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Evaluation of pancreatin stability through enzyme activity determination

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Pancreatin is a biotechnological product containing an enzyme complex, obtained from porcine pancreas, that is employed in treating pancreatic diseases. Experiments regarding the stability of the pharmaceutical formulation containing pancreatin were performed using standard binary mixtures with 6 excipients in a 1:1 ratio (*m*/*m*) and a commercial formulation. To accomplish these goals, samples were stored for 1, 3 and 6 months at 40 ± 1 °C and 75 ± 5 % relative humidity (RH) and 40 ± 1 °C and 0 % RH. Stress testing was also performed. All samples were analyzed to evaluate the α-amylase, lipase and protease activities through UV/Vis spectrophotometry. The results revealed that the excipient proprieties and the storage conditions affected enzyme stability. Humidity was a strong influencing factor in the reduction of α-amylase and protease activities. Stress testing indicated that pH 9.0 and UV light did not induce substantial alterations in enzyme activity.

Keywords: α-amylase, lipase, protease, stress testing, pancreatin stability, biopharmaceutical, UV/VIS spectrophotometry

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Stability of pharmaceutical products depends on various factors, including environmental factors (*e.g.*, temperature, humidity and light) and other factors related to the product itself, such as the physicochemical properties of active substances and pharmaceutical excipients, the shape and composition of the pharmaceutical, the fabrication process, and the type and properties of packaging materials (1).

For pharmaceutical products, the stability of biopharmaceuticals could require special attention because of their inherent physicochemical complexity. In this sense, stability tests are performed to determine the expiration date, the utilization period and the storage conditions. During the various fabrication stages of these biopharmaceuticals, aggregates and degradation products might form and compromise the stability, quality and security of the formulations (2).

Pancreatin is a biopharmaceutical obtained from swine pancreas, and according to the *United State Pharmacopoeia*, it is defined as a substance that contains enzymes, mainly

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α-amylase, lipase and protease that show pharmaceutical importance as therapeutic agents in acute and chronic pancreatitis (3).

There are few studies (2, 4) related to the enzyme activity of the pancreatin complex. Thus, the main objective of the present study was to evaluate the stability of formulations containing pancreatin. For this purposes, experiments using standard pancreatin and its binary mixtures with different excipients (ratio of 1:1, *m*/*m*) were performed. In addition, the stability of the commercial formulation containing pancreatin was evaluated. Stress testing was also performed on pancreatin.

The activities of the three enzymes (α-amylase, lipase and protease) in all samples were analyzed through spectrophotometric tests, recommended by the ICH Q5C guidelines (5), which include elaborate guidelines concerning assays to determine the quality of biological and biotechnological products.

EXPERIMENTAL

Samples

The influence of excipients and storage conditions on enzyme stability was determined for samples of standard pancreatin (Sigma-Aldrich, USA), the commercial formulation and binary mixtures of standard pancreatin with each excipient (citric acid, magnesium stearate, mannitol, microcrystalline cellulose, sucrose and talc) at a 1:1 ratio (*m*/*m*). The excipients (99 % degree of purity) were provided by Aché Laboratórios Farmacêuticos S.A. Stress testing analysis was only performed for standard pancreatin.

Stability condition

Samples were stored under the conditions recommended by ICH in a stability chamber for 1-, 3- and 6-month periods at 40 ± 1 °C and 75 ± 5 % relative humidity (RH) and at 40 ± 1 °C in the absence of humidity (5).

Preparation of standard pancreatin and binary mixtures

Binary mixtures at a 1:1 ratio (*m*/*m*) were prepared with 30 mg of standard pancreatin and 30 mg of each excipient in a polyethylene container (3 cm height × 2 cm diameter). The samples were homogenized by vortexing for 1 min, and stainless steel balls (2 mm diameter) were used to mix the samples. In addition, 30 mg of standard pancreatin was placed in a polyethylene container. All samples were prepared in triplicate.

After the storage time, all samples were solubilized in 25 mL of TRIS + HCl buffer (50 mmol L^{-1}, pH 7.0) and a stock solution containing 90 U mL^{-1} α-amylase, 7.2 U mL^{-1} lipase and 90 U mL^{-1} protease was obtained. These concentrations were estimated from the specification of the standard pancreatin manufacturer, which indicated that 1 mg of standard pancreatin contained 75.0 U amylase, 6.0 U lipase and 75.0 U protease.

For α-amylase activity determination, a new dilution was prepared using TRIS + HCl buffer (50 mmol L^{-1}, pH 7.0) and a solution of 2.0 U mL^{-1} α-amylase was obtained. For lipase, TRIS + HCl buffer (50 mmol L^{-1}, pH 9.0) was used and a solution containing 6.0 U mL^{-1} of this enzyme was obtained. Finally, TRIS + HCl buffer (50 mmol L^{-1}, pH 7.5) was used to obtain a solution of 14.0 U mL^{-1} protease.

Preparation of commercial formulation

Commercial formulation samples (both whole capsules and the internal powder content) were evaluated. To obtain the powder from the commercial formulation, capsules were opened, the contents were transferred to a mortar and the material was ground and homogenized. The average mass of each capsule was 258 ± 10 mg. According to the label information, each capsule contained 33,200, 10,000 and 37,500 U of α-amylase, lipase and protease, respectively. Then, the enzymes available in 30 mg of homogenized powder were solubilized in TRIS + HCl buffer (pH 7.0) to prepare a solution containing 153.0 U mL^{-1} α-amylase, 46.0 U mL^{-1} lipase and 173.0 U mL^{-1} protease. From this solution, three further dilutions were made using TRIS + HCl buffer to obtain solutions of 2.0 U mL^{-1} α-amylase, 6.0 U mL^{-1} lipase and 14.0 U mL^{-1} protease.

Stress testing

A stress test was performed as recommended by ICH (5). In this case, five conditions were applied: high temperature, acidic pH, basic pH, ultraviolet light and forced oxidation. The analyses were conducted according to the procedures described by Blessy *et al*. and Printz *et al*. (6, 7). Briefly, 2.5 mg of standard pancreatin was weighed and then solubilized in TRIS + HCl buffer (50 mmol L^{-1}, pH 7.0). Such solutions of standard pancreatin, having a final volume of 1.0 mL, were prepared in 1.5-mL microtubes. After solubilization, the solutions were centrifuged for 1 minute at 1,000 rpm and then placed in an ultrasound bath. In the prepared samples, the activities were 187.5, 15.0 and 187.5 U mL^{-1} for a-amylase, lipase and protease, respectively.

For thermal stability testing, the solution containing pancreatin was subjected to 70 °C for 60 minutes in a water bath and then cooled to room temperature. The choice of temperature was based on the optimal temperature for pancreatin activity (70 °C induces degradation of all enzymes in the pancreatin complex) (8).

To test the pH influence, pancreatin samples were subjected to acid (100 mmol L^{-1} citric acid buffer, pH 3.0) and base (100 mmol L^{-1} TRIS + HCl buffer, pH 9.0 and 50 mmol L^{-1} carbonate/bicarbonate buffer, pH 11.0). Activity tests were performed after 60 minutes of exposure. The applied pH conditions had the objective of providing forced degradation conditions and were selected according to the optimal activity pH of the main pancreatin enzymes.

The solution of pancreatin was exposed to a photostability chamber with 1.2 million lux-hours of ultraviolet light at 25 °C for stress tests. Such samples were stored in amber vials and under refrigeration at 4 °C until analysis. Finally, pancreatin solutions were prepared in 50 mmol L^{-1} TRIS+HCl buffer, pH 7.0, and 3 % (*v*/*v*) hydrogen peroxide for forced oxidation. All of stress tests were performed in triplicate and samples were analyzed for activity as soon as the tests were completed.

Spectrophotometric analyses

Spectrophotometric analyses were performed using a spectrophotometer (UV-Vis 1800, Shimadzu, Japan). In these analyses, the limit of quantification (*LOQ*) was calculated as 10 times the ratio between the standard deviation of the blank and the slope of the respective calibration curve.

α-amylase. – The method was based on the measurement of amylolytic activity through quantification of the reducing sugars given off by the starch hydrolysis reaction, catalyzed by amylases. The reducing sugars were quantified at 550 nm. The α-amylase activity was determined based on the methodology described by ANVISA and Russel (9, 10).

Lipase. – The lipase activity was determined through quantification of the product formed by the lipase-catalyzed hydrolysis of *p*-nitrophenyl palmitate. The chromogen product was quantified at 410 nm. The lipase activity analyses were performed based on the method described by Kanwar (11) and Abd-Elhakeem (12).

Protease. – The proteolytic activity was determined by quantification of the azo compound released during the hydrolysis of the chromogenic substrate azocasein. The protease activity was determined at 400 nm based on the methodology described by ANVISA (9).

RESULTS AND DISCUSSION

Each enzyme of the pancreatin complex showed a different degradation profile, which was strongly influenced by distinct storage conditions. This profile could be accompanied by changes as a function of time (initial, 1-, 3- and 6-months), allowing a detailed analysis of the behavior of enzyme activities during storage.

In stability analyses, one hundred % of the activity of each enzyme was assumed from the absorbance obtained from the standard pancreatin solution containing 2.0 U mL^{-1} α-amylase, 6.0 U mL^{-1} lipase, and 14.0 U mL^{-1} protease.

Stability of α-amylase

When stored at 40 °C and 75 % RH (Table I), a total decline in activity of this enzyme was observed in just one month when the pancreatin was stored in the absence and presence of all excipients. This finding shows that humidity exerts a strong influence on α-amylase stability, inducing its degradation. Thus, none of the evaluated excipients was able to provide a protective effect for α-amylase. In the commercial formulation sample, the activity (only 12.5 % of activity was found both in the capsule and powder) decreased considerably, showing that the combination of temperature and humidity exerts a strong negative influence on α-amylase stability, inducing a distortion of the enzyme structure. The same profile was observed when the commercial formulation was analyzed after 3 months.

When α-amylase was analyzed after 1 month of storage at 40 °C and 0 % RH (Table I), its activity was 61.0 % of the activity when pancreatin was stored without excipients. However, sucrose and mannitol showed protective effects on α-amylase activities (79.5 and 68.0 %, respectively). The other excipients did not provide effective protection. After 3 months, α-amylase activities were low in all samples. However, sucrose still showed a protective effect on this enzyme, maintaining its activity at 52.0 %. After 6 months, α-amylase showed activity lower than the *LOQ* for the pancreatin standard stored without excipients. In the commercial formulation sample, higher preservation of activity was observed compared to that for the samples stored with humidity.

Table I. Activity of α-amylase (%) in pancreatin samples stored at 40 °C and 75 % RH and 40 °C and 0 % RH after different times of incubation[a]

Sample	Initial value	With humidity			No humidity		
		1-month	3-months	6-months	1-month	3-months	6-months
Pancreatin	98.3 ± 0.9	≤ *LOQ*	≤ *LOQ*	≤ *LOQ*	61.0 ± 0.7	33.0 ± 0.7	≤ *LOQ*
P + citric acid	99.1 ± 0.9	≤ *LOQ*	≤ *LOQ*	≤ *LOQ*	13.3 ± 0.5	≤ *LOQ*	≤ *LOQ*
P + sacharose	97.6 ± 0.9	≤ *LOQ*	≤ *LOQ*	≤ *LOQ*	79.5 ± 0.9	52.0 ± 0.7	11.0 ± 0.8
P + mannitol	98.7 ± 1.1	≤ *LOQ*	≤ *LOQ*	≤ *LOQ*	68.0 ± 0.6	33.0 ± 0.6	4.5 ± 0.5
P + cellulose	98.4 ± 1.3	≤ *LOQ*	≤ *LOQ*	≤ *LOQ*	2.5 ± 0.1	≤ *LOQ*	≤ *LOQ*
P + Mg-stearate	99.5 ± 1.1	≤ *LOQ*	≤ *LOQ*	≤ *LOQ*	43.5 ± 0.7	17.5 ± 0.6	4.5 ± 0.5
P + talc	98.1 ± 1.2	≤ *LOQ*	≤ *LOQ*	≤ *LOQ*	17.0 ± 0.7	4.5 ± 0.3	≤ *LOQ*
Capsule	92.5 ± 0.6	12.5 ± 0.4	5.7 ± 0.6	–	69.0 ± 0.7	26.6 ± 0.8	–
Powder	90.2 ± 0.4	12.5 ± 0.3	3.2 ± 0.5	–	55.0 ± 0.6	23.5 ± 0.7	–

P – Pancreatin standard
[a] Mean ± SD, $n = 3$.
[b] ≤ *LOQ* – 2.2 % of activity.
[c] Period of storage not evaluated.

Stability of lipase

Lipase had a very similar stability profile under both conditions; this enzyme showed higher activity levels in binary mixtures and the pancreatin standard. After 1 month at 40 °C and 75 % RH (Table II), lipase showed activity of 66.3 ± 0.9 % in the absence of excipients. The only excipient that showed a protective effect was sucrose, which maintained the enzymatic activity at 88.7 ± 0.9 %. The other excipients (citric acid, mannitol, cellulose, magnesium stearate and talc) led to lower enzymatic activity than the activity of standard pancreatin. After 6 months, the mixtures with mannitol, magnesium stearate and talc showed a decrease in lipase activity (*LOQ* ≤ 3.3 %).

In the commercial formulation samples, a drastic decrease in lipase activity was observed for the storage at 40 °C and 75 % RH, further demonstrating that humidity exerts a strong negative influence on enzyme structure. This finding indicates that the effectiveness of the commercial formulation is compromised under such conditions.

In contrast to the results for humidity conditions, sucrose did not show a protective effect on lipase activity. Independently of incubation time, samples with magnesium stearate stored at 40 °C and 0 % RH showed the highest lipase activities compared to the standard pancreatin sample. The magnesium stearate effect on lipase activity is interesting and could be attributed to enzyme affinity for hydrophobic excipients, such as stearate, which interact with the enzyme active site and reduce its three-dimensional distortion (13). When lipase activities were determined for the commercial formulation after 1 month, there was better preservation of this enzyme (75 and 64 % in the capsule and powder, respectively). After 3 months, a low level of activity was observed (14.5 and 16.3 % in the same previ-

Table II. Activity of lipase (%) in pancreatin samples stored at 40 °C and 75 % RH and 40 °C and 0 % RH after different times of incubation[a]

Sample	Initial value	With humidity			No humidity		
		1-month	3-months	6-months	1-month	3-months	6-months
Pancreatin	100.0 ± 0.2	66.3 ± 0.9	24.4 ± 0.7	7.6 ± 0.6	60.6 ± 0.5	21.0 ± 0.9	7.6 ± 0.6
P + citric acid	97.7 ± 0.2	24.9 ± 0.7	9.6 ± 0.7	≤ *LOQ*	43.6 ± 0.5	15.0 ± 0.5	2.8 ± 0.4
P + sacharose	99.3 ± 0.7	88.7 ± 0.9	50.1 ± 0.4	15.0 ± 0.9	29.7 ± 0.7	12.7 ± 0.9	3.4 ± 0.6
P + mannitol	98.2 ± 0.4	27.5 ± 0.2	7.4 ± 0.4	≤ *LOQ*	24.9 ± 0.8	9.9 ± 0.5	≤ *LOQ*
P + cellulose	98.3 ± 0.9	48.4 ± 0.2	15.0 ± 0.9	3.4 ± 0.5	37.7 ± 0.9	17.6 ± 0.2	7.6 ± 0.5
P + Mg-stearate	98.2 ± 0.9	23.2 ± 0.5	7.1 ± 0.8	≤ *LOQ*	66.0 ± 0.8	47.3 ± 0.6	23.7 ± 0.6
P + talc	98.1 ± 0.9	24.6 ± 0.8	7.1 ± 0.7	≤ *LOQ*	44.7 ± 0.7	23.8 ± 0.3	9.1 ± 0.3
Capsule	90.3 ± 0.3	26.0 ± 0.6	13.0 ± 0.8	–	75.0 ± 0.3	16.3 ± 0.5	–
Powder	91.3 ± 0.8	13.5 ± 0.7	8.2 ± 0.3	–	64.0 ± 0.4	14.5 ± 0.7	–

P – Pancreatin standard
[a] Mean ± SD, $n = 3$.
[b] ≤ *LOQ* – 3.3 % of activity.
[c] Period of storage not evaluated.

ously mentioned samples). Further, in this case, the capsules did not have an adequate protective effect on the active pharmaceutical ingredient.

Stability of protease

After 1 month at 40 °C and 75 % RH (Table III), the protease activity in the pancreatin standard decreased considerably (only 8.3 ± 0.5 % of the activity was detected). Sucrose and cellulose did not significantly affect the protease activity (no significant differences were found after applying the *t*-test at the 95 % confidence level for comparison with standard pancreatin). This effect is likely due to the chiral centers of enzymes (14). The presence of mannitol, magnesium stearate and talc further decreased protease activities. However, citric acid increased the protease activity to approximately two times the value of pure standard pancreatin. Thus, citric acid exerted a protective effect under conditions of humidity storage. In the commercial formulation, the detected activity was 77.9 and 48.4 % in the capsule and powder, respectively.

When this enzyme was analyzed after 1 month at 40 °C and 0 % RH (Table III), a total decrease in activity was observed in the pancreatin standard (*LOQ* ≤ 2.6 %). The same behavior was observed when the sample was mixed with citric acid. However, some excipients, such as cellulose and talc, showed a slight protective effect. After 3 and 6 months, a total decrease in protease activity was observed for both storage conditions. Amino acid residues (mainly cysteine) can interfere with enzyme activity. These structures are likely to combine with essential amino acids, thus decreasing the enzyme conformational stability and consequently reducing its activity (15).

Table III. Activity of protease (%)[a] in pancreatin samples stored at 40 °C and 75 % RH and 40 °C and 0 % RH after different times of incubation

Sample	Initial value	With humidity			No humidity		
		1-month	3-months	6-months	1-month	3-months	6-months
Pancreatin	100.0 ± 0.3	8.3 ± 0.5	≤*LOQ*	≤*LOQ*	≤*LOQ*	≤*LOQ*	≤*LOQ*
P + citric acid	98.5 ± 0.4	19.8 ± 0.5	≤*LOQ*	≤*LOQ*	≤*LOQ*	≤*LOQ*	≤*LOQ*
P + sacharose	99.5 ± 0.7	8.8 ± 0.5	≤*LOQ*	≤*LOQ*	2.9 ± 0.6	≤*LOQ*	≤*LOQ*
P + mannitol	98.5 ± 0.8	3.4 ± 0.6	≤*LOQ*	≤*LOQ*	4.6 ± 0.4	≤*LOQ*	≤*LOQ*
P + cellulose	98.5 ± 0.8	8.0 ± 0.5	≤*LOQ*	≤*LOQ*	11.0 ± 0.5	≤*LOQ*	≤*LOQ*
P + Mg-stearate	99.0 ± 0.6	2.9 ± 0.6	≤*LOQ*	≤*LOQ*	2.9 ± 0.6	≤*LOQ*	≤*LOQ*
P + talc	98.3 ± 0.8	5.4 ± 0.5	≤*LOQ*	≤*LOQ*	5.4 ± 0.5	≤*LOQ*	≤*LOQ*
Capsule	96.3 ± 0.6	77.9 ± 0.9	27.0 ± 0.8	–	91.7 ± 0.3	43.0 ± 0.4	–
Powder	96.4 ± 0.5	48.4 ± 0.4	24.5 ± 0.2	–	82.3 ± 0.7	37.9 ± 0.3	–

Pancreatin – P
[a] Mean ± SD, *n*=3.
[b] ≤*LOQ* – 2.6 % of activity.
[c] Period of storage not evaluated

Table IV. Activity of enzymes in pancreatin samples subjected to stress testing

Sample	α-amylase		Lipase		Protease	
	DF [a]	Activity (%)[b]	DF [a]	Activity (%)[b]	DF [a]	Activity (%)[b]
No stress	100	100.2 ± 0.3	2.5	102.3 ± 0.8	13	100.3 ± 0.4
pH 3.0	–	0.3 ± 0.1	–	15.4 ± 0.4	–	4.1 ± 0.2
pH 9.0	100	101.2 ± 0.9	2.5	94.4 ± 0.5	13	73.1 ± 0.3
pH 11.0	–	0.1 ± 0.02	–	10.9 ± 0.3	–	3.6 ± 0.1
Temperature	–	2.1 ± 0.03	–	7.5 ± 0.2	–	1.8 ± 0.1
Oxidation	–	0.3 ± 0.02	–	32.2 ± 0.2	13	54.1 ± 0.4
UV light	100	100.2 ± 0.2	–	5.9 ± 0.2	–	2.1 ± 0. 2

[a] DF – dilution factor.
[b] without dilution.
[c] Mean ± SD, *n*=3.

Activity of samples subjected to stress testing

As shown by the data in Table IV, the α-amylase activity was completely maintained at pH 9.0 and when subjected to UV light. No significant differences between the sets of results were found after applying the *t*-test at the 95 % confidence level. Further, other stress testing conditions were extremely harmful, resulting in activity lower than 2.1 %.

In contrast, significant alterations in lipase activity were observed under all stress testing conditions, as indicated by the same statistical parameters. It is important to emphasize that lipase is the main enzyme of the pancreatin complex.

The conditions that best preserved protease activity were pH 9.0 and oxidation. Other conditions (acid pH, temperature, UV light and pH 11.0) were extremely harmful to protease activity (activities lower than 4.1 % were detected).

For three enzymes, acidic pH, highly basic pH (pH 11.0) and high temperature (70 °C for 60 minutes), exposure induced considerable losses in activities. Therefore, attention should be paid to the appropriate pH and temperature during the preparation and storage of formulations containing pancreatin.

CONCLUSIONS

The α-amylase and protease activities decreased significantly after 1-month storage at 40 °C and 75 % RH. The protease activity also decreased without humidity; thus, this enzyme was most susceptible to degradation. In contrast, lipase showed the highest stability (indicated by higher activity levels). The results demonstrate that the proprieties of chemical excipients can affect enzyme stability and, consequently, its activity. Sucrose was the excipient with the best protective effect on α-amylase and lipase in samples stored with humidity. In addition, the enzymatic activity study of the commercial formulation after 3 months under both storage conditions showed a substantial decrease in all enzyme activities. This result indicates that the efficiency of the commercial formulation is compromised.

The stress testing data indicate that exposure to pH 3.0, pH 11.0 and temperature induces considerable losses in the activities of the three enzymes. Attention should be paid to pH, temperature and humidity during the preparation and storage of pharmaceutical pancreatin formulations.

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