Supplementary Information for

Development of a Certified Reference Material of Human Serum Albumin: Certification and Value Assignment via Amino Acid Analyses

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Experimental condition for amino acid analyses

For the analysis of liquid phase hydrolysate, the hydrolysate was modified using *N*-butylnicotinic acid *N*-hydroxysuccinimide ester iodide, and injected into an LC-MS/MS system with a C30 reversed-phase column (Develosil C30-UG-5, 5 μm particle size, 2 mm I.D.× 250 mm. Nomura Chemical, Japan) and a TSQ Quantum triple quadrupole mass spectrometer (Thermofisher Scientific, USA) with an electrospray ion source. Solvent and gradient conditions: mobile phase A (0.05% trifluoro acetic acid and 0.5 % formic acid in water) and mobile phase B (0.05% trifluoro acetic acid and 0.5 % formic acid in acetonitrile) under the gradient condition of 5 to 20 %B in A for 10 min then 20 to 55 %B in A for 10 min at 0.2 mL/min. The SRM transitions were as follows, Asp: 295.13 > 205.13; Glu: 309.15 > 263.14; Pro: 277.16 > 233.17; Lys (+2): 235.15 > 207.64; Val: 279.17 > 233.17; Leu and Ile: 293.19 > 247.18; Phe: 327.17 > 281.17. Asp*:300.14 > 208.138; Glu*: 315.16 > 268.15; Pro*: 283.17 > 233.17; Lys*(+2): 239.15 > 211.15; Val*: 285.19 > 238.18; Leu* and Ile*: 300.20 > 253.20; and Phe*: 337.20 > 290.19, where "*" indicates an isotopically labeled amino acid.

For the analysis of gas phase hydrolysate, the resulting hydrolysate was modified with 1-bromobutane, and injected into the same LC-MS/MS system as that used during the liquid phase hydrolysis. The chromatographic separation was performed using a C18 reversed-phase column (Kinetex core-shell XB-C18 2.6 μm particle size, 2.1 mm I.D.× 75 mm, Phenomenex). Solvent and gradient conditions: mobile phase A (1 % acetic acid in water) and mobile phase B (1 % acetic acid in acetonitrile) under the gradient condition of 1 to 75 %B in A for 20 min at 0.3 mL/min. The SRM transitions were as follows, Asp: 302.2 > 186.2; Glu: 316.2 > 216.2; Pro: 228.2 > 126.2; Lys : 371.2 > 242.2; Val: 230.2 > 128.2; Leu and Ile: 244.2 > 142.2; Phe: 278.2 > 176.2. Asp*:307.2 > 189.2; Glu*: 322.2 > 219.2; Pro*: 234.2 > 131.2; Lys*: 383.2 > 248.2; Val*: 236.2 > 133.2; Leu* and Ile*: 250.2 > 145.2; and Phe*: 284.2 > 185.2, where "*" indicates an isotopically labeled amino acid.

Indicative value: concentration determination using the biuret method

In clinical chemistry, the biuret method is frequently used to determine the total protein concentration in serum using BSA as the calibrant. We assigned the indicative value determined by the biuret method using the BSA CRM NIST SRM927d as the calibrant according to NIST method.

The reagents, $CuSO_4 \cdot 5H_2O$ and NaOH were purchased from Wako pure chemical, and potassium sodium tartrate and KI were purchased from Sigma-Aldrich. The absorbance was measured using a UV-2550 spectrophotometer (Shimadzu).

The quantitation of the candidate CRM via the biuret method was performed by evaluating three independent samples per day on three separate days. The result obtained was 77.28 g/L. The associated uncertainty related to inhomogeneity used the result obtained by HPLC analysis for the certification by amino acid analysis, which was 0.33 %. The stability was monitored for up to 358 days at 4 °C by measuring the concentration using the biuret method. The trend analysis of these data performed according to ISO Guide 35 indicated no significant changes over the examined period. The relative standard deviations of the nine measured concentrations over the examined period were determined as the uncertainty associated with instability, which was 1.38 %. The associated uncertainty of the quantitation obtained using the biuret method was 1.37 %. The uncertainty associated with the calibrant, NIST SRM927d, was 0.53 % as shown on the certificate. The overall uncertainty budget is summarized in Table S1.

Table S1 Uncertainty budget for the indicative value of HSA CRM 6202-a determined by biuret method

Uncertainty components (%)		
Value-assignment	1.37	
Inhomogeneity		0.33
Instability		1.38
BSA standard solution		0.53
Combined standard uncertainty (Rel, %)		2.05
Combined standard uncertainty (g/L)	1.58	

The indicative value was determined to be (77.3 ± 3.2) g/L, the number following "±" represents the expanded uncertainty (k = 2).

Electrophoresis of the material

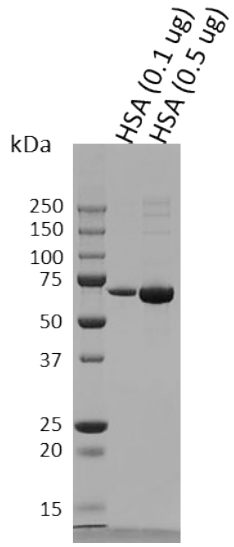

Fig S1 Sodium dodecyl sulfate polyacrylamide gel-electrophoresis of a candidate HSA CRM stained by Coomassie Brilliant Blue.

Peptide mapping

The identified peptides covered 86.5 % of the secreted form of HSA sequence in number of the amino acids. Protein modifications such as oxidation and deamidation were not considered by the database search, except the cysteine carbamidomethylation resulted by the alkylation of cysteine residues by iodoacetamide treatment. The result is shown in Table S2.

Table S2 The results of peptide mapping of a candidate HSA CRM after trypsin digestion.

Sequence	Charge	MH+ [Da]	ΔM [ppm]	# Missed Cleavages
TcVADESAENcDKSLHTLFGDKLcTVATLR	3	3411.59836	0.13	2
SHcIAEVENDEMPADLPSLAADFVESK	2	2974.34648	0.62	0
SHcIAEVENDEMPADLPSLAADFVESKDVcK	3	3476.56821	0.74	1
EFNAETFTFHADIcTLSEK	2	2260.02226	-0.22	0
KVPQVSTPTLVEVSR	2	1639.93767	-0.09	1
EFNAETFTFHADIcTLSEKER	2	2545.16753	0.41	1
TcVADESAENcDK	2	1498.57915	0.42	0
TcVADESAENcDKSLHTLFGDK	2	2497.09795	0.34	1
QNcELFEQLGEYK	2	1657.75261	-0.06	0
AAFTEccQAADKAAcLLPK	2	2124.98662	-0.46	1
LVRPEVDVMcTAFHDNEETFLK	2	2650.26470	0.22	0

LVAASQAALGL	2	1013.59838	-0.65	0
RPcFSALEVDETYVPKEFNAETFTFHADIcTLSEKER	3	4437.08164	0.28	2
VPQVSTPTLVEVSR	2	1511.84258	-0.18	0
VHTEccHGDLLEcADDR	2	2086.83623	-0.67	0
SLHTLFGDKLcTVATLR	2	1932.03496	-1.17	1
YKAAFTEccQAADK	2	1662.72490	-0.13	1
RHPDYSVVLLLR	3	1467.84354	0.29	1
RHPYFYAPELLFFAK	2	1898.99578	0.27	1
NEcFLQHKDDNPNLPR	2	1996.92998	0.26	1
YIcENQDSISSK	2	1443.64226	0.13	0
LVRPEVDVMcTAFHDNEETFLKK	3	2778.35575	-1.20	1
VFDEFKPLVEEPQNLIKQNcELFEQLGEYK	3	3683.83261	0.62	1
RPcFSALEVDETYVPKEFNAETFTFHADIcTLSEK	3	4151.93991	0.78	1
AVMDDFAAFVEK	2	1342.63445	-0.30	0
VFDEFKPLVEEPQNLIK	2	2045.09184	-1.77	0
QNcELFEQLGEYKFQNALLVR	2	2599.29936	0.74	1
VHTEccHGDLLEcADDRADLAK	2	2585.11797	0.04	1
LVNEVTEFAK	2	1149.61406	-0.88	0
ETYGEMADccAK	2	1434.53313	-0.24	0
LcTVATLRETYGEMADccAK	2	2349.03520	0.39	1
LKEccEKPLLEK	2	1546.79643	-0.26	1
RMPcAEDYLSVVLNQLcVLHEK	2	2674.31621	0.42	1
ADDKETcFAEEGKK	2	1627.72490	-1.24	2
ccAAADPHEcYAK	2	1552.59844	0.41	0
RPcFSALEVDETYVPK	3	1910.93314	0.72	0
AAFTEccQAADK	2	1371.56633	-0.36	0
HPDYSVVLLLR	2	1311.74175	-0.19	0
KLVAASQAALGL	2	1141.69402	0.01	1
QTALVELVK	2	1000.60356	-0.21	0
FKDLGEENFK	2	1226.60491	-0.27	1
KQTALVELVK	2	1128.69878	0.03	1
QEPERNEcFLQHKDDNPNLPR	2	2636.22881	0.63	2
DAHKSEVAHR	2	1149.57598	-0.01	1
SLHTLFGDK	2	1017.53575	-0.65	0
KVPQVSTPTLVEVSRNLGK	2	2052.18071	-0.27	2

YKAAFTEccQAADKAAcLLPK	3	2416.14530	-0.25	2
LDELRDEGK	2	1074.54277	0.12	1
HPYFYAPELLFFAK	3	1742.89414	0.00	0
LDELRDEGKASSAK	2	1518.77629	0.25	2
SLHTLFGDKLcTVATLRETYGEMADccAK	3	3347.55246	-0.12	2
LcTVATLR	2	933.52001	1.45	0
ccTESLVNR	2	1138.49761	-0.35	0
KYLYEIAR	2	1055.58818	-0.24	1
QEPERNEcFLQHK	2	1714.79875	1.23	1
LVTDLTK	2	789.47173	0.06	0
HPYFYAPELLFFAKR	3	1898.99594	0.36	1
EccEKPLLEK	2	1305.61760	-0.15	0
DLGEENFK	2	951.44139	-0.48	0
VTKccTESLVNR	2	1466.70964	0.39	1
YLYEIAR	2	927.49352	0.06	0
NYAEAKDVFLGMFLYEYAR	2	2300.10576	0.03	1
VGSKccKHPEAK	2	1400.67803	0.46	2
TPVSDRVTK	2	1002.55828	0.39	1
TYETTLEK	2	984.48845	0.01	0
LSQRFPK	2	875.51030	0.57	1
AWAVAR	2	673.37810	0.06	0
TPVSDR	2	674.34673	-0.12	0
AEFAEVSK	2	880.44127	0.17	0
FQNALLVR	2	960.56230	-0.28	0
SEVAHR	2	698.35808	0.06	0
ccKHPEAK	2	1029.46013	-0.35	1
LAKTYETTLEK	2	1296.70500	0.31	1
ADDKETcFAEEGK	2	1499.63213	0.12	1
ccTESLVNRRPcFSALEVDETYVPK	3	3030.40995	-0.65	1