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Note

Separation of vitamin B_6 components by high-performance liquid chromatography

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Vitamin B_6 occurs naturally in foods mainly as pyridoxol (POL), pyridoxal (PAL), pyridoxamine (PAM) and their corresponding phosphates. Available quantitative methods for vitamin B_6 have several disadvantages such as lengthy procedure time, variability in growth responses in microbiological methods[1–3] and interfering impurities which can seriously affect the accuracy of fluorometric[4,5] and spectrophotometric[6] methods.

Although several researchers[7–10] have reported on the separation of the various forms of vitamin B_6, reports on the quantitation of vitamin B_6 components in foods by high-performance liquid chromatography (HPLC) are lacking. Two research groups have quantitated the vitamin B_6 compounds in simulated or extracted food substances; Gregory and Kirk[10] used a dehydrated model food system simulating breakfast cereal and Wong[9], enriched food extractives monitored at 210 nm. Major advantages offered by HPLC for the analysis of vitamin B_6 include (1) direct analysis without derivatization, (2) minimum of sample clean-up and (3) use of non-destructive detectors which permit recovery of the compounds in their original forms and quantity. As part of a larger study on the quantitation of vitamin B_6 in various foods, a rapid separation via HPLC of the various forms of vitamin B_6 has been developed. This report describes this system and its application to milk samples.

EXPERIMENTAL

Materials and methods

Reagents. Acetonitrile was glass-distilled HPLC grade obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.); hydrochloride forms of POL, PAL, PAM and 4'-deoxypyridoxine (DPOL) were obtained from Sigma (St. Louis, Mo, U.S.A.). Sep-Pak C-18 cartridges were obtained from Waters Assoc. (Milford, Mass., U.S.A.). All other reagents were of analytical-reagent grade.

Mobile phase. Potassium phosphate, monobasic (0.033 *M*), was prepared using deionized, distilled water, the pH adjusted to 2.2 with orthophosphoric acid; this solution vacuum filtered through sintered glass. A mixture of acetonitrile and phosphate buffer (1:99, v/v) was prepared. The mobile phase was pumped at a rate of 1 ml/min at 1100 p.s.i.g. at ambient temperature.

Analytical system. The HPLC apparatus was constructed from commercial components including a Milton Roy (Ivyland, Pa., U.S.A.) 5000 p.s.i. minipump, a Laboratory Data Control (Riviera Beach, Fla., U.S.A.) Model 709 pulse dampener and a Valco (Houston, Texas, U.S.A.) 7000 p.s.i.g. Universal Inlet injection system with a 8-μl loop. The detector was a Bausch and Lomb (Rochester, N.Y., U.S.A.) Spectronic 700 variable-wavelength spectrophotometer set at 280 nm and connected to a Tracor (Austin, Texas, U.S.A.) Model 100 equipped with 254 nm fixed-wavelength absorbance detector. The column was a Spherisorb ODS 25 cm × 4.6 mm I.D., 10 μm particle column obtained from Laboratory Data Control and fitted with a Guard column packed with Co: Pell ODS (Whatman, Clifton, N.J., U.S.A.). Recording was done with a Varian Aerograph (Palo Alto, Calif., U.S.A.) series A-25 dual-channel 25.4-cm strip chart recorder.

RESULTS AND DISCUSSION

Standard solutions of mixtures containing PAL, POL, PAM and DPOL were prepared in deionized, distilled water and protected from light. The standards were stored at 5° when not in use.

Good separation of the four compounds was obtained (Fig. 1). A resolution of 1.1 was calculated for the PAL and POL peaks, the only peaks not baseline resolved. A theoretical plate value of 386 and a height equivalent to theoretical plate value of 0.065 cm for a 25-cm column were obtained for the peak representing POL. The retention time of PAM was 2.5 min. The retention ratios of the other compounds relative to PAM were: PAL, 1.72; POL, 2.16 and DPOL, 3.44. Separation of all four components was complete in about 12 min.

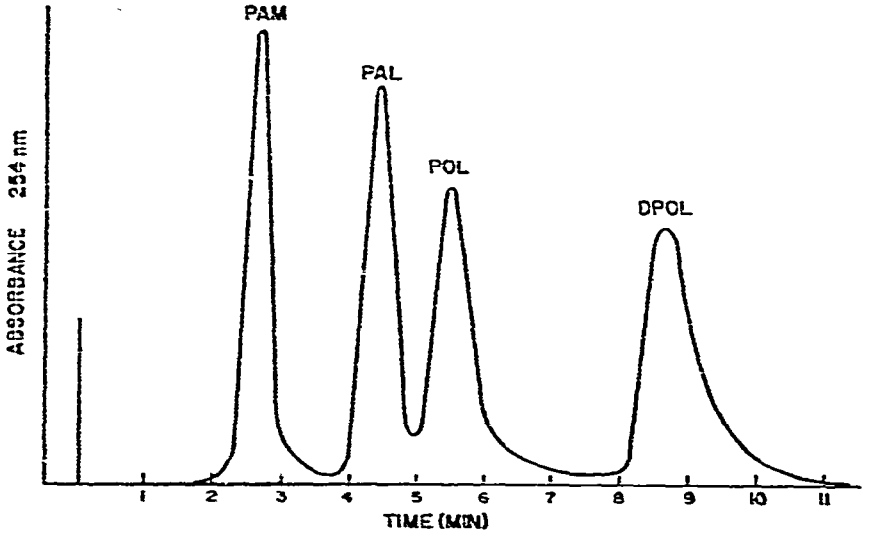

Fig. 1. HPLC separation of vitamin B_6 components. For experimental conditions see text.

The calibration curves for PAM and PAL were linear between 50 and 1000 ng and for POL and DPOL, between 50 and 1400 ng (Fig. 2). The minimum detectable quantities for the four compounds were found to be in the 5-ng range. The dose–response values of the calibration curves were found to be reproducible.

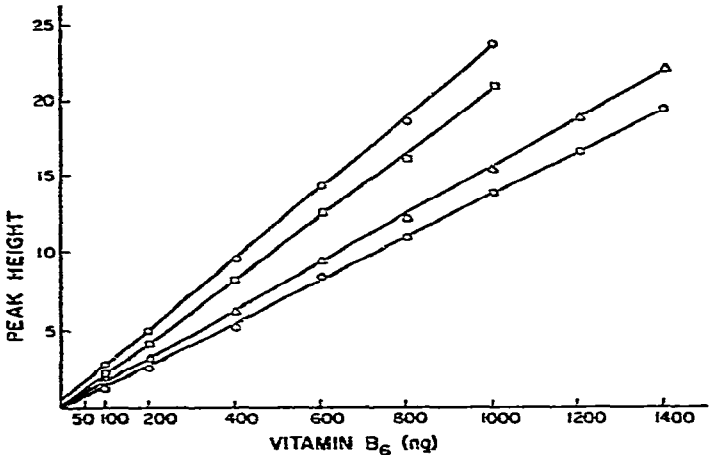

Fig. 2. Calibration plot for the various vitamin B_6 forms at UV 254 nm. ○ = PAM, □ = PAL, △ = POL, ◇ = DPOL.

The use of an internal standard mode tends to minimize errors resulting from sample preparation, apparatus, and technique. The internal standard tested in this study, DPOL, was completely resolved from the other three compounds while at the same time eluting near the peaks of interest. Furthermore, DPOL is not found naturally in foods, making this vitamin antagonist ideal as an internal standard for vitamin B_6 quantitation in foods. From Fig. 2 the following response factors (weight in ng: peak height in cm) were obtained: PAM, 4.16; PAL, 4.78; POL, 6.27 and DPOL, 7.11. The relative response factors (response factor of vitamer:response factor of the internal standard, DPOL) were as follows: PAM, 0.585; PAL, 0.672 and POL, 0.882.

Utilizing the above system and with the UV detector set at 280 nm, a successful qualitative separation of PAM, PAL and POL was obtained on a sample of commercial homogenized and pasteurized skim milk. A Sep-Pak C-18 was used in the clean-up of the milk extract to remove compounds of high polarity. The chromatogram of the milk extract (Fig. 3) exhibited a large PAM peak followed by smaller PAL and POL peaks. The peaks were tentatively identified by co-chromatography of the milk extract with a blend of PAM, PAL and POL.

Yasumoto *et al.*[11] have reported on the quantitation of naturally occurring vitamin B_6 forms in several biological materials employing ion-exchange column chromatography with diazide of 5-chloroaniline 2,4-disulfonyl chloride as a color-producing reagent. Our method, however, is capable of detecting 5 ng of the four compounds as compared to a lower detection limit of 2 μg using the method of Yasumoto *et al.* Other improvements associated with our HPLC method over the

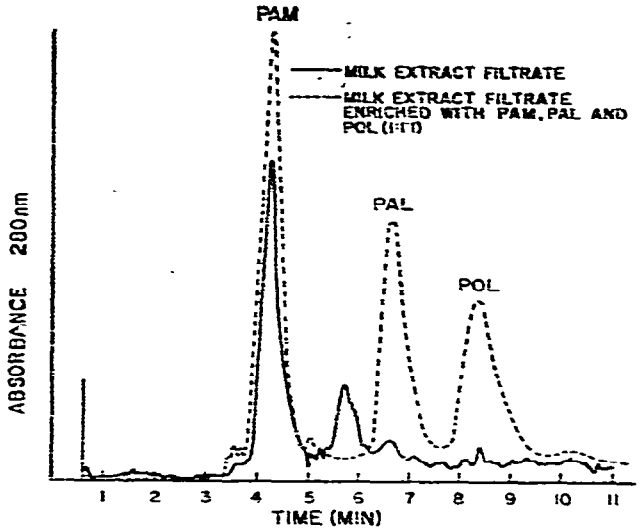

Fig. 3. HPLC chromatogram of milk extract. ——, Milk extract filtrate; – – –, milk extract filtrate enriched with PAM, PAL and POL (1:1:1).

method of Yasumoto *et al.* are: (1) shorter analysis time per sample (12 min *versus* 2 h), (2) simpler instrumentation, (3) simple mobile phase, and (4) the use of a UV detector foregoes the use of a color-producing reagent.

Further work utilizing the described system for quantitation of vitamin B_6 components in several selected foods is being carried out and the results will be reported later.

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