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LABELING OF FATTY ACIDS WITH 4-BROMOMETHYL-7-METHOXYCOUMARIN VIA CROWN ETHER CATALYST FOR FLUORIMETRIC DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the fluorescence labeling of fatty acids with 4-bromomethyl-7-methoxycoumarin using 18-crown-6 as a catalyst is described. The procedure is rapid, simple and requires little or no experience in derivatization techniques. The amounts of the derivatives are linearly related to the amount of the starting fatty acids and the procedure can therefore be used in the quantitative analysis of these solutes. Using reversed-phase chromatography, detection levels as low as 9 pmole could be realized. The separations of several derivatives using a reversed-phase system are discussed.

INTRODUCTION

Many examples of chemical derivatization for detection purposes have been recorded in the literature and a comprehensive discussion of this subject will not be given here. Several reviews on derivatization in gas chromatography (GC)[1–3] and high-performance liquid chromatography (HPLC)[4–9] have clearly demonstrated the significance of the methodology. In HPLC studies, derivatization has been used for the modification of chromatographic properties and selectivities, and also for detection enhancement.

Derivatization for the modification of chromatographic properties can be best illustrated by the work of Mikes *et al.*[10], who separated unsaturated fatty acid methyl esters on a silver-impregnated column. The use of a more polar solvent for the separation of the free acids would be harmful to the above stationary phase.

An improvement in the selectivity occurs when the derivatives interact differently with either the mobile or stationary phases, therefore eliminating the difficulties of co-elution. An example is the separation of optical isomers by derivatization with another chiral agent. The formation of diastereomers promotes the selectivity which results in optical resolution.

Low detection limits are essential in the chromatography of trace amounts. In LC, reasonable detection levels can be accomplished by using UV or fluorescence detectors. For compounds that are transparent to these detectors, proper labeling with absorbing or fluorescing derivatives offers a suitable solution. The improved

detection limits of compounds such as amino acids, fatty acids, biogenic amines and sugars via derivatization proved to be one of the major attractions of HPLC in many of the health and biomedical applications.

The chromatographic analysis of physiological samples is important in providing new insights into metabolic reactions that occur in complex life forms. Fatty acids, a physiologically important class of compounds, have been studied extensively by GC (ref. 11 and references therein). Urinary acids that absorb UV light or fluoresce have been analyzed by HPLC[12]. UV detection of all free fatty acids is possible with variable-wavelength detectors monitored at 210 nm or below[13]. Most frequently, however, the detection of fatty and other organic acids by HPLC involves the formation of UV-absorbing derivatives[14–17]. The most commonly used derivatizing agents for organic acids are the phenacyl esters. They have been widely applied in the analysis of both short-chain[18] and long-chain fatty acids[18,19], dicarboxylic acids[20], unsaturated fatty acids[21,22], prostaglandins[23] and penicillins[24].

Fluorescence labeling of fatty acids has not been used extensively. Recently, fatty acids were reacted with 4-bromomethyl-7-methoxycoumarin (Br-Mmc) to form fluorescence derivatives[25] which were then separated by thin-layer chromatography. The procedure was promising for various fatty acids with the exception of dicarboxylic acids. The drawbacks associated with the reported work are the need for an excess of the expensive derivatization reagent, the long reaction time and the need to perform the reaction in the dark[26]. The purpose of this paper is to describe an improved procedure, using a crown ether catalyst, which eliminates the above shortcomings. The derivatives can be formed easily and economically and can be separated on a reversed-phase HPLC system.

EXPERIMENTAL

Reagents and chemicals

All organic solvents (Fisher Scientific, Pittsburgh, Pa., U.S.A.) were commercial analytical-reagent grade materials. Acetonitrile and acetone were dried with molecular sieves. Methanol was distilled before use. The distilled water used for the mobile phase was passed through an ion-exchange column (Barnstead Sybron, Boston, Mass., U.S.A.). All chromatographic solvents were de-aerated by bubbling dry nitrogen through them. Br-Mmc was prepared according to a publised procedure[27]. 18-Crown-6 was purchased from Aldrich (Milwaukee, Wisc., U.S.A.) and was used without purification.

Apparatus

A Milton Roy Model 396 mini pump (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) with no pulse-damping facility was used for solvent delivery. Typical sample sizes of 5–10 μl were introduced with a 25-μl Hamilton GC syringe (Hamilton, Reno, Nev., U.S.A.) via a Rheodyne Model 70-10 injection valve obtained from Altex Scientific (Berkeley, Calif., U.S.A.). The fluorescence detector was a Gilson (Middleton, Wisc., U.S.A.) Spectra/Glo fluorimeter with a flow cell volume of 5 μl. The excitation filter had maximum transmitance at 360 nm and the emission filter had a cut-off value of 400 nm. Data were collected on a Heath recorder, which has variable voltage imput from 10 mV to 10 V. The reversed-phase packing was

prepared by reacting trimethoxyoctadecylsilane with Partisil 10 (Whatman, Clifton, N.J., U.S.A.). A 250 × 4.2 mm I.D. column was packed using a home-made device with a methanol slurry. All chromatographic studies were performed at ambient temperatures.

Derivatization procedures

The following stock solutions were prepared: (1) 0.07 g of 18-crown-6 in 100 ml of acetonitrile; (2) 0.11 g of Br-Mmc in 100 ml of acetone (protected from light by wrapping the container in aluminum foil); and (3) 10% potassium hydroxide dissolved in methanol. Three different procedures were examined, as described below.

Method A. The fatty acids (2–4 mg, depending on the chain length) were neutralized to a phenolphthalein end-point with the 10% methanolic potassium hydroxide solution in a 50-ml round-bottomed flask. The potassium salt was obtained after the solvent had been removed by aspirator vacuum. Crown ether (1 ml) and Br-Mmc (5 ml, corresponding to a 10–20% molar excess relative to the fatty acid) were added and the mixture was refluxed for 15 min. The molar ratio of the derivatization agent to crown ether was 8:1. The final product was either purified by filtration through a disposable pipet packed with silica gel, or injected directly into the chromatograph.

Method B. The same ratios of fatty acids, crown ether and derivatization agent as in method A were used. However, instead of neutralizing the fatty acid with a base, 20–30 mg of anhydrous potassium carbonate were added to the reaction flask. The reaction mixture was refluxed for 25 min. The final mixture can be treated as in method A.

Method C. This procedure was used only when studying reaction rates. The difference between methods A and B and method C lies in the catalyst. Instead of the crown ether, 10 drops of trimethylamine were used. No base is needed in this procedure.

Reaction rate and quantitation experiments

For the reaction rate studies, a stock solution containing capric acid (0.14 g) and anthracene (0.09 g), as the internal standard, in 50 ml of acetone was prepared. For each of the above derivatization procedures, 1 ml of the sample stock solution was used. Reaction mixtures were withdrawn at given times, and the yields were monitored by the ratios of the peak height of the internal standard to that of the capric acid Mmc ester. The mobile phase in this study was 15% water in methanol at a flow-rate of 1.96 ml/min.

In the quantitation study, weighed amounts of a fatty acid were derivatized by method B. The amount of Br-Mmc was always in excess of the sample. Five millilitres of internal standard, consisting of 0.12 g of anthracene in 50 ml of acetone, were added to each reaction. After refluxing for 25 min the derivatized product was chromatographed in a mobile phase consisting of 15% water in methanol at a flow-rate of 0.83 ml/min.

Preparation of a sample for fluorescence studies

Lauryl acid-4-methyl-7-methoxycoumarin ester was synthesized on a semi-preparative scale. Lauryl acid (0.59 g) was neutralized with methanolic potassium

hydroxide solution. After the solvent had been evaporated, 0.5 g of potassium carbonate, 3 ml of the crown ether stock solution and 0.8 g of Br-Mmc were added and the mixture was allowed to reflux in 50 ml of acetone for 2 h. The lauryl acid derivative was recrystallized three times from ethanol to yield a highly pure reference compound for both the fluorescence spectra measurements and the detection limit studies. The purity was checked by HPLC. The melting point of the lauryl acid derivative was 100–102°. The yield after recrystallization was only 56%; however, we did not try to minimize sample loss in the recrystallization steps. The derivative structure was confirmed by mass spectrometry.

Fluorescence measurement

Samples of lauryl acid 4-methyl-7-methoxycoumarin ester were prepared in three solvents: $4.4 \cdot 10^{-4}$ *M* in methanol, $3.5 \cdot 10^{-4}$ *M* in 20% water in methanol and $3.6 \cdot 10^{-4}$ *M* in acetonitrile. An Aminco-Bowman spectrophotofluorimeter (American Instrument, Silver Springs, Md., U.S.A.) was used. The excitation wavelength was set at 360 nm and the emission wavelength at 410 nm. The fluorescence intensity is given in arbitrary units.

RESULTS AND DISCUSSION

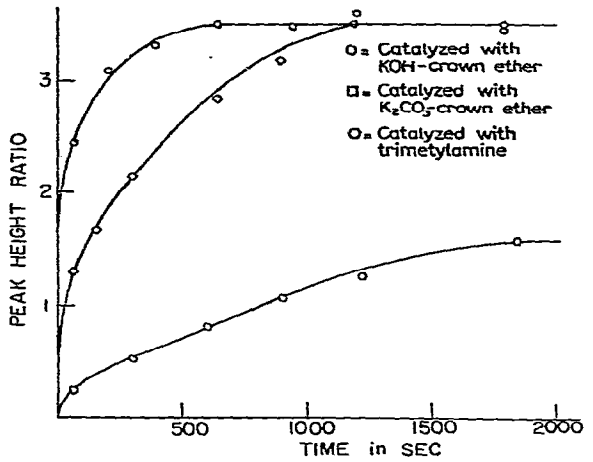
Derivatization studies

The ability of crown ethers to form complexes with metal ions is well known (ref. 28 and references therein). They have been used in many analytical derivatization procedures involving phase-transfer catalysis of organic salts[18,20,21,23,29]. This type of phase-transfer catalyst solvates the metal cations of organic salt in aprotic solvents, leaving the organic anions in a weakly solvated environment. Such anions, especially carboxylate anions, are reactive and can be derivatized rapidly and efficiently. Fig. 1 shows the amount of capric acid Mmc ester formed (relative units) *versus* the reaction time for the three different derivatization procedures. The amount of ester formed is proportional to the peak-height ratio, *i.e.*, the height of the Mmc ester peak relative to that of the internal standard. The reaction rates are dependent on the kind of base present. With potassium hydroxide as the base, the reaction proceeds to completion in 10 min, 20 min are needed when potassium carbonate is used, and with triethylamine the reaction is not completed after 30 min. Therefore, method C is not recommended for the preparation of fatty acid Mmc esters. Method A, although faster than method B, requires extra manipulation steps. Method B is easy to perform and is recommended for workers with little or no experience in derivatization techniques.

The choice of solvent in the derivatization procedures is not critical, provided that it is aprotic, as discussed previously[18]. The solvents used in this study were chosen according to the solubilities of the reagents.

The yield of the reaction was not investigated. However, several reports have demonstrated that crown ether catalyzed reactions proceed essentially to completion[18,24,29]. As will be discussed in a later section, a linear relationship exists between the reaction yields and the amount of fatty acids present in the solution. Therefore, the present procedure is suitable for quantitation purposes.

The superiority of the present method over the previously reported work on the derivatization of fatty acids with Br-Mmc is demonstrated by the fact that only



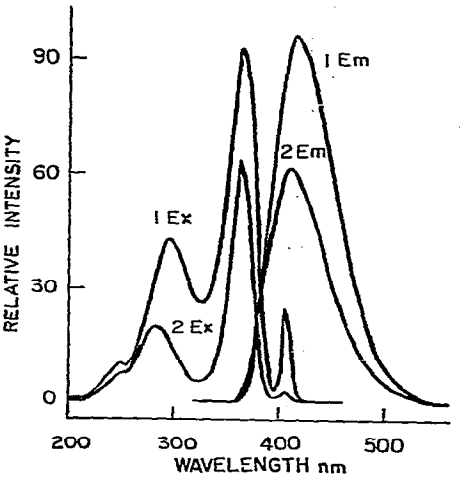


Fig. 1. Dependence of the reaction yield of capric acid 4-methyl-7-methoxycoumarin ester.

Fig. 2. Fluorescence spectrum of lauryl acid 4-methyl-7-methoxycoumarin ester. Solvent: 1, 20% water in methanol; 2, acetonitrile. Ex = Excitation; Em = emission.

a slight excess of the expensive reagent is required here rather than a three-fold excess[25]. Also, the short reaction time obviates the need to perform the reaction in the dark. The short reaction times and small amounts of Br-Mmc used eliminate interferences in the chromatogram due to excess of derivatizing agent and decomposition products that might be formed during longer reaction times.

Fluorescence properties

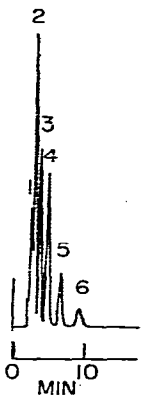
The fluorescence spectra of the lauryl Mmc ester were obtained in solvents most often encountered in reversed-phase chromatography. For the three solvent systems examined, namely methanol, 20% water in methanol and acetonitrile, the excitation maximum appeared at 362 nm and the emission maximum at 415 nm. Fig. 2 shows the excitation and emission spectra for the ester in 20% water in methanol and in acetonitrile. Note that the excitation maximum at 285 nm in acetonitrile is shifted to 295 nm in the 20% water–methanol mixture. The fluorescence showed a marked increase in intensity in the latter solvent.

The fluorescence properties of the Mmc esters are very favorable for reversed-phase work as a signal can be observed in aqueous mobile phases.

Chromatographic separations

The fatty acid derivatives should be soluble in the commonly used mobile phases and elute at different retention times in order to be chromatographically useful. In the present work, reversed-phase separation of some fatty acid Mmc derivatives were attempted. Fig. 3 shows the separation of C_1–C_6 derivatives and Fig. 4 the separation of C_6–C_{16} derivatives. The retention orders are typical of those found in reversed-phase systems. No attempts were made to optimize the resolution. However, it is clear that a gradient elution would allow the separation of most of the fatty acids in a short time.

The ease of derivative formation coupled with the simple chromatographic separation indicates the potential of the method for the routine analysis of fatty acids.



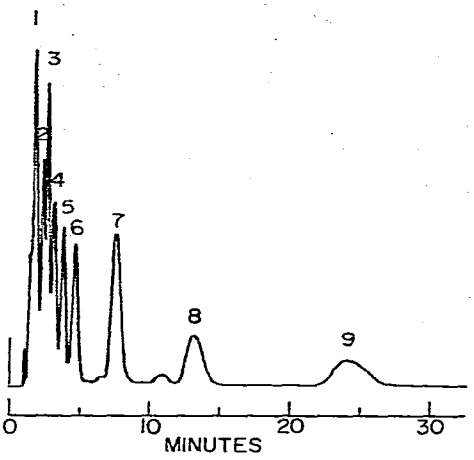


Fig. 3. Separation of C_1–C_6 fatty acid Mmc esters. 1 = C_1; 2 = C_2; 3 = C_3; 4 = C_4; 5 = C_5; 6 = C_6. Detector sensitivity: 50. Mobile phase: 15% water in methanol. Flow-rate: 1.96 ml/min.

Fig. 4. Separation of C_6–C_{16} fatty acid Mmc esters. 1 = Decomposed derivatization reagent; 2 = C_6; 3 = C_7; 4 = C_8; 5 = C_9; 6 = C_{10}; 7 = C_{12}; 8 = C_{14}; 9 = C_{16}. Detector sensitivity: 50. Mobile phase: 15% water in methanol. Flow-rate: 1.96 ml/min.

As an example, a simple but realistic chromatographic profile is shown in Fig. 5. A sample of partially stripped coconut oil was derivatized using method B and chromatographed. The chromatogram shows the presence of C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16} and oleic acids. As expected, C_{12}, C_{14} and C_{16} are the major fatty acid components of the coconut oil. The peaks were identified by the retention times, as well as by the knowledge of the fatty acid percentage composition of the oil. This study demonstrates the ability of the present method to derivatize a complex biological mixture.

The impurity peaks in Fig. 4 can interfere in the determination of some short-chain acids. The impurity peaks resulted when using Br-Mmc stock solution that was exposed continuously to light. When the derivatizing reagent was protected from light, the fatty acid Mmc esters obtained were almost completely free of impurities, as can be seen in Figs. 3 and 5. It should be emphasized that the derivatization reaction was carried out without protection from light.

Fig. 6 is an example of the separation of the ester of two α-hydroxy acids and a furancarboxylic acid. Lactic acid involved in inborn error of metabolism is presently under intensive investigation, and glass capillary columns have helped to identify the defective metabolic pathway[30,31]. A similar procedure is possible with HPLC.

The "unknown" peak in Fig. 6 was present whenever furoic acid was derivatized. We suspect that the peak corresponds to some impurity present in the furoic acid. No attempts were made to identify the impurity since this is outside the scope of this work.

Quantitative and detection limits

To be useful in quantitative analysis, the amount of ester formed in the derivatization reaction should be related to the amount of the fatty acid. A typical result for

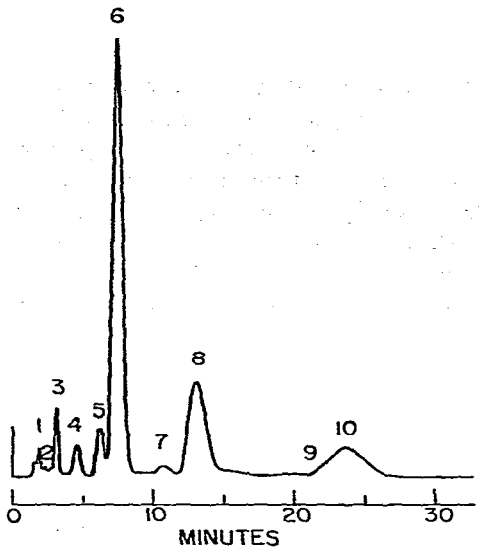
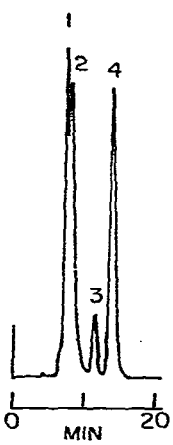


Fig. 5. Separation of partially stripped coconut oil mixture. 1 = Unknown; 2 = C_6; 3 = C_8; 4 = C_{10}; 5 = unknown; 6 = C_{12}; 7 = unknown; 8 = C_{14}; 9 = oleic acid; 10 = C_{16}. Conditions as in Fig. 4.

the present reaction is shown in Fig. 7, which is a plot of the peak height of capric acid Mmc ester, relative to the anthracene standard, *versus* the amount of the fatty acid introduced into the reaction mixture. The linear relationship indicates that the procedure described here can be used to quantitate fatty acids.

The detection limits were found to be a function of the mobile phase and the flow-rate. Typical detection limits were between 9 and 90 pmole. A further reduction in the detection limit is possible with an optimized chromatographic system.



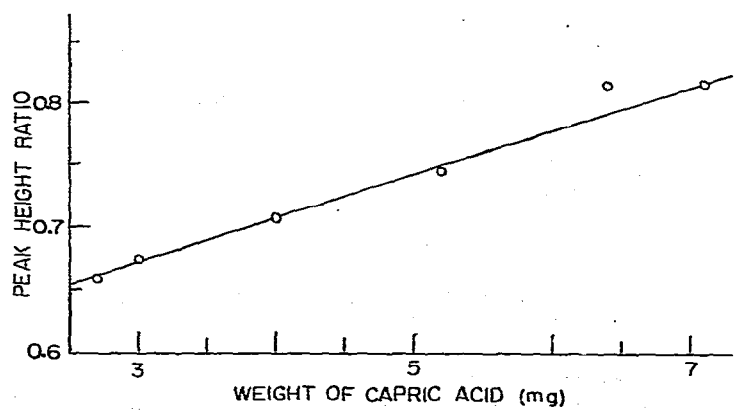


Fig. 6. Separation of α-hydroxy acids and furancarboxylic acid. 1 = Glycolic acid; 2 = lactic acid; 3 = unknown; 4 = 2-furoic acid. Detector sensitivity: 20. Mobile phase: 30% water in methanol. Flow-rate: 0.47 ml/min.

Fig. 7. Quantitation of capric acid: peak-height ratio of capric acid Mmc ester *versus* weight of capric acid used in the derivatization reaction.

CONCLUSION

The procedure for the formation of fatty acid Mmc esters is promising. It is rapid, simple and can be adapted for routine fatty acid analysis. The Mmc esters show excellent chromatographic properties, allowing the ready separation of long- and short-chain acids as well as hydroxy acids. The fluorescence of the derivatives allows one to detect small amounts of fatty acids. This can have significant implications in the analysis of complex biological samples, *e.g.*, the correlation between fatty acid profiles in human excretion and the bacteria associated with some infections[32–34]. Work is continuing to derivatize dicarboxylic acids and some biologically important α-keto acids.

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