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Short Communication

Derivatization of saturated long-chain fatty acids with phenacyl bromide in non-ionic micelles

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ABSTRACT

The derivatization of saturated long-chain fatty acids (LCFA) with a UV chromophore, 1,4-dibromoacetophenone, using non-ionic micelles to facilitate the reaction is described. Synperonic NP-12 was used as a non-ionic surfactant and tetrakis(decyl)ammonium bromide as an ion-pair agent. The reaction was carried out in the dark at 60°C for 20 min. After adding 1 ml of chloroform to the reaction mixture and centrifugation for 30 s, the mixture was ready for high-performance liquid chromatographic analysis on an RP-18 column. The derivatization rate constant for LCFA was found to be in the range $6 \cdot 10^{-4}$–$9 \cdot 10^{-4}$ s^{-1}. The use of micelles overcomes solubility difficulties with highly lipophilic compounds such as LCFA and provides a simple derivatization procedure.

INTRODUCTION

Lipophilic compounds such as fatty acids lack a chromophore or a fluorophore that permits their detection and they therefore require derivatization steps prior to their high-performance liquid chromatographic (HPLC) analysis. Most often these derivatization reactions are carried out in aprotic solvents. Micellar solutions have been used to enhance the reactions by extractive separation similarly to liquid–liquid extraction [1]. Among the compounds that have been made to react in such solutions are amino acids [2], fatty acids [3] and others [4].

Recently, Van der Horst and co-workers [5,6] reported the advantages of micellar systems in the derivatization of carboxylic acids with fluorphore reagents such as 4-bromomethyl-7-methoxycoumarin and 9-bromomethylacridine [7]. The theory and practice of micellar phase-transfer catalysis (MPTC) have been discussed extensively (*e.g.*, [8]).

Derivatization in an aqueous micellar system, without the need for isolation of the analyte prior to the derivatization reaction, is likely to result in good yields and to be less time consuming. In addition, the use of MPTC permits the derivatization of long-chain fatty acids (LCFA) directly in aqueous solutions. The aim of this study was to apply the recently developed micellar-aided derivatization technique to the analysis of LCFA.

The procedure used is based on that of Van der Horst *et al.* [8,9], which has been extended to allow the determination of LCFA with the use of a common UV label bromophenacyl bromide, which has not been used before in MPTC. The micellar system consisted of an aqueous solution containing Synperonic NP-12 as a non-ionic surfactant. A non-ionic surfactant was chosen to allow for better interactions with, and therefore better solvation of, the hydrophobic LCFA [10]. Tetrakis(decyl)ammonium bromide (TDeABr) was used as an ion-pair

agent and 1,4-dibromoacetophenone (bromophenacyl bromide) (Br-Ph-Br) [11] as derivatizing reagent.

EXPERIMENTAL

Chemicals

Synperonic NP-12 non-ionic surfactant [a polyoxyethylene(12) nonylphenyl ether], was a gift from Professor Nissim Garti (Casali Institute of Applied Chemistry, Jerusalem, Israel). TDeABr was purchased from Fluka (Buchs, Switzerland) and Br-Ph-Br from Sigma (St. Louis, MO, USA).

Standard fatty acids (C16:0, C18:0, C20:0, C22:0, C24:0, C25:0, C26:0, C27;0 and C28:0) were purchased from Fluka. HPLC-grade chloroform, methanol and acetone were obtained from BioLab (Jerusalem, Israel) and were filtered through an RC-55 membrane filter from Tamar (Jerusalem, Israel). Silica gel (63–100 μm) was purchased from Woelm (Eshwege, Germany).

Solutions

Synperonic NP-12 surfactant was prepared at a concentration of 0.025 *M* in 0.01 *M* phosphate buffer (pH 7.0) (the critical micelle concentration is about 0.1 m*M* in phosphate buffer). A 0.006 *M* solution of the ion-pair reagent, TDeABr, was prepared in 0.01 *M* phosphate buffer (pH 7.0). Fatty acid stock solutions were prepared by dissolving 5 mg of each acid in 100 ml of chloroform. A Br-Ph-Br stock solution was prepared daily by dissolving 0.525 g in 25 ml of acetone; it was stored in the dark at 4°C.

Derivatization reaction

To dried fatty acids mixtures (which were evaporated under nitrogen) the following solutions were added: 300 μl of Br-Ph-Br, 560 μl of Synperonic NP-12 and 140 μl of TDeABr. The mixture reacted, protected from light, by refluxing at 60°C for 20 min while stirring.

The fatty acid esters in the reaction mixture were initially extracted using one of three techniques: (1) purification by filtration through a silica gel column (protected from light) and extraction with chloroform; (2) direct extraction with chloroform and absorption of the aqueous phase with anhydrous sodium sulphate, followed by filtration; (3) extraction with chloroform and separation by centrifugation. The third method was the easiest and gave the best yield, and was therefore adopted. Chloroform (1 ml) was added to the reaction mixture while stirring. Centrifugation for 30 s resulted in two separate phases. The lower chloroform phase was injected into the HPLC system directly without any further treatment.

HPLC system

The analyses were made on a Spectra-Physics (Santa Clara, CA, USA) Model 8700 liquid chromatograph equipped with 20-μl loop injector and a Model D-2000 Chromato Integrator (Merck, Darmstadt, Germany).

Separations of the fatty acid esters were done on an RP-18 reversed-phase column (LiChrosorb cartridge, 12.5 cm × 0.4 cm I.D.) protected by a 2.5 cm × 0.4 cm I.D. guard column (Merck). Both columns were packed with 5-μm particles.

All organic solvents were filtered through Type RC-55 0.45-μm membrane filters (Tamar). The mobile phase was methanol at a flow-rate of 1.5 ml/min. The analytical column was kept in a water-bath at 16°C. Detection of the fatty acid esters was performed with a Spectra-Physics UV detector operated at 254 nm.

RESULTS AND DISCUSSION

As the analysis of fatty acids is hindered by the lack of a chromophore or a fluorophore, derivatization is necessary. Most of the derivatization procedures require first the preparation of fatty acid salts, followed by extraction into an organic phase with a phase-transfer catalyst such as an 18-crown-6-ether [11,12]. The derivatization reaction takes place in the organic phase and is difficult to combine with reversed-phase (RP) HPLC. In the micellar system, "pseudo-phases" exist and the combination with RP-HPLC is easier. In addition, as the aqueous micellar solution is buffered at pH 7, the fatty acids are converted into their salts, which aids in the derivatization reaction. Therefore, the derivatization procedure is simple and requires minimum volumes of reagent and solvents. The derivatization scheme used is a modification of the method described by Van der Horst *et al.* [8] for other substances and allows the satisfactory separation of LCFA by HPLC.

Reaction conditions

Several experimental conditions were examined to find the optimum for preparing the derivatives. Derivatization of LCFA was done with excess of Br-Ph-Br at 60°C. The reaction time was varied from 5 to 70 min and it was found that 20 min represent a suitable compromise between reaction time and completion of reaction.

Fig. 1 shows a three-dimensional surface diagram of LCFA reaction rates. Chromatographic peak areas are plotted *versus* reaction time and *versus* chain length of the acid. Each crossing point on the surface represents one set of experimental conditions. Fig. 1 shows an initial increase in the reaction completion (larger peak areas) with increasing reaction time. After 40 min the reaction reaches a plateau.

Using the approach of van der Horst *et al.* [7] and of Schmid and Sapunov [13], the data in Fig. 1 allow the calculation of the derivatization reaction rate constants of the fatty acids. Table I gives these constants for all the acids studied, and they vary between $6 \cdot 10^{-4}$ and $9 \cdot 10^{-4}$ s^{-1}. As expected [8], the k_{obs} values for LCFA from C16:0 to C28:0 were found to be in the same range, and the general trend seems to be a slight decrease with increasing chain length.

Influence of the surfactant

We examined the effect of the non-ionic surfactant concentration (0.025, 0.05, 0.1 and 0.125 *M*) on the derivatization reaction. It was found that the best yield of the derivative was obtained with a 0.025 *M* surfactant concentration.

Extraction was necessary prior to injection for HPLC analysis in order to obtain a homogeneous solution, avoid blocking of the RP-18 column and obtain reproducible results. Separation by centrifugation was found to be adequate for this purpose.

Chromatographic separations of LCFA

The HPLC separation of LCFA derivatives from C16:0 to C28:0 in 24 min is shown in Fig. 2. Limits of detection for the nine fatty acids, as calculated at a signal-to-noise ratio of 3, are given in Table I. As only 1/40th of the reaction mixture was injected into the HPLC system, the detection limits could be lowered by dissolving the dried reaction mixture in a smaller volume of chloroform.

Reproducibility and linearity

Reproducibility of the method was evaluated using standard fatty acids at a certain concentration (30 μg of each) in four replicates. The data are given in Table I. The reproducibility ranges between 4.5 and 9% (relative error).

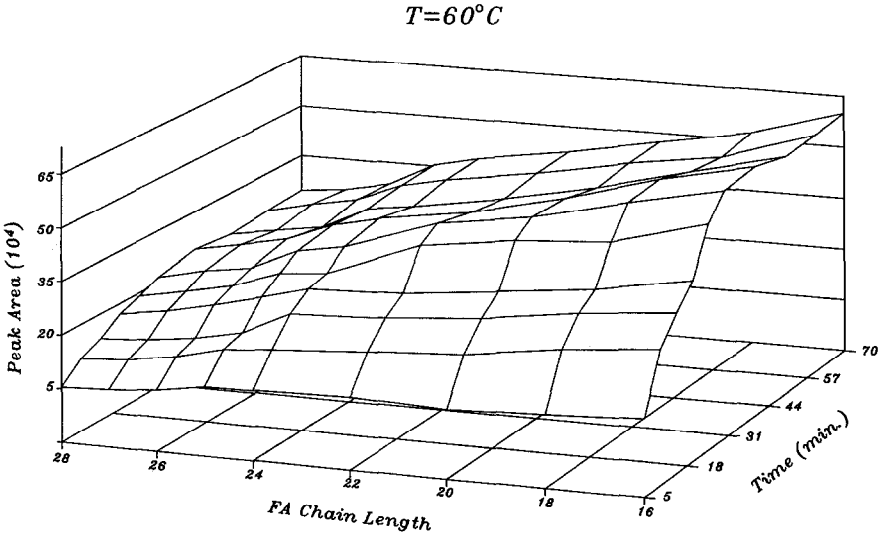

Fig. 1. Three-dimensional surface diagram of LCFA reaction rates.

TABLE I

DERIVATIZATION RATE CONSTANTS AND DATA ON REPRODUCIBILITY AND DETECTION LIMITS OF LCFA PHENACYL ESTERS IN MICELLAR PHASE-TRANSFER CATALYSIS

Fatty acid	Rate constant, k_{obs} (10^{-3} s^{-1})	Reproducibility (%)[a]	Detection limit (nmol)
C16:0	0.963	9.0	0.293
C18:0	0.836	8.6	0.282
C20:0	0.760	8.5	0.321
C22:0	0.778	6.9	0.368
C24:0	0.825	4.5	0.544
C25:0	0.788	8.9	0.589
C26:0	0.783	6.8	0.631
C27:0	0.800	7.7	0.646
C28:0	0.644	6.2	0.649

[a] Relative error for four identical samples injected into the HPLC system five times.

Calibration graphs in the range 2.5–30 μg (*ca.* 5.9–117 nmol) of LCFA showed a correlation coefficient above 0.99.

CONCLUSIONS

The method described offers a solution for the solubility problems of LCFA in aqueous media. Using a non-ionic surfactant, Synperonic NP-12, and a cationic ion-pair agent, TDeABr, derivatization was carried out with a UV reagent, 1,4-dibromoacetophenone, without the need for multi-step purification procedures of the analyte prior to HPLC analysis.

The use of MPTC allows the derivatization of LCFA in only one step. Hence, it can be applied to automated analyses of plasma, as discussed recently by Van der Horst *et al.* [14]. The use of MPTC may be of great interest for the determination LCFA which are present in the plasma of patients with genetic diseases.

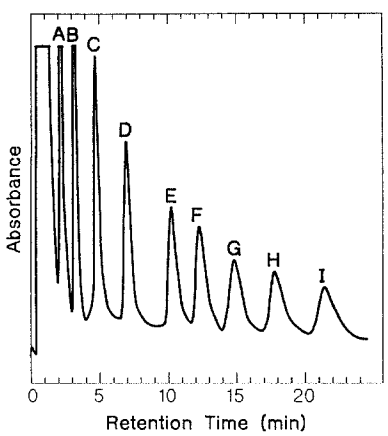

Fig. 2. Chromatogram of LCFA phenacyl esters. (A) C16:0; (B) C18:0; (C) C20:0; (D) C22:0; (E) C24:0; (F) C25:0; (G) C26:0; (H) C27:0; (I) C28:0.

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