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Liquid chromatographic monitoring of pseudocholinesterase activity: comparison of methods

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ABSTRACT

In order to study the activity of pseudocholinesterase *in vitro*, two liquid chromatographic techniques have been developed. One is based on phase transfer-catalyzed (PTC) esterification of the carboxylic acid formed during hydrolysis of the substrate, and the other on the use of a radioisotopically labeled substrate. In both cases, the substrate used was a long-chain choline ester. The PTC method, utilizing (N-9-acridinyl)bromoacetamide as a fluorogenic labeling reagent in an aqueous–organic two-phase system, gives esters with very high fluorescence intensity. The radiochromatographic method makes use of on-line radioactivity monitoring of the substrate and product in order to follow the hydrolysis reaction. In both methods reversed-phase liquid chromatography is used. A method for the synthesis of ^{3}H-labeled choline esters is also described. Both techniques are compared with regard to sensitivity, reproducibility and practical considerations.

INTRODUCTION

Pseudocholinesterase, or butyrylcholine esterase (EC 3.1.1.8), hydrolyzes choline esters and thiocholine esters of varying chain length. The most commonly used methods for the determination of cholinesterase activity, namely titrimetric, electrometric and spectrophotometric methods, have been extensively reviewed by Augustinsson [1]. The substrates used in these methods are short-chain choline esters and thiocholine esters. However, Gomori [2] found that pseudocholinesterase can hydrolyze even long-chain choline esters at different rates, depending on the source of the enzyme. For some time we have been interested in lauroylcholine, which can be successfully used as a substrate for this enzyme (Fig. 1). Lauroylcholine is hydrolyzed at a rate low enough to study the reaction by a chromatographic method, and the technique can further be used for the determination of the enzyme activity. Although lauroylcholine cannot be detected as such, the liberated lauric acid can be derivatized with concomitant introduction of an analytically useful chromophore or fluorophore. Methods of chromatographic determination of carboxylic acids are numerous [3], but only those that are based on precolumn derivatization with UV-absorbing or fluorescent ester groups are useful for this application. A large number of fluorescent esters, as well as their preparation, have been reported [4]. Some of these are based on reactions with aryldiazomethanes [5–7] or with activated halogen compounds [8–16]. In the latter

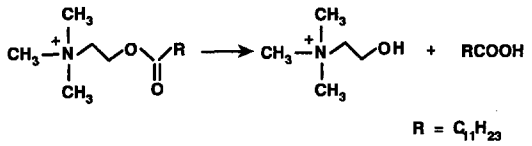

Fig. 1. Hydrolysis reaction of lauroylcholine.

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case, the carboxylic acid must be present as an anion in the reaction medium. This requires transfer of the anion to an organic solvent as an ion pair with a phase-transfer agent. Techniques using a solid potassium carbonate–crown ether–acetonitrile system [7,10,12–16] have been developed. We recently described esterification of carboxylic acids using tetrabutylammonium ions as phase-transfer catalysts (PTC) [17] and found N-(9-acridinyl)bromoacetamide to be a useful reagent for derivatization with the PTC technique [18]. Assuming that the presence of choline ester in the water phase does not influence the derivatization of the acid, this method could be used to follow the enzymatic hydrolysis of lauroylcholine. The method is very sensitive, so even extremely low concentrations of lauric acid (in the femtomole range) can be detected.

An alternative way of studying the reaction is to incorporate an isotope into the substrate molecule and to follow the increasing product concentration and/or decreasing substrate concentration with radioactivity flow detection. The separation of substrate from product is performed on a reversed-phase analytical column. Although hydrolysis reactions of long-chain quaternary ammonium compounds have been successfully followed with this type of method [19], the technique has not been applied to enzyme activity studies. However, it is rather difficult to obtain a specific activity of the labeled compound high enough to permit applications of this method at low substrate concentrations.

In this paper, we describe a fluorimetric and a radiomonitoring liquid chromatographic (LC) method for following the enzymatic hydrolysis of lauroylcholine. The sensitivities, reproducibilities and experimental difficulties are compared. We also describe a method of radioisotope incorporation into lauroylcholine.

EXPERIMENTAL

Chemicals and reagents

2-(N,N-Dimethylamino)ethanol and tetrabutylammonium (TBA) hydrogensulfate were from Fluka (Buchs, Switzerland). Lauric acid and lauroyl chloride were from Janssen Chimica (Beerse, Belgium). Methyl bromide was from BDH (Poole, UK), and [^{3}H]methyl iodide (25 mCi) was from Amersham (Little Chalfont, UK). Butyrylcholine esterase (EC 3.1.1.8), 1000 U, from horse serum was from Sigma (St. Louis, MO, USA). All solvents used were from Merck (Darmstadt, Germany).

The fluorescent reagent, N-(9-acridinyl)bromoacetamide (ABA), was synthesized according to a method described earlier [18]. The reagent was used as a 5 m*M* stock solution in chloroform. The PTC reagent, $TBA^+HSO_4^-$, was used as a 10 m*M* water solution. Lauric acid was stored as a 10 m*M* solution in 95% ethanol. Butyrylcholine esterase was stored in 50 m*M* phosphate buffer, pH 8.0, at −70°C as 200-μl samples containing 1 U each. ^{3}H-Labeled lauroylcholine bromide was synthesized as described below and used as a 2 m*M* stock solution in water. The unlabeled ester was used as a 10 m*M* solution in water.

Synthesis of ^{3}H-labeled lauroylcholine bromide.

The synthetic route is given in Fig. 2. Lauroyl chloride (23.1 ml, 100 mmol) was dissolved in 200 ml of dry diethyl ether and added dropwise under stirring to a solution of 10.3 ml (100 mmol) of dimethylaminoethanol in 150 ml of dry diethyl ether. When the addition was complete, the precipitate, 2-(N,N-dimethylamino)ethyl laurate hydrochloride, was filtered off, washed with diethyl ether and then recrystallized from warm ethyl acetate. The amount obtained was 26.8 g (87.3%). This product was further used for the synthesis of both labeled and unlabeled lauroylcholine bromide.

The amino ester hydrochloride (1 g, 3.25

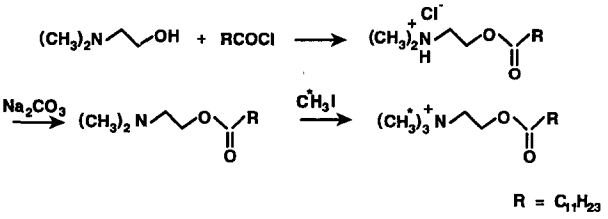

Fig. 2. Synthesis of radiolabeled choline esters.

mmol) was dissolved in 50 ml of water, and 15 ml of 1.5 *M* Na_2CO_3 were added to liberate the free base. The solution was then transferred to a separatory funnel and extracted twice with diethyl ether, 50 and 10 ml, respectively. The combined ether phases were dried with $MgSO_4$ and filtered off.

A 2.5-ml portion of the ether phase containing the amino ester was transferred to a glass vial with a screw cap. Then, 150 μl of a toluene solution of [^{3}H]methyl iodide (25 mCi in 13.3 ml of toluene) were added, and the vial was thoroughly closed and left for 2 h at room temperature. An excess of methyl bromide was then added and the mixture left for three days. The precipitate was filtered off and washed with diethyl ether, giving 13.5 mg. The specific activity of this product was determined to be $4.5 \cdot 10^7$ dpm/mg, which gave a radiochemical yield of nearly 100%. The radiochemical purity of the product was verified by liquid chromatography (HPLC), which showed the isolated product to be >95% pure.

Synthesis of unlabeled lauroylcholine bromide

A modification of the above procedure was used on a three times larger scale. Extraction of the free amino ester was performed with ethyl acetate. Only the excess of methyl bromide was added and the mixture was kept at 37°C for 1 h, and then at room temperature overnight. The product was recrystallized from warm ethyl acetate. An 98% yield was obtained.

Apparatus

System A. The LC–fluorimetric system consisted of an LKB Model 2150 constant-flow pump, a Carnegie Model CMA/200 autoinjector and a Shimadzu RF-535 fluorescence LC monitor coupled to a Shimadzu C-R5A Chromatopac electronic integrator–recorder. A 20-μl injector loop was used, and the separation was carried out on a 150 mm × 4.6 mm I.D. analytical column packed with 7-μm Kromasil C_{18} (Eka Nobel, Surte, Sweden).

System B. The radiochromatographic instrumentation consisted of an LKB Model 2150/2152 dual-pump gradient solvent delivery system, a Rheodyne 7125 injector valve with a 100-μl loop, an analytical column, 150 mm × 4.6 mm I.D., packed with 7-μm Nucleosil C_{18} (Macherey-Nagel, Düren, Germany) and a Radiomatic A-250 radioactivity monitor including a computerized data reduction system, video monitor and an Epson LQ-800 printer unit. The detector was equipped with a 500-μl liquid scintillation cell. The scintillation medium used was Ready-Flow III (Beckman Instruments, Fullerton, CA, USA).

Chromatography

System A. The mobile phase used was 55% acetonitrile and 10% methanol in water with phosphoric acid added to 0.2%. Detection was performed at 357.5/482 nm using a fixed 13-nm bandwidth for both the excitation and emission wavelengths. The flow-rate was 1.0 ml/min.

System B. The mobile phase used was 40% acetonitrile in water adjusted to pH 3.2 with hydrochloric acid, at a flow-rate of 1.0 ml/min. The scintillator fluid/mobile phase flow ratio was 4:1.

Derivatization procedure

The procedure described in ref. 18 and shown in Fig. 3 was modified at some points. The reaction was carried out in small glass vials equipped with septa, and was run in 20 m*M* phosphate buffer, pH 7.2. The stock solution of lauric acid and/or lauroylcholine bromide was diluted with buffer concentrations from 5 to 100 μ*M*. To each vial typically 100 μl of the analyte solution, 10 μl of the PTC reagent and 100 μl of the ABA reagent were added. The vials were closed and placed in a water bath at 80°C for 15 min, on a magnetic stirrer. After cooling, a 10-μl sample was transferred from each vial to a small glass tube, the chloroform was evaporated by application of vacuum and then the sample was diluted with 100 μl of the mobile phase.

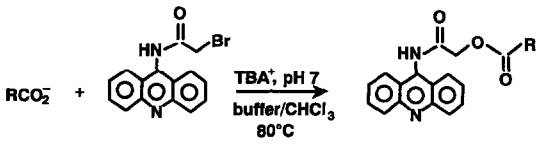

Fig. 3. Derivatization reaction used.

Kinetic studies

The lauroylcholine bromide solution was diluted with 20 m*M* phosphate buffer, pH 7.2, to a concentration of 100 μM and volume of 990 μl. The sample was then kept at 37°C in a thermostat. A 90-μl sample was transferred (t_0) to a vial containing 10 μl of TBA solution. A 100-μl volume of the enzyme solution (0.5 U) was added to the reaction mixture. Samples of 100 μl were transferred at different time points to vials each containing 10 μl of TBA solution in order to stop the reaction. For studying the hydrolysis of the radiolabeled substrate, the samples were diluted with 90 μl of acetonitrile and injected into HPLC system B. In the study of the formation of lauric acid from the unlabeled substrate, 100 μl of the ABA solution were added to each sample, and the derivatization procedure was performed as described above.

RESULTS AND DISCUSSION

Since use of unlabeled substrate requires derivatization of the liberated lauric acid, a prerequisite for a successful application of the technique is that the ester substrate is not hydrolyzed and that the results are not in any other way influenced by the presence of the substrate.

First, the linearity of the calibration curve, obtained from a series of varying concentrations of lauric acid, was investigated and the result is shown in Fig. 4. The calculated correlation coefficient (r^2) is 0.998. The result is in good agreement with our earlier reported data [18]. Next, the possible influence of the substrate was studied by using a fixed acid concentration (50 μM) and varying the concentration of added ester (0–100 μM). As shown in Fig. 5, the substrate does not influence the lauric acid determination in any way (slope = 0.00022, mean value = 50.25 ± 0.44 μM).

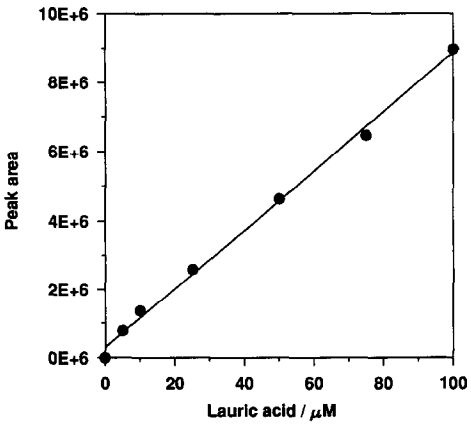

Fig. 4. Calibration curve showing the linear concentration dependence obtained.

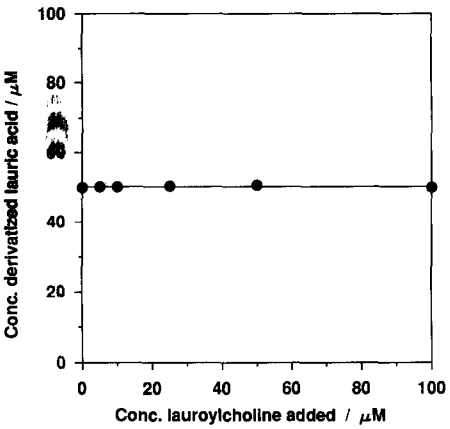

Fig. 5. Plot showing the lack of substrate hydrolysis during the derivatization reaction.

Chromatographic performance and sensitivity.

The difference in chromatographic output from the two techniques, when applied to the monitoring of enzyme activity, is shown in Fig. 6. While the radiochromatogram (Fig. 6a) gives both substrate and product concentrations, fluorescence detection (Fig. 6b) of the derivatized product yields a higher column efficiency and thereby an improved detectability. In the radiochromatographic method, the limit of detection is primarily determined by the specific activity of the substrate. The detection limit can therefore be made very low if the specific activity is increased, which, on the other hand, has certain disadvantages associated with the protection needed during synthesis and sample handling. With the specific activity used in this study, the limit of detection (signal-to-noise ratio = 2) was estimated to be *ca.* 20 pmol of substrate. On a concentration

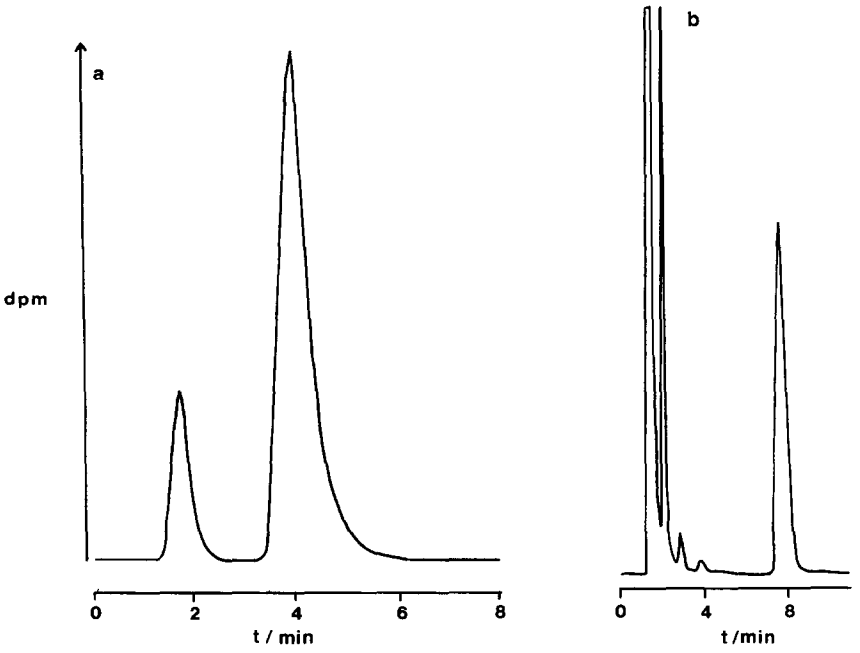

Fig. 6. Chromatograms of samples taken from the enzymatic hydrolysis of lauroylcholine. (a) Radioactivity monitoring (elution order: choline, lauroylcholine); (b) fluorescence detection after derivatization with ABA [lauric acid (as derivative) has a retention time of ≈ 8 min].

basis this means that with the method used and assuming that the product (choline) should be determined at 10% conversion of the substrate with a signal-to-noise ratio ≥ 5, the substrate concentration used in the reaction should be 10 μM or higher.

For the fluorescent ester of lauric acid, a detection limit (signal-to-noise ratio = 2) of 10 fmol has been found previously [18] under the same conditions as used here. A similar calculation gives as a result that a substrate concentration ≥ 0.125 μM is needed in this case using the same assumptions as above.

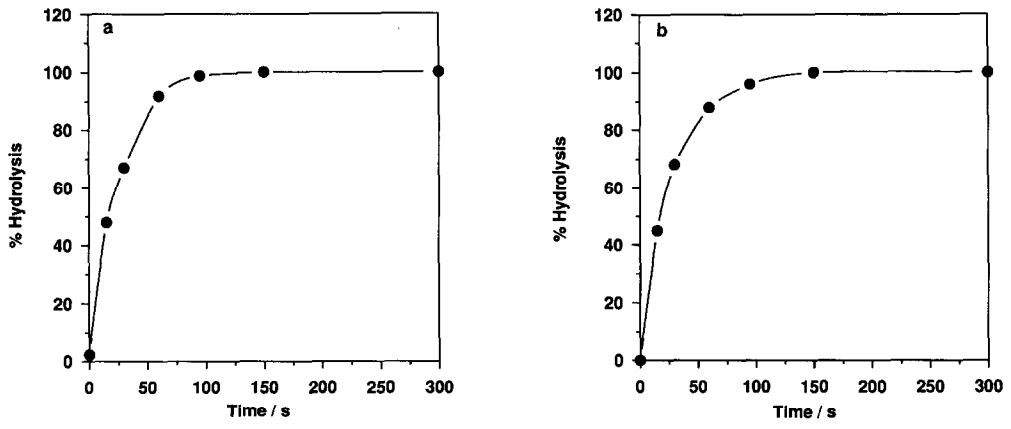

Fig. 7. Time course of the enzymatic reaction as studied by the two different methods under otherwise identical conditions. (a) Via the use of labeled substrate; (b) via PTC-induced fluorescent derivatization of the acid formed. Reaction conditions used: 93 μM substrate, 0.5 U enzyme, 37°C.

Enzyme kinetic monitoring

Pseudocholinesterase is known to be active towards a wider range of acylcholines [20], but detailed, mechanistic studies on the higher amphiphilic esters are lacking in the literature. We have searched for a method suitable for these higher esters, taking lauroylcholine as representative substrate. The kinetic information that can be obtained via sampling from the reaction mixture for subsequent analysis by LC is demonstrated in Fig. 7, which also shows the excellent agreement between the two different methods used. Conditions were selected under which the hydrolysis is essentially complete within a couple of minutes, in order to demonstrate that even relatively fast kinetics can be adequately handled by both techniques.

The results show that the derivatization conditions used do not cause any changes in the composition of the sample taken from the enzymatic reaction. In conclusion, the derivatization method can be used as a favourable substitute for radioisotope monitoring, since it can be easily performed and does not require the expensive instrumentation necessary for β-emission counting.

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