Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, No. 5, pp. 427–431, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00
Pergamon Press plc

Short Communication

High-performance liquid chromatographic assay of pancreatic lipase activity

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Keywords: *Pancreatic lipase; β-naphtyllaurate; HPLC.*

Introduction

Lipases or acylglycerol acylhydrolases (E.C. 3.1.1.3.) are esterases hydrolysing esters of glycerol with long chain fatty acids. In intermediate steps they have as the substrate diglycerides and monoglycerides [1]. They are widely diffused enzymes in nature. Lipases of different origin show different affinities for tri-, di- and monoglycerides and for various aliphatic alcohols [2] and acyl chain lengths [3].

Lipases have many industrial uses [4, 5]. Animal, plant and microbial forms are known. Pancreatic lipase is the most important one [6]. It is studied for diagnostic and pharmacological purposes [7]. As a pharmaceutical aid it is used prevalently as a digestive aid, in a pure state or, more frequently, as a crude dried pancreas powder where amylase and proteolytic enzymes are also present. For pharmaceutical uses it is very important to utilize sterilized material. In order to monitor the residual activity during the different sterilization processes we have carried out a new HPLC method. The sensitivity is lower in respect to the FIP (Federation International of Pharmaceuticals) method [3] because of less substrate specificity, but shows obvious operative advantages.

The activity determination methods used up to now are difficult and time consuming, because of water immiscibility of the natural fat and oil substrates used. The homogeneity of emulsion may limit the precision and accuracy of results [8]. A volumetric method is most frequently used for titrating the fatty acids produced from olive oil [9] or triolein hydrolysis [10], but they are precise and simple only when a pH-stat apparatus is available. The sensitivity was improved in the method involving formation of copper soap [11]. A conductimetric method was also proposed [7]. Using olive oil or triolein as substrate, glycerol formed may be also determined instead of fatty acids [12]. Several methods are proposed for improving sensitivity or simplicity of analysis. Different substrates are used but it is always difficult to combine the two benefits. The absence of a universally valid method explains the large number of procedures proposed by researchers in the hope of finding an optimum, at least for their own purposes [13–15]. As regards chromogenic substrates, fatty acid esters of *p*-nitrophenol [16], β-naphtol [17], eosin, umbrelliferone [18], salicylic acid [19], dimercaptoethanol [20], or acylglycerols esterified with fatty acids containing a coloured or chromogenic probe are most frequently used. The majority of colorimetric substrates are not commercially available, although conditions for their synthesis are published.

Naphtyllaurate was already used as a substrate for lipase [17] but in conditions that did not show satisfactory activity. The measurement of activity needs, moreover, a reaction with tetrazonium salt and extraction of azo dye.

The use of HPLC, together with some

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modifications in incubation medium, permits these problems to be overcome.

Experimental

Materials

Lipase from porcine pancreas (1 mg = 37.03 FIP units declared activity using olive oil as substrate; the unit of lipase activity is contained in that amount of the standard preparation which under the conditions of the assay liberates one micro-equivalent of fatty acid per minute) was supplied by the Commission on Pharmaceutical Enzymes (Centre for Standards, State University, Gent, Wolterslaan 12, B-9000 Gent, Belgium); thaurocholic acid sodium salt, β-naphtyllaurate and β-naphtol were obtained from Fluka AG (Switzerland); Phenacetin from BDH Chemicals Ltd (Poole, UK); and Tris (hydroxymethyl)aminomethane from Sigma Chemical Co. (St. Louis, MO, USA).

Acetonitrile HPLC grade was supplied by Hoechst (Riedel de Haen); sodium chloride, hydrochloric acid and sodium dihydrogen orthophosphate, all analytical grade, by Carlo Erba (Milano, Italy).

All reagents were used without further purification. The acetonitrile and water were filtered with suction through filters obtained from Millipore (HAFT, 0.45 μm).

Reversed-phase HPLC

Analyses were performed on a high-performance liquid chromatograph (Perkin–Elmer, series 4) equipped with an injector Rheodyne model 7125 with a 20-μl loop and connected to a variable wavelength UV detector Perkin–Elmer (LC 75). A 25-cm, 4.6 mm i.d. C18 reversed-phase column with a 10-μm particle size was used. Chromatograms were recorded on a strip-chart recorder.

Analyses were carried out at room temperature. The flow rate was adjusted to 2 ml min^{-1}. The detector sensitivity was 0.02–0.08 AUFS; the chart speed was 0.5 cm min^{-1}; and the detector wavelength was 230 nm.

The mobile phase was a 50:50 (v/v) mixture of water and acetonitrile.

Two internal standard stock solutions of phenacetin in ethanol were used: 0.25 and 0.2 mg ml^{-1}, respectively. The reference standard solution was so obtained: 100 μl of a 0.3 mg ml^{-1} β-naphtol solution, 130 μl of a 1 mg ml^{-1} phenacetin solution and 0.6 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

β-Naphtyllaurate (10 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 mM sodium taurocholate, 10 ml of 70 mM NaCl and 20 ml of water.

Enzymatic assay

The incubation was carried out for 30 min at 37°C in a final volume of 1.4 ml containing 1.2 ml substrate suspension and 0.2 ml of enzyme solution (5 μg ml^{-1} in 0.1 M phosphate buffer pH 7.4). Reaction was stopped by adding 0.1 ml of 5 N HCl; at this point 50 μl of internal standard solution (0.20 mg ml^{-1}) was also added. The zero time was performed by incubating a sample in the absence of the enzyme. The samples were filtered through a 0.45-μm Millex-HV filters (Millipore). Aliquots of the incubation medium and standard solution were alternatively injected into the liquid chromatograph and β-naphtol was quantified by the measurement of chromatographic peak heights. One unit of activity is defined as the amount of enzyme that produces 1 μmol of β-napthol per minute under described conditions. The specific activity is expressed as the number of units per milligram of enzyme preparation [21].

Results and Discussion

In Fig. 1(A–C) a typical chromatogram of standard solution, zero time and of an incubated sample are shown. β-Naphtol enzymatically liberated from the substrate may be calculated through direct comparison with the standard solution. Standard samples were made up by the addition of known amounts of β-naphtol and internal standard to the incubation medium. The ratios between the peak heights of β-naphtol and phenacetin versus β-naphtol concentrations were subjected to linear regression. The resulting equation was used to calculate the β-naphtol concentration in the samples. To determine the precision and accuracy of the assay method, five replicate samples were analysed for each of the eight concentrations. The results are summarized in Table 1.

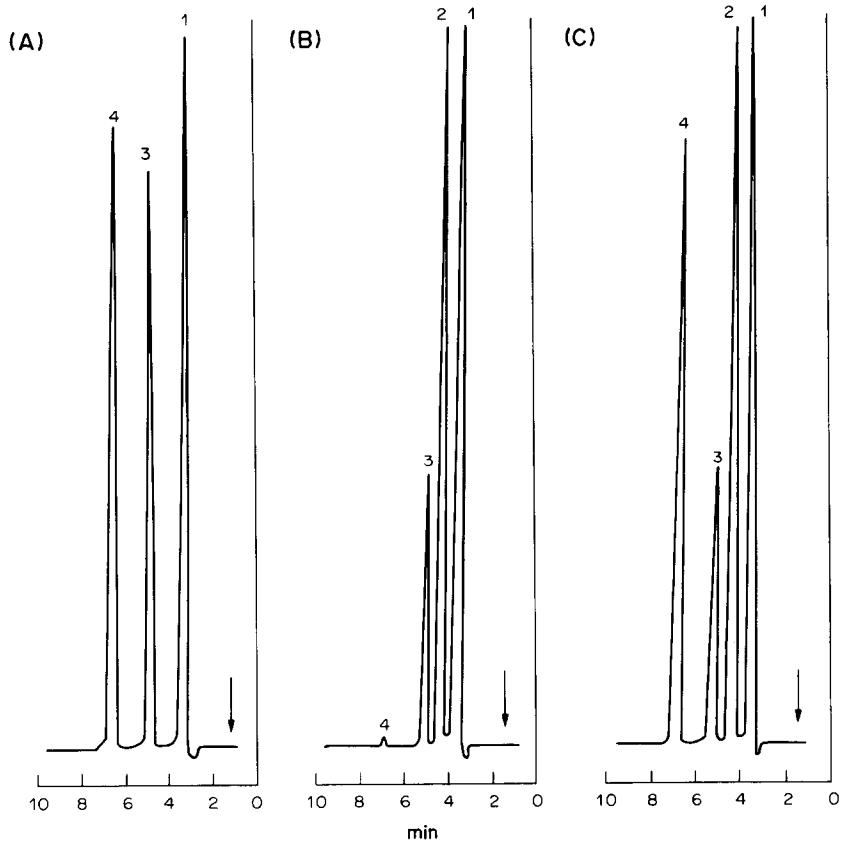

Figure 1
Representative chromatogram of the reference standard solution (A), a zero time (B) and a sample (0.75 μg of lipase) incubated as described in Experimental (C). Key: (1) solvent front; (2) aceton; (3) phenacetin as internal standard; (4) β-naphtol.

Table 1
Recovery of β-naphtol after sample preparation

Amount added (μg)	Amount found (μg)	Recovery (%)	Relative standard deviation
0.2	0.21	105.0	3.5
0.4	0.42	105.0	3.8
0.6	0.63	105.0	2.2
1	1.01	101.0	1.5
2	2.05	102.5	1.3
3	3.1	103.3	1.6
5	5.1	102.0	0.9
10	9.9	99.0	1.1
		Mean	1.98

The samples were prepared as described under "Enzymatic assay", except that the enzyme was omitted. The β-naphtol of "zero time" was subtracted from the measured values, and recovery of β-naphtol was calculated as a percentage of that added.

The lower limit of detection was about 0.15 μg ml^{-1} by injecting a 20-μl sample and operating the detector sensitivity at an attenuation of 0.02 AUFS. In this case the mean recovery of the five analysed samples was 105 ± 3.5%.

The peak of β-naphtyllaurate is not present in the chromatogram. The β-naphtyllaurate is soluble in acetonitrile but it is very insoluble in water. By mobile phase of 50:50 it was not possible to determine the retention time of β-naphtyllaurate; by 90:10 acetonitrile–water the substrate is eluated after 10 min; by 80:20 acetonitrile–water it is eluated after 30 min.

For this reason, washing the column with acetonitrile after every series of samples assayed is recommended.

The zero time was performed incubating the β-naphtyllaurate in absence of the enzyme. No β-naphtyllaurate is hydrolyzed during incubation, the traces of this substance are present as substrate impurity; β-naphtol is also stable for some hours after HCl addition.

Different buffers at different concentrations were compared with sodium barbital used in the Nachlas method [17]. Obtained results are reported in Table 2. The 0.1 M phosphate buffer was found to give better activity conditions. Higher barbital buffer concentrations were not used because diethylbarbituric acid precipitated at the pH used in the system.

In Fig. 2(A) the amount of hydrolysed product is plotted against the amount of enzyme added whilst Fig. 2(B) shows the linearity in respect to time.

The experiments were performed with 0.26 mM substrate concentration, that is below that required for maximal activity. This concentration was preferred because of better reproducibility. Heterogeneity of oil–water emulsion may be the cause of problems at higher substrate concentrations. Linearity is, however, obtained because of the low quantity of enzyme used. In order to confirm the validity of the method 15 samples at concentrations of 1.56–16 μg ml^{-1} were prepared utilizing a standard FIP pancreatic lipase preparation.

The medium specific activity with this method is 1.487 U mg^{-1} with a relative standard deviation of 1.85%, the 95% confidence limits lie between 1.43 and 1.46, i.e. 0.0148 that corresponds to 2.94%.

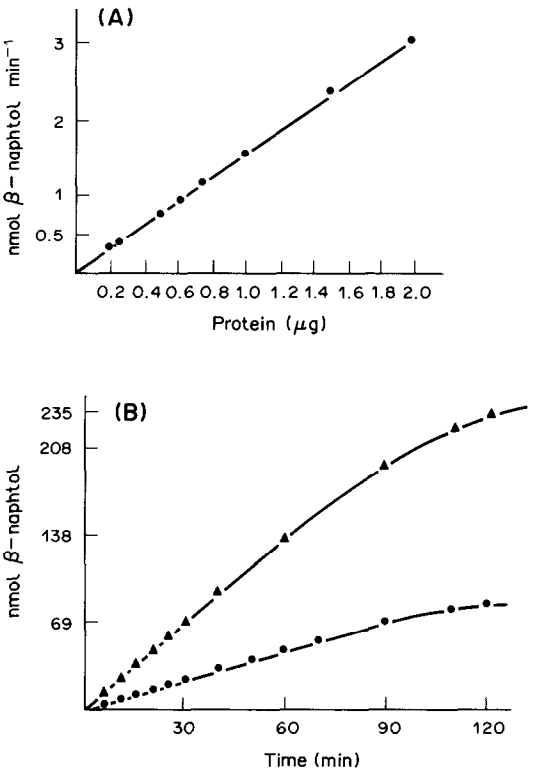

Figure 2
Effect of incubation time and enzyme concentration on lipolytic activity. (A) β-Naphtyllaurate hydrolysed versus enzyme concentration. Incubation was performed as described in Experimental section. (B) β-Naphtyllaurate hydrolysed versus time incubation at 37°C: two different enzyme concentrations are considered: 0.5 μg (●) and 2 μg (▲).

Conclusions

It is believed that this method can merit a place in the great number of methods related to lipases. Every method, at the moment, has both disadvantages and advantages. β-Naphtyllaurate was first used by Nachlas [17]. It did not find much favour because of low specificity as a substrate for lipase. We demonstrated that part of the problem was the buffer utilized by previous authors. Phosphate buffer allows nearly 70% enhancement of activity to be obtained. The problem of the substrate

Table 2
Buffer concentrations

Buffer concentrations (mM)		β-Naphtol liberated (μg) Enzyme, 0.25 μg	Enzyme, 0.5 μg
Phosphate	200	1.351 (1.5)	2.712 (1.4)
	100	1.513 (1.3)	3.073 (1.2)
Tris	100	0.696 (0.4)	1.181 (1.3)
	60	0.454 (2.1)	0.923 (1.1)
Veronal	29	0.895 (1.5)	1.771 (1.1)

Values in parentheses are relative standard deviations of five samples analysed.

homogenization is not overcome, but the extraction of the product before determination is avoided.

Furthermore, despite the low substrate specificity (1.49 instead of 37 U mg^{-1} for the FIP method) small concentrations of enzyme may be determined with good precision because of the high sensitivity of the HPLC method for β-naphtol (0.15 μg ml^{-1}).

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[*Received for review 7 February 1990*]