Optimization of Methods to Analyze Enzymatic Mixture in Oral Gavage Formulation

Archana Akalkotkar, Ophelia Whitmore, Robyn Eggleston, Kallol Biswas
Charles River Laboratories, Ashland, OH, USA

1 Purpose

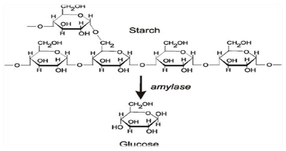
A stable mixture of three pancreatic enzymes including lipase, protease and amylase was formulated to be administered as an oral gavage. The purpose of this study was to transfer the currently available USP assays for each enzyme and to optimize the assays to analyze the enzymes individually in an oral gavage formulation containing sodium acetate buffer. Freeze-thaw stability as well as long term frozen (-20°C) stability following 0.5, 1.0 and 2.0 months was assessed to determine the shelf life of these individual enzyme formulations in sodium acetate buffer.

2 Method

The principle of analysis of lipase is based on hydrolysis of olive oil into di- and monoglycerols and fatty acids. Each bond hydrolyzed produces one fatty acid and causes acidification of the solution and thus the pH drop. Sodium Hydroxide is added to neutralize the free H+ ions and the pH of solution is maintained at 9.0. The enzyme kinetics of the test sample is analyzed by comparing it with the reference standards. The protease activity is measured by its ability to digest casein. The digested proteins are then quantified by measuring the absorbance value on the spectrophotometer. From the standard curve, the activity of the protease samples can be determined in terms of Units, which is the amount of micromoles of tyrosine equivalent released from casein per minute. Amylase activity is measured by the digestion power of starch by breaking the glycosidic linkage. Starch reacts with iodine to form a blue to black complex and the solution is titrated against sodium thiosulfate until the blue color disappears and all iodine is consumed. The volume required of the titrant is used to back-calculate the activity of the amylase enzyme.

AMYLASE

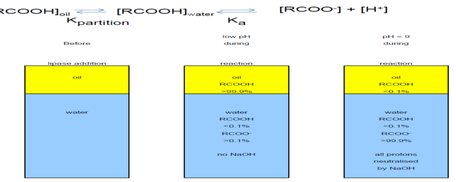
- Amylase is starch hydrolyzing enzyme that converts starch into sugars.
- It is present in the saliva of mammals as well as in pancreatic fluids.
- One USP unit of amylase activity is contained in the amount of pancreatin that decomposes starch at an initial rate such that 0.16 µEq of glycosidic linkage is hydrolyzed per minute under the conditions of the assay (pH 6.8 and 25 °C).
- The amount of 0.1 N sodium thiosulfate consumed in the titration of a soluble starch substrate is measured and compared to the USP reference standard.
- Reaction: 25 mL of starch substrate + 10 mL of pH 6.8 phosphate buffer+ 1.0 mL of sodium chloride+ 1.0mL of Enzyme. Add 1.0 mL of 1N hydrochloric acid to stop the reaction.
- Titrate it against 0.1 N sodium thiosulphate after adding 10 mL of 0.1N iodine + 45 mL of 0.1N sodium hydroxide.



LIPASE

- Lipase is a water-soluble enzyme that acts at the oil-water interface to catalyze the hydrolysis of ester bonds in lipid substrates into free fatty acids and glycerol.
- It is used in several industries including pharmaceuticals, detergents, cosmetics, leather processing, etc.
- Sources of Lipase could be animal, microbial or fungal.
- Titrimetric assay could be used to analyze lipase activity.
- One USP Unit of Pancreatin Lipase activity is contained in the amount of pancreatin that liberated 1.0µEq of acid per minute under the conditions of the assay (pH 9.0 and 37°C). The amount of 0.1N sodium hydroxide titrated to keep the olive oil/acacia emulsion substrate at pH 9.0 is measured and compared to the USP reference standard.
- pH-stat Method is a standard method that could be used. It involves titrating free fatty acids released by lipase breakdown of triglycerides using sodium hydroxide solution.

- Reaction: 10 mL Emulsion + 2.0 ml Bile Salt solution + 8.0 mL Buffer + 9.0 mL distilled water. Adjust pH to 9.2 and add 1.0 mL of Enzyme (Total Volume 30 mL) .



PROTEASE

- One USP Unit of Protease activity is contained in the amount of pancreatin that hydrolyzes casein at an initial rate such that there is liberated per minute an amount of peptides not precipitated by trichloroacetic acid that gives the same absorbance at 280 nm as 15nmol of tyrosine under the conditions of the assay (pH 7.5 and 40°C). The hydrolysate from the casein substrate is measured spectophometrically and compared to the USP reference standard.
- Reaction: potassium phosphate buffer pH 7.5 (1.0, 1.5 and 2.0 mL) +1.5 mL of enzyme+ 2.0 mL of casein+ 5.0 mL of trichloroacetic acid to stop the reaction. Filter and read the absorbance at 280 nm.

	Amylase	Lipase	protease
Method Transfer (%Label Claim)	90.1	81.3	102
Freeze-thaw stability (% Time-zero)	106	107	99.3
0.5 Month (-20°C) frozen stability (% Time-zero)	96.5	110	98.3
1.0 Month (-20°C) frozen stability (% Time-zero)	102	112	91.1
1.0 Month (-20°C) frozen stability (% Time-zero)	94.3	113	92.6

3 Results

- The USP methods were transferred successfully for analyzing the enzymes in an oral gavage formulation containing sodium acetate buffer.
- The lipase method was optimized in terms of techniques used to formulate the olive oil substrate formulation as well as for the use of a manual titrator compared to an autotitrator used typically.
- The olive oil substrate was homogenized followed by sonication in order to obtain the desired particle size necessary for the assay.
- Important points to be considered for lipase assay were substrate preparation, concentration of substrate, size of lipid droplets, consistency of temperature, stirring rate, uniform enzyme preparation time and concentration of olive oil.
- The important factors for the amylase assay was to ensure uniform reference standard stock formulations.
- For the protease assay, the important factors were constant temperature and the preparation of casein substrate.
- It was reported that the minimum volume of formulation needed for each enzyme to be prepared for analyses was 50 mL in sodium acetate buffer. Any volume lower than that would result in a non-homogenous suspension.

4 Conclusion

- The results of the enzyme method transfer as well as the freeze-thaw and long term frozen (-20°C) stability met the acceptable criteria, i.e., the %LC should be 100±25% of the target concentration and for stability, the mean-post storage concentration should not be <90% of the pre-storage concentration.
- Long term stability following 0.5, 1.0 and 2.0 months of frozen(-20°C) storage was also established for all three individual enzymes.

5 Reference & Acknowledgements

- www.USP.org
- I want to thank the staff of the Analytical Chemistry group at Charles River Laboratories Ashland LLC for their contribution towards the study.