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Short Communication

A high-performance liquid chromatographic method for the determination of amylase (*p*-nitrophenyl-α-maltosidase) activity

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Introduction

α-Amylase (1,4-α-glucan glucanohydrolase, EC 3.2.1.1.), an endoenzyme, hydrolyses α-linkages of 1,4-maltopolysaccharides in a random manner [1].

Porcine pancreatic α-amylase forms the active component of various pharmaceutical preparations used in the treatment of pancreatic deficiencies, such as amylaceous dyspepsias. Among the various methods described in literature for the assay of the amylase activity, the most recent ones are those utilizing as substrates, blocked starch molecule [2], maltotetraoside [3, 4], *p*-nitrophenyl-α-maltoside [5], *p*-nitrophenylmaltoheptaoside [6, 7], a mixture of *p*-nitrophenylmaltopentaoside and *p*-nitrophenylmaltohexaoside [8, 9], β-2-chloro-4-phenylmaltopentaoside [10], 2-chloro-4-nitrophenyl-β-D-maltoheptaoside [11, 12].

In this work a reversed-phase HPLC method for the determination of amylase activity in pharmaceutical preparations containing pancreatin is proposed. Two *p*-nitrophenyl-α-maltaosides are used as substrates, which amylase cleaves to shorter chain *p*-nitrophenyl maltosides [13], the latter then yielding *p*-nitrophenol from the action of α-glucosidase. Enzyme activity is determined directly by monitoring the formation of *p*-nitrophenol by means of absorption measurements at 280 nm. A HEPES buffer of pH 6.95 is used, containing chloride as the activator and α-glucosidase (EC 3.2.1.20) as the auxiliary enzyme. The method proposed is rapid (assay time, 10 min) and can be used successfully in various routine analyses.

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Experimental

Apparatus

A Perkin–Elmer (series 4) high-performance liquid chromatograph, equipped with a Rheodyne model 7125 injector with a 20 μl loop, a Lichrosorb RP18 (250 × 4.6 mm, i.d., particle size 10 μm) column, and a Perkin–Elmer (LC 75) variable wavelength UV detector and a Perkin–Elmer strip chart recorder, was used throughout the work.

Materials

p-Nitrophenyl-α-maltopentaoside (PG_5) and *p*-nitrophenyl-α-maltohexaoside (PG_6) were purchased from Calbiochem-Boering (Scoppito (AQ) 67019, Italy); *p*-nitrophenol (PNP) from Janssen (2340 Beerse, Belgium); amylase (1 mg = 32.6 FIP units) by the Commission on Pharmaceutical Enzymes, Centre for Standards (State Univ., Ghent, Wolterslaan 12, B-9000 Ghent, Belgium); α-glucosidase and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) from Sigma Chemical Co. (St. Louis, MI, USA). Acetonitrile HPLC grade and isopropylantipyrine were supplied by Hoechst (Riedel de Haen); sodium chloride, hydrochloric acid, dihydrogen sodium phosphate, 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.

Chromatographic conditions

The mobile phase was prepared with 600 ml of water and 400 ml of acetonitrile, adjusted to pH 3. The operating conditions were: ambient temperature, flow rate 2 ml min^{-1}, detector wavelength 280 nm, chart speed 0.5 cm min^{-1}, sensitivity 0.04–0.32 a.u.f.s. The analytical column was 250 × 4.6 mm, i.d. packed with Lichrosorb RP 18, particle size 10 μm obtained from Perkin–Elmer.

Internal standard stock solution

A solution of 1 mg ml^{-1} isopropylantipyrine in ethanol was used.

Reference standard solution

A 15 ml vol of a 0.56 mg ml^{-1} solution of PNP was added to 18 ml of the internal standard solution and made up to a final volume of 100 ml with water.

Calibration curve

Accurately pipetted volumes of 1, 2, 5, 10, 15, 20 and 25 ml of PNP stock solution (0.56 mg ml^{-1}) and 18 ml of the internal standard stock solution (1 mg ml^{-1}) were transferred into 100-ml volumetric flasks and made up to volume with ethanol. Aliquots of 20 μl vol of each solution were injected into the chromatograph.

A calibration curve was prepared by plotting the peak height ratios for PNP and internal standard against the concentrations of PNP in mg ml^{-1}. The calibration curve was constructed to verify the linearity to the detector response; this was confirmed over the range 5.5–137.5 μg ml^{-1}. Regression analysis gave a slope of 0.01425 and an intercept of 0.05437 and a correlation coefficient of 0.9994.

Enzymatic assay and recovery

The concentrations of the relative components in incubation medium for the enzymatic assay were: PG_5 0.84 mM; PG_6, 0.64 mM, NaCl, 49 mM; α-glucosidase, 34

kU l^{-1}; in buffer HEPES, 49 mM, pH 6.95. The reaction was started by adding to 460 μl of the incubation medium, 50 μl of a 0.652 U FIP ml^{-1} amylase solution in HEPES. Smaller quantities can be used, provided the incubation medium is brought to volume with a HEPES buffer. The solution was incubated at 37°C for 10 min in a shaking water bath (Dubnoff metabolic shaking incubator). After incubation, 50 μl of 0.5 M HCl was added to stop the reaction and subsequently 50 μl of internal standard solution was introduced. Aliquots of the incubation medium (20 μl) were directly injected into a liquid chromatograph and the PNP liberated was measured. Simultaneously a "zero time" assay was performed by incubating a sample in the absence of the enzyme. The samples and reference standard solution were chromatographed alternately using the chromatographic conditions already described. Peak height ratios were used to evaluate the resulting chromatograms. The enzyme unit (U) is the activity to produce 1 μmol PNP/min. The specific activity is the number of units/mg protein. The specific activity was calculated as follows:

$$\text{specific activity} = \frac{\mu g\ PNP \cdot 10^3}{131.11 \cdot 10 \cdot \mu g\ E},$$

where

μg PNP = μg of PNP in the sample;
139.11 = molecular weight of PNP;
10 = incubation time (min);
μg E = quantity of protein in the sample (μg).

Recovery

Nine PNP samples were added to the incubation medium prepared as described in *Enzymatic assay and recovery* except that amylase was omitted. After 10-min incubation, 50 μl HCl 0.5 M, and subsequently 50 μl of internal standard solution, were added. The quantization of PNP was carried out as described in *Enzymatic assay and recovery*.

Results and Discussion

All the analyses were performed in duplicate. The present HPLC method requires no elaborate sample preparation procedures; it is accurate and reproducible. The enzyme was denatured with 0.5 M HCl, and PNP thus liberated was determined at 280 nm. Enzymatic activity was also measured by substituting HEPES buffer with phosphate buffer 50 mM without causing any significant differences.

In Fig. 1(a), a chromatogram of standard solution is shown, whilst in Fig. 1(b) a chromatogram corresponding to a "zero time" is reported. A chromatogram of a sample is shown in Fig. 1(c).

The recovery of PNP added to incubation medium was found to be 99.75% over the range 8.2–131.1 μg ml^{-1}. The RSD % of four estimations for each sample analysed, ranges between 0.8–1.1. Moreover, five samples of concentrations <5 μg ml^{-1} were analysed, which indicates a limit of detection of 0.8 μg ml^{-1} (the recovery was found to be 100.1% with an RSD of 1.4%).

The study of linearity between incubated enzyme and PNP liberated was carried out with FIP amylase, and the relationship was found to be linear over the range 0–40 mU FIP of enzyme. For this purpose seven samples of amylase with concentrations ranging

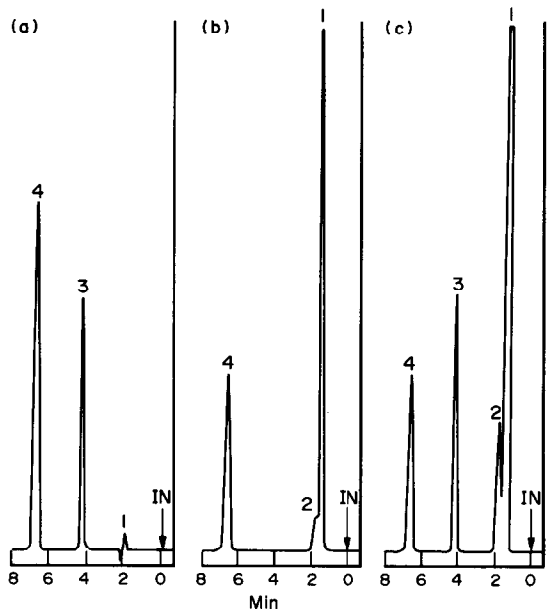

Figure 1
(a) Chromatogram of the reference standard solution containing PNP 0.56 mg ml^{-1} (peak 3) and isopropylantipyrine 1 mg ml^{-1} (peak 4) [0.32 a.u.f.s.]. (b) Chromatogram of the "zero time" solution. Key: (1) solvent + PG_5; (2) PG_6; (4) isopropylantipyrine (0.08 a.u.f.s.). (c) Chromatogram of a sample (0.8 μg of amylase) incubated as described in Experimental. Key: (1) solvent + PG_5; (2) PG_6; (3) PNP liberated (46.8 μg); (4) isopropylantipyrine (0.08 a.u.f.s.).

between 0.65–1.96 μg ml^{-1} were analysed. The kinetics were observed to be linear up to 25 min when the assay was carried out with 0.8 μg protein. The analysis of 12 amylase standard FIP samples further confirmed the validity of the method; the mean activity found was 41.45 U mg^{-1} with the RSD of 1.03%; the 0.95 confidence limits lay between 41.177–41.723, i.e. ±0.273 or 0.66%.

For the recovery with real samples, pancreatin preparations were also analysed and evaluated under the conditions previously described, with the exception of substrate and the addition of 40 μl of 2 μg/100 ml pancreatin solution. The concentration of PNP added was between 8.2–131.1 μg ml^{-1}. The recovery mean was 100.48% and RSD % for four estimations of each sample analysed, ranged between 0.85–1.12.

In conclusion, this method offers good practicability, easy adaptation to analyst and short incubation time; it can be used as against the FIP method, which utilizes the starch–iodine reaction as substrate [14]. The method also has wider technological interest and presently is used in the authors' laboratory to study the time degradation of γ-ray sterilized enzymes for use in pharmaceutical preparations.

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