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A continuous fluorimetric method to monitor the enzymatic hydrolysis of medicinal esters

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Abstract

This paper describes a new spectrofluorimetric assay for continuously monitoring the enzymatic hydrolysis of medicinal esters. The procedure is based on the stoichiometric quantity of protons generated by the hydrolysis of the substrate, which produces changes in the fluorescence of a pH-sensitive dye. The pH indicator, 2′, 7′-bis(carboxyethyl)-5(6)-carboxyfluorescein, was selected due to its favourable pK_a for studies under physiological conditions. Moreover, the presence of a domain in the spectra (< 442 nm) where fluorescence intensities are independent of pH allows measurements of wavelength ratios that cancel artifacts and lower sample-to-sample variability. The indicator did not affect the catalytic activity of purified hog liver carboxylesterase or human serum albumin. This assay is easy to perform and appears to be especially useful for studying enzymatic reactions with half-lives of the order of minutes or hours.

Keywords: BCECF; Esters; Hog liver carboxylesterase; Human serum albumin; Hydrolysis; pH indicator; Prodrugs; Spectrofluorimetric assay

1. Introduction

A large number of drugs and prodrugs contain an ester linkage in their molecular structure. As a consequence of having such a metabolically-labile moiety, hydrolytic cleavage is often found to be a major route in the biotransformation of such compounds. Thus for both active ester drugs and inactive ester prodrugs, a good understanding of their rates of hydrolysis under a variety of conditions is a prerequisite for the development and rationalization of therapy.

Medicinal esters are cleaved chemically and/or enzymatically by hydrolases or proteins displaying an esterase-like activity (e.g. human serum albumin, HSA). While in-vitro studies with isolated enzymes can only yield partial information on the fate of ester prodrugs or drugs, they are nevertheless indispensable when investigating structure–hydrolysis relationships and designing such therapeutic agents. In order to facilitate the experimental screening of therapeutic esters, there is a need for a simple, accurate, sensitive and reliable method applicable under a variety of hydrolytic conditions (non-enzymatic or enzymatic) and to structurally diverse substrates.

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Several authors have studied the hydrolysis of a variety of esters, for example esters of indomethacin [1], nipecotic acid [2], hydrocortisone [3], metronidazole [4] or nicotinic acid [5,6], using a chromatographic method. The disadvantages of these discontinuous assays are the difficulty of following fast kinetics, the necessity to optimize the analytical conditions for each ester, and a danger of error accumulation during sample work-up, e.g. extraction, evaporation and centrifugation. Accurate measurements of the initial rate of hydrolysis can be performed more rapidly and conveniently by monitoring the progress of the reaction in a "continuous" manner, i.e. without sample work-up and at very short time intervals. Spectrophotometric methods can be used conveniently when the reaction is accompanied by a marked change in the spectrum, but this condition is often not fulfilled. Restricting the study to chromogenic substrates [7–11] can lead to results not generalizable to common substrates (the so-called "*p*-nitrophenyl ester syndrome" [12]). Another approach is to use a chromogenic indicator. This is the method described here, which can be used to monitor any reaction involving the release or uptake of protons. The reaction catalyzed by an esterase can be represented by the following equation:

$$RCOOR' + H_2O \rightarrow R'OH + RCOO^- + H^+ \quad (1)$$

When the analysis is carried out at a pH considerably higher than the pK_a of the acid liberated, the hydrolysis of the substrate results in stoichiometric production of protons which can be measured by a pH indicator present in the reaction mixture. For maximum sensitivity the pK_a of the dye should be close to the pH of the assay. Furthermore, the pH variation should remain small enough to avoid influencing detectably the rates of chemical and enzymatic hydrolysis. A preliminary knowledge of enzyme activity versus pH allows the tolerable change in pH to be defined. For years, phenol red [13,14], neutral red or *p*-nitrophenol [15] have been used as pH indicators in combination with spectrophotometric measurement. The main limitation of these indicators is their putative interaction with proteins which may inhibit enzyme activity [15,16]. Here, the successful use and validation of a spectrofluorimetric pH indicator, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), is reported. The higher sensitivity of the fluorescence assay allows dye concentrations to be minimized and renders enzyme–indicator interactions negligible, should they occur at all. BCECF has a suitable pK_a of 6.97 [17] and exhibits a linear relationship between fluorescence and pH, at least in the convenient pH range of 6.0–7.8.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of analytical grade, and the solutions were prepared with demineralized and purified water obtained with the system Seralpur Pro 90 C (Seral, E. Renggli AG., Rotkreuz, Switzerland).

The buffer MPS (morpholinopropanesulfonic acid) was obtained from Merck AG (Darmstadt, Germany). The phosphate buffer components, dimethylsulfoxide (DMSO) and acetylcholine chloride purum were purchased from Fluka AG (Buchs, Switzerland). Acetonitrile (MeCN) was of HPLC grade and obtained from Romil Chemicals (Loughborough, UK). Ethyl nicotinate was purchased from Fluka, 2-butoxyethyl nicotinate was kindly donated by Dr. Karl Thomae GmbH (Biberach an der Riss, Germany), and *n*-propyl, *n*-butyl and 2-methoxyethyl nicotinates were synthesized in this laboratory according to known methods [18].

BCECF came from Calbiochem Co. (La Jolla, CA). An aqueous solution (10 μg ml^{-1}) was prepared and kept at 4°C in the dark.

Essentially fatty-acid-free HSA (quality A-1887, lot 118F9311) was obtained from Sigma Chemical Co. (St Louis, MO). A standard solution (33.5 mg ml^{-1}) was prepared in phosphate buffer [19] (pH 7.4 ± 0.1; 6.1 mM), divided into portions of 0.9 ml and kept at −80°C, so that the same protein solution was used for all experiments.

Purified hog liver carboxylesterase (EC 3.1.1.1) was purchased from Boehringer (Mannheim, Germany) and stored at 4°C.

2.2. Equipment

Fluorescence was monitored with a Perkin-Elmer luminescence spectrometer LS 50B (Perkin-Elmer Ltd., UK). All measurements were made using a standard 1 cm quartz cell (111 QS, Hellma Swiss SA, Basle, Switzerland), sealed with a stopper. The cell was maintained at 37 ± 0.2°C with a water-bath circulator (Haake D8, Digitana, Lausanne, Switzerland). The fluorescence was recorded with the FL Data Manager (Perkin-Elmer Ltd., England). The Excel 5.0 software (Microsoft Co.) was used for the data processing and GraphPad Prism 1.03 (GraphPad Software, Sorrento Valley, San Diego, CA) software was used as a convenient graphical package. Fluorescence is given without spectral correction. A Metrohm 654 pH-meter (Buchs, Switzerland) was used for all pH measurements.

2.3. Calibration procedure

Fig. 1 shows the excitation spectra of BCECF at different pH values in a calibration solution comprising the pH indicator, the enzyme, different concentrations of HCl corresponding to 0, 10, 20, 30, 40 and 50% of hydrolysis, and the possible co-solvent. These spectra had similar shapes and their amplitudes depended only on pH. Lowering the pH decreased the fluorescence at 505 nm, with a spectral domain (< 442 nm) not being affected by pH variations.

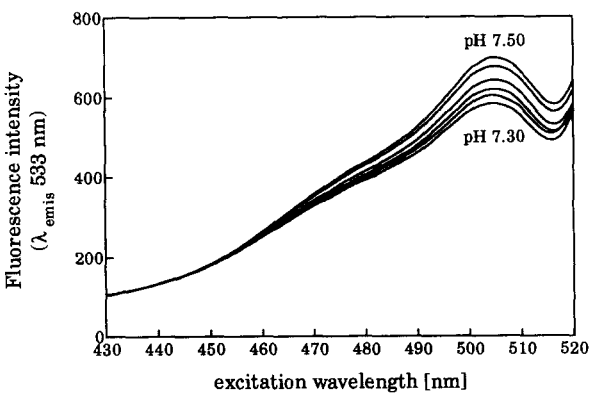

Fig. 1. Excitation spectra of 96 nM BCECF in phosphate buffers (6.1 mM) of pH 7.50 (highest fluorescence intensity), 7.46, 7.42, 7.38, 7.34 and 7.30 (lowest fluorescence intensity). The solution also contained 10 μM HSA and DMSO 1%. Emission was measured at 533 nm.

Fluorescence intensity was measured alternately at 505 and 442 nm, with 533 nm emission and slit widths of 10 and 15 nm respectively. The fluorescence excitation ratio (F_{505}/F_{442}) was calculated as the mean of two 505 nm excitation measurements divided by the mean of two 442 nm excitation measurements. However, the ratio was calculated from one measurement at each wavelength when the pH changed rapidly. All fluorescence intensities were corrected by subtracting background before calculating ratios. The use of the fluorescence excitation ratio provides a measurement that is unaffected by changes in the dye concentration or illumination intensity [20].

The ratio of fluorescences of BCECF (F_{505}/F_{442}) varies linearly in the pH range 6.0–7.8. Since the pK_a values of the dye and buffer are similar, the change in fluorescence (F_{505}/F_{442}) is related to the change in proton concentration by a factor Q [13]:

$$\frac{d(F_{505}/F_{442})}{dt} = \frac{d(F_{505}/F_{442})}{d[H^+]} \cdot \frac{d[H^+]}{dt} = \frac{1}{Q} \cdot \frac{d[H^+]}{dt} \quad (2)$$

The factor Q is constant in the pH range of linearity and depends on the difference between the pK_a values of dye and buffer. It can be determined by a calibration curve using HCl. The fluorescence ratio of BCECF can then be converted to pH values in the pH range 4–9 by the Henderson–Hasselbalch equation (Eq. (3)) [21], taking the pK_a to be 6.97 [17]:

$$pH = pK_a - \log\left[\frac{(R_{max} - R)}{(R - R_{min})} \cdot \frac{F_{base442}}{F_{acid442}}\right] \quad (3)$$

Here, R is the ratio of the fluorescence (F_{505}/F_{442}) when emission is measured at 533 nm, and R_{max} and R_{min} are the ratios (F_{505}/F_{442}) when the fluorescence is at its maximum under alkaline and acidic conditions respectively. $F_{base442}$ and $F_{acid442}$ are the fluorescence intensities at 442 nm under basic and acidic conditions to obtain R_{max} and R_{min} respectively. Experimentally, this approach is feasible because R_{max} and R_{min} are constant over a broad range.

The sensitivity of the method is influenced by the buffer capacity of the reaction mixture, and is

limited by the susceptibility of an "unbuffered" system to such external factors as CO_2 absorption. The buffer concentration was chosen so that 50% reaction would correspond to a drop in pH of 0.20 units.

2.4. Kinetic studies with purified hog liver carboxylesterase

Depending on the activity towards a given substrate, several dilutions of the carboxylesterase were prepared to allow measurement of the initial rate. The enzyme (4.17×10^{-4}–16.67×10^{-4} mg ml^{-1}) and the pH indicator BCECF (96 nM) were incubated for 60 min, at 37 ± 0.2°C in MPS buffer (pH 7.4 ± 0.1; 1–25 mM; ionic strength = 0.163 adjusted with KCl). Triplicates of six concentrations of each substrate (final concentrations in the reaction mixture ranged from 0.0875–7.0 mM) were dissolved in acetonitrile. Enzymatic reactions were initiated by the addition of the ester solution (30 μl) to the enzyme solution, so as to limit to 1% (v/v) the amount of organic solvent in the assay. The temperature was 37 ± 0.2°C. Chemical hydrolyses were performed under the same experimental conditions, replacing the enzyme solution with MPS buffer. At appropriate time intervals determined by the rate of reaction, fluorescence intensities (emission at 533 nm; excitation at 505 and 442 nm, slits of 10 nm × 15 nm) were recorded (about 20 points for very fast reactions, and over 100 points for slow reactions). The method is considered continuous in that it always uses the same sample and monitors its fluorescence at discrete but small time intervals.

2.5. Kinetic studies with HSA

Triplicate samples of HSA (3.35 mg ml^{-1}) were preincubated with BCECF (96 nM) in phosphate buffer [19] (pH 7.4 ± 0.1; 6.1 mM; ionic strength = 0.173 adjusted with KCl) at 37 ± 0.2°C for 60 min. Hydrolysis was started by the addition of a phosphate buffer solution of the ester (30 μl). The final concentration of substrate in the reaction mixture was 1 mM. The poorly soluble substrates were added to the reaction medium as a DMSO solution, but final co-solvent concentration was limited to 1% (v/v). The chemical hydrolyses were performed under the same experimental conditions, replacing the HSA solution with a phosphate buffer. The fluorescence intensities were recorded as described for the kinetic studies with carboxylesterase.

2.6. Kinetic analyses

The pseudo-first-order rate constants (min^{-1}) were obtained by linear regression from the semilogarithmic plot of the decay of C_t versus time, where C_t is the substrate concentration at time t. The velocity (mmol min^{-1} mg^{-1} protein) was determined by linear regression from the initial linear part of the plot of the decay of C_t versus time. The standard error of the regression slope in a single hydrolysis experiment was about 0.5–2%. The kinetic parameters V_{max} and K_m were calculated by iterative non-linear optimization using the equation of a rectangular hyperbola corresponding to Michaelis–Menten kinetics.

The fluorescence ratio of BCECF in the reaction medium did not tend towards a fixed value at infinite time, but rather drifted at a constant rate which became non-negligible for very slow enzymatic or chemical hydrolyses. Data analysis took this drift into account.

3. Results and discussion

3.1. Selectivity and precision

Since many biological macromolecules such as albumin show a strong fluorescence at excitation wavelengths below 360 nm, it is advantageous to use an indicator such as BCECF whose excitation and emission maxima in a phosphate buffer (pH 7.4 ± 0.1; 6.1 mM; ionic strength = 0.173) are at 505 and 533 nm respectively. No shift was observed when HSA or a co-solvent (e.g. DMSO or MeCN) was added. At these wavelengths, the fluorescence of the substrates, enzymes and other component (e.g. co-solvents) was negligible, rendering the measurements sensitive and specific. The precision of the method when determining initial rate constants (three replicates) expressed

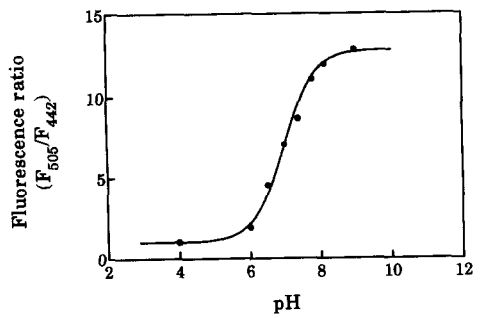

Fig. 2. Fluorescence ratios (F_{505}/F_{442}) of BCECF as a function of pH (4–9) in 6.1 mM phosphate buffer, 50 μM HSA, 1% DMSO. Emission was measured at 533 nm.

as RSD was 0.4–2.5% (for $t_{1/2} < 0.5$ h) and 0.4–5.7% (for $t_{1/2} < 8$ h).

3.2. Stability of the pH indicator

To evaluate the effects of the reaction components on the stability of BCECF, its fluorescence ratio was monitored as a function of time. Under the conditions of this study, BCECF fluorescence decreased with pseudo-first-order kinetics, the half-life depending on such experimental conditions as concentrations of enzyme and co-solvent. For example, under the conditions used to study chemical hydrolysis, the $t_{1/2}$ was 400 ± 30 h (1% DMSO); under the conditions used to study enzymatic hydrolysis, the $t_{1/2}$ was 85 ± 3.6 h (50 μM HSA, 1% DMSO). The chemical nature of this decrease in fluorescence is unknown, but it has no influence on pH, as verified potentiometrically. It was thus easy to calculate the true rate of hydrolysis by correcting for the decrease in fluorescence of the indicator. It was estimated that the minimum rate of reaction that can be determined with confidence must be twice as fast as the degradation of the indicator, but this implies a rate of hydrolysis too slow to be monitored meaningfully by a continuous assay.

3.3. Fluorescence ratio of BCECF as a function of pH

Fig. 2 shows the fluorescence ratio (F_{505}/F_{442}) of BCECF in the pH range 4–9. Under the present experimental conditions, the ratio tends to a value of 1 below pH 4, and to a value of 13 above pH 9. The theoretical curve was calculated from a simplified form of the Henderson–Hasselbalch equation using a pK_a value of 6.97. In fact, the ratio $F_{base442}/F_{acid442}$ is approximately equal to 1, since the fluorescence intensity at this wavelength is independent of pH. The linear relationship between fluorescence ratio and pH over the pH range 6.0–7.8 was verified ($r^2 = 0.998$; $n = 5$).

3.4. Hydrolytic activities of a purified hog liver carboxylesterase

Five nicotinate esters (Table 1) were chosen among a large set of analogues already investigated in this laboratory [22]. The criterion of selection was a large variety and a regular distribution in their V_{max}/K_m ratios as measured by an HPLC method [5]. Fig. 3 shows an example of Michaelis–Menten kinetics as obtained with the present spectrofluorimetric assay. The kinetic parameters V_{max} and K_m of these esters in the presence of purified hog liver carboxylesterase were calculated from the Michaelis–Menten rectangular hyperbola and are reported in Table 1 together with the values previously obtained by an HPLC method [5]. The correlation between the V_{max}/K_m ratios obtained by the two methods is excellent ($r^2 = 0.998$; $n = 5$; slope $= 1.24 \pm 0.03$; intercept $= -17 \pm 2$). The fact that the slope and intercept are not equal to 1 and 0 respectively, can be explained by minor differences in the experimental conditions, and mainly because the enzyme preparation was not from the same batch. Thus the present spectrofluorimetric method appears to yield results very similar to those obtained by a traditional, more time-consuming, discontinuous chromatographic method.

3.5. Hydrolysis of esters catalyzed by HSA

A number of authors have reported that HSA possesses esterase-like activity towards esters [11,23,24], amides [25], and phosphates [26]. It is also well established that HSA has a very broad capacity to bing reversibly a very large number of compounds [27–30]. In order to use a pH indicator to study the esterase-like activity of HSA, it

Table 1
Kinetic parameters of nicotinate esters in the presence of purified carboxylesterase

Nicotinate	Spectrofluorimetry			HPLC		
	$V_{max} \pm SD$ (mmol min^{-1} per mg protein)	$K_m \pm SD$ (mmol l^{-1})	V_{max}/K_m	$V_{max} \pm SD$ (mmol min^{-1} per mg protein)	$K_m \pm SD$ (mmol l^{-1})	V_{max}/K_m
Ethyl	28 ± 2.3	0.6 ± 0.13	50	18.4 ± 0.4	0.353 ± 0.024	52
n-Propyl	35 ± 4.1	0.4 ± 0.11	90	29.8 ± 0.8	0.346 ± 0.028	86
n-Butyl	75 ± 3.9	0.62 ± 0.081	120	70.6 ± 4.2	0.633 ± 0.087	110
2-Methoxyethyl	45 ± 2.6	1.7 ± 0.32	26	42.4 ± 3.6	1.16 ± 0.16	37
2-Butoxyethyl	31 ± 1.4	0.57 ± 0.070	54	87.2 ± 17.2	1.52 ± 0.49	57

must first be ascertained that the latter does not bind the former. It has been suggested that phenol red could be used as a spectrophotometric pH indicator with any enzyme whose catalysis produces or consumes protons [13]. However, this suggestion is invalidated by the fact that phenol red binds to HSA [31,32]. Furthermore, the low sensitivity of the spectrophotometric technique requires concentrations of the pH indicator that are sufficiently high to inhibit the enzyme.

In contrast, the spectrofluorimetric method allows the dye concentrations to be markedly reduced and consequently minimizes its interactions with the enzyme. Indeed, the fluorescence spectra of BCECF as a function of HSA concentration (0–100 μM) showed no shift of the excitation peak, only a decrease of the peak intensity that can be explained by a quenching phenomenon and the inner filter effect. Also, the initial rate constants of hydrolysis of acetylcholine catalyzed by HSA (30 μM) did not change significantly as a function of BCECF concentration (9.6–192 nM). Finally, fluorescence intensity measurements with different phosphate buffer concentrations (1–67 mM) showed that the amplitude of the change in fluorescence depends on buffer capacity. These criteria have already been used [33] to verify that no binding occurs between the pH indicator and the enzyme.

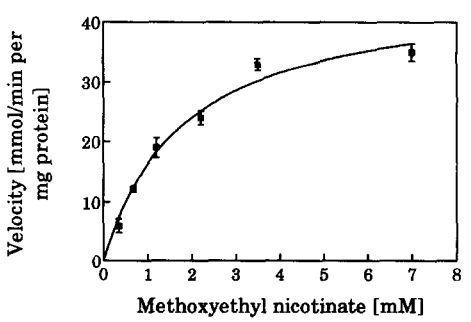

Fig. 3. Velocity of hydrolysis of methoxyethyl nicotinate as a function of substrate concentration, as catalyzed by purified hog liver carboxylesterase (16.67×10^{-4} mg ml^{-1}).

4. Conclusions

The proposed continuous spectrophotometric method is sensitive, specific, simple and easy to perform. The use of ratio measurements cancels many artifacts associated with sample-to-sample variability. The method does not require any extraction or separation optimization, and a calibration with HCl is sufficient. Enzymatic hydrolyses can be monitored with a large variety of substrates even when neither substrate nor product is detectable by UV photometry of fluorescence. The "*p*-nitrophenyl ester syndrome", rightly critized by Menger and Ladika [12], can thus be avoided.

Michaelis–Menten parameters or initial rate constants can be determined in the presence of an esterase or even HSA since no indicator–protein interaction seems to occur. The method is applicable to reactions with half-lives of the order of minutes or hours, but it could be adapted to rapid reaction kinetics, its resolution factor allowing "stopped-flow" experiments [34]. However, one limitation of the method is with very slow hydrol-

ysis reactions, be they enzymatic or non-enzymatic, since the fluorescence intensity decreases slowly with time. In this case, a discontinuous assay appears preferable. Work is now in progress to study the hydrolytic activity of HSA using the present spectrofluorimetric method.

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