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DERIVATIZATION OF FATTY ACIDS WITH 2-BROMOACETYLTRIPHENYLENE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract : A method for UV labeling of fatty acids with 2-bromoacetyltriphenylene using 18-crown-6-ether as a catalyst is described. The procedure is rapid, simple, quantitative and applicable to the HPLC analysis of fatty acids with UV detector. They have high molar absorptivity and their detection limit was about 1ng level. Nine derivatives of saturated fatty acid(C_{12}-C_{22}) were separated on reverse-phase column(μ-Bondapak C-18) using acetonitrile-water gradient.

요약 : 지방산을 UV 검출기를 사용하여 HPLC로 분석하기 위하여 2-bromoacetyltriphenylene을 유도체화제로 응용하였다. 18-crown-6-ether를 촉매로 한 유도체화반응은 매우 신속, 간편하고 정량적으로 진행되었으며, HPLC상에서 지방산의 검출한계가 1ng 수준으로 그 감도가 양호하였다. 또한 C_{12}부터 C_{22}까지 9종류의 포화지방산이 역상컬럼상에서 acetonitrile-물 gradient 조건으로 동시에 분리가 가능하였다. 이를 응용하면 각종 생체 시료로부터 미량의 지방산들에 대한 동시분석이 가능하리라 기대된다.

Key words : fatty acid, derivatization, HPLC

1. Introduction

Applications of high pressure liquid chromatography(HPLC) has been widely employed in the analysis of many kinds of biological materials. In the area of lipid analysis, the application to HPLC has some limitation, because most lipids possess little or no useful absorption in the UV range for the UV detection which is the widely available device.

Recently, many kinds of UV labeling agents have been studied for the purpose of separation and detectability enhancement. The most commonly used derivatizing agents for organic acids are phenacyl ester which have been widely applied in the analysis of both short-chain[1] and long-chain fatty acids[2], dicarboxylic acids[3], unsaturated fatty acids[4], prostaglandins[5], and penicillins[6]. Durst *et al*[1] reported the derivatization method of fatty acids with crown ether as catalyst. This method made the derivatization more efficient with respect to the applicability of variety of aprotic solvents, short reaction time, no need for an excess of the expensive derivatization reagents and the removal of the artifact formation[7], etc.

We applied 2-bromoacetyltriphenylene as a UV-derivatization agent of fatty acids using 18-crown-

6-ether as catalyst. The derivatives can be formed easily and economically, and can be separated on a reverse phase HPLC system.

2. Experimental Procedure

Reagents and Apparatus

All organic solvents(Fisher Scientific, Pittsburgh, PA, USA) were commercial analytical-reagents grade materials. The distilled water used for the mobile phase was passed through an ion-exchanger (Millipore Corp., Bedford, MA, USA). All chromatographic solvents were de-aerated by bubbling dry nitrogen through them. 2-bromoacetyltriphenylene(BATP) was prepared according to a published procedure[8]. Fatty acids were purchased from Sigma Chemical Co., St. Louis, MO, USA. 18-crown-6-ether was purchased from Aldrich (Milwaukee, Wisc., USA) and was used without purification.

Derivatization of Fatty Acids

The following stock solutions were preapred : (1) 0.026g of 18-crown-6-ether in 25ml of DMF, THF or acetonitrile ; (2) 0.0035g of BATP in 25ml of DMF, THF or acetonitrile and protected from light by wrapping the container in aluminum foil ; (3) 0.066g of potassium hydroxide dissolved in methanol and protected from the atmosphere. (4) the fatty acids were dissolved in methanol(0.001mM) and the derivatization was carried out as follows.

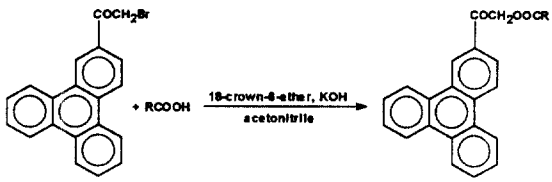

Fig. 1. Esterification of fatty acid with 2-bromoacetyltriphenylene using 18-crown-6-ether as catalst

A sample of fatty acids was neutralized by the potassium hydroxide solution and the solvents were removed by N_2 purging. An excess solution of 18-crown-6-ether was then added and sonicated for the complete solubilization, and BATP solution was further added. The solution was allowed to incubate in a 40℃ water bath for one hour and it is then ready for the chromatographic analysis. The optimization of derivatization was performed by using myristic acid. For the structural confirmation of derivatives, myristic acid-2-bromoacetyltriphenylene ester(0.3g) was prepared on a semipreparative scale, and purified on silicagel column chromatography by using hexane/benzene(1:2) as eluent and recrystallized in acetonitrile.

Myristic acid-2-bromoacetyltriphenylene ester
$C_{34}H_{40}O_3$, mp:88~90℃
UV(acetonitrile) λ_{max} at 268nm($\log \varepsilon = 4.57$)
IR ν_{max}^{KBr} cm^{-1} : 3040(=C-H arom), 1730(C=O conjugated), 1690(C=O arom), 1600(C=C arom), 1197(C-O ester 750, 720(arom)
Mass(EI) m/z : 496(M^+, 33.8%), 255(M-$C_{15}H_{29}O_2$, 100%), 227(M-$C_{16}H_{29}O_3$, 23.7%)
NMR(DMSO-d_6, 80MHz) δ : 0.9(3H), 1.3(22H), 2.5(2H, t), 5.5(2H, s), 7.7(3H, m), 8.1(1H, dd), 8.6(6H, m), 9.2(1H, d)

Chromatographic Procedures

All analysis were performed with Pye-Unicam HPLC model PU 4030 controller equipped with PU4011 pump, PU 4020 UV detector and PU 4810 computing integrator.

UV spectrophotometer was Pye-Unicam PU 8800 model. The column used was a 4.0 mm i.d. × 30cm μ-Bondapak C-18. The mobile phase used for the optimization of derivatization procedures was 80% acetonitrile in water, and for the separation of mixed fatty acid derivatives and calibration was the gradient from 80% acetonitrile to 100% acetonit-

rile.

3. Results and Discussion

Derivatization Studies

The most important advantages in using crown ether to catalyze the derivatizing of fatty acids is that very small amouns of the compounds are needed for the reaction. And the crown ether process gave quantitative yields of derivatives with no byproducts. The choice of solvent in the derivatization procedures was critical and in this study acetonitrile was chosen from the results of *Fig.* 2 according to the solubilities of the products and the completions of reactions. Tetrahydrofuran and N,N-dimethylformaldehyde could not completed the derivatizatizing reactions. The reaction rates are dependent on the base used. With potassium hydroxide as the base, the reaction proceeds to completion in 30 minutes, while 60 minutes are needed for potassium bicarbonate(*Fig.* 3).

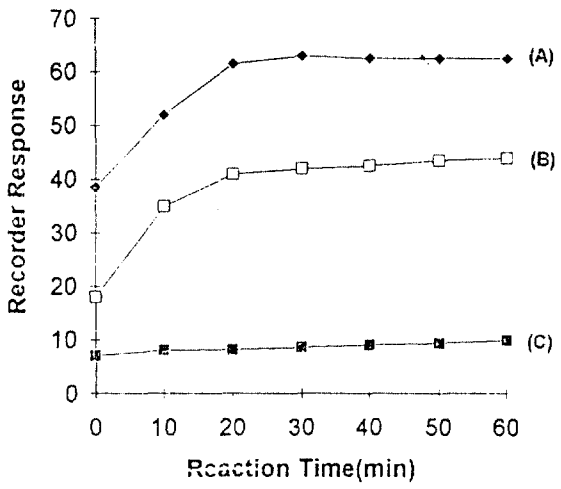

Fig. 2. Effects of solvent in derivatization of fatty acid with BATP (reaction temperature : 40℃, potassium hydroxide as base)
key : (A) acetonitrile, (B) tetrahydrofuran, (C) N,N-dimethyformaldehyde

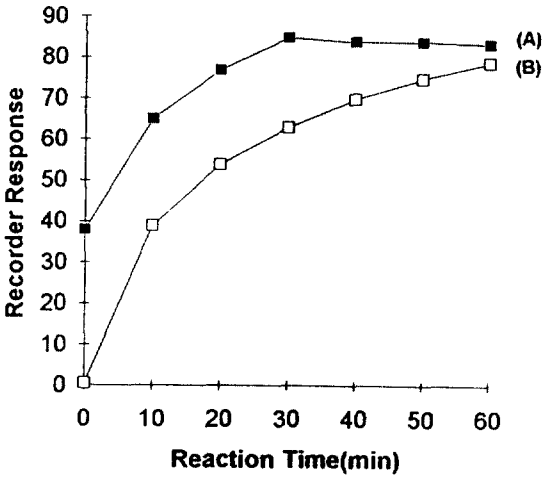

Fig. 3. Effects of base source on the overall reaction yield of fatty acid with BATP using 18-crown-6-ether as catalyst(solvent : acetonitrile, reaction temperature : 40℃)
key : (A) potassium hydroxide, (B) potassium bicarbonate

The crystalline product of derivatization from myristic acid has very high molar absorptivity. Absorption maxima occured at 268nm in acetonitrile solvent and the molar absorptivity was 37,000 $M^{-1}cm^{-1}$. *Fig.* 4 shows the time course of reaction with various reaction temperature based on HPLC analysis. The best condition of derivatization was found as 40℃ for 30 minutes which gave quantitative yields without any byproduct formation. Under the reaction temperature of 70℃, the reaction was completed within 10 minutes but several peaks which seems to be degradated products were appeared.

The mole ratio of potassium hydroxide/fatty acid and BATP/fatty acid was varied from 1 to 9, and the ratio of 4 and 3 showed the best results, respectively(data not shown). At the higher ratio of potassium hydroxide above 7, the yield of derivatization was decreased accompanying with the formation of byproducts. At the higher ratio of BATP, the BATP peak was overlapped with the derivat-

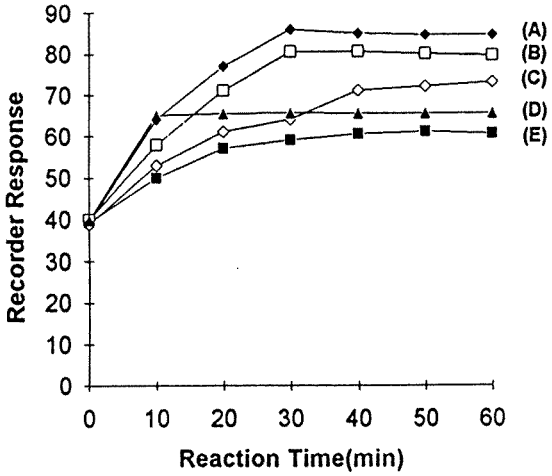

Fig. 4. Effects of reaction temperature(solvent : acetonitrile, potassium hydroxide as base)
key : (A) 40℃, (B) 30℃, (C) 50℃, (D) 70℃, (E) 25℃

ized ester, especially with those from the short chain fatty acids. All those derivatives are stable over 24 hours at room temperature.

Chromatographic Separations

All the separations were done at room temperature. The detector was set at 0.02 AUFS, 268nm wavelength and the derivatives were separated on reverse phase columns. Using a linear acetonitrile-water gradient starting from 80% acetonitrile, complete separation of nine saturated fatty acids between C_{12} and C_{22} was achieved in less than one hour(*Fig.* 5). The retention orders are typical of those found in reverse phase systems. The ease of derivative formation coupled with the simple chromatographic separation indicates the potential of the method for the routine analysis of fatty acids.

Calibration curves

To be useful in quantitative analysis, the amount

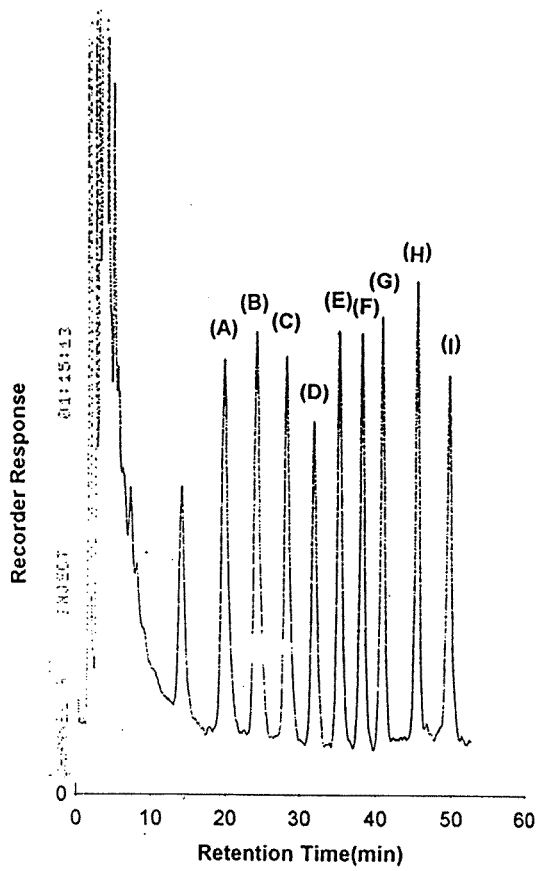

Fig. 5. Chromatogram of derivatized standard fatty acids. Column, μ-bondapak C-18, 4mm i.d. × 30cm : mobile phase, acetonitrile-water gradient, starting with 80% acetonitrile, 10min isocratic, then linear increase of acetonitrile (1%/min) upto 100% acetonitrile. Flow rate, 2ml/min : detection wavelength, 268nm, 0.02AUFS.
key : (A) lauric acid, (B) tridecanoic acid, (C) myristic acid, (D) pentadecanoic acid, (E) palmitic acid, (F) margaric acid, (G) stearic acid, (H) arachidonic acid, (I) behenic acid.

of ester found in the derivatization reaction should be related to the amount of the fatty acid. A typical result for the present reaction is showen in *Fig.* 6, which is a plot of the peak area of esters versus the amount of the fatty acid introduced into the re-

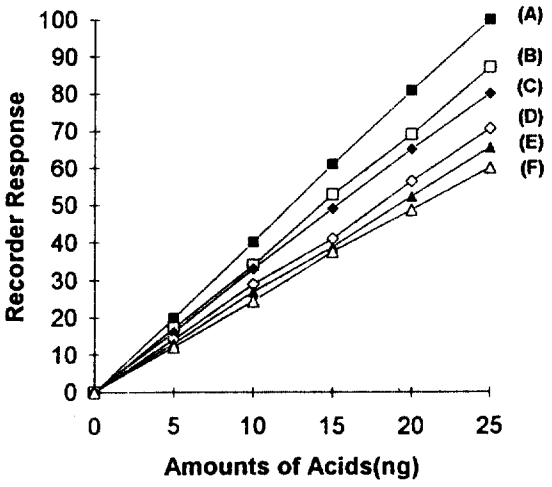

Fig. 6. Calibration curve of fatty acid-BATP ester mixtures

key : (A) lauric acid, (B) myristic acid, (C) palmitic acid, (D) margaric acid, (E) arachidonic acid, (F) pentadecanoic acid

action mixture. The linear relationship indicates that the procedure described here can be used to quantitate fatty acids. The typical detection limit of this procedure was about 1ng and it could be further reducible with an optimized chromatographic system.

CONCLUSIONS

The derivatization of fatty acid by 2-bromoacetyltriphenylene (BATP) is promising. It is rapid, simple and can be adopted for routine fatty acid analysis. The BATP esters show excellent chromatographic properties, allowing the ready separation of long and short chain fatty acids. They offer a high levels of sensitivity for fatty acids down to 1ng level and good linearity for the quantitative application. This method can be applied in the analysis of complex biological samples related to the fatty acid-containing materials and carboxyl-substituted drugs.

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