Vitamin B_6. Ion Exchange Chromatography of Pyridoxal, Pyridoxol, and Pyridoxamine

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The three major components of vitamin B_6, pyridoxal, pyridoxol, and pyridoxamine, have been separated by means of ion exchange chromatography. Systems are described utilizing both single buffer and gradient elution techniques for the resolution of these compounds. Ultraviolet light absorption provides a sensitive and quantitative means of detection.

There has been, for a number of years, a need for a convenient, accurate, and specific technique for the determination of pyridoxal, pyridoxol, and pyridoxamine. Mixtures of these three compounds are frequently difficult to resolve and quantitate by techniques presently available. Historically, the compounds have been determined as a single microbiological growth response even though the three forms of the vitamin have been known to elicit variable growth response of the test organism (Parrish et al., 1955). For this reason, it is frequently desirable to determine these compounds individually rather than as a single biological activity.

Toepfer and Lehmann (1961) described a technique whereby the three compounds present in foodstuffs were subjected to ion exchange chromatography and the fractions containing the individual vitamin components were assayed by conventional microbiological techniques (AOAC, 1970).

Other procedures for the determination of pyridoxal, pyridoxol, and pyridoxamine have been reported. Two widely used methods are described by Sheppard and Prosser (1970) and by Korytnyk (1970). These methods, while quite satisfactory for many purposes, are based upon gas chromatography of volatile derivatives of the vitamin moieties which precludes recovery of the compounds in their original form and quantity.

The development of high-resolution ion exchange resins and techniques of high-pressure chromatography has resulted in satisfactory analytical procedures for many compounds which have proved intractable to the conventional low-pressure, low-resolution systems.

A system employing small bore columns of high-resolution cation exchange resin operated at high pressure has been found to offer considerable promise as a means of analyzing mixtures of pyridoxal, pyridoxol, and pyridoxamine.

The unsaturated ring structure, common to all three of the compounds, lends itself well to detection by ultraviolet light absorption. The relatively high extinction coefficients of these materials allow detection and quantitation in the nanogram range.

MATERIALS AND METHODS

The resin, Aminex A-5 (Bio-Rad Laboratories, Richmond, Calif.), was washed with 2.0 *M* NH_4OH and allowed to settle and the supernatant decanted. This procedure was repeated three times. The resin was then washed on a Buchner funnel with distilled water, until the excess NH_4OH had been removed, and finally suspended in 0.70 *M* ammonium formate buffer (pH 5.60).

A glass column, 0.3 × 150 mm (Chromatronix, Inc., Berkeley, Calif.), in which the stainless steel bed support was replaced by a plug of Pyrex glass wool was thoroughly cleaned and filled with the aminex A-5 suspension. The column was attached to a high-pressure pump and formate buffer passed through the column at a rate sufficient to produce approximately 600 psi of pressure. When the resin had settled the supernatant buffer was removed and the procedure repeated until the column was filled. The buffer was allowed to flow overnight at a rate of 0.30 ml/min.

The column was attached to a Waters ALC 202 liquid chromatograph with an ultraviolet absorption detector. Relative peak areas were determined by means of an Infotronic CRS-101 electronic integration system. The buffer used for elution, 0.7 *M* ammonium formate (pH 5.60), was degassed by agitating in a vacuum until no further evolution of gas was evident. Standard solutions of pyridoxal, pyridoxol, and pyridoxamine were prepared by dissolving the hydrochloride salts in water to give a final concentration of 1.00 mg of free base/ml. Any further dilution of these standards was made with the formate buffer.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram obtained from 100.0 ng of pyridoxal (peak 1) and 200.0 ng each of pyridoxol and pyridoxamine (peaks 2 and 3, respectively). The relatively high buffer concentration (0.70 *M*) used for elution resulted in symmetrical, well-defined peaks which could be easily quantitated by manual or electronic integration methods. These peaks, however, eluted rather rapidly resulting in considerable interference when present in mixtures of other ultraviolet absorbing compounds. The retention times of these compounds were increased significantly by lowering the buffer concentration to 0.40–0.50 *M*. This caused peak spreading, particularly of pyridoxamine (peak 3). When interfering materials are present in the injection mixture, it is frequently desirable to utilize the advantages of gradient elution. Figure 2 shows a gradient elution chromatogram obtained from 70.0 ng of pyridoxal (peak 1) and 140.0 ng each of pyridoxol and pyridoxamine (peaks 2 and 3, respectively). In this case the column was equilibrated with 0.01 *M* ammonium formate buffer (pH 4.5). Thirty milliliters of the 0.01 *M* buffer was placed in a mixing chamber and 1.0 *M* ammonium formate (pH 6.6) was added to the mixing chamber at a rate of 0.15 ml/min. The column was eluted by pumping from the mixing chamber at the rate of 0.30 ml/min (Burtis and Stevenson, 1971). Under these conditions pyridoxal, pyridoxol, and pyridoxamine exhibited retention times of 35, 54, and 78 min, respectively. This procedure, while requiring more time for elution and re-equilibration of the column, had a distinct advantage over the single buffer system in that interference due to contaminating materials was considerably reduced. The peaks obtained by gradient elution were somewhat broader than those from the single buffer system. However, while the peak width at half-height doubled, the retention times were approximately ten times as great in the gradient system.

Figure 3 shows the relationship between quantity of vita-

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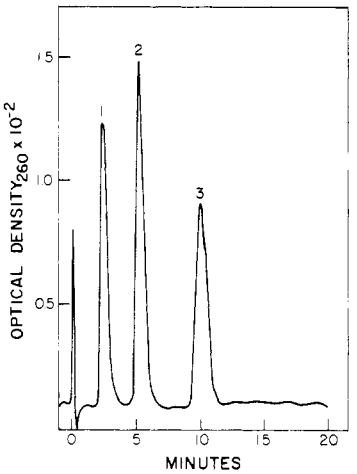

Figure 1. Chromatography of vitamin B_6.

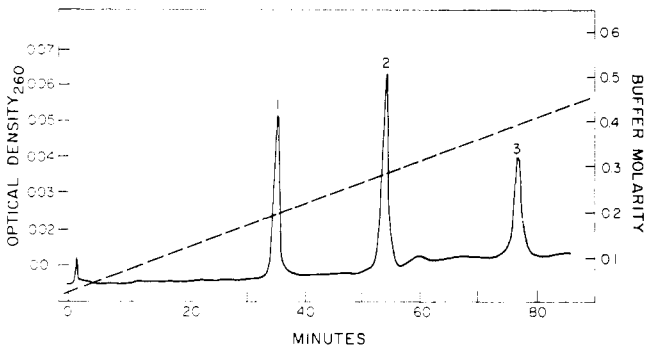

Figure 2. Gradient elution chromatography of vitamin B_6: solid line, optical density; dashed line, buffer concentration.

min and peak area. This relationship is virtually linear in the range of 40.0–300.0 ng of vitamin base. The linear range of the chromatographic system is much wider than that indicated here. The lower limit of the procedure is determined by the noise level of the detector and by pulsation generated by the pumping system. It has been noted that if the column is not completely filled with resin, considerable mixing occurs in the buffer stream immediately preceding the column. This mixing frequently results in broad peaks and tailing. This difficulty can usually be overcome by adjusting the sample solvent to a density slightly higher than that of the eluting buffer by the addition of sucrose.

This method has been applied to the analysis of pyridoxol in commercially manufactured vitamin capsules. Chromatography of a water extract revealed the presence of pyridoxol in amounts corresponding very closely to the analysis on the label. Pyridoxal and pyridoxamine were absent. Attempts to analyze foodstuffs such as chicken muscle, dried beans, and whole wheat were not successful due to many overlapping peaks which precluded quantitation and in many cases, identification of the vitamins. Since our purpose was to analyze the vitamin B_6 moieties in relatively simple mixtures, cleanup of these natural materials was not pursued.

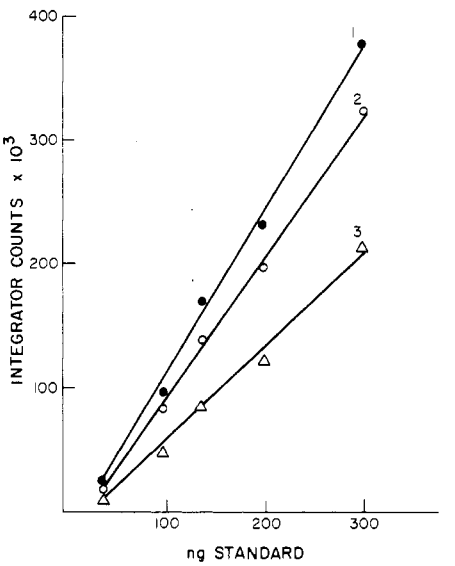

Figure 3. Dose–response plots for pyridoxal, pyridoxol, and pyridoxamine: (●) pyridoxal; (○) pyridoxol; (△) pyridoxamine.

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