National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material 968

Fat-Soluble Vitamins in Human Serum

Standard Reference Material (SRM) 968 is intended for use in validating methods for determining retinol, α-tocopherol, and ß-carotene in human serum and plasma. This SRM can also be used for assigning values to in-house control materials. A unit of SRM 968 consists of six vials of lyophilized human serum, two each of three concentration levels for the three fat-soluble vitamins. The serum in each vial is to be reconstituted with 1.00 mL of HPLC quality water.

Certified Concentrations

The certified concentrations of retinol, α-tocopherol, and total ß-carotene (all-*trans* plus *cis* isomers) in reconstituted SRM 968 are provided below in Table 1. Information values for all-*trans* ß-carotene and γ-tocopherol are given in Table 2. The certified concentration values are derived from the concordant results of analyses performed at NIST and seven collaborating institutions. An alphabetized listing of these laboratories is provided in Appendix A. The values provided by the various laboratories are shown in Appendix B and descriptions of the various methods used are provided in Appendix C. The certified concentrations apply to solutions obtained after the sera have been reconstituted using the procedure described in "Instructions for Use".

Table 1

Certified Concentrations of Fat Soluble Vitamins in Reconstituted SRM 968 (μg/mL)

	Low Level	Medium Level	High Level
Retinol	0.313 ± 0.010	0.477 ± 0.019	1.15 ± 0.05
α-Tocopherol	4.94 ± 0.16	7.67 ± 0.29	12.4 ± 0.5
Total ß-Carotene	0.123 ± 0.017	0.677 ± 0.022	1.49 ± 0.06

The uncertainties listed represent two standard deviations of the certified concentrations and include analytical imprecision, and any vial-to-vial variability, and imprecision associated with the reconstitution process. The uncertainties do not include possible unrecognized bias due to the extraction process and the purity assessment of the reference compounds.

The overall direction and coordination of the preparation and technical measurements leading to the certification of this SRM were performed by W. E. May, M. C. Kline, and R. Schaffer, Organic Analytical Research Division.

Analytical measurements at NIST were performed by J. M. Brown-Thomas, R. G. Christensen, N. E. Craft, and W. A. MacCrehan of the Organic Analytical Research Division.

Statistical consultation was provided by R. C. Paule, National Measurement Laboratory.

The technical and support aspects involved with the certification and issuance of this SRM were coordinated through the NIST Office of Standard Reference Materials by R. Alvarez, and through the Division of Cancer Prevention and Control, National Cancer Institute, by H. Pierson.

April 21, 1989
Gaithersburg, MD 20899

Stanley D. Rasberry, Chief
Office of Standard Reference Materials

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Notice to Users

Storage: Until required for use, the freeze-dried serum should be stored between -20 and -80 °C in the dark.

Expiration of Certification: This certification is valid within the specified uncertainty limits for one year from the date of shipment when the SRM is stored properly. In the event that the certification becomes invalid prior to that time, users will be notified by NIST. Please return the attached registration form to facilitate notification.

Warning: The supplier of this serum has reported testing the source materials used to prepare this product and found them to be non-reactive when tested for Hepatitis B Surface Antigen (HB_sAg) and for human immunodeficiency virus (HIV) by FDA required tests. However, because no test method can offer complete assurance that HIV, hepatitis B virus, or other infectious agents are absent, these specimens should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 1984, 11-13.

Instructions for Use

SRM 968 is provided as a set of two vials of lyophilized serum for each of three concentration levels for the certified analytes and must be reconstituted before use. Prior to reconstitution, the vials should be allowed to stand at room temperature for at least 30 minutes, but no longer than 16 hours. Since the vials were sealed under pressure, it is recommended that the vials be vented by insertion of an empty syringe needle prior to opening. To achieve the certified concentrations, the analyst must reconstitute the freeze dried serum with 1.00 mL of HPLC quality water. Dissolution can be facilitated by ultrasonic agitation for 3 to 5 minutes or by intermittent swirling for at least 15 minutes. Vigorous shaking or mechanical swirling causes the formation of foam and should be avoided. After reconstitution, the contents should be used immediately or stored at refrigerator temperature. After reconstitution, the contents must be used within 24 hours for the certified values to be valid.

Preparation of SRM Serum Pools

Plasma for preparing three one-liter pools of materials for analysis and certification as SRM 968 was obtained from Plasma Biological Services, Inc., Memphis, TN. At NIST, plasma was converted to serum by several freeze-thaw, clotting processes, followed by filtration through a 0.45-μm filter to remove fibrin. Once processed, the plasma-converted serum was clot-free. Several serum sub-pools were obtained from the blood of volunteers whose diets had been supplemented to varying extents with ß-carotene. The "nutritionally fortified" ß-carotene pools were blended at NIST to obtain three desired levels of ß-carotene. This blending process yielded the desired levels for retinol and α-tocopherol in the low and medium pools, but not in the high pool. The desired levels of retinol and α-tocopherol in the high pool were achieved by supplementing the levels naturally present through addition of 2.5 mL of an ethanolic solution containing 210 μg/mL retinol and 2.7 mg/mL α-tocopherol to 1 liter of serum. The three serum pools were filtered and dispensed as 1.00 mL aliquots into 3.5-mL serum vials. The samples were then lyophilized, sealed under slight positive pressure with argon, and stored at -80 °C.

Analytical Measurements for Value Assignment

The certified concentrations for this SRM represent the pooled results from analyses performed at NIST and seven collaborating institutions. All analyses involved precipitation of protein with ethanol followed by extraction of the supernate with a lipophilic solvent (e.g. hexane or petroleum ether). The extracts were then analyzed by liquid chromatography using various stationary phase-mobile phase combinations, detectors, and internal standards. Since the maintenance of pure and stable primary reference compounds for retinol, α-tocopherol and ß-carotene is not technically feasible, detector responses were calibrated against solutions whose concentrations were determined spectrophotometrically based on the following extinction coefficients in absolute ethanol: 1850 $dL \cdot g^{-1} \cdot cm^{-1}$ at 324 nm for retinol; 75.8 $dL \cdot g^{-1} \cdot cm^{-1}$ at 292 nm for α-tocopherol; 2560 $dL \cdot g^{-1} \cdot cm^{-1}$ at 452 nm for ß-carotene. The values provided by each laboratory are given in Appendix C and descriptions of the methods used for the determination of each analyte are provided in tabular form in Appendix D.

Two different procedures were used at NIST for the determination of each of the analytes certified in this SRM. In both procedures, the serum was extracted by precipitating the protein with ethanol and extracting the supernate with hexane. NIST procedure A was based on gradient elution of the extract from a 300 Å pore, polymerically-bonded C-18 column using a methanol:butanol:acetic acid mobile phase. Ten randomly selected vials from each of the three levels were analyzed in duplicate (extraction of two aliquots from each vial; one injection per aliquot) to determine the variability associated with fill weights throughout each lot. No vial-to-vial variability was detected. NIST procedure B involved separate isocratic analyses of the extracts for retinol/α-tocopherol and ß-carotene on different monomerically-bonded C-18 columns. Retinol and α-tocopherol were monitored at 300 nm and ß-carotene was monitored at 450 nm. Three vials from each level were analyzed in duplicate.

Table 2

Information Values

Values for γ-tocopherol and all *trans* ß-carotene are provided below for information only. These values are derived from a limited number of analyses using methods that have not been validated for these particular analytes.

	Concentration (μg/mL)		
Analyte	Low Level	Medium Level	High Level
γ-tocopherol[a]	2.3	2.4	3.0
all *trans* ß-carotene[b]	0.115	0.55	1.2

[a]Values represent the mean from measurements made using NIST method B and values submitted by Laboratory #4. The data provided by each laboratory is shown in Appendix B and descriptions of the methods used are provided in Appendix C.

[b]Most HPLC columns used for determination of fat-soluble vitamins do not separate all-*trans* ß-carotene from its *cis* isomers. Two different columns that are capable of separating all-*trans* ß-carotene from its *cis* isomers were used at NIST to provide data for both total-and all-*trans* ß-carotene.

APPENDIX A

The institutions listed below performed measurements that contributed to the certification of SRM 968.

Name	Analytes Measured
Dr. Phyllis E. Bowen University of Illinois Health Science Center Chicago, IL	Retinol, α-Tocopherol
Dr. Lawrence A. Kaplan University of Cincinnati Medical Center Cincinnati, OH	ß-Carotene
Dr. Jarmo Pikkarainen National Public Health Institute Helsinki, Finland	Retinol, α-Tocopherol, ß-Carotene
Dr. Willy Schuep F. Hoffmann-La Roche & Co. Basle, Switzerland	Retinol, α-Tocopherol

Prof. Tsuguyoshi Suzuki University of Tokyo Tokyo, Japan	Retinol, α-Tocopherol, ß-Carotene
Dr. Govind T. Vatassery Veterans Administration Hospital South Minneapolis, MN	α-Tocopherol, γ-Tocopherol
Prof. Chung S. Yang Rutgers State University Piscataway, NJ	Retinol, ß-Carotene

APPENDIX B

The average values provided by each of the institutions that participated in the certification of SRM 968 are listed in the following tables. The laboratory numbers given do not follow the alphabetized listing shown in Appendix B. Concentrations are given as µg/mL.

Low Level

Institution	Retinol	Tocopherol		ß-Carotene	
		α-	γ-	total	all-*trans*
NIST A	0.313	4.98		0.112	0.109
NIST B	0.317	4.77	2.45	0.159	0.119
1	0.291	4.69			
2	0.334	5.03		0.136	
3				0.105	
4		5.26	2.16		
5	0.312			0.110	
6	0.323	5.10		0.119	
7	0.305	4.75			

Medium Level

NIST A	0.479	7.32		0.660	0.582
NIST B	0.469	7.26	2.49	0.655	0.513
1	0.472	7.50			
2	0.518	7.97		0.730	
3				0.674	
4		8.20	2.22		
5	0.463			0.674	
6	0.498	8.03		0.670	
7	0.438	7.42			

High Level

NIST A	1.156	11.97		1.445	1.342
NIST B	1.175	11.81	3.06	1.348	1.115
1	1.04	12.41			
2	1.201	12.32		1.537	
3				1.577	
4		13.40	2.86		
5	1.143			1.518	
6	1.238	12.94		1.482	
7	1.073	11.86			

APPENDIX C

Methods Used for Certification of Fat-Soluble Vitamins in Human Serum SRM

Lab #	Analytical Column			Mobile Phase Conditions		Detection		Standardization	
	Packing Type	MFG Model, dp	Dimensions (cm)	Elution	Solvent Composition	Type (instrument design)	Wavelength in nm	Type	Internal Standard
Retinol									
NIST A	C-18	Vydac 201TP, 5 μm	25 X 0.46	Gradient	67:10:23//86.5:10:3.5 MeOH:BuOH:H_2O 10 mM HOAc pH 5.5	UV/Vis Absorbance (variable wavelength)	325	Internal	Tocol
NIST B	C-18	Beckman Ultrasphere 5 μm	25 X 0.46	Isocratic	100 MeOH	UV/Vis Absorbance (variable wavelength)	300	Internal	Tocol
1	C-18	Waters Novapak, 5 μm	15 X 0.39	Isocratic	50:45:5 MeOH:CH_3CN:THF	UV/Vis Absorbance (rapid scanning)	325	External	
2	C-18	HP Hypersil, 3 μm	6 X 0.46	Isocratic	100 MeOH	UV/Vis Absorbance (diode array)	320	Internal	Retinyl Acetate
5	C-18	Waters Radial Pak, 5 μm	10 X 0.80	Isocratic	55:22:11.5:11.5 CH_3CN:MeOH:CH_2Cl_2:Hexane	UV/Vis Absorbance (rapid scanning)	325	Internal	α-Tocopheryl Acetate
6	C-18	YMC A-302, 5 μm	15 X 0.46	Isocratic	80:19.9:0.1 MeOH:BuOH:H_2O 10 mM HOAc pH 3.6	UV/Vis Absorbance (variable wavelength)	325	External	
7	Silica	Merck Licrosorb, 5 μm	25 X 0.40	Isocratic	97:3 Hexane:2-Propanol	UV/Vis Absorbance (fixed wavelength)	313	External	
α-Tocopherol									
NIST A	C-18	Vydac 201TP, 5 μm	25 X 0.46	Gradient	67:10:23//86.5:10:3.5 MeOH:BuOH:H_2O 10 mM HOAc pH 5.5	UV/Vis Absorbance (variable wavelength)	292	Internal	Tocol
NIST B	C-18	Beckman Ultrasphere 5 μm	25 X 0.46	Isocratic	100 MeOH	UV/Vis Absorbance (variable wavelength)	300	Internal	Tocol
1	C-18	Waters Novapak, 5 μm	15 X 0.39	Isocratic	50:45:5 MeOH:CH_3CN:THF	UV/Vis Absorbance (rapid scanning)	294	External	
2	C-18	HP Hypersil, 3 μm	6 X 0.46	Isocratic	100 MeOH	UV/Vis Absorbance (diode array)	292	Internal	Retinyl Acetate
4	Silica	Dupont Zorbax-Sil, 5 μm	25 X 0.46	Isocratic	99:1 Hexane:MeOH	Fluorescence (dual monochrometer)	295/340	External	
6	C-18	YMC A-302, 5μm	15 X 0.46	Isocratic	80:19.9:0.1 MeOH:BuOH:H_2O 10 mM HoAc pH 3.6	UV/Vis Absorbance (variable wavelength)	292	External	
7	Silica	Merck Licrosorb, 5 μm	25 X 0.40	Isocratic	93:7 Hexane:EtAc	Fluorescence (dual monochrometer)	298/328	External	
β-Carotene									
NIST A trans,total	C-18	Vydac 201TP, 5 μm	25 X 0.46	Gradient	67:10:23//86.5:10:3.5 MeOH:BuOH:H_2O 10 mM HOAc pH 5.5	UV/Vis Absorbance (variable wavelength)	450	Internal	Tocol
NIST B trans,total	C-18	Analytichem Sepralyte, 5 μm	25 X 0.46	Isocratic	70:20:10 CH_3CN, CH_2Cl_2, MeOH	UV/Vis Absorbance (variable wavelength)	450	Internal	Tocol
2 total	C-18	HP Hypersil, 3 μm	6 X 0.46	Isocratic	100 MeOH	UV/Vis Absorbance (diode array)	450	Internal	Retinyl Acetate
3 total	C-18	BAS Biophase, 5 μm	25 X 0.46	Isocratic	78:16:3.5:2.5 CH_3CN:$CHCl_3$:1-Propanol:H_2O	UV/Vis Absorbance (fixed wavelength)	460	Internal	Ret + α-Toc Acetate
5 total	C-18	Waters Radial Pak, 5 μm	10 X 0.80	Isocratic	55:22:11.5:11.5 CH_3CN:MeOH:CH_2Cl_2:Hexane	UV/Vis Absorbance (rapid scanning)	450	Internal	α-Tocopheryl Acetate
6 total	C-18	YMC A-302, 5 μm	15 X 0.46	Isocratic	80:19.9:0.1 MeOH:BuOH:H_2O 10 mM HOAc pH 3.6	UV/Vis Absorbance (variable wavelength)	450	External	
7 total	Silica	Merck Licrosorb, 5 μm	25 X 0.40	Isocratic	99:1 Hexane:Dioxane	UV/Vis Absorbance (fixed wavelength)	436	External	