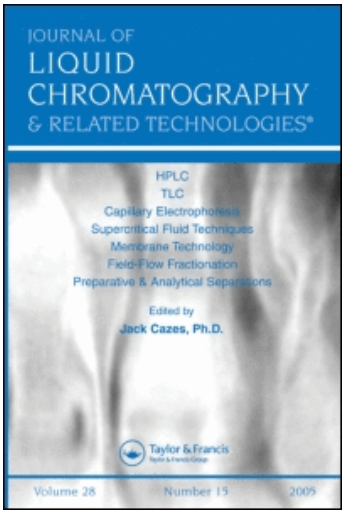

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A Reverse Phase HPLC Assay for the Determination of Calcium Pantothenate Utilizing Column Switching

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A REVERSE PHASE HPLC ASSAY FOR THE DETERMINATION OF CALCIUM PANTOTHENATE UTILIZING COLUMN SWITCHING

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ABSTRACT

A reverse phase HPLC assay utilizing column switching has been developed and validated for the determination of calcium pantothenate (CP) in several multivitamin tablet formulations. The reverse phase system utilizes a DuPont Zorbax C-8 analytical column, an automatically switched and backflushed Brownlee RP-18 guard column for the elimination of a highly retained excipient peak, 88:12 0.25M phosphate buffer:MeOH mobile phase, and 214 nm detection. Sample preparation and the switched column chromatography cycle each require approximately 15 minutes. A spiked recovery study showed linearity over the 50-150% of theory concentration range. Average recovery was 99.7%. Assay precision studies yielded sample RSD's ranging from 0.8 to 2.3%. Results obtained by this method are comparable to those obtained by the USP method.

INTRODUCTION

Calcium pantothenate (CP) is the calcium salt of vitamin B_3. It is a component of a variety of multivitamin formulations. The USP microbiological assay for CP (1) requires a lengthy, labor intensive sample preparation, a 16 to 24 hour incubation and the measurement of turbidity of samples and standards. The time consuming nature of the USP assay provided the incentive to develop an HPLC assay capable of giving equivalent results. We

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utilized column switching to minimize chromatography run time and a blender to simplify sample preparation.

Technical notes published by Hewlett-Packard (2) and DuPont (3) had demonstrated that it is possible to separate CP from other vitamins by HPLC. Quantitation of components in a tablet formulation often requires the masking or removal of interfering materials. Column switching has been utilized to remove interfering materials from chromatographic systems by a number of researchers (4-9). The development and validation of a sensitive, selective, reproducible column switching HPLC assay for CP in Upjohn multivitamin tablets is the subject of this report.

EXPERIMENTAL

Instrumentation

The HPLC system included the following components; a Varian 5060 programmable ternary chromatograph interfaced to a Varian 8055 autosampler with programmable 110V AC external events, a Valco AH60 six port injector, a Rheodyne 7000 six port valve with air actuator, an Altex 110A HPLC pump, a 110V AC event controlled Humphrey solenoid valve and AC outlet device, an LDC Spectromonitor III variable wavelength detector, and a Sargent-Welch UKR single pen recorder. Chromatographic traces were integrated on an in-house PDP 11/40 based computer system.

Reagents

Methanol (Burdick and Jackson), double distilled water, reagent grade NaH_2PO_4 and phosphoric acid were used to prepare the mobile phase. Reagent grade (99+%) adipic acid (Aldrich) was used as the internal standard. Tablet excipient materials were obtained in-house from production stocks. A representative tablet placebo was prepared by mixing appropriate quantities of excipients.

Backflush Flow Rate:	0.7 ml/min
Detector:	214 nm at 0.05 AUFS
Injection Volume:	10 μl
Internal Standard:	1 g adipic acid/liter in 25% MeOH:75% water solution. Each sample preparation requires 250 ml

Reference Standard

Solution: Accurately weigh approximately 13.2 g USP Reference Standard CP and transfer to a 50 ml volumetric flask. Dissolve in internal standard solution and dilute to volume.

Valve Switching - Backflush Sequence:

Time 0:00	Sample injected, guard column in line with analytical column, backflush pump off.
Time 4:00	Guard column switched off line, backflush pump turned on.
Time 16:00	Guard column switched in line with analytical column, backflush pump off.
Time 17:00	Load autosampler for next injection.

Sample Preparation

Accurately measure 250 ml of internal standard solution and transfer to a Waring blender. Weigh accurately a number of tablets equivalent to 55 to 80 mg of CP and add to the blender. Cover the blender and homogenize at low speed for about 5 minutes. Transfer about 25 ml of the resulting slurry to a centrifuge tube and centrifuge at 2000 rpm for 5 minutes. Filter through a 0.45 μm membrane filter. Inject the filtrate.

HPLC System Survey

A variety of HPLC column/mobile phase combinations were examined to establish conditions under which CP was resolved from

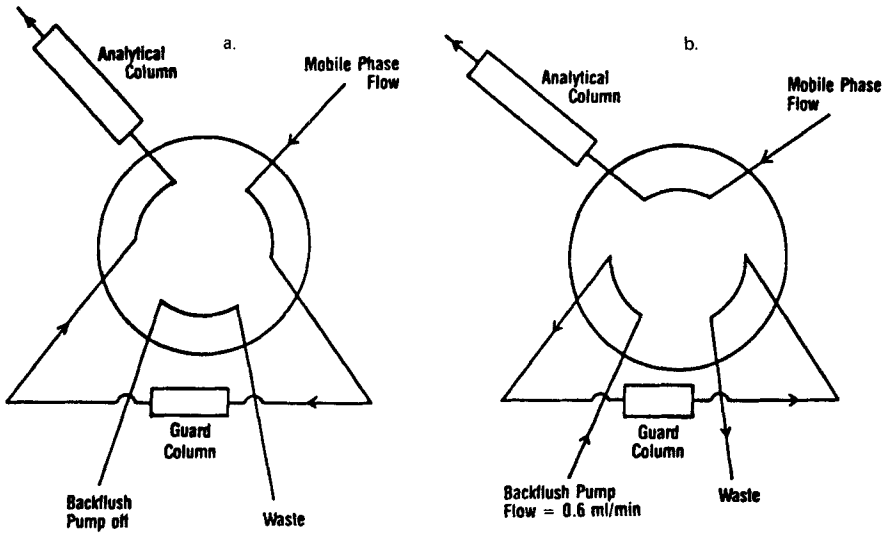

Figure 1. Valve configurations for column switching system.
a) When a sample is injected the mobile phase flows through the guard and analytical columns.
b) After 4 minutes CP and the internal standard have eluted from the guard column onto the analytical column while the highly retained excipient remains within the guard column. The system switches the guard column off line and simultaneously activates a backflush pump to remove the highly retained excipient from the system.

excipient materials (Table 1). An internal standard was chosen from a group of polar organic molecules which were soluble in the mobile phase. The specificity of the system was checked by examining chromatograms of placebo preparations. The retention behavior of CP, adipic acid and excipients was examined on the Brownlee guard column to establish the correct timing for valve position selection and control of the backflush pump.

HPLC Assay Validation

Validation experiments included a spiked placebo recovery/linearity study spanning approximately 50% to 150% of

Table 1

HPLC Systems Investigated

Column	Mobile Phase	Comments
DuPont Zorbax NH_2	pH 3.8 0.005M KH_2PO_4:ACN (20:80)	Resolves CP, ascorbic acid and thiamine; problems with ruggedness
DuPont Zorbax CN	pH 3.8 0.005M KH_2PO_4:ACN (20:80)	CP poorly retained even with weak mobile phase
DuPont Zorbax TMS	pH 3.5 0.25M NaH_2PO_4:MeOH (95:5)	CP unresolved
DuPont Zorbax C-8	pH 3.5 0.25M NaH_2PO_4:MeOH (88:12)	Resolves CP
DuPont Zorbax ODS	pH 3.5 0.25M NaH_2PO_4:MeOH (88:12)	Resolves CP
Waters μBondapak C-18	pH 3.5 0.25M NaH_2PO_4:MeOH (88:12)	Resolves CP
Regis Workhorse ODS	pH 3.5 0.25M NaH_2PO_4:MeOH (88:12)	Resolves CP

tablet potency. The final concentrations in the sample preparation in this study were 0.12 to 0.46 mg CP/ml. Multiple assays of a variety of lots of five products were performed over two days to examine precision. Results obtained by the HPLC assay were compared to those obtained by the USP assay (1).

Recommended HPLC Conditions

Analytical Column:	DuPont Zorbax C-8 25.0 cm x 4.6 mm i.d.
Mobile Phase/Backflush:	1000 ml 0.25M phosphate buffer (pH 3.5) mixed with 135 ml methanol. Filter and degas before using.
Guard Column:	Brownlee MPLC 3 cm cartridge column packed with RP-18 Spheri-5. See Figure 1

for mounting configuration on the Rheodyne 7000 valve

Analytical Flow Rate: 1.5 ml/min

RESULTS AND DISCUSSION

HPLC System Survey

Results of the survey of HPLC column/mobile phase combinations are summarized in Table 1. Chromatography on the Zorbax-NH_2 column was sufficient to resolve CP and ascorbic acid, but the retention and resolution of these compounds decreased drastically over a period of 6-8 hours. Flushing of this column with 0.1 M (NH_4) H_2PO_4 only temporarily returned resolution and retention. The Zorbax-CN and Zorbax-TMS columns did not adequately retain or resolve CP from excipients under the conditions studied.

The octyl and octadecyl modified silica columns surveyed were all capable of resolving CP from excipients under the conditions studied. The Zorbax-C8 column provided slightly better resolution of CP from niacinamide and the adipic acid internal standard and is therefore the column of choice. Tables 2-4 summarize the retention and resolution of niacinamide, CP and adipic acid on the reversed phase columns as MeOH concentration, buffer concentration and pH of the mobile phase were varied. The retention of CP and adipic acid increased with decreasing MeOH, decreasing pH and increasing buffer concentration. The retention of niacinamide increased with decreasing MeOH, increasing pH and increasing buffer concentration. This data suggests some limiting mobile phase conditions. Our desire to keep the chromatography run time to about 17 minutes sets the lower limit on MeOH concentration to about 10% on Zorbax-C8, 12% on Zorbax-ODS and 8% on Waters C-18 μBondapak columns. Mobile phase pH must be maintained at pH 3.5 or less to obtain good resolution of CP and niacinamide within 16 minutes. Adjustment of the buffer concentration has relatively little effect on the chromatography between 0.05 and 0.4M. A representative sample chromatogram is shown in Figure 2.

Table 2

The Effect of Percent Methanol on Retention and Resolution

% MeOH	T_{rn} (min)	t_{rcp} (min)	t_{rad} (min)	R_1	R_2
DuPont Zorbax C_8 25 cm					
14	4.7	6.1	9.5	1.8	4.9
12	5.9	9.3	14.0	4.0	5.9
10	6.6	11.8	17.2	5.5	5.7
8	7.7	15.9	22.1	7.8	5.0
6	9.3	21.7	28.5	9.5	4.4
DuPont Zorbax ODS 25 cm					
15	4.2	6.1	9.5	3.4	4.7
13	4.5	7.3	11.3	5.1	6.7
12	5.9	11.4	16.6	5.0	6.1
11	6.1	13.0	18.8	6.9	5.8
10	6.8	14.6	20.6	6.5	5.2
9	7.5	16.7	23.0	6.8	5.0
7	8.7	22.6	29.6	9.0	4.1
Waters μBondapak C-18 30 cm					
14	5.7	7.4	10.5	2.3	4.8
12	5.9	8.4	11.7	3.6	5.1
10	6.6	9.8	13.7	4.6	5.6
8	7.1	11.7	16.2	6.6	5.6
6	7.9	14.4	19.3	6.5	4.7

where: t_{rn} is the retention time of niacinamide

t_{rcp} is the retention time of calcium pantothenate

t_{rad} is the retention time of adipic acid

R_1 is the resolution between niacinamide and CP calculated by:

$$\frac{2\,(t_{rcp}-t_{rn})}{W_{cp}+W_n}$$

R_2 is the resolution between CP and adipic acid calculated by:

$$\frac{2\,(t_{rad}-t_{rn})}{W_{ad}+W_{cp}}$$

Table 3

The Effect of pH on Retention and Resolution

pH	t_{rn} (min)[a]	t_{rcp} (min)[a]	t_{rad} (min)[a]	R_1[a]	R_2[a]
DuPont Zorbax C_8 25 cm					
4.3	9.2	9.2	11.5	0	--
4.1	8.2	9.6	13.1	1.6	5.4
3.8	7.4	10.8	15.0	3.6	4.9
3.5	6.4	11.5	16.5	5.1	4.8
3.4	5.6	11.6	17.0	6.3	5.4
3.1	4.9	11.6	17.1	7.9	5.5
3.0	4.5	11.8	17.6	7.3	5.8
DuPont Zorbax ODS 25 cm					
4.2	10.8	7.9	10.8	CP eluted before niacinamide	
4.0	9.9	8.5	11.7	CP eluted before niacinamide	
3.8	9.2	9.5	13.5	0	--
3.5	7.9	10.5	15.0	2.6	5.0
3.4	7.0	10.8	15.7	3.4	4.9
3.2	6.5	11.1	16.1	4.1	5.0
3.0	5.6	11.2	16.2	5.9	5.3
Waters μBondapak C-18 30 cm					
3.5	6.8	10.5	14.9	3.7	4.0
3.4	6.3	10.6	15.2	4.3	3.8
3.2	6.1	10.8	15.6	4.3	3.7
3.1	5.6	10.8	15.6	4.8	3.7
3.0	5.0	10.8	15.8	5.0	3.7

[a]See Table 2 for definitions

Placebo chromatograms exhibited no interfering peaks near the CP or adipic acid peaks (Figure 3). A highly retained excipient peak was observed to elute approximately 2 hours after the injection of the excipients, however, and this peak interfered with subsequent chromatograms as shown in Figure 4. Attempts to remove this interfering excipient by modifying the extraction conditions of the sample preparation were not successful.

Table 4

The Effect of Buffer Concentration on Retention and Resolution

Conc. NaH_2PO_4 (M)	t_{rn} (min)[a]	t_{rcp} (min)[a]	t_{rad} (min)[a]	R_1[a]	R_2[a]
DuPont Zorbax C-8 25 cm					
0.05	6.7	10.1	15.8	3.2	6.3
0.10	7.0	10.5	15.9	3.9	6.4
0.20	6.3	10.4	15.6	4.3	5.8
0.25	6.5	11.3	16.8	4.6	6.1
0.30	6.3	11.2	16.2	5.8	5.9
0.40	6.8	11.1	15.9	6.2	5.0
DuPont Zorbax ODS 25 cm					
0.15	7.0	8.7	13.1	1.8	4.9
0.20	6.8	8.7	13.0	2.1	5.7
0.25	6.5	8.8	13.1	2.3	4.8
0.30	7.8	12.9	17.9	4.6	4.2
0.40	7.9	13.6	18.8	5.2	4.3
Waters μBondapak C-18 30 cm					
0.15	5.1	9.0	13.2	4.9	4.2
0.20	4.9	9.0	13.3	6.0	4.3
0.25	4.9	9.2	13.3	5.0	4.1
0.30	4.8	10.7	15.4	6.2	3.8
0.35	5.5	10.1	14.5	4.2	3.5

[a]See Table 2 for definitions.

The use of a guard column, switching valve, and backflush configuration to trap highly retained excipients has been reported previously (9). We decided to use a commercially available guard column to remove the need to pack our own. See Figure 1 and the Experimental section for the configuration and conditions. CP, niacinamide and adipic acid eluted within 4 minutes from the Brownlee guard column and the highly retained excipient eluted in about 25 minutes. Switching the guard column off line at 4 minutes trapped the excipient peak about one-sixth of the way into the guard column. Backflushing the guard column for 12 minutes at

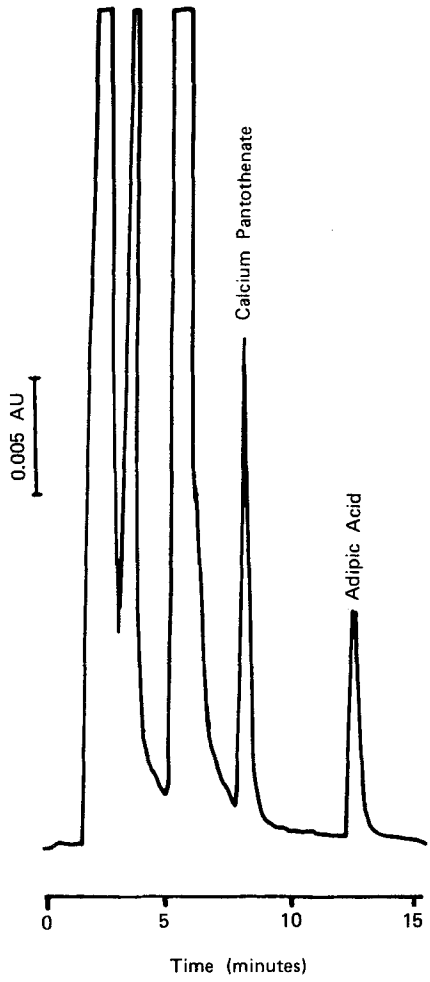

Figure 2. Typical chromatogram of a multivitamin tablet formulation. Eluted with phosphate buffer:methanol (88:12) at 1.5 ml/min, detection at 214 nm.

0.7 ml/min flushed this excipient peak out with 2.4 ml more mobile phase than was pumped forward in the loading phase, i.e. 6 ml pumped forward and 8.4 ml backflushed. The excess flush was used to assure the guard column was thoroughly cleaned. The use of the programmable event controls on the Varian 5000 pump to turn the backflush pump on and off reduced mobile phase consumption.

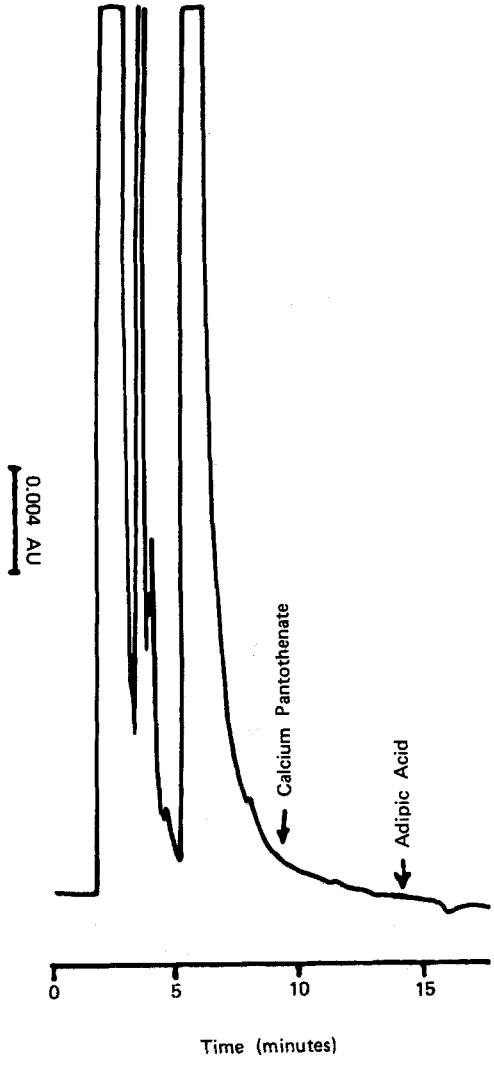

Figure 3. Placebo chromatogram.

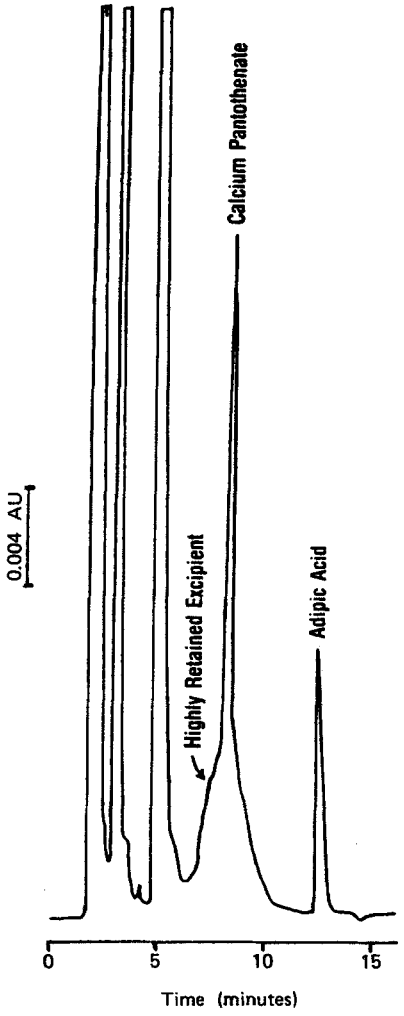

Figure 4. Chromatogram showing the highly retained excipient eluting 2 hours after injection and interfering with a later sample.

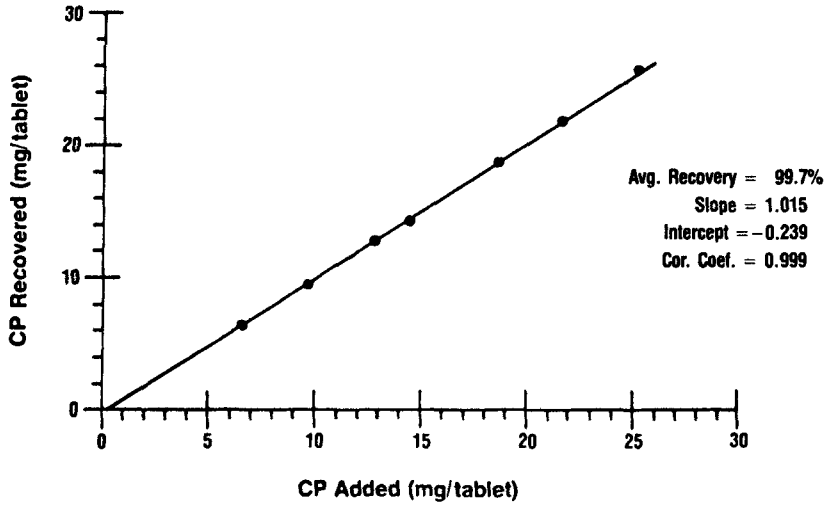

Figure 5. Universal placebo spiked recovery results, concentration found vs. concentration added using peak heights.

HPLC Assay Validation

The plot of the data from the spiked placebo recovery study is shown in Figure 5. Peak height calculations were used to obtain the amount recovered because peak height results were more precise than peak area results. No significant bias or deviation from linearity was observed over the range of concentrations studied. Average recovery was 99.7%.

The results of the precision study are summarized in Table 5. Pooled RSD's for two runs of triplicate assays of two lots each of five products ranged from 0.83% to 2.32%. The RSD for six injections of a single sample preparation was 1.58%. The RSD of the standard factors for 6 to 8 injections of duplicate reference standard preparations ranged from 0.52% to 0.93%. This data indicates that the method is precise.

The CP potencies obtained by the USP method (1) and the HPLC method were in good agreement. It must be noted that the HPLC

Table 5

Precision Study Results

Product	Pooled RSD
A	1.68%
B	1.42%
C	0.83%
D	2.32%
E	1.35%

assay is not specific for the biologically active d-isomer of CP, but rather measures total CP content of the tablets. A method specific for the d-isomer of CP, such as the microbiological assay, is performed on bulk drug CP before production of the tablets to insure the active form is present.

SUMMARY

The HPLC assay described provides a rapid, accurate, precise method for the determination of CP in multivitamin tablet formulations. This HPLC assay gives results which are comparable to those generated by the USP microbiological procedure in a substantially shorter period of time. The use of a column switching arrangement shortens chromatography run time and the use of a blender simplifies sample preparation.

ACKNOWLEDGEMENTS

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