Note

Determination of carboxylic acids by liquid chromatography after phase-transfer-catalysed fluorogenic labelling

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Owing to the biological importance of carboxylic acids there is a great need for sensitive methods for their determination. For liquid chromatography a number of methods of preparing fluorescent ester derivatives have been reported[1], many based on reactions either with aryldiazomethanes[2–4] or with activated halogen compounds[5–13]. In the latter instance the analyte has to be present in ionized form, which means that solubilization of the carboxylate and the reagent has to involve either a mixed solvent system or a two-phase system incorporating a phase-transfer agent such as a crown ether or tetraalkylammonium ion. Although extractive alkylations of the latter type have been used for preparative purposes, investigations on their application to the analytical-scale derivatization of carboxyl groups seem to be limited to the fundamental studies published by Gustavii and Furängen[14–18]. A paper describing the dansylation of phenolic compounds via phase transfer of this kind has also recently appeared[19].

In general, however, very little use has been made of this type of pre-column derivatization, despite great progress in the synthesis of new fluorogenic reagents. The method of choice is the use of a solid potassium carbonate–crown ether–acetonitrile system[4,7,9–13].

This paper describes how the tetrabutylammonium-mediated transfer of the analyte as an anion into ethylene dichloride containing the fluorogenic reagent can be applied for pre-concentration with simultaneous derivatization.

EXPERIMENTAL

Reagents and chemicals

Alkanoic acids, tetrabutylammonium hydrogen sulphate (TBA) and 2-naphthacyl bromide (NABr) were obtained from Fluka (Buchs, Switzerland). 2-(Bromoacetyl) fluorene was prepared by direct bromoacetylation of fluorene in the presence of aluminium chloride in carbon disulphide at $-5°C$ according to Sargent and Small[20]. The crude product was repeatedly recrystallized from acetone; thin-layer chromatography [silica gel plate; toluene–ethyl acetate (70:30)] gave a single spot; m.p. 142–143°C (lit.[20] m.p., 147–149°C).

The following reagent solutions were prepared in order to study the derivatization kinetics. Alkanoic acids were used as solutions in 50 mM phosphate buffer

(pH 8.1) of varying concentrations. TBA was used as a 10 mM solution in water. NABr and 2-(bromoacetyl) fluorene were used as 10 mM solutions in ethylene dichloride (b.p. 84°C).

Procedure for extractive derivatization with formation of 2-naphthacyl esters

The kinetics of the 2-naphthacyl ester formation were determined by the use of 10-ml screw-capped vials to each of which were added 2 ml of a solution of the alkanoic acid(s) (50μM–5 mM) in 50 mM buffer, 1 ml of TBA solution and 2 ml of NABr solution. The vials were stirred vigorously at the reaction temperature chosen and taken for further processing at different times. After rapid cooling, the vial was centrifuged to separate the phases and 10 μl of the clear organic phase were diluted with acetonitrile to 0.1–1.0 ml prior to injection into the liquid chromatographic (LC) system. The degree of dilution was dependent on the initial alkanoic acid concentration.

The concentration experiments were performed by using varying concentrations of 10 μmol of alkanoic acid in each derivatization. The initial concentrations used were 0.1–5.0 mM, corresponding to volumes between 100 and 2 ml. These reactions were performed at 60°C for 1 h.

Procedure used to obtain other ester derivatives

By the use of the same technique of extractive alkylation, derivatization with two other aracyl bromides was tried, *viz.*, with 9-anthracyl bromide and 2-(α-bromoacetyl) fluorene. Derivatives were readily obtained only with the latter reagent.

Reduction of the aracyl esters obtained in the organic phase was performed by shaking the sample with an excess of solid sodium borohydride for a few minutes at room temperature prior to dilution.

Liquid chromatography

The LC apparatus was composed of an LKB 2150 high-pressure pump, a Rheodyne 7120 injection valve equipped with a 10-μl loop, a 100 × 2.6 mm I.D. stainless-steel column packed with Nucleosil-100 C$_{18}$ (5 μm), an ISCO V[4] variable-wavelength UV detector and a Waters 740 electronic integrator. Fluorimetric detection was carried out by means of a Shimadzu RF-510 LC spectrofluorimeter equipped with a 10-μl flow cell. In this instance, the mobile phase was delivered by a Waters Model M 45 high-pressure pump.

The mobile phase was acetonitrile (HPLC grade, Rathburn Chemicals, Walkerburn, U.K.)–water (9:1) at a flow-rate of 1.5 ml/min, unless indicated otherwise.

Removal of excess of reagent by thiolate–silica

Preparation of the sorbent. A modification of the method of Pirkle and House[21] was used. Silica (Polygosil 60Å, 63–100 μm; Macherey, Nagel & Co., Düren, F.R.G.) was first treated in boiling toluene for azeotropic removal of water. Then, an excess of 3-mercaptopropyltrimethoxysilane (Serva, Heidelberg, F.R.G.) was added and the mixture kept at *ca.* 100°C for 5 h with stirring. After cooling the thiol–silica was isolated by filtration, washed carefully with toluene and diethyl ether and dried. To activate the sorbent, *i.e.*, to convert it into the sodium thiolate form, it was treated with an excess of saturated sodium carbonate (pH 10.4), filtered and washed succes-

sively with water, acetone and diethyl ether. The capacity of the dry sorbent was *ca.* 300 μmol/g.

Elimination of reagent. Solutions containing different concentrations of bromomethyl reagent and ester in ethylene dichloride were shaken with an excess of the thiolate–silica for 5 min at 50°C. The concentration changes were followed by determination of peak areas after injection of the supernatant into the LC system.

RESULTS AND DISCUSSION

Effect of pH, phase-transfer reagent concentration and temperature on the yield of labelled product

As preliminary experiments showed that the extractive labelling was preferably carried out in a buffer–ethylene dichloride two-phase system, the effect of buffer pH was then investigated. Under the conditions used it was found that the rate of ester formation increased with increasing pH, and that phosphate or borate buffers of pH between 8 and 9.5 were suitable. Control experiments without a phase-transfer reagent yielded almost no ester.

As expected, the rate of the overall reaction increased with increasing concentration of the phase-transfer reagent. However, at a reaction temperature of 80°C and a buffer pH of 8.05, complete derivatization of 10μmol of palmitic acid was obtained after 10 min even when the molar ratio of phase-transfer reagent to acid was reduced from 1:1 to 1:5.

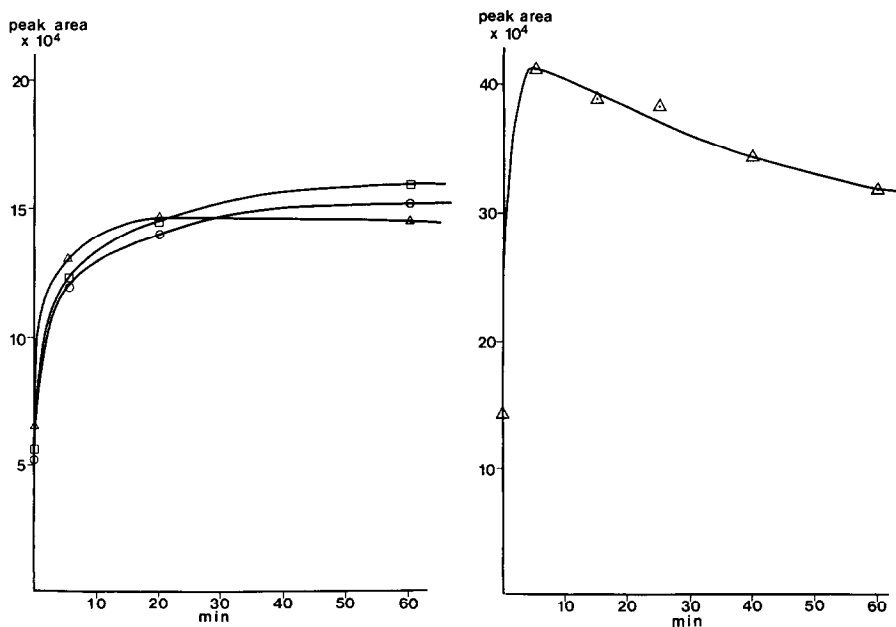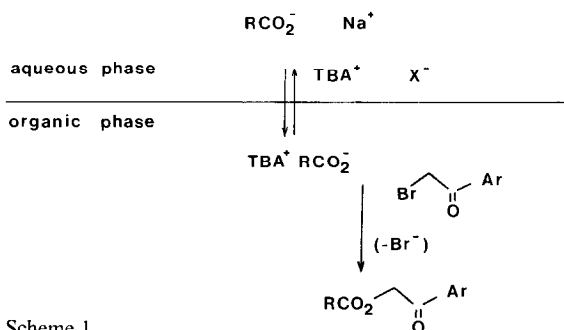Fig. 1. Rate of 2-naphthacyl ester formation at different temperatures. Left: 60°C, ($\triangle$) C_{12}, ($\square$) C_{14}, ($\bigcirc$) C_{16} acids, equimolar mixture of total concentrations 5.0 mM. Right: 100°C, C_{16} acid, 5.0 mM. The aqueous to organic phase volume ratio was 1.5 (see Experimental) and a 100-fold dilution with acetonitrile prior to injection was used.

Kinetic studies of the ester formation at 60 and 100°C showed that the reaction was complete after 60 and 5 min, respectively (Fig. 1). However, a slight decrease in ester concentration is observed on prolonged heating at 100°C. Therefore, a reaction temperature of 80°C was considered optimal and the derivatization was best carried out in a closed reaction vial with efficient mixing by warming for 15 min on a water-bath kept at 80°C.

The concentration decrease found at 100°C is probably due to a competing hydrolysis reaction caused by the alkaline medium. Compared with the rate of ester-ification, however, this hydrolysis rate is slow enough to be tolerated under the deriv-atization conditions suggested here.

Extractive concentration of the analyte from dilute solution

When a series of palmitic acid solutions of decreasing concentration was stud-ied with respect to the formation of 2-naphthacyl esters using the technique described, it was found that quantitative yields of ester were obtained even at phase ratios of buffer to ethylene dichloride as high as 100 or more. Therefore, provided that ade-quate mixing between the phases is ensured, a substantial concentration effect can be achieved. It is obvious that the reaction takes place in the organic phase, where it is highly accelerated owing to the desolvation of the carboxylate anion. The total process is outlined in Scheme 1. Owing to the preference of both the reagent and the ester for the organic phase, the carboxylate anion will be continuously removed from its equilibrium between the phases by its fast reaction in the organic phase.

$$RCO_2^- \quad Na^+$$

aqueous phase $\qquad\qquad TBA^+ \quad X^-$

organic phase

$$TBA^+ \ RCO_2^-$$

$(-Br^-)$

$$RCO_2\diagdown\diagup_O^{Ar}$$

Scheme 1.

The reaction in the organic phase is kinetically of second order and therefore dependent on the actual concentration of the anion (as the TBA ion pair). This is determined by the partition coefficient of the ion pair which, in turn, depends on the lipophilicity of the anion part. Consequently, the derivatization should be slower for the lower homologues in a series. However, from the applications point of view, this effect will be unimportant as long as the reaction conditions are chosen such as to ensure completeness of the reaction.

Thorough investigations of the kinetics of the tetraalkylammonium-mediated alkylation of carboxylic acids in two-phase systems at 25°C have been published[16,18].

Certain other advantages are inherent in the technique. Often, when deriv-atizations of protolytes are carried out in water or mixed aqueous solvents, there is a strong competition between the analyte and hydroxide ions for the reagent. At low analyte concentrations this may lead to substantial by-product formation even if a

pH is chosen to minimize solvolysis of the reagent. Owing to the low extractability of hydroxide ions even as TBA ion pairs into an organic phase, such by-product formation is negligible with the present method.

Elimination of excess of derivatization reagent

Kinetic studies of the nucleophilic substitution reaction between the solid-phase reagent, thiolate–silica, and the derivatization reagent showed the reaction to be fast enough to be useful for convenient and complete removal of the excess of the latter. This very simple procedure consisted in adding an excess of the thiolate–silica to the ethylene dichloride phase after completion of the derivatization reaction, followed by stirring at 50°C for 5 min. Dilution of the supernatant with acetonitrile and injection on to the column gave a reagent-free chromatogram with no loss of ester product.

Because in our system the reagent peak did not interfere with the analytes, reagent peak elimination was not essential. The technique should be most useful, however, when dealing with less retained analytes.

Fluorogenic labelling and sensitivity

The 2-naphthacyl esters possess excellent UV-absorbing properties ($\varepsilon = 1.2 \cdot 10^4$ at 248 nm)[22], but give no useful fluorescence. The sensitivity achieved by UV detection is illustrated in Fig. 2, which shows a chromatogram of the C_{12}, C_{14} and C_{16} acids obtained by the technique described. The detection limit (signal-to-noise ratio = 2) was calculated to be 1–3 pmol. However, for many applications fluorogenic ester derivatives are desirable because of the gain in both sensitivity and selectivity. On attempting to extend the procedure to the use of other aryl bromomethyl ketones
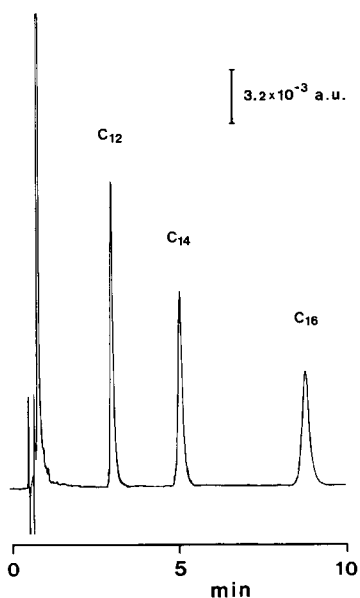

Fig. 2. Chromatogram showing the 2-naphthacyl esters of an equimolar mixture of C_{12}, C_{14} and C_{16} acids. UV detection 235 nm. Each peak represents 167 pmol. Mobile phase: acetonitrile–water (9:1).

as reagents, we found that 9-anthracyl bromide and 2-(α-bromoacetyl) fluorene, both of which are relatively readily available, could not be used with success. Application of the first of these gave only traces of ester derivative. This might be due to a too low reactivity, possibly because of steric hindrance. This assumption is supported by earlier results, showing the failure of this compound to react with amine nucleophiles[23]. Application of the second reagent gave a quantitative yield of the corresponding ester under the conditions given. The fluorescence yield of this product was low, however, owing to the perturbation of the fluorene π-electron system by the conjugated carbonyl group. This effect was demonstrated by reduction of the carbonyl group with sodium borohydride. The ester alcohol produced had excellent fluorescence properties but, unfortunately, two products were obtained from each non-reduced species, thus giving two peaks for each analyte. The result is shown in Fig. 3. The reason for this behaviour has not yet been elucidated.

In order to make efficient use of aracyl esters of the type described, a larger aromatic π-system is desirable. Therefore, the recently described use of 1-bromoacetylpyrene as reagent[11] is of great interest as strong fluorescence (excitation at 360 nm, emission at 450 nm) has been reported for these ester derivatives.

It also seems very reasonable that substituted bromomethylcoumarins[10] and related reagents[13] can be successfully used in this phase-transfer reagent mediated derivatization procedure. Work is in progress to sustantiate this point.
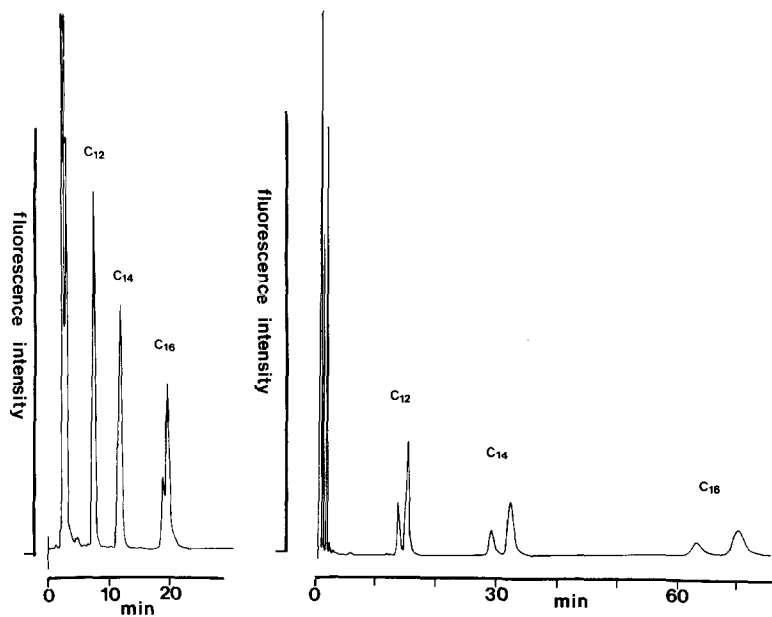

Fig. 3. Chromatograms of an equimolar mixture of C_{12}, C_{14} and C_{16} acids after derivatization with 2-(bromoacetyl)fluorene and reduction with sodium borohydride. Mobile phase: left, acetonitrile–water (9:1); right, acetonitrile–water (7:3). $\lambda_{ex.}$ and $\lambda_{em.}$ = 271 and 313 nm, respectively.

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