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Drug Standards

Standardization of Papain Activity

Report of a Collaborative Study

By EDGAR A. LAZO-WASEM

Methods of assay for the enzyme papain were evaluated, and those endorsed are presented. A procedure which measures the hydrolysis of casein under standardized conditions was found to be the method of choice.

PAPAIN, a crude or purified proteolytic enzyme derived from the tropical plant *Carica papaya*, has been used in the pharmaceutical and food industries for over half a century. Twenty years ago, a monograph for papain was included in the eighth edition of the "National Formulary" (1). The then official assay procedure consisted of a limit test based on digestion of beef muscle.

After deletion of papain from the "National Formulary," many procedures came into use for the standardization of commercial papain. For pharmaceutical and food grade papain, the most widely used procedures have been milk-clotting (2), casein digestion (3), and digestion of hemoglobin (4, 5). For crystalline papain, most laboratories have, at least recently, relied on the initial rate of hydrolysis of synthetic peptide substrates such as *N*-benzoyl-L-arginine ethyl ester hydrochloride.

In an attempt to bring about unification in methods of assay throughout United States laboratories, a committee was established within the Quality Control Section of the Pharmaceutical Manufacturers Association in the fall of 1962. This group was to study current prevailing methods and recommend a generally acceptable method for use throughout the industry. This report describes the findings and recommendations of that committee.

PLAN OF STUDY AND RESULTS

Member firms of the Pharmaceutical Manufacturers Association, representing manufacturing suppliers and pharmaceutical firms marketing papain in dosage forms, were invited to supply their procedures. The methods received involved either milk-clotting, casein digestion, or hemoglobin digestion. From the procedures received, three assays based on the above principles were prepared and forwarded to eight laboratories for collaborative study. "Standard" and "unknown" papain preparations were also forwarded, and thus an effort was initiated whereby

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A study by the Committee on Papain, Quality Control Section, Pharmaceutical Manufacturers Association. Committee Membership: J. E. Giesemann, Brayten Pharmaceutical Co.; N. Kartinos, Baxter Laboratories, Inc.; G. F. McCutcheon, S. B. Penick & Co.; C. F. Peterman, Kremers-Urban Co.; J. V. Saenger, Warner-Chilcott Laboratories; I. S. Shupe, Winthrop Laboratories; L. A. Underkoffler, Miles Chemical Co.; and E. A. Lazo-Wasem, Strong Cobb Arner, Inc. (Chairman).
After the study reported here was underway, it was learned that efforts toward uniformity of enzyme assays, including papain, were being made by the International Commission for the Standardization of Pharmaceutical Enzymes, Fédération Internationale Pharmaceutique. Since then this writer has been kept informed of the efforts of this predominantly European group, the initial studies of which have been excellently summarized in the commission's First Report (6). For papain, the commission has endorsed a method based on the initial rate of hydrolysis of a synthetic substrate, *N*-benzoyl-L-arginine ethyl ester hydrochloride, for both crystalline papain and less purified preparations. A comparison of the unit of activity reported here with that adopted by the commission will be the subject of a future report.

the potency of the preparations using each procedure was compared. An analysis of intra- and inter-laboratory variation showed all three procedures to be equally reliable. The milk method (visual observation of the clotting of milk by the enzyme, an initial step in the proteolysis of casein) was found to require personal experience by the analyst to correctly time the corresponding subjective end point, but was found to be the simplest of the methods tried, and thus, worthy of further work. Difficulty was encountered at some laboratories in the duplication of the hemoglobin procedure studied, and thus no further study with this procedure was recommended. The milk-clotting and casein digestion methods were adopted as choice methods. Details of the two adopted assay procedures, as used by the committee, are described below.

Casein Digestion Assay

Reagents.—*Sodium Phosphate, 0.05 M.*—Dissolve 7.1 Gm. of anhydrous disodium phosphate in sufficient water to make 1000 ml. Add a drop of toluene as preservative.

Citric Acid, 0.05 M.—Dissolve 10.5 Gm. of citric acid monohydrate in sufficient water to make 1000 ml. Add a drop of toluene as preservative.

Casein Substrate.—Disperse 1 Gm. of Hammersten type casein in 50 ml. of 0.05 *M* sodium phosphate. Place in a boiling water bath for 30 min. with occasional stirring. Cool to room temperature and add 0.05 *M* citric acid to pH 6.0 ± 0.1. Solution must be stirred rapidly and continuously during addition of the 0.05 *M* citric acid to prevent precipitation of the casein. Dilute to 100 ml. with water. Prepare fresh daily.

Phosphate–Cysteine Disodium Ethylenediaminetetraacetate Buffer Solution.—Dissolve 3.55 Gm. of disodium phosphate in 400 ml. of water in a 500-ml. volumetric flask. Add 7.0 Gm. of disodium ethylenediaminetetraacetate and 3.05 Gm. of cysteine hydrochloride monohydrate. Adjust to pH 6.0 ± 0.1 with 1 *N* HCl or 1 *N* NaOH solutions, and dilute to volume with water. Prepare fresh daily.

Trichloroacetic Acid, 30% (TCA).—Dissolve 30 Gm. of reagent grade trichloroacetic acid in water and dilute to 100 ml. with water.

Standard Test Dilution (Standard).—Accurately weigh 100 mg. of N.F. papain reference standard in a 100-ml. volumetric flask and add buffer solution to dissolve. Dilute to volume with buffer solution. Further dilute 2 ml. of this solution to 50 ml. with buffer solution. Use within 30 min. after preparation.

Assay Test Dilution (Unknown).—Accurately weigh an amount of sample containing an activity equivalent to 100 mg. of reference standard and proceed exactly as in the preparation of the *Standard Test Dilution.*

Procedure.—Into each of 12 test tubes (18 × 150 mm.) pipet 5.0 ml. of casein substrate. Place in a 40° water bath and allow 10 min. to reach bath temperature. Into each of two of the tubes (tests are run in duplicate except for the blanks) labeled S_1, pipet 1 ml. of standard and 1 ml. of buffer solution, mix by swirling, note zero time, stopper, and replace in the bath. Into each of two other tubes labeled S_2, pipet 1.5 ml. of standard and 0.5 ml. of buffer solution, and proceed as before. Repeat this procedure for two tubes labeled S_3, to which 2 ml. of standard is added, and for two tubes labeled U_2, to which 1.5 ml. of unknown and 0.5 ml. of buffer solution are added. After exactly 60 min., add to all 12 tubes 3 ml. of 30% TCA and shake vigorously. With the four tubes to which no standard or unknown solutions were added, prepare blanks by pipetting, respectively: 1 ml. standard plus 1 ml. buffer solution, 1.5 ml. standard plus 0.5 ml. buffer solution, 2 ml. standard, and 1.5 ml. unknown plus 0.5 ml. buffer solution. Replace all tubes in the 40° bath for 30–40 min. to allow to fully coagulate the precipitated protein. Filter through Whatman No. 40 or equivalent filter paper, discarding the first 3 ml. of filtrate (filtrates must be completely clear). Read the absorbance at 280 mμ of the filtrates of all solutions against their respective blanks. Plot the readings for S_1, S_2, and S_3 against the enzyme concentration of each corresponding level. By interpolation from this curve, taking into consideration dilution factors, calculate the potency of the sample in units/mg.

Calculation of Potency.—

$$C \times \frac{100}{\text{sample wt. (mg.)}} \times \frac{50}{2} \times \frac{10}{1.5} \times A = \text{units/mg.}$$

where C = mg./ml. obtained from the standard curve, and A = activity of reference standard in units/mg.

Milk-Clotting Assay

Reagents.—*pH 4.5 Buffer Solution Concentrate.*—Mix 2 vol. of 1 *M* acetic acid with 1 vol. of 1 *M* sodium hydroxide. Check pH and adjust.

Dilute Buffer Solution.—Dilute 2 vol. of the pH 4.5 concentrated buffer solution to 15 vol. with water.

Milk Substrate.—Mix thoroughly 50 Gm. of milk powder with 215 ml. of dilute buffer solution in a Waring blender or similar device. Add a trace of octyl-alcohol (2-ethyl-1-hexanol) to decrease foaming during the blending operation. Filter through cheesecloth into a clean bottle. Add a few drops of toluene as a preservative. Allow to stand a few hours before use. Use the day of preparation. Keep refrigerated.

Phosphate–Cysteine–Disodium Ethylenediaminetetraacetate Buffer Solution (P–C–EDTA Buffer).—Dissolve 3.55 Gm. of disodium phosphate in 400 ml. of water in a 500-ml. volumetric flask. Add 7.0 Gm. of disodium ethylenediaminetetraacetate and 3.05 Gm. of cysteine hydrochloride monohydrate. Adjust to pH 6.0 with 1 *N* HCl or 1 *N* NaOH solutions, and dilute to volume with water. Prepare fresh daily.

Standard Test Dilution (Standard).—Accurately weigh 100 mg. of N.F. papain reference standard in a 50-ml. volumetric flask and add P–C–EDTA buffer solution to dissolve. Bring to volume with more P–C–EDTA buffer solution. Use within 30 min. after preparation.

Assay Test Dilution (Unknown).—Accurately weigh about 100 mg. of sample and dilute with P–C–EDTA buffer solution to obtain an activity approximately equivalent to that of the standard test dilution.

Procedure.—Pipet 25 ml. of milk substrate into each of a series of test tubes (25 × 150 mm.), close with rubber stoppers, place in a water bath at 40 ± 0.5°, and allow the contents of the tubes to reach bath temperature. (The assay is quite sensi-

tive to temperature. Variations of 1.0° can introduce errors of about 10%.) At zero time, pipet 2 ml. of standard and discharge the contents into one of the tubes. Stopper the tube, shake briefly but gently, so as to avoid the inclusion of air bubbles, and return to the bath. Using a stopwatch, measure the time from the addition of the standard test dilution until clotting of the milk begins. Roll the tube gently back and forth in a horizontal position while in the bath. (Less than 1 min. prior to clotting, the milk will appear to thicken somewhat and will no longer drain readily from the walls of the tube.) Watch the smooth film of milk closely from this point on. The end point is the almost instantaneous appearance of a granular character in the milk film. The time required for the end point to be reached should not be less than 100 sec. and not more than 150 sec. If not, the test should be repeated using a higher or lower enzyme concentration level. Once the right time limit of activity has been found, the test should be repeated at least once, and the average time recorded. Repeat the determination using the assay test dilution in the same volume as used for the standard.

Calculation of Potency.—Determine the relative potency of the sample in terms of the reference standard preparation by the equation:

$$\frac{w^S \times t^S}{w^U \times t^U} \times D = \text{units/mg.}$$

where

w^S = mg. of reference standard added to milk,
t^S = time in seconds for standard to produce clotting,
w^U = mg. of sample added to milk,
t^U = time in seconds for sample to produce clotting,
D = activity of reference standard in units/mg.

A second collaborative study further comparing milk-clotting and casein digestion again showed total equivalence of the two procedures. Interlaboratory variation in determining relative potency of two preparations by either method was below 5%. At this point, the committee felt that in view of the objective end point of the casein procedure (spectrophotometric measurement of released products of casein digestion), this method was the one of choice.

After selection of the assay procedure, the committee turned its attention to the selection of a suitable reference standard preparation. This study involved assay of preparations of varying potency, type, and origin.

The adopted standard preparation was tested for its tyrosine-releasing activity when allowed to act on a standard casein substrate. Definition of a unit of activity was done essentially following the conditions recommended by the International Union of Biochemistry, which states that an enzyme unit should be defined as the amount which catalyzes transformation of a stated amount (1 μm.) of substrate per unit time under defined conditions.

After experimental study at various laboratories, the committee agreed on the following definition of a papain unit: one unit represents the activity which releases the equivalent of 1 mcg. of tyrosine from a standard casein substrate, under specified conditions and at the enzyme concentration which liberates 40 mcg. of tyrosine per ml. of test solution. Using this unit definition, the preselected reference standard was found to contain 6600 units per mg. This N.F. papain reference standard is being made available through the offices of the National Formulary, American Pharmaceutical Association, Washington, D. C. The method of assay has been adopted by the Food Chemicals Codex. The committee agreed that papain for pharmaceutical use should have a potency of not less than 6000 papain units in each mg., when tested by the above described method.

SUMMARY AND CONCLUSIONS

1. Prevailing methods in the U. S. pharmaceutical and food industry for papain standardization have been studied by a Committee of the Pharmaceutical Manufacturers Association. Of the methods evaluated, that which measures hydrolysis of casein under standardized conditions was endorsed.

2. A preparation to be used as the reference standard in conjunction with the assay procedure was selected.

3. The papain reference standard has been submitted to the National Formulary for storage and distribution. The casein digestion method of assay has been adopted by the Food Chemicals Codex.

4. It is hoped that as many segments of industry as possible will begin using the recommended method and unit for routine quality control and for the labeling of papain activity.

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