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# STUDY OF THE INFLUENCE OF AQUEOUS MICELLAR SYSTEMS ON THE DERIVATIZATION OF UNDECYLENIC ACID WITH 4-BROMOMETHYL-7-METHOXYCOUMARIN

F. A. L. VAN DER HORST*, M. H. POST and J. J. M. HOLTHUIS*
*Department of Pharmaceutical Analysis, Faculty of Pharmacy, University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht (The Netherlands)*

## SUMMARY

The effects of aqueous micellar systems on the derivatization of a fatty acid, 10-undecenoic acid (UA), with a fluorophore, 4-bromomethyl-7-methoxycoumarin (BrMMC), were examined. High derivatization rates were obtained in solutions of the non-ionic surfactants Triton-X 100 and Arkopal N in the presence of cationic ion-pair reagents such as tetrahexylammonium bromide. The derivatization mechanism is probably based on phase-transfer catalysis. Especially high reaction rates are obtained in turbid non-ionic micellar solutions. This opaqueness is connected with an important optimizing parameter of the derivatization rate, the so-called cloud temperature of a micellar system. Under the optimal conditions the derivatizaton of UA with BrMMC is complete within 45 min at 70°C.

## INTRODUCTION

High-performance liquid chromatography (HPLC) is a versatile technique for the analysis of drugs that are present in biological matrices. However, HPLC analysis can be hampered by the fact that the drug may be difficult or impossible to detect. This problem can be overcome by derivatizing the drug with, *e.g.*, a fluorescence label[1,2]. Unfortunately, with carboxylic acids the derivatization reactions are often incompatible with water[3]. This means that in bioanalysis the substrate has to be extracted from the aqueous matrix into a suitable organic solvent. Extraction procedures are often tedious and can cause problems concerning the drug recovery and the reproducibility of the analysis[4,5].

A possible alternative to the extraction of a substrate into an organic solvent could be to introduce an organic "pseudo"-phase into the aqueous matrix by means of micelles. Micelles are small, more or less spherical aggregates of amphiphilic molecules[6–8]. The core of the micelle may be more or less deprived of water[9,10] and the properties are similar to those of hydrocarbons[10]. If the substrate and the reagent are solubilized in the micellar core then the necessary derivatization conditions may be

* Present address: EuroCetus B.V., Strawinskylaan 357, 1077 XX Amsterdam, The Netherlands.

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fulfilled. In this manner it could be possible to circumvent the tedious procedures for extraction of the drug.

Micelle-enhanced reactions have been studied extensively[11–13]. It is surprising, however, that very few studies have dealt with the labelling of bio-active compounds (*e.g.*, amines[14,15]) prior to HPLC analysis.

The need for an alternative, more convenient, derivatization procedure is most marked with carboxylic acids that are present in aqueous biological matrices. Therefore, we have extended our investigations to the derivatization of carboxylic acids with a fluorescence label in aqueous micellar systems. As a fluorescence label we selected 4-bromomethyl-7-methoxycoumarin (BrMMC)[16–20]. In general, the derivatization of carboxylic acids with BrMMC has to be carried out in water-free[19,20] aprotic dipolar solvents such as acetone to which 18-crown-6 ether and potassium carbonate have been added to increase the reaction rate[17].

This paper describes part of a study on the mechanism of the derivatization of aliphatic carboxylic acids with BrMMC in the presence of aqueous micellar systems.

We report here on the influence of the micellar system [type and concentration of the surfactant and presence of additives (such as ion-pair reagents)] on the derivatization of a model substrate, undecylenic acid (10-undecenoic acid, UA), with BrMMC. The influence of substrate-related factors on the derivatization reaction in the micellar system will be discussed elsewhere[21].

Micelle-mediated labelling with BrMMC has recently been applied to plasma samples of patients treated with an anti-epileptic drug, valproic acid[22]. The ultimate aim of our studies is to develop a fully automated on-line labelling procedure for carboxylic acids that is based on micelle-enhanced derivatizations and which can be applied to biological matrices.

## EXPERIMENTAL

### *Chemicals and solutions*

Millipore water (Milford, MA, U.S.A.) was used throughout. Except for the non-ionic surfactants the chemicals were of analytical-reagent grade.

The non-ionic Arkopal N surfactants (condensates of nonylphenol with polyoxyethylene) were kindly supplied by Hoechst Holland (Amsterdam, The Netherlands). These surfactants are mixtures of molecular species varying in their polyoxyethylene (POE) chain length. The Arkopal N surfactants contained a POE moiety with averages of 8, 9, 10, 11, 13, 15, 18 and 23 oxyethylene units, and are denoted N-80, N-90, N-100, N-110, N-130, N-150, N-180 and N-230, respectively. The mean POE chain lengths are used to calculate the molecular weights of the non-ionic surfactants.

The non-ionic surfactants of the Brij type (alkoxy-POE condensates) were purchased from Sigma (St. Louis, MO, U.S.A.) and Servo (Delden, The Netherlands).

Triton X-100, cetyltrimethylammonium bromide (CTAB) and sodium dodecylsulphate (SDS) were purchased from Merck (Darmstadt, F.R.G.). Tetrabutylammonium bromide (TBuABr), tetrapentylammonium bromide (TPeABr), tetrahexylammonium bromide (THxABr) and 4-bromomethyl-7-methoxycoumarin (BrMMC) were supplied by Fluka (Buchs, Switzerland). Acetonitrile, acetone, methanol and 18-crown-6 ether were obtained from Merck. Undecylenic acid (UA)

was supplied by OPG (Utrecht, The Netherlands). All chemicals were used as obtained.

In a sealed amber-coloured flask the BrMMC reagent was added to acetone at the level of 8 mg/ml. This saturated solution was stored at 4°C and prepared freshly every week. Prior to incubation the BrMMC was completely dissolved in acetone by heating to *ca.* 50°C. Undecylenic acid was dissolved in acetone at a concentration of 5 m*M* and stored at 4°C. The 18-crown-6 ether was dissolved in acetone at a concentration of 3 mg/ml and stored at room temperature.

*General incubation procedure*

The micellar solutions were prepared by dissolving known amounts of the surfactant and the ion-pair reagent in 10 m*M* phosphate buffer adjusted to pH 7.0 with sodium hydroxide. Other additives, such as organic solvents, salts and urea, were dissolved in the micellar solution prior to the incubation. Of the incubation solutions 965 μl were pipetted into type 3814 reaction vessels (Eppendorf, Hamburg, F.R.G.) and 10 μl of undecylenic acid solution (final concentration 50 μ*M*) were added. This mixture was pre-incubated for 5 min in a water-bath at the incubation temperature. The incubation was started by the addition of 50 μl of BrMMC stock solution. The incubations were carried out protected from light and were performed at 70 ± 1°C, if not stated otherwise. At given times (depending on the derivatization rate) 75-μl samples were taken from the incubation mixture and diluted with 75 μl of acetonitrile in a type 3810 Eppendorf vessel. These samples were stored at −20°C. The derivatization product of UA with BrMMC obtained from the micellar systems was tentatively identified by comparing the retention time and the excitation and emission spectra of the product with those obtained from incubations in acetone.

The incubations in acetone were carried out according to ref. 17. A 10-μl volume of the undecylenic acid solution was added to a type 3814 Eppendorf vessel that contained 935 μl of acetone, 30 μl of 18-crown-6 ether solution and *ca.* 10 mg of fine-grained potassium carbonate. After the addition of 20 μl of the BrMMC solution, the reaction was carried out at 60°C for 30 min. Next, samples were taken as described above.

*Chromatographic system*

Samples of 10 μl of the diluted incubation mixture were injected into a fully automated HPLC system, using a laboratory-filled 10-μm LiChrosorb RP-18 column (300 × 4.6 mm I.D.) (Merck). The HPLC system consisted of two M 6000 A pumps and an automated gradient controller (Waters Assoc., Milford, MA, U.S.A.), controlled by a Model 231 automatic sampler injector (Gilson, Villiers le Bel, France). A linear gradient was run in 10 min from methanol–water (60:40, v/v) to 100% methanol. All solvents were filtered through a 0.2-μm filter and deaerated ultrasonically prior to use. A Model 650 fluorescence detector (Perkin-Elmer/Hitachi, Tokyo, Japan) was used. The optimized excitation and emission wavelengths were 330 and 395 nm, respectively. Retention times and peak areas were measured with an SP 4270 integrator (Spectra-Physics, Santa Clara, CA, U.S.A.).

*Data analysis*

The apparent pseudo-first-order rate constant, $k$, was calculated from[23]

$$I(t) = I^* (1 - e^{-kt}) \quad (1)$$

where $I(t)$ is the peak area of the derivative at a given time, $t$, and $I^*$ is the peak area after complete derivatization of the substrate. A non-linear Marquardt optimization[24] of eqn. 1 through at least eight data points was used to calculate the best approximation of the rate constant.

*Micelle size determination*

Dynamic light scattering was used to determine the apparent mean diameter of the micellar aggregates. The surfactants and ion-pair reagent were dissolved at various concentrations in 10 m*M* phosphate buffer. All solvents were filtered through a 0.2-$\mu$m polycarbonate filter (Nucleopore, Pleasanton, CA, U.S.A.) prior to use. Triplicate determinations were carried out at 50°C (instrumental limitation) by the use of Malvern 7027 particle analyser controller and additional equipment (Malvern, U.K.). A 100-mW He–Ne laser ($\lambda$ = 632.8 nm) (NEC, Tokyo, Japan) was used as a light source.

*Cloud point determination*

To determine the cloud temperature ($T_c$) of the various micellar solutions, 3-ml aliquots of the micellar solutions were pipetted into stoppered glass-walled tubes. By means of a temperature-controlled water-bath the temperature dependent change from clear to opaque solutions was determined (in duplicate) by eye.

## RESULTS AND DISCUSSION

*Preliminary experiments*

Preliminary derivatization experiments were carried out in pure surfactant systems without any additives except buffer. Irrespective of the type and concentration of the surfactant used, only insignificant amounts of MMC derivatives were formed. This can be explained as follows.

It is generally accepted that nucleophilic substitution ($S_N2$) reactions involving carboxylic acids are inhibited in aqueous solutions owing to solvation of the carboxylic acid[3,25].The aqueous bulk phase and the strongly hydrated interface of the micelles[26], therefore, can be ruled out as possible reaction sites.The micellar core may be more or less deprived of water[9,10]. This means, in principle, that the derivatization reaction can occur in the micellar core. In the micellar solution the acid will be present in a protonated (HA) and a deprotonated ($A^-$) form. It is likely that only the uncharged species, HA, can penetrate into the hydrocarbonaceous environment of the micellar core. However, this species lacks the nucleophilicity that is necessary for an $S_N2$ reaction[3,25]. Therefore, only the $A^-$ species present in the core of the micelle can be derivatized in the micellar system. However, it is unlikely that the ionic $A^-$ can penetrate into the hydrocarbonaceous core of the micelle. The species $A^-$ can be extracted, however, into the micellar core using a cationic ion-pair reagent (*e.g.*, a quaternary ammonium salt). Following extraction, the reaction with the label (BrMMC) could take place in the micellar core. In non-micellar two-phase systems this principle is known as phase-transfer catalysis (PTC)[27–29].

Two equilibria mainly determine the derivatization rate in conventional PTC

systems. The first is the extraction of the analyte, $(A^-)_w$, with an ion-pair reagent, $(Q^+)_w$, from the aqueous phase into the organic phase. In the organic phase the analyte is present as the electro-neutral complex, $(QA)_0$. The extraction equilibrium is described by

$$[(QA)_0] = K_{ex}^{qa} [A^-_w] \cdot [Q^+_w] \quad (2)$$

where $K_{ex}^{qa}$ is the extraction constant. The value of $K_{ex}^{qa}$ depends strongly on the hydrophobicity of the ion-pair reagent and of the analyte[27–29].

The second equilibrium is the derivatization rate in the organic phase. If the label (BrMMC) is present in excess, then the derivatization rate, $v_0$, can be described as a pseudo-first-order equation[30]:

$$v_0 = k[(QA)_0] \quad (3)$$

where $k$ is the pseudo-first-order reaction constant.

We have studied the possible use of ion-pair reagents in the derivatization of carboxylic acids in aqueous micellar systems. To our knowledge, no study has been reported on phase-transfer catalysis in micellar systems.

The addition of TBuABr to SDS solutions led to the precipitation of the surfactant with the ion-pair reagent. When the ion-pair reagent was added to CTAB only a slight improvement in the reaction rate was found. In contrast, the addition of TBuABr to non-ionic micellar systems, especially Triton X-100 and Arkopal N, led to a marked improvement in the reaction rate and was, therefore, studied in more detail.

*Influence of the ion-pair reagent*

Fig. 1 shows the influence that the concentration of TBuABr, TPeABr and THxABr has on the derivatization rate of UA with BrMMC at 70°C in the presence 50 m*M* Triton X-100 in 10 m*M* phosphate buffer (pH 7.0). The Triton X-100 concentration was chosen arbitrarily. However, a surfactant concentration range of 20–100 m*M* is often used in micellar catalytic systems[11–13].

Comparison of Fig. 1A–C illustrates that the derivatization rate is strongly affected by the type and concentration of the ion-pair reagents. At an equimolar concentration of the ion-pair reagents the derivatization rate increases sharply from TBuABr to THxABr. This phenomenon is generally seen in PTC, and is related to the fact that the extraction constant, $K_{ex}^{qa}$ (eqn. 2), increases with increasing number of carbon atoms in the ion-pair reagent[29].

In PTC the derivatization rate gradually increases with increasing ion-pair reagent concentration[29]. At low analyte concentrations the derivatization rate initially increases linearly with increasing ion-pair reagent concentration. Finally, it reaches asymptotically a certain plateau value[21]. This can be explained as follows. Eqn. 3 shows that, when the label is present in excess, the derivatization rate is proportional to the concentration of the ion-pair complex, $(QA)_0$, in the organic phase[30]. With a fixed analyte concentration the amount of the analyte extracted into the organic phase initially increases linearly with increasing concentration of the ion-pair reagent (eqn. 2). When the concentration of the ion-pair reagent is increased still further it will become increasingly difficult to extract the last traces of analyte into the organic phase.

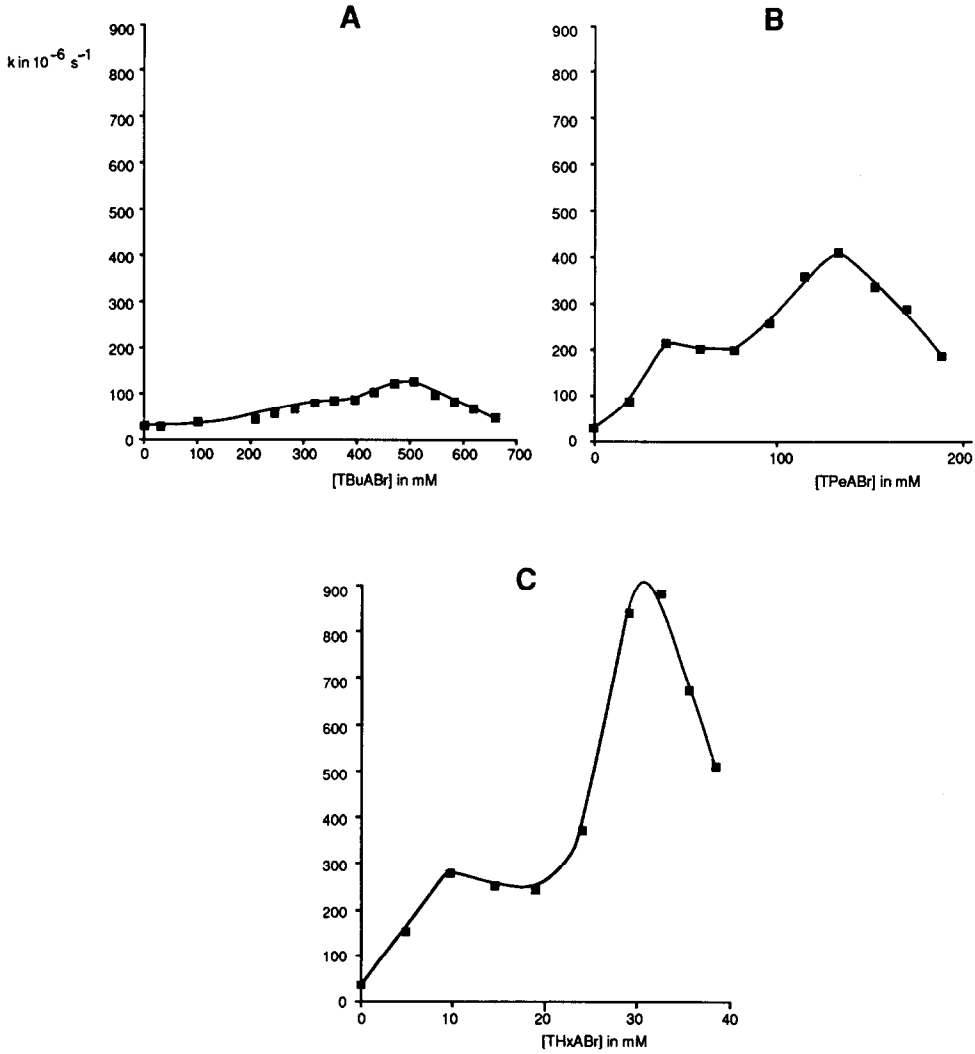


Fig. 1. Influence of the concentration of the ion-pair reagents (A) TBuABr, (B) TPeABr and (C) THxABr on the apparent rate constant ($k$) of the reaction between UA and BrMMC at 70°C in Triton X-100 (50 m$M$) in 10 m$M$ phosphate buffer (pH 7.0).

Finally, the concentration of $(QA)_0$ and, therefore, the derivatization rate will reach a certain plateau value. A more quantitative description of the relationship between the ion-pair reagent concentration and the derivatization rate can be found elsewhere[21].

The relationship described can also be observed in Fig. 1 at lower ion-pair concentrations (*e.g.*, $<80$ m$M$ TPeABr or $<20$ m$M$ THxABr). Initially the derivatization rate increases with increasing concentration of the ion-pair reagent. With a further increase in the ion-pair reagent concentration the derivatization rate

levels off. However, in a particular concentration range of the ion-pair reagents (*e.g.*, 100–180 m*M* TPeABr or 25–40 m*M* THxABr) higher, deviant, derivatization rates were observed compared with the calculated values[21]. This aberrant behaviour is caused by changes in the properties of the micellar system and is discussed in the following section.

*Effect of cloud temperature*

Non-ionic micellar systems are often clear solutions. Most of the incubation mixtures that were used for the set-up shown in Fig. 1 also looked clear. However, at the incubation temperature some of these incubation solutions were more or less opaque. In some of these solutions (*e.g.*, 32–40 m*M* THxABr; Fig. 1C) the deviant, high derivatization rates were observed. Obviously there is a relationship between the high derivatization rates and the opaqueness of the solutions. This relationship was studied in more detail.

The opaqueness of non-ionic surfactant solutions is connected with the so-called cloud temperature ($T_c$), at which there is a transition from normal to very large micellar aggregates[31]. It is these large structures that are responsible for the turbid appearance of the micellar solutions. $T_c$ can be affected by the presence of additives such as salts and organic solvents[31]. At higher temperatures than $T_c$ the solution begins to flocculate and finally separates into two phases. One layer is surfactant-rich whereas the other is deprived of micelles[31,32].

The influence of the concentration of the ion-pair reagents on the $T_c$ of Triton X-100 (50 m*M*) is shown in Fig. 2. At low ion-pair reagent concentrations the $T_c$ of Triton X-100 gradually increases with increasing concentration of the ion-pair reagents. The steepness of the increase in $T_c$ clearly depends on the number of carbon atoms in the ion-pair reagent. Similar results have been reported for other hydrophobic additives such as hydrocarbons[31]. Fig. 2. shows that the micellar systems become turbid again at low temperatures within a small concentration range of the ion-pair reagents, *e.g.*, 30–40 m*M* THxABr (Fig. 2C). This uncommonly steep change in $T_c$ probably coincides with the saturation curve of the ion-pair reagents. This notion is based on the observation that, at room temperature, above *ca.* 40 m*M* THxABr the surplus ion-pair reagent remained present as small droplets in the more or less turbid micellar solutions.

A discrepancy is observed between the ion-pair reagent concentration at which a maximum derivatization rate is obtained in the more or less turbid incubation solutions (Fig. 1) and the concentration at which a $T_c$ of 70°C was determined (Fig. 2). The discrepancy can be explained by the presence of BrMMC in the incubation mixtures. This causes a further decrease in the $T_c$ of these mixtures. The decrease in the derivatization rate, *e.g.*, > 150 m*M* TPeABr (Fig. 1B), occurred in the region in which phase separation was observed (Fig. 2B). One should therefore try to prevent phase separation.

From the above experiments we concluded that the cloud temperature is a very important parameter in optimizing the derivatization rate in micellar systems.

It must be emphasized that the deviant, high derivatization rates are not restricted to a particular composition of the incubation solution, *e.g.*, 50 m*M* Triton X-100 and 33 m*M* THxABr (Fig. 1C). Additional experiments indicated that similar derivatization rates can also be obtained with different compositions of the micellar

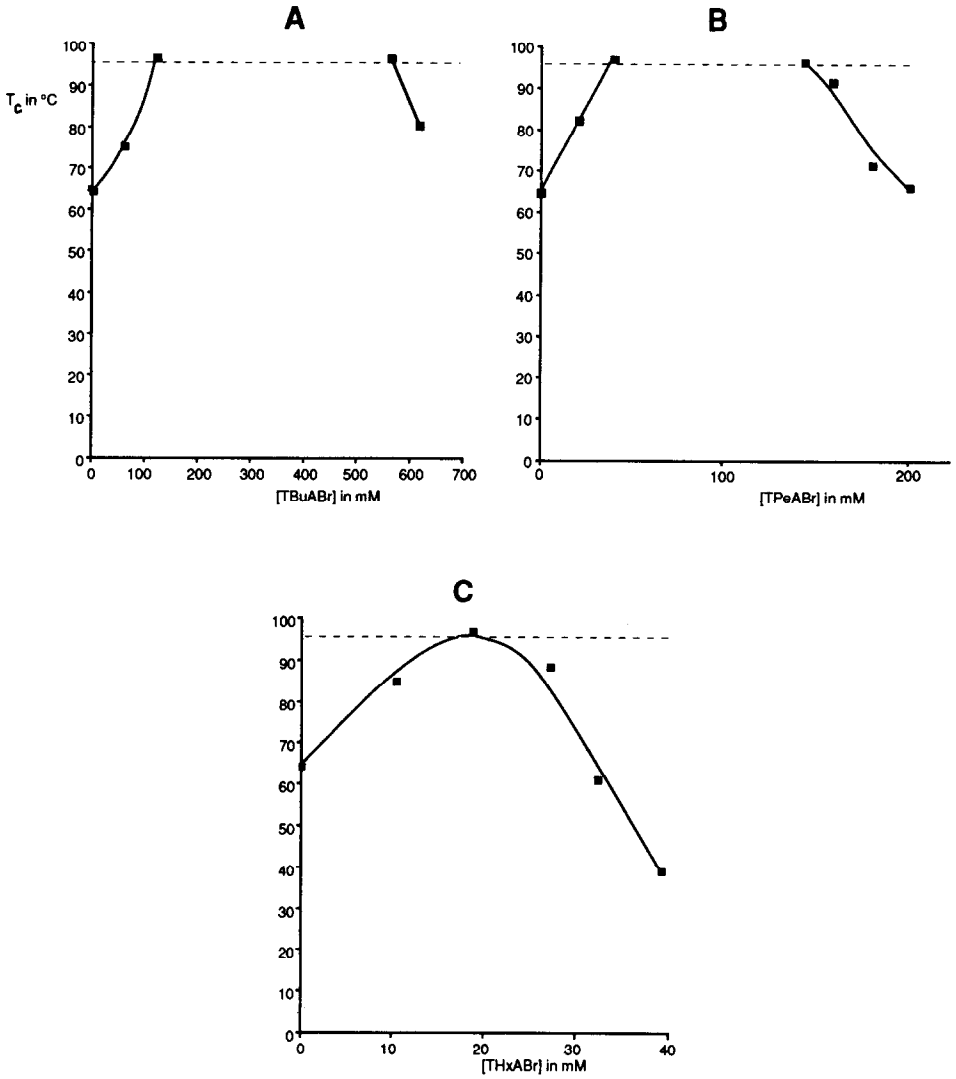


Fig. 2. Influence of the concentration of the ion-pair reagents (A) TBuABr, (B) TPeABr an (C) THxABr on the cloud temperature ($T_c$) of Triton X-100 (50 m*M*) in 10 m*M* phosphate buffer (pH 7.0). The broken line indicates the experimental limit.

system (*e.g.*, 70 m*M* Triton X-100 and 40 m*M* THxABr). The only important requirement for obtaining a high derivatization rate in a particular micellar system is, as far as we know, that the solution must be turbid at the incubation temperature.

$T_c$ can easily be adjusted by changing the surfactant or the ion-pair reagent concentration. One should try, however, to prevent phase separation.

*Effect of micellar size on reaction rate*

In the previous section it was concluded that the cloud temperature plays an important role in the derivatization rate in micellar solutions. At the cloud temperature very large micellar aggregates form. Obviously, the size of the micellar aggregates might be an important parameter affecting the reaction rate. This was therefore investigated in more detail. These and subsequent experiments were performed with Arkopal N surfactants, because a better derivatization performance (*e.g.*, reaction rate) was obtained in the Arkopal N systems than in Triton X-100. It is conceivable that in general the results with the Arkopal N systems can be applied to other nonionic surfactant systems.

Fig. 3 shows the effect of the THxABr concentration on the apparent mean micelle size ($\Phi_{app}$) of Arkopal N-130 at a fixed concentration of 50 m*M* in buffer at 50°C (experimental limitation). The determined value of the apparent mean micellar size of the pure micellar solution is in reasonable agreement with ref. 33. Fig. 3 shows that the micellar size is almost constant at lower ion-pair reagent concentrations (< 20 m*M* THxABr). After a small decrease the micellar size increases almost asymptotically beyond 30 m*M* THxABr. Above *ca.* 36 m*M* the micellar solutions were opaque, as a result of which the size of the very large micellar aggregates was indeterminable.

At a concentration of 50 m*M* Arkopal N-130 and 36 m*M* THxABr a cloud temperature is reached at *ca.* 50°C (Fig. 4). The difference between the THxABr concentrations that induce a $T_c$ of 50°C and 70°C (incubation temperature), respectively is small (Fig. 4). Therefore, it is conceivable that a similar relationship to that depicted in Fig. 3 would be found if the micellar size could be determined at 70°C. Fig. 3 also shows the relationship between the apparent rate constant and the

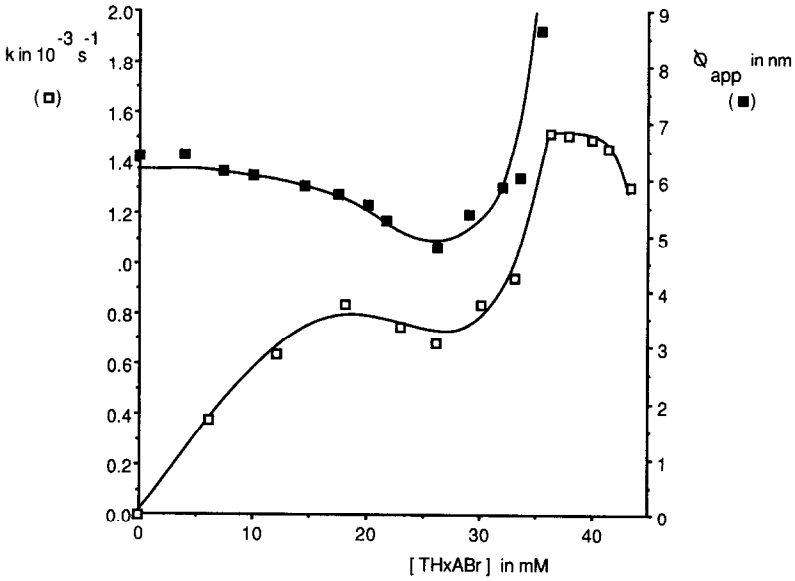


Fig. 3. Influence of THxABr concentration on the apparent mean micellar size, $\Phi_{app}$ (■), at 50°C and the apparent rate constant, *k* (□), of the reaction between UA and BrMMC at 70°C in a surfactant system of 50 m*M* Arkopal N-130 in 10 m*M* phosphate buffer (pH 7.0).

concentration of the ion-pair reagent in the same micellar system at 70°C. This rate profile is similar to that found in Triton X-100 (Fig. 1). However, higher derivatization rates are obtained. Fig. 3 shows that the derivatization rate increases up to 20 m*M* THxABr. Obviously, in this concentration range the increase in the derivatization rate is not related to changes in the micellar size. However, as argued previously, this increase can be related to the amount of the acid that is extracted into the micellar core[21]. Between 25 and 36 m*M* THxABr a clear relationship can be observed between the increase in the derivatization rate and the increase in the size of the micelles.

It is not known why the derivatization rate increases with increasing micelle size. One possible explanation is that the increase in the micelle size leads to a decrease in the water content within the micellar aggregates. It is reported that the reaction with BrMMC is strongly inhibited by the presence of water[19,20].

Also of interest is the observation that the optimum in the rate profile in the Arkopal N-130 system (Fig. 3) is less critically related to the THxABr concentration than in the Triton X-100 system. This means that it is more advantageous to use the Arkopal N-130 surfactant than Triton X-100.

We studied whether the presence of large micellar aggregates alone with the $T_c$ of 70°C, hence without the presence of ion-pair reagents, could be responsible for the increase in the derivatization rate. However, insignificant reaction rates were found in ion-pair reagent-free micellar solutions in which the presence of large micelles was induced by the presence of 100 m*M* sodium chloride. This finding also indicates a PTC-like derivatization mechanism in the micellar systems.

*Influence of polyoxyethylene chain length*

It is well known that the POE chain length of non-ionic surfactants strongly affects their properties. If the POE content increases, then the aggregation number ($N$)[26,34] and the micellar size[26,34] decrease, whereas the critical micelle concentration (CMC)[34,35] and $T_c$[31] increase. Therefore the influence of the POE content on the catalytic properties of the micellar system was investigated.

Fig. 5 shows the influence that the concentration of several Arkopal N surfactants has on the reaction rate of UA with BrMMC in the presence of a fixed THxABr concentration of 36 m*M* at pH 7.0 and 70°C. It illustrates that on increasing the surfactant concentrations the rate constants in the various micellar systems approach an almost constant value.

On decreasing the surfactant concentration the derivatization rate increases more or less steeply to high values. With a further decrease in the surfactant concentration flocculation occurs and/or the solubility limit of the ion-pair reagent is exceeded. The surfactant concentration at which the optimal derivatization rate is obtained depends on the POE chain length of the surfactant. This might be related to a different solubility of the ion-pair reagent in the Arkopal N solutions. Fig. 4 shows that the solubility limit of the ion-pair reagent increases with increasing POE chain length. This notion is based on the previous arguments that the solubility limit is connected with the ion-pair concentration at which $T_c$ decreases.

Fig. 5 demonstrates that the maximum derivatization rate that can be obtained in a micellar system is apparently independent of the POE chain length. Obviously the POE chain length does not have strong influence on the maximal derivatization rate in opaque solutions. Fig. 5 shows that in the non-opaque solutions the derivatization rate

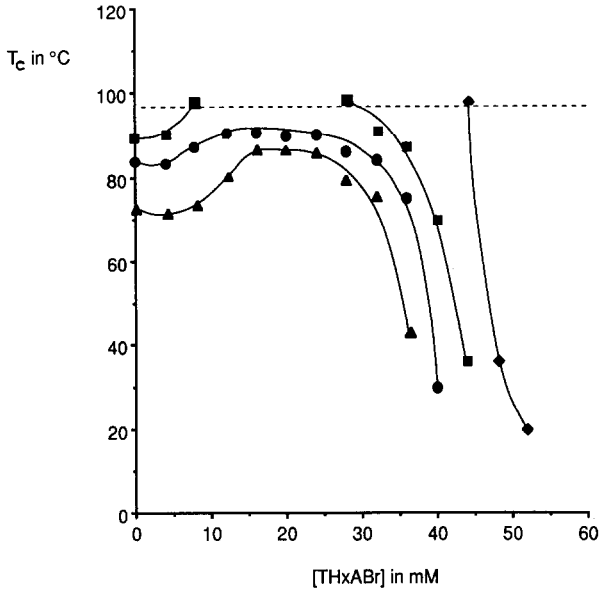


Fig. 4. Influene of THxABr concentration on the cloud temperature ($T_c$) of different Arkopal N surfactants (50 m$M$) in 10 m$M$ phosphate buffer; ▲, N-110; ●, N-130; ■, N-150; ◆, N-230. The broken line indicates the experimenal limit.

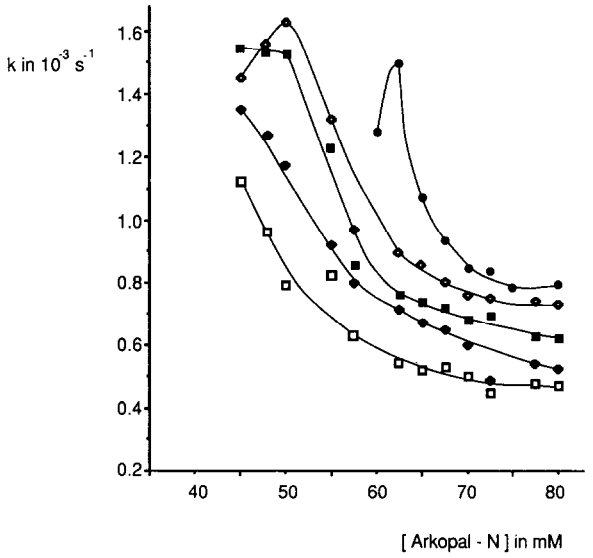


Fig. 5. Influence of the concentration of different Arkopal N surfactants on the apparent rate constant ($k$) of UA with BrMMC at 70°C in the presence of THxABr at a fixed concentration of 36 m$M$; ●, N-80; ◇, N-110; ■, N-130; ◆, N-150; □, N-230.

increases with a decrease in the POE chain length of the surfactant. The possible reason for this is that the micellar size increases with a decrease in the POE chain length[26]. It was concluded in a previous section that the derivatization rate increases with increasing size of the micelle. Further experimental evidence for this assumption is given by Fig. 6, which shows that in non-opaque Arkopal N systems the derivatization rates (70°C; $n=3$) increase with the apparent micellar size ($\Phi_{app}$; 50°C) of the five surfactants. The concentration of the surfactants was 70 m*M* in all instances and that of THxABr was 32 m*M*.

*Influence of organic solvents*

To ensure high derivatization rates the BrMMC must be present in excess. We investigated how the label could be added to the incubation solutions. The BrMMC is almost insoluble in aqueous solution and slightly soluble (*ca.* 2.5 m*M*) in the micellar solutions. Thus aqueous stock solutions of BrMMC could not be used. Crystalline BrMMC dissolves only slowly in the micellar solution. Therefore, it was necessary to dissolve BrMMC at high concentration in an organic solvent before adding it to the micellar system. Originally acetone was used for the preparation of the stock solutions of BrMMC. The solubility of BrMMC in acetone is *ca.* 3 mg/ml at 25°C. To ensure the presence of an excess of BrMMC, relatively large amounts of acetone had to be added to the micellar solution. This was considered to be undesirable because organic solvents could strongly influence the properties of micellar systems[8,36]. Therefore, we tested several other solvents to dissolve BrMMC. The solubility of BrMMC in

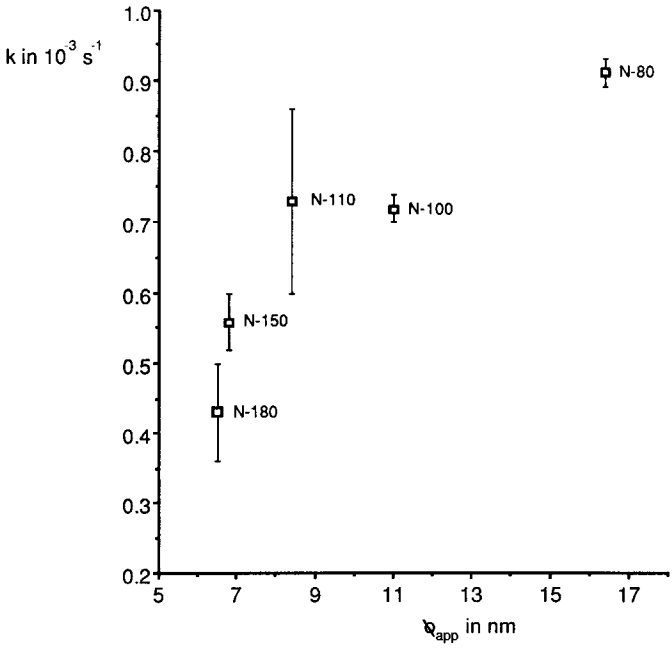


Fig. 6. Relationship between the apparent mean micellar size ($\Phi_{app}$) at 50°C and the apparent rate constant ($k$) at 70°C of various Arkopal N surfactant systems. The micellar systems were composed of 70 m*M* Arkopal N surfactant and 32 m*M* THxABr in 10 m*M* phosphate buffer (pH 7.0).

acetonitrile and ethyl acetate was similar to that in acetone and no further consideration was given to their use. Although BrMMC could be solubilized more easily in dioxane (*ca.* 8 mg/ml at 25°C), this solvent could not be used because it strongly inhibited the reaction between UA and BrMMC in the micellar system even at low percentages. The reason for this behaviour is unknown. However, dioxane shows a similar behaviour to incubations performed in acetonitrile[20].

Because of these poor alternatives we investigated how much acetone could be added to the micellar systems without affecting the derivatization rate. Fig. 7 shows that with the addition of up to 6% acetone no significant changes in the reaction rate could be seen in a micellar system of 50 m*M* Arkopal N-130 and 36 m*M* THxABr. At higher acetone percentages the derivatization rate decreased sharply. Above *ca.* 35% acetone the derivatization reactions were almost completely inhibited because the micelles were no longer present under this condition[36]. Obviously, unimpaired micellar aggregates are a prerequisite for the catalytic properties. On the other hand, the rate-inhibiting effect of organic solvents was utilized to terminate the derivatization reaction by the addition of an equal volume of acetonitrile to the micellar solutions.

With regard to the addition of the reagent, it was concluded that the percentage of acetone should be kept low (<6%). However, the addition of 60 µl of BrMMC stock solution (3 mg/ml) would result in only 0.7 m*M* BrMMC in the incubation solution (1 ml). Therefore the solubility of BrMMC in acetone was increased to *ca.* 8 mg/ml by heating the BrMMC stock solution prior to the addition of the reagent. An

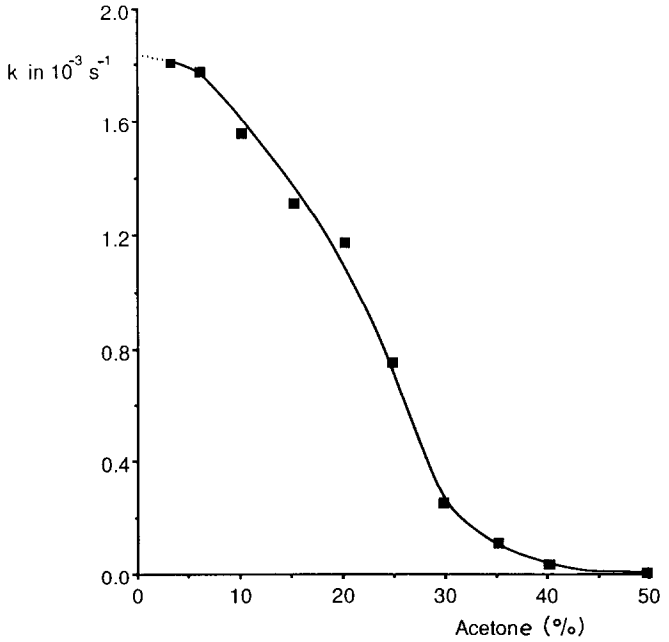


Fig. 7. Influence of the percentage of acetone present in the incubation mixture on the derivatization rate constant ($k$) of UA with BrMMC at 70°C. The micellar system consisted of 50 m*M* Arkopal N-130 and 36 m*M* THxABr in phosphate buffer (pH 7.0).

alternative and more convenient method of adding the BrMMC has been reported recently[22].

*Influence of salts*

The final aim of this study is to develop micelle-mediated derivatizations of carboxylic acids in biological matrices, *e.g.*, plasma and urine. These matrices contain salts and urea, which will affect the properties of the micellar system. Salts increase the aggregation number[8,37] and decrease the CMC[36,38] and $T_c$ of non-ionic surfactants ($ClO_4^- \gg Br^- > Cl^- >$ urea)[8,39]. Anions can also compete with UA for the extraction into the micellar core [$ClO_4^- \gg Br^- > Cl^-$ (refs. 27–29)], which may result in lower derivatization rates.

Fig. 8 shows the influence of $ClO_4^-$ (a well known protein precipitant), $Cl^-$ and urea (plasma constituents) on the derivatization rate in 50 m*M* Arkopal N-130 and 36 m*M* THxABr at pH 7.0. Fig. 8 clearly illustrates that the perchlorate ion strongly inhibits the derivatization reaction, whereas chloride anion and urea do this to a much lesser extent. It is difficult to say for certain that the decrease in the derivatization rate is caused entirely by the competition of the anions with extraction of the acid[22]. Also, the induced flocculation of the micellar systems could have affected the derivatization rate. Owing to the the marked decrease in the derivatization rate it can be concluded, however, that perchloric acid cannot be used as a protein precipitant in combination with the micellar derivatization systems.

Derivatizations were carried out in a 50% Hanks balanced salt solution (BSS)[39] at pH 7.0 in order to study whether the micelle-enhanced derivatization reactions are

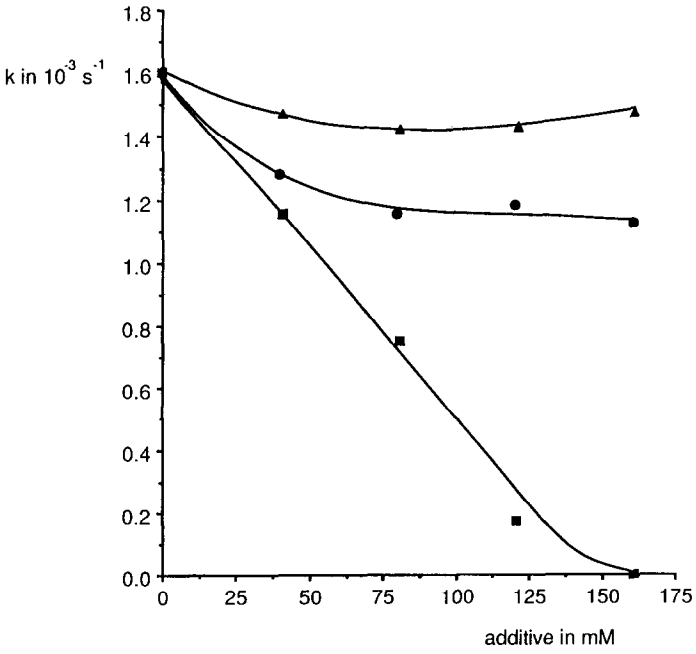


Fig. 8. Influence of the concentrations of urea (▲), NaCl (●) and $NaClO_4$ (■) on the apparent rate constant ($k$) at 70°C. The micellar solution consisted of 50 m*M* Arkopal N-130 and 36 m*M* THxABr in 10 m*M* phosphate buffer (pH 7.0).

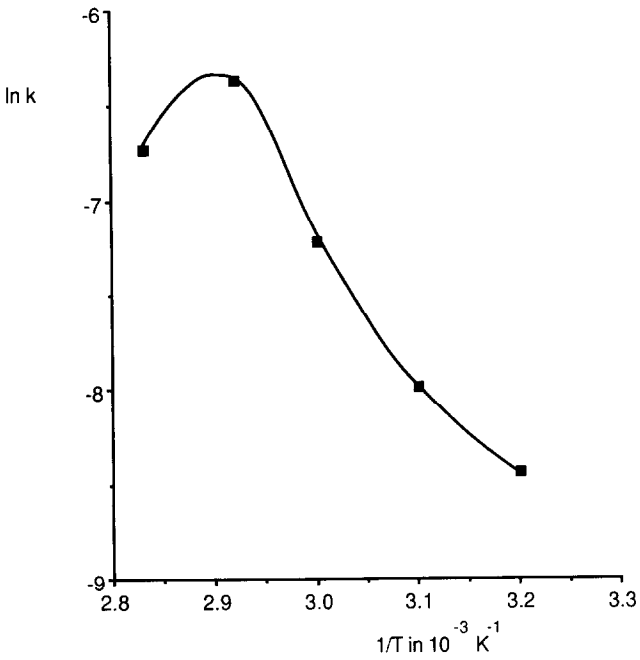


Fig. 9. Arrhenius plot of the influence of temperature on the derivatization rate in a micellar solution of 50 m*M* Arkopal N-130 and 36 m*M* THxABr in 1 m*M* phosphate buffer (pH 7.0).

compatible with the salt composition of biological fluids. With a 50% BSS solution it is assumed that the biological matrix is diluted with an equal volume of concentrated micellar solution. The apparent rate constant found for the reaction of UA with BrMMC was $1.4 \cdot 10^{-3}$ s$^{-1}$. In a micellar solution with 10 m*M* phosphate buffer (pH 7.0) a rate constant of $1.8 \cdot 10^{-3}$ s$^{-1}$ was obtained. In other words, the salt composition of biological fluids will not seriously limit the use of micelle-enhanced derivatizations in biological matrices.

*Influence of temperature*

Fig. 9 shows the Arrhenius plot[23] of the influence of temperture on the derivatization rate in a micellar system that consisted of 50 m*M* Arkopal N-130 and 36 m*M* THxABr. This micellar system has a $T_c$ of 60–70°C (Fig. 4). The relationship is not linear, which indicates that mechanisms other than purely kinetic ones affect the reaction rate in the micellar system[23]. An explanation of this behaviour is that micellar size increases with increase in temperature[8]. The micellar size increases particularly near $T_c$ and, therefore, so does the derivatization rate. The decrease in the reaction rate at 80°C is caused by phase separation.

We investigated whether the derivatization rate at 40°C could be improved by lowering the $T_c$ of the micellar system to *ca.* 40°C by the addition of extra THxABr. However, in these turbid solutions the derivatization rates found at 40°C were not significantly higher than those in clear solutions. Obviously, high derivatization temperatures are still required in order to overcome the enthalpy of reaction in the presence of large micelles.

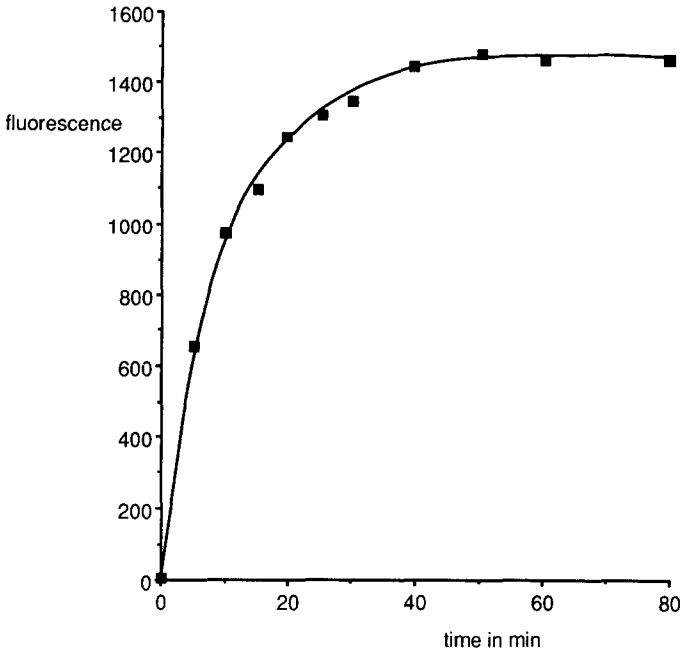


Fig. 10. Relationship between the derivatization time and the peak area of the 10-undecenoic derivative obtained in an incubation solution of 50 m*M* Arkopal N-130 and 36 m*M* THxABr in 10 m*M* phosphate buffer (pH 7.0) at 70°C.

*Validation of the micelle-mediated derivatizations*

Fig. 10 illustrates that the derivatization of 50 $\mu M$ UA with BrMMC in a micellar system of 50 m*M* Arkopal N-130 and 36 m*M* THxABr is complete within 45 min at 70°C.

The chromatogram of the MMC derivative of UA after derivatization for 60 min in the same micellar solution is shown in Fig. 11. Although several hundred injections were performed on a single HPLC column, no deterioration in the performance of the column could be observed. This demonstrates that the injected surfactant and the ion-pair reagent do not affect the chromatographic process in the long term[40].

The linearity of the determination of 10-undecenoic acid in 10 m*M* phosphate buffer solution that contained 50 m*M* Arkopal N-130 and 36 m*M* THxABr was satisfactory from 2 n*M* to 2 m*M* UA ($r = 0.997$; $n = 17$). The reproducibility of the determination of 50 $\mu M$ UA in the same solution was 3.7% ($n = 6$).

These tentative results indicate that the derivatization performed in the micellar system is satisfactory compared with other pre-column derivatization procedures for carboxylic acids that are present in aqueous solutions[20,41,42].

## CONCLUSIONS

This study has demonstrated that carboxylic acids can be derivatized in aqueous solutions using micelles. The proposed derivatization procedure involves the use of a non-ionic surfactant (*e.g.*, Arkopal N or Triton X-100) and a cationic ion-pair

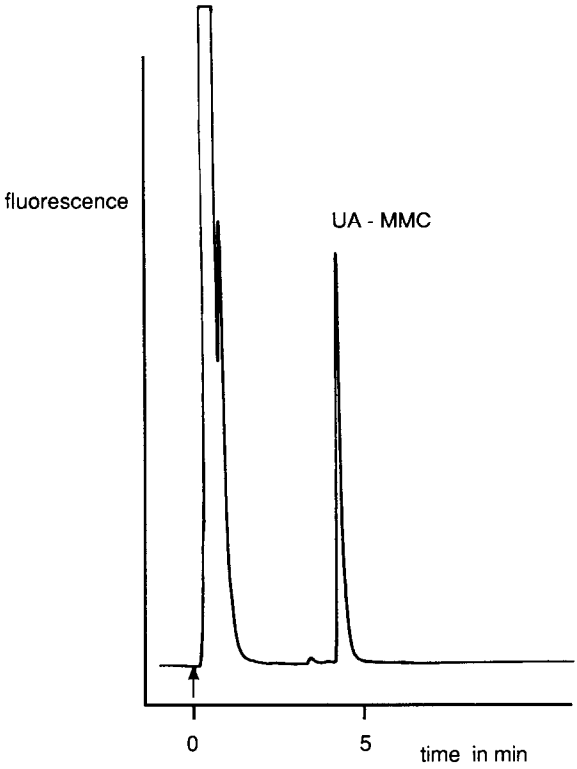


Fig. 11. Chromatogram of 20 $\mu$l of the 10-undecenoic derivative (UA-MMC, 50 $\mu M$) obtained from incubation in 50 m$M$ Arkopal N-130 and 36 m$M$ THxABr at pH 7.0 and 70°C.

reagent (*e.g.*, quaternary ammonium salt). The mechanism of the derivatization of the acid is likely to be related to phase-transfer catalysis. The micelles act here as an organic "pseudo"-phase, in which the actual derivatization reaction occurs.

The derivatization rates increase with increasing size of the micellar aggregates. Especially high reaction rates are observed if the cloud temperature of a particular micellar system, at which giant micellar aggregates are formed, approaches the incubation temperature.

In a subsequent paper we shall discuss the influence that the acid-related factors have on the derivatization rate of aliphatic carboxylic acids in the micellar systems[21]. In addition, a model will be presented of the mechanisms involved in the derivatization of the acids in the micellar systems.

It has been demonstrated recently that with the use of the micellar systems the tedious extraction steps that are involved in conventional derivatization procedures can be circumvented[22]. This could make the principle of micelle-mediated derivatization reactions very well suited for an on-line pre-column derivatization procedure for carboxylic acids that are present in aqueous biological matrices.

The interesting new approach of the use micellar systems in the automated on-line pre-column derivatization of bio-active acids that are present in plasma samples is currently under investigation.

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REFERENCES

1 J. R. Lawrence and R. W. Frei, *Chemical Derivatization in Liquid Chromatography*, Elsevier, Amsterdam, 1976.
2 H. Lingeman, W. J. M. Underberg, A. Takadate and A. Hulshoff, *J. Liq. Chromatogr.*, 8 (1985) 789.
3 P. Sykes, *A Guide Book to Mechanism in Organic Chemistry*, Longman, London, 4th ed., 1975.
4 S. Chakir, P. Leroy, A. Nicolas, J.-M. Ziegler and P. Labory, *J. Chromatogr.*, 395 (1987) 553.
5 D. E. M. M. Vendrig, J. J. M. Holthuis, V. Erdélyi-Tóth and A. Hulshoff, *J. Chromatogr.*, 414 (1987) 91.
6 D. Attwood and A. T. Florence, *Surfactant Systems*, Chapman and Hall, London, 1983.
7 B. Lindman and H. Wennerström, in F. L. Boschke (Editor), *Micelles*, Springer, Berlin, 1980.
8 M. J. Schick (Editor), *Nonionic Surfactants*, Marcel Dekker, New York, 1967.
9 K. A. Dill, D. E. Koppel, R. S. Cantor, J. D. Dill, D. Dendedouch and S. H. Chen, *Nature (London)*, 309 (1985) 42.
10 A. Ben-Shaul and W. M. Gelbart, *Annu. Rev. Phys. Chem.*, 36 (1985) 1755.
11 I. V. Berezin, K. Martinek and A. K. Yatsimirskii, *Russ. Chem. Rev.*, 42 (1973) 787.
12 J. H. Fendler and E. J. Fendler, *Catalysis in Micellar and Macromolecular Systems*, Academic Press, New York, 1975.
13 K. L. Mittal (Editor), *Micellization, Solubilization and Microemulsions*, Vols. 1 and 2, Plenum Press, New York, 1977.
14 F. A. L. van der Horst and J. J. M. Holthuis, *J. Chromatogr.*, 426 (1988) 267.
15 K. A. Connors and M. P. Wong, *J. Pharm. Sci.*, 68 (1978) 1470.
16 W. Dünges, A. Meyer, K. Müller, R. Pietschman, C. Plachetta, R. Sehr and H. Tuss, *Fresenius Z. Anal. Chem.*, 288 (1977) 361.
17 S. Lam and E. Grushka, *J. Chromatogr.*, 158 (1978) 207.
18 W. Voelter, R. Huber and K. Zech, *J. Chromatogr.*, 217 (1981) 491.
19 P. Leroy, S. Chakir and A. Nicolas, *J. Chromatogr.*, 35 (1986) 267.
20 J. H. Wolf and J. Korf, *J. Chromatogr.*, 436 (1988) 437.
21 F. A. L. van der Horst, M. H. Post, J. J. M. Holthuis, A. Bult and U. A. Th. Brinkman, *J. Chromatogr.*, submitted for publications.
22 F. A. L. van der Horst, G. G. Eikelboom and J. J. M. Holthuis, *J. Chromatogr.*, 456 (1988) 191.
23 R. Schmid and V. N. Sapunov, in H. F. Ebel (Editor), *Monographs in Modern Chemistry*, Vol. 14, Verlag Chemie, Weinheim, 1982.
24 D. W. Marquardt, *J. Soc. Ind. Appl. Math.*, 11 (1963) 431.
25 A. Hulshoff and A. D. Förch, *J. Chromatogr.*, 220 (1981) 275.
26 P. Becher, in M. J. Schick (Editor), *Nonionic Surfactants*, Marcel Dekker, New York, 1967, p. 478.
27 E. V. Dehmlov and S. S. Dehmlov, *Phase Transfer Catalysis*, Verlag Chemie, Weinheim, 1980.
28 W. P. Weber and G. W. Gokel, *Phase Transfer Catalysis in Organic Synthesis*, Springer, Berlin, 1977.
29 C. M. Starks and C. Liotta, *Phase Transfer Catalysis, Principles and Techniques*, Academic Press, New York, 1978.
30 C. M. Starks and R. M. Owens, *J. Am. Chem. Soc.*, 95 (1973) 3613.
31 T. Nakagawa, in M. J. Schick (Editor), *Nonionic Surfactants*, Marcel Dekker, New York, 1967, p. 558.
32 P. H. Elworthy and C. McDonald, *Kolloid Z. Z. Polym.*, 195 (1963) 16.
33 Z. Bedö, E. Berecz and I. Lakatos, *Colloid Polym. Sci.*, 265 (1987) 715.
34 M. J. Schick, S. M. Atlas and T. R. Eirich, *J. Phys. Chem.*, 66 (1962) 1326.
35 Z. Bedö, E. Berecz and I. Lakatos, *Colloid Polym. Sci.*, 264 (1986) 267.
36 P. Becher, *J. Colloid Sci.*, 20 (1965) 728.
37 L. Hsiao, H. N. Dunning and P. B. Lorenz, *J. Phys. Chem.*, 60 (1956) 657.
38 L. Marszall, *Langmuir*, 4 (1988) 90.
39 N. W. Blacow (Editor), *Martindale, the Extra Pharmacopoeia*, Pharmaceutical Press, London, 26th ed., 1973.
40 M. F. Borgerding and W. Hinze, *Anal. Chem.*, 57 (1985) 2183.
41 H. Miwa, M. Yamamoto, T. Nishida, K. Nunoi and M. Kikuchi, *J. Chromatogr.*, 416 (1987) 237.
42 R. A. Kelly, D. S. O'Hara and V. Kelley, *J. Chromatogr.*, 416 (1987) 247.