Reaction of Piperine with Nitric Acid. Adaptation to Quantitative Assay of the Piperine Content of Pepper

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Piperine reacts with concentrated nitric acid to give an unstable yellow color which changes to an unstable red color on the addition of alkali. This color has been stabilized by aqueous 5 per cent thiourea. The absorbance at 490 mμ is proportional to the amount of piperine present, and the reaction has been adapted to the quantitative determination of the piperine content of pepper. The concentration of and reaction time with nitric acid, the temperature of the reaction with nitric acid, the concentration of potassium hydroxide, and the time and temperature of heating the mixture after the addition of the potassium hydroxide are important variables for obtaining reproducible results. Water, ethyl and methyl alcohols, and other solvents inhibit the reaction. The red is stable for as long as 2 hours and obeys Beer's law over the range of 0.02 to 0.20 μmole of piperine/ml. of reagent.

PIPERINE, when treated with concentrated nitric acid, forms a yellow resinous mass. If strong alkali is added to this yellow resin, a red color develops (1). Attempts to use this color reaction for the quantitative determination of piperine have met with little success due to its gross instability (2). With proper control of the reaction variables, the red color can be stabilized enough to permit a rapid and simple quantitative assay of the piperine content of pepper.

EXPERIMENTAL

Materials.—Piperine, m.p. 129–130° (Inland Alkaloid, Tipton, Ind., and K and K Laboratories, Long Island, N. Y.), absolute ethyl alcohol and methyl alcohol, 40% aqueous potassium hydroxide, and 5% aqueous thiourea U.S.P. were employed. Piperine, isolated from black pepper according to the procedure of Marion (3), also was used. The purity of this and that of the commercial samples was checked by determining its melting point (130°) after three recrystallizations from ethyl alcohol (2) and by the ultraviolet spectrophotometric method (4).

Equipment.—A Beckman DU spectrophotometer, 40-ml. Pyrex glass-stoppered test tubes, permanently etched at the 10-ml. mark, or 10-ml. volumetric flasks, and 10-ml. and 50-ml. burets were utilized.

General Procedure.—One milliliter containing 0.0–2.0 μmole of piperine dissolved in absolute ethyl alcohol or methyl alcohol was placed in consecutive glass-stoppered test tubes or 10-ml. volumetric flasks. The tubes or flasks were placed in a boiling water bath for 5 minutes to evaporate the alcohol. After cooling in tap water, 0.5 ml. of concentrated nitric acid was added to each test tube and the tubes incubated in a water bath at 30° for 5 minutes. Four milliliters of 40% KOH was then added to each tube and the tubes placed in a boiling water bath (100 ± 0.5°) for 5 minutes. After cooling to room temperature (29 ± 1°), the contents of the tubes were made up to 10 ml. by adding 5% aqueous thiourea. The mixtures then were allowed to stand at room temperature (29 ± 1°) for 30 minutes, after which the intensity of the red color developed was measured at 490 mμ against a reagent blank similarly treated.

Influence of Variables on the Reaction.—A constant quantity (1.0 μmole) of piperine was treated according to the general procedure, except that the variables listed in Table I were tested over the ranges indicated. Efforts to stabilize the red color led to the selection of 5% aqueous thiourea which was added after the heating of the piperine–HNO_3–KOH mixture.

Quantitative Response of Piperine.—After the influence of the several variables was established, the quantitative response of piperine was tested. For this, 0.0 to 2.0 μmole of piperine was treated according to the general procedure, and the absorbance of the color developed was measured at 490 mμ *versus* a reagent blank. A linear relationship was obtained from a plot of absorbance *versus* amount of piperine added (Fig. 2).

Determination of Piperine Content of Pepper and Comparison to Other Methods.—Piperine extracted from black pepper by the method of Lee (2) was determined according to the general procedure, by the ultraviolet spectrophotometric method (4), and the chromotropic acid method (2). The results are shown in Table II. Recovery data are shown in Table III.

DISCUSSION

Several variables are critical for reproducible quantitative results. The strength of the nitric acid used is rather limiting and, below 15 *M*, color production will be reduced. Although the amount of acid used can be varied between any desired limits, as the amount is increased the quantity of alkali subsequently added must likewise be increased to ensure maximum color development. The amounts and strengths of acid and alkali selected (Table I) must be adhered to; otherwise, maximum color development will not be achieved or, on cooling, precipitation of salts formed may occur. If the method is scaled up or down, consistent results can be achieved, provided the acid and alkali proportions are retained.

The piperine–HNO_3 reaction time is extremely critical. If it is less than 5 minutes, maximum color development will not be achieved due apparently to incomplete nitration. Beyond 10 minutes, degradation probably ensues, and low results will be obtained.

The temperature of the piperine–HNO_3 reaction is also critical. At 10° or lower, nitration probably is not rapid enough, even after extended periods, to allow for maximum color development. Above 40°, degradation seems to occur rapidly, and low results will be obtained. For convenience, a temperature of 30 ± 1° was chosen.

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TABLE I.—INFLUENCE OF VARIABLES ON THE DETERMINATION OF PIPERINE BY THE HNO_3–KOH–THIOUREA METHOD

Variable Investigated	Range Investigated	Conditions for Reproducibility Min.	Conditions for Reproducibility Max.	Value Selected
Concn. of HNO_3 used, *M*	7.99–15.99	15.99	15.99	15.99
ml. concn. HNO_3 added to system	0.5–2.0	0.5	0.5	0.5
Reaction time for piperine–HNO_3 reaction, min.	0–60	5	10	5
Presence of solvents in HNO_3–piperine reaction	Alcohol and other solvents inhibit			None
Temp. of reaction of HNO_3–piperine, ° C.	10–100	10	40	30
ml. 40% KOH added	1–10	3.5	4	4
Temp. of heating after adding KOH, ° C.	5–100	80	100	100
Time of heating at 100° C. after adding 40% KOH, min.	2–30	4	6	5
Concn. of aqueous thiourea added, % w/v	0–10	3.5	6	5.0
Stability of red piperine–HNO_3 color, hr.	0.1–24	0.5	2	0.5
Solvent for red color	H_2O and many other solvents tried			5% thiourea in H_2O

TABLE II.—COMPARISON OF THE DETERMINATION OF PIPERINE CONTENT OF PEPPERS BY DIFFERENT METHODS

Method	Piperine Content of Peppers Analyzed, %[a] P_1	P_2	P_3 (White pepper)
Ultraviolet spectrophotometric	6.7	5.7	5.0
Chromotropic acid	7.8	7.4	6.35
HNO_3–KOH–thiourea	7.6	7.1	6.26

[a] Reported as percentage of original material analyzed. Average of eight different determinations.

TABLE III.—RECOVERY OF PIPERINE ADDED TO SAMPLE OF BLACK PEPPER

Piperine Added, μmole	Piperine Recovered, μmole	Recovery,[a] %
10	9.92	99.2
20	20.1	100.5
30	30.2	100.7
40	39.8	99.5

[a] Average of five different determinations.

Several solvents for piperine were tried, and all inhibited the reaction between the alkaloid and HNO_3. Therefore, prior to the addition of the HNO_3, solvents were removed by heating over a steam bath.

Heating for more than 6 minutes or less than 4 minutes at 100° will lead to low results. Below 80°, heating this mixture for 5 minutes will not lead to maximum color production.

The red color developed in the piperine–HNO_3 reaction is highly unstable (1, 2); its intensity decreases on aging. Of several chemicals tried as "stabilizers," aqueous 5% thiourea proved to be the most effective. It must be added after the heating of the piperine–HNO_3–KOH mixture. Color development will not occur if it is added before.

The method gives results approximately like those obtained by the chromotropic acid method (Table II). Both methods measure not only piperine but also piperic acid and piperonal. Piperettine and chavicine (3, 5) probably would be measured also.

Although piperine, piperic acid, and piperonal all gave the typical red color and absorbed at around 490 mμ (Fig. 1), their responses differed widely. On a molar basis, if the response of piperine is taken as 1.0, then that of piperic acid was 0.95 and that of piperonal 0.08. This reflects the ease of nitration of the compounds. A similar trend is found in the xanthoproteic reaction of the aromatic amino acids, where tyrosine and tryptophan are readily nitrated, but phenylalanine is not. Tryptophan responded but gave a comparative value of 0.56, an indication that the optimum conditions for piperine may not hold for other responsive aromatic compounds or that side chains in the molecule may influence color intensity.

Although not so sensitive, the total time involved for an analysis is less than that needed in the chromotropic acid procedure, and the method is fairly precise, with a standard deviation established from 10 different determinations of ± 1.86%, when applied to commercial piperine, and of ±1.92% to ±1.98% when applied to the determination of

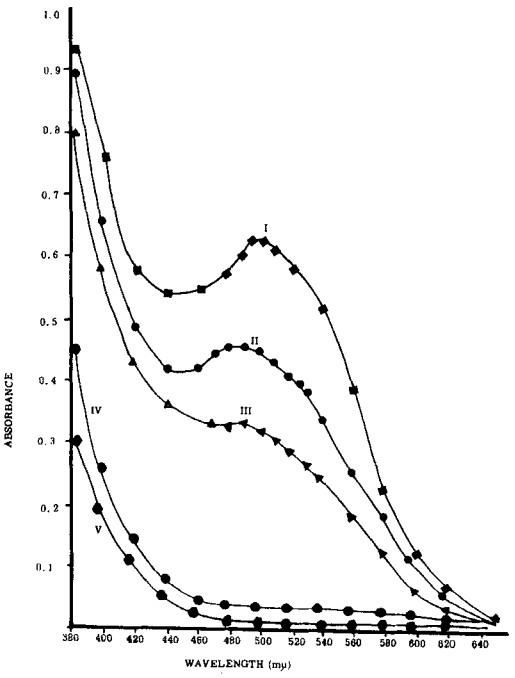

Fig. 1.—Absorption spectra of colors produced by piperine and its degradation products in the HNO_3–KOH–thiourea method. Key: I, piperine; II, piperic acid; III, piperonal; IV, piperidine; V reagent blank.

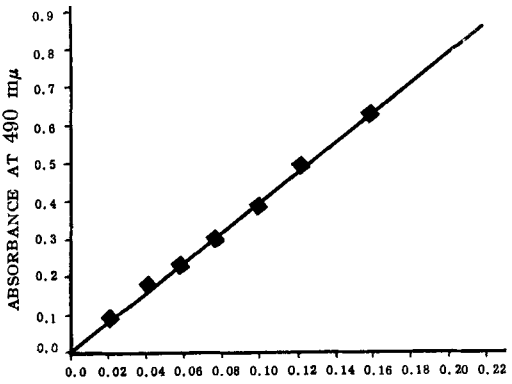

Fig. 2.—Quantitative response of piperine in the HNO_3–KOH–thiourea method.

piperine in pepper samples. With four levels of piperine added to black pepper, good recoveries of 99.2% to 100.7% (Table III) were obtained.

The standard curve for piperine (Fig. 2) obeys Beer's law between the levels of 0.02 to 0.2 μmole of piperine per ml. of reagent. It can be described by:

$$X = 0.25785\ Y + 0.00123$$

where X = micromoles of piperine per milliliter of reagent and Y = absorbance at 490 mμ.

Therefore

$$\%\ \text{piperine in sample} = \frac{X(0.28533)(\text{dilution factor})}{\text{sample wt. (mg.)}} \times 100$$

The red chromogen is stable for as long as 2 hours and has a molar extinction coefficient of 5.299×10^3. It is unstable in acids, strong alkalies, water, alcohols, chloroform, and many solvents tried.

All compounds with the benzene nucleus will interfere; the severity of interference depends on the ease of nitration.

Reproducibility of absorbance values from day to day was good. Over a period of 20 consecutive days, runs at three levels of 0.05 to 0.15 μmole of piperine and pepper extracts containing 5.0–7.2% piperine showed absorbance variations of only 0.01 to 0.02.

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Effect of Ultrasound Energy on Hydrolysis of Aspirin

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The effect of ultrasonic energy on the hydrolysis of aspirin solutions at various temperatures and pH values is described. The reaction kinetics followed a pseudo first-order rate, both with and without the influence of ultrasound. The rate of hydrolysis was increased in all cases by applying sound energy. A mathematical treatment of the data disclosed that the effect of ultrasonic energy on the hydrolysis rate is relatively constant and can be compared to the effect obtained by increasing the reaction temperature.

THE VERSATILITY of ultrasonic energy, particularly as it has been employed to increase the rate of a number of chemical reactions, has been demonstrated by several investigators in the field (1–5); however, no references to the use of ultrasound in accelerated drug stability studies were found in the literature. Since such an application might offer advantages over the use of traditional physical means for effecting acceleration of degradation, this study was undertaken. The extensive work reported on aspirin–water systems and their degradation without the influence of ultrasound (7) led to the selection of this system for such a study.

EXPERIMENTAL

Equipment.—The ultrasonic energy was supplied by a 100-kc. generator[1] operated at the maximum plate voltage of 1000 v. The transducer consisted of a mounted barium titanate crystal. Fitted to the inner wall of the ultrasonic bath was a round copper coil connected through an inlet–outlet pump arrangement to a separate constant-temperature water bath,[2] and controlled so that the temperature of the two baths was both constant and identical, within the limits of ±0.2°, during the individual runs.

Procedure.—Two sets of duplicate samples of aspirin in appropriate buffer solutions were used for all degradations. Duplication was accomplished by preparing a stock solution containing 100 mg. of aspirin per 100 ml. of water. Dissolution of the aspirin was aided by the addition of 5.0 ml. of 95% ethanol. Aliquots were pipeted from the freshly prepared stock solutions and brought to volume with the appropriate buffer in 50-ml. volumetric flasks. When the reaction was followed under conditions of high aspirin stability, it could only be allowed to go to about 10% completion, since the instrument

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[1] McKenna model 100 Generator, McKenna Laboratories, Santa Monica, Calif.

[2] Catalog No. 3052, Labline Instruments Inc., Chicago, Ill.