



Laboratory Procedure Manual

Analyte: **Vitamin B₆ (pyridoxal 5'-phosphate; 4-pyridoxic acid)**

Matrix: **Serum**

Method: **HPLC-fluorescence**

Method No: 4002.05

Revised:

as performed by: Nutritional Biomarkers Branch (NBB)
Division of Laboratory Sciences (DLS)
National Center for Environmental Health (NCEH)

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Important Information for Users

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

Data file name	Variable name	SAS Label
VIT_B6_E	LBX4PA	4-pyridoxic acid (nmol/L)
	LBXPLP	Pyridoxal 5'-phosphate (nmol/L)

1. Summary of Test Principle and Clinical Relevance

A. Clinical relevance

First identified in 1934 by György as the preventative factor for rat pellagra [1], vitamin B₆ presently refers to a collective of six biologically interconvertible 3-hydroxy-2-methylpyridine compounds: pyridoxal (PL); pyridoxine (PN); pyridoxamine (PM) and their respective 5'-phosphates (PLP, PNP, PMP) [2]. Of these compounds, PLP is the primary biologically active form of vitamin B₆, serving as a coenzyme in numerous biologic processes that include tryptophan-niacin conversion (kynureninase), heme synthesis (δ -aminolevulinic acid synthetase), gluconeogenesis (glycogen phosphorylase), neurotransmitter synthesis, and amino acid metabolism (various transaminases and decarboxylases) [3]. In addition, PLP acts as a coenzyme in the catabolism of homocysteine to cystathionine (cystathionine β -synthase) and ultimately cysteine (cystathionine γ -lyase) [3]. As a result, vitamin B₆ plays a role in lowering homocysteine levels and its associated risk with atherosclerosis and coronary heart disease [4,5], although the clinical evidence of this is somewhat inconsistent. Additionally, low PLP status may be associated with an increased risk for stroke and transient ischemic attack, irrespective of homocysteine levels [6].

Clinical vitamin B₆ status typically is assessed by measuring the level of one or more of the B₆ vitamers in vivo. Plasma or serum PLP concentration traditionally has been used as a status indicator because it is the active coenzyme form of vitamin B₆. Circulating PLP levels were also believed to correlate with indicators of long-term B₆ storage in the body, such as the amount of glycogen phosphorylase-bound PLP in skeletal muscle [7] but more recent evidence suggests that this is not the case [8,9]. As a result, the use of PLP alone as a status indicator has been contested, and the measurement of PLP along with other B₆ vitamers and indicators has been proposed [10]. An example is the measurement of the end product of vitamin B₆ catabolism, 4-pyridoxic acid (4PA), in either urine [11] or plasma/serum as an indicator of short-term status.

B. Test principle

Serum is prepared for analysis by first combining the specimen 1:1 with a 5% solution of metaphosphoric acid to precipitate proteins out of the sample. After vortex mixing the sample is centrifuge filtered and ready for high-performance liquid chromatographic (HPLC) analysis.

Vitamin B₆, in the form of PLP, and the metabolite 4PA are measured by reversed-phase HPLC using fluorometric detection at 325 nm excitation and 425 nm emissions. Post-column introduction of a sodium chlorite derivatization reagent is incorporated into the HPLC system to improve the PLP signal. Quantitation is based on analyte peak area interpolated against a five-point calibration curve obtained from aqueous standards [12,13].

2. Safety Precautions

Consider all plasma or serum specimens potentially positive for infectious agents including HIV and the hepatitis B virus. We recommend the hepatitis B vaccination series for all analysts working with whole blood and/or plasma. Observe universal precautions; wear protective gloves, laboratory coats, and safety glasses during all steps of this method. Discard any residual sample material by autoclaving after analysis is completed. Place disposable plastic, glass, and paper (pipet tips, autosampler vials, gloves, etc.) that contact plasma in a biohazard autoclave bag and keep these bags in appropriate containers until sealed and autoclaved. Wipe down all work surfaces with 10% bleach solution when work is finished.

Handle acids and bases with extreme care; they are caustic and toxic. Handle organic solvents only in a well-ventilated area or, as required, under a chemical fume hood.

Reagents and solvents used in this study include those listed in Section 6. Material safety data sheets (MSDSs) for these chemicals are readily accessible as hard copies in the lab. If needed, MSDS for other chemicals can be viewed at <http://www.ilpi.com/msds/index.html> or at <http://intranet.cdc.gov/ohs>.

3. Computerization; Data System Management

During sample preparation and analysis, samples are identified by their sample ID. The sample ID is a number that is unique to each sample that links the laboratory information to demographic data recorded by those who collected the sample.

The raw data file and respective batch file from the HPLC instrument are collected using the instrument software and stored on the instrument workstation. Results are typically generated by auto-integration, but may require in some cases manual integration. The results file (including analyte names, peak areas, retention times, sample dilution factor, data file name, acquisition time, etc.) is imported into a LIMS database for review of the patient data, statistical evaluation of the QC data, and approval of the results. See **"4002.05 SOP for Computerization and Data System Management"** for a step-by-step description of data transfer, review, and approval.

For NHANES, data is transmitted electronically. Abnormal values are confirmed by the analyst, and codes for missing data are entered by the analyst and are transmitted as part of the data file. NCHS makes arrangements for the abnormal report notifications to the NCHS Survey Physician.

Data processing is typically performed at the instrument workstation. Data files from the instrument workstation are typically copied to the CDC network on a periodic basis. This is the responsibility of the analyst under the guidance of the team lead and/or supervisor. Files stored on the CDC network are automatically backed up nightly by ITSO support staff.

4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

For best results, a fasting sample should be obtained.

Specimens for vitamin B₆ analysis may be fresh or frozen serum. In the absence of serum, heparinized plasma may be used. EDTA plasma should be avoided due to a potential interference with the PLP signal.

A 350- μ L sample of plasma or serum is preferable to allow for repeat analyses; a volume of 100 μ L is required for analysis.

The appropriate amount of plasma or serum is dispensed into a Nalgene 2.0-mL cryovial or other plastic screw-capped vial labeled with the participant's ID.

Specimens collected in the field are frozen, and then shipped on dry ice by overnight carrier. Frozen samples are stored at -70°C. Samples are stable for at least 5 years if stored at -70°C. They can withstand at least 5 freeze/thaw cycles [12].

Specimens generally arrive frozen. Refrigerated samples may be used provided they are kept cold and brought promptly (within 2 hours) from the site of collection.

Specimens that have been through more than five freeze-thaw cycles, been refrigerated for more than one week, or undergone hemolysis may give inaccurate results.

Specimen handling conditions are outlined in the DLS Policies and Procedures Manual. The protocol discusses collection and transport of specimens and the special equipment required. In general, serum should be transported and stored at no more than -20°C. Samples thawed and refrozen less than five times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of serum should be transferred into a sterile Nalgene cryovial labeled with the participant's ID.

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable for this procedure

6. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

A. Reagent Preparation

Prepare all reagents using deionized water with resistance of at least 15 megaOhm-cm.

- (1) 5% metaphosphoric acid (MPA) solution

CAUTION! Metaphosphoric acid is corrosive. It is a contact and inhalation hazard. Consult MSDS before use.

Verify the actual MPA content (%) of the solid reagent you are using. This is stated on the label, and is usually around 35%. Prepare the 5% MPA solution using the following formula:

$$MPA\ required(solid, g) = \left(\frac{volume\ of\ solution\ being\ made\ (mL)}{actual\ MPA\ content\ of\ reagent\ (\%)} \right) \times 5\ \%$$

For example, if the actual MPA content of the reagent was 35% and you wanted to make 30 mL of reagent, you would add 4.29 g of the MPA reagent to 30 mL of water.

Prepare 30 mL of 5% MPA according to the formula above by adding the appropriate amount of MPA reagent to 30 mL of water in a 50 mL polypropylene screw-top centrifuge tube. Mix vigorously using a vortex mixer until the solid reagent dissolves. Refrigerate immediately and store at 4 °C at all times. Solutions maintained at 4 °C are good for 5 days.

- (2) 50 mM phosphate buffer, pH 3.2, 0.1% acetonitrile (mobile phase)

Weigh 6.9 g of sodium phosphate, monobasic, monohydrate (NaH₂PO₄(H₂O)) into a 1-L glass beaker or bottle. Add 1000 mL of water using a graduated cylinder, and add 1 mL of acetonitrile using a 1-mL micropipette twice. Mix the solution using a magnetic stir bar until all of the sodium phosphate dissolves. Continue stirring the solution and measure its pH using a digital pH meter set to read to two decimal places. The pH should be around 4.6. Slowly add concentrated (85%) orthophosphoric acid drop wise until the pH of the solution is 3.2 (3.18–3.22 is acceptable). Vacuum filter the solution through a 0.45 µm polyvinylidene fluoride filter into a clean 1-L amber glass bottle and cap tightly. This solution will be good for 5 days.

- (3) 2 g/L sodium chlorite solution (post-column derivatization solution)

CAUTION!!! Sodium chlorite is a strong oxidant. Contact with combustible material may cause fire. Do not heat sodium chlorite with organic matter or strong reducing agents. Contact with acids releases toxic fumes. Sodium chlorite is toxic in contact with the skin and if swallowed. Consult MSDS before use.

Weigh 2.0 g of sodium chlorite into a 1-L glass beaker or bottle. Add 1000 mL of water, and mix the solution with a magnetic stir bar until all of the sodium chlorite dissolves. Vacuum filter the solution through a 0.45 µm polyvinylidene fluoride filter into a clean 1-L amber glass bottle and cap tightly. This solution will be good for two weeks.

B. Preparation of standards

NOTE: Vitamin B₆ is light sensitive! Perform all sample preparation under yellow lighting and in the absence of any direct sunlight. Use amber colored glassware and plastic ware whenever possible.

(1) Individual stock solutions

(a) Pyridoxal 5'-phosphate (PLP) – 100 ppm:

Using an electronic balance readable to 0.1 mg, transfer ~10 mg of PLP into a weighing boat and record the mass. Quantitatively transfer the PLP to a 100-mL volumetric flask and dilute with some water. Mix the solution thoroughly until the 4PA dissolves. Dilute to the mark with water.

(b) 4-Pyridoxic acid (4PA) – 100 ppm:

Using an electronic balance readable to 0.1 mg, transfer ~10 mg of 4PA into a weighing boat and record the mass. Quantitatively transfer the 4PA to a 100-mL volumetric flask and dilute with some water. Mix the solution thoroughly until the 4PA dissolves. Dilute to the mark with water. NOTE: 4PA dissolves very slowly in water. A sonicating bath can be used to help bring the 4PA into solution. Avoid exposing the solution to excessive heat when doing this.

(2) Formulas to calculate the concentration (μmol/L) of the stock solutions

(a) PLP:

If the free form of PLP is used:

$$PLP \text{ stock solution } (\mu\text{mol} / L) = \frac{(\text{mass of PLP used (mg)}) \times (1000)}{(\text{final volume (L)}) \times (247.14 \text{ g} / \text{mol})}$$

If the monohydrate form of PLP is used (PLP·H₂O):

$$PLP \text{ stock solution } (\mu\text{mol} / L) = \frac{(\text{mass of PLP used (mg)}) \times (1000)}{(\text{final volume (L)}) \times (265.2 \text{ g} / \text{mol})}$$

(b) 4PA:

$$4PA \text{ stock solution } (\mu\text{mol} / L) = \frac{(\text{mass of PLP used (mg)}) \times (1000)}{(\text{final volume (L)}) \times (183.16 \text{ g} / \text{mol})}$$

(3) Working solutions

(a) PLP – 100 ppb:

Prepare a 1:1000 dilution of the stock solution by transferring 1000 µL of the stock solution with a micropipette to a 1-L volumetric flask and diluting to the mark with water. Mix thoroughly. Aliquot the solution (~1.5 mL) into pre-labeled 2-mL cryogenic vials. Approximately 200 vials will be needed for one year of analyses. Store vials at –70°C. These solutions are stable for at least one year.

(b) 4PA – 100 ppb:

Prepare a 1:1000 dilution of the stock solution by transferring 1000 µL of the stock solution with a micropipette to a 1-L volumetric flask and diluting to the mark with water. Mix thoroughly. Aliquot the solution (~1.5 mL) into pre-labeled 2-mL cryogenic vials. Approximately 200 vials will be needed for one year of analyses. Store vials at –70°C. These solutions are stable for at least one year.

(4) Formulas to calculate the concentration (nmol/L) of the working solutions

(a) PLP:

$$PLP \text{ working solution (nmol / L)} = \frac{(PLP \text{ stock conc.}(\mu\text{mol / L})) \times (\text{vol. of stock used}(\text{mL}))}{\text{final volume (L)}}$$

(b) 4PA:

$$4-PA \text{ working solution (nmol / L)} = \frac{(4-PA \text{ stock conc.}(\mu\text{mol / L})) \times (\text{vol. of stock used}(\text{mL}))}{\text{final volume (L)}}$$

(5) Calibration standards

When a run is performed, thaw one vial of the PLP working solution and one vial of the 4PA working solution. Mix the working solutions thoroughly prior to use. Using a micropipette, combine 550 µL of the PLP working standard and 450 µL of the 4PA working standard in a 1.5-mL microcentrifuge tube with a snap-top closure. Close the tube and mix its contents thoroughly using a vortex mixer. This will be referred to as the mixed standard in steps (a) through (e) that follow.

(a) Level 1 calibration standard (~4 nmol/L PLP, 4PA):

Using a micropipette, combine 10 µL of the mixed standard and 590 µL of water in a 1.5-mL microcentrifuge tube with a snap-top closure. Close the tube and mix its contents thoroughly using a vortex mixer.

- (b) Level 2 calibration standard (~11 nmol/L PLP, 4PA):

Using a micropipette, combine 30 µL of the mixed standard and 570 µL of water in a 1.5-mL microcentrifuge tube with a snap-top closure. Close the tube and mix its contents thoroughly using a vortex mixer.

- (c) Level 3 calibration standard (~37 nmol/L PLP, 4PA):

Using a micropipette, combine 100 µL of the mixed standard and 500 µL of water in a 1.5-mL microcentrifuge tube with a snap-top closure. Close the tube and mix its contents thoroughly using a vortex mixer.

- (d) Level 4 calibration standard (~75 nmol/L PLP, 4PA):

Using a micropipette, combine 200 µL of the mixed standard and 400 µL of water in a 1.5-mL microcentrifuge tube with a snap-top closure. Close the tube and mix its contents thoroughly using a vortex mixer.

- (e) Level 5 calibration standard (~225 nmol/L PLP, 4PA):

Using a micropipette, combine 600 µL of the mixed standard and 0 µL of water in a 1.5-mL microcentrifuge tube with a snap-top closure. Close the tube and mix its contents thoroughly using a vortex mixer.

- (f) Calibration blank:

Using a micropipette, transfer 600 µL of water to a 1.5-mL microcentrifuge tube with a snap-top closure and close.

- (6) Formulae to calculate the concentrations (nmol/L) of the calibration standards

$$PLP \text{ calibration std. (nmol/L)} = \frac{(\text{vol. mixed std. used } (\mu\text{L})) \times (PLP \text{ working conc. (nmol/L)} \times 550)}{600 \times 1000}$$

$$4\text{-PA calibration std. (nmol/L)} = \frac{(\text{vol. mixed std. used } (\mu\text{L})) \times (4\text{-PA working conc. (nmol/L)} \times 450)}{600 \times 1000}$$

The exact calculated concentrations of the calibration standards should be used in the instrument method.

C. Preparation of Quality Control Materials

Quality control materials for this assay are prepared in-house from blood products acquired from blood banks or from other volunteer blood donors. The low QC pool is prepared by selecting and pooling serum to have a final concentration approximately 20 nmol/L for both PLP and 4PA. The medium QC pool is prepared by pooling serum to have a final concentration approximately 50 nmol/L for both PLP and 4PA. The medium QC pool is prepared by pooling serum to have a final concentration approximately 100 nmol/L for both PLP and 4PA. These concentration targets satisfy the DLS Policies and Procedures requirements for placement of quality control material concentrations relative to clinical decision points and population distribution curves. If no serum with elevated PLP and/or 4PA levels is available, spiking the serum with known amounts of PLP or 4PA may be a useful alternative.

All serum pools are filtered through gauze before being dispensed. Serum (400 µL) is dispensed into pre-labeled 2.0-mL Nalgene cryovials, capped, and frozen. The QC pools are stored at -70 °C and are stable for at least 2 years. Means plus range limits for all pools are established by analyzing duplicates for at least 20 consecutive runs.

D. Other Materials

With some exceptions, a material listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of standards, internal standards, chemicals and reagents, the chemical and/or isotopic purity of the substituted must meet or exceed that of the listed product. In the case of the HPLC column and guard cartridge, equivalent performance must be demonstrated experimentally in accordance with DLS policies and procedures.

(1) Chemicals and solvents

- Acetonitrile, HPLC-grade (Burdick & Jackson, Muskegon, MI, USA).
- Methanol, HPLC-grade (Burdick & Jackson, Muskegon, MI, USA).
- Deionized water, 0.45 µm filtered lab source (Aqua Solutions, Jasper, GA, USA).
- Pyridoxal 5'-phosphate (Sigma, St. Louis, MO, USA).
- 4-pyridoxic acid (Sigma, St. Louis, MO, USA).
- Metaphosphoric acid (HPO₃) (Mallinkrodt, Paris, KY, USA).
- Orthophosphoric acid (H₃PO₄) (Fisher, Fair Lawn, NJ, USA).
- Sodium phosphate, monobasic, monohydrate (NaH₂PO₄ · H₂O) (Sigma, St. Louis, MO, USA).
- Sodium chlorite (NaClO₂) (Aldrich, Milwaukee, WI, USA).

- Serum samples (for QC pools) (Tennessee Blood Service, Memphis, TN, USA).

(2) General consumables

- Hypersil BDS C18 HPLC column, 150 × 3-mm, 5- μ m particle, 130-Å pore (Thermo Hypersil-Keystone, Bellefonte, PA, USA).
- Security Guard HPLC guard cartridge holder, 4 × 2-mm (Phenomenex, Torrance, CA, USA).
- Security Guard HPLC C18 guard cartridges, 4 × 2-mm (Phenomenex, Torrance, CA, USA).
- 1.5-mL polypropylene microcentrifuge tubes (VWR, Suwanee, GA, USA).
- 2.0-mL polypropylene cryovials (Nalgene, Rochester, NY, USA).
- 5.75" Disposable glass Pasteur pipettes (Kimble, Toledo, OH).
- 0.22- μ m nylon microcentrifuge filters (Costar Spin-X, Corning, Corning, NY).
- 0.22- μ m nylon 96-well filtration plate (P/N 5052, Pall Life Sciences, Ann Arbor, MI, USA).
- Sealing mat for 96-well filtration plate (P/N 5230, Pall Life Sciences, Ann Arbor, MI, USA).
- 0.22- μ m × 47-mm dia polyvinyl difluoride vacuum solvent filters (Millipore, Billerica, MA, USA).
- 1.5-mL, high-recovery, 12 mm × 32 mm amber HPLC vials with PTFE/silicone caps (Lab Depot, Dawsonville, GA).
- 1-mL 96-well plate (P/N 260252, Nalgene, Rochester, NY, USA).
- Pre-slit sealing mat for 1-mL 96-well plate (P/N 03-396-52, Fisher Scientific, Suwanee, GA, USA).
- 100-1000 μ L air-displacement pipette tips (Eppendorf) (Brinkmann, Westbury, NY, USA).
- 10-100 μ L air-displacement pipette tips (Eppendorf) (Brinkmann, Westbury, NY, USA).
- 5-mL repeater pipette tips (Eppendorf Combi-Tip Plus) (Brinkmann, Westbury, NY, USA).
- 10–100 μ L positive-displacement pipette tips (Gilson Pipetteman) (Rainin, Woburn, MA, USA).
- 50–250 μ L positive-displacement pipette tips (Gilson Pipetteman) (Rainin, Woburn, MA, USA).
- Hamilton standard volume (300 μ L) tips without filter (P/N 235902, Hamilton, Reno, NV, USA)
- Nitrile laboratory gloves (Best Manufacturing, Menlo, GA, USA).
- Various glass beakers, volumetric flasks, graduated cylinders, and bottles, class A glassware.

E. Instrumentation

In the case of simple laboratory instrumentation (e.g., pipettes, vortex mixer, analytical balance, etc.) a product listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of analysis instrumentation (e.g., HPLC components, tandem quadrupole mass spectrometer) equivalent performance must be demonstrated experimentally in accordance with DLS policies and

procedures if a product substitution is made. Equivalent performance must also be demonstrated in accordance with DLS policies and procedures when multiple analysis systems are used in parallel, even if they are of the exact same type.

- (1) Agilent Technologies HPLC system (Agilent, Palo Alto, CA, USA).
 - G1311A quaternary pump (×2)
 - G1367B high performance autosampler
 - G1330B autosampler thermostat
 - G1316A thermostatted column compartment (×2)
 - G1321 fluorescence detector with 8- μ L flow cell
 - ChemStation workstation and software (version B.03.02 or higher)

- (2) Hamilton Starlet 8-channel with auto-load arm (Hamilton, Reno, NV, USA)
 - TIP_CAR_480_A00 tip carrier
 - SMP_CAR_32_A00 sample carrier (×3)
 - PLT_CAR_L5AC_A00 plate carrier
 - RGT_CAR_5X50_C reagent carrier

- (3) Other laboratory instrumentation
 - Microfiltration assembly, 47 mm, with 1 L solvent bottle and fritted glass support (Kimble-Kontes, Vineland, NJ, USA).
 - Vortex mixer, single sample (Daigger, Vernon Hills, IL).
 - Vortex mixer, multiple samples (Glas-Col, Terre Haute, IN).
 - Magnetic stir plate (Fisher, Fair Lawn, NJ, USA).
 - Refrigerated microcentrifuge (Labnet, Edison, NJ, USA).
 - Eppendorf Centrifuge (5810R, Brinkmann Instruments Inc., Westbury, NY).
 - Digital pH/temperature meter (Beckman, Fullerton, CA, USA).
 - Sonicating bath (VWR, Suwanee, GA, USA).
 - Digital balance, 0.001 g accuracy (Mettler Toledo, Columbus, OH, USA).
 - Digital balance, 1 mg accuracy (Mettler Toledo, Columbus, OH, USA).
 - 100–1000 μ L adjustable air-displacement micropipette (Eppendorf) (Brinkmann Instruments Co., Westbury, NY)
 - 10–100 μ L adjustable air-displacement micropipette (Eppendorf) (Brinkmann Instruments Co., Westbury, NY)
 - 10–100 μ L adjustable positive-displacement micropipette (Gilson Pipetteman) (Rainin, Woburn, MA, USA).
 - 50–250 μ L adjustable positive-displacement micropipette (Gilson Pipetteman) (Rainin, Woburn, MA, USA).

- Digiflex diluter (Micromedic Division, ICN Biomedical, Costa Mesa, CA).

7. Calibration and Calibration Verification Procedures

A. Method calibration

At the beginning of each run, five calibrators and a blank are prepared as described in this document. Sample preparation for the calibrators and blank is identical to the sample preparation used for patient samples and QCs.

Calculation of calibration curves for PLP and 4PA, and interpolation of standards, QCs and patient samples against these equations is performed in ChemStation software. After determining the peak areas for PLP and 4PA in the standards, a five-point calibration curve is generated by performing a 1/x-weighted linear regression (not forced through zero). At the end of each run, the two calibrators are reanalyzed as unknowns. The measured concentrations of these calibrators should agree within 15% of their set values.

This method uses aqueous calibrators. Matrix based calibration was tested by comparing the average slope and intercept parameters of three 10-point calibration curves prepared using stripped serum with three 10-point aqueous calibration curves. A <5% difference in the average calibration curve slope was observed between serum and water calibrations for all analytes. Differences observed were of a similar magnitude to slope variability observed within and between individual calibration curves of a particular matrix.

Calibration verification is performed twice yearly using “NIST SRM 3950 – Vitamin B₆ in Frozen Human Serum.” This two-level material has certified values for PLP and information values for 4PA. Additional details can be found in “**4002.05 SOP for Calibration and Calibration Verification.**”

No known external proficiency testing programs exist for vitamin B₆ in serum. Proficiency testing is according to a program developed in house, details of which can be found in “**4002.05 SOP for In-House Proficiency Testing.**”

An inter-laboratory round-robin study involving 10 internationally-recognized laboratories [14] was organized by our laboratory in 2004. Results from our laboratory were found to satisfy optimal performance requirements in terms of method bias and imprecision when evaluated against empirically-derived performance criteria based on biological variation for vitamin B₆ [15].

Method figures of merit are presented in **Appendix A.**

Results from a series of in-house ruggedness testing experiments designed to assess how much method accuracy changes when certain experimental parameters are varied are presented in **Appendix B.**

B. Instrument calibration

A wavelength verification test is performed on the fluorescence detector as part of a preventative maintenance schedule or whenever major service is performed (e.g., lamp replacement). If the excitation and/or emission wavelength deviates from the allowable specification a wavelength calibration is performed. Other performance verification checks (e.g., pump leak test, pressure test, etc.) are performed as part of a preventative maintenance schedule. Any instrument component requiring recalibration should be addressed by a qualified service engineer.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

A. Preliminaries

Allow frozen patient serum specimens, QC serum specimens, and PLP and 4PA working solution aliquots to thaw and reach ambient temperature. If needed, prepare the 5% MPA and refrigerate. If already available, keep the 5% MPA solution refrigerated until ready to use. Turn on the refrigerated microcentrifuge and set the thermostat to 4 °C. Turn on all components of the HPLC system including the computer and start the instrument software. Refer to “**4002.05 SOP for Instrument Preparation**” for additional details.

B. Sample preparation - manual

A typical run consists of one reagent blank, 5 calibrators, 3 QC samples, 60 patient samples, and 3 QC samples, followed by reinjection of the blank and calibrators.

NOTE: Vitamin B₆ is light sensitive! Perform all sample preparation under yellow lighting and in the absence of any direct sunlight. Use amber colored glassware and plastic ware whenever possible.

- Prepare the five calibrators and blank as described in this document.
- Label one set of microcentrifuge filters, one tube per sample in each set, and place them in racks.
- Label one set of high-recovery amber HPLC vials, one vial per sample in each set and place them in racks.
- Using a repeater pipette (positive displacement recommended), transfer 100 µL of the cold 5% MPA solution to each of the microcentrifuge filters in the first set.
- Using a manual pipette or automated pipettor (positive displacement recommended), transfer 100 µL of the blank, standards, QCs and patient samples into their respective microcentrifuge filters in the first set.
- Tightly cap all of the microcentrifuge filters in the first set and mix the tubes for 5 min at 2000 rpm or greater with a multitube vortexor.

- Transfer the first set of filters to a refrigerated microcentrifuge set to 4 °C and centrifuge at 10,000 *g* for 5 min.
- For each tube in the set:
- Verify that a clear filtrate was obtained in the bottom of the filtration assembly. If not, transfer the filtrate to a new microcentrifuge tube and repeat step 8.b.(6) above.
- Transfer the filtrate to a high-recovery amber HPLC vial.
- Cap the HPLC vials and gently tap them to ensure that there is no air trapped at the bottom of the vial.
- If the HPLC is ready, transfer the HPLC vials to their appropriate positions in the autosampler tray; otherwise, refrigerate the samples until the HPLC is ready for the analysis. If a significant delay prior to analysis is anticipated (e.g., 8 hours), store the HPLC vials at -70 °C.

C. Sample preparation – automated

“**4002.05 SOP for Automated Sample Preparation**” describes automated sample preparation using the Hamilton Starlet system. These steps directly mimic those described above for manual sample preparation with most pipetting actions being performed by the Hamilton Starlet. In brief: A 96-well 0.2 µm nylon centrifuge filter plate collecting into a 96-deep well plate is used for all sample preparation. 100 µL of cold 5% MPA is transferred into each of the filter plate wells designated for use in the run. 100 µL of the blank and each calibrator, QC and patient sample is then transferred into its designated well. The 96-well filter plate is then sealed with a sealing mat and the stacked filter plate and well-plate assembly is secured with several rubber bands. The entire assembly is then vortex mixed with a multitube vortexor and centrifuge filtered. Lastly, the filter plate is carefully removed and the collection plate sealed with a pre-slit sealing mat prior to HPLC analysis.

All precautions observed in manual sample preparation should be observed when performing automated sample preparation.

The instructions given in the SOP reflect the custom program developed for performing sample preparation that is currently being used. Certain non-critical elements of this program (e.g., positions of samples, wording of user messages) may be modified and differ from the exact instructions given in the SOP. The user is strongly encouraged to be familiar with the exact program being used.

A liquid handling system other than the Hamilton Starlet may be used for this purpose provided that it is able to perform these steps with accuracy and precision that meets or exceeds that of the Hamilton Starlet.

D. Instrument Preparation

(1) Solvent Reservoir Filling

The HPLC system has two quaternary pumps, identified herein as Pump 1 and Pump 2. Pump 1 controls solvent delivery for the actual HPLC separation, while Pump 2 is responsible for delivering the post column derivatization reagent. Each pump has four 1-L solvent reservoirs designated A through D. The solvent reservoirs should be filled as follows:

For Pump 1:

- A: 50 mM phosphate buffer, pH 3.2, 0.1% acetonitrile
- B: Acetonitrile
- C: Methanol
- D: Water

For Pump 2:

- A: Water
- B: 2 g/L sodium chlorite solution
- C: Methanol
- D: not used

Make sure that all reservoir bottles and solvent lines are clean and free of foreign matter (particulates, algae, etc.). If dirty, clean or replace these components as necessary.

Fill all reservoirs with the appropriate solvent or reagent. For the 50 mM phosphate buffer (Pump 1, Reservoir A), use an amber colored reservoir. Once the reservoirs have been filled, replace the solvent lines, making sure that they are in the correct reservoirs and reach the bottom of each bottle. Track how much solvent is left in each reservoir and take preventative measures (i.e., shutdown the system) before running out of solvent. Additional instrument-specific information on solvent reservoir filling can be found in **“4002.05 SOP for Instrument Preparation.”**

(2) Solvent Priming

The HPLC solvent lines should be primed before every use, especially if there are signs of air present in a solvent line, or the instrument has not been used for several days. Affected lines should be run with the solvent flow diverted to waste at a flow rate of 5 mL/min for 5 min or longer until there are no signs of air present in the lines. This can be done manually or the user may wish to develop an instrument method for priming all solvent lines.

Prime each solvent line separately. When priming, avoid switching directly from solvent lines containing high amounts of dissolved salts (buffer, post column reagent) to lines containing 100% organic solvents (acetonitrile, methanol) and vice versa. Doing so may cause precipitates to form in the solvent lines and the pump. To prevent this, briefly purge (30 s) a solvent line

containing water between purging lines containing dissolved salts and organic solvents. Additional instrument-specific information on solvent priming can be found in “**4002.05 SOP for Instrument Preparation.**”

(3) Guard Cartridge Replacement

The HPLC system uses a guard column to protect the integrity of the analytical column by blocking out particulates and other potential contaminants. The guard column uses a holder that connects directly to the inlet of the analytical column and disposable drop-in cartridges. The guard cartridge should be replaced before each new sample run. Always wear clean, powder-free gloves when working with the guard cartridge holder. Specific guidance on how to replace the guard cartridge is given in “**4002.05 SOP for Instrument Preparation.**”

(4) Analytical Column Replacement

The analytical column should be replaced when its chromatographic performance has been compromised. Problem areas include, but are not limited to, sudden changes and/or poor reproducibility in peak shape, retention time, resolution, and/or column backpressure.

Analytical column replacement is at the sole discretion of the operator, but it is recommended that the column is replaced after 2000 sample injections, even if it is still apparently in good working order. To replace the analytical column, disconnect the guard cartridge holder and the HPLC outlet line from the old column and reconnect a new column in its place. New analytical columns are shipped in 95% acetonitrile / 5% water, so there is no need to purge the column as is done when the guard cartridge is replaced.

E. Setting Up and Running a Sequence

Once the HPLC system has been primed and the guard and analytical columns have been replaced (if needed), a sequence is set up for running the samples. A sequence can be used to perform all steps of the analysis. A typical sequence starts with a startup method that gradually switches the HPLC solvents over from its standby conditions to starting conditions for analysis injections, while allowing the HPLC system components adequate time for warming up. Once the HPLC is ready for analysis, a series of test injections (typically the blank, two calibrators and two QCs) are done to further equilibrate the HPLC system and ensure that the instrument is operating properly. The analysis stage typically consists of injecting the following in order: the blank and calibrators, a set of QC samples, patient samples, a second set of QC samples, and reinjection of the blank and calibrators. Lastly, a shutdown method that gradually switches the HPLC solvents over from its analysis conditions to standby conditions is run before returning the instrument to its standby mode.

Information is entered in the form of a sequence table – a line-by-line set of analysis steps to be entered by the analyst. Rows in the sequence table are numbered 1 through n and indicate the

order in which the analysis steps will be executed. Columns in the sequence table represent fields for variables that can be set for each analysis step. All necessary information for the analysis is entered into the sequence table and its related menus. This includes: type of vial/well plate used, the physical positions of all samples, sample IDs, sample types (calibrator, unknown, etc.) calibration levels, dilution factors, etc. Refer to “**4002.05 SOP for Setting Up and Running a Sequence**” for specific details.

F. Data Review

Once the sequence has finished, the peak integration has to be reviewed and calibration equations updated before it can be exported for QC verification. This is done by reviewing the batch file generated when the sequence is run.

For each chromatogram the analyst should verify that the peaks for PLP (retention time ~ 3.2 min) and 4PA (retention time ~ 5.6 min) are being integrated properly (i.e., verify that the entire peak is being integrated and that no extraneous signals are being integrated in the process). The integration parameters can be adjusted if necessary. If any peaks other than PLP and 4PA have been integrated, these can be manually removed if desired. Note: leaving integrated peaks other than PLP and 4PA in the chromatogram will have no deleterious effects on the sample processing. Removing them is done simply to clean up chromatogram appearance. If the peaks for PLP or 4PA do not appear to be integrated properly, manual integration may be performed, but should only be done as a last resort.

Calibrations curves should be visually inspected for linearity and calibration curve settings verified (i.e., a linear calibration model with 1/x weighting). Calibration curves should have a correlation coefficient ($r \geq 0.999$).

“**4002.05 SOP for Data Review**” provides instrument-specific information on performing the data review process. Once the data review process has been completed, the instrument data can be transferred reviewed and submitted for QA/QC approval as specified in “**4002.05 SOP for Computerization and Data System Management.**”

G. System Maintenance

The system maintenance consists of following components:

(1) Per run:

- Inspection of the solvent reservoirs, inlet lines and filters. Replenishment or replacement of solvents as needed. Cleaning or replacement of components as needed.
- Replacement of the guard column cartridge.
- Replacement of the wash vial.
- Inspection and replacement of column fittings, tubing (as needed).

(2) Per annum:

- Preventative maintenance (performed by a service engineer):
 - Pump pressure and leak tests
 - Replacement of pump seals, inlet filters, inlet valves (if needed).
 - Fluorescence detector dark current and wavelength calibration test.
 - Replacement of fluorescence bulb (if needed).

(3) Other:

- Analytical column replacement (after 2000 injections or as needed depending on chromatographic performance).

H. Special Method Notes

If the HPLC is to remain idle for a week or longer, purge the solvent lines containing buffers and dissolved salts with water followed by methanol to prevent salt precipitation or algae growth from occurring in the HPLC components.

I. CDC Modifications

This method is based on the method described by Rybak et al. [12] with modifications as described in Rybak et al. [13]

9. Reportable Range of Results

This method is linear for PLP and 4PA for the range of LOD–250 nmol/L. Samples with PLP or 4PA concentrations greater than the concentration of the highest calibrator (~220 nmol/L) are diluted such that the appropriate analyte falls within its linear range.

10. Quality Control (QC) Procedures

A. Blind Quality Controls

Blind QC specimens can be inserted into the mix of patient specimens. These QC specimens are generally prepared at two levels that would be encountered in patient samples; the labels used are identical to those used for patient samples. One blind QC specimen randomly selected for concentration is included at a randomly selected location in every 20 specimens analyzed.

B. Bench Quality Controls

Bench QC specimens are prepared from three serum pools that represent low, medium and high levels of serum vitamin B₆ vitamers. Samples from these pools are prepared in the same manner as patient samples and analyzed in duplicate as part of each run.

The results from the pools are checked after each run using a multi-rule quality control system [16] based their characterization data, namely: the pool mean; the pooled within-run standard deviation associated with individual QC results measured in the same run (S_w); the standard deviation associated with individual QC results (S_i); and the standard deviation associated with run mean QC results (S_m). QC rules have been designed to accommodate the use of 1–3 different QC pools during a run, the use of 1–2 measurements of each pool per run, and as many instruments as needed. In the case of three QC pools per run with two QC results per pool:

- (1) If all three QC run means are within $2 S_m$ limits and individual results are within $2 S_i$ limits, accept the run
- (2) If one of the three QC run means is outside a $2 S_m$ limit, reject run if:
 - (a) 1 3S Rule—Run mean is outside a $3 S_m$ limit or
 - (b) 2 2S Rule—Two or more of the three run means are outside the same $2 S_m$ limit or
 - (c) 10 Xbar Rule—Current and previous nine run means are on the same side of the characterization mean
- (3) If one of the six QC individual results is outside a $2 S_i$ limit, reject run if:
 - (a) Outlier—One individual result is beyond the characterization mean $\pm 4 S_i$ or
 - (b) R 4S Rule—two or more of the within-run ranges in the same run exceeds $4 S_w$ (i.e. 95 per cent range limit).

A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared “out of control” for that analyte as assessed by internal (bench) QC.

The initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated quarterly. When necessary, limits are updated to include more runs.

While a study is in progress, QC results are stored in a LIMS database. For runs that are not imported into the database (i.e., R&D, troubleshooting, research-type runs), QC results are stored electronically in the analyte-specific folder on the DLS network. A hardcopy of the QC results from each run is also maintained by the analyst.

11. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

Check to make sure that the hardware is functioning properly, and verify that all instrumental settings are correct. Check the autosampler to make sure the injections are being made as programmed.

Look for sample preparation errors.

Check the calibrations of the pipettes.

If the steps outlined above do not result in correction of the "out of control" values for QC materials, consult the supervisor for other appropriate corrective actions. Do not report analytical results for runs not in statistical control.

12. Limitations of Method; Interfering Substances and Conditions

If serum is not available, heparinized plasma may be used as a substitute [12]. EDTA plasma should be used only when necessary due to possible interference with the signal for PLP.

Increases in the following activities may increase (↑) or decrease (↓) serum PLP and 4 PA levels as indicated [10]:

- Use of vitamin B₆ supplements: ↑ PLP, ↑ 4PA
- Protein intake: ↓ PLP
- Glucose: ↓ PLP
- Plasma volume: ↑ PLP
- Physical activity: ↑ PLP

13. Reference Ranges (Normal Values)

The Second National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population [17] has reference ranges for serum PLP and 4PA. The serum PLP and 4PA data in the Nutrition Report were generated by our laboratory using this method. For the total U.S. population aged 1 year and older, NHANES 2005-2006:

- PLP: 11.3–302 nmol/L (2.5th–97.5th percentile; *n* = 8311)
- 4PA: 8.73–385 nmol/L (2.5th–97.5th percentile; *n* = 8312)

14. Critical Call Results ("Panic Values")

Serum and plasma PLP values of 30 nmol/L [10] and 20 nmol/L [18] have been proposed as cutoff values for normal status. There is no consensus on a critical call value for PLP in serum. There is no critical call value for 4PA in serum.

15. Specimen Storage and Handling during Testing

Serum specimens are allowed to reach room temperature during preparation. Prepared samples are held at 4 °C during the HPLC analysis. The unused portion of the serum specimen is returned to the freezer.

16. Alternate Methods for Performing Test of Storing Specimens if Test System Fails

Because the analysis of serum vitamin B₆ is inherently complex and challenging, there are no acceptable alternative methods of analysis in the NHANES laboratory. If the analytical system fails, we recommend that the extracted specimens be stored at -20°C until the analytical system is restored to functionality.

17. Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)

Test results are reported to the collaborating agency at a frequency and by a method determined by the study coordinator. Generally, data from this analysis are compiled with results from other analyses and sent to the responsible person at the collaborating agency as an ASCII text file or Excel file, either through electronic mail or on a diskette.

For NHANES 1999+, all data are reported electronically periodically to the Westat ISIS computer and then are transferred to NCHS. For some smaller studies, hard copies of a data report are sent, as well as the results in electronic format.

18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

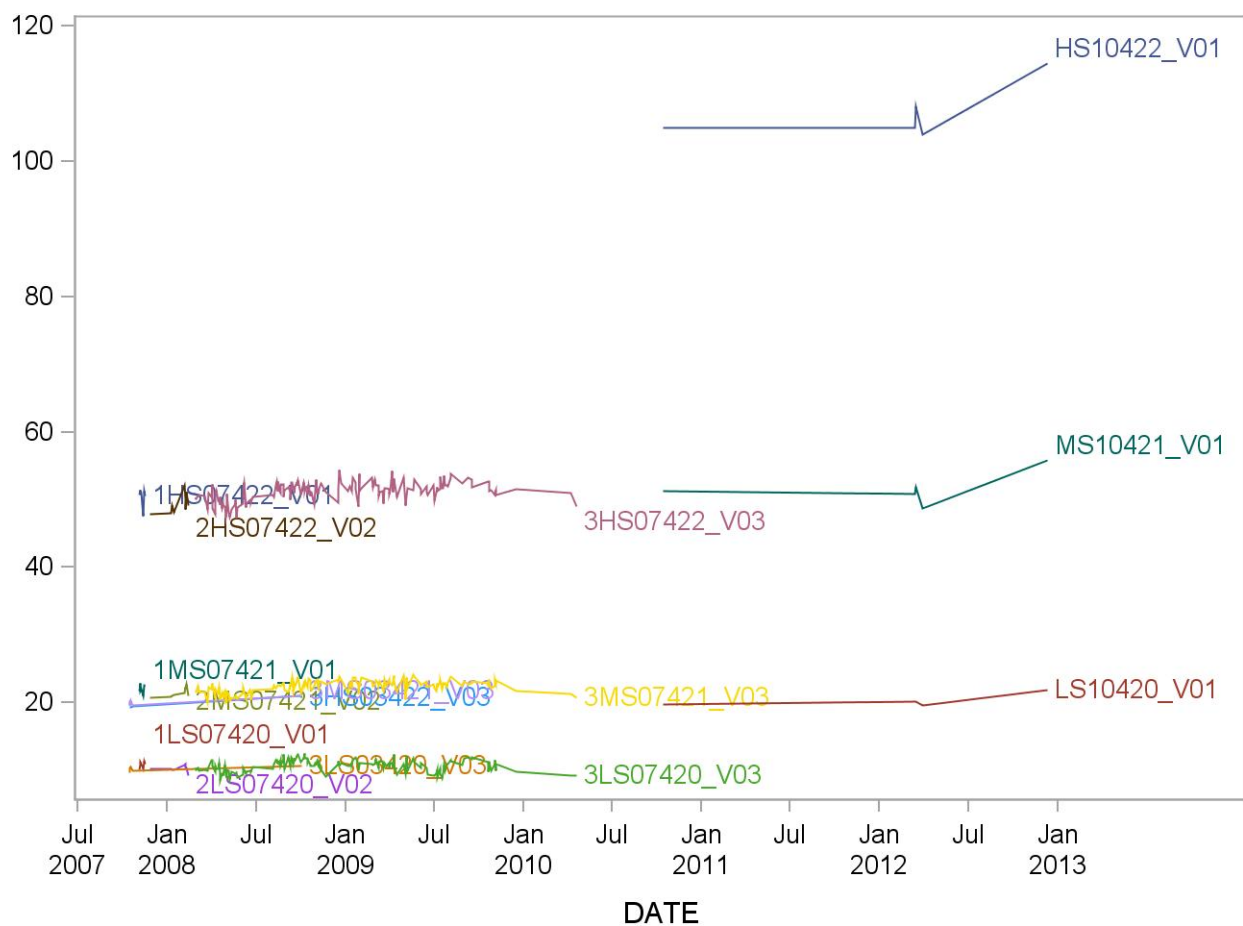
The LIMS database is used to keep records and track specimens for NHANES 1999+. If serum vitamin B₆ analyses are used for smaller, non-NHANES studies, records may be kept in Excel files on the network.

We recommend that records, including related QA/QC data, be maintained for 10 years after completion of the NHANES study. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual serum from these analyses for non-NHANES studies may be discarded at the request of the principal investigator, or may be transferred to the CDC CASPIR facility for use by other investigators. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens stored in a freezer at -70°C. The specimen ID is read off of the vial by a barcode reader attached to the computer used to prepare the electronic specimen table for the

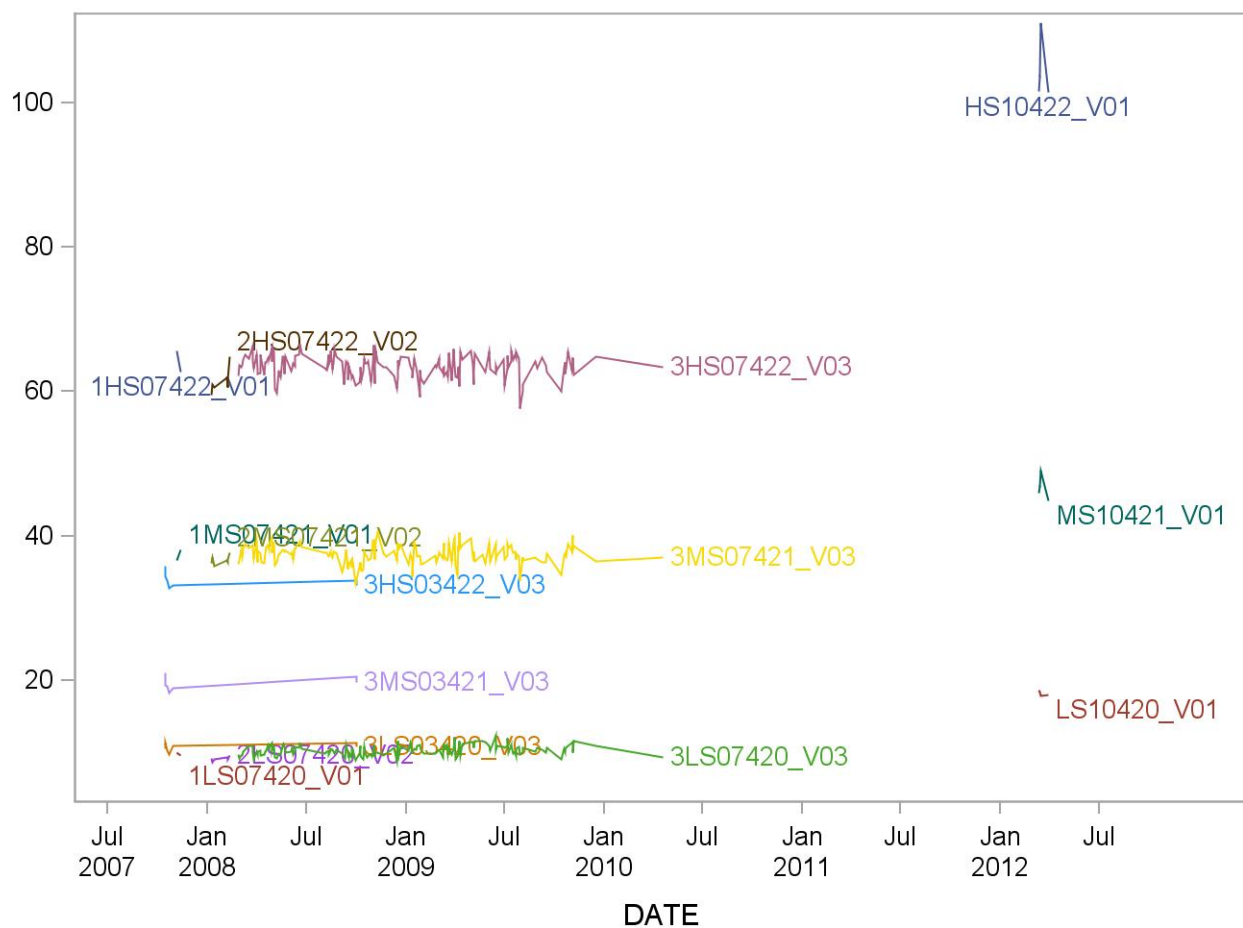
2007-2008 Summary Statistics and QC Chart for 4-pyridoxic acid (nmol/L)

LBCPNME	Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LBC4PH4	3HS03422_V03	6	15OCT07	02OCT08	19.84	0.77	3.9
LBC4PL4	3LS03420_V03	6	15OCT07	02OCT08	10.31	0.40	3.9
LBC4PM4	3MS03421_V03	6	15OCT07	02OCT08	20.42	0.87	4.3
LBC4PH5	1HS07422_V01	7	05NOV07	16NOV07	50.51	1.35	2.7
LBC4PL5	1LS07420_V01	7	05NOV07	16NOV07	10.82	0.49	4.5
LBC4PM5	1MS07421_V01	7	05NOV07	16NOV07	22.10	0.66	3.0
LBC4PH5	2HS07422_V02	8	26NOV07	08FEB08	49.36	1.59	3.2
LBC4PL5	2LS07420_V02	8	26NOV07	08FEB08	10.38	0.33	3.2
LBC4PM5	2MS07421_V02	8	26NOV07	08FEB08	21.54	0.75	3.5
LBC4PH5	3HS07422_V03	164	27FEB08	19APR10	51.19	1.40	2.7
LBC4PL5	3LS07420_V03	164	27FEB08	19APR10	10.66	0.87	8.2
LBC4PM5	3MS07421_V03	164	27FEB08	19APR10	22.40	0.92	4.1
LBC4PH6	HS10422_V01	6	13OCT10	10DEC12	106.91	3.96	3.7
LBC4PL6	LS10420_V01	6	13OCT10	10DEC12	20.28	1.00	4.9
LBC4PM6	MS10421_V01	6	13OCT10	10DEC12	51.57	2.40	4.7



2007-2008 Summary Statistics and QC Chart for Pyridoxal 5'-phosphate (nmol/L)

LBCPNME	Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LBCPLH4	3HS03422_V03	7	15OCT07	02OCT08	33.91	0.99	2.9
LBCPLL4	3LS03420_V03	7	15OCT07	02OCT08	10.79	0.58	5.4
LBCPLM4	3MS03421_V03	7	15OCT07	02OCT08	19.63	0.94	4.8
LBCPLH5	1HS07422_V01	3	06NOV07	13NOV07	64.42	1.51	2.3
LBCPLL5	1LS07420_V01	3	06NOV07	13NOV07	9.85	0.22	2.2
LBCPLM5	1MS07421_V01	3	06NOV07	13NOV07	37.32	0.75	2.0
LBCPLH5	2HS07422_V02	7	09JAN08	11FEB08	61.49	1.72	2.8
LBCPLL5	2LS07420_V02	7	09JAN08	11FEB08	9.24	0.31	3.4
LBCPLM5	2MS07421_V02	7	09JAN08	11FEB08	36.69	0.63	1.7
LBCPLH5	3HS07422_V03	168	27FEB08	19APR10	63.48	1.54	2.4
LBCPLL5	3LS07420_V03	168	27FEB08	19APR10	10.38	0.69	6.7
LBCPLM5	3MS07421_V03	168	27FEB08	19APR10	37.49	1.23	3.3
LBCPLH6	HS10422_V01	5	12MAR12	29MAR12	104.29	4.38	4.2
LBCPLL6	LS10420_V01	5	12MAR12	29MAR12	18.31	0.53	2.9
LBCPLM6	MS10421_V01	5	12MAR12	29MAR12	46.73	2.07	4.4



analytical system. When the analyses are completed, the DIF file containing the electronic copy of the results is loaded into the database, and the analytical results are linked to the database by ID number. The analyst is responsible for keeping a notebook containing the ID numbers of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies.

19. Summary Statistics and QC Graphs

See following pages

Acknowledgement

We gratefully acknowledge the contributions of: Michael E. Rybak, Ph.D. for developing and validating this method and preparing this document; Donna LaVoie, M.T. (A.S.C.P.), Janette Collins-Mitchell, M.S., R.N., M.T. (N.C.A.) and Mary Xu, B.S. for their assistance in the validation and day-to-day performance of this method; and Christine M. Pfeiffer, Ph.D. for her assistance and advice provided.

Appendix A. Method Figures of Merit

Analyte	Fluorescence excitation/emission (nm)	Sensitivity		Between run Imprecision		Spike recovery (% mean \pm SD) ¹
		LOD (nmol/L) ²	Detection frequency (%) ³	Serum QC pool (nmol/L) ⁴	CV (%)	
PLP	325/425	0.3	100	19.1 49.5 108	5.0 6.2 3.6	100 \pm 4
4PA	325/425	0.3	97	20.0 51.1 109	5.1 5.1 4.3	96 \pm 3

¹ Three serum specimens were spiked in triplicate at a concentration approximately equal to the endogenous analyte level.

² Determination of the limit of detection (LOD) was conducted by serially diluting a "low" serum QC pool with water and by estimating the SD at a concentration of zero (σ_0) by extrapolating repeat analyte measurements ($n = 9$) made near the detection limit in these dilutions (LOD defined as $3 \sigma_0$).

³ Based on measurements performed for NHANES 2005-2006. $n = 8311$ (PLP); 8312 (4PA).

⁴ Applies only to imprecision.

Appendix B. Ruggedness testing

A. Experiments

(1) Sample preparation

Principle: The coenzyme form of vitamin B₆ (pyridoxal 5'-phosphate – PLP) is typically protein bound in serum. A protein precipitation step with metaphosphoric acid is used to free PLP from proteins as well as precipitate out proteins to remove any potential interferences. Incomplete protein precipitation or PLP deconjugation may result in erroneous results.

Proposal: To vary the concentration of the precipitating reagent and the mixing time allowed for the protein precipitation step.

(2) HPLC-fluorescence conditions

Principle: Samples are analyzed using reversed-phase HPLC with fluorescence detection using a post-column derivatization reaction (to improve PLP sensitivity). The mobile phase pH will affect the retention time of PLP, which may cause partial overlap with neighboring peaks. The post-column derivatization reaction is highly temperature dependent.

Proposal: To vary select HPLC conditions known to exert measurable effects on analyte sensitivity.

B. Findings

(1) Sample preparation

Factor	Method specification		Test condition #1		Test condition #2	
	Value	Results (nmol/L)	Value	Results (nmol/L)	Value	Results (nmol/L)
Metaphosphoric acid concentration	5%	PLP: 37.4 4PA: 23.0	2.5%	PLP: 37.7 4PA: 22.7	10%	PLP: 38.5 4PA: 23.5
Vortexing time	5 min	PLP: 37.1 4PA: 22.6	<1 min	PLP: 35.8 4PA: 21.5	10 min	PLP: 36.6 4PA: 22.5

Metaphosphoric acid concentration: Varying the precipitating reagent concentration does not appear to affect PLP or 4PA concentrations.

Vortexing time: Varying the precipitating reagent concentration does not appear to affect PLP or 4PA concentrations.

(2) HPLC-fluorescence conditions

Factor	Method specification		Test condition #1		Test condition #2	
	Value	Results (nmol/L)	Value	Results (nmol/L)	Value	Results (nmol/L)
Mobile phase pH	3.2	PLP: 37.1 4PA: 23.3	3.0	PLP: 41.3 4PA: 22.2	3.4	PLP: 31.5 4PA: 22.1
Post-column reagent (sodium chlorite) concentration	2 g/L	PLP: 36.9 4PA: 22.1	1 g/L	PLP: 29.7 4PA: 22.7	4 g/L	PLP: 37.9 4PA: 20.8
Post-column reaction temperature	75 °C	PLP: 37.1 4PA: 23.3	55 °C	PLP: 32.3 4PA: 24.1	65 °C ⁵	PLP: 34.7 4PA: 23.2

Mobile phase pH: Varying the mobile phase pH over the specified range has a significant effect on PLP concentration. This is due to a pH-dependent retention time shift for PLP that results in coelution with other chromatographic peaks (see detailed information). Mobile phase pH has no effect on 4PA concentrations or retention times. Mobile phase pH must be 3.20 ± 0.02 .

Post-column reagent concentration: At the low end of the post-column reagent concentration test range, approximately 80 % of the anticipated PLP concentration is observed. At the high end of the test range, approximately 95 % of the anticipated 4PA concentration is observed. Post-column reagent concentration must be 2.0 ± 0.1 g/L.

Post-column reaction temperature: Post-column reaction temperature is positively correlated with PLP concentration. No effect on 4PA concentrations was observed though an inverse correlation with temperature is suspected (Rybak et al., Anal. Biochem. 333 (2004) 336-344). Post-column reaction temperature must be 75 ± 2 °C.

⁵ Maximum temperature setting is 80°C, ∴ two lower levels were tested in this case.

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