Journal of Chromatography, 375 (1986) 225—231
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 2922

SEPARATION AND QUANTITATION OF FATTY ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received May 20th, 1985, revised manuscript received October 16th, 1985)

SUMMARY

To facilitate the determination of the fatty acid composition of tissues and the investigation of fatty acid metabolism, we developed a method for the rapid separation by high-performance liquid chromatography and quantitation (by ultraviolet light absorption) of *p*-bromophenyl esters of fatty acids which vary in chain length from 10 to 22 carbon atoms. The utility of the method was demonstrated by evaluating the fatty acid composition of human uterine decidua vera tissue and human endometrial stromal cells that are maintained in monolayer culture

INTRODUCTION

Prostaglandins (PGs), prostacyclin, thromboxane, and leukotrienes are synthesized from essential fatty acids [1—3], linoleic acid (18 2), linolenic acid (18·3) and arachidonic acid (20 4) Importantly, these fatty acids are present in cells principally in esterified form with glycerophospholipids and triacylglycerols A major regulatory mechanism in the rate of PG synthesis is believed to be the rate of release of arachidonic acid from these lipid storage forms Thus, the amount and distribution of these fatty acids among tissues and lipid species is of central importance in the evaluation of the capacity for and the regulation of prostaglandin synthesis in various tissues

To facilitate the evaluation of the fatty acid composition of tissues as well as the metabolism of fatty acids, a method for the rapid separation and quantitation of these compounds is desirable Heretofore, gas chromatography was the method of choice for separation and quantitation of nanogram quantities of

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these compounds. Recently, methods for rapid separation, by high-performance liquid chromatography (HPLC), of derivatives of fatty acids that absorb ultraviolet light [4—6] or that are fluorescent [7–10] have been developed.

Herein, we describe a method for the rapid separation and quantitation by UV absorption of *p*-bromophenacyl esters of fatty acids which vary in chain length from 10 to 22 carbon atoms, and one that can be used with extracts of tissues or cells maintained in monolayer culture. These derivatives are formed in a reaction of the carboxylate anion of the fatty acid with α-*p*-dibromoacetophenone in which dicyclohexyl-18-crown-6 ether serves as a catalyst.

EXPERIMENTAL

Materials

The authentic fatty acids used as standards were obtained from Serdary Research Labs (London, Canada). Potassium hydroxide was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A) The *p*-bromophenacyl ester derivatization kit was obtained from Applied Sciences Labs (State College, PA, U.S.A). Methanol (HPLC grade) was obtained from J.T Baker (Phillipsburg, NJ, U.S A.). Light petroleum (boiling point 38—56°C) and acetonitrile were obtained from Mallinckrodt (Paris, KY, U S A.).

Tissue preparation

Human endometrial tissue was obtained on day 23 of the ovarian cycle from the uterus immediately after hysterectomy that was performed for reasons other than endometrial disease. Endometrial stromal cells were obtained after enzymatic digestion of the tissue as described previously [11], the cells were placed in 100-mm culture dishes Confluent endometrial stromal cells in monolayer culture were scraped from the culture dishes into a solution of 0.15 *M* sodium chloride and were centrifuged at 1000 *g* for 15 min. The pellet of cells was suspended in 2 ml of water and was sonicated in a W-375 sonicator (Heat Systems-Ultrasonics, Plainview, NY, U.S.A) at a setting of 4 with a 30% duty cycle and a continuous pulsar cycle for 10 s.

Human decidua vera tissue was obtained at the time of elective cesarean section, conducted at term, but before the onset of labor. The decidua vera tissue was separated from the chorion laeve as described previously [12]. Decidua vera tissue (approx. 0.5 g) was homogenized in a solution of 0.15 *M* sodium chloride (5 vols) in a Potter-Elvehjem homogenizer and a 2-ml aliquot was sonicated as described above. The protein content of aliquots of sonicates was determined by the method of Lowry et al. [13] with bovine serum albumin as the standard.

The lipids from an aliquot (1 6 ml) of each tissue sonicate were extracted by the method of Bligh and Dyer [14]. The lipid extracts were reduced to dryness at 25°C under a stream of nitrogen. The residue was dissolved in a solution of 1.3 *M* potassium hydroxide in methanol (95%) and refluxed at 95°C for 45 min. Thereafter, water (2 ml) was added and the non-saponifiable lipid material was extracted with light petroleum (5 ml) The samples were acidified (pH < 4) with 2 *M* hydrochloric acid, and the free fatty acids were extracted twice with light petroleum (5 ml) The light petroleum fractions were combined and

reduced to dryness under a stream of nitrogen at 25°C, the residue was dissolved in acetonitrile (4 ml)

The *p*-bromophenacyl esters were prepared by alkylation by the method of Durst et al [4] as modified by Pei et al [6]

Derivative formation

Fatty acid extracts of tissues or authentic fatty acids in acetonitrile (4 ml) were mixed with potassium carbonate (15 mg), 0 2 *M* α-*p*-dibromoacetophenone (0.1 ml) and 0.02 *M* dicyclohexyl-18-crown-6 ether (0.1 ml). The mixture was boiled under reflux, with vigorous stirring, for 45 min, and the yellowish solution was filtered through a 0 5-μm filter (Millipore, Bedford, MA, U S A) to remove potassium bromide and unreacted potassium carbonate After evaporation of the solvent at 25°C under a stream of nitrogen, the residue was reconstituted in 1 ml acetonitrile

HPLC

Fatty acid analyses were conducted with a DuPont Model 850 liquid chromatograph (DuPont, Wilmington, DE, U S A) that was equipped with a C_{18} μBondapak column 30 cm × 3 9 mm, 10 μm particle size, Waters Assoc (Milford, MA, U.S.A.) and UV (254 nm) detector The retention times, peak areas and percentage distribution of the compounds were computed by use of an HP 3380A integrator (Hewlett-Packard, Avondale, PA, U S A) An aliquot (0 05 ml) of the sample was injected and chromatographed at ambient temperature in methanol—water (23 2) The flow-rate was 1 3 ml/min at a pressure of 103 bar. The attenuation for the detector and recorder were set at 0 01 and 32 a u f s , respectively. The recorder sensitivity was 3 mV/min.

RESULTS

A chromatogram that is illustrative of the separation of the *p*-bromophenacyl esters of a variety of authentic fatty acid standards is presented in Fig 1 In the reversed-phase HPLC procedure, the fatty acid derivatives were eluted in an order of decreasing polarity and increasing chain length The lower limit of detection for short-chain fatty acids (C_{10} to C_{12}) was 25 ng and that for long-chain fatty acids (C_{20} to C_{22}) on average, was 90 ng. The most commonly occurring compounds with an intermediate chain length (C_{14} to C_{18}) could be detected at concentrations as low as 60 ng, on average The average upper limits of detection were 550, 2800 and 2100 ng, respectively Although the sensitivity could be increased five- to fifteen-fold by setting the detector attenuation to 0 002 a u f s or by expanding the recorder range to 1 mV/min, this was not useful for the quantitation of fatty acids in tissues since the evaluation of the compounds in smaller quantities was complicated by the fact that the detector background was considerably greater

By decreasing the polarity of the solvent, the separation of arachidonic acid (20 4) and linoleic acid (18·2) was facilitated. The retention times of other fatty acids, however, were reduced considerably even at a flow-rate of 0 5 ml/min because of underutilization of the stationary phase such that some fatty acids (10 0, 11 0, 12 0, 14 1) could not be separated On the other

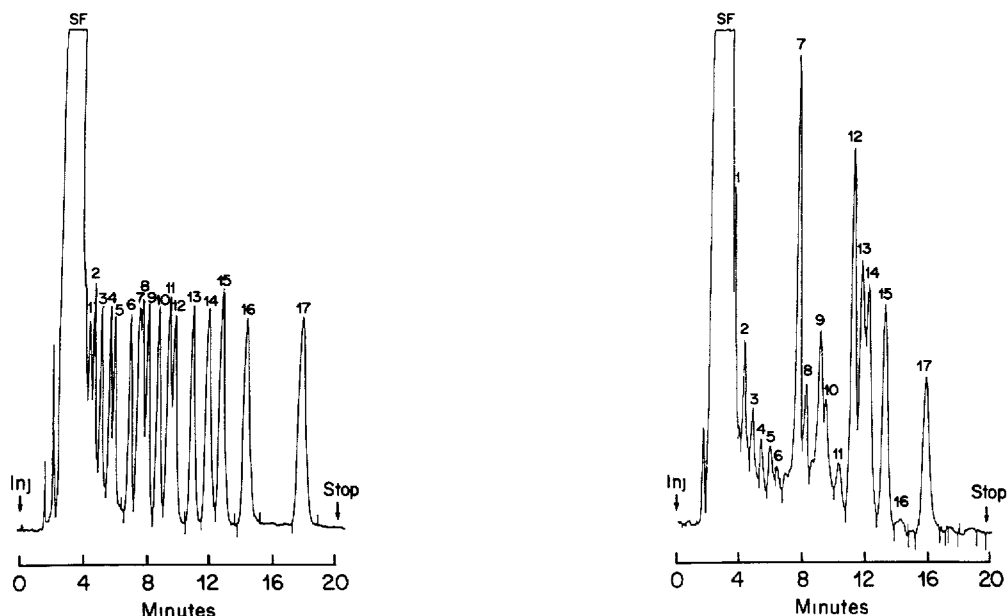

Fig 1 HPLC profile of fatty acids A 50-μl aliquot of the fatty acid standard mixture was injected Peaks Inj = injection, SF = solvent front, 1 = α-*p*-dibromoacetophenone, 2 = 10 0 (*n*-decanoic acid, 117 ng), 3 = 11 0 (undecanoic acid, 200 ng), 4 = 12 0 (*n*-dodecanoic acid, 200 ng), 5 = 14 1 (*cis*-9-tetradecenoic acid, 250 ng), 6 = 18 3 (all-*cis*-9,12,15-octadecatrienoic acid, 450 ng), 7 = 16 1 (*trans*-9-hexadecenoic acid, 450 ng), 8 = 20 4 (all-*cis*-5,8,11,14-eicosatetraenoic acid, 666 ng), 9 = 18 2 (all-*trans*-9,12-octadecadienoic acid, 666 ng), 10 = 20 3 (all-*cis*-8,11,14-eicosatrienoic acid, 750 ng), 11 = 16 0 (*n*-hexadecanoic acid, 616 ng), 12 = 18 1 (*cis*-9-octadecenoic acid, 666 ng), 13 = 17 0 (heptadecanoic acid, 616 ng), 14 = 22 3 (all-*cis*-7,10,13-docosatrienoic acid, 1117 ng), 15 = 18 0 (*n*-octadecanoic acid, 1167 ng), 16 = 22 2 (all-*cis*-10,13-docosadienoic acid, 1283 ng), 17 = 20 0 (*n*-eicosanoic acid, 1366 ng)

Fig 2 HPLC profile of fatty acids Fresh decidua vera tissue was extracted and saponified, and derivatization was accomplished as described in the text Peaks 1 = α-*p*-dibromoacetophenone, 2 = 10 0, 3 = 11 0, 4 = 12 0, 5 = 14 1, 6 = unknown, 7 = 18 3, 8 = 16 1, 9 = 20 4, 10 = 18 2, 11 = 20 3, 12 = 16 0, 13 = 18 1, 14 = unknown, 15 = 17 0, 16 = 22 3, 17 = 18 0 For abbreviations, see legend to Fig 1

hand, by increasing the polarity of the solvent and by adjusting the flow-rate to 2 ml/min, the resolution of palmitic acid (16 0) and oleic acid (18 1) was improved at the expense of separation of arachidonic acid and linoleic acids, longer retention times and higher back-pressure The saponification of fatty acid standards, either separately or as the mixture, did not give rise to other peaks on the chromatogram, this finding is indicative that the formation of isomers was not a problem.

An example of the application of the method described is illustrated in Fig. 2 There was no detectable fatty acid derivative compound with a longer retention time than that of 18 0. It should be noted that the separation and quantitation of 16.1, 20 4 and 18 2, and of 16 0 and 18 1 fatty acids, may be difficult due to tailing when the differences in the amounts of these fatty acids are great This can be resolved, however, by collecting and recycling these compounds with appropriate adjustment of the eluent composition or the flow-rate as described, or by a combination of both procedures

TABLE I

FATTY ACID COMPOSITION OF DECIDUA VERA TISSUE AND ENDOMETRIAL STROMAL CELLS IN MONOLAYER CULTURE

Fatty acid*	Concentration (μg/mg of protein)	
	Decidua vera tissue	Endometrial stromal cells
10 0	0 78 ± 0 07	0 76 ± 0 06
11 0	0 85 ± 0 06	0 83 ± 0 07
12 0	0 71 ± 0 05	0 51 ± 0 04
14 1	0 95 ± 0 07	0 61 ± 0 05
18 3	19 45 ± 1 5	6 72 ± 0 42
16 1	5 76 ± 0 42	1 9 ± 0 11
20 4	14 22 ± 0 81	12 08 ± 0 95
18 2	7 66 ± 0 61	5 76 ± 0 62
20 3	4 04 ± 0 22	0 73 ± 0 04
16 0	16 72 ± 1 21	6 76 ± 0 31
18 1	15 7 ± 1 02	8 11 ± 0 49
22 3	1 68 ± 0 11	0 14 ± 0 01
18 0	15 30 ± 0 98	7 21 ± 0 41
22 2	N D**	N D
20 0	N D	N D

*In order of increasing retention time
**N D = Not detectable

The fatty acid concentrations of fresh decidua vera tissue and endometrial stromal cells in monolayer culture are presented in Table I. Standard curves were prepared for each fatty acid and linearity was established within the lower and upper limits of detection The amount of fatty acid standard was plotted against the area under the peak and the concentration of fatty acid was calculated by use of linear regression analysis. The results represent mean values ± standard error of the mean (S.E M.) of five different experiments conducted in triplicate The overall inter- and intra-assay variabilities were 6 and 4%, respectively Thus, quantitation of fatty acids could be achieved with as little as 0 1 g wet weight of uterine decidua vera tissue Since the retention times in HPLC for fatty acids in tissue homogenates are altered somewhat when compared with those of a mixture of authentic fatty acids, the identity of the fatty acids was determined by the addition of authentic fatty acids to tissue homogenate

To monitor losses during the extraction and saponification procedure, heptadecanoic acid (17 0) was used as the internal standard since this fatty acid occurs naturally only in trace amounts, the recovery of this standard was 85 ± 5% (mean ± S.E M , n = 12)

Derivatives of the following fatty acids could not be resolved by the method described (a) *cis*-9,12,15-octadecatrienoic acid or linoleic acid (18 3), all-*cis*-6,9,12-octadecatrienoic acid (18 3) and *n*-tetradecanoic acid (14 0), (b) *cis*-9-octadecenoic acid or oleic acid (18 1), *trans*-9-octadecenoic acid (18 1) and *cis*-6-octadecenoic acid (18·1), (c) *n*-octadecanoic acid or stearic acid (18 0) and *cis*-11-eicosenoic acid (20 1), (d) *trans*-9-hexadecenoic acid (16 1) and all-*cis*-4,7,10,13,16,19-docosahexenoic acid (22 6)

DISCUSSION

For the investigation of fatty acid metabolism, a means of separation and quantitation of a number of saturated and unsaturated fatty acids is required The analysis of fatty acids as dibromophenyl esters by HPLC is a rapid method of high resolution and sensitivity We find that this method is appropriate for analysis of fatty acids in very small amounts of tissue although the analysis of fluorescent derivatives [7—10] may be necessary for detection of minute quantities of fatty acids One major advantage of HPLC is that the separated compounds are detected by use of optical instruments in a manner that does not necessitate destruction of the compound, thus, further analyses may be conducted [15]

In their original studies, Durst et al [4] separated a number of fatty acids by use of a C_9 reversed-phase column packing Their conditions were not optimized, however, and this resulted in relatively long retention times and the requirement of different eluents or a gradient elution technique. Pei et al [6] were able to separate the geometrical isomers of palmitoleic, oleic and linoleic acids as the *p*-bromophenacyl esters thereof by use of a 5-μm particle size C_{18} reversed-phase bonded support, but they did not include other compounds Borch [16], who used a C_{18} column packing and a gradient elution technique with acetonitrile—water, effected the separation of 24 fatty acids as phenacyl esters with excellent resolution The analysis time for all compounds, however, was 4 h and for the most commonly occurring fatty acids 70 min.

We found that C_{18} reversed-phase bonded support with methanol—water (23 2) as the eluent gives excellent separation of a variety of saturated and unsaturated fatty acids as the *p*-bromophenacyl esters These include members of the linoleic acid (18 2, $n - 6$) and linolenic acid (18 3, $n - 3$) families, which are precursors of all PGs and thromboxanes The separation of the compounds can be accomplished by elution in the isocratic mode, which is a distinct advantage over gradient elution techniques The analysis time for eighteen fatty acids (25 min) was relatively short

Importantly, this method is applicable to extracts of tissues and cells in monolayer culture (Fig 2). The distribution of fatty acids in fresh decidua vera tissue and in endometrial stromal cells in monolayer culture was similar (Table I) In women, the decidua vera of pregnancy develops from the endometrial stromal tissue in response to the action of estrogen and progesterone Moreover, the distribution of the commonly occurring fatty acids, viz the 14 1, 16 0, 16 1, 18 0, 18 1, 18 2, 18 3 and 20 4 acids, is comparable to that found in other mammalian tissues [17] The separation and quantitation of a number of fatty acids, together with the achievement of high sensitivity and resolution, short analysis time and convenience of preparation, are such that this method is well suited for investigations of fatty acid metabolism

ACKNOWLEDGEMENTS

The authors thank Jess Smith, Brent D Hartsell and Aena Kim for skilled technical assistance and Rosemary Bell for expert editorial assistance. This

investigation was supported, in part, by USPHS Grant Nos 5-P50-HD11149 and 2-P01-AG00306. K K. was a postdoctoral trainee supported, in part, by DFG Grant No 1-3 K 772/1-2 (F.R.G.)

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