) was further developed into instruments such as the IATROSCAN utilized to determine quantitatively the lipid fractions in serum.

In our work, an adsorbent coated quarz rod called a CHROMAROD (Iatron,JAPAN)was passed through the jet flame nozzles, and basic experiments were performed to determine serum lipid fractions by the instrument

The purpose of the present paper is to determine serum cholesterol esters, free cholesterol, triglyceride and phospholipids using the IATROSCAN and also to report that the method was sufficiently effective as a routine laboratory test.

METHOD

1. Principle

The main components of the apparatus are the jet flame nozzle with a traveling stage, the collector electrode, current amplifier, integral amplifier and recorder, as shown in Figure 1. The TLC-FID strip is a thin quartz rod (0.9-mm in diameter x 152-mm in length) covered with an adsorbent such as silica gel or alumina. After development and separation of the specimen on the rod, the rod is moved with constant speed in the hydrogen flame and then each fraction is burnt continuously. The ions formed in the flame are collected between the collector electrode and the burner. The signal and its integrated value are recorded automatically by a recorder

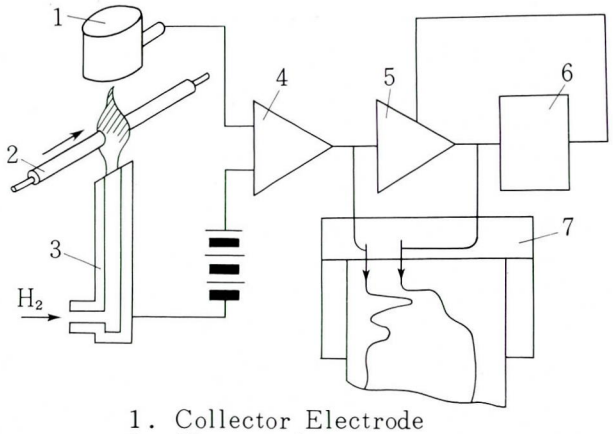
2. Apparatus and Reagents

1) FID scanner: A TLC autodetector (IATROSCAN TH-10 analyzer).
2) Folch solvent: A mixture of chloroform (Wako, JAPAN): methanol (Wako, JAPAN) = 2:1 (V/V)
3) Internal Standard Solution: Dissolve 10ml of cholesterol acetate (Tokyo Kasei, JAPAN) in 100ml of Folch solvent.
4) Developing solvent: A mixture of n-hexane (Wako, JAPAN): ethylether (Wako, JAPAN) = 9:1 (V/V)
5) Thin-Layer rod: Sintered quartz rod covered with silica gel (CHROMAROD) activated by burning with the jet flame of the FID.
6) Developing chamber: A compact cubic jar (190mm in height × 130mm in width × 48mm in depth).

3. Procedure

1) Transfer 0.25ml of serum to a glass-stopperd test tube, add 5.0ml of Folch solvent containing the internal standard and shake 30 seconds with a Thermo-Mixer.
2) Add 1.0ml of distilled water and mix for 15 seconds by hand.
3) Centrifuge at 2500rpm for 5 minutes and then remove the upper layer by an aspirator.
4) Filter the Fluff layer (white upper layer) through filter paper (Toyo No.5, 7.5cm in diameter) and wash the residue with 3.0ml of Folch

Schematic principle of TLC - FID detection method



1. Collector Electrode
2. Chromarod
3. Burner
4. Current Amplifier
5. Integral Amplifier
6. Integral Reset Circuit
7. Recorder

Fig. 1 Schematic Principle of TLC-FID detection method

solvent.

5) Collect whole filtrate and evaporate by a reduced pressure evaporater in 40°C water.
6) Redissolve in 0.25ml of Folch solvent and spot 3μl aliquots of the solution on the CHROMAROD.
7) Develop it in a developing chamber for about 30—40 minutes.
8) After developing, dry it at 110°C for 5 minutes and let the CHROMAROD run through the FID to detect each serum lipid fraction.

4. Conditions for detection

1) Hydrogen pressure to FID:1.4kg/cm²
2 Air flow-rate to FID: 2000-2500ml/min
3) Scanning speed: Gear 40T
4) Chart Speed: 240/min
5) Recorder range: 100mV

RESULTS

1. Calculations and units:

The areas under each peak were estimated with the recorder. To convert them to concentrations, the internal standard (cholesterol acetate) and the following formula were used:

$$\frac{\text{area under unknow peak}}{\text{area under internal std. peak}} = \text{IS-U (Internal Standard Unit)}$$

2. Normal serum lipid pattern:

Figure 2 shows a typical pattern obtained for the serum from a healthy individual without any abnormalities in laboratory tests. The order of larger Rf values to smaller ones was cholesterol esters, triglyceride, free cholesterol, diglyceride and phospholipids. The internal standard was separated between cholesterol esters and the triglyceride fraction. No esterified fatty acid was detected because so little was present.

3. Normal values with different mean ages:

Normal values for lipid fractions of 141 healthy male subjects with different mean ages were obtained. The subjects were classified into four age groups: 20—29, 30—39, 40—49, and 50—59. The mean values ± 1.S.D. for each lipid fraction were calculated and are shown in Table 1.

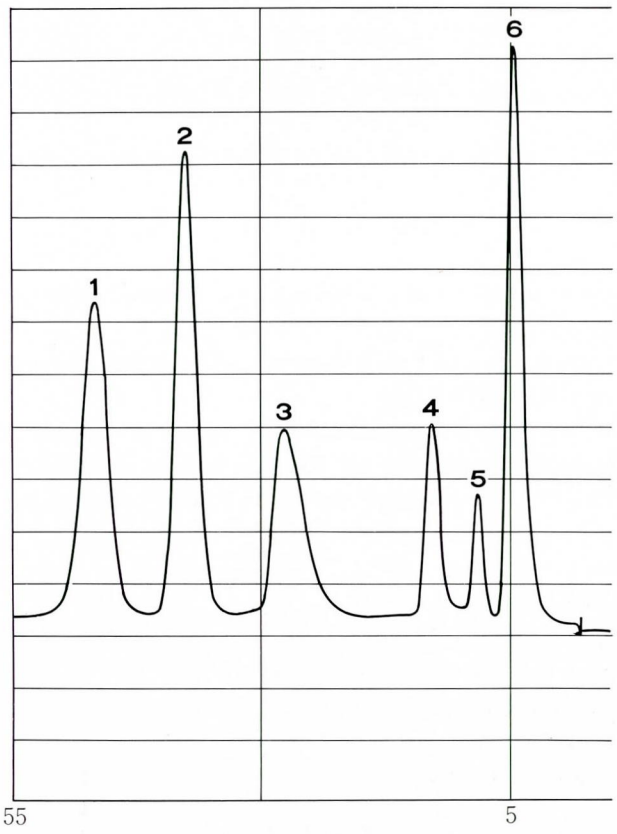


Fig. 2 Tracing of the pattern obtained from normal serum by TLC-FID

*Abbreviations in all of the Tables and Figures of this paper are as follows

1. CE: cholesterol esters
2. IS: Internal Standard
3. TG: triglyceride
4. FC: free cholesterol
5. DG: diglyceride
6. PL: phospholipids

Table 1. Normal lipid content of serum of subjects with different mean ages by TLC-FID

Age	CE	FC	TC	TG	PL	n
20–29	1.01±0.21	0.29±0.08	1.30±0.27	0.31±0.11	1.35±0.29	15
30–39	1.10±0.26	0.29±0.05	1.39±0.29	0.34±0.23	1.10±0.22	24
40–49	1.09±0.24	0.25±0.06	1.35±0.28	0.39±0.22	1.00±0.18	59
50–59	1.15±0.29	0.27±0.07	1.44±0.37	0.42±0.24	1.05±0.28	43

TC: total cholesterol (M±1.S.D.)

Table 2. Reproducibility of serum lipid fractions by TLC-FID.

Run-No.	CE	FC	TG	PL
1.	0.68	0.11	0.13	0.67
2.	0.70	0.15	0.15	0.79
3.	0.68	0.14	0.19	0.87
4.	0.57	0.17	0.18	0.81
5.	0.60	0.12	0.15	0.89
6.	0.51	0.15	0.19	0.72
7.	0.51	0.14	0.17	0.78
8.	0.54	0.16	0.12	0.99
Mean	0.60	0.14	0.16	0.82
S.D	0.07	0.02	0.02	0.09
C.V (%)	12.14	12.14	15.25	11.57

(Internal Standard Unit)

4. Reproducibility of the method:

The simultaneous reproducibility of the method on a single sample is given in Table 2. In the present method, variation may be due to inherent errors in the TLC method.

5. Correlation between the colorimetric analysis data and that obtained by IATROSCAN:

Figures 3, 4 and 5 illustrate the values found for the various fractions separated by IATROSCAN and compare them to those obtained by chemical procedures. Thirty random patient samples from a general

hospital population were assayed. The percent concentrations of IS-U obtained by IATROSCAN are on the X-asis. The results obtained by the colorimetry are on the Y-axis. As indicated in Fig. 3, a linear relationship was found between the Liebermann-Burchard method and IATROSCAN for choresterol ester determination. In case of triglyceride, the regression equation was $Y = 165.1x + 27.4$, and the correlation coefficient was $r = 0.861$, as seen in Fig. 4. Fig. 5 showns the correlation between the colorimetric method and IATROSCAN for phospholipid determination. The regression equation was $Y = 88.73x + 93.22$, and the correlation coefficient was $r = 0.878$.

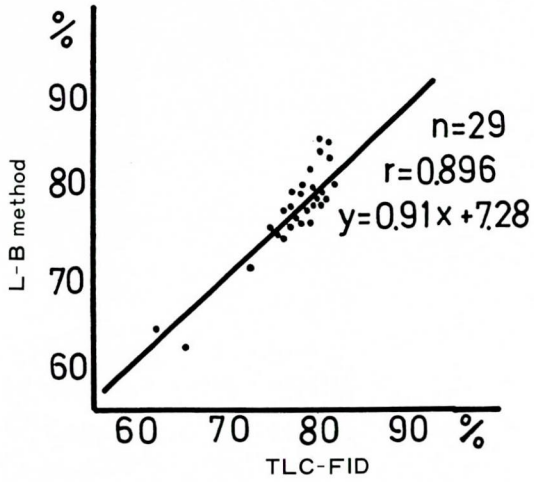


Fig. 3 Correlation between TLC-FID and the L-B method for cholesterol esters

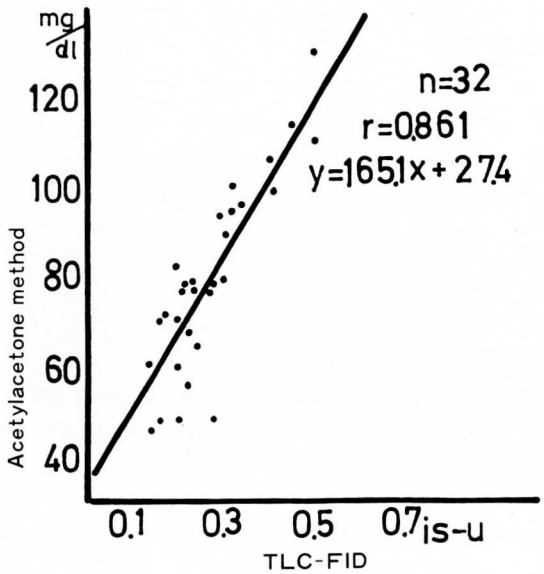


Fig. 4 Correlation between TLC-FID and the acetylacetone method for triglycerides

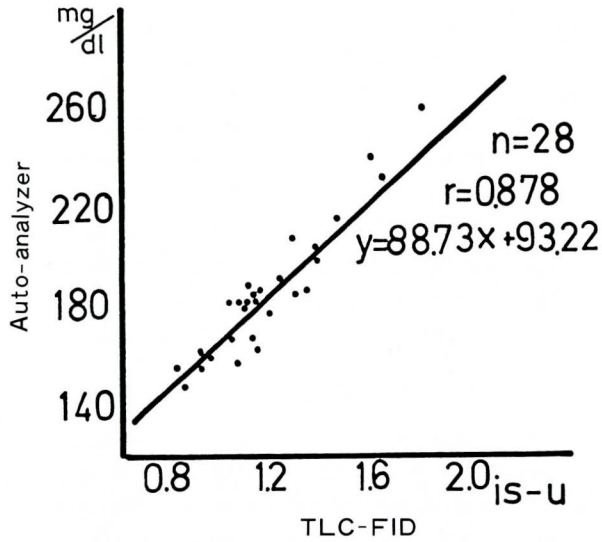


Fig. 5 Correlation between TLC-FID and the auto-analyzer for phospholipids

Table 3. Normal lipid contents of serum of subjects with different mean ages by colorimetric method

Age	CE	FC	TC	TG	PL	n
20–29	134.5±29.2	37.8± 9.4	172.3±36.5	76.1±19.8	213.7±29.4	15
30–39	146.8±26.3	50.7±16.6	199.8±31.4	97.8±54.0	218.3±36.9	24
40–49	151.2±31.8	48.7± 8.5	199.4±35.3	109.2±45.2	178.8±43.3	59
50–59	169.5±38.6	49.4± 8.5	218.1±40.9	122.2±61.3	190.9±50.5	43

(mg/dl, M±1.S.D.)

6. Value of the lipid composition by colorimetric analysis:

The results of analyses of lipid composition of the same serum sample by the colorimetric method are shown in Table 3. Free cholesterol was determined by the Liebermann-Burchard method, triglyceride by the Fletcher method and phospholipids by an auto-analyzer (Kessler method). The results of t-tests for each fraction value by age group were as follows. In the case of cholesterol esters, the value for the subjects in their fifties was higher by 0.14 IS-U ($P<0.05$) than that for subjects in their twenties. In the case of free cholesterol, the value for subjects in their forties was lower by 0.04 IS-U on the average than that of those in their twenties ($P<0.05$), and also lower

by 0.04 IS-U than that of those in their thirties ($P<0.01$). In the case of triglyceride, the value for subjects in their fifties was higher by 0.11 IS-U than that of those in their twenties ($P<0.05$). In the case of phospholipids, the value for subjects in their thirties was lower by 0.25 IS-U on the average than that of those in their twenties ($P<0.01$), and the value for those in their forties was lower by 0.35 IS-U on the average than that of those in their twenties ($p<0.001$). Also the value for those in their fifties was lower by 0.30 IS-U on the average than that for those in their twenties ($P<0.01$). Thus, phospholipids decreased as the age increased, while the variations of cholesterol values by age groups were not statistically significant.

DISCUSSION

The quantitative evaluation of thin-layer chromatograms can be accomplished by a variety of procedures either directly on the plate or after extraction from the plate. Components must be estimated rapidly by densitometry or by spot area measurements. These techniques have the disadvantage that a reasonable number of standard materials must be run with the unknown samples to obtain quantitative results.

Methods for detecting lipid spots depend mainly on charring, either with heat or with strong acid such as sulfuric acid. A charring technique is impractical when a plastic plate is used. Even when they are glass backed, charred plates tend to absorb moisture or the color fades away. The strong acid procedure has the disadvantage that the plates cannot be stored.

However, the thin-layer chromatography-FID method (TLC-FID) has various advantages (9, 3). It permits quantitative TLC separation and accurate FID detection of lipids. Unlike thin-layer densitometry, detection with the FID is easily performed without tiresome detection by reagents such as sulfuric acid.

In general, the most important requirement is the detection since the FID is not a specific but a general detector. The problem of evaluating the data quantitatively was solved by introducing an internal standard. A major requirement of the internal standard compound was that it be well separated from the major lipid components and the peak of the chromatogram should be recorded individually and clearly. Of many compounds tested, we found (8) that cholesterol acetate satisfied this requirements, and we calculated the peak area of 10mg of cholesterol acetate as an Internal Standard Unit (IS-U).

The use of the internal standard corrects for errors in the volume of the material applied. For instance, it is difficult to apply a minute amount of the sample quantitatively on the TLC rod because a low boiling point solvent is used. However, based on the peak area of the internal standard as expressed by IS-U, each lipid fraction can be expressed in a relative manner, so that the amount of the sample applied does not matter. Also, calculations using the internal standard are carried out for quality control to determine and check the values for the various factors of error.

The results obtained by TLC-FID indicate that relative responses of the individual lipids are different. In this study, triglyceride gave a lower response and the introduction of a correction factor was required for a

quantitative analysis. A comparison of lipid profiles obtained by the TLC-FID technique and those obtained by the classic chemical analysis is underway to establish practical correction factors.

In spite of these difficulties, the TLC-FID method has various advantages. For example, triglyceride is determined by the modified Fletcher method, and glycerol is used as a standard solution, which is calculated as tri-oleate found abundantly in normal sera. Serum triglyceride determination by the modified Fletcher method is affected by phospholipids or glucose since these substances form a colored complex. On the other hand, TLC-FID does not detect each constituent fatty acid individually but whole triglyceride can be determined graphically like the electrophoresis pattern of serum proteins.

Variations in the lipid fractions by age group were observed for cholesterol esters and triglyceride. This fact also coincides with the results by the conventional colorimetric method. However, in the case of phospholipids, a slight decrease was observed as the age increased, differing from the report (6) that the value hardly varied by age group. In the case of free cholesterol, triglyceride and phospholipids, differences by sex have been reported, but we could not investigate this because our subjects were all male.

The differences in the results by age group using TLC-FID were smaller than those using the colorimetric method. This was caused by peculiarities of TLC-FID, i.e., the difference of fractions from rod to rod, the problem of tailing and the stability of developing conditions. These will be eliminated by technical improvements in the future.

In conclusion, the TLC-FID method (IATROSCAN) is a rapid simple screening procedure applicable to epidemiological studies such as the screening of hyperlipidemia or to prevention studies on atherosclerosis. Also, the technique, like electrophoresis of serum proteins, is most useful for demonstrating, graphically and quantitatively, the lipid distribution in the serum of patients.

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