Determination of Free Fatty Acids in o/w Injectable Emulsions by HPLC with Fluorescence Detection Using 4-(*N*,*N*-Dimethylaminosulfonyl)-7-*N*-piperazino-2,1,3-benzoxadiazole

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HPLC using the fluorescent reagent, 4-(*N*,*N*-dimethylaminosulfonyl)-7-*N*-piperazino-2,1,3-benzoxadiazole (DBD-PZ) was applied to the determination of free fatty acids in an o/w injectable emulsion. This fluorescence HPLC method was highly sensitive and the free fatty acids could be directly derivatized without any previous preparation such as solvent extraction and elimination of water. In addition, the derivatized fatty acids could be adequately separated from interfering peaks, *i.e.*, reagent and by-product peaks.

The detection limit of this HPLC method was *ca.* 5 fmol in the case of $C_{18:0}$ fatty acid. The mean recovery of 2.5 nmol of each fatty acid ($C_{8:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$) added to the o/w injectable emulsion was 101.6% ($n=3$). This fluorescence HPLC method with DBD-PZ was applied to commercial nonaqueous injection preparations to study their stability, and each free fatty acid in these preparations could be determined successfully. These results indicate that the proposed fluorescence HPLC method can be adequately used for the determination of free fatty acids in an o/w injectable emulsion.

Key words o/w injectable emulsion; fluorescence HPLC; the fluorescent reagent; fatty acid

In the development of an o/w injectable emulsion and an oil-soluble injection, several excipients have been used to improve drug solubility and stability. In particular, medium or long-chain fatty acid esters are generally used as solubilizing agents. However, their esters cause hydrolysis and fatty acids are liberated during the manufacturing process or under relatively high temperature storage conditions, *e.g.*, 40 °C for 6 months. In addition, the degree and nature of the toxicity of a fatty acid depends on its chain length.[1] Therefore, the determination of the individual free fatty acids in nonaqueous injection preparations is important for the quality control and investigation of the optimal preparation when fatty acid esters are used in nonaqueous injection preparations.

For the determination of free fatty acids, several methods using enzyme,[2,3] titration[4—7] and high-performance liquid chromatography (HPLC)[8—20] have been reported. HPLC is better at determining each fatty acid, although enzymatic and titrimetric methods are used to determine total fatty acids. Various fluorescent reagents have been proposed[8—16] for the highly sensitive determination of fatty acids using HPLC. However, the application of HPLC to the determination of fatty acids in injection preparations has not been reported. 4-(*N*,*N*-dimethylaminosulfonyl)-7-*N*-piperazino-2,1,3-benzoxadiazole (DBD-PZ) is a more sensitive reagent for fatty acids than other reagents (detection limit; from 3 to 14 fmol), and the derivatization of fatty acids with DBD-PZ is extremely easy to achieve under mild conditions.[17] In addition, DBD-PZ is not an intrinsic impurity, and these derivatives can be adequately separated from the reagent peak and by-products.[18] The by-products presumably result from the side-reaction of DBD-PZ with diethyl phosphorocyanidate (DEPC) (the condensing agent).

In this study, a fluorescence HPLC method with DBD-PZ was used to determine each fatty acid in a nonaqueous injection preparation with their minimal pre-treatment (only dilution). This method was applied to a commercial o/w injectable emulsion and an oil-soluble injection preparation.

Experimental

Materials and Reagents Caproic acid ($C_{6:0}$), caprylic acid ($C_{8:0}$), capric acid ($C_{10:0}$), lauric acid ($C_{12:0}$), tridecanoic acid ($C_{13:0}$), myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dimethylsulfoxide, ethanol, *n*-heptane, isopropanol, sodium sulfate anhydrous, phenolphthalein, 0.05 M alcoholic potassium hydroxide, DEPC and HPLC grade acetonitrile were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DBD-PZ was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Determiner® NEFA was from Kyowa Medex Co., Ltd. (Tokyo, Japan). The silica gel column (Mega Bond Elut®; SI, 20 ml/5 g) for sample pre-treatment was from Varian (CA, U.S.A.). Injection preparations were commercial products. The composition of the products is shown in Table 1.

Apparatus HPLC was carried out using an "intelligent" pump (L-6200; Hitachi, Tokyo, Japan) equipped with an auto-sampler (655A-40; Hitachi). DBD-PZ fatty acids were separated on an octadecylsilanized slica gel (ODS) column (YMC-Pack Pro C18, 150×4.6 mm i.d., spherical 5 μm, 120 Å; YMC Co., Ltd., Kyoto, Japan) and detected fluorometrically at 557 nm, with excitation at 444 nm (821-FP; JASCO, Tokyo, Japan). The results were analyzed using a chromato-integrator (D-2500; Hitachi). Linear gradient elution from 50% to 98% acetonitrile in water was performed for 60 min to separate the DBD-PZ fatty acids.

Fluorescence spectra were measured by a fluorescence spectrophotometer (F-4010; Hitachi, Tokyo, Japan) and absorbance was measured by a UV-VIS spectrophotometer (U-3210; Hitachi, Tokyo, Japan).

Sample Preparation (Derivatization Conditions) A nonaqueous injection preparation (1 ml) was diluted with acetonitrile to 20 ml. This solution (100 μl), internal standard solution ($C_{13:0}$ in acetonitrile, 10 μM, 500 μl), DBD-PZ solution (DBD-PZ in acetonitrile, 50 mM, 500 μl) and DEPC (50 μl)[17] were mixed, and then diluted with acetonitrile to 5 ml. After 6 h at

Table 1. Composition of o/w Injectable Emulsions

Composition	Product A	Product B
Soybean oil	100.0 g	200.0 g
Medium chain triglyceride	100.0 g	—
Glycerol	25.0 g	25.0 g
Egg yolk phospholipid	12.0 g	12.0 g
Sodium oleate	Adequate	—
Sodium hydroxide	Adequate	—
Water for injection	Total 1 l	Total 1 l

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25 °C, 10 μl of the sample solution was subjected to HPLC. A standard mixed solution ($C_{8:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ in acetonitrile; each 10 μM, 500 μl) was prepared and subjected to HPLC under the same conditions as the sample solution. The concentration of each free fatty acid in the nonaqueous injection preparation was calculated as follows:

$$\text{free fatty acids (mEq/l)} = (At/As) \times 100 = At/(aT^2 + bT + c) \times 100$$

where At, As and T are the fluorescence intensity of the DBD-PZ fatty acid in the sample solution, the fluorescence intensity of DBD-PZ fatty acid (1 μM) and the relative retention time, respectively. The equation ($As = aT^2 + bT + c$) was obtained by the non-linear least squares method using the standard mixed solution ($C_{8:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$). Details of the correction value are described in the Results and Discussion section (effect of the fatty acid chain length on fluorescence, Fig. 4).

Effect of Fatty Acid Chain Length on Fluorescence The mixed solution of fatty acids was prepared as described above (derivatization conditions). To investigate the fluorescence of DBD-PZ fatty acids, the eluent corresponding to the fluorescence peak of each DBD-PZ fatty acid was collected following HPLC. An each fraction was diluted with acetonitrile and the fluorescence spectrum measured.

Effect of Acetonitrile Concentration on Fluorescence of DBD-PZ Fatty Acids The fatty acids mixed solution was prepared as described above (derivatization conditions). Linear gradient elution (from 50% to 98% acetonitrile in water for 60 min) and several isocratic elutions (acetonitrile/water, 6/4, 7/3, 8/2, 9/1 for 30 min) were used for the determination of the DBD-PZ fatty acids. The fluorescence intensity (peak area) of the DBD-PZ fatty acids detected by under gradient HPLC conditions was compared with that under isocratic HPLC conditions.

Comparison of Methods Enzymatic Method: The principle of the enzymatic method is based on the measurement of hydrogen peroxide produced from free fatty acids by acyl-CoA synthetase and acyl-CoA oxidase.[3] Methylene blue (the color reagent) was used for the determination of hydrogen peroxide in the presence of peroxidase and 10-*N*-methylcarbamoyl-3,7-dimethylamino-10*H*-phenothiazine. The NEFA kit consists of the color reagent and enzyme reagent. The color reagent was 10-*N*-methylcalbamoyl-3,7-dimethylamino-10*H*-phenothiazine solution, and the enzyme reagent was a mixture of ascorbate oxidase, acyl-CoA synthetase, acyl-CoA oxidase, adenosine pentaphosphoric acid, coenzyme A and peroxidase.

The injection preparation (20 μl) was added to the NEFA reagent (3 ml), vortex mixed and reacted at 37 °C for 5 min. Dimethylsulfoxide (5 ml) was added to the reaction mixture, with cooling in a water bath. After the reaction mixture had been filtered through a membrane filter (0.45 μm), the absorbance (At; at 660 nm) of the supernatant was immediately measured. The standard solution ($C_{8:0}$ in ethanol, 1 mM, 20 μl, As; at 660 nm) was treated in the same way as the injection preparation. The free fatty acids in the nonaqueous injection preparation were calculated as follows:

$$\text{free fatty acids (mEq/l)} = (At - Ab)/(As - Ab) \times M$$

where Ab and M are the blank absorbance at 660 nm and the molarity of $C_{8:0}$ (mM), respectively.

Titrimetric Method: The titrimetric method was performed as described in "Intravenous Fat Emulsion, Free fatty acids in *USP pharmacopeial forum* Vol. 21 No. 5 1995 (in process of revision)." A mixture of heptane, isopropyl alcohol, and water (2 : 2 : 1, v/v) was prepared, the phases allowed to separate, and the lower phase was discarded. Anhydrous sodium sulfate in the upper phase was removed by filtration, and the supernatant was used as the extracting solvent.

The nonaqueous injection preparation (20 ml) was pipetted into a flask, frozen, and lyophilized. The residue was dissolved in 30 ml extracting solvent, and the solution was applied to a silica gel column. The flask was rinsed with 90 ml extracting solvent and the solvent was also passed through the silica gel column (Mega Bond Elut®; SI, 20 ml/5 g). A total of 120 ml effluent was collected, and then phenolphthalein test solution (phenolphthalein in ethanol, 10 mg/ml, 10 drops) was added. The effluent was titrated with 0.05 M alcoholic potassium hydroxide, bubbling nitrogen through the solution during titration. The concentration of free fatty acids in the nonaqueous injection preparation was calculated by the formula:

$$\text{free fatty acids (mEq/l)} = (Vs - Vb) \times M \times 50$$

where Vs, Vb, M and 50 correspond to the titration volume of 0.05 M alcoholic potassium hydroxide (ml) for the sample solution, the blank value, the molarity of alcoholic potassium hydroxide and the dilution factor.

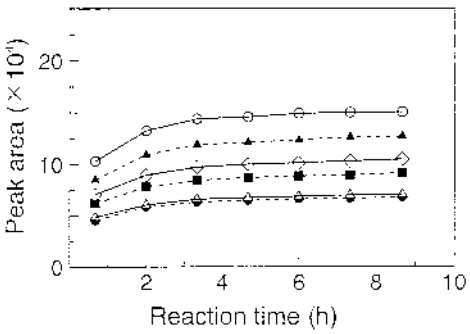

Fig. 1. Time-Course of the Reaction of Fatty Acids with DBD-PZ at 25 °C

○: $C_{18:0}$, ▲: $C_{16:0}$, ◇: $C_{14:0}$, ■: $C_{12:0}$, △: $C_{10:0}$, ●: $C_{8:0}$. The reaction conditions with DBD-PZ are described in the text.

Results and Discussion

Pre-column Derivatization of Free Fatty Acids As Toyo'oka *et al*. reported, acetonitrile and DEPC were used as the solvent in the reaction medium and the condensing agent, respectively.[17,18] Several fatty acids (from $C_{8:0}$ to $C_{18:0}$) were reacted with DBD-PZ in acetonitrile and DEPC. For the simple and accurate determination of fatty acids in the o/w injectable emulsion, the emulsion was directly reacted with DBD-PZ without any pretreatment. As shown in Fig. 1, the reaction was nearly complete about 5 h after the fatty acids and DBD-PZ were vortex mixed at 25 °C.

$C_{13:0}$, which was not contained in the o/w injectable emulsion, was used to measure the recovery of fatty acids in the o/w injectable emulsion and 10 μM $C_{13:0}$ was added to the reaction medium. Up to 1.0% (v/v), the o/w injectable emulsion was added to the reaction medium (total volume; 5 ml), and a constant fluorescence of the $C_{13:0}$ derivative could be observed from 0 to 0.2% (v/v) of the o/w injectable emulsion. The presence of more than 0.2% (v/v) of the o/w injectable emulsion in the reaction medium caused the fluorescence to decrease. These results indicate that less than 0.2% (v/v) of the o/w injectable emulsion has no effect on the derivatization reaction with DBD-PZ.

Considering the results described above, 100 μl of the o/w injectable emulsion in acetonitrile (5.0%, v/v) was added to 5 ml reaction medium, and derivatization was carried out for 6 h at 25 °C.

Effect of Fatty Acid Chain Length on Fluorescence Fluorescence spectra of several DBD-PZ fatty acids in acetonitrile (from $C_{8:0}$ to $C_{18:0}$) were measured. The same maximum excitation (443—445 nm) and maximum fluorescence wavelength (556—558 nm) were obtained from all the DBD-PZ fatty acids (from $C_{8:0}$ to $C_{18:0}$).

Effect of Acetonitrile Concentration on Fluorescence of DBD-PZ Fatty Acids To investigate the effect of acetonitrile concentration in the mobile phase on the fluorescence of the DBD-PZ fatty acids, four sets of isocratic HPLC conditions (acetonitrile/water; 6/4—9/1, v/v) were used for the separation of the DBD-PZ fatty acids. Fig. 2 shows the relationship between the acetonitrile concentration in the mobile phase and the fluorescence of DBD-PZ fatty acids. The fluorescence spectrum of each peak fraction collected by HPLC had almost the same excitation and maximum fluorescence wavelength, although the fluorescence intensity depended on the acetonitrile concentration in the mobile phase.

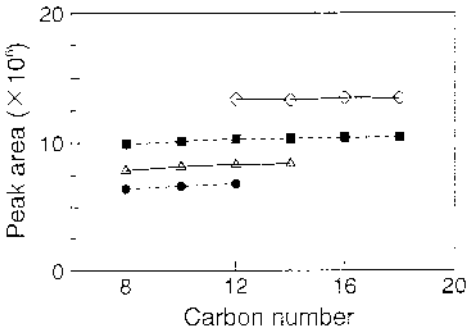

Fig. 2. Relationship between the Acetonitrile Concentration in the Mobile Phase and the Fluorescence of the DBD-PZ Fatty Acid

Acetonitrile/water (v/v), ◇: 9/1, ■: 8/2, △: 7/3, ●: 6/4.

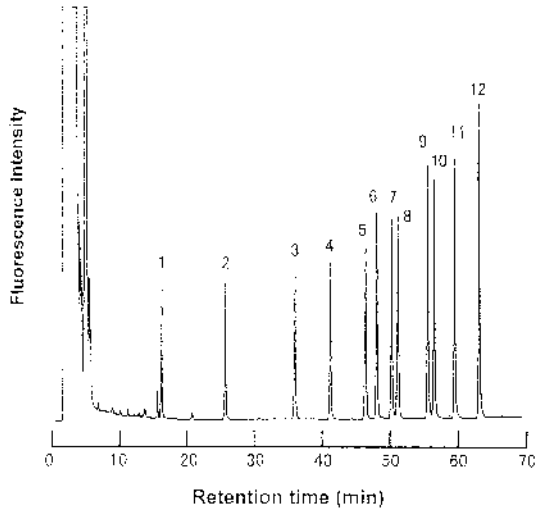

Fig. 3. Typical Chromatogram of DBD-PZ Fatty Acids

Each peak corresponds to 10pmol fatty acid. 1: $C_{8:0}$, 2: $C_{10:0}$, 3: $C_{12:0}$, 4: $C_{13:0}$, 5: $C_{14:0}$, 6: $C_{16:1}$, 7: $C_{18:2}$, 8: $C_{15:0}$, 9: $C_{16:0}$, 10: $C_{18:1}$, 11: $C_{17:0}$, 12: $C_{18:0}$. HPLC conditions are described in the text.

A gradient method was used to achieve a good separation of all fatty acids in the o/w injectable emulsion, and Fig. 3 shows a typical chromatogram obtained with a gradient of acetonitrile in water from 50 to 98% (v/v) for 60 min. However, an appropriate correction factor must be applied to the fluorescence intensity of the DBD-PZ fatty acids, because the fluorescence intensity of the DBD-PZ fatty acids increased in parallel with the acetonitrile concentration in the mobile phase. Fig. 4 shows the relationship between the relative retention times of 7 DBD-PZ fatty acids (the retention time of $C_{13:0}$ was taken as 1) and their fluorescence intensity, and the experimental data (shown as dots) were in good agreement with the calculated values (shown as a line).

Based on these results, the equation shown in Fig. 4 was used to calculate the fluorescence intensity of the DBD-PZ fatty acids in this study. In this HPLC method, a good separation of 12 DBD-PZ fatty acids (9 saturated fatty acids; from $C_{8:0}$ to $C_{18:0}$, 3 unsaturated fatty acids; $C_{16:1}$, $C_{18:1}$, $C_{18:2}$) could be achieved, although the short chain fatty acids ($C_{6:0}$, $C_{4:0}$) could not be separated. The retention time of fatty acids was closely correlated to their chain length, as Lee *et al*. reported (MDC-fatty acid, monodansyl cadaverine).[16] Therefore, the hydrophobicity of the fatty acid remained, even if it was derivatized with DBD-PZ.

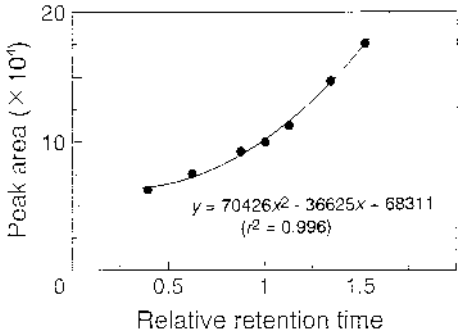

Fig. 4. Relationship between the Relative Retention Time and the Fluorescence of the DBD-PZ Fatty Acid

●: experimental data, —: calculated by the nonlinear least-squares method. The retention time of the $C_{13:0}$ DBD-PZ fatty acid was taken as 1.

Table 2. Precision of the Determination of Free Fatty Acids in the o/w Injectable Emulsion (Product A) (Unit: mEq/l)

Fatty acid	Mean (n=6)	S.D.
C8 : 0	0.36	0.012
C10 : 0	0.21	0.012
C14 : 0	0.09	0.018
C18 : 2	0.29	0.032
C16 : 0	0.59	0.066
C18 : 1	1.19	0.045
C18 : 0	0.26	0.026

Accuracy of the Fluorescence HPLC Method The calibration curves, recovery and accuracy of this HPLC method were determined using four DBD-PZ fatty acids; $C_{8:0}$, $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ (the standard fatty acid). As far as the relationship between the fatty acid concentration (from 0.1 to 3.0 μM) and the fluorescence intensity at Ex 444 nm and Em 557 nm, was concerned good linearity was obtained for all the DBD-PZ fatty acids ($C_{8:0}$; $y=61238x+1375$, $r=0.9998$, $C_{14:0}$; $y=115474x+1352$, $r=1.0000$, $C_{16:0}$; $y=136170x+7783$, $r=0.9999$ and $C_{18:0}$; $y=164892x+5078$, $r=0.9999$). The detection limit of $C_{18:0}$ fatty acid added to acetonitrile containing 0.1% (v/v) water was 5 fmol (1 pM) using the gradient mode (S/N; 5). The mean recovery of 2.5 nmol (0.5 μM in product A) of each fatty acid, $C_{8:0}$, $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$, was 103.7, 102.1, 98.3, and 105.8%, respectively (mean; 101.6%, n=3). The precision of this fluorescence HPLC method using product A is shown in Table 2.

Comparison of Methods Regarding the total amount of free fatty acids, the results obtained from the fluorescence HPLC method were compared with those from the enzymatic and titrimetric methods (Table 3). The results from the fluorescence HPLC method were good agreement with that of the titrimetric method, although the enzymatic method gave a slightly lower result than the other methods, as Shimizu *et al*. reported.[20]

Application of the Fluorescence HPLC Method to Nonaqueous Injection Preparations The fluorescence HPLC method with DBD-PZ was applied to commercial nonaqueous injection preparations. As shown in Table 4, two oil-soluble injection preparations and three o/w injectable emulsions were used, and each free fatty acid in the nonaqueous injection preparations could be determined successfully.

Two types of o/w injectable emulsions were subjected to a

stability study using this fluorescence HPLC method. Fig. 5 shows HPLC chromatograms of o/w injectable emulsions (initial and stored at 50 °C for 4 weeks). From the compositions shown in Table 1 (product A, B), free fatty acids seem to form during the manufacturing process, presumably in the heat sterilization step. In the o/w injectable emulsion A, there were appreciable amounts of medium chain free fatty acids ($C_{8:0}$, $C_{10:0}$) and $C_{18:1}$. The medium chain fatty acids were liberated from migriol, and the $C_{18:1}$ peak was due to sodium oleate being added for pH adjustment. In this way, the free fatty acids present as degradation products, added reagents and ingredients could be individually measured.

Table 3. Free Fatty Acids in the o/w Injectable Emulsion (Product A)

Method	Free fatty acids (mEq/l)	
HPLC method	C8 : 0	0.36
	C10 : 0	0.21
	C14 : 0	0.09
	C18 : 2	0.29
	C16 : 0	0.59
	C18 : 1	1.19
	C18 : 0	0.26
	Total	2.99
Enzymatic method		2.80
Titrimetric method		2.94

Generally fatty acids can not be derivatized with fluorescence reagents in the presence of water. However, in this fluorescence HPLC method, fatty acids in nonaqueous injection preparations could be derivatized even if water was present, and there were no peaks interfering with the determination of each fatty acid. In conclusion, the fluorescent determination

Table 4. Free Fatty Acids in Nonaqueous Injection Preparations

Products	A[a]	C[b]	D[c]	E[d]	F[e]
Ingredients[f]	Soybean oil Migriol[g] Phospholipid Glycerol	Soybean oil Migriol[g] Phospholipid Glycerol	Soybean oil Migriol[g] Phospholipid Glycerol	Migriol[g]	Migriol[g]
C8 : 0	0.36	0.32	0.22	0.10	0.15
C10 : 0	0.21	0.22	0.17	0.08	0.11
C12 : 0	0.00	0.07	0.06	0.04	0.06
C14 : 0	0.09	0.08	0.09	0.05	0.05
C16 : 1	0.00	0.05	0.04	0.00	0.00
C18 : 2	0.29	0.28	0.32	0.00	0.00
C15 : 0	0.00	0.00	0.07	0.00	0.00
C16 : 0	0.59	0.36	0.47	0.12	0.15
C18 : 1	1.19	0.78	0.78	0.00	0.00
C18 : 0	0.26	0.18	0.26	0.09	0.11
Total	2.99	2.34	2.48	0.48	0.63

Unit: mEq/l. *a*—*c*) injectable emulsion for i.v. infusion. *d*,*e*) injection for i.m. *f*) ingredients assumed to liberate fatty acids. *g*) medium chain triglyceride.

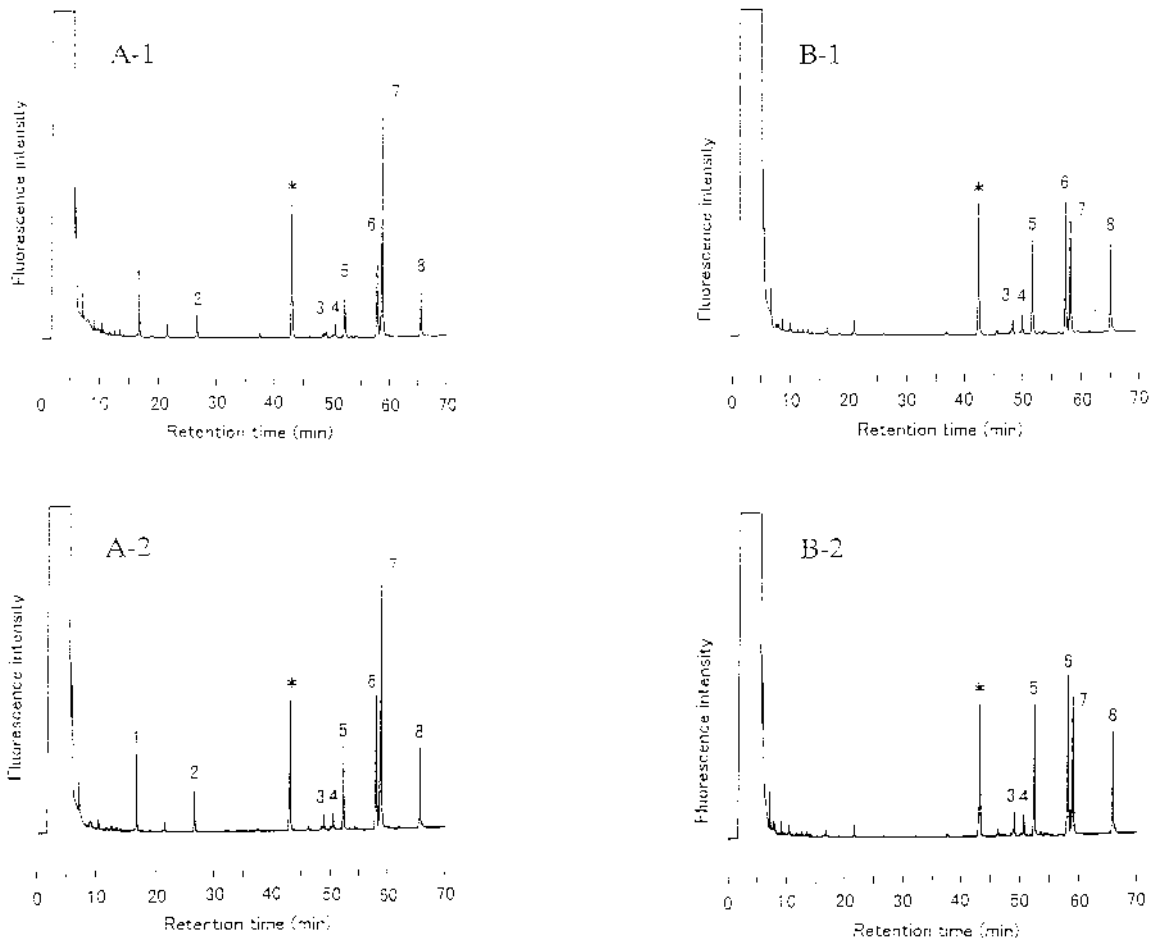

Fig. 5. Typical Chromatograms of o/w Injectable Emulsions

A-1: Product A (initial). A-2: Product A stored at 50 °C for 4 weeks. B-1: Product B (initial). B-2: Product B stored at 50 °C for 4 weeks. 1: $C_{8:0}$, 2: $C_{10:0}$, 3: $C_{14:0}$, 4: $C_{16:1}$, 5: $C_{18:2}$, 6: $C_{16:0}$, 7: $C_{18:1}$, 8: $C_{18:0}$, *: $C_{13:0}$ (Internal standard). HPLC conditions are described in the text.

using DBD-PZ was simple and readily applicable to the determination of free fatty acids in o/w injectable emulsions.

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