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Liquid Chromatographic Method for Determination of Calcium Pantothenate Preparations and Related Stability Studies

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ABSTRACT

This report describes a fast, simple and reliable HPLC method for the assay and quantitative determination of calcium pantothenate in commercial products. The samples were analyzed on a C18 column with the mobile phase of acetonitrile and potassium dihydrogen phosphate solution (adjusted pH = 2.5 with phosphoric acid) at a flow rate of 1.0 mL/min and UV absorbance detection at 204 nm. Ampicillin was used as an internal standard. The retention times of calcium pantothenate and ampicillin were 5.3 and 6.5 min, respectively. An equation was presented for linear relationship between peak height ratios of calcium pantothenate to ampicillin and the calcium pantothenate concentration over a range of 10-50 μg/mL (r = 0.9999). Standard addition recoveries were greater than 98.96% with twelve commercial products. The relative standard deviations were between 0.1 and 0.9% in inter-day assays, 0.1 and 0.7% in intra-day assays. The results obtained from the HPLC assay method which we developed and the microbiological assay of USP were paired at 95% confidence level. There were no significant differences between these two methods. The proposed HPLC method was a suitable substitute for microbiological method for quantitative assays of calcium pantothenate in commercial products.

Key words: pantothenic acid, bioassay, pharmaceutical preparation

INTRODUCTION

Pantothenic acid, one of the B vitamin complexes, is usually found as calcium pantothenate in solid pharmaceutical preparations because of its stability and handling properties. The United States Pharmacopeia (USP) XXIII[(1)] describes two official methods for analytical assay of calcium pantothenate: microbiological method and HPLC method. The retention time of the HPLC method described in USP XXIII is more than 20 min, but this method is not presented in USP XXV[(2)]. On the other hand, the greatest disadvantages of the microbiological method are long preparation time and unreliable values. Alternative methods have been used to determine calcium pantothenate. Colorimetry, spectrophotometry, fluorometry, GC[(3,4)], thin layer chromatography[(5)], over-pressure layer chromatography[(6)] have been utilized. However, these methods often have poor recovery, reproducibility or are time-consuming. ELISA[(7)] and micellar electrokinetic capillary chromatographic[(8)] method have high specificity and sensitivity, but are not commercially available.

Several capillary electrophoresis[(9)] and LC methods[(10-17)] for the determination of calcium pantothenate in commercial products have been reported. However, the drawback of these procedures is either lack of internal standard or lack of comparison with the results of a microbiological method.

In order to establish an acceptable HPLC method, it is important to determine whether it is robust enough for assaying samples kept under extreme conditions. Degradation of calcium pantothenate should be equally reflected by microbiological and HPLC assays. This paper describes a comparison of a proposed HPLC method with a microbiological assay for the determination of calcium pantothenate in commercial formulations. Further, calcium pantothenate was kept at strong acidic, alkaline solutions and elevated temperatures as part of accelerated degradation experiments and assayed by microbiological and HPLC methods.

MATERIALS AND METHODS

I. *Instruments*

LC-10AD liquid chromatograph (Shimadzu, Kyoto, Japan), Shimadzu SPD-M10A diode array detector, Shimadzu CAM-10A communication bus module, Shimadzu CTO-10A column oven and Shimadzu DGU-4A degasser were employed during the study. The mobile phase was pumped through a reversed-phase column (Alltima C18; 25 cm × 4.6 mm i.d.; particle size, 5 μm;

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Alltech) with a B-gradient flow rate of 1.0 mL/min. The detector was set at 204 nm. Chromatography was performed at 38°C. Injections of 50 μL of all solutions to analyze were made.

II. *Reagents and Materials*

Acetonitrile (LC grade) was purchased from BDH Lab. (Poole, England). Potassium dihydrogen phosphate and calcium-D (+) pantothenate were purchased from E. Merck (Darmstadt, Germany). Ampicillin was supplied by Taiwan Biotech Co. Ltd. (Taoyuan, Taiwan). Commercial tablets were obtained from commercial sources.

III. *Mobile Phase*

The mobile phases were A and B solutions. A solution for A pump was 0.025 M potassium dihydrogen phosphate (adjusted pH = 2.5 with phosphoric acid). B solution for B pump was 0.025 M potassium dihydrogen phosphate/acetonitrile (50/50, v/v, adjusted pH = 2.5 with phosphoric acid). The elution of mobile phase used the gradient method by different ratio of A and B solution. The gradient time is shown in Table 1.

IV. *Internal Standard Solution*

Internal standard (ampicillin, 50.0 mg) was diluted to 500.0 mL with deionized water to form the internal standard solution.

V. *Calcium Pantothenate Standard Solution*

Calcium pantothenate (50.0 mg) was diluted to 500.0 mL with deionized water to form the stock solution. Different amounts of calcium pantothenate stock solution (5.0, 10.0, 15.0, 20.0, and 25.0 mL) were added to 5.0 mL of internal standard solution to form calcium pantothenate standard solution. Each solution was brought up to 50.0 mL with deionized water.

VI. *Sample Preparations*

At least 10 tablets of sample was weighed, powdered and accurately weighed portion of the powder equivalent to about 5.0 mg of calcium pantothenate, then the volume was brought up to 50.0 mL with deionized water. Stirred and filtered, the clear filtration was used as the sample stock solution. To prepare sample solution, 5.0 mL of internal standard solution was added to 15.0 mL of sample stock solution and the volume was brought up to 50.0 mL with deionized water.

Table 1. Gradient time

Time (min)	A solution/B solution
0	80/20
7	80/20
10	50/50
15	50/50
17	80/20
45	80/20

VII. *Solution for Linearity Response*

Five concentrations of calcium pantothenate (10, 20, 30, 40 and 50 μg/mL) were prepared. Each concentration was chromatographed six times.

VIII. *Solution for Recovery Studies*

Ten-mililiter sample stock solutions of commercial preparations were added to different amounts of calcium pantothenate stock solution and 5.0 mL of internal standard solution. Each solution was made up to 50.0 mL with deionized water and chromatographed in triplicate.

IX. *Microbiological Assay Procedure*

The analysis protocol was described in USP XXV. *Lactobacillus plantarum* was used in the microbiological assay. Sodium acetate (1.0 g/60 mL) and 0.2 N acetic acid were added to standards and test drugs, and diluted to 500 μg/mL concentrate with water (adjusted to pH = 6.7 with sodium acetate or acetic acid). The solution is further diluted to 0.02 μg/mL with deionized water on the day of analysis. After incubation for 16 to 24 h at 37°C, the actual concentration was measured by the turbidity. The turbidity was measured by spectrophotometer at 600 nm.

RESULTS AND DISCUSSION

The linearity of the peak-height ratio (calcium pantothenate versus internal standard) was verified by injection of five calcium pantothenate solutions with concentration from 10 to 50 μg/mL. A straight line with correlation coefficient of 0.9999 ($y = 0.0317x + 0.0100$) [x = calcium pantothenate concentration (μg/mL); y = peak height ratio of calcium pantothenate/ampicillin] was obtained when the ratios of height counts of the calcium pantothenate to height counts of the internal standard were plotted against concentration of calcium pantothenate.

Reproducibility for both inter-day assay and the intra-day assay was evaluated. The relative standard deviations (RSDs) based on the peak-height ratio of six replicate injections in the intra-day assay were between 0.1 and 0.9%. The RSDs in the inter-day assay were 0.1 and 0.7%.

The results of standard addition recovery studies of calcium pantothenate from sample composites of commercial preparations are shown in Table 2. The average recovery was greater than 98.96%.

Typical chromatograms of calcium pantothenate in commercial dosage forms are shown in Figure 1. The retention time was about 6.5 min for the internal standard

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Table 2. Recovery of calcium pantothenate from various commercial composites

Sample	Amount added (mg)	Amount found (mg)	Recovery (%)	Average recovery (%)
	0.5	0.500	100.02	
A	1.0	0.999	99.92	99.89 ± 1.53
	1.5	1.496	99.72	
	0.5	0.503	100.59	
B	1.0	1.001	100.10	100.14 ± 0.43
	1.5	1.496	99.73	
	0.5	0.502	100.41	
C	1.0	0.998	99.83	99.92 ± 0.46
	1.5	1.493	99.51	
	0.5	0.503	100.55	
D	1.0	1.004	100.35	100.59 ± 0.26
	1.5	1.513	100.86	
	0.5	0.499	99.76	
E	1.0	1.003	100.26	100.05 ± 0.26
	1.5	1.502	100.14	
	0.5	0.515	102.95	
F	1.0	1.007	100.75	101.55 ± 1.22
	1.5	1.514	100.95	
	0.5	0.498	99.64	
G	1.0	0.997	99.69	99.52 ± 0.24
	1.5	1.489	99.24	
	0.5	0.502	100.33	
H	1.0	0.999	99.99	100.03 ± 0.28
	1.5	1.497	99.78	
	0.5	0.495	98.98	
I	1.0	0.992	99.16	98.96 ± 0.21
	1.5	1.481	98.74	
	0.5	0.500	100.03	
J	1.0	1.001	100.07	99.93 ± 0.20
	1.5	1.495	99.70	
	0.5	0.498	99.64	
K	1.0	0.993	99.30	99.26 ± 0.40
	1.5	1.483	98.84	
	0.5	0.505	101.04	
L	1.0	1.010	101.04	101.06 ± 0.04
	1.5	1.517	101.11	

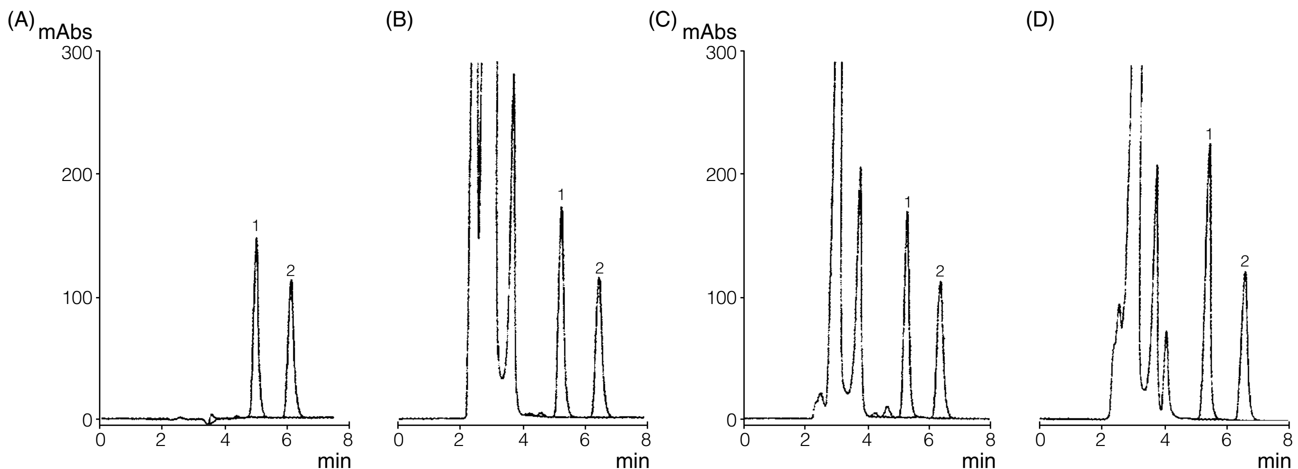

Figure 1. Chromatograms of calcium pantothenate preparations. (A) standard; (B) tablet containing water-soluble vitamins; (C) tablet containing water- and fat-soluble vitamins; (D) tablet containing water-, fat-soluble vitamins and minerals. Peaks: 1 = calcium pantothenate; 2 = ampicillin.

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(ampicillin) and 5.3 min for calcium pantothenate. Excipients from commercial formulations did not interfere.

To ensure the extraction of calcium pantothenate from commercial products by water and by extracted solution of USP were the same, four samples extracted by water and by the extracted solution were assayed by HPLC. A paired *t*-test was applied to the data and showed no significant difference at 95% confidence level. Hence, it was not critical to extract the calcium pantothenate from commercial formulations by water or extracted solution.

When calcium pantothenate was degraded by acid, none interfered with the interpretation and measurements of the chromatographic peaks for calcium pantothenate and ampicillin. Chromatograms of 2-, 6- and 10-hr degraded solution of calcium pantothenate (500 μg/mL) in 2 N hydrogen chloride (pH = 0.7) showed calcium pantothenate disappeared with increasing acidic degradation time (Figure 2). The same results were found in calcium pantothenate (500 μg/mL) degradation using 0.1 N sodium hydroxide (pH = 12.7) (Figure 3). The degradation rate of acid and alkali were nearly 5.6% and 9.2% per hour. Chromatograms of 2-, 6- and 14-day degraded solutions of calcium pantothenate powder (500 μg/mL) at 80°C showed calcium pantothenate did not disappear with increasing incubation time (Figure 4). These results revealed calcium pantothenate had tolerance for heat but not for acid and alkali.

Studies were initiated to ascertain suitability of the proposed method for stability studies. The degraded solutions were taken for microbiological and HPLC assays. The assay results are given in Table 3. The paired values of

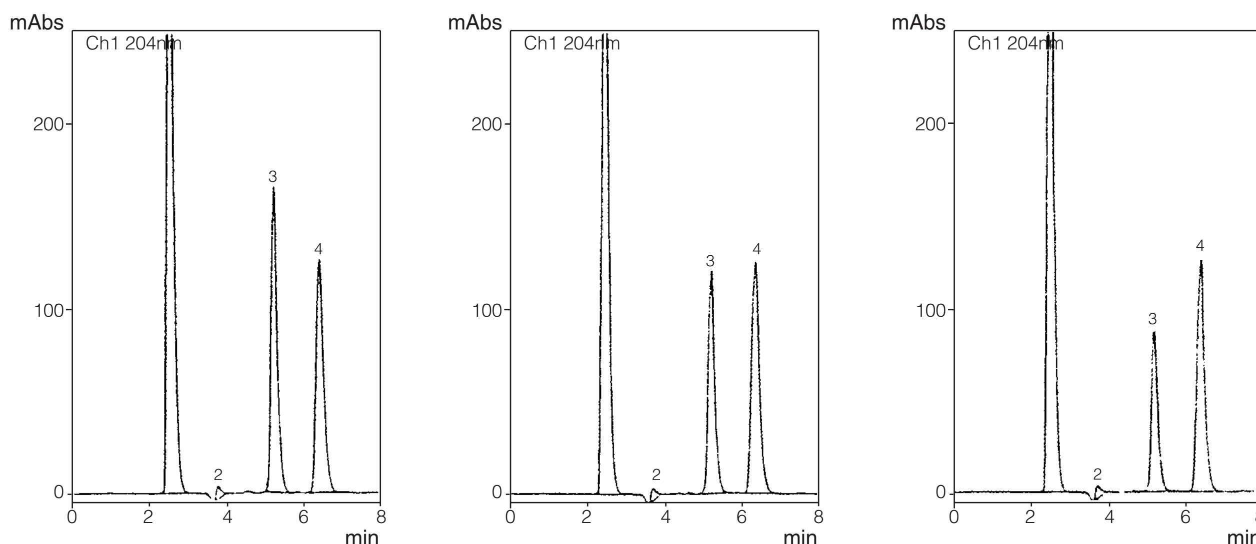

Figure 2. Chromatograms of 2-, 6- and 10-hr degraded solutions (from left to right) of calcium pantothenate in 2 N hydrogen chloride. It has been shown that calcium pantothenate (peak 3) disappeared with increasing degradation time. Peak 4 is ampicillin.

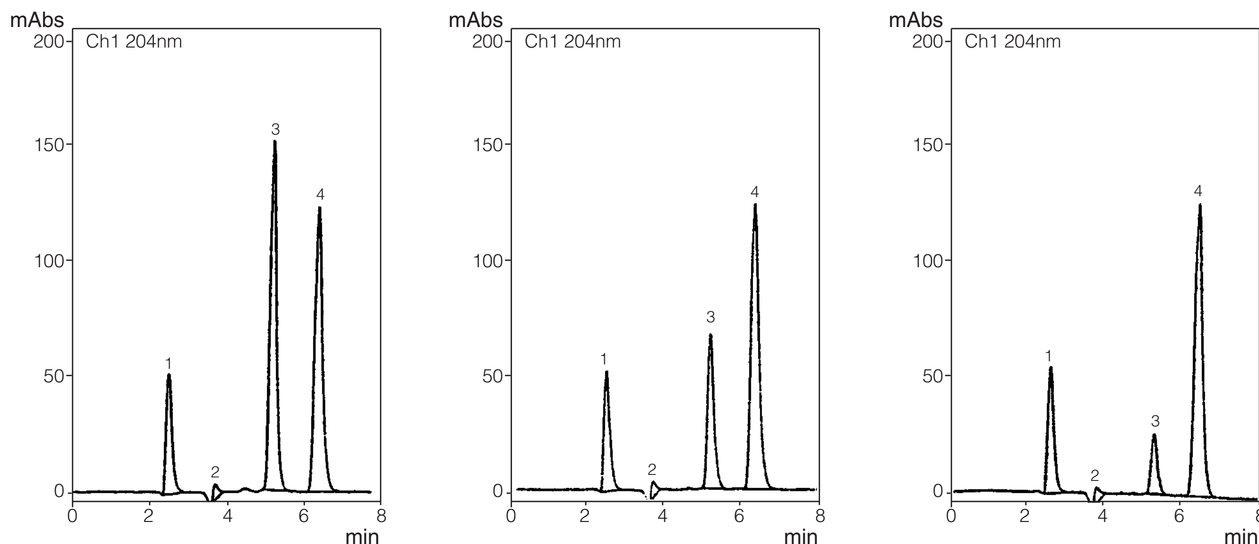

Figure 3. Chromatograms of 2-, 6- and 10-hr degraded solutions (from left to right) of calcium pantothenate in 0.1N sodium hydroxide. It has been shown that calcium pantothenate (peak 3) disappeared with increasing degradation time. Peak 4 is ampicillin.

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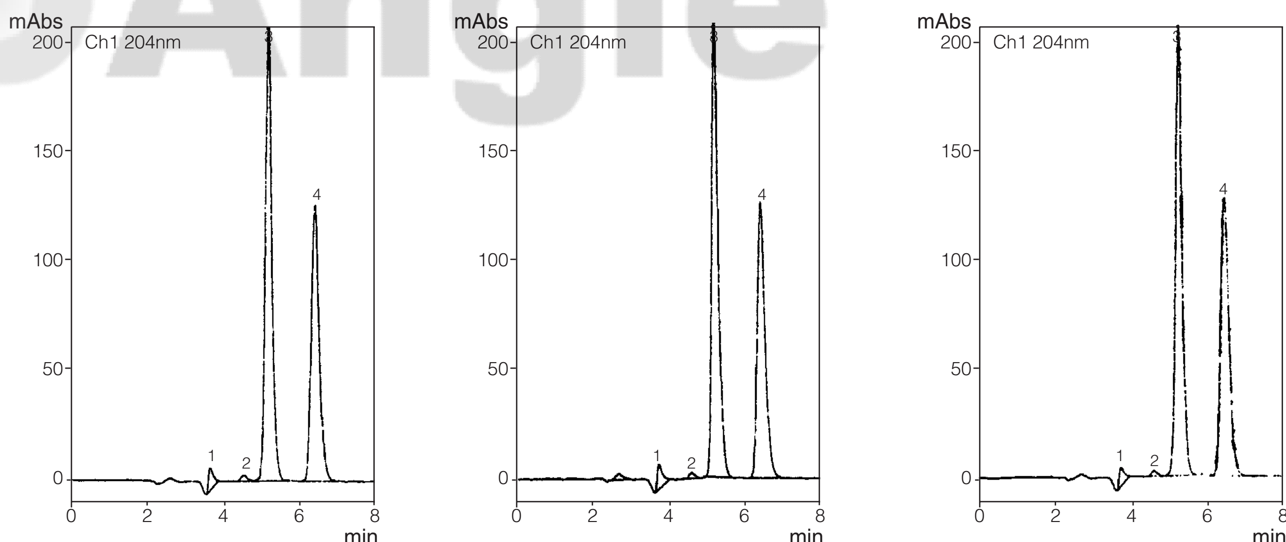

Figure 4. Chromatograms of 2-, 6- and 14-day degraded solutions (from left to right) of calcium pantothenate in 80˚C. It has been shown that calcium pantothenate (peak 3) did not disappear with increasing incubation time. Peak 4 is ampicillin.

Table 3. Comparison of microbiological and HPLC assays for the calcium pantothenate solution degraded by acid, alkaline and heat

Condition	Degradation time	% of declared concentration by	
		Microbiological method	HPLC method
Acidic	0.3 hr	99.16 ± 0.58	92.77 ± 6.47
	2 hr	83.05 ± 0.61	89.96 ± 5.48
	4 hr	72.99 ± 0.60	74.85 ± 3.42
	6 hr	60.63 ± 1.46	57.78 ± 18.88
	8 hr	52.73 ± 4.09	57.19 ± 1.73
Alkaline	0.3 hr	97.31 ± 2.92	98.87 ± 5.53
	2 hr	77.78 ± 0.51	84.10 ± 6.76
	4 hr	53.45 ± 0.43	50.99 ± 10.00
	6 hr	33.52 ± 0.23	32.75 ± 3.56
	8 hr	20.96 ± 0.90	19.33 ± 1.66
Heated	2 day	102.20 ± 0.18	102.14 ± 2.55
	6 day	103.03 ± 0.13	95.73 ± 4.45
	14 day	100.07 ± 0.34	98.82 ± 19.00

Table 4. Comparison of microbiological and HPLC assays for calcium pantothenate

Sample	Declared dosage form (mg/tab)	%[a]	
		HPLC method	Microbiological method
A	5	88.28 ± 2.81	94.42 ± 6.87
B	5	110.26 ± 3.35	103.26 ± 7.86
C	5	115.57 ± 2.37	100.79 ± 10.22
D	2	91.92 ± 0.35	94.50 ± 9.35
E	5	167.59 ± 4.14	158.40 ± 14.13
F[b]	15	122.56 ± 1.52	117.20 ± 10.26
G	3	104.82 ± 6.35	106.25 ± 10.44
H	5	72.59 ± 4.10	70.53 ± 8.09
I	10	14.00 ± 0.60	4.96 ± 8.22
J	2	57.62 ± 0.03	49.37 ± 7.25
K	5	107.40 ± 5.12	106.33 ± 6.43
L[c]	10	146.03 ± 8.51	130.78 ± 20.33

[a]The percent was determined as found/declared dosage. Values for microbiological and HPLC are averages of triplicate.
[b]Contains vitamins A, D, E.
[c]Contains vitamins A, D, E, K and minerals.

acid, alkaline and heat degraded solutions are shown in Table 3. There was no significant difference at 95% confidence level by the paired *t*-tests. Hence, no significant difference in the assay values obtained by the two analytical methods was found for degraded or non-degraded calcium pantothenate.

Twelve calcium pantothenate commercial products were analyzed by HPLC. These samples were also assayed by the microbiological method. The results are shown in Table 4. A paired *t*-test was applied to the data; analysis showed no significant difference at 95% confidence level for any of the preparations when assayed by microbiological or HPLC method.

In order to compare the HPLC method of USP XXIII with the proposed HPLC method, one sample was assayed by both two methods. The result of the HPLC method of USP XXIII was 110.81% and that of the proposed HPLC method was 110.26%. However, our method can save for 13 min as compare to the method of USP XXIII. No difference between two methods has been shown. Furthermore, the proposed HPLC method can analyze not only the samples that contain water-soluble vitamins but also fat-soluble vitamins and minerals. The proposed method is an alternative method.

CONCLUSION

This study demonstrates the applicability of the proposed HPLC method for the determination of calcium pantothenate. The method can be successfully used for routine quality control and stability assays, and also offers advantages in speed, simplicity and reliability. Furthermore, the proposed HPLC method is a suitable sub-

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stitute for microbiological method for quantitative assays of calcium pantothenate in commercial products.

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