Journal of Chromatography, 344 (1985) 304—308
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2744

Note

Application of silica gel column chromatography in the assessment of non-esterified fatty acids and phosphoglycerides in myocardial tissue

Th.H.M. ROEMEN and G.J. VAN DER VUSSE*

Department of Physiology, Biomedical Centre, University of Limburg, P.O. Box 616, 6200 MD Maastricht (The Netherlands)

(First received March 12th, 1985; revised manuscript received June 4th, 1985)

Recent studies have shown that the endogenous content of non-esterified fatty acids (NEFAs) in normoxic myocardial tissue is very small in comparison with the content of phosphoglycerides [1, 2]. During ischaemia, the tissue level of total NEFAs increases, particularly that of linoleic and arachidonic acid, most likely due to the degradation of phosphoglycerides [1, 3]. Attempts to investigate a possible stoichiometric relationship between the increase in tissue NEFAs and changed levels of the various phosphoglyceride subclasses prompted us to explore whether silica gel column chromatography was a useful application in our assay system for the determination of myocardial lipids.

EXPERIMENTAL

Lipid standards were obtained from Sigma (St. Louis, MO, U.S.A.), silica gel 60 (230—400 mesh) and methanol and chloroform, for residue analysis, were purchased from Merck (Darmstadt, F.R.G.). Before use, the silica gel was washed successively with methanol, chloroform—methanol (1:1) and chloroform. After washing with chloroform, the silica gel was sucked dry in a Buechner funnel with the aid of a slight negative pressure. The funnel remained covered with a plastic cap throughout the procedure, and a stream of purified nitrogen under the cap helped to prevent absorption of contaminants from the atmosphere by the silica gel. Glass columns (10 cm × 9 mm I.D.), equipped with a sintered-glass disk at the bottom, were made to our specifications. After meticulously cleaning the glass columns, they were filled with 0.5 g of silica gel and covered with a small disk of chloroform—methanol washed filter

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paper, to prevent dislodging of the silica gel into the solvent. The columns were placed on a vacuum manifold and a slight negative pressure was achieved by means of a water aspirator.

The lipid extracts to be separated were dried in test-tubes under a stream of nitrogen at 40°C, and the residues were dissolved in 0.1 ml of chloroform–methanol (17:3). These lipid mixtures were carefully pipetted onto the columns. The test-tubes were rinsed twice each time with 0.1 ml of chloroform–methanol and these volumes were also pipetted onto the columns. Subsequently, the lipids were left to be absorbed by the silica gel for 5 min. Elution of the neutral lipids was carried out with 3 ml of chloroform–methanol (39:1) using a slight negative pressure. Elution of the phosphoglycerides was carried out with 3 × 2.5 ml of chloroform–methanol–water (5:5:1). Each 2.5 ml of the elution mixture was entirely sucked through the column before the next 2.5 ml were added. The separated fractions were collected in glass vials and the solvents dried at 40°C under a gentle stream of nitrogen. The eluted phosphoglyceride-containing fraction was subsequently subjected to two-dimensional thin-layer chromatography (TLC) [4] to obtain the various phosphoglyceride subclasses. The fatty acid moieties and dimethylacetals (DMAs) were transmethylated, according to Morrison and Smith [5]. NEFAs were separated from the other neutral lipids with the use of one-dimensional TLC, methylated and quantitatively assessed with gas–liquid chromatography (GLC) [6]. Aliquots of tissue corresponding to 150–300 mg of wet tissue were extracted as described previously [6].

RESULTS AND DISCUSSION

To conclude that silica gel column chromatography is a reliable technique, the following requirements should be fulfilled: (1) complete separation between phosphoglycerides and neutral lipids, including NEFAs; (2) good recovery of the various lipid classes; (3) no degradation of the esterified fatty acids leading to artificially elevated amounts of NEFAs; (4) the eluted fractions should be suitable for further treatment, such as evaporation to dryness and TLC, without leading to artificial changes in the lipid moieties; (5) no elevation of the blank value for the assay of NEFAs.

Standard mixtures of NEFAs (myristic, palmitic, palmitoleic, stearic, oleic, linoleic and arachidonic acid), ranging from 4 to 25 nmol per assay, were subjected to silica gel column chromatography. All of the NEFAs were eluted in the first fraction with chloroform–methanol (39:1). The recovery ranged from 98.3 ± 2.4% for myristic acid to 101.7 ± 1.6% for palmitoleic acid. A standard mixture of triacylglycerol, consisting of trimyristate, tripalmitate, tripalmitoleate, tristearate, trioleate and trilinoleate, ranging from 50 to 70 nmol per assay for the individual subclasses, was completely eluted in the first elution fraction. The recovery ranged from 98.0 ± 2.1% for trilinoleate to 104.8 ± 2.5% for tripalmitoleate. A standard mixture of cholesteryl esters, consisting of palmitoyl-, stearoyl- and linoleoylcholesterol, ranging from 150 to 170 nmol per assay for the individual subclasses, was completely eluted in the first fraction. The recovery ranged from 97.9 ± 1.2% for palmitoylcholesterol to 101.7 ± 2.1% for stearoylcholesterol. A standard mixture consisting of purified phosphatidylcholine, phosphatidylethanolamine, phospha-

TABLE I

CONTENT OF NEFAs, PHOSPHATIDYLCHOLINE (PC), LYSOPHOSPHATIDYLCHOLINE (LPC), PHOSPHATIDYLETHANOLAMINE (PE), LYSOPHOSPHATIDYLETHANOLAMINE (LPE), PHOSPHATIDYLINOSITOL (PI), PHOSPHATIDYLSERINE (PS) AND CARDIOLIPIN (DPG) IN NORMOXIC DOG MYOCARDIUM

The total amount (nmol of fatty acid moieties per g wet weight) and percentage fatty acid composition (%) are presented. Myocardial tissue specimens were obtained and extracted as described previously [6]. The various fatty acids are indicated by their chemical notation. DMA refers to the dimethyl acetal form of the corresponding fatty aldehyde. Mean values and S.E.M. of seven experiments are shown.

Fatty acid	Lipid class							
	NEFA	PC	LPC	PE	LPE	PI	PS	DPG
14:0	5.9 ± 0.8	—	2.2 ± 1.3	—	4.6 ± 1.3	—	—	0.5 ± 0.1
14:0 DMA	—	1.9 ± 0.6	—	—	—	—	—	—
16:0	28.0 ± 1.6	14.2 ± 0.7	21.1 ± 2.4	2.2 ± 0.2	5.4 ± 2.4	1.7 ± 0.2	1.2 ± 0.1	1.4 ± 0.3
16:0 DMA	—	20.8 ± 0.9	—	6.9 ± 0.9	—	—	—	—
16:1	4.5 ± 0.9	0.5 ± 0.1	1.1 ± 0.6	0.2 ± 0.1	—	0.2 ± 0.1	0.2 ± 0.1	1.3 ± 0.1
18:0	26.4 ± 0.9	6.8 ± 0.4	32.4 ± 3.3	24.4 ± 0.7	37.4 ± 2.1	50.6 ± 0.4	48.3 ± 0.3	1.4 ± 0.2
18:0 DMA	—	1.5 ± 0.2	—	6.4 ± 0.4	—	—	—	—
18:1	16.3 ± 1.6	23.7 ± 0.6	11.0 ± 1.4	5.5 ± 0.4	11.9 ± 1.9	3.7 ± 0.2	10.2 ± 0.4	5.3 ± 0.3
18:1 DMA	—	2.6 ± 0.2	—	4.4 ± 0.4	—	—	—	—
18:2	11.1 ± 1.8	13.6 ± 0.8	20.4 ± 5.3	5.6 ± 0.3	22.0 ± 1.9	13.3 ± 0.8	14.6 ± 1.0	87.6 ± 0.9
18:3	—	0.3 ± 0.1	—	0.1 ± 0.1	—	—	—	0.7 ± 0.2
20:0	—	—	6.4 ± 1.1	—	—	0.2 ± 0.1	0.4 ± 0.1	—
20:4	6.3 ± 1.1	14.0 ± 1.1	4.8 ± 1.7	42.1 ± 1.4	16.5 ± 1.5	29.8 ± 0.9	22.0 ± 1.0	0.1 ± 0.1
22:4	—	0.2 ± 0.1	—	0.5 ± 0.1	—	0.3 ± 0.1	1.7 ± 0.3	—
22:6	—	0.2 ± 0.1	—	0.7 ± 0.1	—	—	—	—
Total amount	28 ± 4	19 212 ± 992	112 ± 20	13 986 ± 771	85 ± 14	2243 ± 102	1218 ± 67	6212 ± 281

tidylserine, phosphatidylinositol, cardiolipin, lysophosphatidylethanolamine and lysophosphatidylcholine, ranging from 900 to 1350 nmol per assay for the individual subclasses, was completely eluted by the second elution step using chloroform—methanol—water (5:5:1). No traces of phosphoglycerides could be detected in the first chloroform—methanol step. The recovery of the phosphoglycerides ranged from 96.3 ± 0.3% for lysophosphatidylethanolamine to 106.8 ± 0.4% for phosphatidylethanolamine.

Other column chromatographic techniques to separate phosphoglycerides from NEFAs have been described in literature. They seem, however, to be less suitable for the present application. Silicic acid columns showed incomplete recovery of NEFAs [7], whereas DEAE or TEAE cellulose columns, as described by Rouser et al. [8], did not result in a complete separation between phosphoglycerides and NEFAs. Moreover, Wuthier [9] and Garcia et al. [10] have reported that phosphatidylinositol could not be recovered quantitatively from cellulose columns.

The application of silica gel column chromatography did not result in the release of fatty acids from phosphoglycerides and other esterified fatty acid classes. This aspect of chromatographical techniques used in the assay for tissue NEFAs should be considered very carefully, since the amount of NEFAs present in normoxic tissue is very small compared with the amount of esterified fatty acids [1, 2]. For this reason, the use of silicic acid columns seems to be less suitable since Bazan and Joel [11] found a time-dependent generation of NEFAs from esterified lipids during the course of the chromatographical procedure.

A special advantage of silica gel column chromatography is the yield of eluted lipid fractions that can be used for subsequent separation into subclasses of these lipids with the use of TLC. In previous studies, we used one-dimensional TLC to separate total phosphoglycerides from the neutral lipids [1, 6]. Attempts to separate the total phosphoglycerides (after being scraped from the plate and eluted from the silica gel) into the various distinct phosphoglycerides with two-dimensional TLC according to Broekhuyse [4] were not successful. The recovery of phosphatidylethanolamine, for example, became reduced, whereas various unidentified spots were found on the thin-layer plate. These problems were not encountered when silica gel columns were used instead of one-dimensional TLC to separate phosphoglycerides from neutral lipids. A feasible explanation is that unwanted changes occurred in some phosphoglycerides during evaporation to dryness of the eluent prior to two-dimensional TLC. Traces of acetic acid derived from the TLC-developing mixture [6] might promote degradation of phosphoglycerides during evaporation to dryness [12].

Since NEFAs were present in normoxic cardiac tissue in trace amounts, special attention has been given to the blank values obtained by the application of silica gel column chromatography. We have previously reported that our routine assay technique for NEFAs, including Folch extraction, TLC and GLC, resulted in blank values of 3.3 ± 1.3 nmol per assay [6]. Application of the above-described column chromatography with silica gel did not increase the blank value. In contrast, application of commercially available separation devices using silica gel, such as Sep-Pak (Waters Assoc., Milford, MA, U.S.A.) as

used by Hamilton and Comai [13] and Juaneda and Rocquelin [14] and the Baker-10 system (Baker, Phillipsburg, NJ, U.S.A.), resulted in blank values ranging from 10 to 20 nmol per assay. For this reason, these devices are less useful for the determination of NEFAs in myocardial biopsies.

We employed silica gel column chromatography in combination with two-dimensional TLC to assess the content of myocardial NEFAs and various phosphoglycerides. The results are shown in Table I. Both the absolute amount and the relative fatty acid composition of NEFAs are in good agreement with data previously published [1, 6]. The total amount of phosphoglycerides was found to be 43 μmol of fatty acid per g of tissue. Phosphatidylethanolamine and phosphatidylcholine were the main constituents. Lysophosphatidylcholine and lysophosphatidylethanolamine were present in trace amounts, representing 0.5 and 0.4% of the total amount of phosphoglyceride molecules, respectively. The latter observation is in good agreement with data published by others [15] and most likely indicates that the analytical conditions proposed did not lead to breakdown of plasmalogens resulting in artificially elevated lysophosphoglyceride levels in biological material.

To summarize, the application of silica gel column chromatography appears to be a reliable and fast technique to separate phosphoglycerides and neutral lipids, including NEFAs present in a biological specimen such as cardiac tissue. Both the recoveries of the various lipids and the separation were excellent. No artificial changes in the lipids were introduced, and blank values for NEFA could be kept as low as possible when self-made columns were used. Silica gel column chromatography can easily be applied in combination with, for example, TLC for subsequent analysis of phosphoglyceride subclasses.

ACKNOWLEDGEMENT

This investigation is supported by the Foundation for Medical Research (FUNGO), which is subsidized by The Netherlands Organization for the Advancement of Pure Research (ZWO).

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