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ANALYSIS OF TISSUE FREE FATTY ACIDS ISOLATED BY AMINOPROPYL BONDED-PHASE COLUMNS

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SUMMARY

Gas chromatographic analysis revealed that polyunsaturated fatty acids such as arachidonic acid and total tissue free fatty acids isolated from an aminopropyl bonded-phase column yield a two- to three-fold higher recovery of arachidonic acid as compared to those isolated from thin-layer chromatographic plates. This method was further improved by packing the aminopropyl bonded phase in glass columns, since the glass column significantly eliminated the other contaminants (from polypropylene columns) coeluting with fatty acids in both a neutral lipid thin-layer chromatographic system and on a 5% DEGS-PS column of gas chromatographic analysis. In aminopropyl bonded-phase columns, the standard triglycerides and phospholipids were completely separated from free fatty acids as judged by gas chromatographic analysis. These results warrant the use of an aminopropyl bonded-phase column for the isolation of free fatty acids to obtain better recovery of polyunsaturated fatty acids.

INTRODUCTION

The analysis of tissue free fatty acids (FFA) generally involves the following steps: (a) extraction of lipids; (b) separation of FFA from other lipids by thin-layer chromatography (TLC); (c) conversion of FFA to methyl esters; and (d) analysis of methyl esters by gas chromatography (GC) [1,2]. In order to prevent the loss of polyunsaturated fatty acids (PUFA) by oxidative reactions, an antioxidant such as butylated hydroxytoluene (BHT) is added during the extraction procedure [3]. Recently, FFA were separated in our laboratory using Sep-Pak™ columns and quantitated by high-performance liquid chromatographic (HPLC) techniques [4]. Inclusion of BHT during each step of analysis ensured better recovery of tissue free arachidonate (20:4) by HPLC analysis compared to the GC methods, which require separation of FFA by TLC prior to GC analysis [4].

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The Sep-Pak extraction method cannot be used in conjunction with GC analysis, because FFA coelutes with other neutral lipids such as triglycerides (TG) [5], making it unsuitable for GC analysis. Recently, Kaluzny et al. [6] have been successful in separating FFA from other neutral lipids such as TG and diglycerides (DG) on aminopropyl Bond Elut columns. However, the recovery and purity of lipids separated by this column were examined only by employing radioactive compounds and TLC technique [6].

In the present paper, we have used the aminopropyl bonded-phase column for the analysis of PUFA such as arachidonic acid (20:4) from the tissue lipids using GC. This method yielded a three-fold higher recovery of 20:4 as compared to the TLC method. The optimum conditions of bonded-phase aminopropyl column to analyze tissue FFA have also been examined.

EXPERIMENTAL

Materials

Aminopropyl Bond Elut (100 mg phase, 1-ml column with stainless-steel frits), free aminopropyl phase (Separalyte) and stainless-steel frits (20 μm porosity, 6.4 mm diameter), and TLC plates (silica gel G, 250 μm) were purchased from Analytichem International (Harbor City, CA, U.S.A.). Fatty acids were obtained from Nu-Chek Prep (Elysian, MN, U.S.A.). The 5% DEGS-PS columns (1.8 m$\times$2 mm I.D.) were purchased from Supelco (Bellefonte, PA, U.S.A.). All solvents (HPLC grade) were from Bakers (Phillipsburg, NJ, U.S.A.).

Isolation of FFA

Aminopropyl phase (solid) (150 mg) was packed into a glass column (5 ml capacity) using glass wool and two stainless-steel frits placed on the top and bottom of the bonded phase. In this paper, this column will be referred to as "bonded-phase glass column" as opposed to Bond Elut ready-made columns, i.e., aminopropyl packed in polypropylene columns. The fatty acid mixture containing 10 nmol of heptadecenoic acid (17:0) as internal standard and either indicated amounts of 20:4 (0–50 nmol) or myocardial lipid extract from 150 mg wet weight tissue [7] were applied on bonded-phase glass columns to isolate FFA as described by Kaluzny et al. [6]. In this study, all columns were washed in succession with solvent systems under atmospheric pressure. In brief, the columns were prewashed with 3 ml of hexane before loading the sample in 1 ml of chloroform. After adsorption, the columns were washed in succession with 4 ml of chloroform–2-propanol (2:1, v/v) and 3 ml of 2% glacial acetic acid in diethyl ether to elute TG and DG, and FFA, respectively. All solvents used for the development of column contained 0.005% (w/v) BHT. The solvents were dried under nitrogen and the residue was processed for the derivatization of methyl esters either by the method of boron trifluoride–methanol [8] or sulfuric acid–methanol [9]. The two methylation procedures gave identical results.

In the TLC methods, the lipid extracts from 120 mg biopsy were chromatographed on silica gel G plates with light petroleum–diethyl ether–acetic acid

(80:20:1, v/v/v) as the solvent system. The region corresponding to FFA was scraped and methyl esters were prepared as described above.

The methyl esters of fatty acids were separated by a Model HP5890A (Hewlett-Packard) gas chromatograph equipped with 5% DEGS-PS columns. An initial temperature of 120°C was used for 2 min, which was followed by two successive temperature programs: (a) a linear increase of 120 to 180°C at a rate of 10°C/min and a 25-min period at 180°C; and (b) a second linear temperature gradient of 180 to 220°C at a rate of 5°C/min and a 10-min period at 220°C. The fatty acids were identified by equivalent chain lengths. The quantitation of 20:4 and other tissue fatty acids were performed either on the basis of 17:0 (10 nmol), which was added during the extraction of lipids and on the basis of arachidic acid (20:0) added during the methylation step.

RESULTS AND DISCUSSION

Before analyzing FFA by GC, they are usually separated from the other lipids by TLC. During the TLC procedure, PUFA are presumably not in the liquid phase where oxidation of PUFA is minimum, which may result in losses of PUFA in free fatty acid analysis. We recently demonstrated that the HPLC method, which did not require the separation of TG after separating neutral lipids from lipid extract, yielded a higher recovery of 20:4 as compared to the GC analysis, which required the TLC step. Recently, Kaluzny et al. [6] have successfully purified fatty acids using Bond Elut columns. These authors used TLC to check the purity of fatty acids. However, these studies may not reveal the (trace) contamination of other lipids like TG and phospholipids.

To analyze the acyl chain compositions of tissue FFA by GC, it was of utmost importance to examine the contaminants eluted from the blank Bond Elut columns in which no lipid samples were applied. In these columns, the hexane and these chloroform washes did not contain any significant contaminants eluting with the fatty acid methyl esters as judged by the GC analysis (Table I). On the other hand, acidified ether extract showed large amounts of contaminants eluting with the 16:0, 16:1, 18:0, 18:1 and 18:2 fatty acids. No contaminants were coeluted with 20:3, 20:4, 20:5 and 22:6 fatty acids. The acidified ether eluate obtained in blank Bond Elut columns (no lipids added) was subjected to neutral lipid silica gel TLC. The methylated products of the FFA region of TLC showed similar amounts of contaminants as found in the samples which were not chromatographed on TLC (data not shown). These results show that the contaminants comigrated with FFA not only on GC but also on TLC, suggesting they may be similar to fatty acids in nature. It should be pointed out that we used stainless-steel frits in these columns, as suggested by Kaluzny et al. [6] to avoid elution of plasticizer present in polypropylene frits. The total contaminants measured in a typical run of blank Bond Elut column corresponds to the amount of FFA in about 200 mg of myocardial tissue.

In an attempt to eliminate the polypropylene contaminants coeluting with fatty acids in GC analysis, we used aminopropyl bonded phase packed in glass columns, as described under Experimental. In blank columns where no lipids are applied,

TABLE I

QUANTIFICATION OF CONTAMINANTS PRODUCED DURING THE SOLVENT ELUTION OF AMINOPROPYL BONDED-PHASE COLUMNS

The blank polypropylene column (ready-made Bond Elut) and 100 mg of aminopropyl bonded-phase packed in glass columns (to which no lipids were applied) were washed in succession with 3 ml each of (a) hexane, (b) chloroform–2-propanol (2:1, v/v) and (c) 2% acetic acid in diethyl ether. The solvents were evaporated, and 10 nmol of 20:0 was added as the internal standard. The methyl esters were prepared by boron trifluoride–methanol method and analyzed in GC as described under Experimental. Values are expressed in nmol and are mean of three independent determinations.

Retention time equivalent to fatty acids*	Blank polypropylene column (Bond Elut)			Blank glass column		
	a	b	c	a	b	c
16:0	1.5	1.0	20.6	1 6	2.2	1.5
16:1	0.5	0.8	6.88	0.5	0.96	0.30
17:0	0.0	0.0	3.00	0.00	0.00	0.00
18:0	0.66	0.52	40.08	1.30	0.96	0.20
18:1	0.74	0.46	6.88	0.50	1.00	0.65
18:2	0.20	0.20	2.2	0.172	0.30	0.652
20·3	0.00	0.00	0.00	0.00	0.00	0.000
20:4	0.00	0.00	0.00	0.00	0.00	0.000
20 5	0.00	0.00	0.00	0.00	0.00	0.000
22:6	0.00	0.00	0.00	0.00	0.00	0.000

*16:0 = palmitic acid; 16:1 = palmitoleic acid; 17:0 = heptadecenoic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid; 20:4 = arachidonic acid; 20:5 = eicosapentaenoic acid; 22:6 = docosahexenoic acid.

the contaminants in the acidified ether eluate were significantly reduced (Table I). For example, the contaminants coeluting with 16:0, 18:0 and 18:1 were decreased by a factor of 13.3, 200 and 10.55, respectively. These contaminants from a blank bonded-phase packed glass column would correspond to the amount of FFA present in 5 mg of myocardial tissue or 10–15 ml of plasma [10]. Therefore, for further studies we used aminopropyl bonded phase (Sepralyte) packed in glass columns.

The recoveries of arachidonate isolated from bonded phase packed in glass columns and TLC plates are shown in Table II. The results clearly indicate that the recovery in bonded-phase columns was greater than 90% for all concentrations tested. On the other hand, the TLC method yielded lower recoveries at lower concentrations, the maximum recovery being not less than 70%. However, the recoveries of 17:0 were similar in both methods. Since the recovery of 20:4 is based on the 17:0, a saturated fatty acid added before either the column or the TLC procedure, the loss of 20:4 from the TLC procedure appears to arise from the process of peroxidation. Furthermore, a linear increase in the recovery of arachidonate by bonded-phase column is obtained when its concentration was increased from 5 to 50 nmol (Fig. 1).

The TG and DG were eluted in the chloroform–propanol wash prior to FFA by acidified ether [6]. The TLC and radioactivity recoveries of triolein was about 99% in the chloroform–propanol eluate. When a lipid mixture containing FFA,

TABLE II

COMPARISON OF RECOVERIES OF ARACHIDONIC ACID ISOLATED FROM BONDED PHASE PACKED IN GLASS COLUMNS AND TLC PLATES

Indicated amounts of arachidonate were added to 20 nmol of 17:0. The samples were subjected to bonded-phase glass columns or TLC to isolate and measure FFA. After the isolation of fatty acid fraction, 10 nmol of 20:0 was added as the second internal standard. The exact amount of added 20:4 was obtained by direct methylation, followed by its analysis. The nmol of 20:4 was calculated on the basis of 17:0. All values are mean of triplicate measurement

Fatty acid	Amount (nmol)	Recovery of arachidonate			
		Bonded-phase column		TLC	
		nmol	%	nmol	%
Arachidonate	0	0		0	
	5	4.5	90	1.75	35
	10	9.2	92	4.4	44
	15	14.20	94	10.5	70
	20	18.00	90	13.06	65
	25	22.50	90	15.10	60
Heptadecenoic acid (17:0)	20.0	19.50	97.5	19.30	96.5

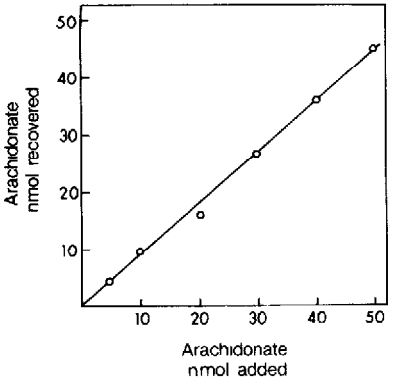

Fig. 1 Recovery of arachidonate by aminopropyl bonded-phase column. Increasing amounts of 20:4 and 10 nmol of 17:0 were taken in 1 ml of chloroform, subjected to bonded phase packed in glass column and analyzed on GC as described under Experimental. The absolute amount of added 20:4 was calculated by measuring an aliquot taken directly without being passed on column.

TG and phospholipid are fractionated on the column and analyzed for methyl esters on GC, no contamination of methyl esters of either TG or phospholipid fatty acids were observed in the FFA fraction (Table III). We have also examined the acyl chain composition of the FFA and TG fractions of myocardial lipids. The TG fraction eluted in the chloroform–propanol wash was rich in oleic acid and palmitic acids (Table IV). This acyl chain composition of heart TG is very similar to that reported by Wood [10] employing TLC and GC methods.

Finally, we compared the acyl chain composition of FFA isolated by bonded-

TABLE III

VALIDITY OF FREE FATTY ACID PURITY ISOLATED FROM A LIPID MIXTURE CONTAINING TRIGLYCERIDES, FREE FATTY ACIDS AND PHOSPHOLIPIDS

A mixture containing 20 nmol each of 17:0, triolein and dipalmitoyl phosphatidylcholine was loaded on aminopropyl bonded-phase columns (glass) as described under Experimental. 20:0 was added as the internal standard. Control with no lipids added to the column was run to subtract the contaminants on GC analysis.

Elute	Amount of acyl chain (nmol)		
	16:0	17:0	18:1
Chloroform–propanol	0.0	0.0	55.0
Diethyl ether–acetic acid	0.0	19.0	0.0
Methanol	35.0	0.0	0.0

TABLE IV

ACYL CHAIN COMPOSITION OF FREE FATTY ACIDS AND TRIGLYCERIDES OF SWINE MYOCARDIUM

The lipid extract (equivalent to 120 mg wet tissue weight) was loaded on a 150-mg bonded-phase glass column previously washed with 4 ml of hexane and 1 ml of chloroform. After loading, the column was eluted in succession with 4 ml each of chloroform–propanol and 2% glacial acetic in diethyl ether. Values are mean of duplicate measurements, and the corresponding values of duplicate samples did not vary more than 5% from each other. Values in parentheses indicate mole percent of each fatty acid.

Elute	Acyl chain composition (nmol/g wet weight)						
	16.0	16:1	18:0	18:1	18:2	20:4	Total
Chloroform–propanol eluate (triglycerides)	440 (29.3)	88 (5.8)	160 (10.6)	536 (35.7)	248 (16.5)	30 (1.9)	1502
Diethyl ether–acetic acid eluate (free fatty acids)	140 (27)	38 (7.3)	112 (21)	143 (27)	63 (12)	23 (4.4)	519

phase column and TLC (Table V). The results clearly indicate that both in control and ischemic-reperfused swine myocardium, the bonded-phase column method yielded a higher recovery of arachidonic acid as compared to that of TLC. However, it should be pointed out that although saturated and monosaturated fatty acids also showed losses in the TLC isolation procedure, these losses were significantly lower compared to those of 20:4. The results obtained in Table III have already indicated that higher losses occur at lower concentrations of 20:4. Although error in measurement could be higher because of very low amounts of fatty acids, it is unlikely that similar losses will occur for both unsaturated and saturated fatty acids when present in very low concentrations.

The increase of FFA in the swine ischemic-reperfused myocardium has also been observed by other investigators [11]. The higher recovery of FFA by bonded-phase column as compared to the TLC method was also observed by Kaluzny et al. [6], who employed radioactive fatty acid. Furthermore, the mole percent of

TABLE V

COMPARISON OF MYOCARDIAL FREE FATTY ACIDS ISOLATED FROM BONDED-PHASE COLUMN AND TLC

The lipid extracts (equivalent to 120 mg wet tissue weight) in 1 ml chloroform were loaded on a 150-mg bonded-phase glass column, and the fatty acids were isolated and analyzed on GC as described under Experimental. Values are nmol/g wet weight.

Acyl chain	Control swine myocardium		Ischemic-reperfused swine myocardium	
	Bonded phase	TLC	Bonded phase	TLC
16:0	90.00	81.00	140.00	114.00
16:1	33.60	16.00	38	17.00
18:0	77.00	66.00	112	84.0
18:1	110.00	63.00	143	89.00
18:2	61.07	46.00	62.0	54.00
20:4	15.00	3.00	24.0	4.00
Total	386*	275	514**	361
Mole percent of 20:4	3.9	1.09	47	1.2

*Control myocardial biopsy.
**Ischemic-reperfused swine myocardial biopsy.

20:4 obtained in this study is identical to that analyzed by HPLC of FFA after isolation by Sep-Pak columns [4]. In the Sep-Pak columns, all the neutral lipids were eluted in a single fraction; but the presence of TG did not interfere with the FFA analysis, probably because of a μBondapak^{TM} C_{18} Guard-Pak ^{TM} precolumn [8]. In this method also, it was possible to use BHT in each step of analysis. Indeed, isolation of FFA by bonded-phase column yielded a better recovery of polyunsaturates.

In summary, the results presented in this paper show that in aminopropyl-packed glass columns (blank columns), insignificant amounts of contaminants are produced which interfere with the analysis of fatty acids by GC. The bonded-phase column packed in glass yields higher recoveries of tissue arachidonate or total FFA as compared to the TLC method. Thus, the use of aminopropyl bonded-phase column packed in glass may serve better than TLC in isolating FFA for subsequent analysis either by HPLC or GC.

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