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Note

Separation of underivatized C_{20} fatty acids by reversed-phase high-performance liquid chromatography

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High-performance liquid chromatography (HPLC) is a suitable alternative to gas–liquid chromatography (GLC) for the analysis of fatty acids because the high temperature employed in GLC tends to decompose or isomerize the unsaturated fatty acid esters. Furthermore, being non-destructive, HPLC provides a convenient method for the purification of radiolabeled fatty acid substrates for biological use. Both normal-phase and reversed-phase HPLC have been employed for the resolution of fatty acids[1–6]. Silver nitrate-impregnated silica gel columns are usually employed in normal phase chromatography and are more useful for the resolution of geometric isomers of unsaturated fatty acid derivatives. Reversed-phase columns, on the other hand, are more sensitive to the length of the carbon chain in the fatty acid and several C_{14}–C_{20} fatty acids have been separated[6].

In all these methods, UV-absorbing derivatives *e.g.*, *p*-bromophenacyl, methoxyphenacyl, phenacyl, 2-naphthacyl, methoxyanilide esters, of fatty acids have been employed[6,7–11]. Although this improves the sensitivity of the method, these derivatized fatty acids cannot be used as standards for GLC and thin-layer chromatography. Furthermore, when required for biological reactions, these esters first have to be hydrolyzed, which adds an extra step in their isolation. Scholfield[4] and Hsieh *et al.*[1] have recently resolved several fatty acid methyl esters by argentation HPLC. The emphasis is now being placed on the separation of the free fatty acids[12–16], since this avoids the derivatization step and the method can be used for preparative isolation of fatty acids. Thus, Aoshima[14] has separated free 13-hydroxylinoleic acid and 13-hydroperoxylinolenic acid from linoleic acid and linolenic acid, respectively, by HPLC on a porous polymer gel, and using two fatty acid analysis columns in series or a LiChrosorb RP-8 column, Baile *et al.*[15] have separated several underivatized C_{14}–C_{18} fatty acids. With a gradient of acetonitrile in aqueous phosphoric acid,

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Aveldano and co-workers[16,17] have resolved several free fatty acids on reversed-phase columns.

During our studies on the metabolism of arachidonic acid by human platelets, we needed ^{14}C-labeled arachidonic acid free from traces of $\Delta^{8,11,14}$-eicosatrienoic acid, since both these fatty acids can be converted into a variety of monohydroxy-eicosenoic acids and prostaglandins by different tissues. Woollard *et al.*[18] have used reversed-phase partition chromatography on Lipidex 5000 for purification of 2–10 mg samples of arachidonic acid and deuterium- and tritium-labeled arachidonic acid. However, the chromatography is very time consuming (more than 20 h) and the retention volume is very high. We used HPLC for the purification of arachidonic acid and in the process have developed a simple method for the separation of several unsaturated eicosanoic acids including $\Delta^{8,11,14}$- and $\Delta^{11,14,17}$-eicosatrienoic acids on reversed-phase HPLC columns. This paper describes the HPLC separation of these C_{20} fatty acids.

EXPERIMENTAL

All fatty acid standards were purchased from Sigma (St. Louis, MO, U.S.A.) and stored at −70°C when not in use. The solvents used were prefiltered HPLC grade and were purchased from Waters Assoc. (Milford, MA, U.S.A.).

HPLC analysis

HPLC analysis was performed with a Waters Assoc. liquid chromatograph Model 202, equipped with two Model 6000 A reciprocating pumps, a Model UK6 septumless loop injector system and a Model 450 variable wavelength UV detector set to monitor absorbance at 214 nm. A Waters Assoc. "Z" module radial compression system fitted with a 100 × 8 mm I.D. Radial-Pak C_{18} cartridge (5 μm) was used for all separations. A guard column (49 × 4.6 mm I.D.) dry packed with 37–50 μm C_{18} Corasil reversed-phase material (Waters Assoc.) was placed before the separation column.

Each fatty acid (25 mg) was dissolved in 100 ml of acetone and 5–15 μl of this solution were injected onto the column for HPLC analysis. The mobile phase consisted of methanol–water (89:11) containing 0.2% acetic acid (system A) or methanol–water (80:20) containing 0.2% acetic acid (system B). The flow-rate was maintained at 1 ml/min (operating pressure, *ca.* 800 p.s.i.) when system A was used, while system B was run through the column at 2 ml/min (operating pressure, *ca.* 1600 p.s.i.).

RESULTS AND DISCUSSION

Fig. 1A shows the HPLC analysis of six unsaturated C_{20} fatty acids with system A as the mobile phase. As is seen, fatty acids differing in the number of double bonds were very well resolved. However, the method failed to resolve the positional isomers of eicosatrienoic acid and the peak due to the $\Delta^{11,14,17}$ isomer was eluted as a shoulder on the peak due to the $\Delta^{8,11,14}$ isomer. These two isomers were partially resolved (Fig. 1B) when their retention volumes were increased by increasing the proportion of water in the mobile phase (system B). As expected, the more polar solvent system resulted in slight broadening of the peaks. However, the retention

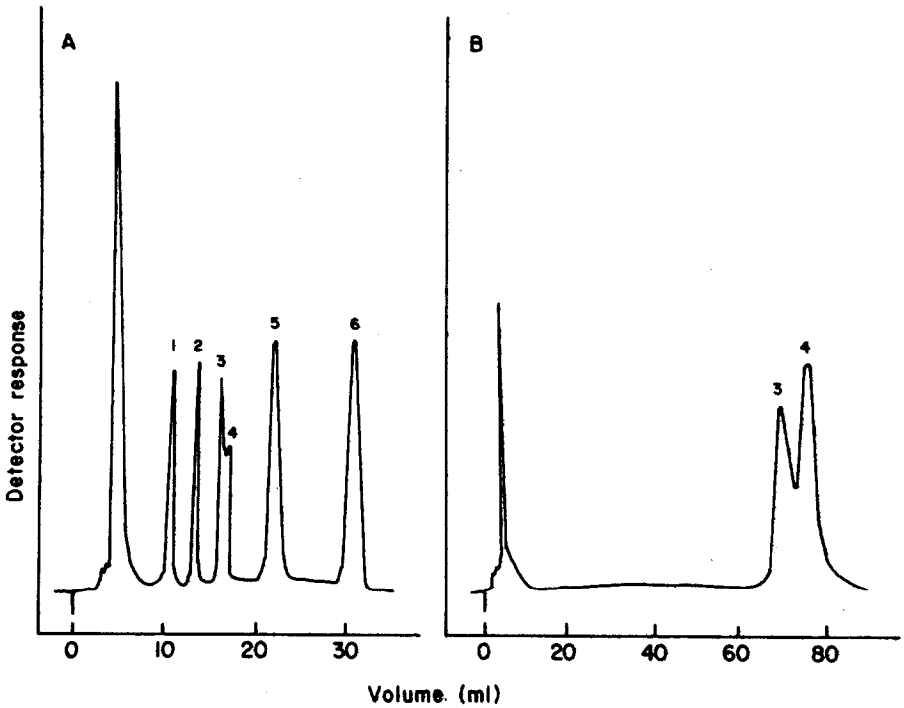

Fig. 1. HPLC separation of fatty acids. Column: 100 × 8 mm I.D. Radial-Pak C_{18} cartridge (5 μm) attached to a guard column (49 × 4.6 mm I.D.) dry packed with 37–50 μm C_{18} Corasil reversed-phase material. Eluents: A, methanol–water–acetic acid (89:11:0.2); B, methanol–water–acetic acid (80:20:0.2). Flow-rates: A, 1.0 ml/min; B, 2.0 ml/min. See Table I for peak identification.

TABLE I

RETENTION VOLUMES OF C_{20} FATTY ACIDS

Free fatty acids were subjected to HPLC on a 100 × 8 mm Radial-Pak C_{18} 5-μm reversed-phase column. Solvent systems: A, methanol–water–acetic acid (89:11:0.2); flow-rate 1 ml/min; B, methanol–water–acetic acid (80:20:0.2); flow-rate 2 ml/min.

*No.**	*Trivial name*	*Positions of double bonds*	*Retention volume (ml)*	
			System A	*System B*
1	Eicosapentaenoic acid	5, 8, 11, 14, 17	10.8	35.8
2	Arachidonic acid	8, 11, 14, 17	13.8	51.5
3	Eicosatrienoic acid	8, 11, 14	16.6	68.8
4	Eicosatrienoic acid	11, 14, 17	17.0	73.6
5	Eicosadienoic acid	11, 17	22.4	–
6	Eicosaenoic acid	11	31.4	–

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

volumes were found to be quite reproducible. Table I lists the retention volumes of these fatty acids in the two mobile phases. Retention volumes were very high in system B, and therefore this system is more useful for identification of the positional isomers of eicosatrienoic acid, whereas system A can be used for the resolution of the various eicosanoic acids with differing degrees of unsaturation.

Unlike the published methods, the method described here is simple, less time-consuming and reliable for the resolution of the C_{20} fatty acids in biological fluids. The fatty acids are resolved without derivatization and the procedure is based on an isocratic constant flow system. Less than 1 μl of the fatty acid can be detected and the retention volumes are small and reproducible. Depending upon whether positional isomers of fatty acids or fatty acids differing in the number of double bonds are to be resolved, a more or less polar solvent system can be used.

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