National Institute of Standards and Technology
U.S. Department of Commerce

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Standard Reference Material® 3950
Vitamin B_6 in Frozen Human Serum
CERTIFICATE OF ANALYSIS

Purpose: This Standard Reference Material (SRM) is intended primarily for use in evaluating the accuracy of procedures for the determination of the vitamin B_6 metabolite pyridoxal 5'-phosphate (PLP) in human serum. It is also intended for use in validating working or secondary reference materials. PLP is the major circulating form of vitamin B_6 and the most common direct measure of this vitamin in serum or plasma.

Description: A unit of SRM 3950 consists of two stoppered vials of frozen human serum, one vial each at two different concentration levels. Each vial contains 1.0 mL of human serum.

Certified Values: The certified concentration values for PLP are provided in Table 1. These values are traceable to International System of Units (SI) [1]. The certified concentration values for each level are based on the agreement of results from isotope dilution liquid chromatography/tandem mass spectrometry (ID LC-MS/MS) at NIST [2] and liquid chromatography/fluorescence detection (LC/FD) at the Centers for Disease Control (CDC) [3]. All values were combined without weighting. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence; it incorporates Type B uncertainty components related to the analyses, and expresses both the observed difference between the results from the methods and their respective uncertainties, consistent with the ISO/JCGM Guide and with its Supplement 1 [4–6]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is the combined uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for each analyte [4]. For the certified values shown below, $k = 2$. The certified concentrations apply only to serum thawed to room temperature, 20 ºC to 25 ºC (see "Storage" and "Use").

Table 1. Certified Concentration Values for PLP

Material	(ng/g)	(ng/mL)[a]	(nmol/L)[b]
Level 1	4.49 ± 0.15	4.59 ± 0.16	18.6 ± 0.6
Level 2	8.81 ± 0.29	9.00 ± 0.29	36.4 ± 1.2

[a] Mass concentrations were calculated from mass fractions using the following measured serum densities: Level 1, 1.02213 g/mL and Level 2, 1.02138 g/mL. The standard deviations of Level 1 and Level 2 were 0.00009 (n=6) and 0.00015 (n=6), respectively. The uncertainty in the serum density measurements was incorporated in values that are reported relative to units of volume.

[b] Molar concentrations were calculated from mass concentrations using the relative molecular mass 247.14 g/mol.

Additional Information: Values of potential interest to users, and additional information are provided in Appendix A.

Period of Validity: The certified values delivered by **SRM 3950** are valid within the measurement uncertainty specified until **31 March 2031**. The certified values are nullified if the material is stored or used improperly, damaged, contaminated, or otherwise modified.

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Maintenance of Certified Values: NIST will monitor this SRM over the period of its validity. If substantive technical changes occur that affect the certification, NIST will issue an amended certificate through the NIST SRM website (https://www.nist.gov/srm) and notify registered users. SRM users can register online from a link available on the NIST SRM website or fill out the user registration form that is supplied with the SRM. Registration will facilitate notification. Before making use of any of the values delivered by this material, users should verify they have the most recent version of this documentation, available through the NIST SRM website (https://www.nist.gov/srm).

Safety: SRM 3950 IS INTENDED FOR RESEARCH USE. THIS IS A HUMAN SOURCE MATERIAL. HANDLE PRODUCT AS A BIOHAZARDOUS MATERIAL CAPABLE OF TRANSMITTING INFECTIOUS DISEASE. The supplier of this serum has reported that each donor unit of serum or plasma used in the preparation of this product has been tested by a FDA-approved method and found non-reactive/negative for hepatitis B surface antigen (HbsAg), human immunodeficiency virus (HIV) 1 and 2 antibodies, and hepatitis C virus (HCV). However, no known test method can offer complete assurance that hepatitis B virus, HCV, HIV, or other infectious agents are absent from this material. Accordingly, this human blood-based product should be handled at the Biosafety Level 2 or higher as recommended for any POTENTIALLY INFECTIOUS HUMAN SERUM OR BLOOD SPECIMEN in the CDC/National Institutes of Health Manual [7].

Storage: The serum is shipped frozen (on dry ice) and, upon receipt, should be stored frozen until ready for use. The material should be stored at or below –60 °C. This SRM should be handled under subdued lighting conditions (i.e., yellow light) [8,9].

Use: SRM 3950 is provided as frozen serum that should be allowed to thaw at room temperature (20 °C to 25 °C) under subdued light. After the material is thawed, it should be used immediately. The contents of the vial should then be gently mixed prior to removal of a test portion for analysis. Precautions should be taken to expose serum only under subdued lighting conditions.

Additional Information: Support for the development of SRM 3950 was provided in part by the National Institutes of Health Office of Dietary Supplements.

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[3] Rybak, M.E.; Pfeiffer, C.M; *Clinical Analysis of Vitamin B6: Determination of Pyridoxal 5'-phosphate and 4-Pyridoxic Acid in Human Serum by Reversed-Phase High-Performance Liquid Chromatography with Chlorite Post Column Derivatization*; Anal. Biochem., Vol. 333, pp. 336–344 (2004).

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[7] CDC/NIH; *Biosafety in Microbiological and Biomedical Laboratories, 6th ed*.; Meechan, P.J.; Potts, J., Eds.; U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention and National Institutes of Health; National Institutes of Health; HHS Publication (CDC) 300859 (2020); available at https://www.cdc.gov/labs/BMBL.html (accessed May 2023).

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Certificate Revision History: 31 May 2023 (Change of period of validity; updated format; editorial changes); **28 December 2015** (Change of expiration date; editorial changes); **20 July 2011** (Original certificate date).

Certain commercial equipment, instruments, or materials may be identified in this Certificate of Analysis to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the Office of Reference Materials 100 Bureau Drive, Stop 2300, Gaithersburg, MD 20899-2300; telephone (301) 975-2200; e-mail srminfo@nist.gov; or the Internet at https://www.nist.gov/srm.

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APPENDIX A

Values of Potential Interest to Users: Values of potential interest to users are provided for the vitamin B_6 metabolite 4-pyridoxic acid in Table A1. Measurement of 4-pyridoxic acid was performed by LC/FD [3] at the CDC.

Table A1. Values of Potential Interest to Users for 4-Pyridoxic Acid

	Concentrations		
	(ng/g)[(a)]	(ng/mL)[(b)]	(nmol/L)
Level 1	21.7	22.2	121
Level 2	36.3	37.1	202

[(a)] Mass fractions were calculated from mass concentrations using the measured serum densities: Level 1, 1.02213 g/mL and Level 2, 1.02138 g/mL.
[(b)] Mass concentrations were calculated from molar concentrations using the relative molecular mass 183.16 g/mol.

ADDITIONAL INFORMATION

Source and Preparation: SRM 3950 was prepared by Aalto Scientific Ltd. (Carlsbad, CA). Level 1 is an unfortified pool of human serum. In order to achieve the desired level of PLP in Level 2, a human serum pool containing a naturally lower level was fortified with additional PLP.

Analytical Methods: NIST determined vitamin B_6 levels as PLP by ID LC-MS/MS [2]. A labeled internal standard (pyridoxal-[$^2H_3$] 5'-phosphate) was added to 0.5 g serum and allowed to equilibrate for 30 min. Serum proteins were precipitated by the addition of aqueous trichloroacetic acid followed by incubation at room temperature. After centrifugation, supernatants were analyzed by LC-MS/MS. The transitions at *m/z* 248.0 → *m/z* 149.9 and *m/z* 251.1 → *m/z* 153.0 were monitored for the unlabeled and labeled forms of the analyte, respectively. The CDC precipitated proteins with metaphosphoric acid, filtered the samples, and determined the vitamin B_6 vitamers pyridoxal 5'-phosphate and 4-pyridoxic acid by LC with chlorite post-column derivatization and fluorescence detection [3,10]. The B_6 vitamers were separated under isocratic conditions on a C_{18} column with a mobile phase comprised of aqueous phosphate buffer (with 0.2 % acetonitrile, volume fraction). The initial mobile phase was comprised of 100 % aqueous buffer, and a linear gradient from 0 % to 30 % methanol was employed after elution of the B_6 vitamers to facilitate column cleanup between injections.

Homogeneity Assessment: The homogeneity of PLP was assessed at NIST using the method and test portion size described above; analysis of variance did not show statistically significant heterogeneity. All analytes have been treated as though they are homogeneously distributed in the material although the homogeneity of the other analytes was not assessed.

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