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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF 5,8,11,14,17-EICOSAPENTAENOIC ACID IN FATTY ACIDS (C_{18} AND C_{20}) BY LABELLING WITH 9-ANTHRYLDIAZOMETHANE AS A FLUORESCENT AGENT*

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SUMMARY

A sensitive high-performance liquid chromatographic method using 9-anthryldiazomethane has been developed for the separation and determination of 5,8,11,14,17-eicosapentaenoic acid (EPA) from C_{18} and C_{20} fatty acids, with a view to its application to the HPLC determination of EPA in fish and human blood or serum and in plankton body fluid. The limit of detection of EPA is about 300 pg and the coefficient of variation is 2.1%.

INTRODUCTION

Eskimos, who feed on the blood of many fish, rarely suffer from heart attacks (myocardial infarction). The main cause of this protection is believed to be the existence of 5,8,11,14,17-eicosapentaenoic acid (EPA), as an unsaturated free fatty acid, in the blood of fish and its possible prevention of thrombosis in human blood vessels.

It was thought that a sensitive high-performance liquid chromatographic (HPLC) method using a suitable fluorescent labelling agent could be developed for the separation and determination of EPA in C_{18} and C_{20} free fatty acids with a view to its application to the HPLC determination of EPA in fish and human blood or serum and in plankton body fluid.

Studies on the fluorescence–HPLC determination of various free fatty acids have already been reported in which the fluorescent labelling agents were 4-bromomethyl-7-methoxycoumarin[1], 9,10-diaminophenanthrene[2], 9-anthryldiazomethane (ADAM)[3–7], 1-bromoacetylpyrene[8] and 1-naphthylamine[9]. However, no detailed

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study on the analytical separation and determination of EPA in other free fatty acids has been reported.

This work was carried out using ADAM[3], because it has the advantages over the other reagents that it is able to effect the esterification of the fatty acids at room temperature in neutral media, without heating the sample solution, the need for any catalysts or preliminary purification of the reagents.

EXPERIMENTAL

Apparatus

The measurements were made with a Shimadzu Model LC-4A HPLC instrument equipped with a Shimadzu Model RF-510LC fluorescence spectromonitor and a Shimadzu Model C-RIA(S) Chromatopac. Excitation and emission spectra were measured with Shimadzu Model RF-503 double-beam difference spectrofluorophotometer. A Taiyo Kagaku Model S-50 automatic mixer was used for mixing the fatty acids and ADAM.

Reagents

Stock standard EPA and the other fatty acid solutions (1000 ppm) were first prepared by dissolving them in *n*-hexane, and working standard solutions were prepared by diluting the stock solutions in methanol to give the desired concentrations for preparing calibration graphs. These solutions were stored in a freezer (at $-10°C$) to prevent decomposition. The stock standard solutions in *n*-hexane as a non-polar solvent were stable in the freezer for several months.

ADAM solution (0.05%, w/v) was prepared by adding one drop of acetone to 1 mg of ADAM and dissolving this in 2 ml of methanol with agitation. This ADAM solution was stored in a freezer (at $-10°C$), but even then it did not keep for more than 10 h.

Analytical procedure

A 50-μl volume of standard EPA solution containing different amounts of EPA ($\leqslant$ 45 ng) is taken in a glass microtube (about 1 ml) and 50 μl of 0.05% ADAM solution are added. In order to carry out the esterification as shown in Fig. 1, the mixed solution is vibrated by means of the automatic mixer for 1 min. After allowing the EPA ester solution to stand for 1 h, a portion of it is analysed by HPLC under the conditions shown in Table I.

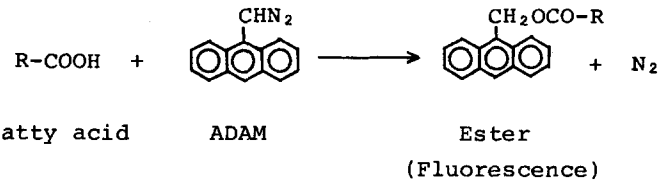

Fig. 1. Esterification of fatty acids with ADAM.

TABLE I

EXPERIMENTAL CONDITIONS FOR THE DETERMINATION OF THE ADAM DERIVATIVE OF EPA

Variable	*Conditions*
Solid phase	Zorbax-C8
Column dimensions	150 × 4.6 mm I.D.
Mobile phase	Methanol-water (9:1)
Flow-rate	1.0 ml/min (44 kg/cm^2)
Temperature	50°C
Detector	Fluorescence spectromonitor, $\lambda_{ex.}$ 345 nm, $\lambda_{em.}$ 416 nm; range 1; band width 10 nm (excitation and emission)
Recorder speed	2 mm/min
Sample solution injected	2, 5 or 10 μl

RESULTS AND DISCUSSION

Chromatographic conditions

For the HPLC analysis of the ADAM derivative of EPA, methanol–water (9:1) was used as the mobile phase with Zorbax-C8, the solid phase, because better separation of EPA from the other fatty acids was obtained than with acetonitrile–water. Fig. 2 shows the effect of the proportions of methanol and water on the peak area and retention time of the ADAM derivative of EPA. The effect of the column temperature was studied (Fig. 3) and the optimum was found to be ca. 50°C.

Stabilities of ADAM and of the ADAM Derivative of EPA

To check the stabilities of the ADAM solution and of the ADAM derivative

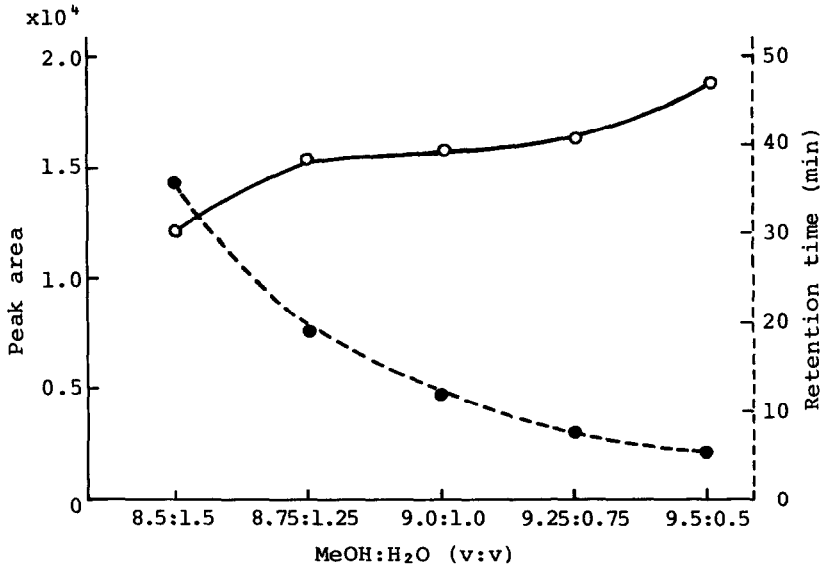

Fig. 2. Effect of composition of mobile phase on the peak area and retention time of the ADAM derivative of EPA. ○, Mixing ratio of methanol and water; ●, retention time. Amount of EPA: 18.8 ng.

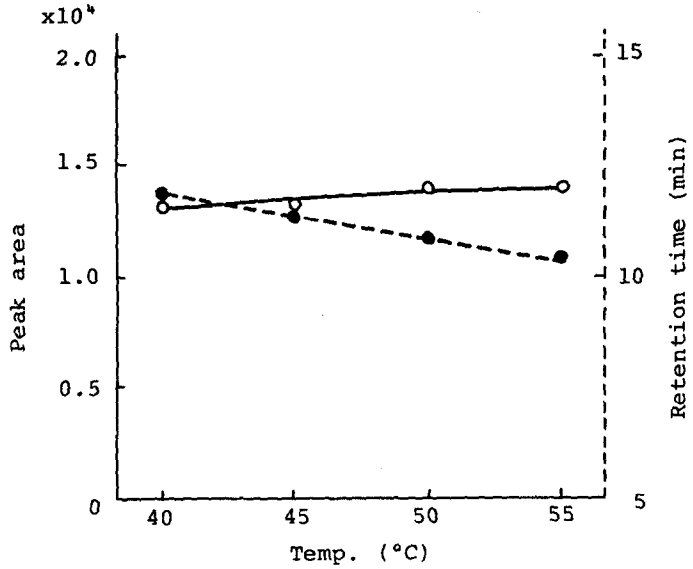

Fig. 3. Effect of column temperature on the peak area (○) and retention time (●) of the ADAM derivative of EPA. Amount of EPA: 9.0 ng.

of EPA, several identical solutions containing the same amount of EPA were previously prepared and stored in a freezer (at −10°C), then 0.05% ADAM solution, at varying times after its preparation, was added at intervals, and each ADAM derivative of EPA was measured by the proposed procedure 1 h after the synthesis.

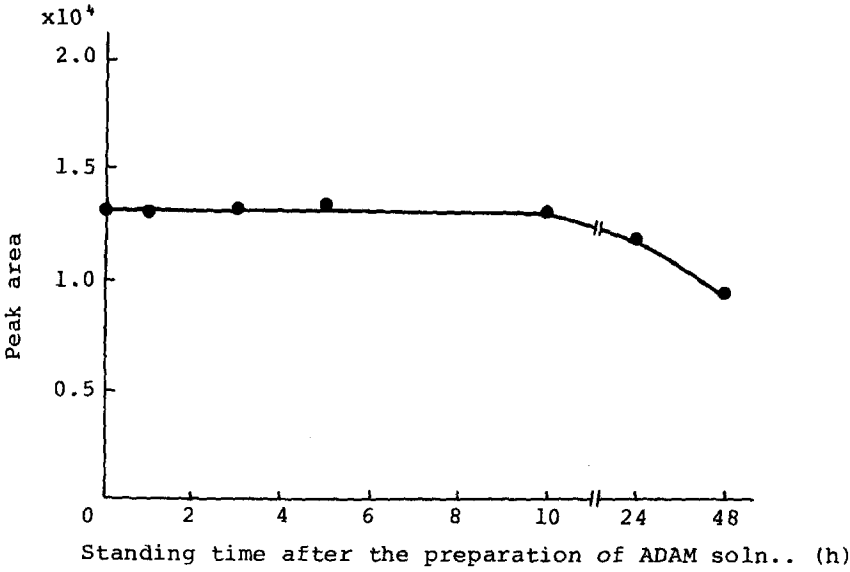

Fig. 4. Stability of ADAM. ADAM solution was kept in a freezer. Each ADAM derivative of EPA was determined by HPLC 1 h after synthesis. Amount of EPA: 8.9 ng.

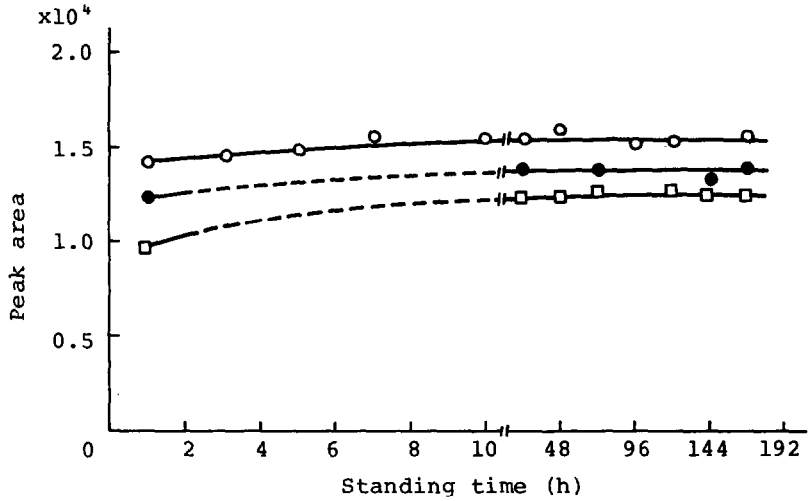

Fig. 5. Stability of the ADAM derivatives of EPA. ○, ADAM prepared just before its use was added to EPA; ●, ADAM 24 h after its preparation was added to EPA; □, ADAM 48 h after its preparation was added to EPA. Amount of EPA: 9.1 ng.

The results in Fig. 4 indicate that the ADAM solution was stable in the freezer for at least 10 h.

Fig. 5 shows stability of ADAM derivatives prepared using the same amount of EPA with ADAM solution at 0, 24 and 48 h after its preparation. It can be seen that, especially when ADAM solution prepared just before the synthesis is used, there are no significant differences in the peak areas for standing times in the range 0–192 h, in diffuse daylight at ambient temperature, although the curve rises slightly. The ADAM derivative of EPA was stable for several months.

Fluorescent properties of the ADAM derivatives of different fatty acids

Excitation and fluorescence emission spectra of the ADAM derivatives of C_{18} and C_{20} fatty acids were measured with the double-beam difference spectrofluorophotometer as contrasted with Rhodamine B which has the known fluorescence spectrum. On the basis of these results, 345 and 416 nm were chosen as the excitation and the fluorescent emission wavelengths, respectively.

Fig. 6 shows the separation from a mixture of the ADAM derivatives of $C_{18}{}^{0-3=}$ or $C_{20}{}^{0-5=}$ fatty acids (see Table II for full name). Generally, the greater the unsaturation in the molecule of fatty acids at a constant carbon number, the shorter the retention time becomes.

Table II shows the formulae of the fatty acids that were considered in these experiments, and their detection limits, relative retention times obtained from Fig. 6 and excitation and fluorescence maxima.

Calibration graph

A calibration graph was prepared by using standard EPA solutions containing different amounts of EPA and by injecting 5 μl of the solution into the chromatograph. It was linear at least in the range 0–45 ng, as shown in Fig. 7.

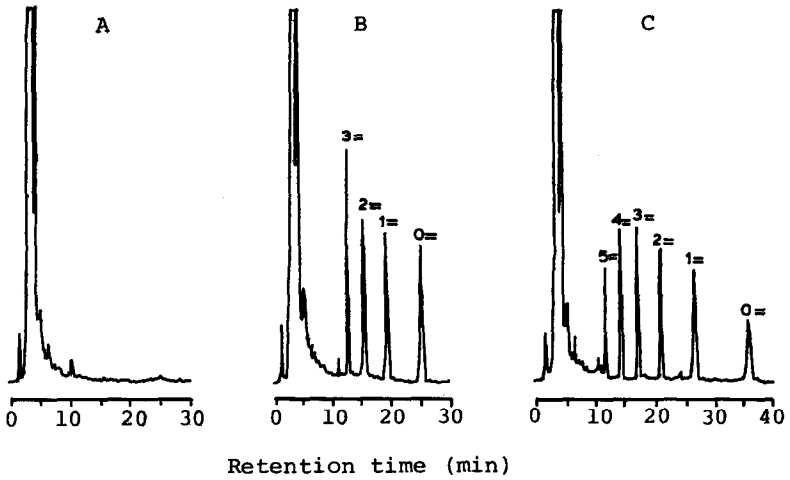

Fig. 6. HPLC traces of the ADAM derivatives of C_{18} and C_{20} fatty acids. A, Reagent blank; B, $C_{18}{}^{0-3=}$; C, $C_{20}{}^{0-5=}$. Full names of fatty acids as in Table II.

TABLE II

FORMULAE OF C_{18} AND C_{20} FATTY ACIDS CONSIDERED AND DETECTION LIMITS AND RETENTION TIMES OF THEIR ADAM DERIVATIVES

Carbon number	*Name and formula*	*Detection limit* (pg)*	*Retention time (min)*	*Excitation/ fluorescence maxima (nm)*
$C_{18}{}^{0=}$	*n*-Octadecanoic acid $CH_3(CH_2)_{16}COOH$	233	24.5	346/416, 439
$C_{18}{}^{1=}$	*cis*-9-Octadecenoic acid $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	293	18.6	345/415, 437
$C_{18}{}^{2=}$	*cis,cis*-9,12-Octadecadienoic acid $CH_3(CH_2)_4\ (CH=CHCH_2)_2(CH_2)_6COOH$	308	14.8	334/416, 438
$C_{18}{}^{3=}$	*cis,cis,cis*-9,12,15-Octadecatrienoic acid $CH_3(CH_2CH=CH)_3\ (CH_2)_7COOH$	255	12.1	344/416, 438
$C_{20}{}^{0=}$	*n*-Eicosanoic acid $CH_3(CH_2)_{18}COOH$	240	36.7	346/416, 438
$C_{20}{}^{1=}$	*cis*-11-Eicosenoic acid $CH_3(CH_2)_7CH=CH(CH_2)_9COOH$	173	27.4	343/415, 437
$C_{20}{}^{2=}$	11,14-Eicosadienoic acid $CH_3(CH_2)_4(CH=CHCH_2)_2(CH_2)_8COOH$	158	21.5	345/415, 437
$C_{20}{}^{3=}$	11,14,17-Eicosatrienoic acid $CH_3CH_2(CH=CHCH_2)_3(CH_2)_8COOH$	150	17.3	345/416, 438
$C_{20}{}^{4=}$	5,8,11,14-Eicosatetraenoic acid $CH_3(CH_2)_4(CH=CHCH_2)_4(CH_2)_2COOH$	188	14.3	344/416, 438
$C_{20}{}^{5=}$	5,8,11,14,17-Eicosapentaenoic acid $CH_3CH_2(CH=CHCH_2)_5(CH_2)_2COOH$	300	11.8	345/416, 438

* Detection limits fatty acids assuming a signal-to-noise ratio of about 3.

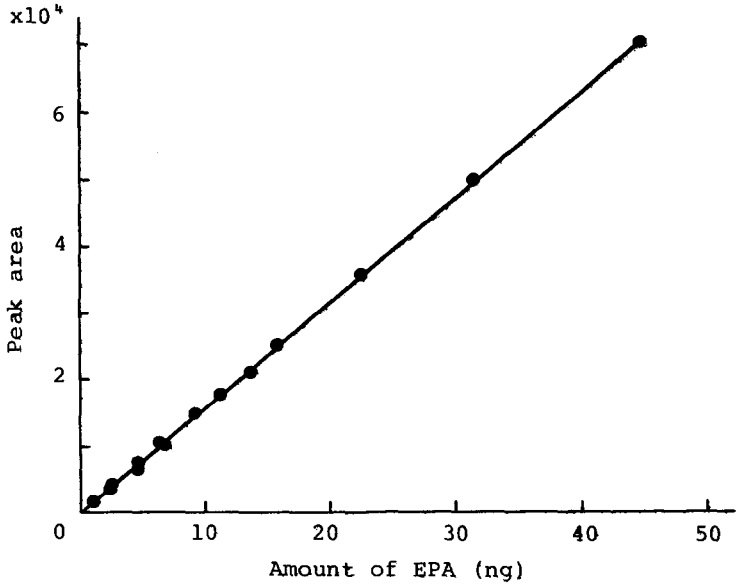

Fig. 7. Calibration graph for EPA.

Precision and limit of detection

The precision of the proposed method was tested by measuring twelve times the peak area of identical pure solutions containing 22.5 ng of EPA. The coefficient of variation was 2.1% and the limit of detection was calculated to be 300 pg.

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REFERENCES

1 W. Dünges, *Anal. Chem.*, 49 (1977) 442.
2 J. B. F. Lloyd, *J. Chromatogr.*, 189 (1980) 359.
3 N. Nimura and T. Kinoshita, *Anal. Lett.*, 13 (1980) 191.
4 S. A. Barker, J. A. Monti, S. T. Christian, F. Benington and R. D. Morin, *Anal. Biochem.*, 107 (1980) 116.
5 M. Hatsumi, S.-I. Kimata and K. Hirosawa, *J. Chromatogr.*, 253 (1982) 271.
6 S. Imaoka, Y. Funae, T. Sugimoto, N. Hayahara and M. Maekawa, *Anal. Biochem.*, 128 (1983) 459.
7 Y. Shimomura, K. Taniguchi, T. Sugie, M. Murakami, S. Kakuta, S. Sugiyama and T. Ozawa, *Rinshokensa*, 27 (1983) 561.
8 Sankyo Co., *Jap. Kokai Tokyo Koho*, JP81, 113,728; *C.A.*, 96 (1982) 52044u.
9 R. F. Borch, *Anal. Chem.*, 47 (1975) 2437.