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A MICROMETHOD FOR THE QUANTITATIVE DETERMINATION OF LIPIDS BY USE OF THIN-LAYER CHROMATOGRAPHY, PHOTODENSITOMETRY AND AN INTERNAL STANDARD

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SUMMARY

Cholesterol, cholesteryl palmitate, palmitic acid and mono-, di- and tripalmitins were separated and quantitatively determined on thin-layer plates. The method includes visualization of the spots by dichromate–sulphuric acid treatment, scanning and planimetry of the peak areas. An internal standard (heptadecanoic acid methyl ester) and a correction factor for the peak areas markedly increased the precision of the TLC method.

Cholesterol, cholesterol esters, free fatty acids and triglycerides could be quantitatively determined in 25–50 μl of blood plasma.

INTRODUCTION

Quantitative thin-layer chromatographic methods for the analysis of lipids can be divided into two principal groups. In the first, separation only is performed by TLC and after elution from the silica gel the quantitation is done by gravimetric or conventional chemical methods[1,2]. This procedure usually requires milligram amounts of lipid and is rather cumbersome.

In the second group, the quantitation is also done on the thin-layer plate either by spot area measurements[3] or by photodensitometry[4–6]. The latter considerably adds speed and sensitivity to the analysis and makes detection of less than one microgram possible. However, the quantitative densitometric determination is subject to errors; the relation between the densitometric peak area and the amount of substance in a chromatographic spot is not linear[7], the correct centering of the spot against the scanning slit is difficult[8] and it is also difficult to quantitatively apply small amounts of highly volatile solvents to the chromatoplates[9].

In the present paper a method is described where use of a correction factor, introduction of an internal standard and application of the sample in rows diminishes the errors and increases the precision of the "on-plate" TLC analysis of micro amounts of lipids.

MATERIALS AND METHODS

Solvents

Acetic acid, benzene, chloroform, diethyl ether, hexane, methanol and sulphuric acid were supplied by Merck or Mallinckrodt (all of reagent grade). All solvents except acetic and sulphuric acids were distilled twice before use.

Reference lipids

Cholesterol was supplied by Difco, cholesteryl palmitate, monopalmitin, 1,2- and 1,3-dipalmitin, heptadecanoic acid methyl ester (HAME), and tripalmitin by Sigma, and palmitic acid by Kebo. The dipalmitins and monopalmitin were separated by preparative TLC from a mixture containing mono-, di, and tri-palmitin[6]. Palmitic acid was recrystallized from hexane. All reference lipids were checked by TLC (system as described below) or GLC[10] for purity before use. The weight relation between the isomers of the dipalmitins was estimated as 1:2 by TLC separation and determination as described below.

Extraction of lipids

Rat blood plasma was obtained from the carotid artery of white Sprague-Dawley rats after they had been killed by a blow on the head. The blood was drawn into heparinized test tubes and plasma was separated from the erythrocytes by centrifugation. Rat blood plasma (50 μl), 5–20 μg HAME (internal standard) and 0.5 ml methanol were pipetted into a dichromate-washed conical centrifuge tube, which was then swirled vigorously. 1 ml of chloroform was added followed by 0.5 ml 0.2 *M* NaH_2PO_4[11–13]. The tube was then left overnight in a refrigerator (4°). The next morning, 0.8 ml of the lower phase was carefully aspirated into an all-glass syringe and transferred into a small (3 ml) silanized conical test tube. The chloroform was evaporated under a stream of nitrogen and the lipid residue redissolved in 25 μl of hexane.

TLC procedure

Silica Gel G containing 15 % $CaSO_4$ (Merck) was refluxed for several hours with methanol, followed by filtering and drying at 50°. The plates (10 × 20 cm or 20 × 20 cm) were prepared using a Camag or Desaga device giving a layer thickness of 0.20 mm. They were run to the top with diethyl ether several times to remove minor contaminants and were then activated at 180° for at least 1 h. The sample was applied 1.5 cm from the bottom of the plate as a row (10 mm) of closely spaced dots (N = 6), distance between the rows being 7 mm. Reference lipids were run on each plate.

The plates were developed twice, first with diethyl ether–acetic acid (99:1) to about 8 cm above the starting point and second, after air drying, with hexane–diethyl ether–acetic acid (85:15:1) to about 15 cm above the starting point. After air drying the plates were sprayed with a saturated solution of potassium dichromate in 70 % sulphuric acid and heated at 180° for 25 min[4]. The plates were scanned in a Vitatron densitometer (slit size 0.2 × 3 mm) and the O.D. registered on a Vitatron recorder. The area under each peak was measured planimetrically and a correction factor (1 + 0.4 *E*) was used[7], *E* being the maximal optical density of the peak.

An internal standard was added to the original sample. HAME was chosen as it does not occur in significant amounts in biological material and is well separated from the other lipids in the TLC system used.

To calculate the amounts of unknown lipid (Q_{xe}) in the samples, the following formula was used:

$$Q_{xe} = \frac{Q_{xr} \cdot A_{sr} \cdot Q_{se} \cdot A_{xe}}{Q_{sr} \cdot A_{xr} \cdot A_{se}} \quad (1)$$

where Q = quantity; A = corrected area; x = lipid to be estimated; s = internal standard; r = reference lane; e = experimental lane. The formula is based on the observation that Q_x/Q_s plotted against A_x/A_s gives a straight line (Fig. 1).

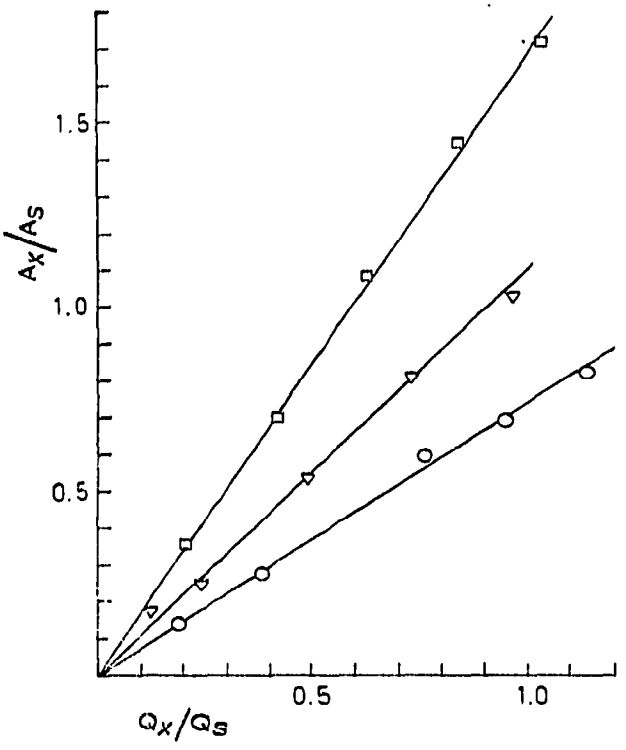

Fig. 1. Relation between the ratio corrected area for a lipid (A_x)/corrected area for the internal standard (A_s) *versus* the ratio amount of lipid (Q_x)/amount of internal standard (Q_s). The lipids included are cholesterol (□—□), 1,3-dipalmitin (▽—▽) and monopalmitin (○—○). Values for mean of duplicates are given.

Ordinary statistical methods have been used[14]. Coefficient of variation (C.V.) has been calculated from (S.D. × 100)/mean.

RESULTS

Reference lipids

The TLC system gave a good separation of the mixed reference lipids as illustrated by the densitogram in Fig. 2.

The error introduced by the planimeter was estimated by making double determinations of 120 peaks. The C.V. was 2 % for areas < 750 mm², 1 % for areas 750–1500 mm² and < 1 % for areas > 1500 mm².

In the preliminary experiments lanes were run where the lipids were applied on the plates as rows, or as spots, respectively. The C.V. for the densitometrically determined areas was in the first case 5.2 % and in the second 13.0 % (corrected areas; N = 10). Application of the lipids as rows thus gave the highest precision. The ratio between the corrected area for a given lipid and the corrected area for the internal standard on the same lane had a C.V. of 2.8 %, indicating that irregularities within each lane were compensated for by the simultaneous scanning of the spot for the lipid of interest and the spot for the internal standard.

The relation between the uncorrected and corrected peak areas, A, *versus* the amount of a given reference lipid, Q, has been plotted in Fig. 3. It is obvious that a more linear relation was obtained when the peak area was corrected with the factor $(1 + 0.4\,E)$. This was particularly the case when $E(\text{O.D.}_{\text{max.}})$ was higher than 1.0.

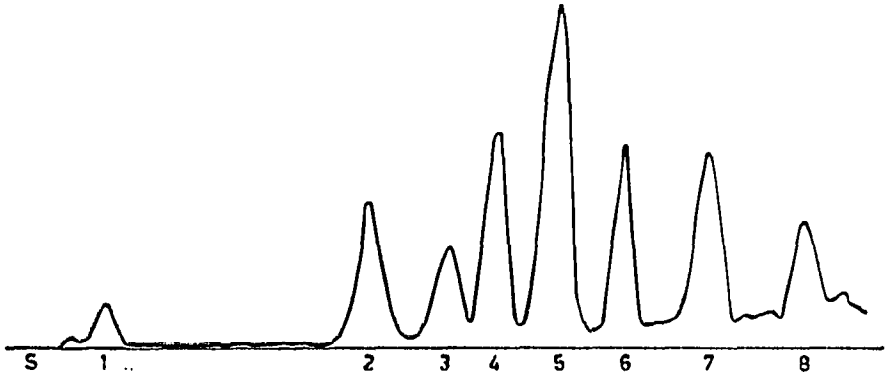

Fig. 2. Photodensitometric pattern from a TLC containing monopalmitin (1), cholesterol (2), 1,2-dipalmitin (3), 1,3-dipalmitin (4), palmitic acid (5), tripalmitin (6), heptadecanoic acid methyl ester (7) and cholesteryl palmitate (8).

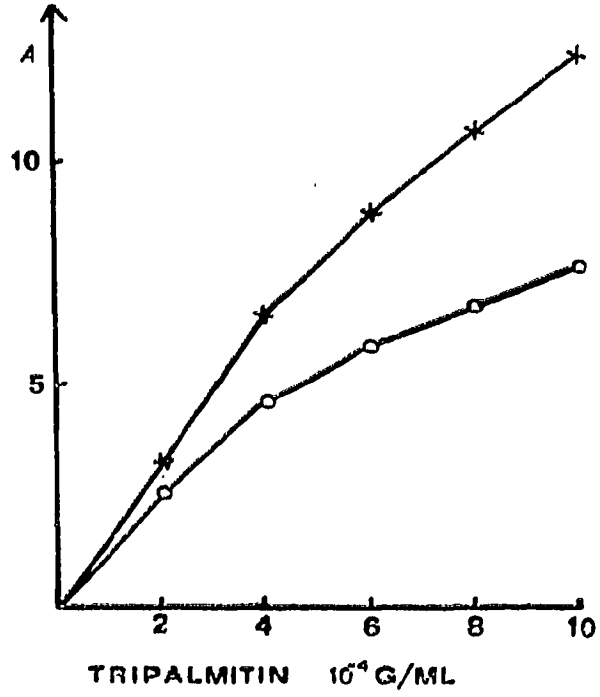

Fig. 3. Relation between peak area before (○—○) and after (×—×) correction with the factor $(1 + 0.4\,E)$ *versus* the concentration of tripalmitin.

TABLE I

REGRESSION COEFFICIENT, y-INTERCEPT AND LINEAR CORRELATION COEFFICIENT

Data relate to ratio — corrected area for lipid *x*/corrected area for int. stand. *versus* the ratio — amount of lipid *x*/amount of int. stand.

Lipid (x)	*Regression coefficient*	*y-Intercept*	*Linear correlation coefficient*
Monopalmitin	0.81	−0.03	0.99
Cholesterol	1.67	0.01	0.98
1,2-Dipalmitin	0.97	0.03	0.98
1,3-Dipalmitin	1.04	0.03	0.99
Palmitic acid	1.17	0.11	0.99
Tripalmitin	0.79	0.07	0.99
Cholesteryl palmitate	1.32	−0.08	0.98

TABLE II

REPEATED DETERMINATIONS OF LIPIDS IN ONE SAMPLE OF RAT BLOOD PLASMA

Area = corrected peak area (arbitrary units). The lower coefficients of variation (C.V.) for the calculated amounts demonstrate that the precision increased ($p < 0.01$) when an internal standard was used. N = 7.

	Cholesterol		*Free fatty acids*		*Triglycerides*		*Wax esters*	
	Area	*mg/100 ml*	*Area*	*mg/100 ml*	*Area*	*mg/100 ml*	*Area*	*mg/100 ml*
Mean	100	16.0	142	21.6	597	130.2	671	89.6
Range	85–118	14.8–17.2	120–198	18.2–25.2	527–627	116.0–137.2	580–856	82.0–98.2
C.V.	12	7	19	13	8	6	14	7

The ratio A_x/A_s was linearly correlated with the ratio Q_x/Q_s, as illustrated in Fig. 1. To make this clearer, only three of the reference lipids have been included, but the linearity was the same for all compounds. The coefficients of correlation between the ratios were in all cases 0.98–0.99 (Table I).

To study the reproducibility of the method triglycerides (TG) and internal standard (I.S.) were spotted onto seven different plates, three lanes on each. For the corrected TG area the C.V. was 8.4 % and for the ratio: corrected TG peak area/corrected I.S. peak area, 5.3 %.

Rat blood plasma

The reproducibility was determined by analyzing seven samples (50 μl) from the same specimen of rat blood plasma. The corrected area and calculated amount for each compound are presented in Table II. As the C.V. is smaller for the calculated amounts than for the areas, the precision has increased by including an internal standard ($p < 0.01$).

The accuracy of the method was studied by adding 5–10 μg of known lipids per 50 μl of rat blood plasma. The recovery was 96 % for cholesterol (N = 10), 97 % for palmitic acid (N = 8), 98 % for tripalmitin (N = 8) and 105 % for cholesteryl palmitate (N = 8).

DISCUSSION

SKIPSKI *et al.*[2] recently discussed the problems involved in the quantitative analysis of lipids separated on TLC. The separation is not in itself a major problem but the precision and accuracy of the quantitative determination have not previously been satisfactory.

In the present study it was demonstrated that application of the sample as a row instead of a spot decreased the variability in the ratio peak area/amount of lipid, when the densitometric peak area was determined planimetrically. This is possibly due to more easy centering of the slit on the row. A more linear relation between peak area and quantity of lipid was obtained by multiplying the peak area with a correction factor $(1 + 0.4\,E)$ as introduced by GOLDMAN AND GOODALL[7]. These authors performed a continuous correction during the scanning by means of a special integrating unit. In this study the maximum E-value for each peak was used. As long as the maximum O.D. (E) was below 1.0 this correction was sufficient, but for higher E-values a gradually larger factor would have been needed. However, the method has been developed mainly for analysis of human seminal plasma and 25–50 μl of this fluid seldom gives E-values greater than unity.

During the procedure there is inevitable loss of material. To correct for this an internal standard was added to the sample. This made it possible to calculate with good accuracy and precision the amount of lipid in both standard solutions and rat blood plasma. Amounts as small as 0.5 μg of lipid could be detected. Preliminary studies have also shown that analysis of 25–50 μl of human blood plasma gives results in close agreement with those obtained with an autoanalyser or standard chemical methods[15].

In Fig. 1 it was demonstrated that the slope of the regression line for area *versus* quantity was different for the various lipids. It is thus necessary to run a reference lane parallel to the experimental lanes.

The precision in determinations of free fatty acids was not as high as for the other groups of lipids. LOUIS-FERDINAND *et al.*[5] also found low reproducibility and significant mean differences for free fatty acids analysed by TLC compared with conventional chemical assay methods. The reason for this was not clear, but a breakdown of glycerides in the solution may be partly responsible.

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REFERENCES

1 R. J. KOMAREK, R. G. JENSEN AND B. W. PICKETT, *J. Lipid Res.*, 5 (1964) 268.
2 V. P. SKIPSKI, J. J. GOOD, M. BARCLAY AND R. B. REGGIO, *Biochim. Biophys. Acta*, 152 (1968) 10.
3 S. J. PURDY AND E. V. TRUTER, *Chem. Ind. (London)*, 11 (1962) 506.
4 M. L. BLANK, J. A. SCHMIT AND O. S. PRIVETT, *J. Amer. Oil Chem. Soc.*, 41 (1964) 371.
5 R. T. LOUIS-FERDINAND, D. G. THERRIAULT, W. F. BLATT AND M. MAGER, *Clin. Chem.*, 13 (1967) 773.
6 E. STAHL, *Dünnschicht-chromatographie. Ein Laboratoriumshandbuch.* Springer-Verlag, Berlin, Göttingen, Heidelberg, 1962.

7 J. GOLDMAN AND R. R. GOODALL, *J. Chromatogr.*, 32 (1968) 24.
8 M. S. J. DALLAS, *J. Chromatogr.*, 33 (1968) 337.
9 J. W. FAIRBAIRN AND S. J. RELPH, *J. Chromatogr.*, 33 (1968) 494.
10 A. KUKSIS, L. MARAI AND D. A. GORNALL, *J. Lipid Res.*, 8 (1967) 352.
11 J. BOBERG, *Clin. Chim. Acta*, 14 (1966) 325.
12 L. A. CARLSON, *J. Atheroscler. Res.*, 3 (1963) 334.
13 J. FOLCH, M. LEES AND G. H. SLOANE STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
14 W. J. DIXON AND F. J. MASSEY, *Introduction to Statistical Analysis*. McGraw-Hill, New York, 1969.
15 B. WIKLUND, unpublished results.