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Note

An improved thin-layer chromatographic method for micro determination of lipids using flame ionization detection

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The Iatroscan is an autoanalyzer which combines the qualitative resolution capabilities of thin-layer chromatography (TLC) with the quantitative sensitivity of flame ionization detection (FID). Since even poorly volatile or colourless materials which are separable on the Chromarod without any chemical modifications for vaporization or visualization can be determined, it has been used as a convenient tool for lipid analysis[1–4]. However, there have been few studies on micro determinations by the Iatroscan. Such determinations are difficult, because aliphatic hydrocarbons are not usually expected to give large FID responses with the Iatroscan[5].

This paper reports a new procedure which enables the micro determination of lipids by the Iatroscan with good precision and reproducibility.

MATERIALS AND METHODS

Organic solvents (Wako Pure Chemicals Co.) were analytical grade and redistilled before use. Sphingomyelin (SM), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were prepared from egg yolk; phosphatidylserine (PS) was obtained from whale brain by the method of Rouser *et al.*[6]. Cholesterol (C), cholesteryl palmitate (CE), cholesteryl acetate (CA), tripalmitin (TG) and palmitic acid (FFA) were from Tokyo Kasei Kogyo Co., and purified by recrystallization before use. The purity of these lipids were >99% according to high-performance TLC.

A standard solution of neutral lipid and phospholipid was prepared as follows: from a stock solution containing 8 mg/ml of each lipid in chloroform–methanol (1:1, v/v), a series of 4, 2, 1, 0.5 and 0.25 mg/ml solutions were prepared in the same solvent. A similar series of lipid mixtures containing 0.96 mg/ml of CA as an internal

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TABLE I

WEIGHT RATIOS OF NEUTRAL LIPIDS TO CA

Each sample contained 0.96 mg/ml of CA (internal standard). Lipid abbreviations as in the text.

Sample No.	*Weight ratio to CA*				
	C	*TG*	*FFA*	*CA*	*CE*
1	0.29	0.23	0.27	1.00	0.31
2	0.57	0.47	0.55	1.00	0.63
3	1.15	0.94	1.10	1.00	1.25
4	2.29	1.87	2.19	1.00	2.50

standard were prepared. The weight ratio of each lipid to CA is shown in Table I. Lipids were extracted directly with chloroform–isopropanol (7:11, v/v) from erythrocytes prepared from 2.5 μl of pooled normal human blood and evaporated to dryness under an atmosphere of nitrogen[7]. The dried lipids were dissolved in 0.2 ml chloroform for analysis.

The rods (Chromarod Type S-II; Iatron Labs. Inc., Tokyo, Japan), newly purchased or contaminated by use (about 30 experiments), were cleaned by dipping in concentrated nitric acid overnight at room temperature, rinsed with distilled water and dried at 100°C for 1 h. Just prior to use, they were activated by passing through the FID scanner, monitoring the baseline for each rod. With these treatments, the rods did not produce any significant signals which would interfere with the determination. A 0.2-μl volume of lipid sample can be applied to each rod by a single injection with a Hamilton Microsyringe 7001N.

The rods were developed by either chloroform–methanol–water–acetic acid (45:15:15:0.2, v/v/v/v) for phospholipid or *n*-hexane–diethyl ether–acetic acid (54:6:0.8, v/v/v) for neutral lipids in a vessel which had been saturated with the same solvent systems.

Determinations were made with an Iatroscan TH-10 analyzer (Iatron Labs. Inc.) equipped with an Intelligent Integrator 7000A (System Instrument Co., Tokyo, Japan) and a Hitachi 056 two-pen recorder (Hitachi, Tokyo, Japan). The flame ionization detector was operated at an hydrogen flow-rate of 200 ml/min. The constant air flow-rate of 2000 ml/min was supplied by an air compressor (Super bebicon, Hitachi) equipped with an air cleaner comprising 300 ml of molecular sieve (3 Å, Wako Pure Chemicals) and 300 ml of activated charcoal (Kishida Chemicals). The scanning speed and chart speed were 30 s per scan and 120 mm/min. The recorder full scale was 5 mV.

RESULTS AND DISCUSSION

To obtain a higher sensitivity and good reproducibility for small sample sizes, ways of increasing the signal-to-noise ratio, S/N, were examined. Fig. 1 shows the effect of the hydrogen flow-rate on the FID sensitivity. The sensitivity was maximum around 200 ml/min, where the most reproducible response was obtained. On the other hand, the sensitivity was not affected by the air flow-rate between 1500 and

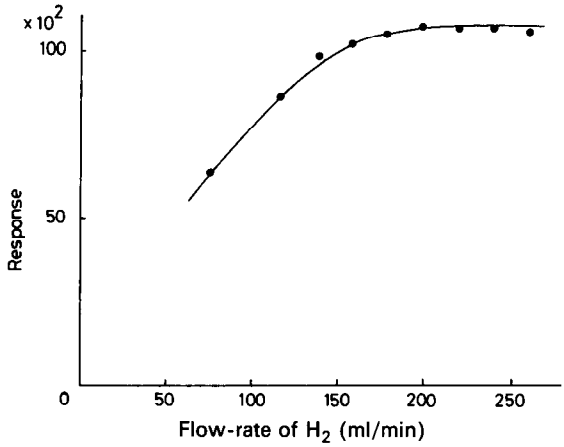

Fig. 1. Effect of hydrogen flow-rate on sensitivity. The air flow-rate was 2000 ml/min. Sample: cholesterol (2 μg).

3000 ml/min. The effect of the distance between the rod and the burner nozzle is shown in Fig. 2. The highest sensitivity was obtained at 0.8 mm, which was virtually the minimum practical distance between the rod and burner nozzle. A vibrator pump, found to cause baseline pulsation (Fig. 3), was replaced by an air compressor equipped with an air cleaner as described above. The noise level was markedly decreased by cleaning the rods with concentrated nitric acid (Fig. 4). This treatment is necessary before the first use of a new rod, and recommended after every 30 experiments.

These modifications increased the sensitivity more than two-fold and decreased the noise level to less than one fifth that usually obtained. As a result, the sample

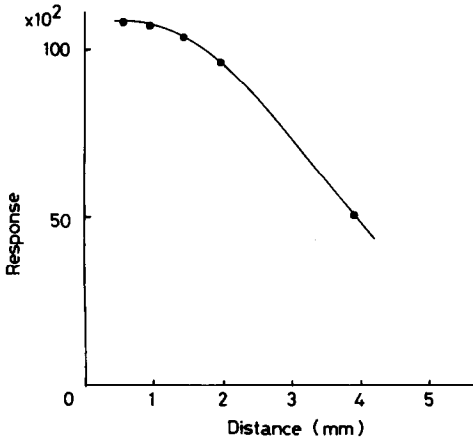

Fig. 2. Effect of the distance from the rod to the burner nozzle on sensitivity. The flow-rates of hydrogen and air were 200 and 2000 ml/min respectively. Sample as in Fig. 1.

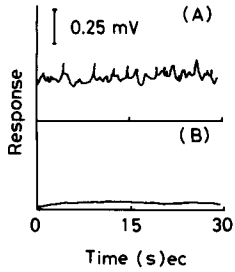

Fig. 3. Elimination of the baseline pulsation. The scan was carried out without the rod. (A) The vibrator pump was used. (B) The air compressor equipped with an air cleaner was used. Operating conditions as in the text.

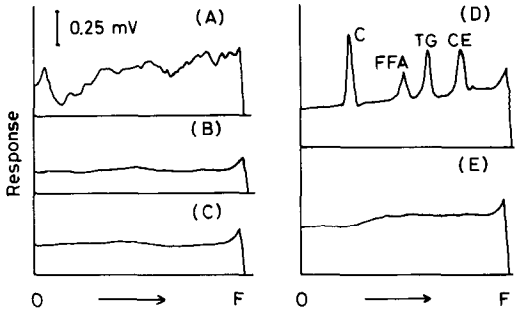

Fig. 4. FID signals obtained: (A) with a new Chromarod but without any treatment; (B) as in (A) but after cleaning the rod with concentrated nitric acid; (C) as in (B) but after a development with *n*-hexane–diethyl ether–acetic acid (54:6:0.8, v/v/v); (D) after a development of cholesterol (C), palmitic acid (FFA), tripalmitin (TG) and cholesteryl palmitate (CE) (0.05 μg of each) with the same solvent as in (B); (E) soon after scanning of (D). O and F respectively represent the origin and front of TLC. Operating conditions as described in the text.

TABLE II

REPRODUCIBILITY OF PEAK-AREA RATIO OF NEUTRAL LIPIDS TO CA

Operating as in the text. Values expressed as means ± standard deviation, with coefficient of variation in parentheses, for six determinations

Sample No.	*Peak area ratio to CA*			
	C	*TG*	*FFA*	*CE*
1	0.18 ± 0.02 (13)	0.16 ± 0.03 (18)	0.18 ± 0.03 (17)	0.25 ± 0.04 (16)
2	0.50 ± 0.05 (10)	0.36 ± 0.06 (16)	0.29 ± 0.04 (15)	0.62 ± 0.10 (16)
3	1.1 ± 0.08 (7.7)	0.88 ± 0.12 (14)	0.70 ± 0.08 (12)	1.4 ± 0.14 (10)
4	3.1 ± 0.14 (4.7)	2.1 ± 0.15 (7.2)	1.7 ± 0.11 (6.6)	3.6 ± 0.28 (7.9)

TABLE III

REPRODUCIBILITY OF PEAK AREA OF EACH LIPID

Operating conditions as in the text. Values expressed as in Table II.

Lipid	*Lipid content (μg)*				
	0.05	*0.1*	*0.2*	*0.4*	*0.8*
C	130 ± 21 (16)	289 ± 20 (6.9)	753 ± 120 (16)	2530 ± 450 (18)	6260 ± 840 (13)
FFA	113 ± 17 (15)	246 ± 29 (12)	688 ± 82 (12)	1590 ± 190 (12)	3810 ± 840 (10)
TG	97 ± 17 (18)	219 ± 25 (11)	561 ± 86 (15)	1480 ± 160 (10)	3960 ± 535 (14)
CE	111 ± 16 (14)	270 ± 37 (14)	788 ± 130 (17)	1960 ± 160 (8.2)	5410 ± 810 (15)
SM	103 ± 17 (16)	196 ± 36 (17)	527 ± 30 (5.7)	1330 ± 180 (13)	3950 ± 420 (11)
PC	80 ± 8 (10)	171 ± 16 (9.5)	370 ± 47 (13)	930 ± 71 (7.6)	2420 ± 170 (7.0)
PS	55 ± 5.5 (10)	109 ± 19 (17)	219 ± 23 (11)	582 ± 63 (11)	1230 ± 150 (12)
PE	68 ± 7 (9.6)	141 ± 16 (11)	283 ± 27 (9.6)	701 ± 85 (12)	1490 ± 60 (4.0)

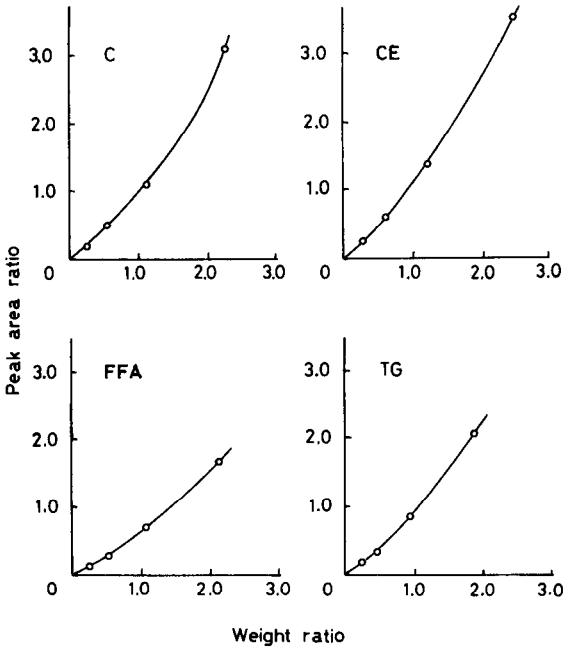

Fig. 5. Standard curves for neutral lipids. CA was used as an internal standard. Abbreviations as described in the text.

amount could be decreased to as little as 0.05 μg of each lipid which were contained conventionally in 0.2 μl of sample solution (Fig. 3). This simplified the sample application and resulted in a sharp sample zone. Furthermore, because of the perfect pyrolysis of each lipid during the scanning, a subsequent determination can be done directly afterwards without reactivating the rods (Fig. 3).

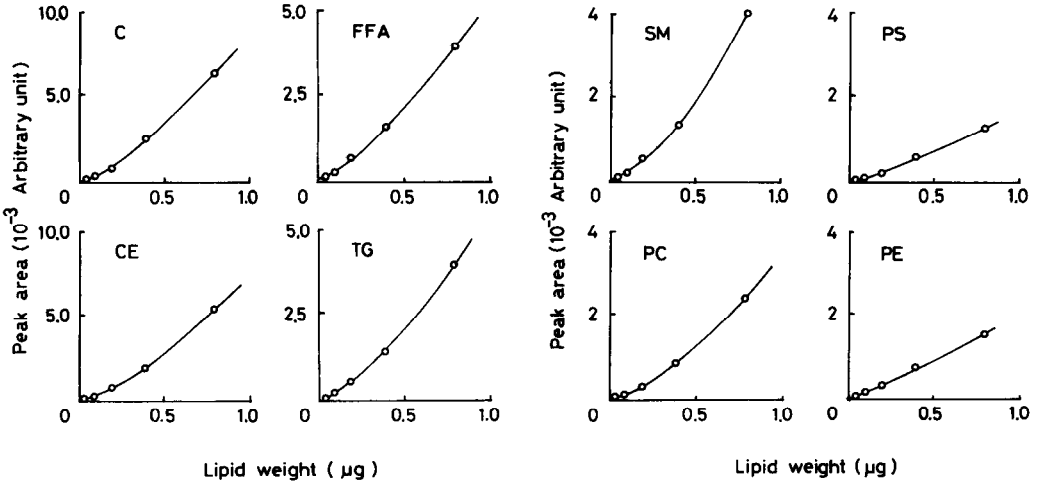

Fig. 6. Standard curves for neutral lipids. Abbreviations as described in the text.

Fig. 7. Standard curves for phospholipids. Abbreviations as described in the text.

TABLE IV

LIPID COMPOSITION OF HUMAN ERYTHROCYTES

Lipid	*Weight (%)*	
	*Present method**	*Usual method***
C	29.9	31.7 ± 3.8
PE	22.3	19.9 ± 2.7
PS	10.7	12.1 ± 2.2
PC	22.1	19.3 ± 2.6
SM	15.7	17.0 ± 2.2

* Values are means of three determinations.
** Values are means ± standard deviation for 23 adults[8].

Tables II and III compare the results obtained with and without the internal standard. The reproducibility and the precision were similar in each case. Although the standard curve for each lipid was not linear (Figs. 5–7), the good reproducibility and precision make this micro method reliable for lipid determination.

The lipid composition of human erythrocytes estimated by this method was in good agreement with that reported by Sibata *et al.*[8], using the normal method with an Iatroscan (Table IV).

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