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Note

Some limitations of plasma lipid analysis in clinical research by thin-layer chromatography with flame-ionization detection[a]

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The method of thin-layer chromatography with flame-ionization dectection (TLC–FID) was first used for the analysis of blood lipids in 1972 [1]. Since then, a number of papers have described the application of this method in clinical lipid analysis [2–8]. Nonetheless, some problems connected with the reproducibility of results still remain unresolved, and thus the application of this method in lipid metabolism research tends to be limited.

EXPERIMENTAL

TLC–FID analyses were carried out on an Iatroscan TH-10 (Iatron Labs., Tokyo, Japan) connected to a Spectra Physics Model 4100 computing integrator. Separations were performed on S-II Chromarods. The scanning speed was 4 mm/s, the hydrogen flow-rate 180 ml/min and the air flow-rate 2000 ml/min.

The calibration mixtures were prepared from pure chemicals: cholesteryl oleate, cholesterol, triolein, racemic 1,2-palmitoylglycerol-3-phosphorylcholine (lecithin), sphingomyelin and racemic 1-palmitoylglycerol-3-phosphorylcholine (lysolecithin), purchased from Sigma (St. Louis, MO, U.S.A.). The internal

[a] This paper is dedicated to the memory of Dr. P. Mareš, Ph.D., a leading scientist in lipid research in Czechoslovakia, who tragically died in August 1989.

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standards, 1-octadecanol (Alfol RD 18) and racemic 1,2-dipalmitoyl-N,N-dimethyl-3-phosphorylethanolamine (dimethylcephalin) were products of Condea (Hamburg, F.R.G.) and Fluka (Buchs, Switzerland), respectively.

Plasma lipids were extracted according to a procedure recommended by the instrument manufacturer. All the samples were spotted in chloroform solutions (1 μl) containing 10 μg of internal standard. Chromarods were activated immediately before development and developed with hexane–diethyl ether–formic acid (52:8:0.1, v/v) for 20 min to separate neutral lipid classes from total phospholipids and with chloroform–methanol–water (40:17:1.5, v/v) for 2 × 20 min to separate individual phospholipids.

RESULTS AND DISCUSSION

In agreement with other authors [2,4,7,9] we found non-linear calibration dependences giving typical sigmoid curves (Fig. 1). The reproducibility of the calibration as well as that of the analysis of biological sample were evaluated statistically (serial, day-to-day and within 30 days). Results are given in Tables I–III. Chromatograms of the calibration mixtures of neutral lipids and phospholipids are shown in Fig. 2A and B, respectively, and a chromatogram of a plasma lipid profile is shown in Fig. 2C.

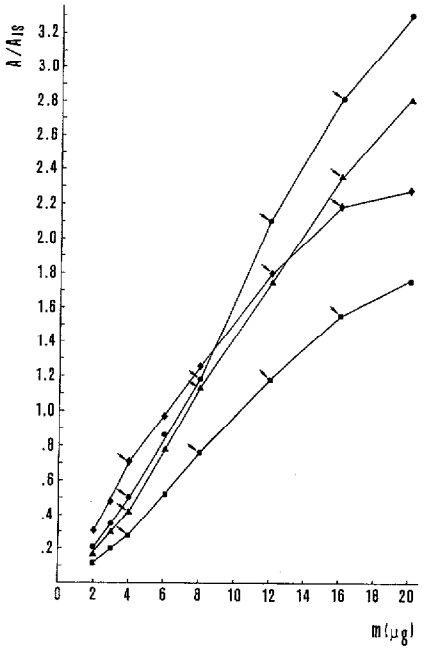

Fig. 1. Calibration curves of the individual lipid classes in multilinear approximation. Slope changes are indicated by arrows. Identification: (●) cholesterol; (▲) cholesteryl esters; (■) triglycerides; (◆) phospholipids.

It is evident from these results that the reproducibility of the measurements is improved when each Chromarod is calibrated individually. This requirement leads to more complicated evaluation of biological sample analyses. Thus, we compared the results obtained for one biological sample evaluated according to the individual and average calibration methods. The higher variability of the results obtained from the average calibration is primarily a result of the difference in the physical properties of the individual rods, as all other parameters that influence the reproducibility [10] were kept constant.

In order to check the accuracy of the results, we compared the levels of plasma total cholesterol (TC), triglycerides (TG) and phospholipids (PL) measured by the TLC–FID and enzymic methods (plasma samples with known lipid levels were obtained from a routine biochemical laboratory). The results given in Table IV show a significant correlation of both methods in the cases of TG and TC. The lower significance in the case of TC is caused by the determination of this value as the sum of free and esterified cholesterol by the TLC–FID method. No correlation was observed in the case of PL, probably because of complications with the evaluation of undeveloped peak areas [10].

As the total PL level cannot be determined in this way, we evaluated the calibration curves for individual phospholipids, the determination of which is more useful for metabolic studies. Similar sigmoid curves as for neutral lipids were observed.

TABLE I

STATISTICAL EVALUATION OF CALIBRATION PARAMETERS (PEAK-AREA RATIO) FOR DIFFERENT CONCENTRATIONS OF CALIBRATION MIXTURE

Internal standard, octadecanol (10 μg); one series includes the set of ten rods.

Spotted amount (μg)	Lipid class	In series		Day-to-day		Within 30 days	
		Mean peak-area ratio	C.V. (%)	Mean peak-area ratio	C.V. (%)	Mean peak-area ratio	C.V. (%)
2	CE	0.19	9.8	0.20	17.7	0.20	21.4
	TG	0.13	9.1	0.14	13.9	0.14	18.2
	FC	0.18	8.2	0.19	13.9	0.20	13.1
	PL	0.31	3.7	0.35	12.2	0.35	11.6
8	CE	1.10	8.4	1.11	8.3	1.12	7.6
	TG	0.76	7.2	0.76	6.6	0.77	6.7
	FC	1.34	3.6	1.34	3.8	1.32	4.1
	PL	1.31	6.4	1.31	6.4	1.32	5.9
16	CE	2.34	6.0	2.36	6.3	2.41	6.8
	TG	1.59	5.7	1.60	5.8	1.65	7.2
	FC	2.86	4.7	2.89	4.8	2.86	4.8
	PL	2.35	7.8	2.40	8.2	2.43	7.9

TABLE II

STATISTICAL EVALUATION OF THREE CALIBRATIONS OF INDIVIDUAL RODS

Spotted amount, 8 μg; internal standard, octadecanol (10 μg). Calibration: 1, new rods; 2, after ten analyses of biological samples; 3, after cleaning in sulphuric acid.

Rod No.	Lipid class	Mean peak-area ratio	C.V. (%)	Rod No.	Lipid class	Mean peak-area ratio	C.V. (%)
1	CE	1.07	1.6	6	CE	1.21	2.0
	TG	0.72	3.6		TG	0.83	1.7
	FC	1.25	1.0		FC	1.28	2.2
	PL	1.22	3.2		PL	1.35	0.4
2	CE	1.26	2.6	7	CE	1.05	2.5
	TG	0.85	1.7		TG	0.75	3.8
	FC	1.36	2.1		FC	1.35	0.3
	PL	1.41	3.6		PL	1.27	2.4
3	CE	1.12	2.2	8	CE	1.12	1.7
	TG	0.75	3.3		TG	0.72	2.6
	FC	1.35	1.2		FC	1.28	3.2
	PL	1.40	4.3		PL	1.29	0.7
4	CE	1.21	3.8	9	CE	1.06	1.2
	TG	0.83	1.1		TG	0.71	3.4
	FC	1.35	3.4		FC	1.34	4.2
	PL	1.38	2.1		PL	1.29	3.8
5	CE	1.00	2.5	10	CE	1.11	1.7
	TG	0.77	1.8		TG	0.76	2.1
	FC	1.37	3.8		FC	1.27	0.6
	PL	1.37	1.4		PL	1.21	2.0

TABLE III

STATISTICAL EVALUATION OF TWO SERIES OF BIOLOGICAL SAMPLES CALCULATED FROM INDIVIDUAL CALIBRATIONS OF EACH ROD AND FROM MEAN CALIBRATION OF TEN RODS

Series No.	Lipid class	Individual calibration (mg/dl)	C.V. (%)	Mean calibration (mg/dl)	C.V. (%)
1	CE	217	2.6	210	6.3
($n=5$)	TG	261	4.1	262	6.5
	FC	53	4.4	54	7.5
	PL	248	4.3	251	6.8
2	CE	214	1.7	208	5.5
($n=5$)	TG	246	4.6	251	6.2
	FC	54	4.2	54	5.6
	PL	224	4.6	229	6.0

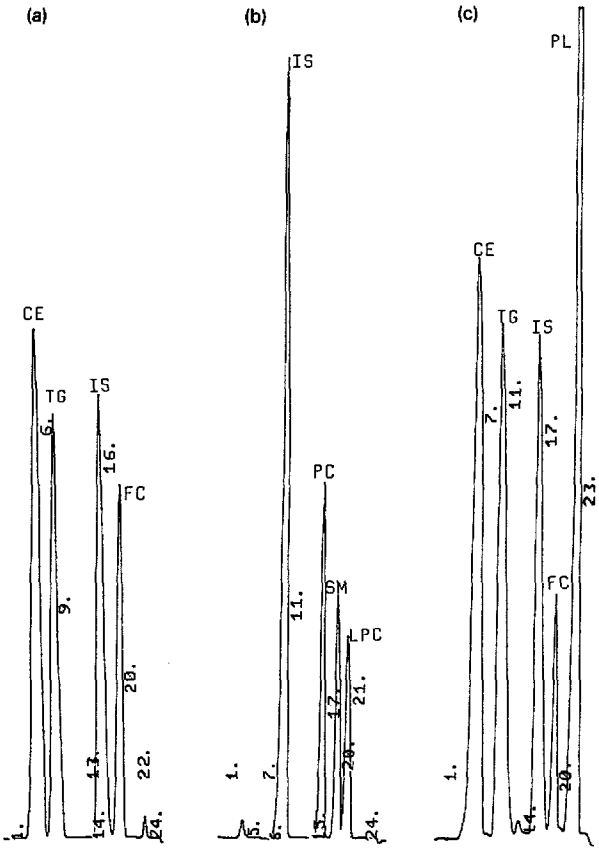

Fig. 2. Examples of chromatograms. (A) A standard mixture of neutral lipids (CE = cholesteryl ester; TG = triglyceride; IS = octadecanol; FC = free cholesterol); (B) a standard mixture of phospholipds (IS = dimethylcephalin; PC = lecithin; SM = sphingomyelin; LPC = lysolecithin); (C) plasma neutral lipids (PL = phospholipids, others as in A).

TABLE IV

COMPARISON OF PLASMA LIPID DETERMINATION BY THE TLC–FID AND ENZYMIC METHODS

$n = 20$; for the enzymic determination, Lachema-Ames (Czechoslovakia) commercial sets were used.

Lipid class	Correlation coefficient	P
Total cholesterol	0.58	0.01
Triglycerides	0.98	0.0001
Phospholipids	0.42	N.S.

From the practical point of view, the evaluation of the results using the non-linear calibration is inconvenient and thus linearization of the calibration dependence is an important criterion for the usefulness of the method. This problem was studied independently by two groups [7,10]. For the lipid classes tested (cholesteryl esters, triglycerides, free fatty acids, cholesterol, diglycerides, monoglycerides and phospholipids), three dependencies seem to be most convenient (correlation coefficient 0.98–0.99): $y = a + bx^{1/2}$, $y = ax^b$ and $y = a + bx + cx^2$. For practical purposes, the dependence $y = ax^b$ is most advantageous for calculations. Using the logarithmic form of this equation, we obtain, after the substitution of variables,

$$\log A/A_{\text{I.S.}} = \log a + b \log m \tag{1}$$

where A and $A_{\text{I.S.}}$ are the peak areas of the component measured and internal standard, respectively, m is the spotted amount of the individual component, and a and b are constants characteristic of each component and the rod. The spotted amount can be easily calculated from the equation:

$$\log m = (\log A/A_{\text{I.S.}} - \log a) \cdot 1/b \tag{2}$$

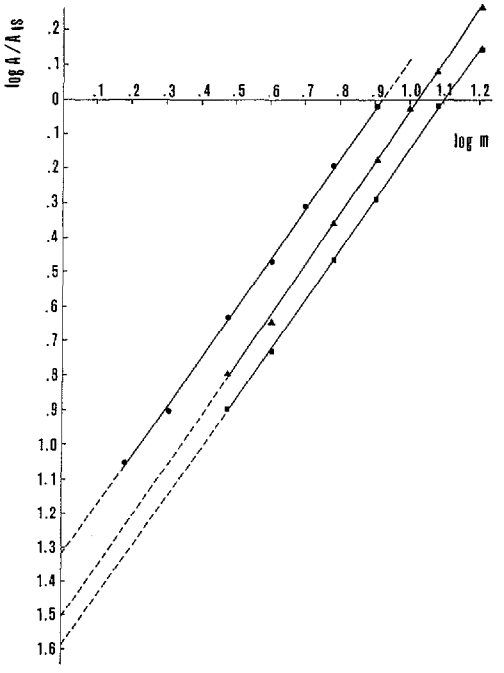

Fig. 3. Calibration curves for (●) cholesterol, (▲) cholesteryl ester and (■) triglyceride on a logarithmic scale.

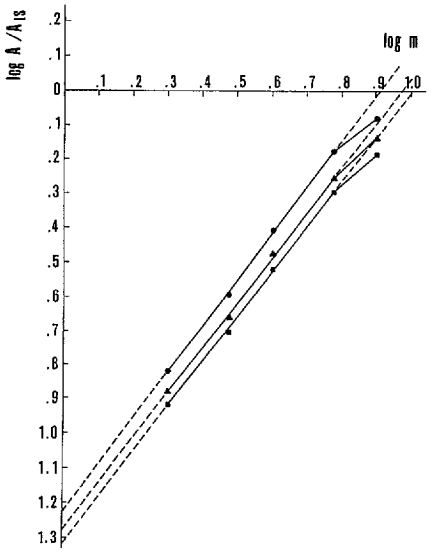

Fig. 4. Calibration curves for (●) lecithin, (▲) sphingomyelin and (■) lysolecithin on a logarithmic scale.

The concentration of the component in the biological sample is then determined by a simple numerical recalculation.

For neutral lipids, the relationship given by eqn. 1 was linear in the concentration range 1.5–16 μg of the spotted amount, for phospholipids in the range 2–6 μg, as shown in Figs. 3 and 4. For each component, as well as for each rod, it is necessary to check the linearity of eqn.1. Determination of the calibration constants and the evaluation of the clinical results can be easily computerized, or they can be calculated manually.

When the peak-area ratio is outside the linear range, it is necessary to repeat the whole analytical procedure using a smaller amount of the biological sample (dilution of the final extract with the internal standard solution leads to inaccurate results), or to calculate the concentration from a non-linear calibration.

The TLC–FID method has as advantages over other chromatographic techniques of lipid analysis in a higher sample capacity and in the possibility of simultaneous determination of both neutral and polar lipids. However, the variability of results leads to some limitations in the application of this method in clinical research.

(1) The method is not suitable for the measurement of subtle changes in the individual lipid levels (*e.g.* lipolysis, LCAT activity, etc.).

(2) The method is not suitable for the measurement of low lipid levels (*e.g.* plasma free fatty acids, lipid profile in high-density lipoprotein, etc.).

(3) The method is suitable for all measurements where a variability of *ca.* 5–8% is compatible with the changes in the value measured.

REFERENCES

1 T. Kawai, S. Hasunuma, E. Nakano, I. Sakurabayashi, N. Okkulo, S. Yoshioka and J. Ishii, *Jpn. J. Clin. Pathol.*, 20 (1972) 186.
2 R. G. Ackman, *Methods Enzymol.*, 72 (1981) 205.
3 D. M. Bradley, C. R. Rickards and N. S. T. Thomas, *Clin. Chim. Acta*, 92 (1979) 293.
4 R. T. Crane, S. C. Goheen, E. C. Larkin and G. A. Rao, *Lipids*, 18 (1983) 74.
5 P. Mareš, M. Ranný, J. Sedláček and J. Skořepa, *J. Chromatogr.*, 275 (1983) 295.
6 G. L. Mills, C. E. Taylaur and L. Miller, *Clin. Chim. Acta*, 93 (1979)173.
7 E. Peuchant, G. Covi and R. Jensen, *J. Chromatogr.*, 310 (1984) 297.
8 D. Vandamme, V. Blaton and H. Peeters, *J. Chromatogr.*, 145 (1978) 151.
9 R. P. Delmas, C. C. Parrish and R. G. Ackman, *Anal. Chem.*, 56 (1984) 1272.
10 C. C. Parrish and R. G. Ackman, *Lipids*, 20 (1985) 521.