Characterization of Crystalline Calcium Pantothenate by Physicochemical Assay Methods

M. J. FRANK, P. G. TRAGER, J. B. JOHNSON, and S. H. RUBIN▲

Abstract □ Crystalline calcium pantothenate was characterized by application of analytical techniques including: (*a*) TLC separation of intact calcium pantothenate from its degradation product β-alanine, (*b*) electrochemical methods using a calcium-specific ion electrode, (*c*) spectropolarimetry, and (*d*) colorimetric determination of pantothenate according to Schmall and Wollish. These measurements complement one another to ensure the specificity and stability of crystalline calcium pantothenate. Analysis of calcium pantothenate materials having varying degrees of purity was accomplished by these physicochemical methods as well as the prescribed microbiological procedure. Data indicative of good agreement between the two procedures, within the precision of the microbiological assay, are shown. The spectropolarimetric technique is not applicable to materials that contain optically active species other than calcium pantothenate. Stability data for the solid-state degradation of calcium pantothenate in the presence of ascorbic acid, benzoic acid, barbituric acid, and succinic acid are presented. The stability results are interpreted in terms of general-acid catalysis.

Keyphrases □ Calcium pantothenate, crystalline—characterization by TLC, potentiometric titrimetry, spectropolarimetry, and colorimetry, compared to microbiological method □ Pantothenates, calcium pantothenate—characterization by combination of physicochemical methods, compared to microbiological method □ TLC—separation, calcium pantothenate from β-alanine □ Potentiometric titrimetry—calcium assay, calcium pantothenate □ Spectropolarimetry—determination, optical rotation of calcium pantothenate □ Colorimetry—determination, pantothenate, ninhydrin reagent

The analysis of pantothenates, in the pure state and in dosage forms, has been the subject of numerous publications in the past 3 decades. Several established methodologies, particularly chemical colorimetry and microbiological methods, have been used to determine pantothenates. The colorimetric methods are based on the color reactions of the products which result from the hydrolytic cleavage of the vitamin in acidic or basic media. Acid hydrolysis of pantothenates produces β-alanine (3-aminopropionic acid) and pantolactone (2,4-dihydroxy-3,3-dimethylbutyric acid γ-lactone); with alkali treatment, pantothenates split into β-alanine and pantoic acid (2,4-dihydroxy-3,3-dimethylbutyric acid) sodium salt.

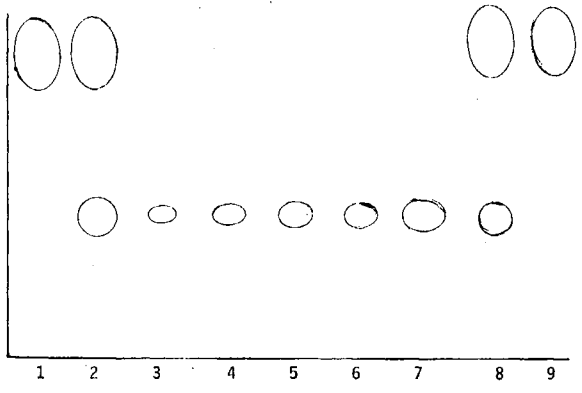

Figure 1—*Thin-layer chromatogram of USP and 80% "stabilized" forms of calcium pantothenate and of its degradation product β-alanine. Key: 1, stabilized form, 250-mcg. experimental sample; 2, stabilized form, 250-mcg. commercial sample; 3, β-alanine, 0.5 mcg.; 4, β-alanine, 1.0 mcg.; 5, β-alanine, 3.0 mcg.; 6, β-alanine, 5.0 mcg.; 7, β-alanine, 10.0 mcg.; 8, USP form, 250-mcg. commercial sample; and 9, USP form, 250-mcg. experimental sample. Note: All samples are initial.*

Colorimetric assay methods described in the literature include the reactions of β-alanine with 2,4-dinitrophenylhydrazine (1), 1,2-naphthoquinone-4-sulfonate (2), or ninhydrin (3); the reaction of 2,7-naphthalenediol with pantolactone (4); the reaction of pantolactone with hydroxylamine and the final development of a purple color with ferric chloride (5); and the chlorination of β-alanine followed by the liberation of iodine on the addition of an iodide solution (6). The colorimetric methods have been modified further by the elimination of interfering substances by solvent extraction and column chromatography prior to colorimetry (7–9), as well as by improvements in the reagents employed in the color-development step (10–12).

The microbiological assay procedures are based on the reaction of a suitable test organism with pantothenate. The microbiological methods are superior to the colorimetric techniques in that the former are specific for the physiologically active *d*-form of the vitamin, whereas colorimetry fails to differentiate between the enantiomorphs of pantothenate. Several microbiological assay methods employing a variety of test organisms have been documented (13–15); the presently recommended procedure described in USP XVIII utilizes *Lactobacillus plantarum* as the test organism. Less publicized analytical methods used to determine pantothenate include the complexometric titration of calcium in calcium pantothenate with disodium ethylenediaminetetraacetate (16), GLC (17, 18), optical rotatory dispersion, and circular dichroism (19).

There appears to be no report in the literature in which a combination of physicochemical assay methods has been applied to characterize crystalline calcium pantothenate [calcium D(+)-pantothenate] completely. The present investigation was initiated to apply existing physicochemical techniques and to develop new methods for the analysis of calcium pantothenate materials having varying degrees of purity. The physicochemical techniques utilized include: (*a*) TLC separation of intact calcium pantothenate from its degradation product β-alanine, (*b*) electrochemical methods using a calcium-specific ion electrode, (*c*) spectropolarimetry, and (*d*) colorimetric determination of pantothenate according to Schmall and Wollish (7). These methods

complement each other to ensure specificity in the analysis of crystalline calcium pantothenate, as evidenced by the excellent agreement of assay results obtained with the physicochemical techniques and the prescribed microbiological procedure (within the limits of reproducibility of the latter).

In addition to the analytical investigation, a study was initiated to determine the kinetics and mechanism of the solid-state degradation of calcium pantothenate, particularly in the presence of such organic acids as ascorbic acid, barbituric acid, benzoic acid, and succinic acid. Due to the complexity of the interactions involved, only preliminary stability data are presented; the entire kinetic study will be published later.

EXPERIMENTAL

Apparatus—*TLC*—Thin-layer plates were prepared by coating 20 × 20-cm. glass plates with 0.5 mm. of a cellulose MN 300 G/UV[1] slurry and were dried overnight at 40°. A commercially available spreader[2] was used to prepare all plates.

Potentiometric Titrimetry—Saturated calomel and calcium-specific ion electrodes[3] were employed as the reference electrode and auxiliary electrode, respectively. The potential changes during the titration were measured with a digital pH meter[4], and all titrations were performed with an automatic constant-rate buret[5] in synchronization with a linear recorder[6].

Spectropolarimetry—Optical rotations were measured with a single-beam digital polarimeter[7] using a cell with a 100-mm. path length.

Colorimetry—Spectra and spectrophotometric assays were determined with a double-beam UV/visible recording spectrophotometer[8] using matched 1-cm. quartz cells.

Reagents—Specific reagents are described under the individual analytical procedures. All other common reagents were ACS certified grade. Distilled, deionized water was used for the preparation and dilution of all solutions unless otherwise specified.

Analytical Procedures—*TLC*—Calcium pantothenate (250 mg.) was dissolved in 10 ml. of water, and 10 μl. (250 mcg.) was applied to the plate. Then 1.000 g. of β-alanine[9] was dissolved in 100 ml. of water, and solutions containing 0.125, 0.25, 0.50, 1.0, 3.0, 5.0, and 10.0 mcg./5 μl. of solution were prepared. Five microliters of each solution was applied to the plate to serve as a comparison standard. The chromatogram was developed in a solvent system consisting of *n*-butanol–ethanol–water (40:30:30), and the solvent front was allowed to ascend 15 cm. above the point of application of the sample.

The detection reagent was prepared by dissolving 300 mg. of ninhydrin[10] in 100 ml. of *n*-butanol and adding 3 ml. of glacial acetic acid. The plate was then sprayed with the ninhydrin reagent and heated for 30 min at 105°. The β-alanine and calcium pantothenate appear as violet bands at R_f 0.40 and 0.65, respectively. Since 2.67 mg. of calcium pantothenate degrades to form 1.0 mg. of β-alanine, the β-alanine standard that corresponds to the β-alanine found in the sample must be multiplied by 2.67 to obtain the amount of calcium pantothenate that has degraded. A typical chromatogram is presented in Fig. 1.

Although the analytical procedure for determining the percent intact drug retained may have a precision of ±2% and the estimation of degradation by TLC may have a precision of only ±20%, it is more accurate to gauge degradation by the amount of decomposition product formed when the amount of degradation lies in the 0–5% range. The direct determination of small amounts of decomposition products by TLC is certainly more meaningful than ascertaining degradation based on small variations in the assay for the intact drug.

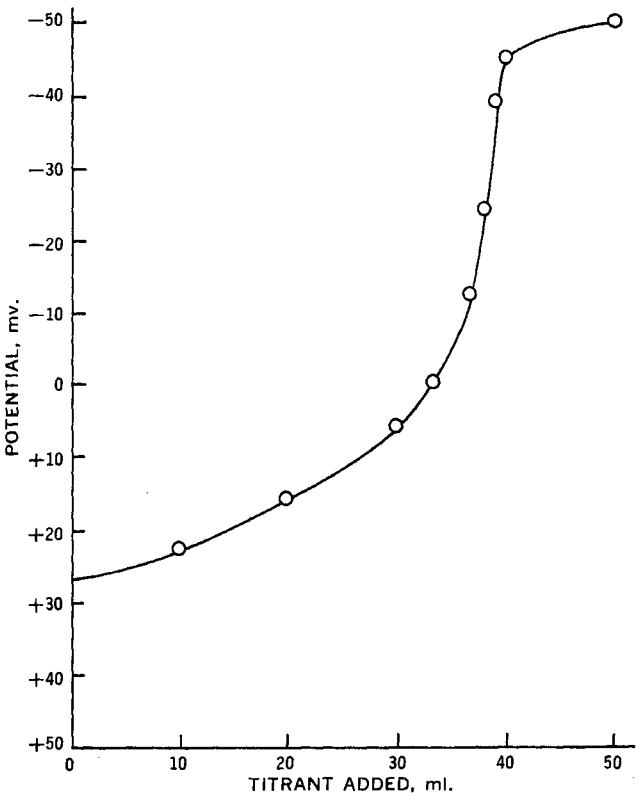

Figure 2—*Titration curve of calcium pantothenate against 0.05* N *disodium ethylenediaminetetraacetate.*

Calcium Assay—*Reagent: pH 10 Buffer*—Ammonium chloride (4.3 g.) and 26.6 ml. of concentrated ammonium hydroxide were dissolved and diluted to 1 l. with water.

Reagent: 0.05 N *Disodium Ethylenediaminetetraacetate, Dihydrate*—Disodium ethylenediaminetetraacetate, dihydrate, 18.61 g., was dissolved in 1 l. of water.

Procedure—Approximately 4.0 g. of calcium pantothenate was weighed accurately and transferred to a 250-ml. volumetric flask. The sample was dissolved in 100 ml. of water and diluted to volume with water. In the event the solution appeared cloudy, Whatman 2^V filter paper was used to filter the solution. A 50-ml. aliquot of the clear solution was pipeted into a 250-ml. beaker, and 100 ml. of the buffer was added. The solution then was titrated with 0.05 *N* disodium ethylenediaminetetraacetate employing the apparatus already described. The titration was repeated on a second 50-ml. aliquot of the stock solution. A characteristic titration profile is illustrated in Fig. 2.

Optical Rotation—The equivalent of 400 mg. of calcium pantothenate was weighed into a 25-ml. volumetric flask and dissolved in 20 ml. of water. The sample was diluted to volume with water and filtered with Whatman 2^V filter paper. If cloudiness persisted, the previously filtered solution was refiltered with 0.45-μ filter paper[11]. The rotation was measured at a suitable wavelength, usually 589 nm., and the specific rotation was calculated. The optical purity was determined by comparing the specific rotation of the sample to that obtained with calcium pantothenate, USP reference standard. Table I shows the specific rotation of calcium pantothenate and pantolactone as a function of wavelength at 20°, using water as the solvent. The specific rotation will vary with the solution composition; *i.e.*, the presence of succinic acid or some other optically inactive solute will affect the specific rotation of the optically active species. The optical rotatory dispersion curves of calcium pantothenate and pantolactone were reported previously in the literature (19). Calcium pantothenate was found to exhibit a positive Cotton

[1] Macherey, Nagel & Co., Duren, West Germany.
[2] Quickfield and Quartz, Ltd., Stone, England.
[3] Orion model 92-20.
[4] Orion Ionalyzer model 801.
[5] Sargent model C.
[6] Sargent model SRL.
[7] Perkin-Elmer model 141.
[8] Beckman Acta III.
[9] Matheson, Coleman & Bell.
[10] Eastman.
[11] Millipore Filter Corp.

Table I –Specific Rotation of Calcium Pantothenate and Pantolactone as a Function of Wavelength at 20°

Wavelength, nm.	Specific Rotation of Calcium Pantothenate	Specific Rotation of Pantolactone
589	+27.4	−50.9
578	+28.8	−53.6
546	+33.0	−62.1
436	+58.8	−121.2
365	+99.1	−227.6

Table II—Precision of Spectropolarimetric Method

Sample	Specific Rotation	Deviation from Mean
1	26.7	−0.4
2	27.4	+0.3
3	26.9	−0.2
4	27.4	+0.3
5	27.4	+0.3
6	26.8	−0.3
7	27.3	+0.2
8	27.1	0.0
9	27.1	0.0
10	27.0	−0.1
Mean	27.1	
SD	0.26	

effect with a peak at 227 nm. (α = 1066), while pantolactone showed a negative Cotton effect with a trough at 233 nm. (α = −6081). The positive Cotton effect in calcium pantothenate and the negative Cotton effect in pantolactone are presumably due to the carbonyl chromophore found in the amide group and the γ-lactone of the two species, respectively.

The precision obtained with the spectropolarimetric method is illustrated in Table II. A standard deviation (*SD*) of 0.26 was obtained in 10 replicate experiments, when the specific rotation was determined at 589 nm. The precision of the spectropolarimetric technique employed in the present study compares favorably to the precision obtained by Schmall and Wollish (7) with the colorimetric methods.

Colorimetry– Calcium pantothenate was determined according to the method of Schmall and Wollish (7), using ninhydrin as the coloring reagent. The procedure and reagents, with the exception of the preliminary column chromatography, are exactly as described in *Reference 7*. The assay consists of the basic hydrolysis of calcium pantothenate under reflux conditions to yield β-alanine and pantoic acid. On heating a dilute solution containing β-alanine and ninhydrin, oxidative deamination results in the formation of a purple species having an absorbance maximum at 570 nm. A known amount of calcium pantothenate is also hydrolyzed and serves as a comparison standard. A Beer's law plot for the β-alanine–ninhydrin complex is presented in Fig. 3.

Microbiological Assay—The microbiological assay was performed as described in USP XVIII (p. 864). When conducted with extreme care, the precision of the assay was ±3%.

Stability Studies—The solid-state stability study was prepared by combining calcium pantothenate with ascorbic, barbituric, benzoic, or succinic acid in a mole ratio of 1:16. This ratio was based

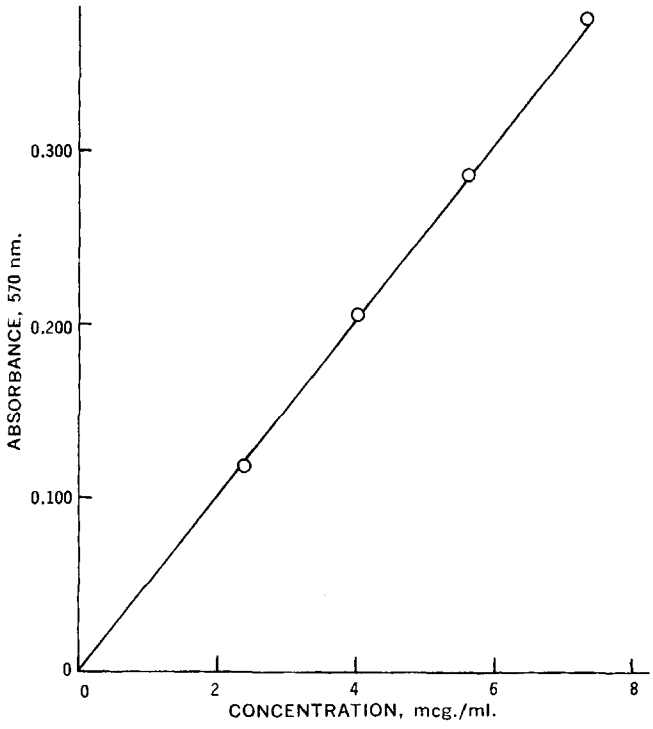

Figure 3—*Beer's law plot for the β-alanine–ninhydrin complex.*

on the recommended dietary allowance of calcium pantothenate and ascorbic acid established by the Food and Nutrition Board of the National Research Council (20). The samples were stored in 10-ml. rubber-stoppered vials at various temperatures and were withdrawn at appropriate times for assay. Whenever possible, the stability was followed spectropolarimetrically at 365 nm., using the specific rotation of calcium pantothenate and that of the degradation product, pantolactone, to determine the amount of intact calcium pantothenate. The microbiological assay method was utilized to assay samples containing ascorbic acid.

The percent of intact calcium pantothenate in a solution originally containing 10 mg./ml., as determined by spectropolarimetry, is given by the following equation:

$$\% \text{ calcium pantothenate} = \frac{A - 0.00545(B)}{0.01(C) - 0.00545(B)} \times 100 \quad \text{(Eq. 1)}$$

where:

A = observed rotation at wavelength λ
B = specific rotation of pantolactone at wavelength λ
C = specific rotation of calcium pantothenate at wavelength λ

RESULTS AND DISCUSSION

The results of the assay of calcium pantothenate obtained with the various analytical methods are summarized in Table III. The percent loss on drying is listed for samples that are theoretically 100% calcium pantothenate in order to provide a better estimation of the accuracy of each method. The data indicate that the precision

Table III—Results of Calcium Pantothenate Assays

Sample	Theoretical Percent Calcium Pantothenate	Percent Loss on Drying[a]	Percent β-Alanine	Assay for Calcium Pantothenate: Titrimetry	Spectropolarimetry	Colorimetry	Microbiological Method
A	80	—	<0.1	82.6	81.1	81.5	82
B	100	2.8	3.8	95.4	92.3	96.4	93
C	100	0.32	<0.05	99.8	98.9	99.5	99
D	80	—	0.05	81.3	81.9	81.7	82
E	100	2.7	3.9	95.0	94.5	96.8	94
F	100	0.78	<0.05	99.7	99.6	99.0	99

[a] Three hours at 105°.

Table IV—Solid-State Stability of Calcium Pantothenate in the Presence of Several Acids[a,b]

Sample	Condition	TLC	Spectropolarimetry	Microbiological Method
Calcium pantothenate + ascorbic acid	4 days, 70°	—	—	10
Calcium pantothenate + barbituric acid	4 days, 70°	25	25	23
Calcium pantothenate + benzoic acid	4 days, 70°	75	73	71
Calcium pantothenate + succinic acid	4 days, 70°	75	76	72
Calcium pantothenate + succinic acid[c]	16 hr., 55°	20	18	20
Calcium pantothenate + ascorbic acid[c]	16 hr., 55°	—	—	6

[a] Mole ratio of calcium pantothenate–acid of 1:16. [b] The pK_1a of each acid is about 4.2. [c] Three percent water.

of the assay for a single sample using the various analytical techniques is extremely good. When considerable amounts of β-alanine are present, the colorimetric method yields consistently high results because the technique is based on the β-alanine–ninhydrin color reaction. This difficulty may be eliminated by separating any preformed β-alanine on a Dowex 50-X4(H) column (7), thus rendering the method rather specific for intact pantothenate.

In view of the excellent agreement between the spectropolarimetric and microbiological methods, particularly in the presence of excess β-alanine, the optical purity of the vitamin appears to be closely approximated by the spectropolarimetric technique. The complexometric titration for calcium complements the assay results obtained with the other methods, as evidenced by the data presented in Table III. The titrimetric method is only for the calcium content and does not reflect the stability of the pantothenate moiety *per se*. The analytical results suggest that these physicochemical techniques, when applied in combination, assure a specificity and precision of assay that allow the accurate determination of the purity, identity, and stability of crystalline calcium pantothenate materials having varying degrees of purity. The spectropolarimetric and TLC methods, however, are not applicable to the assay of calcium pantothenate in multivitamin preparations and in the presence of certain materials, such as ascorbic acid.

To test the applicability of the spectropolarimetric and TLC procedures to stability studies, a limited solid-state compatibility study was initiated. The results of this investigation are tabulated in Table IV. Where spectropolarimetry and TLC are applicable, the agreement between the physicochemical and microbiological assays are excellent. The results are noteworthy in that the amount of degradation observed in the presence of benzoic and succinic acids is much greater than when barbituric acid or ascorbic acid is utilized, although the pK_1a for all the acids is about 4.2. This phenomenon may possibly be due to differences in the nature of the acidic moiety of the acids in question. Benzoic acid and succinic acid are both carboxylic acids, whereas ascorbic acid is a lactone which owes its acidic properties to the presence of an enediol grouping; barbituric acid derives its acidity from acid amide groups. The data suggest that, in the solid state, the effective acidity of benzoic and succinic acids is greater than that of ascorbic and barbituric acids, despite the fact that all exhibit comparable acidity in solution. An extended investigation is presently underway to explain more clearly the above observations as well as to ascertain the fundamental factors affecting the rate and kinetics of the reaction in the solid state.

The data in Table V summarize the stability results obtained on a commercially available "stabilized" calcium pantothenate and an experimental stabilized form tested under a variety of conditions. The stability of Sample A (experimental sample) is clearly superior to that of Sample B under the stress conditions employed; in addition, the amount of β-alanine present initially in Sample B is significant, suggesting that the vitamin should be analyzed for β-alanine by TLC prior to its incorporation into dosage forms.

Table V—Stability of Stabilized Calcium Pantothenate Forms

Condition	Percent β-Alanine A	Percent β-Alanine B
Initial	0.05	5.8
24 hr., 43% relative humidity	0.08	6.0
24 hr., 63% relative humidity	0.11	7.0
24 hr., 75% relative humidity	0.15	8.0
1 week, 55°, as is	0.08	6.0
1 week, 55°, 3% water	0.4	7.0
1 week, 55° + succinic acid[a]	2.0	10.0
1 month, 45° + ascorbic acid[b], 3% water	17	54

[a] Calcium pantothenate + succinic acid (1:4 w/w). [b] Calcium pantothenate + ascorbic acid (1:7.5 w/w).

Although the stability of pantothenates in solution has been well studied (21–23), a detailed stability of the solid state has not been reported. Frost (21) stated that the rate of destruction of calcium pantothenate in the solid state, just as in solution, is dependent upon the effective pH, but he did not define the meaning of this term. The present limited study suggests that the rate of degradation is dependent on the nature of the acid involved and may be related to the concept of general-acid catalysis. Further elucidation of the observed phenomena will be possible upon completion of the detailed investigation in progress.

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