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Note

Improved separation of biologically relevant C_{14}–C_{20} fatty acids by reversed-phase high-performance liquid chromatography

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UV-absorbing derivatives of many fatty acids may be separated by high-performance liquid chromatography (HPLC)[1–10]. However, the presently available methods are not satisfactory for the investigation of naturally occuring C_{14}–C_{20} fatty acids, as some of these derivatives are not separated. This subgroup of fatty acids is of primary interest when dealing with the analysis of lipids in mammalian cells and tissues[11].

We have devised a method for the simultaneous chromatographic separation of nine of the biologically most important C_{14}–C_{20} fatty acids as their *p*-bromophenacyl esters. The method is based on the use of a high-performance 5-μm reversed-phase column and the isocratic three-component eluent methanol–acetonitrile–water (82:9:9).

EXPERIMENTAL

HPLC-grade methanol and acetonitrile were purchased from Rathburn Chemicals (Walkerburn, Great Britain). Ultrapure water was prepared by filtration of distilled water through a Gelman Water I Filtration Unit (Gelman Sciences, Ann Arbor, MI, U.S.A.). *p*-Bromophenacyl bromide, N,N-diisopropylethylamine and dimethylformamide were obtained from Fluka (Buchs, Switzerland). Traces of water were eliminated from the reagents by drying the dimethylformamide over molecular sieve 4A (Pearlform; E. Merck, Darmstadt, G.F.R.) and by adding sodium hydroxide pellets to the N,N-diisopropylethylamine. Margaric acid was purchased from Sigma (St. Louis, MO, U.S.A.) and the other fatty acid standards were obtained from Supelco (Bellefonte, PA, U.S.A.).

p-Bromophenacyl esters of fatty acids were synthesized as described by Jordi[5]. HPLC analyses were carried out on a modular system consisting of a Consta Metric-III HPLC pump [Laboratory Data Control, Riviera Beach, FL, U.S.A. (LDC)]

working at a flow-rate of 1.00 ml/min, and a Consta Metric Spectromonitor-III (LDC) variable-wavelength UV detector set to monitor absorbance at 254 nm. The detector was connected to a Rec-2 recorder (Pharmacia, Uppsala, Sweden). The column system consisted of a 49 × 4.6 mm I.D. guard column, dry packed with 40-μm pellicular reversed-phase material (Pelliguard LC-18) and a 250 × 4.6 mm I.D. Supelcosil LC-18 analytical column, all purchased from Supelco. Reversed-phase columns were also purchased from Gene Tec, Sweden (Nucleosil ODS) and Supelco (Chromosorb LC-7). The Supelcosil LC-18 column was packed with 5-μm spherical packing and the other columns with 10-μm spherical packing. Injection was performed through a Rheodyne fixed-loop (20 μl) injector.

RESULTS AND DISCUSSION

When mixtures of acetonitrile and water were used as eluents, the *p*-bromophenacyl esters of palmitic and oleic acids eluted together as a single peak, and there was a poor separation of myristic and palmitoleic acid derivatives (Fig. 1A). An eluent system based on mixtures of methanol and water gave an incomplete separation of the derivatives of myristic and linoleic acids (Fig. 1B).

The two principal parameters determining the polarity and thereby the elution volume of the fatty acid *p*-bromophenacyl esters are the number of carbon atoms and the number of unsaturated bonds in the fatty acid chain[5,7]. However, as can be seen from the chromatograms (Fig. 1A and B, Table I), the relative importances of these two parameters are different in acetonitrile and methanol. When acetonitrile was used as the eluent the number of unsaturated bonds seemed to be of greater importance in determining the elution volume, whereas with methanol the elution volume was relatively more influenced by the chain length. By mixing acetonitrile and methanol in varying proportions, it was possible to vary the relative retention of the different fatty acid derivatives in a systematic and predictable manner. Thus it was possible to find the proportions of acetonitrile and methanol that resulted in the optimal overall resolution. An eluent consisting of methanol–acetonitrile–water (82:9:9) gave a good separation of all of the fatty acid derivatives investigated, with $R \geqslant 1.5$ for any two neighbouring peaks (Fig. 1C).

Margaric acid (17:0) is suitable as an internal standard for the analysis of the fatty acid composition of samples of mammalian origin. The *p*-bromophenacyl ester of margaric acid was completely separated from the other fatty acid esters used in the study (Fig. 1C, Table I).

The results described were obtained using a high-performance 5-μm reversed-phase column (Supelcosil LC-18). The column-to-column reproducibility was found to be excellent. The two 10-μm columns (Nucleosil ODS-18 and Chromosorb LC-7) did not give satisfactory separations of the fatty acid derivatives in any solvent system tested (data not shown). Our study has demonstrated that for some reversed-phase chromatographic separations, changing from a two-component to a three-component eluent system may result in a substantial increase in chromatographic selectivity[12,13]. Using *p*-bromophenacyl esters of myristic (14:0) and arachidonic (20:4) acids as standards the detection limit was determined to be about 0.1 pmol injected. This corresponds to 20–50 pg of fatty acid, which is of the same order of magnitude as that reported elsewhere[5,9].

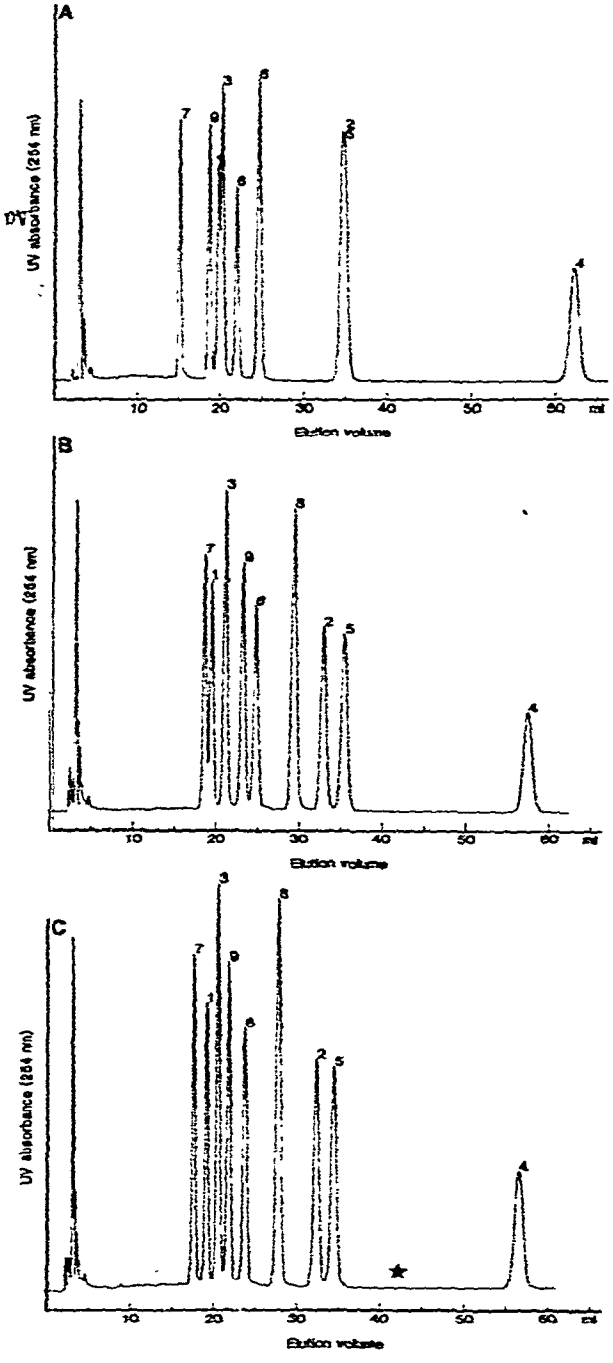

Fig. 1. HPLC of fatty acid *p*-bromophenacyl esters. Columns: 49 × 4.6 mm guard column dry packed with 40-μm pellicular reversed-phase material (Pelliguard LC-18) and a 250 × 4.6 mm Supelcosil LC-18 analytical column. Flow-rate: 1.0 ml/min with pressure *ca.* 1000 p.s.i. Eluents: A, acetonitrile–water (91:9); B, methanol–water (91:9); C, Methanol–acetonitrile–water (82:9:9). ★, Elution volume of margaric acid (17:0). See Table I for peak identification.

TABLE I

RETENTION VOLUMES OF FATTY ACID STANDARDS

p-Bromophenacyl esters of different fatty acids were subjected to HPLC on a 250 × 4.6 mm Supelcosil LC-18 reversed-phase column. Flow-rate, 1.0 ml/min with pressure *ca.* 1000 psi. Eluents: A: acetonitrile–water (91:9); B: methanol–water (91:9); C: methanol–acetonitrile–water (82:9:9).

*No.**	*Trivial name*	*Structure***	*Retention volume (ml)*		
			A	*B*	*C*
1	Myristic	14:0	19.7	19.3	19.3
2	Palmitic	16:0	34.8	32.8	32.8
3	Palmitoleic	16:1	20.2	21.0	20.7
4	Stearic	18:0	62.8	57.4	57.4
5	Oleic	18:1	34.8	35.3	35.0
6	Linoleic	18:2	22.0	24.7	24.0
7	Linolenic	18:3	15.1	18.4	17.8
8	Eicosatrienoic	20:3	24.6	29.3	28.1
9	Arachidonic	20:4	18.6	23.1	22.0
	Margaric	17:0			44.0

* These numbers correspond to the peaks in Fig. 1.

** The number to the left of the colon is the number of carbon atoms; the number to the right represents the number of double bonds present in the molecule.

The method described here has several advantages compared with previously published methods[1,2,4,5,7,9,10]. Good resolution of all the individual fatty acids of interest is obtained. The chromatographic procedure is fast and the elution volumes are very reproducible. The procedure is based on an isocratic constant-flow system, and can easily be adapted to automatic injection. It is thus a simple, sensitive and reliable method for the separation of naturally occuring C_{14}–C_{20} fatty acids, which has been used successfully in our laboratory for the quantification of free fatty acids in human serum and in the analysis of the fatty acid composition of lipids in human monocytes cultured *in vitro*.

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