Journal of Chromatography, 585 (1991) 219–224
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 529

Determination of alcohols by high-performance liquid chromatography after pre-column derivatization with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole

Masatoki Katayama*, Yuichi Masuda and Hirokazu Taniguchi

Meiji College of Pharmacy, 1-35-23, Nozawa, Setagaya-ku, Tokyo 154 (Japan)

(First received March 20th, 1991; revised manuscript received June 3rd, 1991)

ABSTRACT

A sensitive method for the determination of fatty alcohols using high-performance liquid chromatography with fluorescence detection has been developed. The alcohols were derivatized with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole to their esters in the presence of 4-piperidinopyridine and 1-isopropyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate. The resulting esters were extracted with a Sep-Pak ODS cartridge, and then the esters were separated on a reversed-phase column (Zorbax ODS) with methanol–propan-2-ol (85:15, v/v) as the mobile phase. The esters were detected by fluorescence spectrophotometry (excitation 338 nm, emission 428 nm). The limits of detections for alcohols were 0.2–0.4 pg per 20 μl (signal-to-noise ratio of 3) in an acetonitrile solution.

INTRODUCTION

Alipathic alcohols have been determined by gas chromatography [1,2] and high-performance liquid chromatography (HPLC) with derivatization reagents. 3,5-Dinitrobenzoyl chloride [3], phenylisocyanate [4] and trityl chloride [5] were used as pre-column derivatization reagents for the determination of aliphatic alcohols with ultraviolet (UV) detection. 1-Anthroyl and 9-anthroyl nitriles [6], 3-chloroformyl-7-methoxycoumarin [7], 4-diazomethyl-7-methoxycoumarin [8], 7-methoxycoumarin-3- and -4-carbonyl azides [9], 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl chloride [10] and 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl azide [11] have been used for the sensitive determination of aliphatic alcohols and hydroxysteroids with fluorimetric detection.

It is established that 2-phenyl-5,6-dimethylbenzimidazoles fluoresce strongly [12,14], and therefore benzimidazole has been used as a labelling reagent for HPLC analysis. The aim of this study was the development of sensitive HPLC methods for the determination of fatty alcohols. The determination of trace amounts of fatty alcohols with chain lengths from 18 to 27 carbon atoms [15,16] and dialkyl glycerols generated from phospholipids [17,18] can give information about lipid contents and the function of membranes. Fatty alcohols with chain lengths from 8 to 20 carbon atoms are used in cosmetics and as raw materials in surfactants [19]. The determination of residual dodecyl alcohols by HPLC with photometric detection was reported by Czichocki *et al.* [20]. The development of sensitive, simple HPLC methods would therefore be useful for the determination of trace amounts of fatty alcohols from tissues or samples from industrial plants and laboratories.

Acid chloride and azide reagents have been reported in many sensitive pre-column derivatization methods [6–11,17,18]. These reagents must be reacted in anhydrous solvents [17,18] at 100°C for 40 min and then at 130°C for 60 min [10,11]. These reagents are unstable and need to be stored under dry and cool conditions [8–10]. As an alternative

0021-9673/91/$03.50

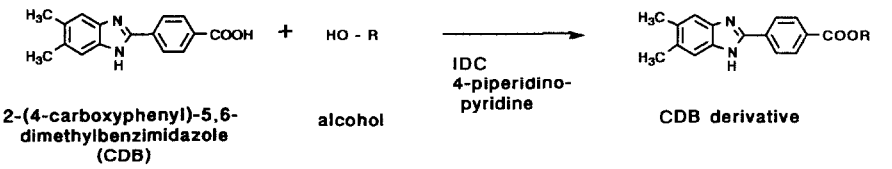

Fig. 1. Derivatization reaction of CBD with alcohol.

reaction condition, it was found that the esterification of fatty acids progressed favourably in the presence of dicyclohexylcarbodiimide and 4-dimethylaminopyridine [21] at room temperature, without the need for an anhydrous solvent. In this work, the analytical reaction proceeded as shown in Fig. 1. Separation conditions for alcohols, cetyl alcohol was selected as a model compound and the reactivities of secondary and tertiary alcohols were also studied.

EXPERIMENTAL

Reagents and materials

A stock solution of alcohol (1000 ng/ml) was prepared by dissolving 10 mg of alcohol (Tokyo Kasei and Nakarai Tesque, Japan) in 0.2 ml of pyridine and diluting to 10 ml with acetonitrile. The 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole (CDB) solution (0.1%, w/v) was prepared by dissolving 10 mg of the reagent (synthesis given later) in 1.0 ml of pyridine and adding 700 mg of 4-piperidinopyridine (Aldrich) and then diluting to 10 ml with acetonitrile. This solution was stable for 3 days in daylight at room temperature. The 1-isopropyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate (IDC) solution (2.0%) the reagent (Wako, Japan) in 10 ml of acetonitrile. This solution was stable for 8 days in daylight at room temperature. All other reagents were of analytical-reagent grade.

Synthesis of CDB

A 13-g mass of telephthalaldehydic acid dissolved in 400 ml of ethanol was added dropwise to 13 g of 4,5-dimethyl-*o*-phenylenediamine dissolved in 400 ml of ethanol in an ice-bath. After 1 h, the mixture was refluxed for 8 h. After cooling to room temperature, the precipitate was collected and then recrystallized three times from methanol–water (1:1, v/v). A white amorphous product (4 g) was obtained: m.p. over 300°C. Analysis calculated for $C_{16}H_{14}N_2O_2 \cdot 2/3H_2O$: C 72.17, H 5.30, N 10.52; found: C 72.23, H 5.50, N 10.39. IR ν_{max} (cm^{-1}) (KBr): 1705. ^1H NMR dimethylsulphoxide (DMSO)-d_6) δ (ppm): 2.33 (6H, s, $-CH_3$), 7.39 (2H, s, Ar-H), 8.07 (2H, t, Ar-H), 8.04 (2H, t, Ar-H), 12.82 (1H, s, $-COOH$); mass spectrometry (MS) *m/z*: 266 (M^+).

Isolation of CDB esters

Masses of 50 mg of CDB, 500 mg of 4-piperidinopyridine and IDC were successively added to 10 ml of 0.1% cetyl alcohol solution (acetonitrile–pyridine, 9:1) in a screw-capped test-tube. The mixture was heated for 1 h at 80°C in an oil-bath and then the reaction solution was applied to a Sep-Pak ODS cartridge. The column was washed with 20 ml of 50% propan-2-ol. The CDB ester was eluted with 20 ml of propan-2-ol. The eluting solution was evaporated to dryness then the obtained white residue (60 mg) was analysed by IR, ^1H NMR and MS. IR ν_{max} (cm^{-1}) (KBr): 1745 (C=O), 1335 (C–O). ^1H NMR (DMSO-d_6) δ (ppm): 0.88 (3H, t, CH_3), 1.14–1.46 [26H, $(CH_2)_{13}$)], 1.62 (2H, m, $COOCH_2CH_2$), 2.37 (6H, s, 5 and 6 CH_3), 4.05 (2H, t, $COOCH_2$), 7.40 (2H, s, Ar-H), 8.10 and 8.17 (2H each, d each, Ar-H); MS *m/z*: 490 (M^+), 267 ($M^+ - C_{13}H_{27}CHCH=CH_2$).

Apparatus and HPLC conditions

Excitation and emission spectra were measured with a Hitachi 650-10S fluorescence spectrophotometer.

The HPLC apparatus and conditions were as follows: pump, Shimadzu LC-6A liquid chromatograph (Shimadzu, Japan); guard-column, Zorbax ODS (50 × 4.6 mm I.D., 7 μm, DuPont); analytical column, Zorbax ODS (250 × 4.6 mm I.D., 7 μm, DuPont); sample solvent, 20 μl; column temperature, room temperature (about 22°C); detector, Shimadzu RF-530 fluorescence spectrophotometer (excitation 338 nm, emission 428 nm); mobile

phase, methanol–propan-2-ol (85:15); flow-rate, 1.0 ml/min.

Pre-column derivatization of alcohols

A 0.1-ml volume each of the CDB and IDC solutions were added to 1.0 ml of sample solution in a screw-capped test-tube. The mixture was heated at 80°C for 20 min then cooled to room temperature. Volumes of 1.0 ml of water and 2.0 ml of 50% propan-2-ol were added to the reaction solution, which was then applied to the Sep-Pak ODS cartridge. The test-tube was washed with 3.0 ml of 50% propan-2-ol and the washing applied to the column. The column was then washed with 3.0 ml of 50% propan-2-ol and the resultant fluorescence derivatives eluted with 2.0 ml of propan-2-ol. A 20-μl aliquot of the eluate was injected into the HPLC system.

Extraction of alcohols in sodium dodecyl sulphate (SDS)

The procedure for the extraction of dodecyl alcohol is that of the Japanese Pharmacopeia XI, with some modifications [22]. Volumes of 3.0 ml of 10 mg/ml SDS (50% methanol) and 3.0 ml of 2.0 μg/ml stearyl alcohol (as an internal standard) were transferred to a screw-capped test-tube. This mixture was then extracted with 3.0 ml of light petroleum (b.p. 30–60°C, as an extraction solvent), centrifuged (1700 *g*, 5 min), and then the organic solvent layer collected. This process was repeated three times. The organic solvent layer was removed under reduced pressure and the residue dissolved with 6.0 ml of acetonitrile. This reaction solution was then used as a sample solution and derivatized with CDB.

RESULTS AND DISCUSSION

Pre-column derivatization

Derivatization solvent. Acetone, acetonitrile, benzene, chloroform, dichloromethane, dioxane, N,N-dimethylformamide, DMSO and pyridine were testred as reaction solvents for the pre-column derivatization of alcohols (Table I). The most intense detector response was obtained with acetonitrile, which was therefore selected as the reaction solvent.

Effect of CDB concentration. When CDB concentrations in the range 0.08–1.5% were used, the highest constant response was obtained; this concentration was therefore used in this procedure.

TABLE I

EFFECT OF REACTION SOLVENT ON THE DERIVATIZATION REACTION

Amount of cetyl alcohol taken, 1000 ng/ml. The derivatization reaction conditions were as follows: CDB 0.1% (w/v), 4-piperidinopyridine 5% (w/v), IDC 2% (w/v); reaction time, 20 min; temperature, 80°C. Average values were obtained from six runs. The detector response of the CDB derivative in acetonitrile was taken as 100.

Solvent	Detector response
Acetone	20
Acetonitrile	100
Benzene	25
Chloroform	75
Dichloromethane	90
Dioxane	10
N,N-Dimethylformamide	2
Dimethylsulphoxide	5
Pyridine	3

Effect of base. The derivatization of alcohols with CDB did not occur without a base catalyst. 4-Dimethylamionopyridine, 4-piperidinopyridine, 4-pyrrolidinopyridine, pyridine, triethylamine, tributylamine and 1,8-diazabicyclo[5,4,0]-7-undecene were tested (Table II). Pyridine derivatives substituted at the 4-position effectively gave CDB derivatives of alcohols. When 4-piperidinopyridine was used, the observed chromatographic interferences

TABLE II

EFFECT OF BASE ON THE DERIVATIZATION REACTION OF CDB AND ALCOHOL

Amount of base catalyst taken, 0.5 *M*; cetyl alcohol, 1000 ng/ml. Average values were obtained from six runs. The detector response of the CDB derivative using 0.5 *M* (*ca.* 8%, w/v) 4-piperidinopyridine was taken as 100. Other derivatization conditions as in Table I.

Base catalyst	Detector response
4-Dimethylaminopyridine	100
4-Piperidinopyridine	100
4-Pyrrolidinopyridine	100
Pyridine	1
Triethylamine	1
Tributylamine	1
1,8-Diazabicyclo[5,4,0]-7-undecene	15

TABLE III

EFFECT OF DCC DERIVATIVE ON THE DERIVATIZATION REACTION OF CDB AND ALCOHOL

Amount of DCC derivative taken, 0.1 *M*; cetyl alcohol, 1000 ng/ml. Average values were obtained from six runs. The detector response of the CDB derivative by 0.1 *M* (*ca.* 2.7%, w/v) IDC was taken as 100. The other derivatization conditions were as in Table I.

Condensing agent	Detector response
Dicyclohexylcarbodiimide (DCC)	3
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride	94
1-Cyclohexyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate	100
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate	100
1-Isopropyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate (IDC)	100
1-Hydroxybenzotriazole	9
N-Ethyl-5-*m*-sulphophenylisoxazolium hydrochloride	–
2-Bromo-1-ethylpyridinium tetrafluoroborate	–

(*e.g.* unknown peaks from the reagent) were lower than with 4-dimethylamino- and 4-pyrrolidinopyridine. The highest constant response was obtained when 4-piperidinopyridine was used in the concentration range 2–10% (w/v); 5% (w/v) 4-piperidinopyridine was therefore selected.

Effect of dicyclohexylcarbodiimide. For the derivatization of alcohols with CDB, the following compounds were tested as condensing agents: dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, 1-cyclohexyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate, 1-isopropyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate (IDC), 1-hydroxybenzotriazole, *N*-ethyl-5-*m*-sulphophenylisoxazolium hydrochloride and 2-bromo-1-ethylpyridinium tetrafluoroborate (Table III).

Good detector responses were obtained with water-soluble carbodiimides and, as a result of its solubility in acetonitrile, IDC was selected as the reagent. The highest constant response was obtained in ther ange 1–10% IDC solution; 2% IDC was therefore used in the procedure.

Reaction time and temperature

The reaction time was varied from 0 to 90 min and the temperature was varied from 25 to 90°C. The results are shown in Fig. 2. The highest detector response was obtained at 80 and 90°C within 20 min. However, the detection limits of the alcohols at 80°C were more sensitive than at 90°C and therefore heating at 80°C for 20 min was selected.

Extraction of CDB derivatives

To avoid interferences, CDB derivatives were extracted from the reaction solution. The extraction of CDB derivatives with the organic solvents dichloromethane, chloroform, ethyl acetate, benzene and hexane was examined under acidic, neutral and alkaline conditions. However, the CDB derivatives were not effectively extracted and it was decided to extract then with a short column. Sep-Pak silica, alumina (acidic, neutral and basic) and ODS cartridges were used with a water–organic mixture solvent. The CDB derivatives were effectively extracted by the Sep-Pak ODS cartridge with water–propan-2-ol and this solvent was selected for further work.

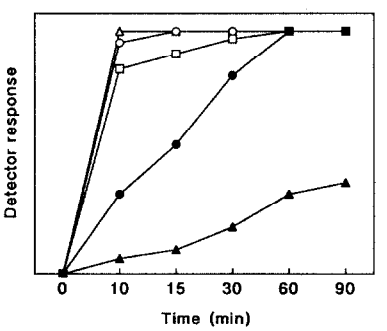

Fig. 2. Effect of reaction time and temperature on the derivatization reaction. Amount of cetyl alcohol used: 1000 ng/ml. ▲ = 25°C; ● = 50°C; □ = 70°C; ○ = 80°C; △ = 90°C; ■ = combination of 80 and 90°C.

Separation of CDB derivatives

The separation of mixtures of the fatty alcohols dodecyl alcohol, tetradecyl alcohol, cetyl alcohol, stearyl alcohol and eicosyl alcohol was studied. Methanol, acetonitrile and tetrahydrofuran were tested as mobile phases. And it was found that methanol–propan-2-ol was the most suitable for the separation. Optimum separation was achieved with a methanol/propan-2-ol ratio of 85:15 (v/v).

Determination of CDB derivatives

The excitation and emission spectra of cetyl alcohol in the HPLC eluent are shown in Fig. 3.

Secondary alcohols, for example, 2-tetradecyl alcohol (C_{14}), were examined in the same manner as the primary alcohols and it was found that the detector response for the secondary alcohol was less than one fiftieth of that of the primary alcohol. However, secondary alcohols in acyl glycerols (α,α'-dilaurin) and at the 3-position in cholesterols gave 50 and 70% reactor response of cetyl alcohol. Other secondary alcohols in steroids (*e.g.* prednisolone acetate and testosterone) did not react; tertiary alcohols also gave no reaction. The excitation maxima of the CDB derivatives from decyl alcohol (C_{10}) toeicosyl alcohol (C_{20}), cholesterols and acyl glycerols were about 338 nm, with emission maxima at about 428 nm. These excitation and emission wavelengths were therefore selected.

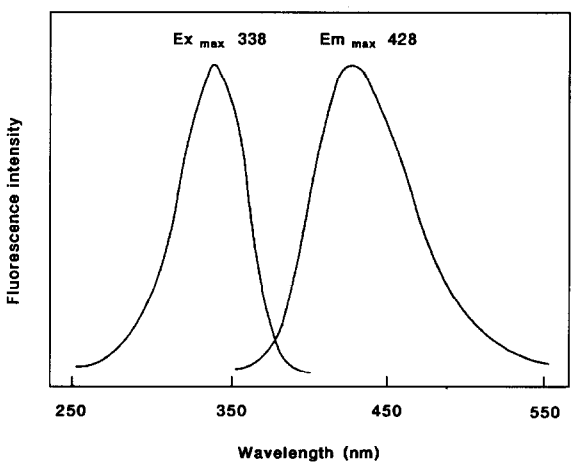

Fig. 3. Excitation and emission spectra of CDB derivative of cetyl alcohol in HPLC eluent. Amount of cetyl alcohol used: 1000 ng/ml.

Fig. 4 shows a chromatogram of five CDB derivatives (C_{12}–C_{20}) obtained by the proposed procedure. The detection limits of the five alcohols were 0.2–0.4 pg per 20 μl (signal-to-noise ratio of 3). The calibration graphs were linear up to 10 000–30 000 ng/ml. The relative standard deviations (n = 6) were 2.4 and 2.9% at 1000 and 10 ng/ml cetyl alcohol, respectively. The fluorescence of the CDB derivative from cetyl alcohol was stable for at least 5 days in daylight at room temperature. The efficiency of the conversion of cetyl alcohol to the CDB derivative was examined by comparing the detector response obtained under derivatizastion conditions with that given by the isolated reaction product (described under Experimental). The conversion rate was 52%. The proposed method is 5 to 800 times more sensitive than other chromatographic methods using fluorescence detection after derivatization [6–11].

Determination of alcohols in SDS

The proposed CDB method was applied to the determination of alcohol impurities in commercial

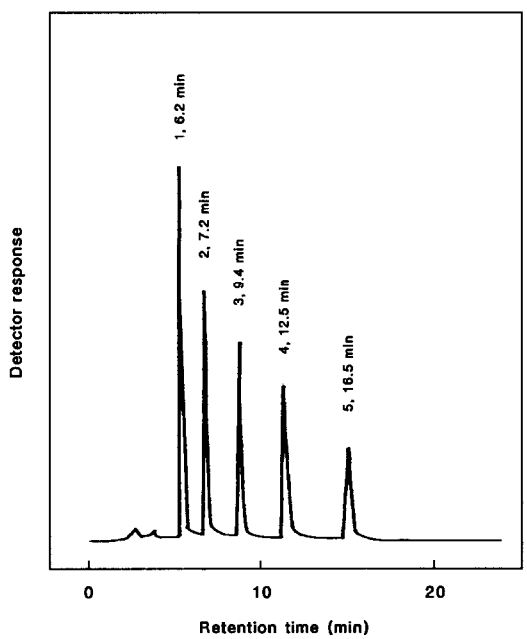

Fig. 4. Chromatogram of CDB derivatives. Amount of alcohol taken: 100 ng/ml. Peaks: 1 = dodecyl alcohol; 2 = tetradecyl alcohol; 3 = cetyl alcohol; 4 = stearyl alcohol; 5 = eicosyl alcohol.

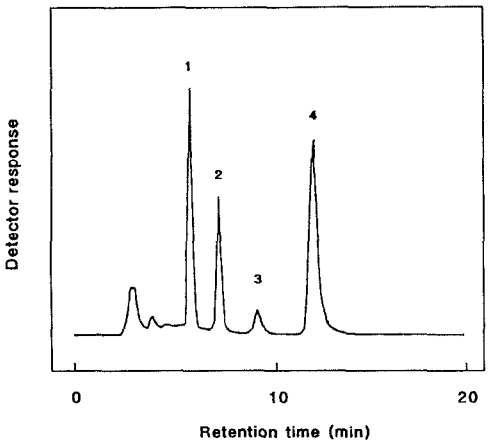

Fig. 5. Chromatogram of alcohols in SDS. Amount of SDS used: 0.1 mg/ml. Peaks: 1 = dodecyl alcohol; 2 = tetradecyl alcohol; 3 = cetyl alcohol; 4 = stearyl alcohol (internal standard).

SDS. SDS is a well known surface-active agent and is widely used as an emulusifier in medicines, cosmetics and as the reagent for SDS polyacrylamide gel electrophoresis. As SDS is made from dodecyl alcohol, trace amounts of alcohols are contained in it and these alcohols affect the formation of micelles [19]. The alcohol residue is defined in the Japanese Pharmacopeia XI [22]. Four commercial SDS samples were tested: A, an analytical standard for anion-type surface-active agent; B, for water analysis; C, for electrophoresis; and D, reagents for synthesis. These four commercial samples contained 0.63, 0.70, 0.81 and 75.10 μg of dodecyl alcohol per 10 mg, respectively. Sample D in particular contained tetradecyl alcohol and cetyl alcohol as other impurities (Fig. 5). The relative standards deviation (n = 6) for the analysis of the samples was about 7.0%.

CONCLUSIONS

The derivatization of alcohols with CDB can be performed at moderate temperatures and is superior to other pre-column derivatization HPLC methods with fluorimetric detection with respect to sensitivity and the stability of the reagent [6–11]. The application of this method to the determination of corticosteroids (*e.g.* cortisone, hydroxycortisone and aldosterone) and phenols (*p*-hydroxybenzoic acid esters) is in progress.

REFERENCES

1 R. J. Argauer, *Anal. Chem.*, 40 (1968) 122.
2 F. K. Kawahara, *Anal. Chem.*, 40 (1968) 1009.
3 J. F. Lawrence and R. W. Frei, *Chemical Derivatization in Liquid Chromatography*, Elsevier, Amsterdam, 1976, p. 151.
4 B. Björkqvist and H. Toivonen, *J. Chromatogr.*, 153 (1978) 265.
5 Y. Suzuki and K. Tani, *Bunseki Kagaku*, 28 (1979) 610.
6 J. Goto, N. Goto, F. Shamsa, M. Saito, S. Komatsu, K. Suzaki and T. Nambara, *Anal. Chim. Acta*, 147 (1983) 397.
7 C. Hamada, M. Iwasaki, N. Kuroda and Y. Ohkura, *J. Chromatogr.*, 341 (1985) 426.
8 A. Takadate, T. Tahara, H. Fujino and S. Goya, *Chem. Pharm. Bull.*, 30 (1982) 4120.
9 A. Takadate, M. Irikura, T. Suehiro, H. Fujino and S. Goya, *Chem. Pharm. Bull.*, 33 (1985) 1164.
10 T. Iwata, M. Yamaguchi, S. Hara, M. Nakamura and Y. Ohkura, *J. Chromatogr.*, 362 (1986) 209.
11 M. Yamaguchi, T. Iwata, M. Nakamura and Y. Ohkura, *Anal. Chim. Acta*, 193 (1987) 209.
12 M. Katayama, Y. Mukai and H. Taniguchi, *Anal. Sci.*, 3 (1987) 369.
13 M. Katayama, Y. Mukai and H. Taniguchi, *Anal. Sci.*, 3 (1987) 565.
14 M. Katayama, Y. Mukai and H. Taniguchi, *Analyst (London)*, 115 (1990) 9.
15 D. J. Harvey, J. M. Tiffany, J. M. Duerden, K. S. Pandher and L. S. Mengher, *J. Chromatogr.*, 414 (1987) 253.
16 D. J. Harvey, *J. Chromatogr.*, 494 (1989) 23.
17 M. Kito, H. Takamura, H. Narita and R. Urade, *J. Biochem. (Tokyo)*, 98 (1985) 327.
18 H. Takamura, H. Narita, R. Urade and M. Kito, *Lipids*, 21 (1986) 356.
19 M. J. Rosen, *J. Colloid Interface Sci.*, 79 (1981) 587.
20 G. Czichocki, H. Much and D. Vollhardt, *J. Chromatogr.*, 280 (1983) 109.
21 F. E. Ziegler and G. D. Berger, *Synt. Commun.*, 9 (1979) 539.
22 *The Japanese Pharmacopeia with Commentary*, Vol. XI, Pharmaceutical Society of Japan, Hirokawa Shoten, Tokyo, 1986, p. D-997.