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Note

9-(Chloromethyl)anthracene: a useful derivatizing reagent for enhanced ultraviolet and fluorescence detection of carboxylic acids with liquid chromatography

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Many carboxylic acids, including the fatty and bile acids, are difficult to monitor spectrophotometrically at low concentrations in liquid chromatography (LC) because the acids have relatively weak absorption bands above 200 nm; however, derivatization of the acids with moieties that either enhance ultraviolet absorption or fluorescence can greatly facilitate detectability[1–4]. 9-Anthrylmethyl (9-AM) esters formed from carboxylic acids with 9-anthradiazomethane (ADAM) show both high absorptivity and intense fluorescence, appear to be as readily separated on reversed-phase LC columns as the products from other derivatization reagents[4] and are very stable[5]. This paper describes the use of 9-(chloromethyl)anthracene (9-ClMA) as an alternative reagent for the preparation of 9-AM esters of carboxylic acids prior to LC separation for those analysts who prefer to avoid the inconvenience of generating the diazoreagent. Spectral properties, detection limits, rates of formation and two examples of separations with fatty acid esters and glycine-conjugated cholates are discussed.

EXPERIMENTAL

Reagents and chemicals

Fatty acids, bile acids and glycine-conjugated bile salts were obtained from Sigma (St. Louis, MO, U.S.A.), and methanol, ethanol, acetonitrile, cyclohexane, dimethylformamide (DMF) and tetramethylammonium hydroxide (TMH) (24% in methanol) were obtained from MCB (Norwood, OH, U.S.A.). Solvents were purified according to standard procedures[6]. Dicyclohexyl-18-crown-6 was obtained from DuPont (Wilmington, DE, U.S.A.). 9-Anthraldehyde and purified anthracene (Gold Label Grade) were purchased from Aldrich (Milwaukee, WI, U.S.A.). Preparation of 9-ClMA from 9-(hydroxymethyl)anthracene gave bright yellow needles with m.p. 137–138°C (lit.[7] 137.5–138°C).

9-AM esters of lauric, myristic, palmitic and stearic acid were prepared in millimolar quantities by the general derivatization procedure using TMH and 9-ClMA described below. The esters were purified (>98% by LC analysis) by chromatography over neutral silica gel and recrystallization from ethanol. Structures of the esters were confirmed by infrared, proton magnetic resonance and mass spectroscopic

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TABLE I

UV ABSORPTION, FLUORESCENCE AND MELTING POINT DATA OF FOUR 9-ANTHRYLMETHYL FATTY ACID ESTERS

The esters contained traces of anthracene estimated to be less than 1% by LC analysis.

Fatty acid ester	*Molar absorptivity (1 mol^{-1} cm^{-1})**		*Fluorescence relative quantum yield*** *ex. 365 nm*	*Corrected m.p. (°C)*
	256 nm	*365 nm*		
9-AM Laurate	$1.98 \cdot 10^5$	$1.06 \cdot 10^4$	0.34, 0.34	51–52
9-AM Myristate	$1.95 \cdot 10^5$	$1.04 \cdot 10^4$	0.34, 0.33	60–61
9-AM Palmitate	$1.94 \cdot 10^5$	$1.03 \cdot 10^4$	0.34, 0.1	67–68
9-AM Stearate	$1.92 \cdot 10^5$	$1.01 \cdot 10^4$	0.34, 0.1	73–74

* In methanol solvent.

** Relative to anthracene which has a quantum yield in ethanol of 0.30. First value in methanol, ethanol, acetonitrile and water–methanol (10:90); second value in water–methanol (20:80).

analysis. Melting points of the pale yellow crystalline substances are given in Table I with key molar absorptivities and quantum yields. The esters of oleic, linoleic and linolenic acid were oily substances and special purification techniques were not attempted.

Instrumentation

LC separations shown in Figs. 1 and 2 were obtained with a Waters LC system (Waters Assoc. Milford, MA, U.S.A.) including a Model 6000A pump, 440 Absorbance Detector, U6K Injector and a 30-cm "Fatty Acid" reversed-phase column. Fluorescence detection limits were determined with a Kratos (Schoeffel) (Westwood, NJ, U.S.A.) FS 970 Fluorescence Detector. Molar absorptivities (Table I) were calculated from absorbance data obtained on a Varian (Palo Alto, CA, U.S.A.) Cary 1605 Spectrophotometer, and relative quantum yields (Table I) were calculated from excitation and emission spectra generated with a Varian SF 330 Spectrofluorometer and a Perkin-Elmer (Norwalk, CT, U.S.A.) MPF 44B Spectrofluorometer fitted with a corrected spectra accessory. No significant differences were ascertained in quantum yields determined from either corrected or non-corrected emission spectra.

Derivatization procedure

The general procedure for the derivatization of carboxylic acids with 9-CIMA was similar to Greeley's method[8], and the procedure using sodium salts of carboxylic acids followed the method of Durst *et al.*[9]. Most reactions were performed in 2-ml graduated test-tubes and heated with a "Temp Block" (Labline Instruments, Melrose Park, IL, U.S.A.). Transfers of solutions and injections into the liquid chromatograph were done with Hamilton (Reno, NV, U.S.A.) microsyringes. Stock solutions of reagents and carboxylic acids were prepared at approximately 2 m*M* concentrations.

For carboxylic acids. Fatty or bile acids in methanol were treated with a slight excess of methanolic TMH. The sample was dried by passing a slow stream of nitrogen over the surface of the liquid. Acetonitrile was then added to give acid salt

concentrations ranging from mM to μM. After addition of enough 9-CIMA (in cyclohexane) to give a two- to ten-fold excess depending on the concentration of the original acid, the mixture was heated for the desired time. At mM concentrations of acid a two-fold molar excess of 9-CIMA was sufficient for rapid and quantitative reactions; however, at μM concentrations, a ten-fold molar excess of 9-CIMA was used. After cooling, the volume was adjusted by the addition of acetonitrile and an aliquot injected into the chromatograph for analysis.

For sodium salts of carboxylic acids. Appropriate quantities of methanolic solutions of the sodium salts of fatty acids, bile acids or glycine-conjugated bile acids were dried by a slow stream of nitrogen. An equal molar quantity of dicyclohexyl-18-crown-6 catalyst in acetonitrile was added and then solvent to give the desired concentrations. 9-CIMA was added to give a two- to ten-fold ratio as described above. After heating the sample was cooled and analyzed.

RESULTS AND DISCUSSION

Key spectral data of the four 9-AM esters of lauric, myristic, palmitic and stearic acid, the four common saturated fatty acids, are summarized in Table I. Molar absorptivities at 256 nm ranged from $1.92 \cdot 10^5$ to $1.98 \cdot 10^5$ and from $1.01 \cdot 10^4$ to $1.06 \cdot 10^4$ l mol^{-1} cm^{-1} at 365 nm, the excitation wavelength used for fluorescence analysis, consistent with literature values for anthracene[10] and 9-methylanthracene[11]. Quantum yields of the 9-AM esters relative to anthracene (lit.[12] 0.30) were 0.34 in the helium-purged solvents, methanol, ethanol and acetonitrile. The intense absorption band at 256 nm facilitated the detection of 0.1 pmol of the four 9-AM esters with a 10/1 signal-to-noise ratio at 0.005 a.u.f.s. after LC separation with 5% water–methanol. Approximately 2 fmol of 9-AM esters were monitored with the fluorescence detector (excitation 365 nm, emission 412 nm) after separation under similar conditions. Using a combination of the two detection systems, signals were linearly dependent on concentration over a 10^7-fold range.

Lloyd[13] considered several complications associated with the use of fluorescent derivatives in LC analysis, including the effects of solvent and oxygen on quantum yields and the photolability of derivatives. Although none of these factors was significant in the reversed-phase separations of 9-AM esters in this study, they could become important in procedures with solvents that quench fluorescence in anthracene[14]. Molecular aggregation, as noted with other derivatives of fatty acids[3,13], appeared significant as the concentration of water increased beyond 10%. With 9-AM esters of palmitic and stearic acids, the quantum yields dropped rapidly from 0.34 to 0.1 as the water concentration increased from 10 to 20%. The myristate ester was only slightly affected by the same change. The presence of oxygen in non-purged methanol led to a 10% decrease in quantum yield, a small factor for LC detectability and eliminated by the use of degassed solvents in the separation.

Carboxylic acids can be alkylated with 9-CIMA by a variety of different routes similar to those described in the literature for reactions of acids with alkyl halides[15]; however, either of the two processes, using quaternary ammonium hydroxide to form a quaternary ammonium salt of the carboxylic acid prior to alkylation[8] or using a "crown" catalyst with the sodium salt of the carboxylic acid during alkylation[9], gave excellent yields of derivatives within a reasonable time. Fatty acids in 10 μM concen-

trations were 98% alkylated in 15 min with a ten-fold excess of 9-CIMA in acetonitrile at 75°C. Lower concentrations of acids required longer heating periods or higher temperatures in sealed vials. Although the reaction was slightly faster in DMF solvent and could tolerate higher temperatures in open systems without solvent loss, absorbance signals from the DMF interfered with the signals of the lower-molecular-weight esters at 10 μM ester concentrations. Therefore, acetonitrile, which is transparent at 254 nm, was clearly the solvent of choice for alkylations with low concentrations of carboxylic acids.

For preparing 9-AM esters from carboxylic acids in protic solvents at lower than μM concentrations, ADAM would be the preferred reagent because it reacts more rapidly and selectively in protic solvents than 9-CIMA[4]. ADAM would also be the reagent of choice for base-sensitive carboxylic acids, such as the polyunsaturated fatty acids which may rearrange under the conditions for rapid alkylation with 9-CIMA.

Fig. 1 demonstrates the separation of six fatty acid esters and Fig. 2 shows the separation of three glycocholates and two types of cholates on a reversed-phase column with water–methanol mixtures. Principal by-products of the alkylation procedure, 9-(hydroxymethyl)anthracene and 9-(methoxymethyl)anthracene, eluted rapidly at the start of both separations and did not interfere with signals for the 9-AM

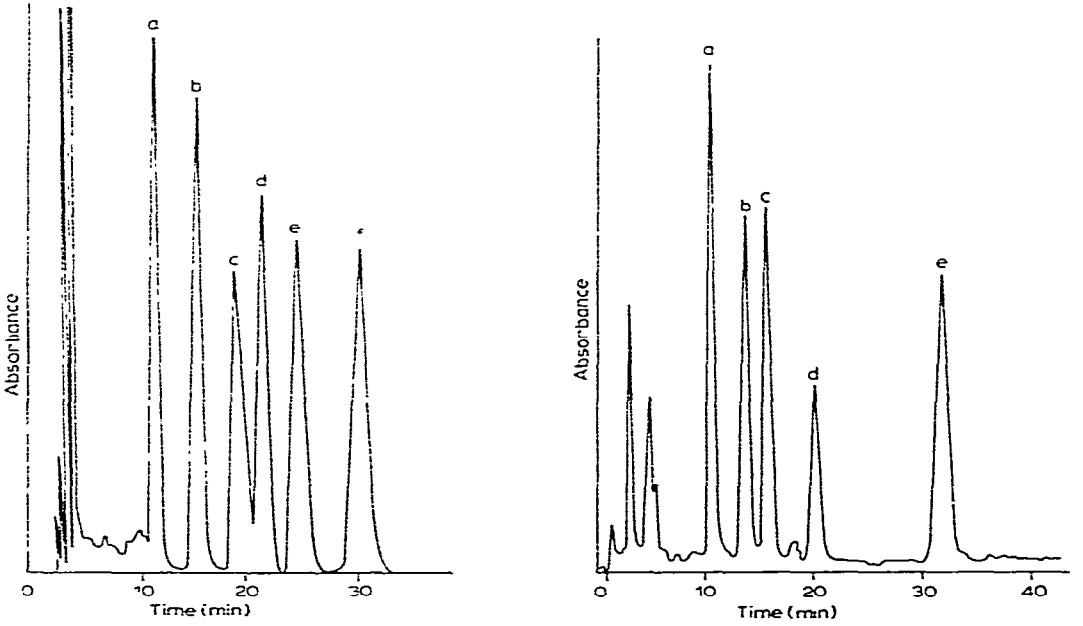

Fig. 1. Chromatogram of six 9-anthrylmethyl fatty acid esters: a = 9-AM laurate; b = 9-AM myristate; c = 9-AM linolenate; d = 9-AM palmitate; e = 9-AM oleate and f = 9-AM stearate. Separation on a Waters 30-cm "Fatty Acid" column with water–methanol (12:88) at 0.75 ml/min.

Fig. 2. Chromatogram of three 9-anthrylmethyl glycocholates and three 9-anthrylmethyl cholates; a = 9-AM glycocholate; b = 9-AM glycochenodeoxycholate; c = 9-AM glycodeoxycholate; d = 9-AM cholate; e = a mixture of 9-AM deoxycholate and 9-AM chenodeoxycholate. Separation on a Waters 30-cm "Fatty Acid" column with water–methanol (18:82) at 0.75 ml/min.

esters. With *p*-bromophenacyl bromide as the alkylating agent, glycocholic *p*-bromophenacyl ester was separated from excess of derivatizing reagent with a preliminary separation by thin-layer chromatography[16]. The by-products and excess of 9-CIMA do not interfere with normal-phase separations of 9-AM cholates[17], so that 9-CIMA is a useful reagent for both normal and reversed-phase separations.

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