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p-Nitrophenyllaurate: a substrate for the high-performance liquid chromatographic determination of lipase activity

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ABSTRACT

Many assay procedures have been devised to measure lipolytic activity, but none is without problems. It is for this reason that new methods are still being proposed. In this work we have investigated the use of two esters of *p*-nitrophenol, the palmitic acid and lauric acid esters, as substrates for a highly sensitive high-performance liquid chromatographic method. Data on recovery, specific activity and reproducibility are reported only for the lauric ester, because the palmitic ester turned out to be a very poor substrate.

INTRODUCTION

Lipases or acylglycerol acylhydrolases (EC 3.1.1.3) are esterases hydrolyzing esters of glycerol with long-chain aliphatic acids. They are enzymes found widely in nature [1]. Lipases of different origin show different affinities for tri-, di- and monoglycerides [2]; they may also hydrolyze esters of other aliphatic alcohols [3] with different specificity for the acyl chain length [4]. Many assay procedures have been devised to determine lipolytic activity in pharmaceutical and industrial enzyme preparations and in biological liquids, but each one presents some kind of problem. Therefore new methods are still being proposed with the hope of finding a generally applicable method that will overcome the currently experienced problems.

Although olive oil is the most commonly used natural substrate for lipases, many other esters and triglycerides, natural and synthetic, have been utilized [5]. The volumetric method is the one most used for titrating the fatty acids produced from olive oil [6] or triolein hydrolysis [7]. Fatty acids can be also converted to Cu soaps, which are measured spectrophotometrically [8].

Several methods of analysis have been proposed in the hope of improving sensitivity or ease of operation. As regards chromogenic substrates, esters of various aromatic alcohols have been used in the spectrophotometric method, but

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the sensitivity is generally quite low. Among these, *p*-nitrophenyl acetate has been used because of its good solubility [9], but it has proved to be unstable and the acidic chain is too short for it to be a specific substrate for lipase activity (also hydrolyzed by esterases) [10]. Because high-performance liquid chromatography (HPLC) is known to be a very sensitive technique, in previous research we used this method to quantify β-naphthol liberated by the action of pancreatic lipase on the substrate β-naphthyllaurate [11]. We have now examined the possibility of finding a more specific substrate to use for HPLC determinations. Two esters of *p*-nitrophenol, the palmitic acid and lauric acid esters, were tested. Data on recovery, specific activity and reproducibility are reported only for the lauric ester because the palmitic ester proved to be a very poor substrate (it had about half the activity of the lauric ester under the same conditions).

EXPERIMENTAL

Materials

A commercial pharmaceutical pancreatin preparation (porcine pancreas lyophilized extract), supplied by Menarini (Florence, Italy), was used as the lipase source. A reference standard lipase preparation was supplied by Federation Internationale Pharmaceutique (F.I.P.), Centre for Standards, State University of Ghent (Ghent, Belgium). The declared activity was 36 F.I.P. units per milligram. Thaurocholic acid sodium salt was from Fluka (Buchs, Switzerland) and *p*-nitrophenyllaurate, *p*-nitrophenylpalmitate, *p*-nitrophenol and 2,4-dinitroaniline were from Sigma (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile was supplied by Hoechst-Riedel de Haen (Milan, Italy), and sodium chloride, hydrochloric acid, sodium dihydrogenphosphate, all analytical grade, by Carlo Erba (Milan, Italy). All reagents were used without further purification. The acetonitrile and water were filtered under vacuum through 0.45-μm Millipore filters.

Instruments

Analyses were performed with a Perkin-Elmer Series 4 high-performance liquid chromatograph, equipped with a Rheodyne Model 7125 injector with a 20-μl loop and connected to a variable-wavelength Perkin-Elmer Model LC 75 UV detector. A 250 mm × 4.6 mm I.D. column packed with 10 μm particle size LiChrosorb RP 18 (Perkin-Elmer) was used. Chromatograms were recorded on a strip-chart recorder.

HPLC conditions

Analyses were carried out at room temperature. The flow-rate was 1.5 ml/min. The mobile phase was a 55:45 (v/v) mixture of water and acetonitrile. The detection wavelength was 300 nm and the detector sensitivity 0.02 0.08 a.u.f.s. The chart speed was 0.5 cm/min.

Standard solutions

Two internal standard stock solutions of 2,4-dinitroaniline in ethanol were used: 0.2 mg/ml for the samples and 0.15 mg/ml for the reference standard solution. The reference standard solution was obtained as follows: 100 μl of a 0.3 mg/ml β-nitrophenol solution, 1 ml of a 0.15 mg/ml 2,4-dinitroanaline solution and 0.625 ml of 5 *M* HCl were added to 5 ml of 0.2 *M* phosphate buffer (pH 7.4) and made up to a final volume of 10 ml with water.

Substrate suspension

p-Nitrophenyllaurate or *p*-nitrophenylpalmitate (8 mg) was dissolved in 10 ml of acetone and added, through a submerged pipette, into an agitated solution containing 50 ml of 0.2 *M* phosphate buffer at pH 7.4, 10 ml of 25 m*M* sodium thaurocholate, 10 ml of 70 m*M* NaCl and 20 ml of water.

Enzymatic assay

Incubation was performed at 37°C for 30 min in a final volume of 1.4 ml containing 1.2 ml of substrate suspension and different volumes of enzyme solution (25 μg/ml in 0.1 *M* phosphate buffer, pH 7.4) as shown in the figures. The volume was adjusted with 0.1 *M* phosphate buffer. The reaction was stopped by adding 0.1 ml of 5 *M* HCl. At this point, 100 μl of internal standard solution (0.20 mg/ml) was also added. After filtration, aliquots of incubated samples and standard solution were alternately injected into the liquid chromatograph, and *p*-nitrophenol was quantified on the basis of chromatographic peak heights [12]. One unit of activity is defined as the amount of enzyme that produces 1 μmol of *p*-nitrophenol per minute under the described conditions. The specific activity is expressed as the number of units per milligram of enzyme preparation.

RESULTS AND DISCUSSION

Fig. 1A shows a typical chromatogram of a standard solution. Fig. 1B is a chromatogram at zero time obtained by incubating the *p*-nitrophenyllaurate in the absence of enzyme. *p*-Nitrophenyllaurate is not hydrolyzed during incubation or after addition of HCl: the traces of *p*-nitrophenol are present as substrate impurity and are to be subtracted from sample values. *p*-Nitrophenol is also stable for several hours after addition of HCl. The peak of *p*-nitrophenyllaurate is not present in the chromatogram. The substrate has good solubility in acetonitrile but is insoluble in water. Therefore, with the mobile phase used, retention time is very high. For this reason we suggest washing the column with acetonitrile after every series of samples assayed to eliminate residues eventually retained by the column.

In Fig. 1C an example of an incubated sample is shown. The amount of *p*-nitrophenol enzymatically liberated from the substrate may be calculated by direct comparison with the standard solution. A calibration curve can also be

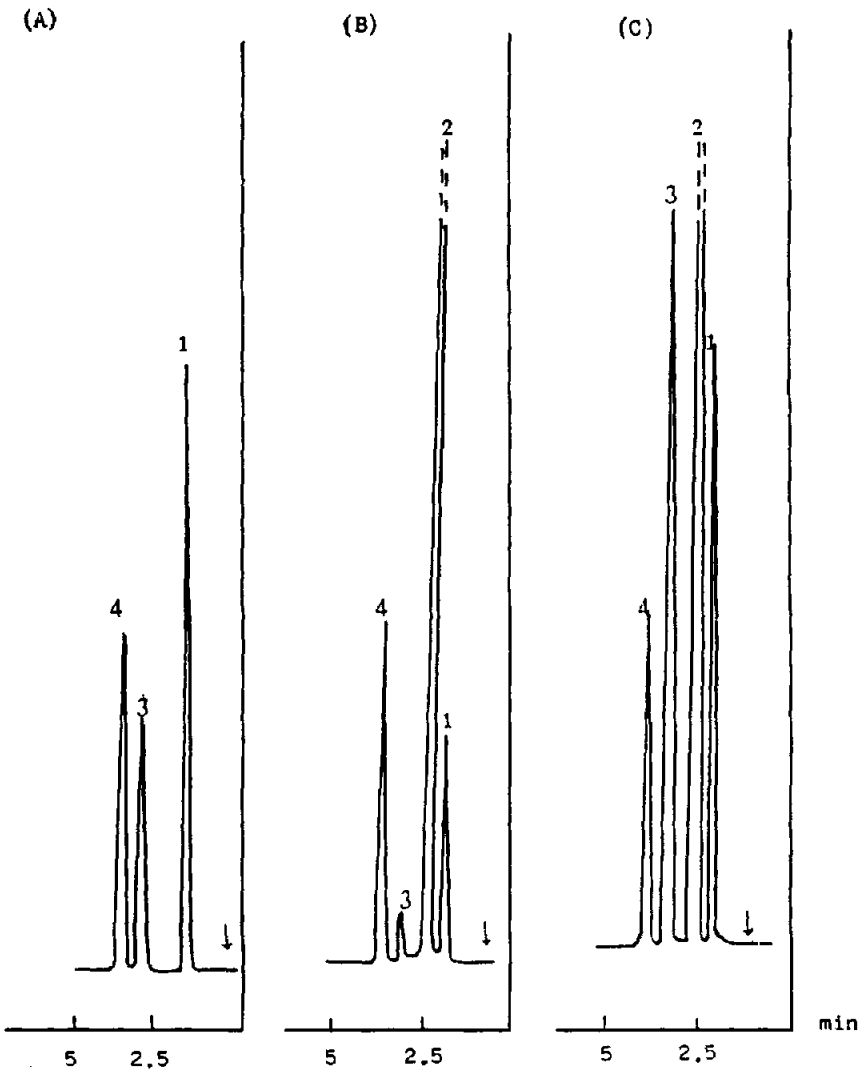

Fig. 1. Typical chromatograms of (A) standard solution, (B) zero time (*p*-nitrophenyllaurate incubated in the absence of enzyme), and (C) an incubated sample (2.5 μg of pancreatin). Peaks: 1 = solvent front; 2 = acetone; 3 = *p*-nitrophenol; 4 = 2,4-dinitroaniline. Mobile phase: water acetonitrile (55:45, v/v). Detection wavelength: 300 nm. Detector sensitivity: 0.04 a.u.f.s.

used: for this purpose, sets of standard samples were made up by the addition of known amounts of *p*-nitrophenol and internal standard to the incubation medium. The ratios of the peak heights of *p*-nitrophenol and 2,4-dinitroaniline *versus* *p*-nitrophenol concentrations were plotted. The linear regression equation was used to calculate *p*-nitrophenol concentration in the samples. The results were comparable to that obtained using the standard solution.

TABLE I

RECOVERY OF *p*-NITROPHENOL AFTER INCUBATION

Data reported correspond to five replicate assays carried out at each of the six different concentrations; the mean relative standard deviation was 2.08%. The samples were prepared and incubated as described in *Enzymatic assay*, except that the enzyme was omitted.

Amount added (μg)	Amount found (μg)	Recovery (mean ± S.D.) (%)
0.75	0.78	104.0 ± 4.1
1.50	1.50	100.0 ± 2.9
3.00	2.97	99.0 ± 1.6
6.00	5.96	99.3 ± 1.4
9.00	8.96	99.5 ± 1.2
12.00	11.98	99.8 ± 1.3

To determine the precision and accuracy of the HPLC assay method, five replicate samples were analyzed for each of six different concentrations of *p*-nitrophenol. The results are summarized in Table I. The lower limit of detection was 0.75 μg/ml. At this concentration the mean recovery of five analyzed samples was 104% with a relative standard deviation (R.S.D.) of 4%.

The amount of hydrolyzed *p*-nitrophenol produced by the enzymatic hydrolysis of *p*-nitrophenyllaurate, plotted against the amount of enzyme added, is shown in Fig. 2. A good linearity is maintained until a rate of splitting of *p*-

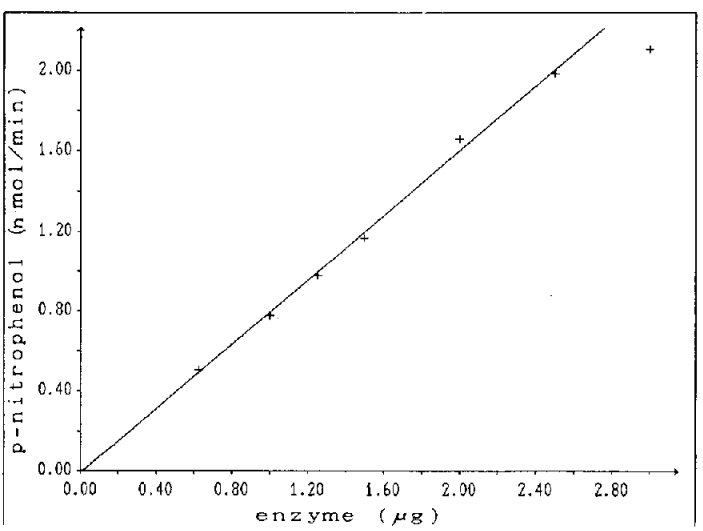

Fig. 2. Linearity of *p*-nitrophenyllaurate hydrolysis in relation to enzyme concentration. Different amounts of the commercial pancreatic preparation were added. Incubation was performed at 37°C for 30 min in a final volume of 1.4 ml, as described under *Enzymatic assay*.

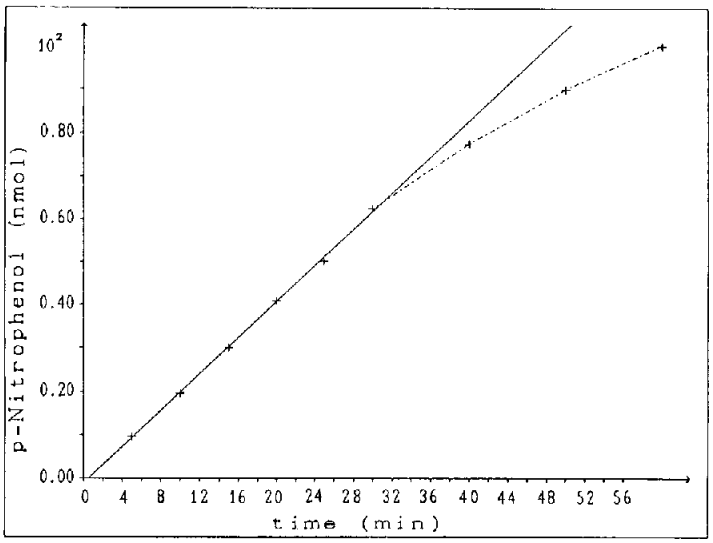

Fig. 3. Linearity of *p*-nitrophenyllaurate in relation to time. A 2.5-μg sample of enzyme was used. Incubation was performed as in Fig. 2, but the reaction was stopped after different incubation times.

nitrophenol of 2 nmol/min, corresponding to 2.5 μg of the examined enzyme preparation. In order to confirm the validity of the method, ten replicate samples were incubated and analyzed for each enzyme value from 0.5 to 2 μg. The average specific activity was 0.798 U/mg with a standard deviation of 0.0119; the 0.95 confidence limits were 0.7895 and 0.8065, *i.e.* 0.0085 or 1.06%.

Fig. 3 shows the linearity of enzymatic reaction with respect to time. The experiments were performed at a substrate concentration of 68 μg/ml, which is slightly below the quantity required for maximal activity. We preferred this concentration because of the better reproducibility. Heterogeneity of oil water emulsion may be the cause of problems observed at higher substrate concentrations. Linearity during the time of incubation is, however, maintained for all the concentrations of enzyme tested.

Some samples were also prepared with F.I.P. standard enzyme in order to compare the units. We found for this preparation an activity of 1.82 U/mg. Therefore with our method one unit corresponds to approximately 20 F.I.P. units.

CONCLUSION

The two substrates presented low specificity compared with the F.I.P. method, but higher than β-napthyllaurate. We believe that *p*-nitrophenyllaurate may be useful because of the high sensitivity, precision and reproducibility found in the HPLC determination of *p*-nitrophenol at low concentrations (up to 0.25 μg/ml by

operating at a detector sensitivity of 0.01 a.u.f.s.). With this substrate, lipase activity may be also evaluated at a large range of concentrations. We can increase this range by shortening incubation time. In this way consumption of substrates is limited, and their quantity is sufficient to maintain linearity even with higher enzyme concentrations.

The method is simple, and the HPLC analysis requires only 5 min for each sample. Therefore this assay can be used routinely for the determination of lipase activity, particularly since the use of an automatic sample injector enables sequential determination of a large number of samples.

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