B_6 vitamers: cation exchange HPLC

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Overview

B_6 vitamers are extracted with trichloroacetic acid and quantitated using ternary gradient cation-exchange liquid chromatography with fluorescence detection. Fluorescence of pyridoxal and pyridoxal phosphate is enhanced through addition of a post-column buffer containing bisulfite.

Reagents

All chemicals are reagent grade. Water must be HPLC quality.

Trichloroacetic acid 40%. Dissolve 20 g solid trichloroacetic acid (Sigma Chemical Co., St. Louis, MO, USA) in water and make to 50 mL volume. This solution should be kept refrigerated and used for up to 2 months.

Trichloroacetic acid 10%. Prepare fresh daily by mixing one volume of 40% stock solution with 3 volumes water.

Ethyl ether. Redistill over ferrous sulfate as necessary to eliminate peroxides.

Chromatographic solvents

Solvent A. 0.02 N hydrochloric acid (HCl). Dilute 1.66 mL concentrated HCl to 1000 mL with water.

Solvent B. Dissolve 5 g anhydrous monobasic sodium phosphate (NaH_2PO_4; Sigma) in 500 mL water and adjust to pH 3.3 by adding concentrated (87%) phosphoric acid (H_3PO_4; J.T. Baker Chemical Co., Phillipsburg, NJ, USA) dropwise.

Solvent C. Dissolve 48 g anhydrous NaH_2PO_4 in 1000 mL water, and add anhydrous dibasic sodium phosphate (Na_2HPO_4; Sigma) to achieve pH 5.9 (usually about 5 g).

Post-column buffer. Dissolve 122 g anhydrous dibasic potassium phosphate (K_2HPO_4; Spectrum Chemical Corp., Gardena, CA, USA) in water and dilute to 200 mL. Adjust to pH 7.8 by adding 3.5 N H_3PO_4 (80 mL concentrated H_3PO_4 to 1000 mL volume).

Post-column reagent. Add 3 mg sodium bisulfite ($NaHSO_3$; J.T. Baker) per mL of post-column buffer. Make fresh daily.

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Standard solutions. Pyridoxine hydrochloride, pyridoxal hydrochloride, pyridoxamine dihydrochloride, pyridoxal 5′-phosphate, pyridoxine 5′-phosphate, pyridoxamine 5′-phosphate hydrochloride, and 4-pyridoxic acid (all from Sigma except pyridoxine-5′-phosphate and pyridoxic acid lactone, which are synthesized in our laboratory) are maintained frozen as 1 mg/mL solutions. Working solutions are kept refrigerated as 10 μg/mL in 0.02 mol/L sodium phosphate buffer pH 7. They are made fresh at least monthly and verified by UV spectroscopy.[1]

Internal standard. The stock solution of 3, 5-diaminobenzoic acid (K&K Laboratories, Plainview, NY, USA) is kept in the freezer as 0.5 mg/mL ethanol. The working solution of 20 μg/mL water is kept refrigerated.

HPLC column packing

Vydac 401 TP cation exchange material (Separations Group, Hesperia, CA, USA) is slurry packed into a 30 × 0.46 cm stainless steel HPLC column using the Micromeritics Model 705 column packer (Alcott, Norcross, GA, USA). The Vydac is slurried in the packer in isopropanol and packed by pumping water through the system. A 5 × 0.46 cm guard column is packed the same way. Columns can also be purchased pre-packed.

HPLC instrumentation

Spectra Physics (San Jose, CA, USA) Model 8700 Solvent Delivery System capable of ternary gradient formation; Micromeritics Model 728 Autosampler with Valco (Houston, TX, USA) Model EC6W electric valve actuator with 500 μl loop; Farrand (Valhalla, NY, USA) Mark 1 spectrofluorometer with 300 μL flow cell; Shimadzu (Kyoto, Japan) C-R3A integrator capable of storing overnight analyses; Harvard (South Natick, MA, USA) Model 975 infusion pump for post-column reagent.

Alternative instrumentation consists of a Shimadzu RF-530 fluorescence monitor, a Hewlett-Packard 3390A integrator, and an Isco (Lincoln, NE, USA) Model 314 syringe pump.

Procedure

Sample preparation

1. Add an equal volume of 10% trichloroacetic acid to plasma, serum, or erythrocyte suspensions to precipitate protein and release B_6 vitamers. Solid samples, such as animal tissues, should be homogenized directly in 5% trichloroacetic acid to avoid shifts in vitamer distribution during processing.[2] Normal urine can be diluted in solvent A without prior treatment with trichloroacetic acid. If possible, solvent to sample ratios are chosen so that the final concentration of the least abundant vitamer of interest will be about 2 ng/mL.
2. Centrifuge at 1000*g* for 10 min.
3. Place supernatant liquid in new tube and add 4–5 volumes ethyl ether. Mix well (Vortex mixer) for 1 min.
4. Centrifuge at 800*g* for 10 min.
5. Aspirate and discard upper ether layer.
6. Add 5 μL of 20 μg/mL internal standard solution to 1.0 mL aqueous sample. Mix.
7. Sample is ready for injection.

Chromatographic procedure

The procedure utilizes 3 solvents to create a gradient of increasing pH and ionic strength at a flow rate of 1.5 mL/min. Post-column reagent is introduced immediately after the column at a flow rate of 1.78 mL/h. A mixing coil of five 2.5 cm diameter coils follows the introduction of the post-

column reagent. Set the excitation wavelength at 330 nm and emission at 400 nm.

Program the gradient as follows:

Time (min)	Solvent	(%)
0	A	100
10	A	100
20	B	100
30	C	100
38	C	100

This produces a linear gradient from 100% A at 10 min to 100% B at 20 min, then a linear gradient from 100% B to 100% C at 30 min. At 38 min the program returns to 0 time and 100% A. The system is allowed to equilibrate with 100% A for 10 min before another sample is injected. The analysis is recorded and quantitated on the Shimadzu integrator using calibrated standard amounts based on the internal standard. The system is flushed with water and stored in methanol when not in use.

Figure 1 shows typical retention times for standards except pyridoxine phosphate, which elutes at about 9 min. The increased ionic strength of biological samples significantly reduces the retention times for pyridoxal phosphate, pyridoxine phosphate, and pyridoxic acid.

Discussion

Work with several laboratories has validated this method against enzymatic,[3,4] microbiological,[3] open column,[4] and reverse phase[2] methods and by use of uniformly labeled tissues.[2] The major impediment to more widespread use of this method seems to be the use of a ternary gradient. However, the cost of the ternary pump used here is comparable to the cost of binary systems.

The changes from our originally published method[5] include different preparation procedures for HPLC solvents to reduce contamination, a change in buffer and concentration for the post-column reagent, a simpler and slightly shorter gradient program, and a different internal standard. The change in internal standard was due to variations from lot to lot in the Vydac that occasionally resulted in interference between the internal standard and pyridoxamine phosphate. We have also used 4-deoxypyridoxine and N-methyl-pyridoxine as internal standards. The present lot of Vydac (#880720-9) is the best material we have received, with sharper peaks and better longevity than previous lots. Retention times, particularly for pyridoxal phosphate, decrease as the column ages requiring gradual adjustments in the gradient.

Pyridoxal phosphate will not fluoresce satisfactorily in solvent A without the post-column reagent. A convenient way to verify that the post-column reagent is flowing is to check the pH of the effluent. The pH should be 6 or higher. If it is not, adjust the post-column pump. The sensitivity and linearity range are dependent on the column, fluorometer, vitamer, and type of sample. Pyridoxal phosphate is the least fluorescent vitamer. With standards injection of 500 μL containing as little as 1.2 pmol (0.3 ng), pyridoxal phosphate will produce a quantifiable peak. For preparative purposes we have injected as much as 5 μmol (1 mg) pyridoxine hydrochloride on the column. For tissues low in vitamin B_6 we can inject trichloroacetic acid extracts equivalent to 50 mg of tissue. Generally pyridoxal, pyridoxal phosphate, and pyridoxic acid are the only vitamers detected in plasma. Apparent pyridoxine and pyridoxamine peaks are usually eliminated if HPLC solvents are kept fresh and clean. A frequent apparent pyridoxamine phosphate peak is usually found to have a slightly different retention time from pyridoxamine phosphate if the plasma is spiked with

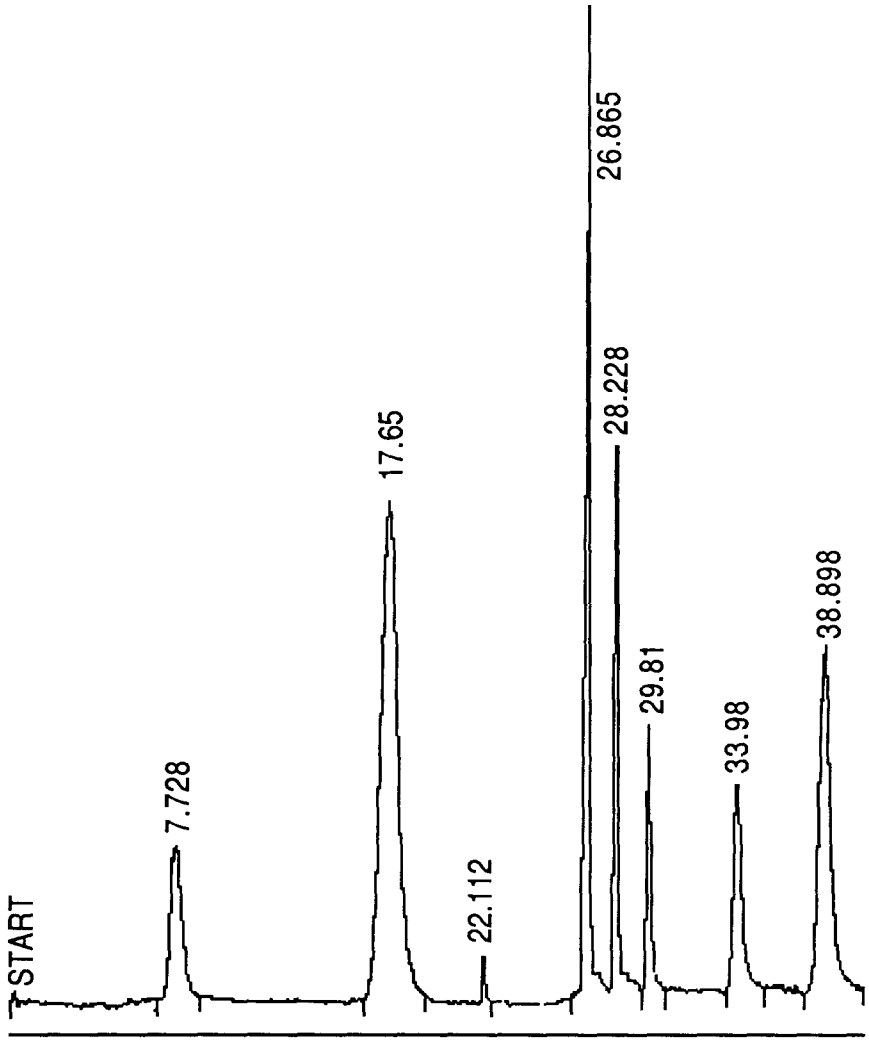

Figure 1 Chromatogram of 10 ng of each B_6 vitamer in 500 μl using sensitivity settings for normal serum (pyridoxal phosphate 7.7 min, pyridoxic acid 17.6 min, pyridoxamine phosphate hydrochloride 26.8 min, pyridoxal hydrochloride 29.8 min, pyridoxine hydrochloride 33.9 min, pyridoxamine dihydrochloride 38.3 min). Peaks at 22.1 min and 28.2 min are solvent change and internal standard, respectively.

pyridoxamine phosphate. Other tissues, however, generally do contain pyridoxamine and pyridoxamine phosphate. We have rarely detected pyridoxine phosphate in tissues. We have encountered interfering peaks in plant products, in plasma of animals consuming forage diets,[3] and in renal tissue.[2] Intestinal tissue may also produce interfering peaks if contaminated with intestinal contents. It is important to remember that elution time alone is not a guarantee of peak identity. Further tests should be done before concluding that unusual peaks are really vitamin B_6 compounds from the sample. Gradient elution may concentrate impurities from the initial solvents during the early part of the run and elute them later. We periodically find interfering substances in our water supply. This can be checked by running the gradient without injecting a sample. Also, the trichloroacetic acid and ether can be a source of spurious peaks and need to be kept fresh. There is continuing controversy over the recovery of B_6 vitamers from erythrocytes. Dilution of erythrocyte suspensions to hematocrits of 50% or less before adding trichloroacetic acid seems to be critical to good extraction.

Pyridoxic acid is easily quantitated in urine using only Solvent A isocratically. Urine is too complex to allow quantitation of other B_6 vitamers

under normal vitamin B_6 intake. If the vitamin B_6 intake is much higher than normal, pyridoxine and pyridoxal can be seen using the usual gradient. With very large vitamin B_6 intakes, additional metabolites such as pyridoxic acid lactone may be detected in plasma and urine. The retention time for pyridoxic acid lactone is about the same as that of 3, 5-diaminobenzoic acid. Therefore, we select one of the other internal standards when running samples with megadoses of vitamin B_6. Under the conditions used here the fluorescence of the lactone is about 10% that of an equal concentration of pyridoxic acid. We have not tested pyridoxine glucoside in this system.

Reverse-phase ion pairing techniques[6] generally give results comparable to our cation exchange procedure but may require a protein precipitant other than trichloroacetic acid.[2] We did encounter some unexplained differences between the reverse phase and cation-exchange procedures in the analysis of cat plasma.[2]

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