Journal of Chromatography, 463 (1989) 207–211
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 018

Note

Analysis of B_6 vitamers in foods using a modified high-performance liquid chromatographic method

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(First received June 10th, 1988; revised manuscript received September 26th, 1988)

In recent years high-performance liquid chromatographic (HPLC) procedures, which are capable of separating and quantifying the various vitamin forms (vitamers), have been accepted as reliable, time sparing and sensitive methods for the determination of the pyridoxine content in foods and other biological materials. HPLC techniques have been presented by numerous authors and the detection limits are comparable to those of microbiological assays[1–15]. Nevertheless, the procedures described so far exhibit some disadvantages, summarized as follows:

(1) separation of the vitamers with the aid of ternary gradient elution techniques, which are poorly reproducible for routine work[1–3];

(2) elution with buffers of relatively high concentrations, *e.g.*, 0.5 *M* phosphate, which are not easy to handle and are able to crystallize within the columns and tubes[1,2,6];

(3) rather long retention times, $\geqslant$ 1 h (refs. 1, 2 and 5);

(4) incomplete separation of the several vitamers, mostly not including pyridoxic acid, which may be present in foods, though vitamin inactive[3,6,9,13,15].

Some procedures[4,5] are hardly reproducible[2]. Considering these problems we have developed a modified HPLC method for the separation of all B_6 vitamers including their phosphorylated metabolites (but without pyridoxine phosphate) as well as the inactive pyridoxic acid. The method is based on the procedure of Gregory and Feldstein[3] who employed reversed-phase chromatography with an ion-pair reagent. The most prominent modifications refer to the mode of elution, by means of a binary gradient, and the extraction medium using perchloric acid to extract also pyridoxic acid.

EXPERIMENTAL

Reagents

All chemicals, reagents and standards were of analytical or HPLC grade. 4-Pyridoxic acid (4-PA) and 4-desoxypyridoxine hydrochloride (DPN) were obtained from Sigma-Chemie (Deisenhofen, F.R.G.), alkaline phosphatase (specific activity 140 U/mg) from Boehringer (Ingelheim, F.R.G.), B_6 vitamers and all other chemicals from Merck (Darmstadt, F.R.G.).

0021-9673/89/$03.50

Chromatographic conditions

An LKB HPLC gradient system (Pharmacia LKB, Freiburg, F.R.G.) consisting of an HPLC pump, syringe-loading sample injector Rheodyne Model 7125 with an 100-μl loop and solvent conditioner for solvent degassing, HPLC controller, gradient mixer and mixing valve for low-pressure gradient mixing was utilized. For detection an Hitachi fluorescence detector Model F 1000 with a 40-μl flow cell (excitation 330 nm, emission 400 nm) was equipped with an Hitachi integrator Model D-2000 (Merck/Hitachi, Darmstadt, F.R.G.).

The analytical column was a LiChrospher RP-18, 5 μm, 125 mm × 4 mm (Merck LiChrocart No. 50943) with a guard column containing LiChrospher RP-18, 4 mm × 4 mm (Merck LiChrocart No. 50803). The separations were performed at 25°C. The mobile phase consisted of methanol (solvent A) and 0.03 *M* phosphate buffer pH 2.7 + 4 m*M* octanesulphonic acid (solvent B), delivered at a flow-rate of 1.5 ml/min. A binary gradient was formed as follows: 90% B and 10% A from 0 to 2 min; linear gradient from 90% B at 2 min to 60% B at 12 min; 60% B and 40% A from 12 to 17 min; 60% B from 17 min to 90% B at 19 min.

For post-column derivatization, 0.5 *M* phosphate buffer pH 7.5 was mixed with sodium hydrogensulphite (10 μl of a 37% aqueous solution per ml buffer) with a flow-rate of 0.07 ml/min. Desoxypyridoxine (DPN) served as an internal standard.

Sample preparation and extraction procedure

All steps for extraction of food samples must be conducted under subdued light and using brown glass vessels. A representative minced food sample, *e.g.*, 0.5–2.0 g, was mixed with 50 nmol DPN as an internal standard and homogenized in an ice-bath with 5 ml ice-cold 0.1–0.5 *M* perchloric acid (dependent on protein content) using an Ultra-Turrax homogenizer. After filtration or centrifugation, the supernatant was adjusted to pH 7.5 with 5 *M* and finally 0.1 *M* potassium hydroxide and kept in an ice-bath for another 10 min. After an additional filtration step, the clear filtrate was then adjusted to pH 4.0 with 0.1 *M* hydrochloric acid and filtered through a 0.45-μm pore size membrane filter (Minisart NML, Sartorius/Göttingen, F.R.G.).

For dephosphorylation of vitamers, 20 μl alkaline phosphatase suspension (10 mg/ml, 140 U/mg) were added to an aliquot of the solution taken after the last filtration step, followed by incubation for 30 min at 25°C. Adjustment to pH 4.0 and filtration through a membrane filter were performed as above.

An 100-μl volume of the clear filtrate before and after dephosphorylation were used for the HPLC analysis.

RESULTS AND DISCUSSION

The chromatographic procedure used enables rather good separations of all vitamers as well as pyridoxic acid (4-PA), but excluding pyridoxine phosphate, which occurs only in minute amounts in foods. The binary gradient employed for elution is well reproducible; separations are performed in a relatively short time of about 30 min.

The chromatogram of a standard mixture (100 n*M* solution in 0.1 *M* acetate buffer, pH 4.0, 400 n*M* DPN) is shown in Fig. 1. The lowest detectable amount of substances separated corresponds to 0.4–0.7 pmol. This is lower than the detection

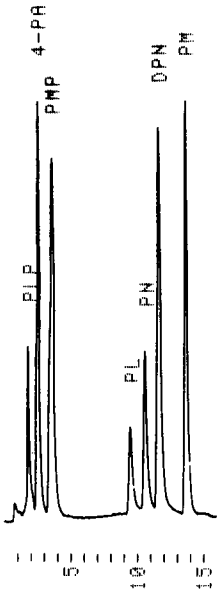

Fig. 1. Chromatogram of vitamin B_6 standard. Time scale in min.

limits reported to date, to the best at our knowledge. Linearity of the dose response between the peak height and the vitamer concentration is achieved up to at least 1 μM.

The within-day coefficients of variation determined for 100 nM solutions ranged from 1.1 (DPN) to 5.0% (PLP), those of the day-to-day variation from 6.2% (4-PA) to 10.3% pyridoxamine 5′-phosphate (PMP). When food samples are analyzed, however, the separation and resolution of PLP, being eluted first, may be inadequate since it is eluted very close to the elution front. For a suitable verification of peak identity, a duplicate of the food sample is treated with alkaline phosphatase. As shown for the case of raw pork liver, the peaks of the phosphorylated derivatives in the sample, PLP and PMP, have disappeared and the peaks of pyridoxal (PL) and pyridoxamine (PM) have increased (Fig. 2).

The technique of post-column derivatization with sodium hydrogensulphite as first described by Coburn and Mahuren[1,2] leads to an enhancement of the fluorescence intensity mainly of PLP and 4-PA. Additionally, the fluorescence of interfering matrix substances that might overlap the peaks of B_6 vitamers is minimized due to the simultaneous shift from an acidic to a weakly alkaline pH. Thus, particularly in food samples with a rather complex matrix, *e.g.*, pork liver, such interfering peaks rarely occur.

In Table I the concentration of the vitamers and of 4-PA in raw liver as well as in pasteurized milk is given. Noteworthy is the relatively high amount of the vitamin inactive 4-PA in milk that was also found by Coburn and Mahuren[1]. Regarding the total content of vitamin B_6, it appears that our own results calculated from the concentration of the single derivatives correspond to the mean value of 40–45 μg B_6/dl given in food tables only after addition of the inactive pyridoxic acid[16,17].

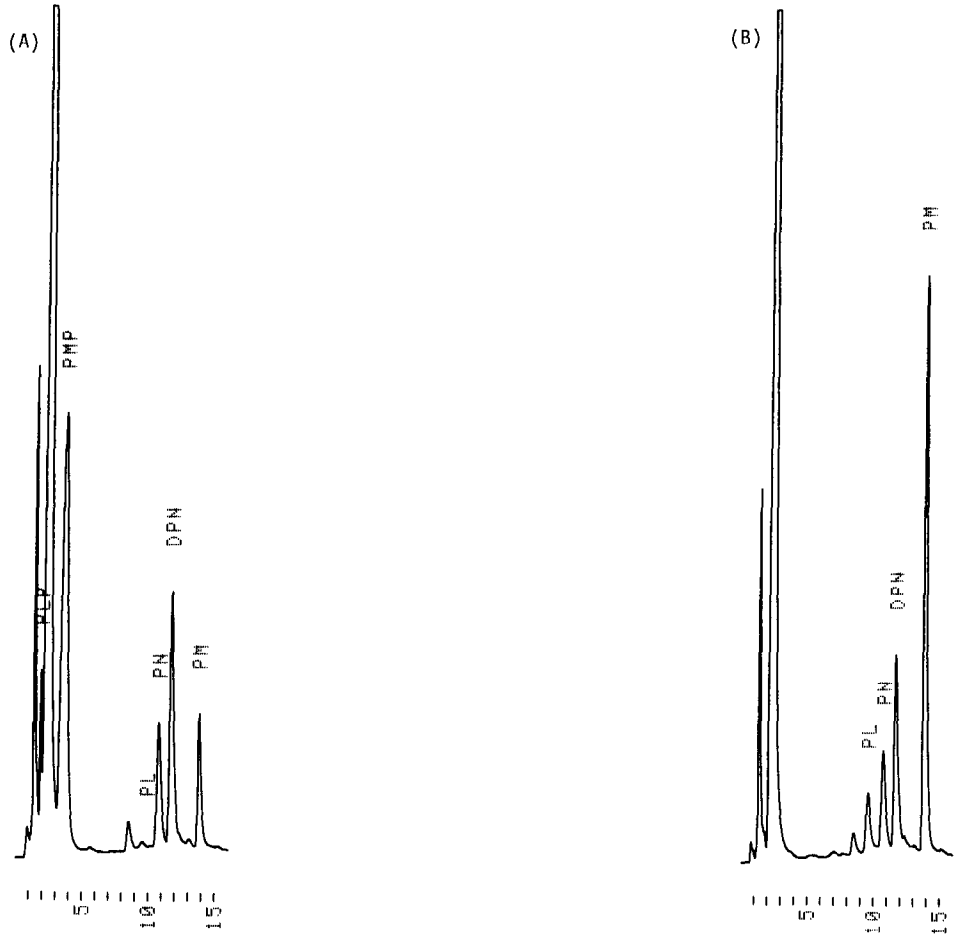

Fig. 2. (A) Separation of B_6 vitamers in a sample of raw pork liver. (B) Chromatogram of a sample of raw pork liver treated with alkaline phosphatase. Time scale in min.

TABLE I

CONTENT (nmol/g) OF B_6 VITAMERS IN RAW LIVER AND PASTEURIZED MILK

n.d. = Not detectable.

	PLP	*4-PA*	*PMP*	*PL*	*PN*	*PM*	*Σ*
Raw liver	7.2 ±0.7	n.d.	24.2 ±0.2	1.0 ±0.1	7.4±0.4	10.8 ±0.4	50.61≡850.3 μg/100 g
recovery (%)	89.2		96.0	95.8	101.8	96.3	
Pasteurized milk	0.35±0.06	0.65±0.03	0.19±0.01	1.18±0.02	n.d.	0.18±0.02	2.55≡ 43.5 μg/100 g
recovery (%)	94.9	101.4	96.8	95.3	96.2	92.4	

Recoveries were determined by spiking of representative food samples (0.5 g liver, 5.0 g milk) with vitamers (5 nmol) before extraction. Values for phosphorylated vitamers are given as their corresponding unphosphorylated compounds after treatment with phosphatase.

The results provide evidence of a reasonable reliability comparable to other HPLC methods.

ACKNOWLEDGEMENT

This research was supported by Deutsche Forschungsgemeinschaft, grant Bi 218/5-1.

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