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Extractions and Determination of Polyunsaturated Fatty Acids in Small Amounts of Plasma

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Introduction

This paper is chiefly concerned with the analysis of small amounts of plasma (1 ml.) for polyunsaturated fatty acid content. Until recently most fatty acid analyses were largely dependent upon isolation of the acids and subsequent oxidation and titration (1). The early spectrophotometric methods were not practical for plasma lipides because of the amount of sample required: 100–200 mg. (2, 3). Also they were inadequate for plasma lipide analysis because certain highly unsaturated fatty acids were shown to be present, for which no spectrophotometric constants were available. Thus the papers of O'Connell, Lipscomb, and Daubert (4) in 1952 and Wiese and Hansen (5) in 1953 were subject to error. They reported the analysis of fatty acids of blood for the di-, tri-, and tetraenoic acids, but the influence of hexaenoic and pentaenoic acids on the spectral absorption values reported was not considered. In 1953, Herb and Riemenschneider (6) published a spectrophotometric method that required 1–10 mg. of fat sample for determination of the polyunsaturated acids including pentaenoic acid. The method was later extended by Hammond and Lundberg (7) to include hexaenoic acid. Holman (8) has recently described a micromethod in which 5–10 ml. plasma was extracted with ethanol and ether (3:1) and the final concentrate was made up to 10 ml. volume. A 1-ml. aliquot was used for

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isomerization which is equivalent to 0.5–1 ml. plasma.[2] The method included spectral constants for hexaenoic and pentaenoic acids. Most workers have used ethanol–ether mixtures (3:1) originally proposed by Bloor (9, 1) for extracting lipides from plasma. Other solvents have been suggested. The use of tetrahydrofurane in the ratio of 20:1 of plasma has been reported (10) to be a good solvent for lipide extraction. Fillerup and Mead (11) used methylal (dimethoxymethane) and methanol mixture (4:1) as indicated by Delsal (12) and reported that it appeared to be better than Bloor's solvent because of its reproducibility and higher yields of lipides.

The present paper describes the results of a comparative study of Bloor's and Delsal's solvents for extraction of lipides, and of the spectrophotometric method for determining the individual polyunsaturated acids from 1 ml. plasma. Further comparison of these results was made with analysis of fatty acids derived from lipides obtained by extraction of 50 ml. of the same plasma.

Experimental Methods

Solvents

All individual components of the solvent mixtures were distilled through efficient columns and their absorbances determined through 1-cm. cells against distilled water. Solvents having approximately the following absorbances at the indicated wavelengths should be satisfactory.

Wavelength	Methanol	Methylal	Isooctane[a]	Ethanol	Ether
220	0.210	0.560	0.333	0.175	0.720
233	0.070	0.262	0.037	0.062	0.252
268	0.00	0.00	0.00	0.00	0.015

[a] Isooctane = 2,2,4-trimethylpentane.

Plasma

The plasma samples were prepared according to Evans *et al.* (13) and were stored at −22°C. in small vials for future analysis.

Methods

(*a*) *Micro.* An accurately measured or weighed sample of plasma (0.5–1 ml. or 0.5–1 g.) was added dropwise to 15 ml. of Delsal solvent (methylal–methanol,

[2] We are informed through correspondence that Holman and co-workers have used a micromethod on as little as 1 ml. plasma or 500 mg. tissue and found this adequate. When samples are small they make up the extracts to 3 or 5 ml.

4:1) in a 50-ml. centrifuge tube with mechanical stirring. After stirring for 30 min.[3] the solids were separated by centrifugation and the clear solution was transferred to another 50-ml. centrifuge tube. The solids were extracted again with 10 ml. of Delsal solvent. After the first extract was taken to dryness in a water bath at 60° in a stream of nitrogen, the clear solution from the second extraction was added to this lipide residue and taken to dryness. This residue was next extracted by adding approximately 10 ml. isooctane and warming on the water bath at 60° for 3 or 4 min. The extraction was aided by scraping the residue from the sides of the tube and mixing with the isooctane by means of a small stainless steel spatula. After centrifugation of the isooctane extract, the clear solution was transferred to a 25-ml. volumetric flask. The residue was extracted twice more with small portions of isooctane, and the combined extracts were made up to 25 ml. volume and thoroughly mixed. Absorbances (spectral densities) at 233, 268, 315, 346, and 374 mμ were determined (before isomerization). Absorptivities (a_0) were calculated at these wavelengths.

$$a_0 = A/cb$$

where A = absorbance; c = milliliters (or grams) of plasma represented by 1000 ml. of lipide solution; and b = length in centimeters of light path through solution in absorption cell.

The isooctane solution of lipides after measuring initial absorbance was transferred quantitatively from the absorption cell and the volumetric flask to a 50-ml. round-bottom flask and taken to near dryness (5 ml.) on the water bath at 60°C. in a stream of nitrogen. This concentrate was then transferred in 2–3-ml. increments with a dropping pipet to an isomerization tube (6) which was placed in a water bath at 60°C., and the solvent was removed under nitrogen. (At least three 2-ml. washes with isooctane were used to transfer the lipides from the round-bottom flask to the isomerization tube, at no time allowing the volume of solution in the isomerization tube to exceed about 3 ml.) When the lipide residue appeared dry, 2 g. (±0.02 g.) of 21% KOH–glycol reagent[4] was added to the isomerization tube containing the residue and to another tube as a blank. The tubes were flushed continuously with nitrogen as described (6) and placed in the isomerization bath at 180°C. for 1 min. and shaken for 5 sec. in the bath with a back-and-forth motion. After 30-sec. intervals the shaking was repeated twice. The total heating time was 16 min., after which the tubes were removed from the bath with nitrogen flowing, cooled in cold water, and the contents transferred quantitatively to 25-ml. volumetric flasks with methanol and diluted to mark.

Absorbances were measured in the analytical regions as in the regular method (6), and the absorptivities were calculated at 233, 268, 315, 346 and 374 mμ again

[3] The Bloor extraction was the same throughout as the Delsal except the initial extraction was limited to 15 min.

[4] Repeated analysis on 1–10-mg. samples of different fats in which isomerizations were conducted with 2 g. and 5 g. of 21% KOH–glycol showed good agreement.

referring c to the milliliters (or grams) of plasma represented by 1000 ml. of lipide solution.

$$a' = A/cb = \text{absorptivity after isomerization.}$$

The initial absorptivity a_0 was then subtracted from a' to give a_c';

Corrected absorptivities (a_c'), multiplied by 1000 were used in place of a, in the following formulae (6, 7) for calculation of the polyunsaturated components in terms of mg./100 ml. (or 100 g.) plasma.

$$\text{Hexaenoic acid} = 3.41a_{374}$$

$$\text{Pentaenoic acid} = 1.449a_{346} - 1.353a_{374}$$

$$\text{Tetraenoic acid} = 1.650a_{315} - 1.667a_{346} - 0.079a_{374}$$

$$\text{Trienoic acid} = 1.105a_{268} - 0.879a_{315} + 0.190a_{346} - 1.251a_{374}$$

$$\text{Dienoic acid} = 1.092a_{233} - 0.573a_{268} - 0.259a_{315} - 0.033a_{346} - 0.260a_{374}$$

(*b*) *Macro.* The macro method of extraction was similar to that already described except that a 50-ml. sample of plasma was used. The ratio of solvents to sample was the same for both methods. After obtaining the isooctane extract the solvent was removed and the lipide residue was saponified. The unsaponifiables were removed and the free fatty acids were recovered and weighed. About 10 mg. of the fatty acids was weighed and isomerized with 5 g. of 21% KOH–glycol reagent, and the polyunsaturated acid content was determined (6). Excellent agreement in results was also obtained when 2 g. of 21% KOH–glycol reagent was used with 5–7-mg. samples of fatty acids. Data were expressed as mg./100 ml. plasma.

Results

In Table I are shown the results of the analysis of beef plasma for polyunsaturated acids by the micro method. The individual determinations made by three different workers show good reproducibility. A comparison is also made between the Delsal solvent and the Bloor solvent concerning the polyunsaturated acid content of the plasma, the use of the latter solvent resulting in slightly higher values for each polyunsaturated fatty acid.

A comparison of results by the micro method and the macro method in which both solvents were employed is shown in Table II. The results by the micro method with either solvent were somewhat higher than those by the macro method. In the spectrophotometric analysis of the fatty acids obtained in the macro extraction, a slight peak in the hexaenoic region (374 mμ) was observed, but was not detected in the analyses by the micromethod. The average values for the total poly-

TABLE I

Polyunsaturated Acids of Beef Plasma (Micro Method)[a]

Acid	Method of lipide extraction							
	Delsal solvent					Bloor solvent		
	mg./100 ml.					*mg./100 ml.*		
	A[b]		*B*		*C*			
Dienoic	44.0	43.8	42.4	42.3	43.3	44.7	45.0	44.9
Trienoic	4.18	4.16	3.95	4.04	3.1	5.4	6.1	5.2
Tetraenoic	11.89	11.96	11.08	10.33	12.5	12.9	13.1	13.2
Pentaenoic	4.05	3.95	4.16	4.12	3.1	6.1	6.4	6.1

[a] One-milliliter sample.
[b] Analysts, *A*, *B*, and *C*.

TABLE II

Micro[a] *vs. Macro Method for Polyunsaturated Acids of Beef Plasma*

Acid	Method of lipide extraction					
	Delsal solvent			Bloor solvent		
	mg./100 ml.			*mg./100 ml.*		
	Micro	Macro		Micro	Macro	
	a[b]	*b*	*c*	*a*[c]	*b*	*c*
Dienoic	43.2	39.9	40.2	44.9	39.4	38.6
Trienoic	3.9	3.5	3.7	5.6	4.2	4.3
Tetraenoic	11.5	9.7	10.2	13.1	9.5	10.5
Pentaenoic	3.9	2.6	3.4	6.2	2.4	2.7
Hexaenoic	—	0.9	1.8	—	1.0	1.3

[a] One-milliliter sample.
[b] Average of five determinations; *b* and *c* are single determinations.
[c] Average of three determinations.

unsaturated acids by the micro method, employing Bloor's solvent, were consistently higher than those by the Delsal solvent.

Table III contains a series of single micro determinations for the analysis of rabbit plasma that show good agreement throughout, for

TABLE III

Polyunsaturated Acids of Rabbit Plasma (Micro Method)[a]

Acid	Method of lipide extraction						
	Delsal solvent				Bloor solvent		
	mg./100 ml.				*mg./100 ml.*		
	a[b]	*b*	*c*	*d*	*a*	*b*	*c*
Dienoic	31.4	30.9	31.7	30.4	33.6	33.1	32.6
Trienoic	3.6	3.0	3.1	2.8	3.5	4.5	4.2
Tetraenoic	5.0	4.6	4.9	4.6	5.2	5.3	5.1
Pentaenoic	2.0	1.7	1.7	1.3	2.7	3.2	3.5

[a] One-milliliter sample.
[b] *a*, *b*, *c*, and *d* are single determinations.

TABLE IV

Polyunsaturated Acids of Human Plasma[a]

Acid	Method of lipide extraction				
	Delsal solvent		Bloor solvent		
	mg./100 ml.		*mg./100 ml.*		
	a[b]	*b*	*a*	*b*	*c*
Dienoic	61.6	62.1	61.2	58.0	59.5
Trienoic	4.8	4.5	7.5	7.1	8.6
Tetraenoic	17.9	19.0	18.2	19.7	19.1
Pentaenoic	3.6	3.8	4.1	4.2	4.1
Hexaenoic	7.2	7.8	8.9	7.9	7.5

[a] Sample, 0.8 ml.
[b] *a*, *b*, and *c* are individual determinations.

both solvents. However, again, the results for the Bloor solvent tend to be slightly higher.

Table IV is similar to Table III except that the determinations were made on human plasma. These results reveal considerable amounts of hexaenoic acid in human plasma. The results with both solvents are in good agreement but those with the Bloor solvent again are higher except for the dienoic acid.

Discussion

The lower results by the macro method may be attributed at least partly to slight losses of fatty acids resulting from the saponification, removal of unsaponifiables, and extraction and washing of the fatty acids. It is generally observed that less than the theoretical amount of fatty acids is recovered by this treatment. In these saponifications of plasma extracts there was always some loss of polyunsaturated fatty acids to the unsaponifiable material amounting to a total of about 2–3 mg./100 ml. as determined spectrophotometrically. The difficulty of obtaining complete saponification of blood lipides has been pointed out by others (14, 15).

Differences in results between the micro method with Delsal solvent, and the macro method can be accounted for by consideration of the losses noted above. Thus it appears that the analyses by the former are substantially correct. The results with the Bloor solvent and the micro method, appear to be slightly high, possibly due to an increase in the spectral absorption of the nonfatty acid portion of the lipide as a result of the isomerization treatment.

Summary

1. A spectrophotometric micro method for the analysis of the polyunsaturated fatty acids in 0.5–1 ml. of plasma has been described.

2. Comparisons based on polyunsaturated fatty acid analyses by the micro method were made with Delsal solvent and Bloor solvent for extraction of lipides from plasma.

3. The analyses by the micro method with Delsal solvent were found to be in better agreement with analyses of fatty acids obtained from large-scale extraction of plasma lipides.

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