Journal of Chromatography, 176 (1979) 280–285

CHROM. 11,940

Note

Liquid chromatographic separation and quantification of B_6 vitamers and their metabolite, pyridoxic acid

JOSEPH T. VANDERSLICE, KENT K. STEWART and MARGARET M. YARMAS

Nutrient Composition Laboratory, Nutrition Institue, Human Nutrition Center, Science and Education Administration, United States Department of Agriculture, Beltsville, Md. 20705 (U.S.A.)

(First received February 27th, 1979; revised manuscript received April 24th, 1979)

The recent upsurge of interest in human nutrition has intensified the search for fast, accurate, reproducible, automated methods of analysis for nutrient in foods[1]. For vitamin B_6 in particular, many of the earlier methods were lengthy, tedious and costly and often lacked precision[2–6]. Until recently, much of the published data on vitamin B_6 composition in foods[7] was based on microbiological assays. Such assays are still considered by some[8] to be the most reliable methods of analysis even though it is acknowledged that results from different laboratories can vary by a factor of five or more[9]. Microbiological assays are subject to the criticism that they fail to distinguish between the various forms of B_6 which can be a hindrance in metabolic work[6]. However, a combination of chemical separation and microbiological assay partially offset this criticism[3]. Enzymatic assays, besides being difficult, detect only one form of the vitamin. Chemical methods of analysis, by themselves, normally lack specificity and require careful extraction procedures to eliminate interfering compounds while still recovering all of the vitamin content[8]. Chemical methods, on the other hand, are relatively inexpensive, can be made accurate and fast, can be automated, and have the potential of measuring each form of the vitamin as well as their metabolite, pyridoxic acid[10].

In recent years, thin-layer chromatography[11,12], thin-layer electrophoresis[13], gas–liquid chromatography (GLC)[14–17], and high-performance liquid chromatography (HPLC)[18–23] have all been suggested as analytical methods, but only the last two appear to offer good potential for quantification and automation. To our knowledge, none of the suggested GLC or HPLC methods are capable of identifying and quantifying all six vitamers plus pyridoxic acid simultaneously. The HPLC separation scheme described in the present paper does this and is inherently much simpler than recently proposed HPLC methods[22,23] in that it requires no pre- or post-column chemistry to achieve the necessary sensitivity. Specificity is achieved by a careful choice of fluorescence filters maximized at the excitation and emission wavelengths of the B_6 vitamers. If one is interested in only the vitamers and not pyridoxic acid, the system is capable of handling 15 samples a day; otherwise, 10 samples a day is the limit.

EXPERIMENTAL*

Vitamin B_6 chromatographic analyses

The apparatus is an HPLC system consisting of a depulsed positive displacement pump[24], an Altec 201-56 PTFE pneumatically actuated sample injection valve with a 0.5-ml sample loop, a 25 cm × 6 mm Glenco column packed with Bio-Rad (Richmond, Calif., U.S.A.) A-25 resin and thermostated at 55°, and two Aminco Fluoromonitor detectors in series (Fig. 1). Both monitor systems use 70-μl flow cells, and the original RCA 931B phototubes were replaced with EMI 9781B tubes. Monitor 1, thermostated at 5° with a Haake water bath, uses a clear General Electric germicidal lamp supplied by Aminco, with a Dell Optics A-4-3100 as a primary filter and a Corning 0-52 secondary filter. Monitor 2, which is used primarily for pyridoxal phosphate measurements, uses a General Electric phosphor coated mercury lamp supplied by Aminco, a Corning 7-51 primary filter, a Wratten 2A secondary filter, and is thermostated at 25° with a Haake water bath. Both monitors are kept in a refrigerator regulated at 5° to avoid water condensation. Areas under chromatographic peaks, which are proportional to concentration, were measured with an Autolab Minigrator connected directly to each monitor. The entire system is connected by 26 AWG plastic tubing and Cheminert™ fittings. The complete apparatus is kept in a dark room and all runs are performed in total darkness.

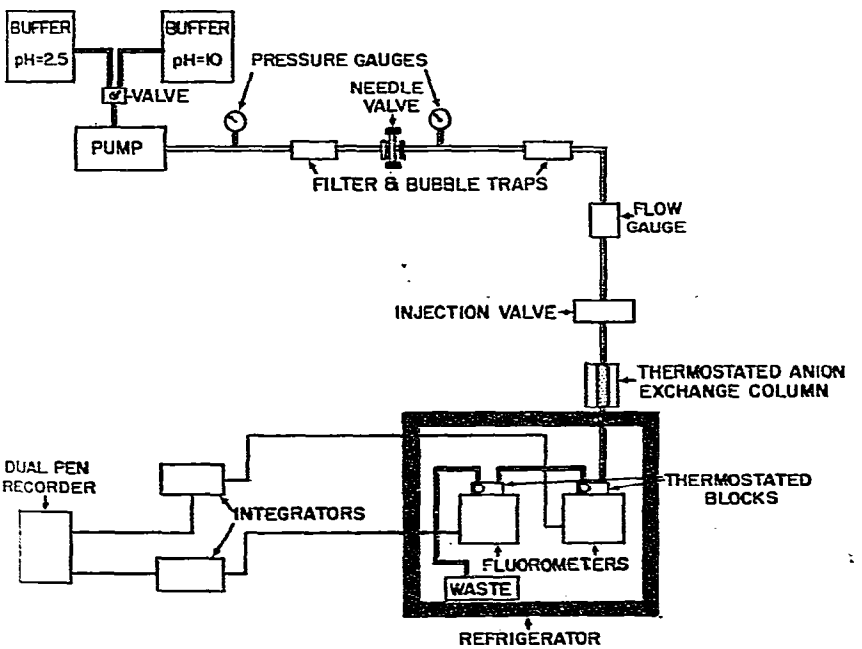

Fig. 1. Flow diagram of HPLC system.

* Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

Chemicals

With the exception of pyridoxine phosphate (PNP), all vitamers —pyridoxamine phosphate (PMP), pyridoxal phosphate (PLP), pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM)— and pyridoxic acid were purchased from Sigma (St. Louis, Mo., U.S.A.), and in the case of PM, also from Calbiochem (Los Angeles, Calif., U.S.A.). PNP was prepared by the method of Peterson and Sober[25]. A convenient internal standard, 3-hydroxypyridine (HOP), was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). All solutions of these standards were stored at pH $\leqslant 6$ in the dark and used for only 1–3 days.

Procedure

Two buffers are used in this method. The first is a 0.4 *M* NaCl–0.01 *M* glycine solution adjusted to a pH of 10.0 with sodium hydroxide and the second is a 0.4 *M* NaCl–0.01 *M* glycine solution with pH adjusted to 2.5 by the addition of hydrochloric acid. After the column is equilibrated with the pH 10 solution, the sample, which in the present case is a water solution containing all six vitamers plus pyridoxic acid, is injected and the flow is continued for 80 min. The eluent is then pumped from the pH 2.5 buffer reservoir for 40 min. The flow-rate is constant at 1.2 ml/min and the working pressure is 300 p.s.i.

RESULTS AND DISCUSSION

Typical chromatographs are shown in Fig. 2a and b. Fig. 2a is the trace from Monitor 1 and Fig. 2b is the corresponding trace from Monitor 2. The filters and lamp

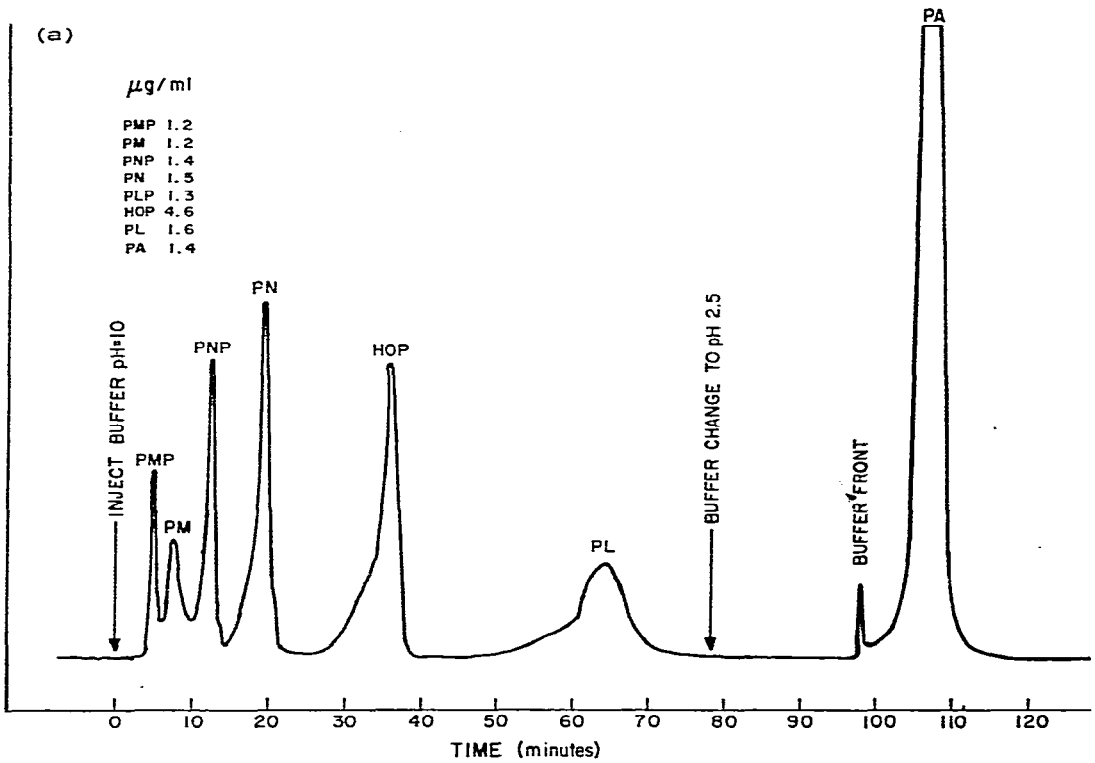

Fig. 2.

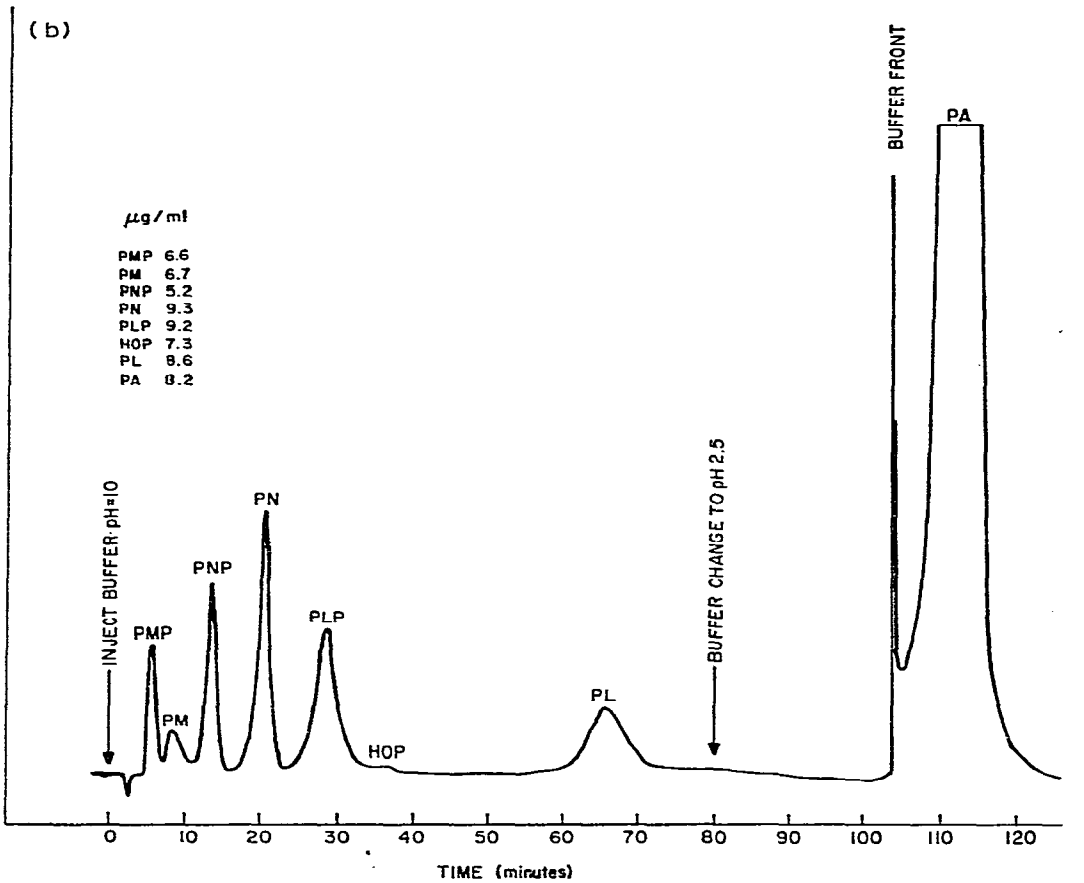

Fig. 2. Typical chromatographs. (a) Detection system uses Dell optics A-4-3100 primary filters and Corning 0-52 secondary filter. (b) Detection system uses Corning 7-51 primary and Wratten 2A secondary filter.

in Monitor 2 were chosen so as to enhance the sensitivity for the measurement of PLP which is a relatively weakly fluorescing compound. The total elapsed time for one run is 120 min. All six vitamers plus 3-hydroxypyridine are eluted within 80 min at pH 10.0 while pyridoxic acid elutes from the column 40 min after the pH has been changed to 2.5.

Standard solutions of various concentrations of all compounds were prepared and injected. The area under the peaks as measured by the Minigrator is linear in concentration for the different compounds in the range from micrograms down to the lower limits of detection. Those limits of detection were determined by the requirement that reproducibility must be 5%. Reproducibility is better than 1% for the higher concentration ranges. The lower limits are 10 ng for PMP, PM, PNP, PN, HOP and PL, 0.1 ng for pyridoxic acid, and 100 ng for PLP. This sensitivity, more than adequate for most food composition work, is achieved through the use of the natural fluorescence of the vitamers without recourse to any pre- or post-column chemistry.

This is the first successful separation of all B_6 vitamers and pyridoxic acid by

HPLC and is attributed to the use of anion exchange. If the various ionic forms of the different vitamers are considered as a function of pH as suggested by Bridges *et al.*[26], all would be expected to behave as anions at pH 10. In contrast, cation and anion behavior is not as clearly defined for all the compounds at low pH values.

The system is free from interference by compounds normally found in vitamin pills as is shown in Fig. 3 where a water extract of a commercial multi-vitamin pill was introduced into the system. The lack of interference is attributed to the choice of filters dictated by the maximum excitation and emission wavelengths for the B_6 vitamers[26] and the weakness of the fluorescence of possible interfering compounds at these wavelengths.

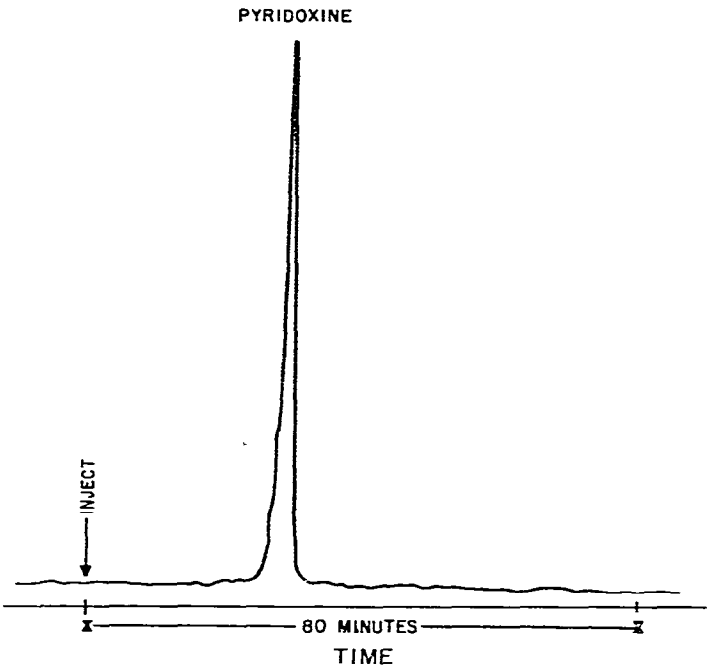

Fig. 3. Chromatograph of a multi-vitamin pill water extract.

The system can be easily automated by the method used in Beecher's[27] amino acid analysis work. For analyses of food, in which only vitamer content is of interest, injected samples could be continuously injected at pH 10 so that fifteen or more samples can be analysed in a 24-h period. However, a daily clean-up at pH 2.5 is recommended. Column clean-up plus regeneration require about 30 min.

Some mention should be made of the choice of 3-hydroxypyridine as the internal standard. For analysis of food, this internal standard should be added in known amounts to the sample prior to any extractions or analyses. This then serves as a check on both the extraction procedures and the behavior of the instruments. The choice of HOP was dictated by the fact that it is stable, does not coelute with the vitamers in the separation procedures and is expected to behave like the B_6 vitamers because of the similarities in their structures.

The system, as described, is simple and can be reproduced in any laboratory at minimal expense. It can be easily automated and the sensitivity is more than adequate for foods of interest. While these sensitivities are not yet adequate for blood

plasma work, it may be possible to achieve the necessary response with other detection methods, such as amperometric methods[28] or with post-column chemical reactions. Such work is in progress.

Experience to date has indicated that different matrices may require different vitamin extraction procedures and that no extraction procedure will be universally applicable to all matrices. Analysts will have to investigate B_6 recoveries for each matrix and for each extraction procedure. We are in the process of developing extraction procedures for various food types and these investigations will be the source of future publications.

ACKNOWLEDGEMENTS

The authors appreciate the instruction and help given by Darla J. Higgs, Robert Doherty, and Gary Beecher of the U.S. Department of Agriculture, and by Lou Heiss of the American Instrument Company.

REFERENCES

1 K. K. Stewart, in H. S. Hertz and S. N. Chester (Editors), *National Bureau of Standards: Proc. Ninth Materials Research Symposium, April 1979*, p. 249.
2 C. A. Storvick, E. M. Benson, M. A. Edwards and M. G. Woodring, *Methods Biochem. Anal.*, 12 (1964) 183.
3 E. W. Toepfer and M. M. Polansky, in R. S. Harris, I. G. Wool and J. A. Loraine (Editors), *Vitamins and Hormones*, Vol. 22, Academic Press, New York, 1964, p. 825.
4 C. A. Storvick and J. M. Peters, in R. S. Harris, I. G. Wool and J. A. Loraine (Editors), *Vitamins and Hormones*, Vol. 22, Academic Press, New York, 1964, p. 833.
5 H. E. Sauberlick, in P. Gyorgy and W. N. Pearson (Editors), *The Vitamins*, Vol. 7, Academic Press, New York, 2nd ed., 1967, Ch. 6, p. 169.
6 Y. H. Loo and W. M. Cort, *Methods Neurochem.*, 2 (1972) 169.
7 M. L. Orr, *Pantothenic Acid, Vitamin B_6 and Vitamin B_{12} in Foods*, Home Economics Research Report, No. 36, U.S. Department of Agriculture Bulletin, Washington, D.C., Aug. 1969.
8 B. E. Haskell, in *The National Research Council: Human Vitamin B_6 Requirements, Proceeding of a Workshop*, Letterman Army Institute of Research, Presidio of San Francisco, Calif., June, 1976, Ch. 4, p. 61.
9 M. Edwards, E. Benson and C. A. Storvick, *J. Ass. Offic. Agr. Chem.*, 46 (1963) 396.
10 J. F. Gregory and J. R. Kirk, in *The National Research Council: Human Vitamin B_6 Requirements, Proceedings of a Workshop*, Letterman Army Institute of Research, Presidio of San Francisco, Calif., June, 1976, Ch. 5, p. 72.
11 S. F. Contractor and B. Shane, *Clin. Chem. Acta*, 21 (1968) 71.
12 J. D. Mahuren and S. P. Coburn, *Anal. Biochem.*, 82 (1977) 246.
13 C. E. Colombini and E. E. McCoy, *Anal. Biochem.*, 34 (1970) 451.
14 W. Korytnyk, G. Fricke and B. Paul, *Anal. Biochem.*, 17 (1966) 66.
15 A. R. Prosser, A. J. Sheppard and D. A. Libby, *J. Ass. Offic. Anal. Chem.*, 50 (1967) 1348.
16 A. K. Williams, *J. Agr. Food Chem.*, 22 (1974) 107.
17 E. M. Patzer and D. M. Hilker, *J. Chromatogr.*, 135 (1977) 489.
18 P. A. Hedin, *J. Agr. Food Chem.*, 11 (1963) 343.
19 R. C. Williams, D. R. Baker and J. A. Schmit, *J. Chromatogr. Sci.*, 11 (1973) 618.
20 K. Caldmer and L. Davies, *Chromatographia*, 7 (1974) 644.
21 A. K. Williams and P. D. Cole, *J. Agr. Food Chem.*, 23 (1975) 915.
22 K. Yasumoto, K. Tadera, H. Tsuji and H. Mitsuda, *J. Nutr. Sci. Vitaminol.* 21 (1975) 117.
23 J. F. Gregory and J. R. Kirk, *J. Food Sci.*, 42 (1977) 1073.
24 K. K. Stewart, *Anal. Chem.*, 49 (1977) 2125.
25 E. A. Peterson and H. A. Sober, *J. Amer. Chem. Soc.*, 76 (1954) 169.
26 J. W. Bridges, D. S. Davies and R. T. Williams, *Biochem. J.*, 98 (1966) 451.
27 G. R. Beecher, in M. Friedman (Editor), *Nutritional Improvement of Food and Feed Protein*, Plenum Press, New York, 1978, p. 827.
28 E. P. Kujawa and S. L. Migdal, *Pittsburgh Conf., Cleveland, Ohio, 1978*, Paper No. 271.