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RELIABLE MEASUREMENT OF NON-ESTERIFIED LONG-CHAIN FATTY ACID PATTERN IN BLOOD PLASMA

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SUMMARY

In this reliable assay for determining the non-esterified long-chain fatty acid pattern in plasma, only 100 μl of sample are needed and a single assay can be done within 40 min. The isolation procedure was performed by adsorption of fatty acids from plasma onto graphitized carbon black (Carbopack B) using a column method. After desorption and removal of the eluting phase, fatty acids are methylated by diazomethane and quantified by packed column gas chromatography. Analytical recoveries ranged between 91% and 103%. Within-run precision gave coefficients of variation of 2.3% and 11% for fatty acid concentrations of 58.2 and 0.6 μmol/l, respectively. Studies of plasma samples under various storage conditions indicated that reliable measurement of the non-esterified fatty acid fraction can be obtained even after 60 days if specimens are conserved at —18°C in the presence of a suitable phospholipase inhibitor.

INTRODUCTION

The pattern of non-esterified long-chain fatty acids (NEFA) in blood is of interest in a wide variety of biochemical and clinical investigations. All of the proposed analytical methods involve solvent extraction and gas chromatography of the methyl esters. Most of them are modifications of the procedures of Dole and Meinertz [1] or Folch et al. [2] which make use of time-consuming multiple purification steps to isolate the NEFA fraction from the lipid extract [3—6]. Recently, Mueller and Binz [7] described a rapid method without further manipulations with a chloroform—methanol extract of buffered (pH 6) serum. However, the sophisticated gas chromatographic apparatus proposed may discourage use of the method in other laboratories.

Recently, Carbopack B has been successfully used as an adsorbing medium

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for isolating acidic compounds [8—12] from blood serum and urine. We describe here a reliable procedure which makes use of 100 μl of plasma and employs Carbopack B for sample purification coupled with a conventional packed column for gas chromatography of NEFA methyl esters. The stability of the NEFA composition in plasma, serum and related extracts under different storage conditions was also investigated.

MATERIALS AND METHODS

Reagents

Solvents of analytical grade from various commercial sources were distilled twice in a glass system. Deionized water was distilled in the presence of permanganate. Diazomethane was generated from N-nitrosomethylurea [13] and the diethyl ether solution was stored at −18°C after addition of solid potassium hydroxide. Under these conditions, the solution is stable for at least two months, whereas working aliquots are stable for about one week if conserved at 4°C during the period of use. Benzenemethanesulphonyl fluoride (BMSF) was obtained from Fluka (Buchs, Switzerland) and a solution of 100 g/l in methanol was used. Carbopack B (80—120 mesh) was kindly supplied by Supelco (Bellefonte, PA, U.S.A.).

Standards

Fatty acid standards (puriss, > 99% GC) were from Fluka. The stock standards were 1 g/l in chloroform and were stable for at least six months at −18°C. For studies of recovery at low and high acid concentrations in serum, *n*-eicosanoic and arachidonic acids were dissolved in methanol—water (50:50, v/v) to give a concentration of 0.5 mg/l. This solution was further diluted to give a concentration of 0.1 mg/l. Two internal standard solutions were used. One was prepared by dissolving *n*-heptadecanoic acid in water—methanol (50:50, v/v) to give a concentration of 0.5 mg/l. The second solution was prepared by a five-fold dilution of the *n*-heneicosanoic acid stock standard. The reference standard solution was prepared by evaporating 10 μl of each individual fatty acid stock solution and derivatizing each residue. All standard solutions were stored at 4°C and replaced every two weeks.

Phospholipids were supplied by Supelco.

Glassware preparation

All the glassware was cleaned with hot chromosulphuric acid and rinsed thoroughly with distilled water.

Gas chromatography

A Model 3800 gas chromatograph equipped with a flame-ionization detector from Dani (Monza, Italy) was used. The glass colum, 2 m × 2 mm I.D., packed with GP 5% DEGS-PS on 100—120 mesh Supelcoport was from Supelco. The chromatographic conditions were: oven temperature 185°C; injection port and detector block temperatures 200°C. Nitrogen was used as carrier gas with a dead time of 30 sec.

Samples

Blood was drawn after overnight fast from apparently healthy volunteers by antecubital venipuncture. The blood samples were collected in centrifuge tubes containing ethylenediaminetetraacetic acid as anticoagulant, chilled immediately in an ice bath, stoppered and centrifuged at 4°C for 15 min at 2000 *g*. Plasma was promptly removed, divided into 100-μl aliquots which were collected in 6-ml air-tight screw-capped glass vials containing 3 μl of the BMSF solution and stored at −18°C if not processed within 1 h.

Procedure

Prepare the Carbopack B bed by introducing 0.25 g of the adsorbent into a 15 × 0.6 cm glass column with a small pledget of glass wool in the bottom. Wash the column with 5 ml of chloroform, 3 ml of methanol and 3 ml of distilled water, in sequence. Remove large bubbles by gently pumping distilled water onto the Carbopack B bed with the aid of a Pasteur pipette. Percolate the biological specimen previously diluted with 5 ml of the methanol—water (50:50, v/v) solution containing *n*-heptadecanoic acid as internal standard. Rinse the vial with two portions of 2.5 ml of 3 mmol/l hydrochloric acid solution and pass the rinsings through the column. Wash the column with 1.5 ml of methanol and elute the long-chain fatty acids with chloroform—methanol (70:30, v/v). Collect 3 ml of the eluate from the moment the eluting solution is applied to the column in a cone-shaped glass vial, add 10 μl of the *n*-heneicosanoic acid solution and evaporate the solvent under a stream of nitrogen at 50°C. Place the vial in an ice bath, add 3 μl of methanol, 30 μl of cold ethereal diazomethane and seal. After about 5 min, eliminate the excess reagents under a nitrogen stream at room temperature, dissolve the residue with 15 μl of chloroform and inject 1 μl.

Quantification was performed by peak height measurement since the fatty acid methyl ester peaks are sharp and symmetrical. To calculate the concentration of each fatty acid, the peak height relative to that of the internal standard is compared with that of the reference standard.

RESULTS AND DISCUSSION

Precision

The within-run precision was evaluated by assaying the same plasma sample nine times. The results are shown in Table I.

Recovery

Under the experimental conditions adopted, arachidonic and eicosanoic acids are, respectively, the first and the last compounds to be desorbed from the Carbopack column.

We determined the recovery of fatty acids from plasma by supplementing plasma samples of known NEFA content with known amounts of selected long-chain fatty acids and re-assaying. With respect to the extraction procedure, we evaluated the relative and absolute analytical recoveries by adding two internal standards, i.e. *n*-heptadecanoic acid and *n*-heneicosanoic acid, the first before and the second after the purification process. Results are given in Table II.

TABLE I

WITHIN-RUN PRECISION FOR THE ANALYSIS OF FATTY ACIDS IN PLASMA SPECIMENS (n = 9)

Fatty acid*	Retention time (min)	Mean ± S.D. (μmol/l)	C.V. (%)
$C_{14:0}$	1.8	3.9 ± 0.17	4.3
$C_{16:0}$	2.8	58.2 ± 1.34	2.3
$C_{16:1}$	3.2	4.1 ± 0.25	6.0
$C_{18:0}$	4.8	19.1 ± 0.63	3.3
$C_{18:1}$	5.4	50.0 ± 1.35	2.7
$C_{18:2}$	6.7	19.0 ± 0.80	4.2
$C_{18:3}$	8.8	4.6 ± 0.22	4.8
$C_{20:0}$	8.3	0.6 ± 0.07	11
$C_{20:4}$	15.3	1.7 ± 0.11	6.2

*The fatty acids are denoted by chain length:number of double bonds.

TABLE II

ABSOLUTE AND RELATIVE RECOVERIES OF FATTY ACIDS ADDED TO PLASMA SPECIMENS

Added (μmol/l)	Found (μmol/l)	Recovery (%)	
		Absolute	Relative*
Arachidonic acid			
81.2 (n = 6)	78.8	97	103
16.2	15.6	97	103
n-Eicosanoic			
79.1	67.2	85	91
15.8	13.7	87	93
n-Heptadecanoic			
92.6	86.3	93	

*Relative to n-heptadecanoic acid.

Specificity

Although diazomethane is a toxic compound, we found it could not be replaced as methylating reagent by methanolic acids or alkalies because of the problem of transmethylation by the latter reagents of any phospholipids which may be coeluted with the free fatty acids from the Carbopack column.

Endogenous lipids and substances present in solvents or Carbopack B were considered as possible interfering compounds.

Under the extraction conditions selected, triacylglycerols and cholesteryl esters were not eluted from the Carbopack B purification column, whereas some phospholipids were partly coeluted with free fatty acids. To ascertain the absence of hydrolysis or transesterification reactions occurring during the analysis, a plasma sample was divided into aliquots, one remained unsupplemented and every single other aliquot was supplemented with one of the following phospholipids: diarachidoylphosphatidylcholine, diarachidoylphos-

phatidylethanolamine, egg lysophosphatidylethanolamine, bovine sphingomyelin. These samples were then submitted to analysis in duplicate. In terms of individual concentrations of free fatty acids, we noted no difference between the blank and phospholipid-supplemented plasma samples.

When the chloroform washing of Carbopack B was omitted, the blank showed the presence of some background interfering peaks. Solvents used as supplied gave high blank values especially for palmitic, stearic and oleic acids. These interferences became negligible when the solvents were bidistilled.

Storage

Five fresh plasma samples from different subjects were each divided in two portions, one of which was supplemented with a phospholipase inhibitor such as BMSF [14]. Each portion was further fractionated into aliquots, stored at either −18°C or 4°C and periodically analysed over a period of 60 days. Table III shows that reliable measurements of free fatty acids in plasma can still be made within 60 days, provided the plasma samples are conserved at −18°C wih a phospholipase inhibitor.

TABLE III

EFFECT OF STORAGE ON PLASMA (n = 5) FATTY ACID CONCENTRATIONS

The data are expressed as percentage variation of the initial concentrations of oleic, linoleic and arachidonic acids which are 121 ± 40, 32 ± 12, 2.4 ± 0.7 μmol/l, respectively.

Temperature (°C)	No. of days	ΔC (%)					
		Oleic		Linoleic		Arachidonic	
		Mean	Range	Mean	Range	Mean	Range
+4	1	+7.5	+2.0 to +18	+24	+15 to +40	+33	+25 to +43
−18	1	+0.5	−3.0 to +3.8	+4.1	−0.5 to +8.0	+3.9	+0.4 to +6.8
−18	5	+6.8	+2.7 to +10.1	+11.4	+4.8 to +18.5	+24	+18 to +29
+4	1*	+1.1	−0.5 to +3.8	−1.5	−3.5 to +0.5	−3.3	−5.0 to −2.1
+4	7*	+4.5	−1.0 to +8.9	+17	+9.5 to +21	+39	+21 to +110
−18	60*	+3.6	+0.1 to +11	+3.6	−1.5 to +9.8	+3.1	−2.7 to +10.3

*Plasma samples supplemented with benzenemethanesulphonyl fluoride.

Some fractions collected after purification of plasma aliquots were not processed further but were stored at 4°C. No significant variation in the NEFA concentrations was observed on periodic analysis of the extracts over a period of two months. Moreover, we observed that extracts were stable even when they were left for one week at room temperature.

A mean 15% higher arachidonic acid concentration was measured when serum instead of plasma was submitted to analysis. Serum was obtained by allowing blood to clot for 1 h at room temperature. The higher amount can be explained by the fact that phospholipids can be hydrolysed to a variable extent and that arachidonic acid in its non-esterified form is particularly abundant in phospholipids.

Extraction column variables

A 50-fold dilution of plasma with methanol—water was essential to ensure quantitative recovery of NEFA. Considerable losses in the effluent, especially

of saturated fatty acids, occurred as the dilution ratio was decreased or water was used as diluting agent. This can be accounted for by the existence of a NEFA—protein complex.

Low recovery of fatty acids was observed when the washing step with acidic water was omitted. This can be ascribed to chemical binding between acidic compounds and some chemical heterogeneities present on the Carbopack surface [15]. However, the binding is readily hydrolysed by acidic washing of the purification column.

Methanol washing eliminates water and some potentially interfering compounds [12]. However, loss of arachidonic acid was observed when the volume of methanol exceeded 5 ml.

We evaluated the effect of the composition of the eluting phase on the recovery of fatty acids. When the chloroform/methanol ratio was decreased, saturated fatty acids were partially lost. A chloroform/methanol ratio higher than 70:30 enhanced the recovery of the last acid to be eluted from Carbopack, i.e. eicosanoic acid. However, under such conditions, other compounds were coeluted which caused the appearance on the chromatogram of peaks with high retention times, thus increasing the analysis time.

Under the experimental conditions used, the flow-rate of the solvents percolating through the Carbopack column was about 3 ml/min. As reported elsewhere [12], attempts to increase the flow-rate by exerting an additional pressure provoked loss of analytes in the plasma effluent.

To improve our procedure in terms of economy and speed, we evaluated the reusability of the purification column. After each extraction, the column was restored by washing it sequentially with 3 ml of chloroform, 3 ml of methanol and 3 ml of water. After five such extractions, the NEFA concentration was unchanged within the precision of the method. However, when the restored column was left unused and filled with water, we observed some increase in the unsaturated fatty acids, probably due to the slow hydrolysis of some phospholipids which are strongly retained by the Carbopack column. An analogous effect occurred when the purification process was casually interrupted after the acidic washing step.

TABLE IV

FATTY ACID CONCENTRATIONS IN PLASMA SAMPLES (n = 5) AS MEASURED BY THREE DIFFERENT PROCEDURES

Values are expressed in µmol/l.

	Our method		Mueller and Binz [7]		Hagenfeldt [3]	
	Mean	Range	Mean	Range	Mean	Range
$C_{16:0}$	83.9	58.1—106	79.4	51.3—104	115	71.4—154
$C_{16:1}$	5.6	4.1— 8.2	4.4	2.1— 7.6	15.4	7.0— 21.1
$C_{18:0}$	26.6	19.1— 38.9	24.5	17.6— 38.8	38.0	22.3— 61.6
$C_{18:1}$	99.0	65.3—122	103	64.4—127	132	80.2—191
$C_{18:2}$	30.3	18.0— 39.8	38.1	21.2— 46.9	58.7	37.2— 82.7
$C_{18:3}$	5.7	4.4— 6.4	5.4	3.2— 6.3	6.4	3.6— 9.8
$C_{20:0}$	0.8	0.6— 1.2	1.0	0.8— 1.2	1.3	1.0— 1.8
$C_{20:4}$	2.2	1.2— 4.0	3.9	1.4— 7.8	7.8	4.3— 13.1

We compared results using our method with those using two previously reported methods [3, 7]. Five fresh serum samples from different subjects were each divided into three aliquots, which were analysed in duplicate following the three extraction procedures. In all cases, final samples were injected into the chromatographic column proposed by us. The results are reported in Table IV. As can be seen, we found a fair agreement between our procedure and that described by Mueller and Binz [7], even though higher values for arachidonic acid were occasionally obtained by the latter procedure. However, we found the purification method reported by Mueller and Binz [7] unsuitable for quantification with a conventional-packed column as it appeared to be seriously deteriorated after about ten injections of the extracted and derivatized sample.

Surprisingly, Hagenfeldt's method [3] gave by far higher values for fatty acids, particularly for those that are relatively more abundant in phospholipids, such as linolenic and arachidonic acids. This could be explained by considering that this method includes an acidic extraction step which may provoke hydrolysis of phospholipids. To obtain experimental evidence for this hypothesis, a serum sample was divided into two aliquots; one was spiked with diarachidoylphosphatidylcholine leaving the other one unsupplemented. These samples were then submitted to analysis in duplicate according to the procedure of Hagenfeldt [3]. The large increase of arachidonic acid concentration in the free fatty acid fraction after addition of the phospholipid unequivocally confirmed the hypothesis.

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