Biologicals (1998) **26**, 331–346
Article No. bg980163

Recombinant Human Albumin as a Stabilizer for Biological Materials and for the Preparation of International Reference Reagents

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Abstract. Recombinant human albumin expressed in *Saccharomyces cerevisiae* was compared with native human serum albumin in its physicochemical properties and in its use as a stabilizer in lyophilized preparations of thyroid-stimulating hormone (TSH), interleukin 15 (IL-15) and granulocyte colony-stimulating factor (G-CSF). Advantages of recombinant albumin include its lack of potential human contaminants and infectious agents. When used at concentrations of 0·1–0·2% (w/v), recombinant albumin was equivalent to native serum albumin in its capacity to protect immunological, biological and biochemical properties of TSH, IL-15 and G-CSF. Physicochemical characteristics of the two forms of albumin including their binding to fatty acids were also similar. The recombinant form of albumin used in this study should be considered as a suitable stabilizer in the preparation of lyophilized products and reference reagents.

Introduction

International Biological Standards and Reference Reagents are usually prepared by freeze-drying aqueous solutions containing the active agents together with buffer salts and/or bulking agents which prevent adherence to absorbent surfaces and which in addition may act as stabilizers of the active agent itself.[1,2] Commonly used bulking agents/stabilizers include carbohydrates, especially αα'-trehalose, and proteins, especially human serum albumin (HSA).[3,4] Both trehalose and HSA are stabilizers for biological materials in nature but, while HSA has in the past proved highly successful for preparing biological standards and reference materials, there are a number of drawbacks to its continued use, including its possible contamination with human pathogens and enzymes, structural heterogeneity and batch-to-batch variability. Recombinant human albumin (RHA) is now available from genetically modified yeast[5–7] and, if employed in the preparation of biological standards, may retain the beneficial stabilizing properties of HSA whilst overcoming the drawbacks associated with serum-derived material.

This report describes the results of a comparative investigation in which the stabilities of freeze-dried preparations, containing either HSA or RHA, were studied. Specifically, recombinant human thyroid-stimulating hormone (rhTSH, a 32-kDa heterodimeric glycoprotein hormone) and recombinant human interleukin 15 (rhIL-15, a 16-kDa peptide cytokine) were used. These materials were chosen because WHO reference preparations for them have been previously established and therefore detailed stability data existed. In the present study the different preparations of albumin were compared

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1045–1056/98/040331 + 16 $30.00/0

using immunological, biological and physicochemical methods. In addition, the ability of HSA and RHA to protect a labile cytokine, granulocyte colony-stimulating factor (G-CSF), from the effects of temperature (including aggregation and adsorption to surfaces) was also studied.

Materials and methods

Materials

Recombinant human albumin (RECOMBUMIN ™-25) batch no. GA950202 (25% w/v), expressed in *Saccharomyces cerevisiae*, was a gift from Delta Biotechnology Limited, (Nottingham, U.K.), and contained 145 mM sodium chloride, 40 mM sodium octanoate and 0·0015% (w/v) Tween-80. Human albumin 20%, 9% and 4·5% w/v solutions (HSA) were obtained from Bio Products Laboratories, (Elstree, U.K.) in both pasteurized and non-pasteurized forms. All albumin solutions were stored at 4°C. For some experiments, non-pasteurized HSA (4·5%) was heated under ''pasteurization conditions'' (60°C for up to 48 h), by incubation in a water bath. For physicochemical experiments, pre-formulated HSA, containing no octanoate, and post-formulated HSA, containing 7 mM octanoate were used. Octanoate titrations were performed by the addition of aliquots of buffered sodium octanoate (Sigma, Poole, U.K.) to the pre-formulated sample. Recombinant hTSH, expressed in Chinese hamster ovary (CHO) cells, was generously donated by Genzyme Corporation (Framingham, MA, U.S.A.). Recombinant hIL-15 was a gift from Immunex (Seattle, WA, U.S.A.) and rhG-CSF from Amgen (Thousand Oaks, CA, U.S.A.).

Endotoxin content of albumin preparations. Endotoxin was determined using the Limulus Amoebocyte Lysate (LAL) test for bacterial endotoxin as detailed in the European Pharmacopoeia.[8]

Physicochemical analysis of albumin preparations

Liquid chromatography–Electrospray mass spectrometry (LC–ESMS). Mass spectra were acquired on a VG Platform II mass spectrometer using pneumatically assisted electrospray ionization (ESI) in positive ion mode. Samples were introduced into the ion source by microbore reversed-phase HPLC. Liquid chromatography instrumentation consisted of two conventional HPLC pumps, model PU-980 (Jasco, Tokyo, Japan) coupled to a model AC-400-VAR microflow processor (LC Packings, Amsterdam, The Netherlands), a Rheodyne valve fitted with a 10-μl loop, a Vydac C_4 column (5 μm, 300X, 15 cm$\times$800 μm i.d.) and a model UV-975 UV-vis absorbance detector (Jasco) equipped with a U-Z capillary flow cell (LC Packings). UV absorbance was monitored at 214 nm and the liquid flow from the detector was directed to the mass spectrometer probe. The flow rate into the mass spectrometer source was 20 μl/min. Samples (45–250 mg/ml) of HSA (with and without sodium octanoate) and RHA were diluted to 1 mg/ml with water, centrifuged at 8500 ***g*** for 3 min (MSE MicroCentaur), and injected (10 μl) onto the C_4 column. The binary solvent system comprised solvent A, H_2O, 0·05 vol % trifluoroacetic acid (TFA), and solvent B, 80% CH_3CN, 0·04 vol % TFA. The column was equilibrated in 40% B and a linear gradient of 40 to 95% B in 20 min started 5 min after sample injection. The probe was not inserted until 9 min into the gradient to allow non-retained material to elute e.g. sodium chloride, Tween 80, and the data was acquired in continuum mode (m/z scan range 700–1700, 10 s/scan). Data acquisition and processing were controlled by the VG MassLynx (version 2·1) data system. The voltage applied on the ESI capillary was +3·5 kV and a sample cone voltage of 45 V was used. The mass spectrometer was calibrated using multiply charged ions of horse heart myoglobin (Sigma, Poole, U.K.).

A previous attempt to analyse HSA using direct flow injection ESI-MS proved unsuccessful. The resolution, irrespective of the purification methodology, was very poor and this was assumed to be due to the high tendency of HSA to bind ligands and form adducts. In this study, LC-MS was initially performed using eluants containing 0·1% TFA and the sample was diluted to 1 mg/ml with 0·1% TFA. Again the resolution was poor and the deconvoluted mass spectrum was greatly complicated by a number of other peaks which were presumed to be TFA adducts (data not shown). Reduction of the percentage of TFA in both sample and eluants as reported here significantly improved the resolution of acquired data.

Tryptic peptide mapping by HPLC

(i) Reduction and carboxymethylation. A 40-μl sample of each albumin (concentration 250 mg/ml) was added to separate solutions of 5 M guanidium chloride in 0·5 M Tris–HCl pH 8·6 (1·0 ml) containing 40 μl of 1 M dithiothreitol and the reduction mixture was incubated for 1 h at 50°C. After this time each solution was treated with 80 μl of 1 M iodoacetic acid in 1 M NaOH and the alkylation allowed to proceed for 20 min at ambient temperature in the dark. The reaction mixtures were then acidified with 50 μl

glacial acetic acid and the proteins recovered by reversed phase chromatography on a column of PLRP-S 300 (20 μm, 250×10 mm i.d.) using a gradient (8–72%) of CH_3CN in 0·1% trifluoroacetic acid over 9 min at a flow rate of 5 ml/min with monitoring at 215 nm on a diode array spectrophotometric UV monitor (Polychrom 9060, Varian). The reduced and carboxymethylated (RCM) albumin peaks which eluted at approximately 8·5 min were collected and lyophilized.

(ii) Tryptic digestions. Each of the lyophilized RCM samples (3 mg) was suspended in 0·3 ml water, and an equal volume of 4% (w/v) ammonium bicarbonate added. The solid was dispersed by sonication and 30 μl of 1 mg/ml trypsin (Sigma, TPCK treated) in 1 mM HCl was added (1/50 by weight trypsin/RCM albumin). The samples were allowed to digest at ambient temperature for 1·5 h (when all the insoluble material had dissolved) and then a further 20 μl of trypsin added and the solutions kept at ambient temperature overnight after which time the reaction was stopped by addition of 20 μl glacial acetic acid (total digestion time 17 h).

(iii) Peptide mapping. Aliquots (100 μl) of the tryptic digests were analysed on a Jupiter 300 X C18 column (Phenomenex) (250×4·6 mm) at a flow of 1 ml/min. The gradient was linear from 8% CH_3CN to 48% CH_3CN in 0·1% TFA over 60 min. The separation was monitored at 215 nm.

Trypsin digestion and MALDI (Matrix-assisted laser desorption Time-of-flight mass spectrometric) Analysis. HSA, RHA and TPCK-treated trypsin (Sigma) were dissolved separately in 50 mM NH_4HCO_3 at concentrations of 0·5 mg/ml. To 100 μl of each albumin solution was added 2 μl of the trypsin solution and following incubation at 37°C for 18 h the digests were analysed by MALDI. This was performed on a Perseptive Voyager Elite mass spectrometer (Perseptive Biosystems Ltd, Framingham, U.S.A.) operating with delayed extraction in positive-ion and reflector modes. Spectra were acquired following irradiation of samples with a nitrogen laser giving a 337-nm output with 3-ns pulse width and molecular ions were accelerated at a potential of 20 kV. For analysis each enzyme digest was mixed with an equal volume of matrix solution and 1 μl was then allowed to dry on the target plate. The matrix was α-cyano-4-hydroxycinnamic acid (Sigma) at a concentration of 10 mg/ml in 0·1% aqueous trifluoroacetoic acid:acetonitrile 1:2 v/v. The instrument was calibrated using des-Arg^1-Bradykinin (904·47), Angiotensin 1 (1296·69) and Glu^1-Fibrinopeptide B (1570·68) (Sigma) as internal standards (monoisotopic masses in parenthesis).

Circular dichroism (CD). Circular dichroism was performed on a Jasco J-720 spectropolarimeter at 25°C ± 1°C. Temperature was controlled using a Neslab RTE-110 circulating water bath. The results were corrected for pathlength and protein concentration and are expressed as molar circular dichroic absorption ($\Delta\varepsilon$) in $M^{-1}\,cm^{-1}$. For near-UV CD (320 nm–260 nm), the molar protein concentration (mol/l) is used; for far-UV CD (250 nm–180 nm), the mean residue molar concentration (mol/l×No. a.a. residues) is used. Each spectrum resulted from the averaging of nine scans and the final spectra were obtained by subtracting a similarly averaged buffer baseline. The instrument was calibrated using ammonium *d*-(+)-camphor-10-sulfonate (Jasco) as a reference standard. The bandwidth employed was 1 nm with a response time of 1 s. Far-UV CD spectra were obtained using solutions suitably diluted with 145 mM NaCl, 40 mM sodium octanoate, 0·0015% w/v Tween 80, pH 7·5 (Buffer 1), resulting in a sample containing approximately 1·3 mg/ml protein in a 0·01-cm demountable cell (Hellma GmbH) holding 50 μl, and, near-UV CD spectra were obtained using a cylindrical cuvette (Hellma GmbH) of 1-cm pathlength containing approximately 1 ml of sample (~1–2 mg/ml). Protein concentrations were determined using a theoretical absorption coefficient based on amino acid content of $A^{0.1\%}_{280\,nm}$ of 0·519 l/g according to the values of Mach.[9] Far-UV CD spectra were analysed for secondary structure elements using the Yang algorithm,[10] the CONTIN program,[10] and VARSELEC software[11] with data from 190–240 nm.

Fluorescence spectroscopy. Fluorescence spectra were obtained with a Spex Fluoromax single-photon counting spectrofluorometer at 25°C ± 1°C in 1-cm quartz cells (Hellma GmbH) with a protein concentration of, typically, 0·04 mg/ml in Buffer 1. Excitation wavelengths of 280 and 295 nm were employed and a band-pass of 4·25 nm used on both excitation and emission monochromators. Fluorescence spectra were corrected for solvent contribution by subtracting an appropriate buffer baseline. λ_{max} values are accurate to ±0·5 nm. Temperature control was maintained with a Techne TE-8D circulating water bath under independent control. The instrument was calibrated using the 435·8 nm mercury line and a water Raman spectrum (λex, 350 nm; $\lambda em_{maximum}$, 397 nm).

Nuclear magnetic resonance spectrometry (NMR). Proton NMR spectra of HSA (with and without

octanoate), RHA (containing octanoate) and sodium octanoate (BDH) were obtained at 500 MHz on a Varian Unity 500 spectrometer. The samples were prepared by diluting the $\geq$4·5% (w/v) albumin solutions with 99·9% D_2O (Apollo Scientific Ltd, Stockport, U.K.) to obtain a final protein concentration of 4 mg/ml. Spectra were recorded at 30°C using presaturation of the solvent peak and were then referenced relative to tetramethylsilane at 0 ppm.

Gel filtration chromatography. An LKB FPLC system (Pharmacia, UK), equipped with a Superdex 200 HR 10/30 gel filtration column (24 ml) was used to analyse the oligomeric state of albumin. Samples of 100 μl containing $\sim$0·1 mg HSA or RHA were chromatographed using phosphate-buffered saline, pH 7·5 at a flow rate of 0·5 ml/min. The absorbance was monitored at 280 nm. The column was calibrated using globular proteins from a Pharmacia MW-GF-1000 kit, cytochrome C and tyrosine. HPLC gel permeation chromatography was also performed on a TSK G3000 SWXL column (7·8 mm $\times$ 430 cm) (Anachem, Luton, U.K.) using a mobile phase of 10 mM phosphate buffer containing 50 mM NaCl and a flow rate of 1 ml/min. Absorbance was monitored at 280 nm.

Gel electrophoresis of albumin samples. RHA and HSA were separated by gel electrophoresis using Laemmli[13] gels at 7%. Samples (50 μg) were run under reducing (10% w/v β-mercaptoethanol) and non-reducing conditions and silver-stained as previously described.[14]

Thiol assay. Free sulfydryl determination of HSA and RHA was performed with Ellman's reagent[15] using the method of Janatova.[16] Briefly, HSA and RHA were diluted to 10 mg/ml with 7·4 mM phosphate buffer pH 7·4 and 600 μl of each solution was treated with 5,5'-dithiobis(2-nitrobenzoic acid) prepared freshly in 37 mM phosphate buffer, pH 8·3. Following 45 min at room temperature in the presence of EDTA, the absorbance of the solution was measured at 412 nm against reagent blanks as described, using a Cecil UV-Vis spectrophotometer, and number of reacting sulfydryls determined using $\varepsilon_{412} = 13\,600\ M^{-1}\ cm^{-1}$.

Preparation of ampouled materials and experimental samples

Recombinant human thyroid-stimulating hormone. For evaluation by ELISA, rhTSH was made up to a concentration of 1 μg/ml in 0·5% lactose and in the presence of either 0·1% (w/v) HSA or 0·1% (w/v) RHA. Each preparation was dispensed into glass ampoules in 1·0-ml volumes which were frozen, freeze-dried and sealed under nitrogen as described for the preparation of International Biological Standards and Reference Reagents.[1,2] Ampoules containing RHA were coded 95/788 and ampoules containing HSA were coded 95/826. The ampouled material was then stored at –20, +4, +20, +37, +45 or +56°C for accelerated degradation studies. For evaluation by RP-HPLC and IEF/immunoblot, ampoules were prepared similarly but at a rhTSH concentration of 25 μg/ml; ampoules containing RHA were coded 95/792 and ampoules containing HSA were coded 95/790.

Recombinant human interleukin 15. Recombinant hIL-15 was made up to a concentration of 1 μg/ml in 0·9% NaCl (Baxter, U.K.) and 0·1% (w/v) trehalose in the presence of either 0·2% (w/v) HSA or 0·2% (w/v) RHA, and ampoules prepared as above; ampoules containing RHA were coded 95/796 and ampoules containing HSA were coded 95/794.

Recombinant human granulocyte colony-stimulating factor. G-CSF at 1000 IU/ml in RPMI 1640 culture medium, was kept at 37°C in plastic Eppendorf tubes for 4 h in the presence of 0·1% HSA or 0·1% RHA or medium alone, prior to biological assay (time 4 h samples). In addition, G-CSF at 1000 IU/ml was made up under similar conditions but entered directly into biological assay (time 0 samples).

Residual moisture content. Moisture content of lyophilized material was determined using a Mitsubishi CA 100 Moisture Meter as previously described.[17]

Stability studies with rhTSH

The immunoactivity of the rhTSH preparations (95/788 and 95/826) was assessed after 60 days at elevated temperatures by ELISA. The two-site ELISA procedure, using monoclonal antibodies (mAb) specific for TSH, was identical to that described for FSH.[18] The capture antibody was specific for the β-subunit whereas the biotin-labelled detector mAb was specific for the α-subunit. Responses from the ELISAs were analysed using the program WRANL.[19] The program carries out iterative weighted regression analysis of the logit-transformed responses on log dose, with low weighting being assigned to responses at or near to the upper and lower asymptotes of the response curve. Potency estimates and slopes are obtained from analysis of multiple doses of the preparations as parallel-line assays. The mean (n = 3–5) and 95% confidence interval of the estimates were calculated

and expressed as a percentage of the activity of the preparations stored at –20°C.

RP-HPLC analysis was performed on the contents of ampoules of 95/790 after storage at elevated temperatures for 60 days. A PLRPS column (Polymer Laboratories PLRP-S 300 A 8 μ 150×4·6 mm) was used, equilibrated with 21·60% acetonitrile in 0·1% trifluoroacetic acid. A load containing 5 μg rhTSH was eluted using a gradient of 1·2% acetonitrile for 20 min, and absorbance was monitored at 210 nm.

Changes in isoform composition after 160 days at elevated temperatures were assessed by IEF/immunoblotting. IEF was carried out on a 7·5% acrylamide gel with ampholine pI range 3·5–10. Separation was carried out at 340 V for 18 h with cooling, using 10 mM H_3PO_4 and 20 mM NaOH. Proteins were transferred onto nitrocellulose membrane at 50 V for 6 h with cooling, using 153 mM glycine and 20 mM Tris buffers. After blocking with milk powder, the membrane was incubated with anti-TSH monoclonal antibody, washed and incubated with biotinylated anti-mouse second antibody (Sigma). After washing, colour was developed using a Vectastian ABC kit (Vector Laboratories, Peterborough, U.K.).

Stability studies with cytokines

Cells and maintenance. The CTLL-2 murine cytotoxic T-lymphocyte cell line was maintained in RPMI 1640 medium (Gibco, BRL Paisley, U.K.) supplemented with 10% FCS (Sigma) 2 mM L-glutamine (Sigma) and 3–5% spleen conditioned medium.[20] Cultures were split 1 in 10 every two or three days, re-fed with conditioned medium and maintained at 37°C in a humidified 5% CO_2 incubator.

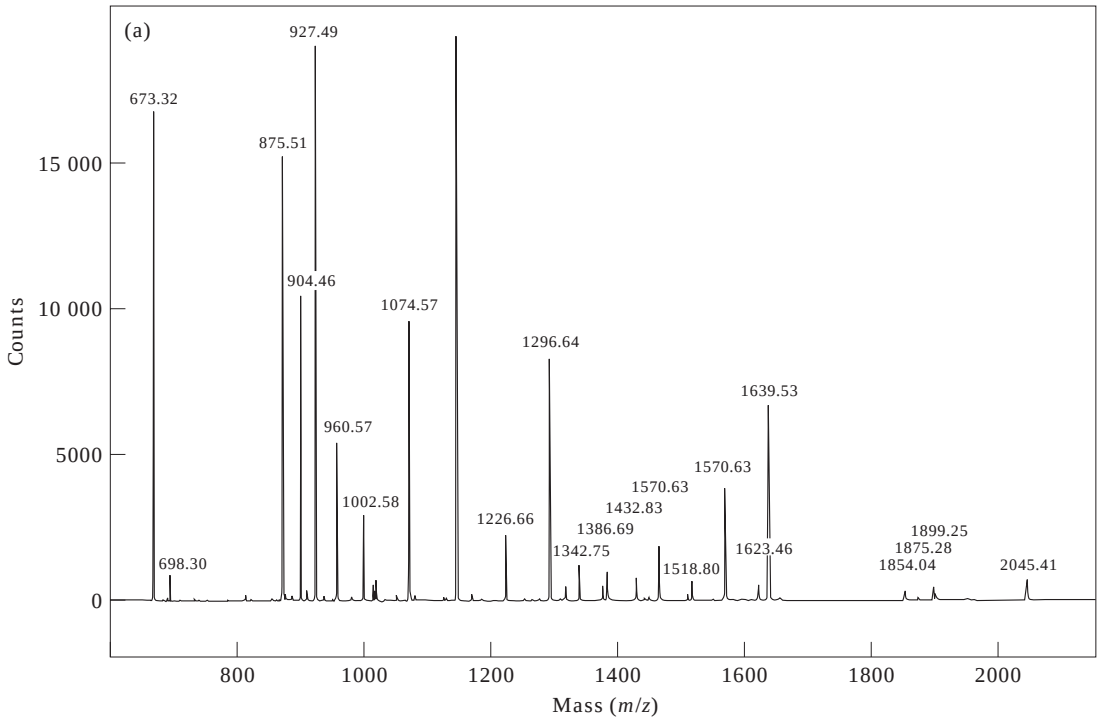
The GNFS-60 murine myeloid leukaemia cell line[21] was maintained in RPMI 1640 medium (Gibco, BRL Paisley, U.K.) supplemented with 5% FCS (Sigma) 2 mM L-glutamine (Sigma) and 10 ng/ml G-CSF (Amgen, Thousand Oaks, U.S.A.). Cultures were split 1 in 5 every two or three days, re-fed with G-CSF and maintained at 37°C in a humidified 5% CO_2 incubator.

IL-15 and G-CSF bioassays. Cell proliferation bioassay procedures for IL-15 and G-CSF were identical.[22] Two-fold dilution series for the construction of standard/sample dose–response curves were prepared on the plate. Negative controls containing 100 μl of medium alone were also included. Cells were used when they had reached a density of 2×10^5 cells/ml. The cells were washed twice in RPMI 1640 medium and re-suspended in RPMI 1640 containing 10% FCS, 100 μg/ml streptomycin and 100 units/ml penicillin at a density of 1×10^5 cells/ml. Aliquots (100 μl) of the cell suspension were added to the microtitre plate continuing the dose–response curve and sample dilution series. The microtitre plate was incubated at 37°C, in a 5% CO_2 humidified atmosphere 18 h. To assess cell proliferation, 0·5 μCi of [^{3}H]thymidine ([^{3}H]Tdr) in 10 μl medium/well was added and the microtitre plate returned to the incubator for a further 4 h. The cells were then harvested onto filter mats and the radioactivity incorporated into DNA was measured using a scintillation counter. Statistically valid quantification of the levels of cytokine in the samples was made by comparison of the sample dilution series to the dose–response curve using parallel line analysis.[23]

Results and discussion

Physicochemical characteristics of HSA and RHA

A number of structural features of RHA and HSA have been previously compared by physicochemical methods.[6] The two proteins showed equivalence with respect to at least six residues from the *N*-terminus and at least eleven from the *C*-terminus. In the same report free thiol[15] content of RHA was found to be close to 1 mol per mol indicating that the single cysteine in RHA is present in an unconjugated state and this was significantly higher than that of HSA. We have confirmed this, and find almost twice the amount of free thiol in RHA than in HSA. In addition, derived tryptic maps of both RHA and HSA were indistinguishable by RP-HPLC and we have also shown very close similarity of the tryptic digests by RP-HPLC (data not shown). These digests have in addition been analysed by MALDI-TOF mass spectrometry and Figure 1(a) shows the MALDI spectrum of the peptide mixture obtained from trypsin-digested RHA. The molecular weights of the peptide signals obtained were entered into the database maintained by the European Molecular Biology Laboratory (EMBL) in Heidelberg, Germany (http://www.mann.embl-heidelberg.de/). Twenty peptides were used in a search and the mass accuracy measurement was set to 0·5 Da [Fig. 1(b)]. The output from the search lists the matched proteins in a ranked order and, the results, presented in Figure 1(c), show that 19 of the 20 peptides entered matched the top ranked proteins, all of which were human serum albumin ''products'', thereby confirming very close similarity in primary structure of RHA to that of HSA. Trypsin-digested



(b) Search parameters

Protein mass range	0–300 kDa
Cleavage agent	Trypsin
Peptide mass accuracy	0.5 Da
Methionine is	Native
Cysteine is	Cys
Peptide charge state	Protonated
Number of peptides required for match	10
Number of uncleavaged sites	1
Number of peptides used in search	20

(c) Search result

Peptides matched	Mass (kDa)	Database accession	Protein name	Digest
19	69.22	trembl:A09561	A09561_1 product: "human" serum	
19	66.47	trembl:A00279	A00279_1 gene: "HSA"; product:	
19	69.23	trembl:M12523	HSALBGC_1 gene: "ALB"; product:	
19	69.37	trembl:L00132	HSALBAF1_1 gene: "ALB"; product	
19	69.37	swissnew:P02768	ALBU_HUMAN SERUM ALBUMIN PRECUR	

Figure 1. (a) MALDI mass spectrum of peptides derived from trypsin digestion of RHA. Molecular weights correspond to monoisotopic masses of peptides observed as $[M+H]^+$ ions. Internal standards (see Materials and methods) are at 904·46, 1296·74 and 1570·63. (b) Molecular weights of the peptides present in the digest are entered into database with these additional parameters. (c) Ranked output of proteins identified in the searched database.

HSA gave virtually the same results (data not shown). Electrospray mass spectra of the intact proteins were also determined and in this case it was demonstrated that HSA and RHA preparations each possessed a major component of mass expected from human albumin monomer however some differences were also apparent (see below). LC–ESMS analysis was performed on HSA (with and without octanoate) and RHA and each eluted as a single, broad peak from a reversed phase C_4 column with 51–52% acetonitrile. The m/z spectrum scans across the peak in the total ion current (TIC) chromatogram were combined and the deconvoluted mass spectra obtained. The spectra for HSA (no octanoate) and RHA are shown in Figure 2. The results obtained for HSA, with and without sodium octanoate (not shown), demonstrated a major ion in each case differing by 1 mass unit, the presence of sodium octanoate in the formulation, as expected, having no significant effect on HSA detectable by ESI-MS. The measured molecular masses were 66 440 Da (no octanoate) and 66 439 Da (with octanoate). The ESMS of RHA contained a single ion at 66 444 Da which although 4 Da greater than that determined for HSA is within the range of precision ($\pm$0·01%) expected from Electrospray mass spectrometry and these values are close to the theoretical mass of human serum albumin (66 438·21 Da–66 439·21 for $[M + H]^+$).[24] For HSA significant additional ions were also observed at mass 66 559 Da (M + 119) consistent with the cysteine adduct (at cysteine-34),[25] and at mass 66 602 Da (M + 162) consistent with mono-glycation which is present in normal serum and which is elevated in diabetics as a result of their raised blood glucose levels.[26] A minor component observed at mass 66 722 Da (M + 282) is probably due to both an additional cysteine and glucose residue. Higher mass ions were not observed in the mass spectrum of RHA indicating that adducts are not present in this preparation. It can be concluded from ES–MS analysis that the major species in RHA and in HSA possess very similar molecular masses and consequently very similar primary structures.

Circular dichroism spectroscopy was used to compare elements of secondary and tertiary structure of human albumin. The far-UV CD spectrum of non-pasteurized HSA [Fig. 3(a)] indicated a highly helical molecule, in agreement with crystallographic results (67% α-helix)[27] although the helical component calculated using different 2° prediction programs did vary (65% helix using CONTIN, 52% using VARSLC1, 45% α-helix using the Yang method) when using data from the same wavelength range (190 nm–240 nm) for each, although using wavelengths >180 nm can lead to errors in estimations. Very similar estimations of α-helix were determined for the recombinant and natural forms of albumin. Similar CD spectra of the same materials were also reported by Dodsworth *et al.*.[6] Far-UV CD performed on HSA before and after heating (60°C for up to 10 h) showed only a slight loss in helicity as a result of heating as did heating a sample from 25°C to 60°C, which is in agreement with Wetzel *et al.*[28] The shape of the spectra remained unchanged (data not shown).

Fatty-acid binding of HSA and RHA

Albumin has the ability to bind many ligands, and is the principal transporter of fatty acids in the blood. HSA binds medium- and long-chain fatty acids with high affinity (K_a, 10^6–10^8 M^{-1}) in subdomains IIIA and IIA.[27,29] The short-chain fatty acid, octanoic acid, is a common additive in therapeutic preparations of HSA; it stabilizes the protein against aggregation which may occur at high concentrations[30] or during pasteurization[28] Optical and NMR spectroscopy were used to compare octanoate binding in albumin samples.

Intrinsic fluorescence spectra, performed using 295 nm as the excitation wavelength, will be indicative of the environment of the single tryptophan-214 in the binding crevice of subdomain IIA, which has been implicated in the binding process along with Lys 199 and Tyr 411.[27] The tryptophan fluorescence in HSA is sensitive to octanoate binding. A significant fluorescence enhancement and blue-shift of 10 ± 1 nm from a fluorescence emission maximum of 342 nm to 332 nm is observed in HSA upon the addition of 10 mM octanoate to 15 μM albumin (Table 1) and is indicative of a less solvent-exposed environment for the Trp, either due to internalization of the Trp side-chain in the protein upon binding, or the non-polar nature of the fatty acid at the binding site which would shield the Trp. An identical fluorescence spectrum was observed for RHA, which contains octanoate.

Evidence of a change in the environment of the tryptophan side chain is also seen from near-UV CD spectra. The Trp band which occurs at around 290 nm in a near-UV CD spectrum of HSA, is not seen upon octanoate binding in both HSA and RHA [Fig. 3(b)]. Further additions of octanoate up to 40 mM did not cause any further change in the tryptophan as determined by fluorescence and

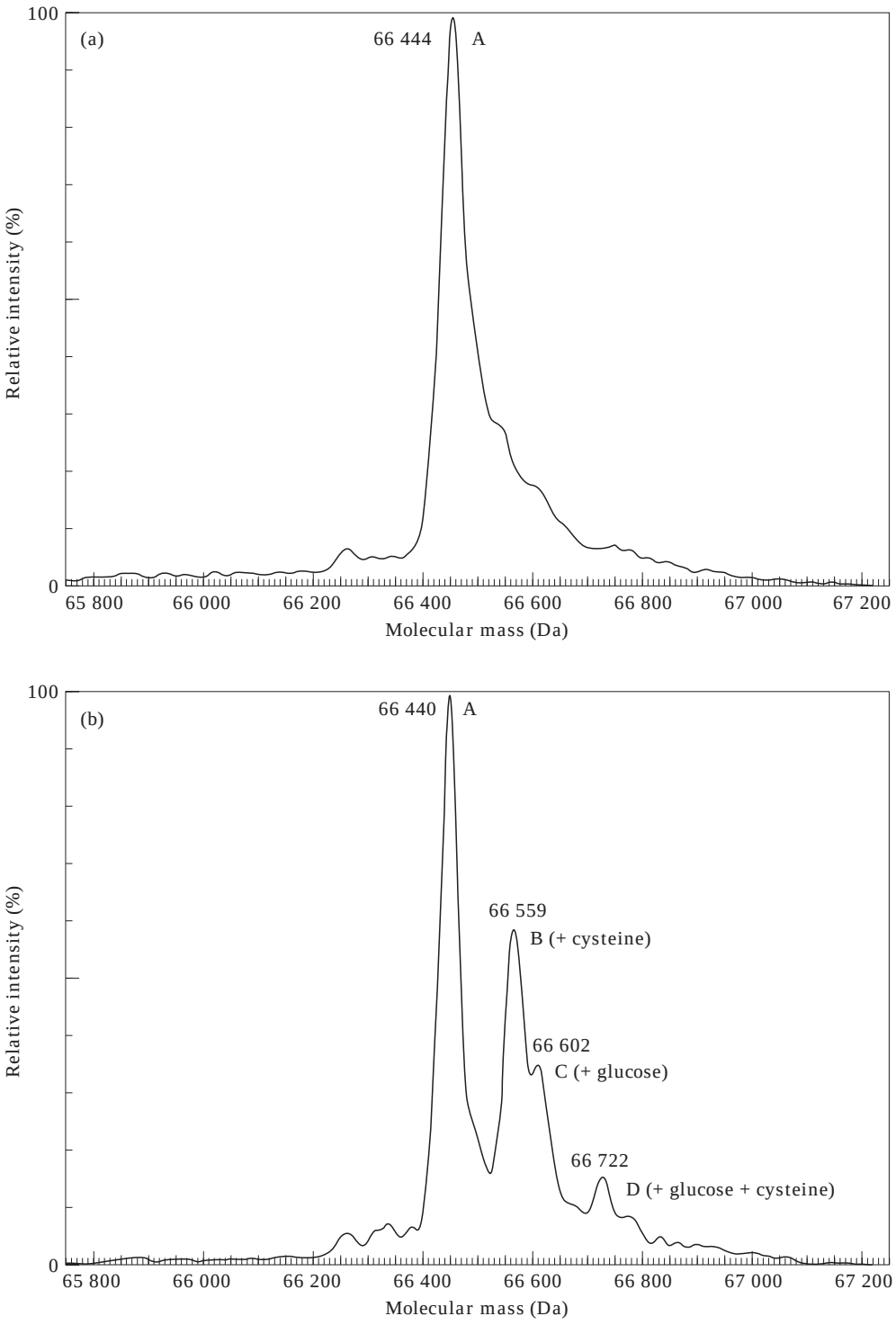


Figure 2. Deconvoluted ES–MS analysis of RHA (a) and HSA (b). A single main peak of mass 66 444 Da was determined for RHA. For HSA a main monomer peak (A) is flanked by minor peaks B, C and D.

near-UV CD, or in far-UV CD spectra. The 290 nm band is also seen in non-pasteurized samples; the near-UV CD spectrum did not change after pasteurization (data not shown).

One and two-dimensional NMR spectroscopy have been used previously to gain structural information on HSA[31,32] and its complexes with long chain fatty acids.[33] We examined ^{1}H-NMR spectra of HSA, HSA spiked with octanoate, RHA containing octanoate and sodium octanoate solution. (RHA without the presence of octanoate was not available). The presence of octanoate in HSA samples which had been spiked with octanoate could be clearly established with additional peaks at 0·77,

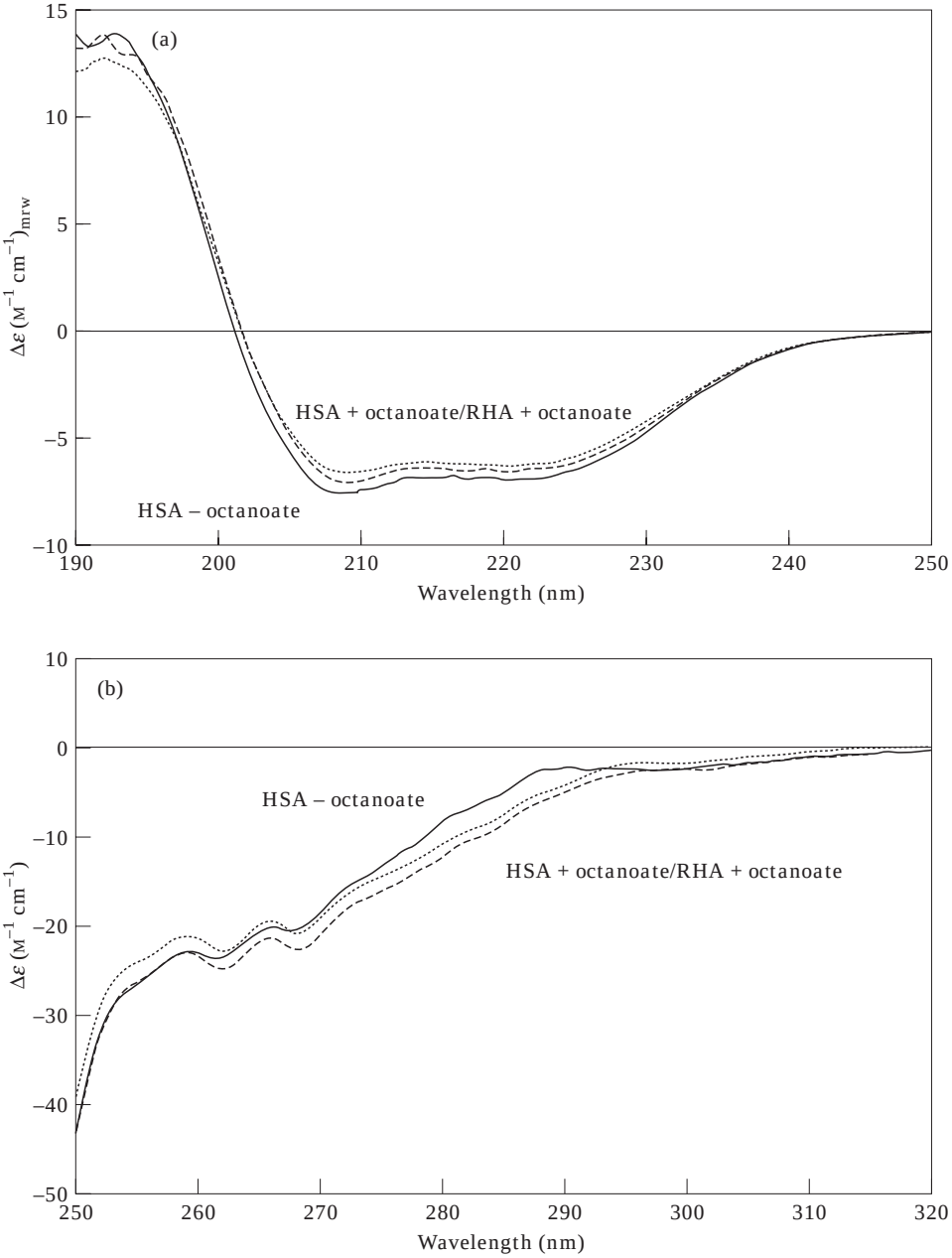


Figure 3. Far-UV (a) and near-UV (b) CD spectra of RHA and HSA. Spectra of RHA and HSA (solid line) of approximately 1·3 mg/ml in 145 mM NaCl, 0·0015% (w/v) Tween 80, pH 7·5 were recorded at 25°C using a 0·01-cm (far-UV) or 1-cm (near-UV) pathlength cuvette. Samples designated ''plus octanoate'' (HSA, dashed line; RHA, dotted line) contain, in addition, 40 mM sodium octanoate. Data are expressed as molar circular dichroic adsorption ($\Delta\varepsilon$, in $M^{-1}\,cm^{-1}$) mrw, mean residue weight for far-UV, and $\Delta\varepsilon$ (in $M^{-1}\,cm^{-1}$) for near-UV CD.

Table 1. Intrinsic fluorescence emission λ maxima values of albumin samples

	F_{max} (nm)	
Sample	ex 280 nm	ex 295 nm
HSA (no octanoate)	334–337	342–344
HSA (+octanoate)	325	332
RHA (+octanoate)	325	332

1·19, 1·51 and 2·17 ppm (Fig. 4). These corresponded with similar peaks in the octanoate-containing RHA sample. The linewidths of these peaks were broader than in a sample of octanoate alone, suggesting some degree of association between octanoate and HSA.

While offering several advantages, recombinant albumin, like its natural form, does form higher oligomers and may aggregate, and conditions of manufacturing require attention to avoid these. The use of the short-chain fatty acid, octanoate, as an additive can aid maintenance of the monomeric form. Both RHA and HSA show similar effects upon octanoate binding.

Oligomeric state of HSA and RHA

FPLC gel filtration chromatography using a Superdex 200 column was used to assess the aggregation state of non-pasteurized HSA (stored with and without octanoate) and RHA (stored with octanoate). Albumin samples (0·1 mg) were eluted in PBS, pH 7·5. All samples eluted as one sharp peak at M_r, 60 K with no evidence of dimers or higher molecular weight oligomers or aggregates (data not shown). HPLC chromatography using approximately ten times the amount of albumin showed that oligomeric species were present in RHA and HSA.[6] The concentration dependence of oligomerization and aggregation has been documented.[30]

The presence and quantitation of dimeric and higher oligomers was demonstrated by size-exclusion-chromatography (SEC) and electrophoresis. SEC-HPLC on a TSK 3000 column indicated that unpasteurized HSA contained an additional small amount (ca. 1%) of dimer but higher oligomers were not detected. Pasteurized HSA also contained dimer (1%) but also significant amounts ($>3·5$%) of higher oligomers. The higher oligomers result from the pasteurization process (60°C/10 h) and are in fact present in significant amounts (>2%) within 15 min of commencement of this treatment (data not shown).

Gel electrophoresis of HSA and RHA preparations under reducing and non-reducing conditions (Fig. 5) also illustrated the presence of multimers in both recombinant and serum-derived samples. Under reducing conditions (10% β-mercaptoethanol), many of the aggregated species were better resolved though apparently high molecular weight forms were still present. Gel filtration and SDS-PAGE analysis demonstrated that the oligomerization of albumin observed under different conditions may be independent of its source.

Endotoxin content

No detectable amounts of endotoxin ($\leq 0·3$ IU/ml) were evident in the RHA preparation using the Limulus Amoebocyte Lysate test.

Moisture content

After lyophilization, the corresponding HSA- and RHA-containing preparations had similar moisture contents as shown in Table 2.

rhTSH stability

When compared with ampoules stored at –20°C, ampoules of rhTSH in the presence of either HSA or RHA kept for 60 days at +4 or +20°C showed no significant loss of immunoactivity. However, loss of immunactivity was observed at higher temperatures [Fig. 6(a)], although degradation rate plots were not linear. If it is assumed that potencies at +20°C and +45°C are representative of both preparations, the predicted degradation rate at –20°C (using an in-house program[34] based on an Arrhenius equation model) was approximately 0·1% loss in immunoactivity per year with either albumin preparation. A similar predicted rate of degradation was seen for rhTSH, filled in the presence of HSA, in a recent international collaborative study set up to establish a reference reagent for rhTSH.

RP-HPLC analysis of the rhTSH content of ampoules of 95/790 (with HSA) and 95/792 (with RHA) stored for 60 days at elevated temperatures showed a similar temperature-related loss of intact rhTSH. However, comparison of the peak profiles of TSH in the presence of either of the albumin preparations showed no apparent differences [Fig. 6(b)].

Examination of the immunoblot of the degra-

dation products of rhTSH after IEF of ampoules of 95/790 and 95/792 stored at elevated temperatures for 160 days showed a temperature-related change in the isoform composition but with no discernible difference between HSA- and RHA-containing ampoules [Fig. 6(c)].

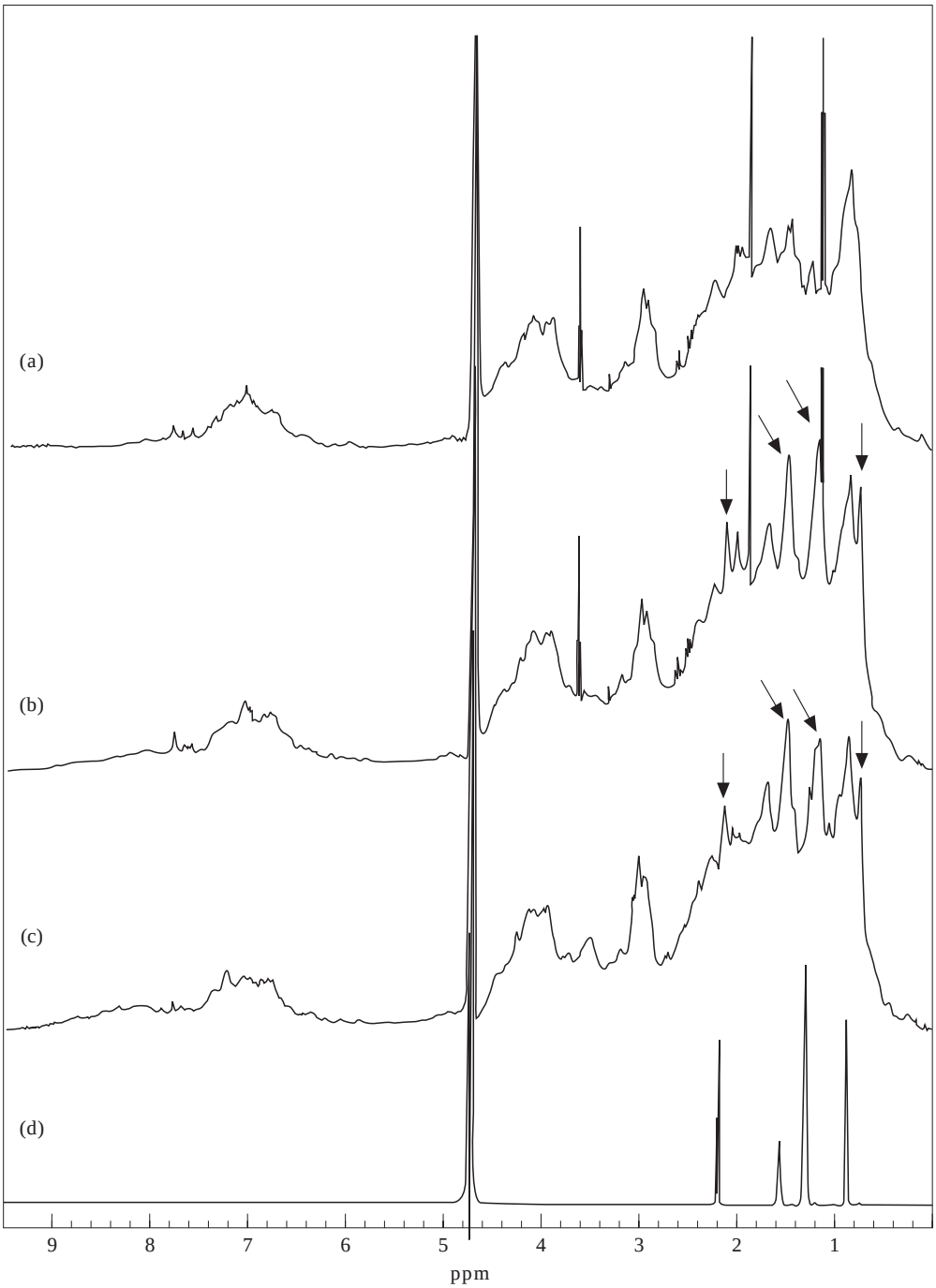


Figure 4. Effect of sodium octanoate on NMR spectrum of HSA and RHA. 500-MHz ^{1}H-NMR spectra of: (a) HSA without octanoate; (b) HSA spiked with octanoate; (c) RHA containing octanoate; (d) sodium octanoate solution in D_2O. The peaks characteristic of the octanoate in the albumin preparations are indicated by arrows. The following signals are also visible, 1·91 ppm: acetate anion; 1·06 ppm and 3·53 ppm: ethanol; 4·72 ppm: residual HDO.

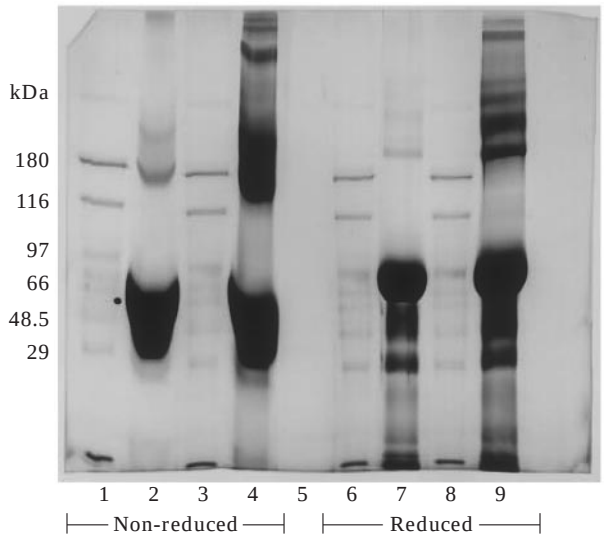


Figure 5. Reducing and non-reducing SDS-PAGE of HSA and RHA, 50 μg per lane, silver-stained. Lanes 1, 3, 6 and 8 are molecular weight markers; lanes 2 and 4 are non-reduced HSA and RHA respectively; lanes 7 and 9 are reduced HSA and RHA respectively.

Stability of rhG-CSF to temperature

Incubation of G-CSF at 37°C for 4 h in culture medium alone results in a dramatic loss of biological activity [Fig. 7(a)]. Addition of either HSA or RHA was able to prevent such loss. There was no statistical difference in the performance of the two materials in preventing loss of G-CSF bioactivity ($P < 0{\cdot}001$).

Stability of IL-15 to lyophilization and accelerated degradation

Lyophilization of IL-15 in the presence of either 0·2% HSA or 0·2% RHA did not result in any significant loss of bioactivity compared to ampouled material that was not freeze-dried, but stored at –150°C (frozen baseline) ($P < 0{\cdot}001$) [Fig. 7(b)]. Exposure of ampouled material to elevated temperatures for a period of six months, even at 56°C, did not result in any significant loss of activity of either formulation ($P < 0{\cdot}001$) [Fig. 7(b)].

Conclusion

World Health Organization (WHO) guidelines for the preparation of International Biological Standards and Reference Preparations recommend the addition of ''inert'' excipients/stabilizers to solutions of the active agent before dispensing aliquots and freeze-drying.[34] Available excipients include carbohydrates, amino acids and proteins, and for this last class, human serum albumin (HSA) has often been used, particularly in parenteral formulations.[35] Over many years its use has proved highly satisfactory in the preparation of biological standards including those for recombinant human interleukin-15 (rhIL-15, ampoule code 95/554) and recombinant human thyroid stimulating hormone (rhTSH, ampoule code 95/674). This property of HSA is perhaps to be expected since it functions *in vivo* by binding, stabilizing and transporting serum components. No significant differences were observed in the biological, immunological and physicochemical properties of the materials used in this study (rhTSH, rhIL-15, and rhG-CSF) when ampouled in the presence of either serum-derived or rDNA-derived albumin, indicating that RHA may be considered as a suitable alternative to HSA in the preparation of standards of these materials. It further suggests that the use of RHA for this purpose is likely to be applicable to other substances. Additional support for the future replacement of HSA by RHA is provided by the results of physicochemical analyses of the albumin preparations, reported here and previously,[6,7,32]

Table 2. Moisture contents of freeze-dried preparations

Material	Code No.	Moisture/ampoule* (μg)
TSH + HSA	95/826	12·5 (10·6–14·3)
TSH + RHA	95/788	7·6 (6·5–8·7)
IL-15 + HSA + phosphate	95/798	180·2 (154·3–207·6)
IL-15 + RHA + phosphate	95/800	160·6 (150·1 + 180·7)
IL-15 + HSA + saline	95/794	62·7 (61·4–65·4)
IL-15 + RHA + saline	95/796	69·5 (64·5–77·7)

*Mean of triplicate determinations with the range in brackets.

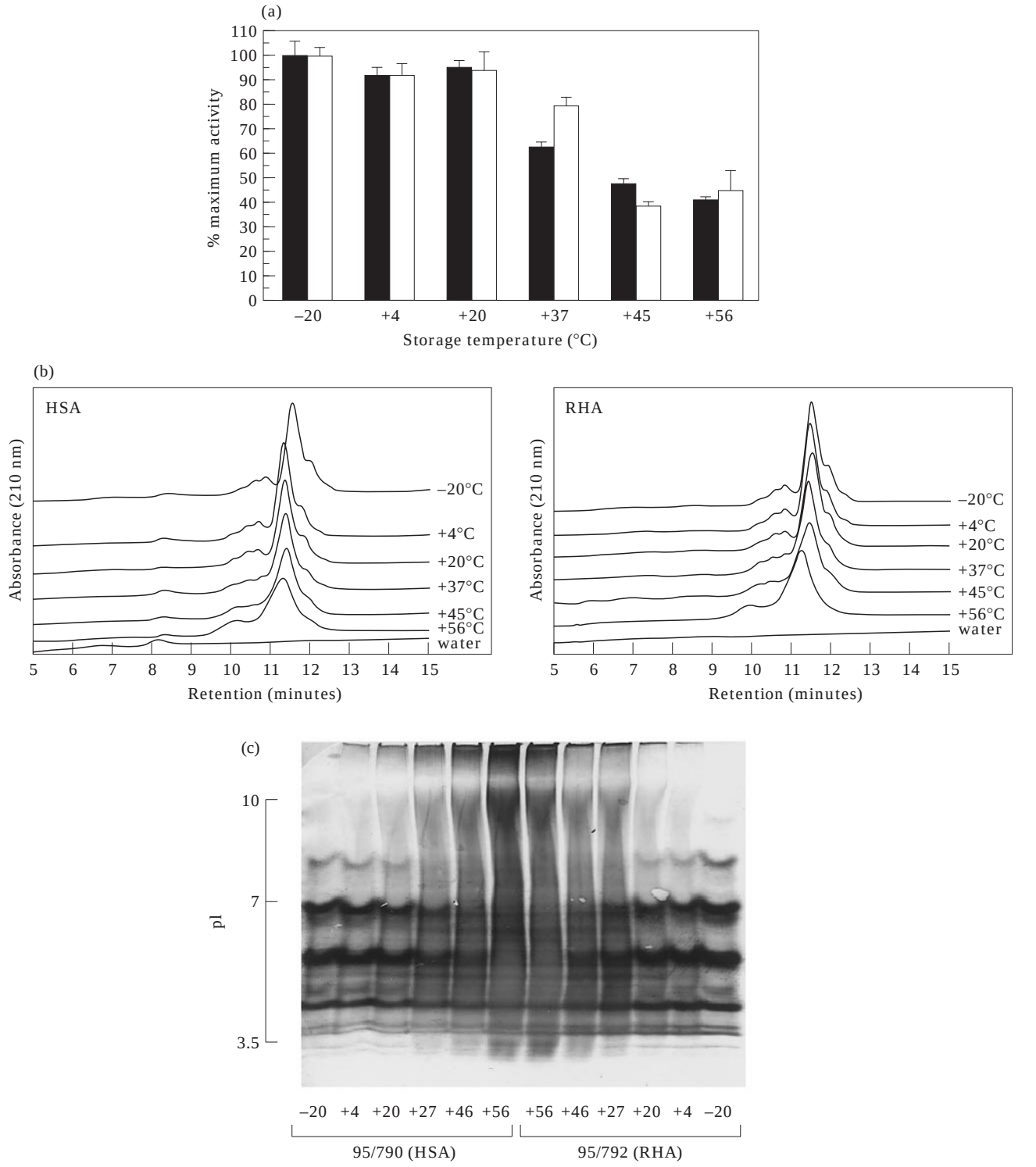


Figure 6. (a): Estimates of immunoreactive rhTSH in ampoules coded 95/826 (HSA) (■) and 95/788 (RHA) (□) after storage at elevated temperatures for 60 days. (b): RP-HPLC analysis of ampoules of rhTSH (25 μg) with either HSA (95/970) or RHA (95/792) as excipient, after storage at elevated temperatures for 60 days. TSH peak profiles are shown between 9·5–12·5 min. (c): Immunoglot, using anti-TSH monoclonal antibody, of ampoules of rhTSH (25 μg) with either HSA (95/790) or RHA (95/792) as excipient, after IEF of preparations stored at elevated temperatures for 60 days.

which reveal close structural similarities between their major components. The advantages of using RHA include absence of pathogenic human material and less batch-to-batch variability. Other advantages are probable as a result of the greater homogeneity of RHA over HSA, including the lack of naturally occurring ligands or adducts.

In selecting additives, consideration needs to be

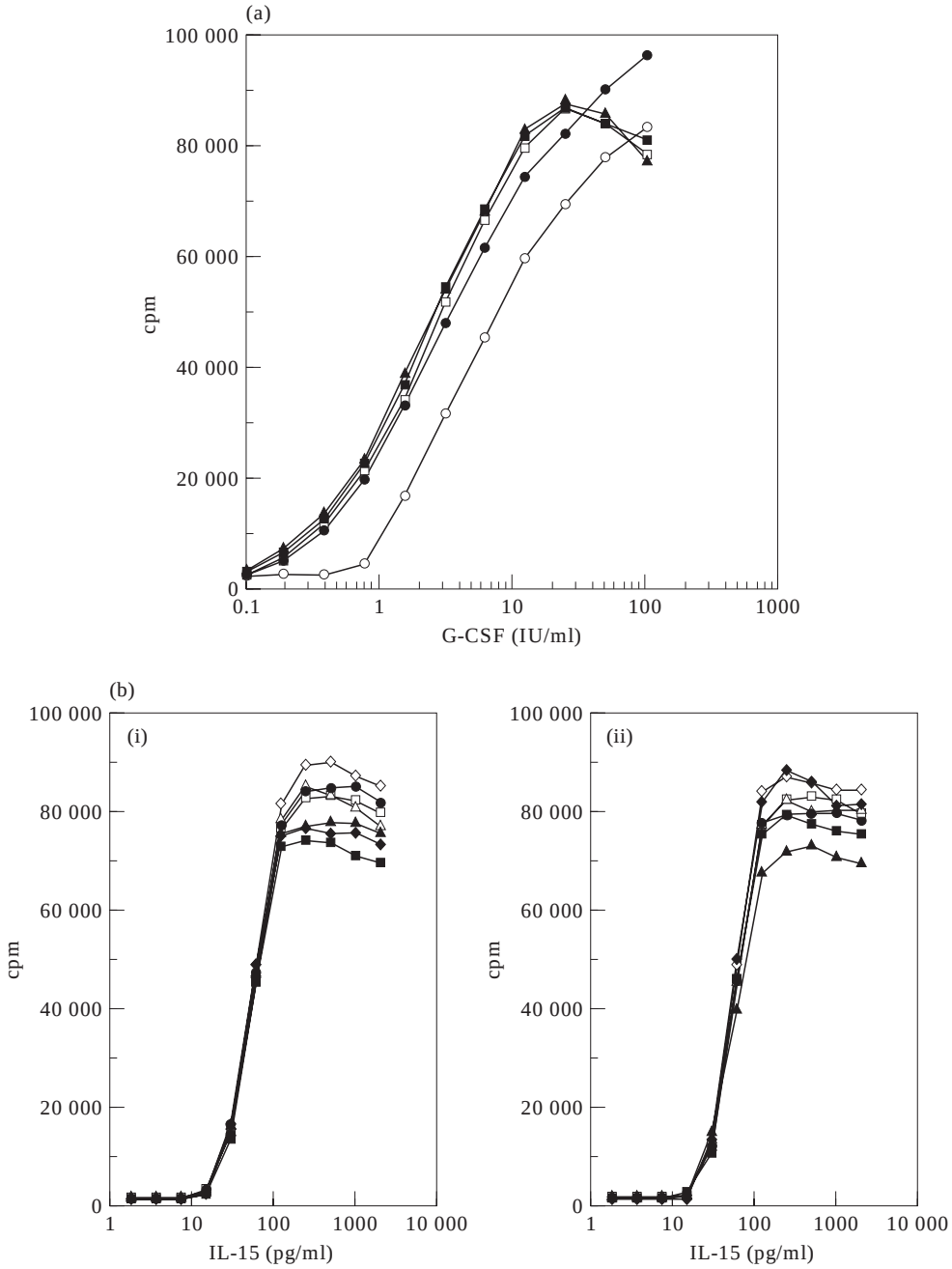


Figure 7. Ability of HSA or RHA to protect: (a). G-CSF from heat inactivation and adsorption. (●) G-CSF in medium alone at time 0, (○) G-CSF in medium alone after 4 h at 37°C, (■) G-CSF in medium/HSA at time 0 (□), G-CSF in medium/HSA 4 h at 37°C, (▲) G-CSF in medium/RHA at time 0, (▼) G-CSF in medium/RHA 4 h at 37°C. (b). The biological activity of IL-15 during lyophilization and accelerated degradation. (i) HSA-containing formulation, (ii) RHA-containing formulation. Ampoules were exposed to the following temperatures: (●), –20°C; (■) +4°C; (□), +20°C; (▲), +37°C, (△), +45°C; (◆), +56°C and (◇), the frozen baseline of ampouled, non-lyophilized material stored at –150°C.

given to possible adverse interactions between the bulking agent and the active agent. Components (covalent adducts or perhaps those resulting from degradative processes) additional to the main molecular species may be present, albeit in relatively small amounts. These small amounts however often translate into large excesses with respect to the biologically active agent. In the test preparations described here, for example, there are 1000:1 and 2000:1 ratios by weight of albumin to IL-15 and TSH. These convert to molar ratios 500:1 and these are typical of the ratios used in preparing biological standards. Consequently the presence of even small amounts of reactive species in an albumin preparation would produce a major adverse effect on the biologically active material and although such affects are apparently not observed (at least in the short term) in the present cases the possibility always needs to be considered. Albumin adducts could be detrimental in some instances. Glycated proteins are known to lead to the formation of reactive carbonyl compounds and to intramolecular protein cross-links whilst oligomeric species significantly increase the viscosity of the medium which may affect biological assays. The absence of such species in RHA is therefore a further reason why it should be considered as a replacement to HSA when preparing biological standards. Further studies may demonstrate a general applicability of RHA as an additive/stabilizer for biological molecules. At this time with limited data, however, it would be wise to evaluate its use on a product-by-product basis.

Acknowledgements

The staff of the Standards Processing Division are thanked for their assistance in preparing ampouled materials and for carrying out the moisture analyses. We are grateful to Yogesh Mistry and Richard Tiplady of the Division of Endocrinology for performing the endotoxin assays and IEF procedures respectively and to Dr Helen Byers (King's College, London) for the MALDI data. We also thank Peter Rigsby, Informatics, for stability data analysis, and Dr Colin Longstaff, Division of Haematology for helpful discussions.

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Received for publication 28 January 1998; accepted 1 December 1998