Application of Thin-Layer Chromatography to the Quantitative Estimation of Tissue Triglycerides. I. Triglyceride Distribution in the Livers of Calf, Pig and Rat[1]

MADHU R. SAHASRABUDHE, Food and Drug Directorate, Ottawa, Canada

Abstract

A multiple technique (TLC-GLC) has been applied to the quantitative estimation of triglycerides in the livers of calf, pig and rat. The results are in general agreement with the triglyceride pattern calculated from the fatty acid distribution as suggested by Vander Wal (15).

Introduction

DURING RECENT YEARS considerable emphasis has been placed on the distribution of triglycerides in natural fats. Thin-layer chromatography (TLC) has been successfully applied to the separation of triglycerides by several workers (1–8). Application of the multiple technique employing TLC and GLC for the determination of glyceride structure has been demonstrated recently by Jurriens and Kroesen (9) and by Blank et al. (10). In this report, results of an investigation on the triglyceride distribution in livers of calf, pig and rat are presented.

Experimental

Fresh livers of pig and calf were obtained from the local slaughterhouse. Thirty rats of Wistar strain from Food and Drug Colony were maintained on a Commercial diet (protein 20%, fat 7%), for 4 weeks after weaning. At the end of this period the animals were anaesthetised with chloroform and bled through the abdominal aorta. Livers were excised and pooled in groups of 5 for analysis.

Extraction of Lipids

Thirty to 40 g of tissue was extracted with methanol-chloroform as described by Bligh and Dyer (11). The separated chloroform solution of the lipids was evaporated to dryness on a rotary evaporator. The lipids were then dissolved in 10 ml of acetone to remove a major portion of the phospholipids and the neutral lipid solution evaporated to dryness.

Fractionation of Lipids

The lipids were fractionated by TLC on 500 μ silica gel-G plates with benzene and ethyl ether (99:1). The lipids were dissolved in chloroform (1:1) and applied in a uniform streak with an Agla brand micrometer syringe assembly mounted on a movable stage (12). After the plate was developed and dried, the silica layer containing the triglyceride fraction was scraped off and extracted with ethyl ether. In order to accumulate a sufficient quantity of triglyceride, 4 plates were used for each analysis. This triglyceride fraction was used for further analysis of (a) fatty acids by GLC and (b) triglyceride classes by TLC.

Methyl esters were prepared by the conventional procedure using HCl-methanol. Fatty acids in the 2-position of the glyceride were estimated by the micro modification of the lipase hydrolysis technique (13).

A Perkin-Elmer model 800, Gas Chromatograph equipped with dual six feet, 1/8 in. stainless steel columns packed with 6% butane-diol-succinate on Chromosorb W, and with flame ionization detector was programmed at 120C to 190C at 4C/min.

Triglyceride Separation by TLC

The triglycerides were separated on silica gel-G impregnated with 12% silver nitrate (w/w) into different classes based on unsaturation (3). Quantitative estimation of the spots was obtained by (a) densitometry and (b) fatty acid analysis of the separated fractions by GLC.

Densitometry

Five to 20 μl of sol were applied on 100 μ silica gel-G-$AgNO_3$ plates and developed in benzene + ether (10:1). The spots were made visible by charring with H_2SO_4 on a hot plate. Spot densities for each application were recorded on a Photovolt Densitometer equipped with a Varicord Recorder. Percent distribution was calculated from the areas under each peak.

Fatty Acid Analysis

Two hundred to 500 μl triglyceride sol in chloroform (1:1) was applied on a 500 μ silica gel-G-$AgNO_3$ plate in a uniform streak. A selected standard mixture was also applied as a spot at one end of the plate. After development, the plate was dried and covered with a paper leaving a 2 cm vertical strip of plate exposed. The exposed surface which contained the standard was sprayed with dibromo (R) fluorescein. With the help of the applied standards, the plate was divided into 6 horizontal areas corresponding to the degree of unsaturation. The entire horizontal band corresponding to each degree of unsaturation was then scraped off and the triglycerides extracted with ether. The fractions were then analysed for (a) total fatty acids and (b) fatty acids in the 2-position. Relative quantities of each fraction were calculated by adding to the methyl ester solution, a known volume of methyl

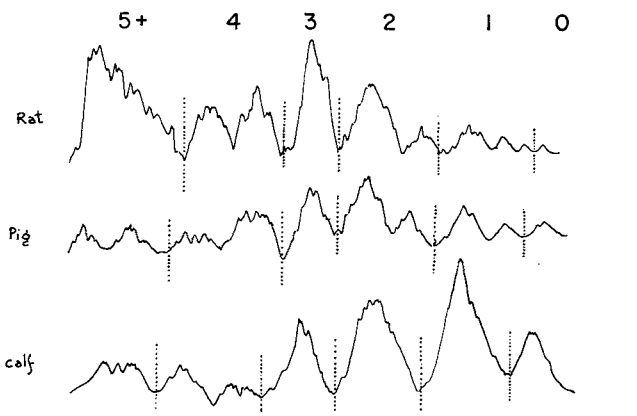

FIG. 1. Selected densitometric curves ($AgNO_3$- TLC) showing triglyceride separation by degree of unsaturation. Numerals on top represent the number of double bonds. 5+ indicates triglycerides with 5 or more double bonds.

[1] Presented at the AOCS meeting in Houston, Texas, April 1965.

TABLE I

Fatty Acid Distribution in Liver Triglycerides

Fatty acids	Calf		Pig		Rat	
	a	b	a	b	a	b
$C_{12:0}$	t	t	0.2	0.6	0.6	0.3
$C_{14:0}$	2.6	1.5	1.1	0.9	0.5	1.0
$C_{15:0}$	2.6	t	t	t	—	—
$C_{16:0}$	38.7	19.1	26.5	40.0	20.8	9.7
$C_{17:0}$	0.8	0.9	0.2	0.1	0.2	t
$C_{18:0}$	3.8	2.4	9.6	2.9	2.8	0.8
$C_{20:0}$	0.2	0.2	0.3	0.2	0.2	t
$C_{14:1}$	0.3	0.9	t	t	0.5	0.4
$C_{16:1}$	6.0	7.8	3.4	3.6	0.9	0.4
$C_{17:1}$	—	—	0.2	t	0.4	t
$C_{18:1}$	42.1	57.0	39.4	35.0	25.5	26.0
$C_{20:1}$	0.1	t	0.2	t	0.5	0.2
$C_{18:2}$	2.3	4.6	15.6	15.4	41.8	58.1
$C_{18:3}$[1]	2.4	5.3	0.2	0.2	1.1	0.6
$C_{20:4}$	0.2	0.2	3.2	1.1	4.4	1.5

a = total fatty acids mole %.
b = Fatty acids in 2-monoglycerides mole %.
[1] includes C $_{16:3}$ and $C_{20:3}$.
1 includes $C_{16:3}$ and $C_{20:3}$.

pentadecanoate as an internal standard. A special methylation flask (14), in which the volume of the methyl ester solution can be measured, was used for the purpose. The methyl esters were made up to a constant volume in the flask and the same quantity of standard was added for all analyses. Triglyceride composition for individual fractions having 0 to 3 double bonds was calculated from the fatty acid distributions in 1, 3 and 2 positions as suggested by Jurriens and Kroesen (9).

Results and Discussion

Fatty acid distribution in the total triglycerides of calf, pig and rat livers is shown in Table I.

The glyceride composition was calculated from the overall fatty acids and those in the 2-position (Table I) as suggested by Vander Wal. These values are presented in Table II along with those calculated from individual fractions. In the last fraction, because the separation was not clearcut, all glycerides containing 5 double bonds and more were pooled. The 6 fractions correspond to the total number of double bonds. Values for all saturated acids are grouped together for the sake of convenience. The major saturated acid in all liver fats was $C_{16:0}$. Monoenoic (M) fatty acids represent all fatty acids with one double bond from $C_{14:1}$ to $C_{22:1}$. Similarly dienoic (D) and trienoic (T) include all fatty acids with 2 and 3 double bonds, respectively. Remaining fatty acids with 4 double bonds in which $C_{20:4}$ is the major constituent are grouped in 'X'.

The calculations are based on the assumptions that (a) each fraction is exclusively composed of triglycerides having the same number of double bonds and (b) that the saturated acyl groups found at 1 and 3

TABLE II

Glyceride Type distribution in Liver Fats

		Calf			Pig			Rat		
		A	B	C	A	B	C	A	B	C
0	SSS	8.1	8.9	10.8	5.3	5.4	5.4	1.1	0.9	1.5
1	SSM	11.1	13.5		14.0	13.2		2.0	2.5	
	SMS	22.1	18.1		4.5	4.3		2.8	2.0	
		33.2	31.6	32.3	18.5	17.5	16.8	4.8	4.5	4.8
2	SMM	30.3	28.4	30.4	12.0	13.2	20.3	4.8	5.0	4.5
	MSM	3.8	4.6		9.2	8.0		0.9	1.1	
	SSD	0.3	0.4	2.1	4.8	5.0	10.1	2.4	2.5	9.0
	SDS	1.5	1.2		1.8	1.8		5.9	5.8	
		35.9	34.6	32.4	27.8	28.0	30.3	14.0	14.4	13.5
3	SMD	0.9	1.2	3.0	4.0	3.9	13.7	5.7	6.2	15.4
	SDM	2.1	1.5		4.8	4.6		10.4	8.6	
	MSD	0.3	0.3		6.3	6.1		2.1	1.6	
	MMM	10.4	9.8		7.9	8.0		2.1	2.0	
	SST	0.3	0.3	12.0	++	++	8.2	0.1	0.1	5.6
	STS	1.8	1.6		+	+		++	++	
		15.8	14.8	15.0	23.0	22.6	21.9	20.4	18.5	20.0
4	MMD	0.6			5.5			5.1		10.6
	MDM	0.7		1.5	3.2		9.7	4.5		
	SDD	++			0.8			12.4		
	DSD	+			1.1			1.3		12.4
	MST	+			++			0.1		
	STM	2.4			++			0.1		
	SMT	0.7			+		1.2	0.3		
	SSX	+		2.5	1.3			0.1		
	SXS	++			0.1			0.1		
		4.5	6.0	3.5	12.0	11.5	10.9	24.3	25.0	24.0
5	MDD	+			2.1			10.8		16.1
	DMD	+			0.9			3.0		
	SMX	+		2.3	1.2		6.0	1.0		
	SXM	++			0.4			0.1		
	MSX	+			1.8			0.4		
	DDD	+			0.4		8.7	6.5		20.1
	Others	1.8		3.7	6.6			13.6		
		2.5	4.1	6.0	13.5	15.0	14.7	35.4	37.2	36.2

A, Calculated according to Vander Wal.
B, Calculated from fatty acid analysis of fractions.
C, Densitometry.
S = Saturated, M = Monoenoic, D = Dienoic, T = Trienoic, X = Others.
+ = less than 0.05, ++ = 0.05 to 0.10.

positions are randomly distributed in these positions. Some minor corrections of up to 1% were necessary in the fatty acid analysis of individual fractions in order to conform to the first assumption. Fatty acid distribution in individual fractions with 4 and more double bonds, separated on silica gel-G-$AgNO_3$ plates is shown in Table III. For fractions with 4 double bonds and over, no attempt was made to calculate the different glyceride types. Fatty acid distribution values were computed from the glycerides as calculated by Vander Wal procedure (Table II, column A) and found to correspond remarkably well with those obtained from the analysis of the fractions (Table III).

Jurriens and Kroesen (9) calculated the relative quantities in the fractions by glycerol analysis. The present results are based on the addition of a known standard of the methyl esters. This procedure was found to be simpler and less time-consuming. Similar observations were made by Blank et al. (10).

TABLE III

Fatty Acid Distribution in Selected Triglyceride Fractions

Fatty Acids	Pig				Calf				Rat			
	Found		Calculated[1]		Found		Calculated		Found		Calculated	
	4 double bonds											
	A	B	A	B	A	B	A	B	A	B	A	B
S	15.9	20.2	13.3	20.2	24.6	2.1	22.4	0.7	19.6	6.4	20.9	7.3
M	56.3	45.1	48.5	45.5	41.3	28.0	43.7	16.8	26.8	18.4	27.0	22.0
D	22.4	33.0	34.3	35.0	10.8	10.8	12.6	21.3	52.6	74.4	50.7	68.8
T	0.6	0.4	0.4	0.5	22.6	53.6	20.6	59.4	0.7	0.4	0.7	1.2
X	4.6	1.22	3.8	0.8	0.6	0.3	0.6	1.7	0.3	0.4	0.7	1.2
	5 or more double bonds											
S	10.2	22.1	12.4	19.9	5.0	1.0	4.6	0.6	6 9	3.2	4.6	2.5
M	35.2	33.2	33.5	34.6	61.1	46.5	59.0	35.6	22.0	19.9	26.3	28.8
D	36.1	38.5	34.5	36.1	9.6	4.0	8.6	6.3	56.2	65.9	58.2	60.1
T+X	18.5	6.2	19.6	9.3	24.3	48.5	27.6	57.5	15.0	10.9	10.9	8.6

[1] = Computed from Vander Wal distribution.
A = mole % of total fatty acids; B = mole % fatty acids in 2-monoglycerides.
S = saturated; M = Monoenoic; D = Dienoic; T = Trienoic; X = others.

Results obtained from the densitometry of the spots are remarkably close to those obtained from fatty acid analysis of the fractions. Figure 1 shows three selected patterns obtained by densitometry. The values in Table II, column C, are averages of 5 to 6 separate determinations made on each plate. The variations in each set ranged from 5% to 10%. Values obtained from densitometric curves represent groups of glycerides rather than one particular type. From the analytical results, it can be concluded the fatty acid distribution in liver fats follows the mathematical pattern of 2 random, 1-3 random, suggested by Vander Wal (15). Some differences were noted in the distribution of SSM and SMS and also between SMM and MSM, yet, the total amounts of glycerides in each fraction were not significantly different.

Jurriens and Kroesen (9) also found a similar agreement with the calculated values in lard, cocoa butter and palm oil. The results on rat liver triglyceride composition in rats presented here are in variance with these reported by Blank et al. (10). The only possible explanation appears to be the differences in the dietary fat and/or strains of experimental animals.

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Application of Thin-Layer Chromatography to the Quantitative Estimation of Tissue Triglycerides II. Influence of Methyl Parathion on the Composition of Liver Triglycerides in the Rat[1]

M. R. SAHASRABUDHE and SHEILA I. READ, Food and Drug Directorate, Ottawa, Canada

Abstract

Methyl parathion fed at 10 ppm in a high protein low fat diet inhibited 46.9% of the total liver carboxylesterase activity. The total fatty acid composition of liver triglycerides was not significantly altered. However, the methyl parathion-fed rats showed a higher percentage of saturated acids in the 2-position of the glyceride molecule. Triglyceride analysis employing the multiple TLC-GLC technique (5) also showed a slightly higher percentage of saturated glycerides and those containing 1 and 2 double bonds than those in the control group. Triglyceride patterns in both groups were in general agreement with those calculated from the fatty acid distribution as suggested by Vander Wal (11).

IN A SERIES OF STARCH GEL electrophoretic studies McKinley and Read (1,2) demonstrated that organophosphorus pesticides inhibit liver carboxylesterases. Since some of the carboxylesterases may act as lipases the authors believe that the presence of an organophosphate might upset the enzyme balance influencing the triglyceride synthesis in the liver.

With the multiple technique employing thin-layer chromatography (TLC) and gas chromatography (GLC) reported by Jurriens and Kroesen (3), Blank et al. (4) and Sahasrabudhe (5) it has been possible to quantitatively determine the glyceride structure of tissue fats.

The experiments reported here were designed to investigate the influence of methyl parathion on the liver triglycerides in the rat.

Experimental

Weanling male rats of an inbred Wistar strain of the Food and Drug colony were divided into 2 groups and housed in individual cages. The two groups were fed ad libitum for 4 weeks a commercial diet containing 20% protein and 4% fat supplemented with 3% corn oil. One diet contained in addition 10 ppm methyl parathion. The pesticide was dissolved in corn oil and dispersed in the diet. At the end of the experimental period, the rats were anesthetised with chloroform and bled through the abdominal aorta. The livers were then excised, pooled in groups of 5 each, frozen at −70C and stored at −20C. The study was carried out in 2 separate series with 30 animals each.

Carboxylesterase Analysis

Representative samples were taken from each group. Liver tissues were homogenized and prepared for analysis as described by McKinley and Read (1). Quantitative estimation of the carboxylesterases was carried out according to the method of Main et al. (6) using a 1:25 dilution of the supernatant and o-nitrophenyl butyrate as the substrate. Liver carboxyl-

[1] Presented at the AOCS meeting in Houston, Texas, April, 1965.