Journal of Chromatography, 196 (1980) 176–179
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,860

Note

Liquid chromatographic separation and quantification of B_6 vitamers at plasma concentration levels

JOSEPH T. VANDERSLICE* and CATHERINE E. MAIRE

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

(Received March 28th, 1980)

A recent article in this journal[1] reports on the separation and quantification of the six forms of vitamin B_6 by a high-performance liquid chromatographic (HPLC) method. The lower limits of detection are 10 ng for all the vitamers, with the exception of PLP*, for which the lower limit of detection is 100 ng. While these sensitivities are adequate for most food composition work, they are less than adequate for the analysis of B_6 in whole blood or blood plasma. Shane[2], for example, reports values of from 10 to 25 ng/ml for the three principal forms (PM, PMP and PLP) of B_6 in whole blood from normal individuals. PLP is frequently used as an indicator of the vitamin B_6 status of an individual, and thus any analytical method used for such purposes must have a sensitivity of at least 1 ng/ml for PLP.

The purpose of this note is to report on modifications of our earlier method which improve the chromatographic separations and enhance the sensitivities so that the lower limits of detection are better than 1 ng/ml for all the vitamers, with an accuracy of 5%.

EXPERIMENTAL**

Vitamin B_6 chromatographic system

The principal components of the apparatus are shown in Fig. 1. It consists of a de-pulsed positive-displacement pump[3], a Valco AH-90 (Houston, TX, U.S.A.) pneumatically activated sample-injection valve with a 0.5-ml sample loop, a Valco AH-90 pneumatically activated six-way switching valve, two Glenco columns packed with Bio-Rad A-25 resin (Richmond, CA, U.S.A.), a Perkin-Elmer 650-40 fluorescence spectrophotometer and a Shimadzu CR-1A Chromatopac computing integrator. The first Glenco column is 6 mm × 24 cm and is thermostated at 50°C; the second is

* Vitamin B_6 compounds have been abbreviated, according to published recommendations (IUPAC–IUB Commission on Biochemical Nomenclature, 1970) as follows: PLP = pyridoxal phosphate; PMP = pyridoxamine phosphate; PNP = pyridoxine phosphate; PL = pyridoxal; PM = pyridoxamine; PN = pyridoxine.

** 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.

0021-9673/80/0000–0000/$02.25

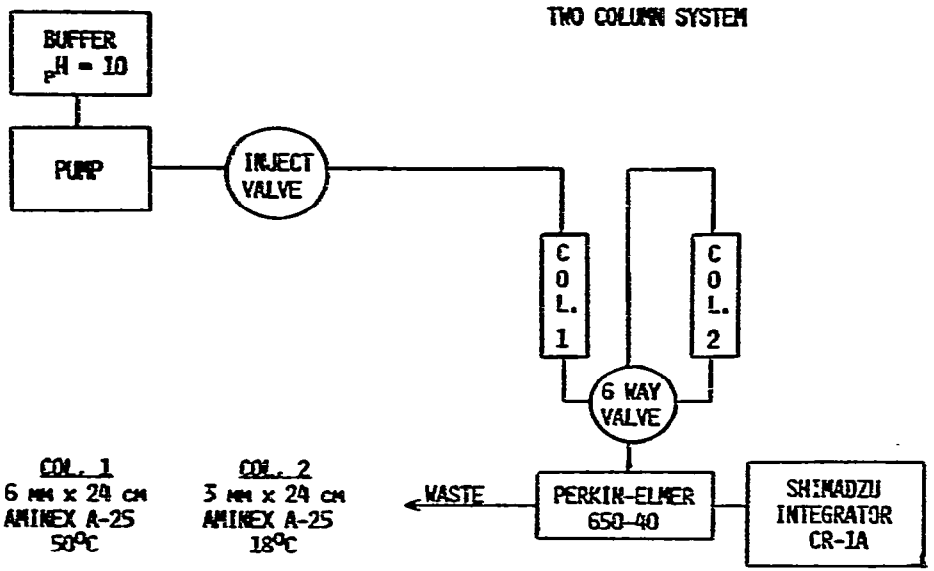

Fig. 1. Flow diagram of two-column HPLC system.

3 mm × 24 cm, and is thermostated at 18°C. The components of the entire system are connected by 26 AWG Teflon tubing and Cheminert™ fittings. The system is kept in a room under yellow fluorescent lights, and the entire procedure (from the initial weighing through to the final analysis) is performed in this room.

Chemicals

With the exception of PNP, all the vitamers were purchased from Sigma (St. Louis, MO, U.S.A.); PNP was prepared by the method of Peterson and Sober[4]. The internal standard, 3-hydroxypyridine (HOP), was obtained from Aldrich (Milwaukee, WI, U.S.A.). All solutions of these standards were stored at a pH between 5 and 6 in the dark and discarded after 24 h.

Procedures

The modified procedure involves the use of two different columns to enhance the separations of the vitamers and changes in excitation and emission wavelengths during the experiment to enhance sensitivities.

A single buffer is used in this procedure. It consists of 0.4 *M* sodium chloride, 0.01 *M* glycine, 0.005 *M* semicarbazide adjusted to a pH of 10 with sodium hydroxide. The flow-rate is 1.25 ml/min. For the first 23.4 min after injection, the buffer passes only through the first column, at a pressure of 250 p.s.i., before entering the detector; during this time, PMP, PM, PNP and PN are eluted. The spectrophotometer excitation and emission wavelengths are set at 310 and 380 nm, respectively (all four forms are detected at these wavelengths). The switching valve then re-directs the flow from the first column through the second column before entering the detector; the working pressure is now 500 p.s.i. At 23.4 min, the excitation and emission wavelengths are changed to 280 and 487 nm to detect PLP. After PLP has been eluted and detected

(39.5 min), the wavelengths are switched back to 310 and 380 nm to detect the internal standard, HOP. At 52.4 minutes, the second column is switched out of the flow, the wavelengths are set at 280 and 487 nm, and PL is detected at 63.4 min as it elutes from the column. Column switching is automatically controlled by an air-actuated valve (Tyna-Myte, Series No. 062-4E1-36, Humphrey Products, Kalamazoo, MI, U.S.A.). This valve is relay-controlled by a Shimadzu programmer PRG-102A, which, in turn, is controlled by the time program on the Chromatopac.

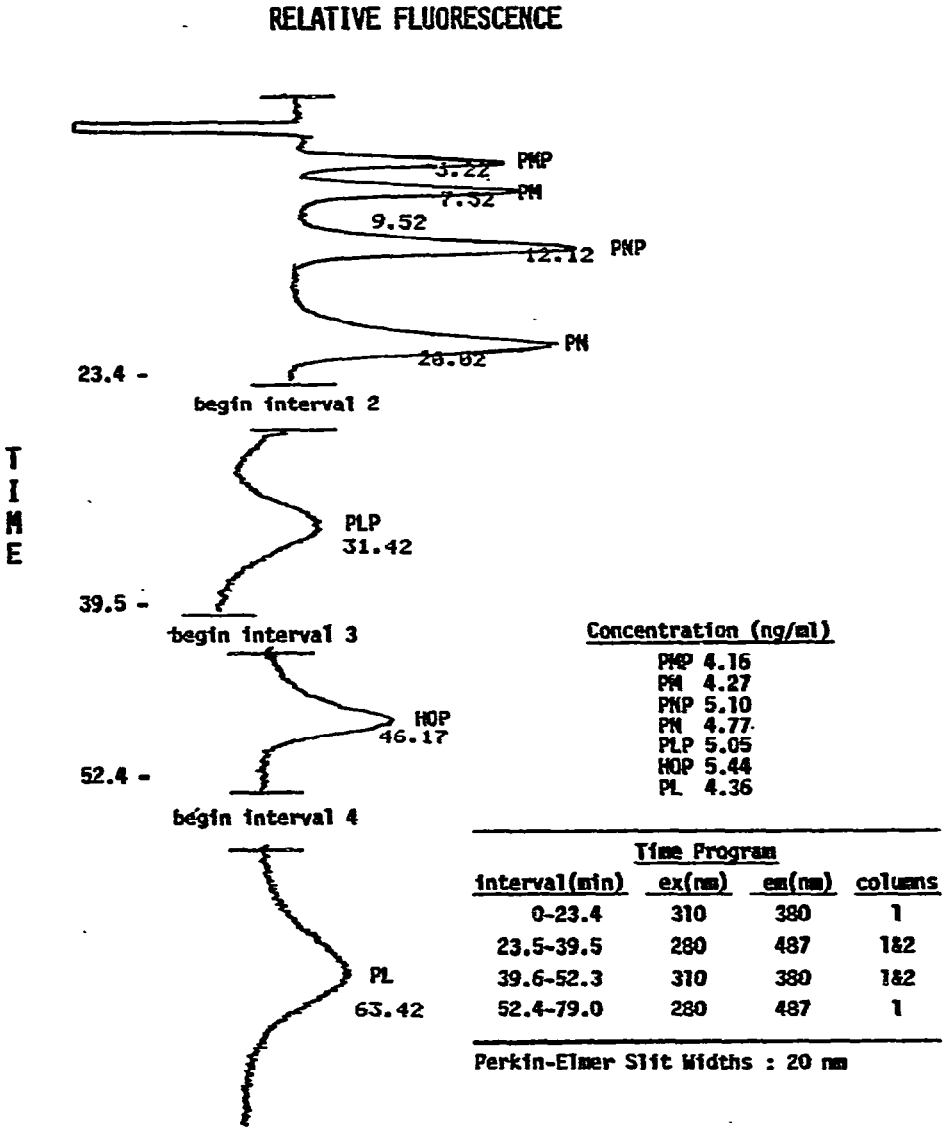

Fig. 2. Typical chromatogram. Concentrations of vitamers are shown, together with the time program controlling the behavior of the system. The time at each peak is the recorder elution time.

RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 2 (note that the concentrations are of the order of 4 to 5 ng/ml for the different vitamers). The lower limits of detection are 0.1 ng/ml for all vitamers except PLP and PL, for which are 0.5 ng/ml. At these limits of detection, reproducibility is 5%.

The improved sensitivity of the over-all method is due to several factors. Not only is the Perkin-Elmer 650-40 more sensitive than the detectors used previously, but it can also be programmed to detect at the maximum excitation and emission wavelengths for the individual vitamers. Secondly, and more importantly, the inclusion of semicarbazide in our buffer system converts both PLP and PL to their respective oximes, which fluoresce orders of magnitude greater than the original compounds. In fact, all the derivatives of ammonia seem to increase the fluorescence of PLP and PL[5]. Extensive work in our laboratory with hydroxylamine has shown that sensitivity is improved, but, as yet, no complete separation of the vitamers has been achieved. It is necessary to maintain this separation for our purposes, as results on different muscle tissues indicate that the concentrations of some other vitamers, such as PMP and PL, are of comparable magnitude to that of PLP[6].

There are several comments that can be made on this system. First, the sensitivity is such that it can be used for the analysis of vitamin B_6 in blood. Second, the metabolite pyridoxic acid can be detected as in our earlier procedure. Namely, if the pH of the buffer is changed to 2.5, the acid will elute in 20 minutes, with a lower limit of detection of 0.01 ng/ml. During this elution, only the first column is on line. Third, the separation of the first five vitamers is significantly improved over the earlier method. Fourth, no interference from other water-soluble vitamers is observed. This was tested, as in our earlier procedure, by introducing an aqueous extract of a commercial multi-vitamin pill into the system.

Finally, it should be mentioned that the system herein described (as well as the earlier one[1]) has been found to be compatible with an extraction procedure (98% recovery) recently developed and successfully applied to cereals, meats, dairy products and fish[6].

ACKNOWLEDGEMENT

The authors are grateful to Darla J. Higgs for her stimulating suggestions on the separation techniques and to James Brown for advice on the relay-controlled switching mechanism.

REFERENCES

1 J. T. Vanderslice, K. K. Stewart and M. M. Yarmas, *J. Chromatogr.*, 176 (1979) 280.
2 B. Shane, in *The National Research Council: Human Vitamin B_6 Requirements, Proceedings of a Workshop*, Letterman Army Institute of Research, Presidio of San Francisco, CA, June 1976, Ch. 7, p. 111.
3 K. K. Stewart, *Anal. Chem.*, 49 (1977) 2125.
4 E. A. Peterson and H. A. Sober, *J. Amer. Chem. Soc.*, 76 (1954) 169.
5 B. E. Haskel, in *The National Research Council: Human Vitamin B_6 Requirements, Proceedings of a Workshop*, Letterman Army Institute of Research, Presidio of San Francisco, CA, June 1976, Ch. 4, p. 61.
6 J. T. Vanderslice, C. E. Maire, R. F. Doherty and G. R. Beecher, *J. Agr. Food Chem.*, submitted for publication.