THE COMPARISON OF VARIOUS EVALUATION METHODS FOR PAPER CHROMATOGRAMS OF DERIVATIVES OF HIGHER FATTY ACIDS*

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(Received March 18th, 1963)

During our study of the fractions of cholesteryl esters and of glycerides in human sera in normal and in pathological conditions, paper chromatography was used. Our system permits differentiation between a series of cholesteryl esters and one of triglycerides. Various methods of detection were examined, which might also lead to quantitative estimation of the saturated and unsaturated derivatives by means of direct photometry.

MATERIALS AND METHODS

The isolation of cholesteryl esters and of glycerides was performed on columns of aluminum oxide. Direct chromatography of the serum extracts was not used, because the spots of some cholesteryl esters and of glycerides are overloaded.

5 ml of serum were extracted with a mixture of ethanol and ethyl ether (1:3) and the extract was dried under nitrogen. The lipids were re-extracted with light petroleum (three times with 5 ml) and applied to the Al_2O_3 column. Cholesteryl esters were eluted with 50 ml of carbon tetrachloride and glycerides with 50 ml of chloroform.

Both fractions were evaporated to dryness under nitrogen. The ester fraction was dissolved in 3 ml of chloroform and the fraction of glycerides in 0.5 ml of chloroform. The solutions were applied in amounts of 50 μl on sheets of Whatman No. 3 paper impregnated with paraffin oil[1].

As the mobile phase, a mixture of acetic acid–chloroform–paraffin oil (80:15:5; v/v/v) was used.

After chromatography with this mixture at room temperature the chromatograms were dried at 80–100° and detected by dipping in 1% aqueous potassium permanganate, then immediately washed in running water. The spots were brown on the white or slightly brownish background.

* A part of this paper was presented at the Conference on Paper Chromatography, Prague, June 21–22, 1961.

Fractions of cholesteryl esters and glycerides of normal human sera were used as reference standards. These fractions were isolated on an Al_2O_3 column as described above. These "normal" extracts were applied on the paper in amounts of 30, 50, 70 and 100 μl.

After detection the chromatograms of the standard mixtures were evaluated: A. In transmitted light; B. In reflected light; C. In transmitted light, after impregnation of the chromatograms with paraffin oil; D. By microphotometry of the negatives of the chromatograms on photographic film.

In all four cases, the densitometric (photometric) curves were registered automatically and evaluated both by measurement of the areas (five times using a polar planimeter; expressed in cm^2) and by measurement of the maximum density (peak heights in mm) of the curves.

In the first three cases the green filter from the Lange colorimeter was used. In cases A and C the densitometer "Chromatometer 3c" (B. Lange, Berlin) and in case B a reflecting densitometer of our own construction[2] was used. Registration was carried out using a mirror galvanometer (Multiflex MG 2, B. Lange) and a recording apparatus ("Nachlaufschreiber 2", B. Lange). Microphotometry was performed with a Czechoslovak recording microphotometer (Keramos, Brno).

The measured values were linearized as a semilogarithmic dependence between the maximum density or the area and the logarithm of the applied volume,

$$y = a + bx,$$

where y = maximum density in mm or area of the curve in cm^2,
x = logarithm of the volume v (μl) of the standard mixture ($x = \log v$),
a, b = constants of the calibration equation.

Semilogarithmic linearization has already been used in the photometric evaluation of chromatograms of many other substances[3] and in spot-tests[4].

The calibration equations were calculated by means of the least-square method.

From the differences between measured and calculated values the standard deviation of y, s_y, was calculated. In order to compare more closely the deviations for various derivatives, the interval Δ_v, corresponding to s_y in the mean value of x ($\bar{v} = 56.9$ μl), was calculated. The scheme of the calculation procedure is demonstrated in Fig. 1.

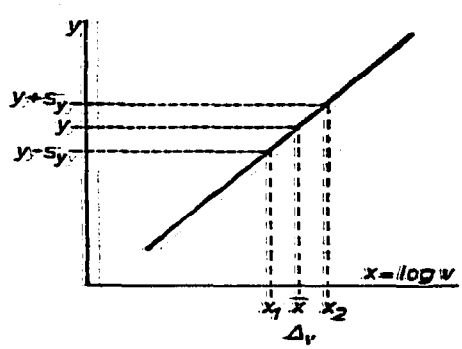

Fig. 1. Comparison of the accuracy of the calibration equations. s_y = Standard deviation of y from the equation $y = a + bx$; $x = \log v$. $s_y = \pm \sqrt{\frac{(y - \bar{y})^2}{n - 2}}$; y = Measured value (cm^2 or mm), $\bar{y}$ = Value calculated from calibration equation. v = Volume of standard mixture, in μl. $\bar{x}$ = Average value of x (corresponding value $v = 56.93$ μl). Δ_x = Interval corresponding to the values $Y + s_y$ and $Y - s_y$.

RESULTS AND DISCUSSION

At the beginning of this work, various methods were tried for separating and detecting saturated and unsaturated derivatives of the higher fatty acids. The methods examined did not give any satisfactory results for the saturated derivatives. The derivatives of unsaturated higher fatty acids (UHFA) were then examined. Densitograms of the chromatograms of glycerides of UHFA showed, however, that the chromatographic method used gave incomplete separation of these derivatives. In addition, the detection method proved unsuitable for quantitative analysis. None of the four methods mentioned gave results of a sufficient degree of accuracy due probably to lack of homogeneity in the coloration of the spots and of the background. The base line showed great variability along the length of the chromatograms. Because of this, the methods of analysis (mainly the detection) must be further perfected, and the results presented are valid only for the cholesteryl esters of UHFA.

The densitometric curves obtained with the four methods of photometry mentioned, evaluated according to their areas and according to their maximum densities, are summarized in Table II.

The relation between the measured values and the logarithms of the applied volumes is shown in Fig. 2.

The results achieved are to some extent surprising. Theoretically (*cf.* FALTA[5]), it could be presumed that the best results would be obtained by photometry of the impregnated chromatograms in transmitted light. However, this method was shown to be less accurate than the other three. The reason for this possibly lies in the

TABLE II

RESULTS OF THE DENSITOMETRY OF CHROMATOGRAMS OF CHOLESTERYL ESTERS

Method*	Ester**	Volume of the standard mixture (μl)				Volume of the standard mixture (μl)			
		30	50	70	100	30	50	70	100
		Height of peaks, in mm (maximum density)				Area of densitograms, in cm² × 5			
A	O	20	45	57	63	65.5	200.3	224.9	291.8
	L	69	88	99	93	420.0	610.3	772.0	771.0
	A	29	42	59	63	111.0	117.1	211.9	222.9
B	O	33	50	60	83	115.2	25.2	361.5	415.7
	L	70	109	132	150	433.4	773.1	977.3	1114.8
	A	33	57	76	93	113.4	24.5	277.3	322.5
C	O	18	24	46	56	44.1	66.7	183.4	222.3
	L	64	74	132	162	300.4	433.8	683.9	900.9
	A	37	17	60	78	9.7	2.1	233.0	244.2
D	O	27	45	67	78	110.5	119.3	311.8	361.4
	L	75	89	153	174	355.9	579.6	922.5	1106.4
	A	29	42	80	96	8.4	111.7	258.7	355.2

* A = Densitometry in transmitted light without impregnation of chromatograms; B = Densitometry in reflected light; C = Densitometry in transmitted light; chromatograms impregnated with paraffin oil; D = Microddensitometry of photographic negatives of the chromatograms.

** Abbreviations: O = cholesteryl oleate; L = cholesteryl linolenate; A = cholesteryl arachidonate.

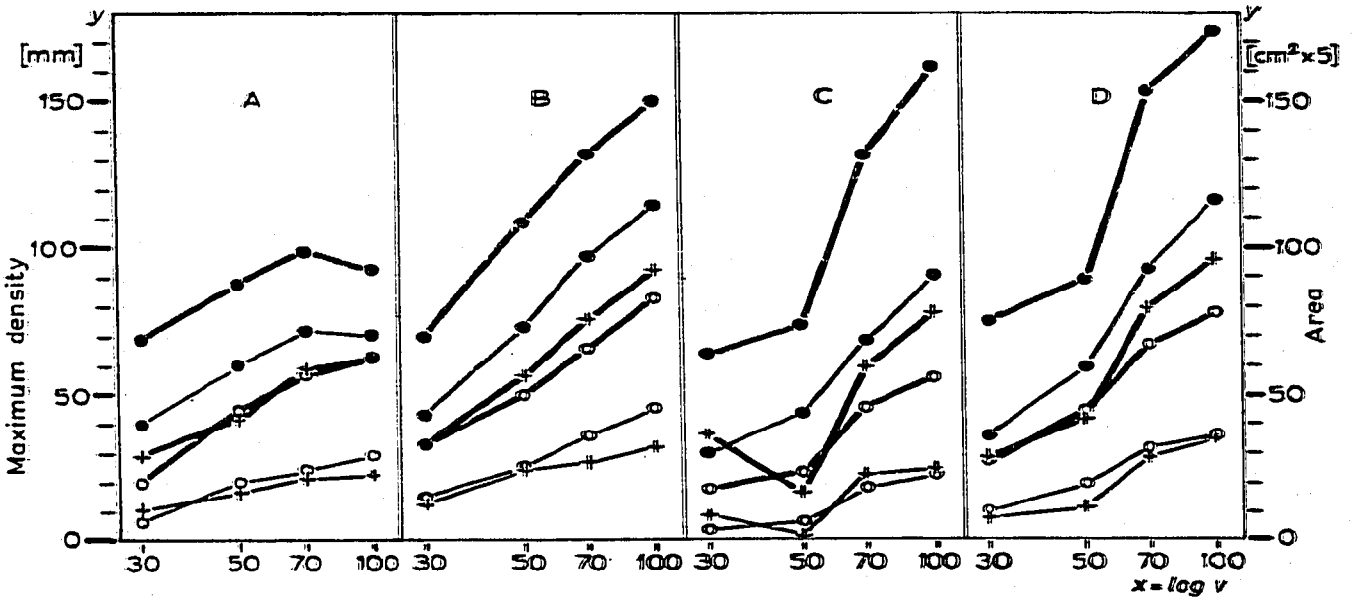

Fig. 2. Results of the photometry of chromatograms of cholesteryl esters. y = Measured photometric values; height of peaks in mm (measurement of maximum density) or area in $cm^2 \times 5$ (measurement of area enclosed by densitometric curves). x = Logarithm of volume of standard mixture applied to chromatograms. Thick lines = measurement of maximum density. Thin lines = measurement of area. Methods of densitometry are indicated by large capitals. Cholesteryl esters: O——O oleate; ●——● linoleate; +——+ arachidonate.

variation in texture of the paper used, which is more evident in transmitted light than in reflected light. The thinner papers (Whatman No. 4 and No. 1) are more homogeneous, but the separation was considerably worse than on Whatman No. 3 paper.

The impregnation procedure carried out prior to densitometry did not affect the colour of the detection reaction. The impregnated chromatograms were registered, extracted with light petroleum and registered again. The resulting densitograms were practically identical with densitograms obtained prior to impregnation.

From Fig. 2 it is evident that method B (measurement of reflectance) is better than the other three methods. This figure alone, however, cannot determine whether it is more accurate to evaluate the densitometric curves according to their areas or according to their maximum densities (*i.e.* peak heights). For this reason we calculated

TABLE II

CALIBRATION EQUATIONS FOR THE EVALUATION OF CHROMATOGRAMS OF CHOLESTERYL ESTERS IN REFLECTED LIGHT

$y = a + bx$; x = log v. y in mm (measurement of maximum density) or $cm^2 \times 5$ (measurement of area).

Ester*	*Measurement of maximum density*				*Measurement of area*			
	a	b	s_y (mm)	Δ_v (µl)	a	b	s_y ($cm^2 \times 5$)	Δ_v (µl)
O	−110.30	95.88	± 2.48	6.80	− 73.48	59.34	± 1.75	7.75
L	−155.95	154.50	± 3.77	6.39	−161.76	138.96	± 2.30	4.33
A	−138.42	115.75	± 1.12	2.64	− 38.18	35.67	± 1.74	11.63

* O = cholesteryl oleate; L = cholesteryl linoleate; A = cholesteryl arachidonate. Other explanations in text.

the calibration equations (the least-square method), s_y, and corresponding values Δ_v for both the measurement of areas and the measurement of maximum densities. The results, shown in Table II, demonstrate the greater accuracy obtained by measuring the maximum density. This method is also much faster than measuring the areas.

On the basis of these results we were able to evaluate the chromatograms of derivatives of UHFA from various sera. The data obtained (some of which have been published[6]) agree with those given in the literature.

The calculated values in μl were converted to weight units or to relative concentrations in the following manner:

If c_s is the concentration of the particular derivative in the standard mixture, then its weight amount in volume v is w:

$$w = v.c_s,$$
$$\text{and } \log w = (y - a)/b + \log c_s,$$
$$\text{or } \log w = (y - a')/b,$$
$$\text{where } a' = a - b \log c_s.$$

If the actual concentration is unknown, we can use the relative concentration (in %). In this case we obtain the results in terms of the relative concentrations.

Thus ZÖLLNER AND WOLFRAM[7] found the relative concentrations of cholesteryl esters in human serum to be:

oleate	28.4%
linoleate	57.1%
arachidonate	14.5%

In this manner we calculated from densitograms (evaluating the reflectance records according to their maximum densities) the concentrations of cholesteryl esters in sera of man and of various animals. Our results conform with the data of other authors obtained by other methods.

The method of photometry used, *i.e.* the photometric evaluation of chromatograms in reflected light, has four main advantages.

(1) It is possible to use thick paper (Whatman No. 3), which is more homogeneous in reflected than in transmitted light.

(2) The method is quick. With our densitometer[2] it is possible to record more than a hundred chromatograms per day.

(3) The method of evaluating the densitograms according to their maxima is faster than measuring areas, and, moreover, in our experiments this method proved to be more accurate than the latter.

(4) Measurement of maxima also allows the evaluation of chromatograms with incomplete separation of the spots.

SUMMARY

Paper chromatography was used in the determination of derivatives of higher fatty acids from biological material. The optimum conditions for quantitative evaluation by photometry of chromatograms were investigated.

The method used did not give any satisfactory resolution of saturated derivatives

and the results of the densitometry of the chromatograms of glycerides demonstrated that the method of chromatography and detection did not permit quantitative evaluation.

Cholesteryl esters of unsaturated fatty acids (cholesteryl oleate, linoleate and arachidonate) were separated to a sufficient degree.

For quantitative evaluation we compared four methods: (1) direct photometry of chromatograms in transmitted light, (2) direct photometry in reflected light, (3) photometry of chromatograms impregnated with paraffin oil, and (4) microphotometry of the photographic negatives of chromatograms.

The resulting densitograms were evaluated (a) according to the areas and (b) according to the maximum density of the curves. Of these four methods, direct photometry in reflected light with evaluation according to the maximum density gave the most accurate results.

The analytical procedure mentioned for the estimation of cholesteryl esters in sera gave results conforming with those obtained by other methods given in the literature.

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