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Determination of pituitary and recombinant human growth hormone molecular weights by modern high-performance liquid chromatography with low angle laser light scattering detection

HANS H. STUTING[a] and IRA S. KRULL*

Department of Chemistry and The Barnett Institute, Northeastern University, 341 Mugar Building, 360 Huntington Avenue, Boston, MA 02115 (U.S.A.)

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ABSTRACT

The determination of molecular weight for pituitary and recombinant human growth hormone (p-hGH/Crescormon and r-hGH/Protropin) has been performed. This has involved on-line coupling of size-exclusion chromatography (SEC) and gradient elution, reversed-phase high-performance liquid chromatography (RP-HPLC) with low-angle laser light scattering (LALLS) detection. A 5-μm, 300 Å, Delta-bond octyl column was used. Traditional specific refractive index increment (dn/dc) and refractive index (n) measurements have been performed in order to derive absolute weight-average molecular weight (M_w) information for p-hGH and r-hGH. Known concentrations of each protein have been separated using reversed-phase gradients utilizing acetonitrile with on-line LALLS determination of excess Rayleigh scattering factors. Accurate M_w data has been obtained for both proteins under conventional RP-HPLC gradient elution conditions.

SEC data of both hGHs were found to be concentration, mobile phase, and column dependent for the particular analyses. Both medium- and high-resolution SEC–LALLS studies were performed, and all of these determinations further confirmed our RP-HPLC results. On-line LALLS provides certain advantages in identifying aggregates that may be present, even in medium-resolution SEC, where incomplete resolution occurs. The on-line coupling of modern RP-HPLC for biopolymers with LALLS detection represents a major step forward in the ability of bioanalytical chemists to determine the nature (monomer *versus* higher-order aggregate) of such materials. Other classes of biopolymers should prove suitable for studies with the same RP-HPLC–LALLS–UV approaches.

INTRODUCTION

The separation and determination of biopolymers by modern high-performance liquid chromatography (HPLC) has become an area of intense interest within the past few years [1–5]. Separation approaches for biopolymers have advanced rapidly; however, methods of detection have lagged somewhat behind these advances [6,7].

[a] Present address: Roche Biomedical Laboratories (RBL), Inc., 1 Roche Drive, P.O. Box 500, Raritan, NJ, 08869, U.S.A.

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Most of the routine detection systems for HPLC, such as ultraviolet–visible (UV–VIS), fluorescence (FL), differential refractive index (DRI), and electrochemistry (ED), offer little in the way of biopolymer information or characterization. Most biopolymers show little or no response under conventional ED conditions, in the absence of some type of derivatization [8,9]. Though UV–FL detectors may provide some information, they rarely provide biopolymer identification or characterization. When different prosthetic groups are present, UV–FL may provide characteristic spectra for certain biopolymers. This is because in UV–FL detection, most biopolymers have very similar absorbance and emission spectra [10,11]. Recent studies using derivative optical spectroscopy illustrate how small differences in protein structure can be used to identify certain materials [12,13]. In the past, chromatographic performance *via* capacity factor determination, or *via* the use of an internal standard, have been the most widely used identification parameters.

The development of recombinant DNA technology has made available substantial amounts of proteins that were formerly available in very limited amounts. These recombinant proteins must be obtained in a state that assures full biological activity. In some cases, the activity is dependent on stoichiometry, mainly that the pure monomer is present, while in others, it may be the dimer or even higher-order oligomer(s). Clearly, it is of practical interest to understand the possible self-association of proteins under a variety of conditions. Association and aggregation have both been used to indicate higher order species greater than monomer that exist(s) on the chromatographic timescale. However, association may be a better word, since it can include both short- and long-lived, higher order species (dimer, trimer, etc.). These higher order species may, or may not, be reversible once they have left the chromatographic system. It is quite possible that associates can form on dissolution of the solid protein, prior to HPLC injection, as a function of the solvents/buffers used and concentrations.

In the HPLC separation of biopolymers, an issue that can arise is the stoichiometry of the eluting species. Many recombinant materials are hydrophobic and can form higher order aggregates either in solution/clean-up prior to chromatography or within the chromatographic column itself [14]. These aggregates are frequently immunogenic and from a regulatory point of view, they require evidence of absence in the final product. Moreover, the formation of aggregates can lead to multiple peaks in a chromatogram, requiring identification of individual peaks. Such behavior has been shown in the hydrophobic interaction chromatography (HIC) of alkaline phosphatase [10] and β-lactoglublin A [15].

In addition, it is usually recommended to inject high concentrations of biopolymer into an HPLC column in preparative-scale chromatography. Such concentration conditions are often conducive to biopolymer–biopolymer interaction, leading to association. Such association can occur without precipitation taking place. Here again, assessment of eluting fractions in terms of molecular weight could be most helpful in deciding on conditions for high sample capacity within the column.

Human growth hormone (hGH) is a single chain polypeptide of 191 amino acids, containing two intramolecular disulfide bonds, where the molecular weight of the monomer is 22 125 dalton [16,17]. The observation of a lower-molecular-weight variant of approximately 20 000 dalton and higher-order aggregates/associates have been reported in the literature [18,19].

In the polymer field, low-angle laser light scattering (LALLS) has been used as a standard technique for measuring the molecular weights in the bulk solution state or in eluting fractions on-line with size-exclusion chromatography [20–29]. Recently, this laboratory has demonstrated that LALLS can be coupled to gradient HIC and reversed-phase (RP) HPLC to yield molecular weight on-line [14,30,31]. An added benefit of LALLS is that it provides an "absolute" measure of molecular weight, since the method of calibration is based on simple geometry, rather than referenced to a different solvent of known Rayleigh scattering characteristics, or to some external standard.

Our previous efforts employed the use of standard biopolymers (proteins) that are commonly available for purposes of thorough system validation [10,14,29–32]. This current research was designed, in part, to further test and extend the usefulness of the gradient LALLS technique to a formulated product made by recombinant DNA technology.

EXPERIMENTAL

Equipment

The RP-HPLC–LALLS and SEC–LALLS systems consisted of two modular arrangements. System 1, used primarily for SEC–LALLS, consisted of an LDC ConstaMetric III analytical metering pump (1/3 speed), a Rheodyne Model 7125 injection valve equipped with a 100-μl loop, a Chromatix (LDC Analytical/Thermo Instruments, Riviera Beach, FL, U.S.A.) KMX-6 LALLS photometer configured for flow analyses, an LDC SpectroMonitor-D variable UV–VIS and a modified RefractoMonitor IV DRI detector. The modifications were performed by the manufacturer within their facilities at LDC Analytical. The DRI modification consisted of replacing the heat blocking filter with an in-line filter (650 nm, $\Delta\lambda = 40$ nm, Corion, part No. S40-650-A-M125, Holliston, MA, U.S.A.), while increasing the light source available from 3.3 to 5.0 V (6.0 V maximum), to compensate for the decreased light (wavelength range) throughput. Detector outputs were all linked to Soltec Model 1242 strip chart recorders (Soltec, Sun Valley, CA, U.S.A.), LDC CMX-10A (analog-to-digital, A/D) converters and to a IBM-AT compatible compute r (Dell Computer, Austin, TX, U.S.A.). The LDC A/D converters were further linked to a DEC (Digital Equipment, Boston, MA, U.S.A.) Micro PDP-11/23+ computer system for digitization of instrumental analog outputs and further graphics/data manipulation. Standard software packages for calculating LALLS molecular weights were from LDC Analytical, versions MOLWT3 (DEC computer) and PCLALLS (IBM-AT compatible computer).

System II, used for RP-HPLC–LALLS studies performed at ambient temperature (25°C), was composed of an LDC Model CM4000 multiple solvent delivery system, a Rheodyne Model 7125 syringe loading injector equipped with either a 20-μl or 100-μl loop, a Chromatix KMX-6 LALLS photometer configured for flow analyses, and an LDC SM4000 programmable UV–VIS detector. Detector outputs were linked to both a Soltec Model 1242 strip chart recorder, and to an IBM PC-AT compatible computer using PCLALLS software.

Mobile phase refractive index (RI) measurements were performed on an Abbe refractometer (Analytical Products Division/Milton Roy, Rochester, NY, U.S.A.)

with the appropriate temperature control. In order to obtain the proper RI of the solvents at the wavelength in use throughout these experiments, the addition of 632.8 ± 0.2 nm narrow band pass filter (Melles Griot, Rochester, NY, U.S.A.) was installed between the traditional light source and the receiving optics.

The specific RI increment measurements, dn/dc values, were performed off-line using a Chromatix Model KMX-16 laser differential refractometer connected to a low-temperature (20°C) water bath (Fisher Scientific, Boston, MA, U.S.A.).

Chemicals and supplies

Pituitary (p-hGH/Crescormon) and recombinant (r-hGH/Protropin) human growth hormones were the kind gift of Genentech. All samples were shipped from Genentech to Northeastern University in dry-ice and were immediately transferred to the freezer (−15°C), for storage. All solutions were prepared daily, without further sample purification, and kept in the refrigerator (5°C) in-between analyses/injections. Before chromatography taking place, samples were allowed to equilibrate to room temperature.

Acetonitrile, methanol, and water were HPLC Omnisolv grade, trifluoroacetic acid (TFA) and ammonium acetate were reagent grade, all from EM Science (Cherry Hill, NJ, U.S.A.). The following reagents and chemicals were used without further purification: TFA, highest purity available, and imidazole, 99% pure, Aldrich Chemical (Milwaukee, WI, U.S.A.); ammonium hydroxide, 30%, Baker Analyzed, J. T. Baker (Phillipsburg, NJ, U.S.A.); N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), Sigma (St. Louis, MO, U.S.A.); EDTA, monobasic phosphate, dibasic phosphate, sodium chloride (all Certified ACS reagent grade); sodium acetate (HPLC buffer salt grade), Fisher Scientific (Springfield, NJ, U.S.A.); sodium azide, Eastman Kodak (Rochester, NY, U.S.A.); non-ionic surfactant, octa(ethylene glycol) mono-N-dodecyl ether, Nikko Chemical (Tokyo, Japan).

Chromatographic columns used were: (RP-HPLC) Deltabond octyl, 5 μm, 300 Å pore size, 10 cm × 4 mm I.D. (Keystone Scientific, State College, PA, U.S.A.); (SEC) TSK-SW-2000 or SWXL-2000, 30 cm × 7.5 mm I.D. (Supelco/Division Rohm & Haas, Bellefonte, PA, U.S.A.), where specified.

Mobile phases

Two mobile phases were prepared for gradient elution RP-HPLC–LALLS–UV studies. The first consisted of 0.15% TFA in water (A), and the second consisted of 0.15% TFA in acetonitrile–water (95:5) (B). Both solvents were adjusted to pH 3.0 with ammonium hydroxide. The column was equilibrated overnight, when not in use, with 90% B at a flow-rate of 0.2 ml/min. The phosphate-buffered saline (PBS) used for the SEC studies consisted of: 25 m*M* of monobasic phosphate, 25 m*M* dibasic phosphate, and 150 m*M* sodium chloride, pH 7.2. The modified protein buffer (MPB) used for the SEC studies consisted of: 18 m*M* HEPES, 7 m*M* imidazole, 1 m*M* EDTA, 3 m*M* sodium azide, 200 m*M* sodium acetate, 0.5 m*M* non-ionic surfactant, octa(ethylene glycol) mono-N-dodecyl ether, pH 7.0.

All mobile phases used throughout this research were first thoroughly degassed with helium, then filtered under vacuum using a 0.22-μm hydrophilic Durapore membrane (Millipore, Bedford, MA, U.S.A.) via standard laboratory vacuum filtration apparatus.

Procedures

One important point that must be considered before performing LALLS analyses is accurate injected mass. Each new loop was carefully custom fitted onto a particular injector and then that loop was dedicated to the injector. Subsequently the loop was removed, and its volume was gravimetrically determined. Injection conditions were performed at least 5 times ($n = 5$, or more).

SEC utilized a bonded diol phase, TSK-SW-2000 (medium-resolution) or SWXL-2000 (high-resolution) SEC column at a constant flow-rate of 0.775 ml/min, using PBS or MPB as the mobile phase, with a fixed injection volume of 100 μl. As is customary in SEC analyses, all protein solutions were prepared in the same buffer as used in the SEC experiment and analyzed the same day. All data are given as the mean (ave) $\pm$ S.D. in kilodalton.

The RP-HPLC gradients consisted of 80% to 20% A, linear, over 30 min (pH 3.0, 25°C), flow-rate 1.13 ml/min (calibrated), injected masses were 20–100 μl of approximately 8 mg/ml using the Deltabond Octyl column. All protein solutions were prepared by dissolving a weighed amount of each protein in mobile phase A at the appropriate pH and allowing to equilibrate for about one hour in the refrigerator at 5°C.

The KMX-6 LALLS detector was configured as for SEC with the 5-mm flow-through cell, 6–7° annulus, 0.2 mm field stop; G_0 (incident intensity) was in the range of 200–600 mV, with 2,3,4 attenuation, transmittance of the attenuators used in measuring the incident illuminating light beam (D) = approximately $4.2 \cdot 10^{-9}$; $G_\varnothing$ (scattered light intensity at initial conditions) ranged from 150–450 mV, and a 0.3-s time constant. Axial dispersion, or band broadening corrections were not performed; however, extra-column effects were minimized by removing the "zero dead volume" filter body, and allowing the eluent to be directly transferred from the column into the light scattering flow cell. This was only possible by using a column that had been excessively flushed with mobile phase in order to minimize particulate (fines) contamination.

All RP-HPLC and SEC–UV measurements were performed at 280 nm. Mobile phase RI (n) determinations were conducted using a modified Abbe refractometer incorporating a 633-nm narrow bandpass filter between the light source and the receiving optics. Experimental values of n were in close agreement with interpolated literature values. RI measurements, with varying percentages of B, were performed in an off-line study over the gradient range of B used in the RP-HPLC separations. Solutions of 0–100% B were prepared volumetrically in final volumes of 10 ml, with the %B increasing successively by 10% in each solution. Refractive indices of those solutions were determined in triplicate.

The dn/dc values for each protein were determined with the Chromatix KMX-16 using concentrations in the range of 3–5 mg/ml. Blanks were prepared identically, with matching supporting buffer (*i.e.*, glycine for p-hGH and mannitol for r-hGH) as in the formulation, side-by-side, to avoid the need for dialysis. Protein dn/dc values were determined by plotting $(n_i - n)/c_i$ *versus* c_i and extrapolating to zero concentration, where n_i is the RI at concentration c_i, and n is the RI of the mobile phase.

Protein solutions for dn/dc determinations used in reversed phase were prepared in the calculated mobile phase composition resulting in peak elution. The weighed portion was first placed in a 20-ml scintillation vial, dissolved in mobile phase A, which

consisted of 0.15% TFA in water (pH adjusted to 3.0 with ammonium hydroxide) and capped. Once the solutions were homogeneous and allowed to equilibrate, roughly one hour later, appropriate amounts of solvent B [0.15% TFA in acetonitrile–water (95:5), pH adjusted to 3.0 with ammonium hydroxide] were added slowly and capped again. These solutions were mildly swirled for about 10 min, then refrigerated at 5°C overnight, and analyzed the very next morning. Blanks were prepared side-by-side. All HPLC–LALLS solutions were allowed to re-equilibrate at room temperature prior to analysis and were filtered through a 0.45-μm, low protein binding, hydrophilic Durapore membrane, just prior to HPLC introduction.

Traditional optical alignment procedures, as are used for isocratic HPLC/SEC–LALLS, were not satisfactory when used for gradient RP-HPLC–LALLS, due to changes in mobile phase RI and solid angle, resulting in actual scattering volume changes [33]. A modification of the alignment procedure was developed, where optical realignment was performed once during an initial blank gradient. This procedure produced consistently stable LALLS baselines and avoided any extra-scattering effects from within the cell that could result in experimental artifacts.

RESULTS AND DISCUSSION

Theory

The basic principles of LALLS pertaining to the simplification of multi-component theory used in this research has been previously described elsewhere [14,29–32]. When the individual solvents and their mixtures employed in the light scattering experiments are considered isorefractive (*i.e.*, their difference is <0.025 RI units), one is able to use the solvent combinations without the need to perform exhaustive dialysis [26]. Ideal solvents do not interact with proteins in solution to produce newer species that scatter light differently than the free biopolymer. This concept allows for a three- (or greater)-component system to be artificially reduced to that of a two-component system. Careful choice of experimental conditions allows one to obtain true weight-average molecular weights, M_w (*vs.* apparent M_w values) under gradient conditions.

Background and operational principles

As with all light scattering methodologies, the specific RI increment (dn/dc) determination is a required quantity for the deduction of molecular weight data. It is one of the most important values that must be determined with utmost precision and accuracy. Even though HPLC–LALLS measurements were made under dynamic conditions, and dn/dc values were measured in an off-line manner, it is clear that different protein stoichiometries can be present in each measurement technique. Accumulated data generally suggests that dn/dc attains a constant value above a certain molecular weight, which is in the range of 10–20 k dalton [24–26]. Therefore, when studying these biopolymers, errors associated with measuring the dn/dc of monomers, dimers, or even higher-order associates in an off-line condition (via the KMX-16), should not significantly affect the measurements made under dynamic conditions (HPLC–LALLS) where higher-order aggregates may be present. All measurements were made at 25°C. The uncertainty of this measurement was approximately $\pm 3\%$, and was our major source of experimental error. This was determined by repetitive measurements under identical conditions.

Experimentally, another consideration that had to be taken into account was constant LALLS baselines, especially for gradient elution HPLC–LALLS. Baselines had to be both highly reproducible and linear for valid quantitation purposes. Factors have been described with regard to solvent combination RI limitations [14,15,26,27]. In addition, the HPLC pumping system must offer excellent on-line, pre-injector solvent mixing in order to minimize local concentration fluctuations in the combined mobile phase RI. For this purpose, we incorporated an analytical HPLC silica column having the following characteristics: 5-μm particle size, 10 cm $\times$ 4.6 mm I.D., into the HPLC–LALLS system just prior to the injector. This system produced consistent LALLS chromatograms throughout this work, in addition to its benefit by serving as a saturator column for protection of the analytical column.

A minimum of five individual chromatograms were generated per protein and condition, with blank gradients in-between, in order to ensure chromatographic and data reproducibility. Proper quantitation of individual peaks was not straightforward, since the commercial software package (PCLALLS) was intended for isocratic HPLC operation, where (in theory) all polymer variables remain constant. This is not the case in gradient HPLC–LALLS operation. Peak molecular weights were performed off-line by measuring the corresponding values for each peak's elution condition at its apex (*i.e.*, RI, scattering volume, and the specific RI increment). The second virial coefficient (a term used to indicate solution non-ideality), A_2, was not determined due to the considerable effect of on-column dilution, and a nominal value of $5 \cdot 10^{-4}$ ml mol/g^2 was used throughout these experiments [10]. Individual peak concentrations via relative peak area and known injected mass were determined on-line using the UV detector. These values were then incorporated into the software, and each peak was quantitated individually. The peak apex M_w was reported and several important M_w values deduced, without the need for computer deconvolution software. Taking into account all sources of error associated with these techniques, we have measured an overall experimental error of about $\pm 5\%$ [34].

Aim and purpose of current studies

This study has been designed to understand various phenomena regarding protein separations under normal (< 2% solvent change per minute) gradient elution RP-HPLC, and under isocratic SEC conditions. This has involved: (1) determination of M_w for each hGH peak as a function of injected sample mass: (2) determination of association, if any, as a function of the injected concentration; (3) biopolymer solution–mobile phase effects; (4) column choice differences; and (5) overall reproducibility, accuracy, and precision of on-line LALLS determinations under certain gradient RP-HPLC and isocratic SEC conditions for hGH.

Our first efforts were in using gradient RP-HPLC–LALLS to deduce dynamic biopolymer M_w values, since the reported and experimentally measured refractive indices for water and acetonitrile, at 25°C and 633 nm, were 1.332 and 1.341, respectively (their difference is 0.009, well below the isorefractive criteria of 0.025). It is well known that when RI *versus* %(w/w) is plotted for acetonitrile in water, the resultant plot is not linear. When the resultant plot of acetonitrile was compared to that of methanol or isopropanol, acetonitrile showed less deviation from linearity when viewing the 20–80% acetonitrile in water range [14]. Reversed-phase gradients using acetonitrile can be used with LALLS detection because of the small change in RI

TABLE I

SUMMARY OF CONCENTRATION DEPENDENT RP-HPLC–LALLS MOLECULAR WEIGHT DETERMINATIONS FOR PITUITARY AND RECOMBINANT HUMAN GROWTH HORMONE

Form/storage (mg/ml) (μl)	Injected mass 10^{-4} (g)	M_w[a] overall	Peak 1		Peak 2	
			M_w[a]	% area[b]	M_w[a]	% area[b]
p-hGH/new (8.35) (20)	1.67	24.1 ± 1.2	22.3 ± 0.4	93.4 ± 0.4	[c]	6.4 ± 0.3
p-hGH/new (4.17) (100)	4.17	24.2 ± 0.8	22.9 ± 0.7	91.6 ± 0,5	[d]	8.3 ± 0.3
p-hGH/new (8.35) (100)	8.35	23.8 ± 0.4	22.0 ± 0.4	91.2 ± 1.0	43.1 ± 1.9[e]	8.9 ± 1.1
r-hGH/5°C (7.52) (100)	7.52	22.3 ± 0.8	22.1 ± 0.8	98.9 ± 0.1	[f]	1.1 ± 0.1
r-hGH/45°C (7.52) (100)	7.52	21.7 ± 0.8	21.6 ± 0.8	98.0 ± 0.0	[f]	2.0 ± 0.0

[a] Expressed as kdalton ± S.D., where $n = 5$.
[b] % determined by UV peak area at 277 nm ± S.D.
[c] LALLS response is below the minimum signal-to-noise (S/N) ratio, M_w is invalid.
[d] LALLS response is just below the minimum S/N ratio, M_w is invalid.
[e] LALLS response is above minimum S/N ratio, M_w is valid.
[f] Insufficient LALLS signal for M_w determination.

with change in weight percent. The LALLS signals were obtained free of any baseline anomalies, and background correction procedures were not necessary.

Reversed-phase chromatography

Pituitary human growth hormone (p-hGH/Crescormon). Table I contains the RP-HPLC–LALLS data for both p-hGH and r-hGH samples stored under different conditions. Chromatography was performed under identical conditions that have been previously shown to deter on-column association of several proteins, that is, a slow solvent changeover, while varying injected mass [14]. It is more than likely that under these particular RP-HPLC conditions, there is little or no on-column association of p-hGH occurring. For all RP-HPLC–LALLS molecular weight determinations, the following constants were determined and used: mobile phase $n = 1.349$ RI units, solid angle $= 646.6$, and $dn/dc = 0.149$ ml/g; resulting in a polymer optical constant, K, of $1.648 \cdot 10^{-7}$ mol cm^2/g^2. The refractive indices for these particular mobile phase combinations over the entire range of chromatographic elution have been previously determined [14]. The dn/dc values of the protein as a function of the water–acetonitrile composition were experimentally determined as described before, and plotted, though not shown here, for at least three individual concentrations of p-hGH.

Initial analysis was performed on p-hGH. Injected mass was determined, based on previous similar analyses, and 20 μl of 8.35 mg/ml (167 μg mass) was used with efforts addressed at characterizing the main protein peak [10,14]. A second peak eluting just after the main peak was obscured, and was proposed to be the p-hGH dimer. In addition, a third peak appearing as a lower retention volume shoulder of the main peak was observed. Quantitation of the minor peaks was impossible, due to the low concentrations present. Injection of 417 μg of p-hGH under the same RP-HPLC–LALLS conditions also did not allow for M_w determinations of the minor peaks. Injection of 835 μg total mass (Fig. 1a) produced adequate LALLS responses for valid integration of the first p-hGH monomer and second, assumed dimer peaks. Based on

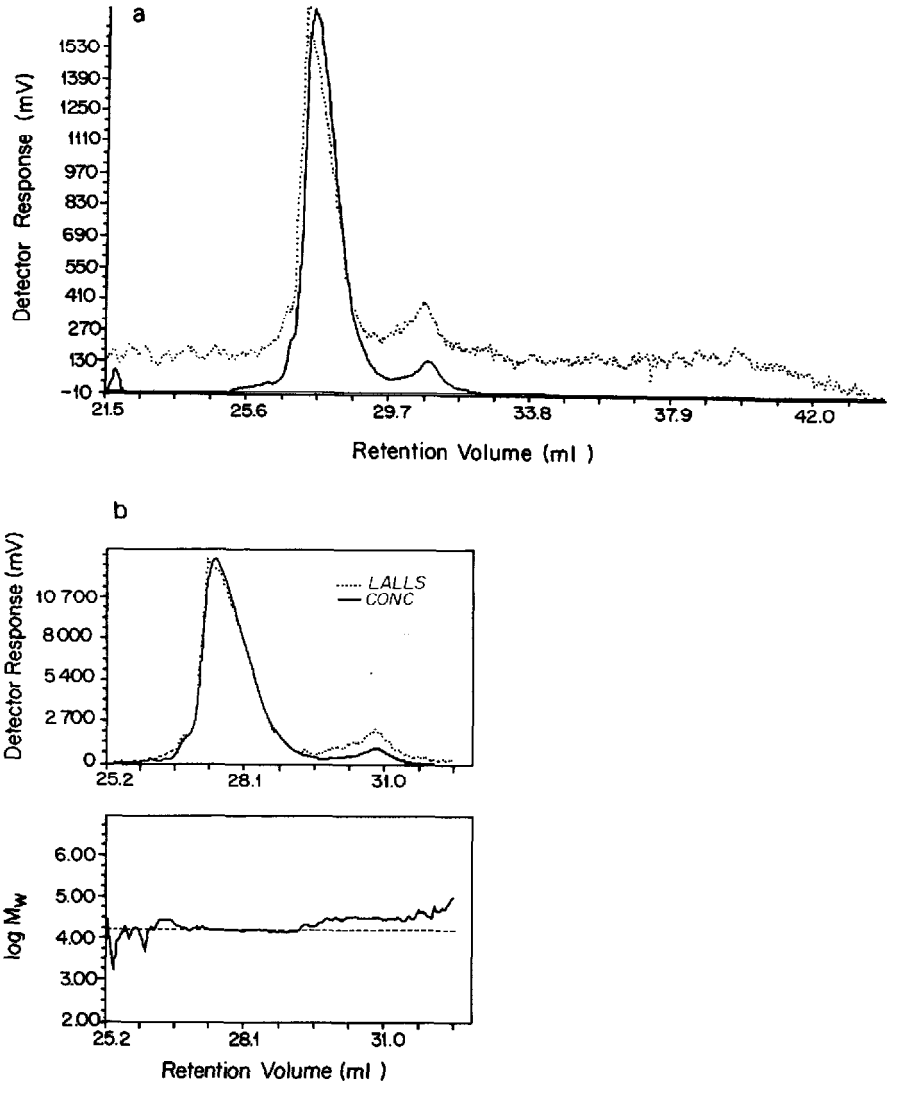

Fig. 1. (a) RP-HPLC–LALLS–UV of p-hGH, 835 μg injected mass, Keystone Deltabond Octyl column. Solid line = UV (280 nm/0.2 a.u.f.s.), dotted line = LALLS. *x*-Axis = retention volume (ml); *y*-axis = detector response (mV). (b) Integrated normalized chromatograms (top) and on-line plot of LALLS determined log M_w *versus* retention volume (bottom), showing two distinct molecular weight species for the two peaks, monomer and dimer, respectively.

previous studies under similar RP conditions, it was assumed here that this second peak was the dimer of p-hGH [12]. Integration of both peaks yielded an average M_w = 23.8 ± 0.4 kdalton, with the first peak M_w = 22.0 ± 0.4 kdalton (monomer) with a % relative UV area = 91.2 ± 1.0%, and peak 2 M_w = 43.1 ± 1.9 (dimer) at a % relative UV area = 8.9 ± 1.1%. The lower M_w peak was even more pronounced, but still of too low concentration for adequate LALLS signal integration (<1%).

Previous studies had suggested that this peak was a variant of p-hGH [12]. Since RP-HPLC baseline fluctuations (noise) were higher than in isocratic SEC, *i.e.* ± 30 mV as compared to ± 10 mV, three times the usual minimum peak height is required for M_w integration. Fig. 1b shows the integrated, normalized chromatograms (top: solid line = UV, dotted line = LALLS) and an on-line plot of LALLS determined log M_w *versus* retention volume (bottom), showing two distinct molecular weight species. From our calculations, it is suggested that in order to properly quantitate the p-hGH dimer, a minimum quantity of 75 μg of this dimer would be required in this RP-HPLC system. We also noticed an increased % relative UV area for the dimer as a function of higher, injected mass/concentration. This suggested, in the absence of on-column association at higher concentrations, increased association on dissolution in the injection medium/solvent. Further results using SEC-LALLS, as below, tended to support these initial conclusions via RP-HPLC–LALLS studies. We do not, at present, believe that association occurred under these particular SEC conditions.

Recombinant human growth hormone (r-hGH/Protropin). When Protropin, stored at 5 and 45°C, was chromatographed under similar conditions (752 μg injected mass) as for p-hGH (835 μg injected mass), some interesting observations were made. For Protropin stored at 5°C (Fig. 2a), the overall M_w was 22.3 ± 0.8 kdalton, with the principle peak having a M_w = 22.1 ± 0.8 kdalton (monomer) and percent relative UV area = 98.9 ± 0.1%. M_w could not be determined for the second peak due to its low percent relative UV area = 1.1 ± 0.1%, Fig. 2b. Based on our previous work with p-hGH, as above, when comparing retention characteristics, we can only presume that this second peak is a dimer. However, without adequate LALLS response due to the low mass of this peak, this can still be in question. Identical chromatographic analysis with Protropin stored at 45°C yielded similar data, with a slight increase in the percent relative UV area of the second peak to 2.0 ± 0.0%.

Size-exclusion chromatography

Much of the literature on the SEC of proteins involves derivatized silica packings, and the TSK-SW series are the most popular [35–37]. In 0.2 *M* ionic strength and neutral pH, numerous globular proteins display ideal chromatographic behavior on SW columns. The primary requirement of the SEC support is that the gel porosity be such that all of the species are well separated. Since resolution affects the accuracy of the results (especially the M_w distribution), by providing a larger change in retention volume, a column should be chosen that offers optimal separation of all components.

Three molecular moments, *viz.*, M_z, M_w, and M_n (z-average, weight-average, and number-average molecular weight) were determined as well as the polydispersity ratio, M_w/M_n, which is an indicator of homogeneity for polymer molecular weight distributions (Table II). A polydispersity value of 1.00 is expected for a monodisperse protein, while using chromatographic band broadening corrections. Previous experiments using this particular SEC–LALLS system have determined that with a pure protein, standard (*e.g.*, ribonuclease A) having a true polydispersity ratio of 1.00, experimentally determined polydispersity was 1.03 [14]. For this reason, band broadening corrections were not applied.

Medium-resolution SEC. Pituitary human growth hormone (p-hGH/Crescormon). In order to define more fully the exact nature of the protein species present on receipt, we initially performed conventional SEC using a TSK-SW-2000 column (medium

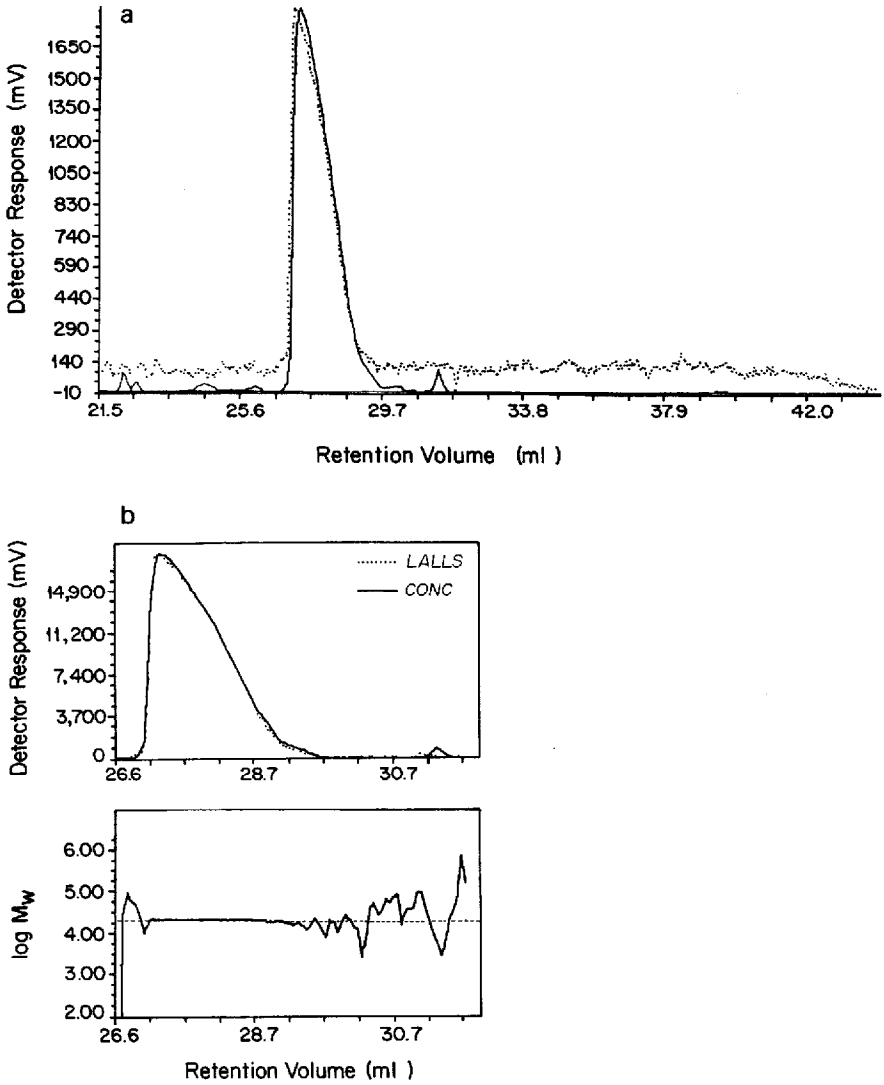

Fig. 2. (a) RP-HPLC–LALLS–UV or r-hGH stored at 5°C for two months, 752 μg injected mass, Keystone Deltabond Octyl column. Solid line = UV (280 nm/0.2 a.u.f.s.), dotted line = LALLS. *x*-Axis = retention volume (ml); *y*-axis = detector response (mV). (b) Integrated normalized chromatograms (top) and on-line plot of LALLS determined log M_w *versus* retention volume (bottom), identifying the major peak as the monomer. Similar conditions as in Fig. 1 where monomer and dimer were identified, but insufficient LALLS signal for proposed dimer characterization.

resolution with LALLS–UV–DRI detection in series for M_w determination in a special buffer (MPB), in addition to PBS. The MPB special buffer has been described and was used to prevent associate formation and dissociation through minimizing ionic and hydrophobic interactions between the protein itself and the mobile or stationary phase [14,29–32]. With many different proteins, we have never found association or

TABLE II

SUMMARY OF MEDIUM RESOLUTION SEC-LALLS MOLECULAR WEIGHT DETERMINATIONS FOR PITUITARY AND RECOMBINANT HUMAN GROWTH HORMONE STORED UNDER DIFFERENT CONDITIONS

Experimental conditions using the TSK-SW-2000 column.

Form/buffer/storage (mg/ml) (μl)	Injected mass (10^{-4} g)	Molecular weight moment[a]			
		M_z	M_w	M_n	M_w/M_n
p-hGH/MPB/new (1.67) (100)	1.67	24.9 ± 1.1	22.2 ± 0.6	18.0 ± 0.6	1.23 ± 0.03
p-hGH/PBS/new (1.67) (100)	1.67	26.2 ± 1.5	23.3 ± 0.4	19.8 ± 0.2	1.18 ± 0.02
p-hGH/PBS/old (1.83) (100)	1.83	427.0 ± 9.2	63.3 ± 1.1	44.6 ± 1.1	1.42 ± 0.01
r-hGH/PBS/5°C (2.20) (100)	2.20	25.8 ± 0.6	22.9 ± 0.6	19.7 ± 0.9	1.16 ± 0.03
r-hGH/PBS/45°C (2.20) (100)	2.20	26.9 ± 0.4	23.1 ± 0.2	20.3 ± 0.5	1.14 ± 0.03

[a] Expressed as kdalton ± S.D., where $n = 5$. M_n = number-average molecular weight; M_w = weight-average molecular weight; M_z = z-average molecular weight.

dissociation of once-formed aggregates to occur in this MPB medium. Under these particular medium resolution SEC conditions, M_w values were determined for p-hGH. The LALLS parameters for integration using MPB as the mobile phase were: mobile phase n was 1.336 RI units and subsequent solid angle = 639.3, dn/dc was 0.160 ml/g, resulting in a polymer optical constant, K, of $1.864 \cdot 10^{-7}$ moles cm^2/g^2. All medium resolution SEC results are summarized in Table II.

With an injected mass of 167 μg, the principle biopolymer peak, M_w = 22.2 ± 0.6 kdalton (monomer), and M_n = 18.0 ± 0.6 kdalton (Fig. 3), showed a leading shoulder which resulted in a non-Gaussian molecular weight distribution (M_w/M_n = 1.23 ± 0.03), incorporating both the monomer and higher M_w species. This can be

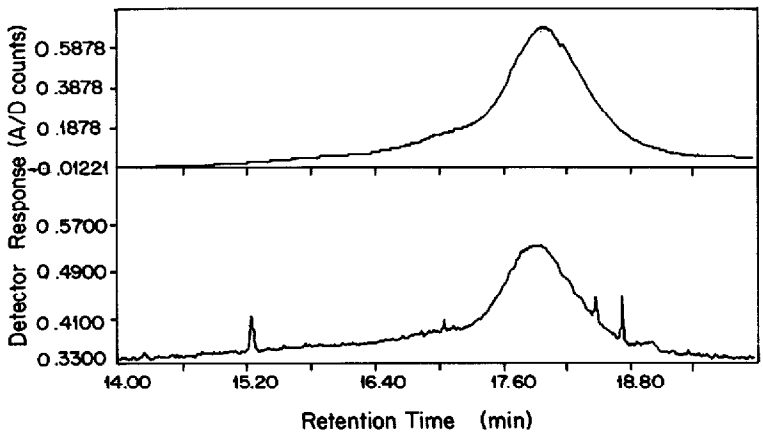

Fig. 3. Medium resolution SEC–LALLS–UV of p-hGH in MPB, new sample, 167 μg injected mass, TSK-SW-2000 column. Top = UV (280 nm/0.1 a.u.f.s.), bottom = LALLS. x-Axis = retention time (min); y-axis = detector response (A/D counts).

seen, both qualitatively and quantitatively, by comparing the normalized peak heights to one another, and by inspection of the processed data, yielding M_{w_i} for each data point at the corresponding elution volume ($M_{w_i} = M_w$ at elution volume i). Both procedures suggested that this peak, though incompletely resolved from the main peak, was the dimer having a percent relative UV area of about 2%, even though at small concentrations relative to the monomer (approximately percent relative UV of 98%). The low M_w (20 kdalton) variant of p-hGH was not readily apparent, under any SEC conditions herein described, most probably due to the inherent lower resolution capability of SEC with respect to RP-HPLC and the small amounts present.

We also investigated the use of the common physiological SEC buffer, PBS. The mobile phase n was similar to MPB having a value of 1.338, solid angle = 640.8. The specific dn/dc was identical to MPB, 0.160 ml/g, using three concentrations for its determination. With an identical injected mass of 167 μg, the M_w was 23.3 $\pm$ 0.4 kdalton and M_n = 19.8 $\pm$ 1.18 kdalton (M_w/M_n = 1.18 $\pm$ 0.02), again indicative of a mixture of species, including a small amount of higher M_w material (co-eluting shoulder on the high M_w side of the peak). These species were identified as primarily monomer (approximately 95% relative UV area) and dimer (approximately 5% relative UV area). When both the PBS and MPB chromatograms were overlaid, they were nearly superimposable.

When a solution of p-hGH was prepared in PBS (183 μg injected mass) and stored in the refrigerator at 5°C of 24 h, it was chromatographically noticed that the biopolymer appeared to randomly "self-associate" producing an increase in the overall M_w and showing a more pronounced high M_w shoulder close to the column exclusion volume, between 13.7–15.1 min (Fig. 4). The elevated LALLS baselines, with respect to the UV response, over the retention times of 13.7–19.3 min suggest that there are small amounts of very high M_w species showing signs of protein mobile/stationary phase competition, resulting in mild, yet reversible adsorption. If this were not the case,

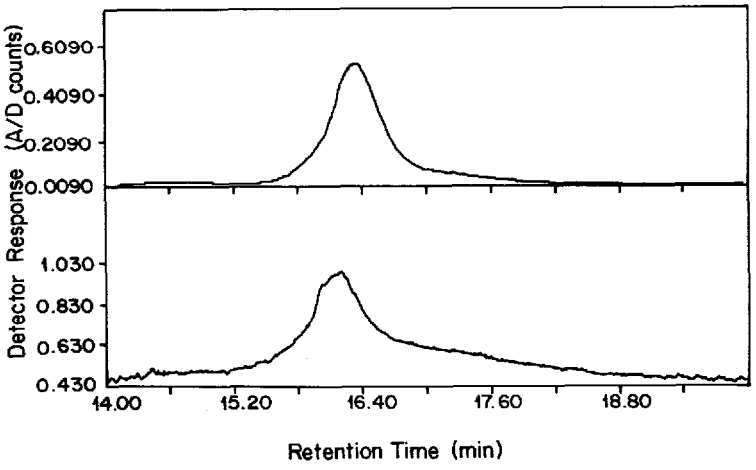

Fig. 4. Medium resolution SEC–LALLS–DRI of p-hGH in PBS, old sample, 183 μg injected mass, TSK-SW-2000 column. Top = DRI (1 V f.s. = $2 \cdot 10^{-4}$ RI units), bottom = LALLS. x-Axis = retention time (min); y-axis = detector response (A/D counts).

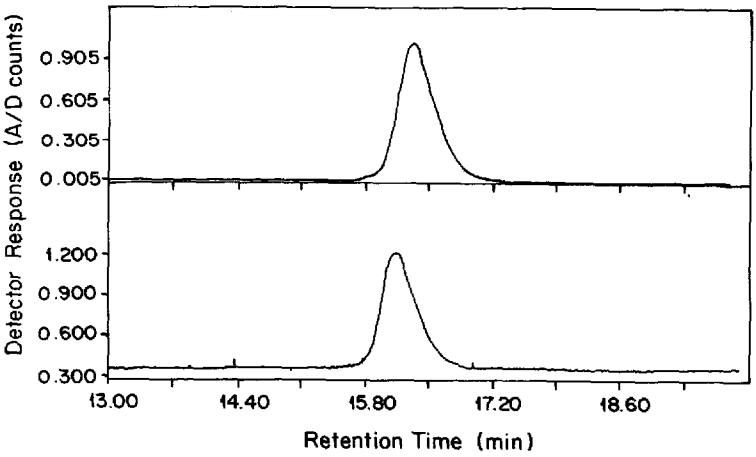

Fig. 5. Medium resolution SEC–LALLS–DRI of r-hGH in PBS, storage at 5°C for 2 months, 220 μg injected mass, TSK-SW-2000 column. Top = DRI (1 V f.s. = 2×10^{-4} RI units), bottom = LALLS. x-Axis = retention time (min); y-axis = detector response (A/D counts).

and the separation was based on purely entropic considerations as SEC should be performed, then one would see similar LALLS and UV responses, but with a large LALLS peak at the column exclusion volume. Molecular weight moments for the major peak(s) were elevated here, with an overall $M_w = 63.3 \pm 1.1$ kdalton and $M_n = 44.6 \pm 1.1$ kdalton ($M_w/M_n = 1.42 \pm 0.01$).

Medium-resolution SEC. Recombinant human growth hormone (r-hGH/Protropin). Similar analyses to the above were performed on two Protropins, stored at 5°C and 45°C for two months, under identical chromatographic conditions. M_w determination procedures were very similar to p-hGH. Fig. 5 illustrates Protropin stored at 5°C, having a $M_w = 22.9 \pm 0.6$ kdalton and $M_n = 19.7 \pm 0.9$ kdalton with a polydispersity $= 1.16 \pm 0.03$. Protropin, stored at 45°C, had a $M_w = 23.1 \pm 1.2$ kdalton and $M_n = 20.3 \pm 0.5$ kdalton with a polydispersity $= 1.14 \pm 0.03$. Both Protropins were chromatographically superimposable. When Protropin was compared to p-hGH in PBS, peak shapes were Gaussian, showing no contribution of the higher M_w dimer. SEC showed only a slight difference between Protropin stored at 5 and 45°C, and when statistically treated, was within experimental error.

We have presented the medium-resolution SEC–LALLS results in order to show that even in the absence of baseline resolution of monomer from dimer hGH species, it is still possible to denote the presence of a higher order species at much lower relative concentrations. Though UV does not easily reveal higher molecular weight species in such situations, the LALLS responses very clearly can and do. Such results should then suggest the use of higher-resolution SEC–LALLS, under identical or similar mobile phase conditions, in order to more fully resolve monomer from dimer or other aggregates that may be present.

High-resolution SEC. Pituitary human growth hormone (p-hGH/Crescormon). High-resolution SEC using a TSK-SWXL-2000 column was utilized in order to separate monomer/dimer. Fig. 6 clearly shows that for p-hGH stored at −15°C for

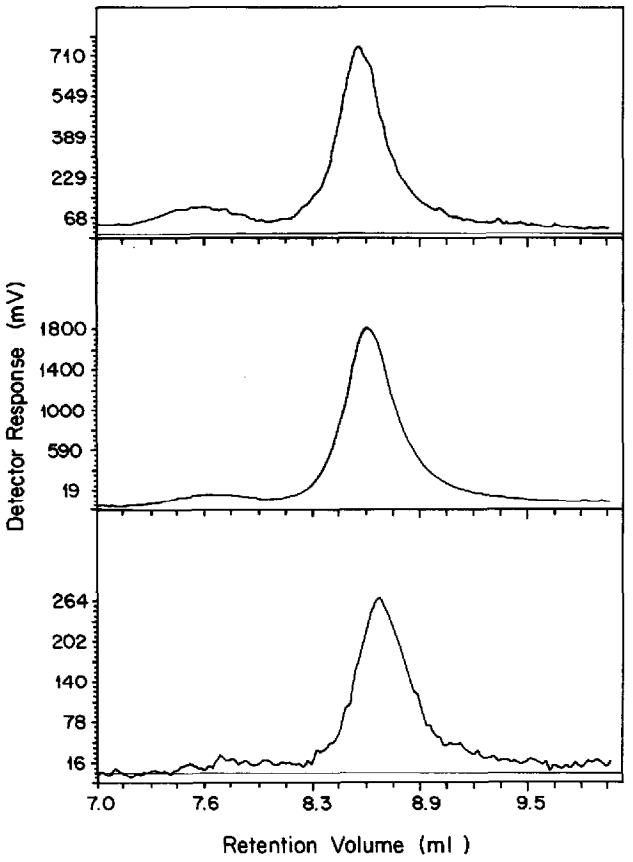

Fig. 6. High-resolution SEC–LALLS–UV–DRI of p-hGH in PBS stored at −15°C for 3 months, 334 μg injected mass, using the TSK-SWXL-2000 column. Top: LALLS; middle: UV (0.2 AUFS = 1000 mV); bottom: DRI ($2.0 \cdot 10^{-4}$ RI units full scale = 1000 mV).

3 months in this laboratory and chromatographed in PBS under similar conditions to the above, the proposed dimer was almost fully resolved from the monomer. Since the RP-HPLC–LALLS experiments suggested that dimer production was concentration related, pre-injection, we again varied concentration in SEC, while keeping the injection volume fixed, for each set of data in Table III ($n = 5$).

Overall M_w values remained constant (*ca.* 25.8 ± 0.5 kdalton) while reducing injected masses from 334–111 μg/injection. However, the percent relative UV area of the monomer increased from 90.3 to 91.8 ± 0.4%, while for the dimer, it decreased from 9.7 to 8.2%. These changes were real, beyond experimental error and fully reproducible. In these SEC experiments, we observed a similar percentage of monomer/dimer as in the RP-HPLC–LALLS experiments for p-hGH (Table I).

We then changed the mobile phase to MPB in order to see if the hGH experienced any solution effects. It was very interesting to note that the M_w values deduced in MPB were identical to those in PBS, but the percent relative UV areas for

TABLE III

SUMMARY OF HIGH RESOLUTION SEC-LALLS DETERMINATIONS FOR PITUITARY AND RECOMBINANT HUMAN GROWTH HORMONE: MOBILE PHASE EFFECTS

Experimental conditions using the TSK-SWXL-2000 column.

Protein[a]/Buffer (mg/ml) (μl)	Injected mass (10^{-4} g)	M_w(overall)[b]	% Monomer[c]	% Dimer[c]
p-hGH/PBS (3.34) (100)	3.34	25.6 ± 0.4	90.3 ± 0.3	9.7 ± 0.1
p-hGH/PBS (2.23) (100)	2.23	25.8 ± 0.4	91.1 ± 0.3	8.9 ± 0.1
p-hGH/PBS (1.11) (100)	1.11	25.8 ± 0.5	91.8 ± 0.4	8.2 ± 0.2
p-hGH/MPB (3.34) (100)	3.34	25.8 ± 0.5	90.1 ± 0.2	9.9 ± 0.1
p-hGH/MPB (2.23) (100)	2.23	25.7 ± 0.5	90.2 ± 0.2	9.8 ± 0.1
p-hGH/MPB (1.11) (100)	1.11	25.7 ± 0.6	90.3 ± 0.2	9.7 ± 0.1
r-hGH/PBS (3.33) (100)	3.33	22.7 ± 0.4	98.0 ± 0.3	2.0 ± 0.1
r-hGH/PBS (2.22) (100)	2.22	22.7 ± 0.4	98.2 ± 0.3	1.8 ± 0.1
r-hGH/PBS (1.11) (100)	1.11	22.7 ± 0.5	98.4 ± 0.3	1.6 ± 0.2
r-hGH/MPB (3.33) (100)	3.33	23.0 ± 0.4	98.3 ± 0.1	1.7 ± 0.1
r-hGH/MPB (2.22) (100)	2.22	23.1 ± 0.4	98.4 ± 0.1	1.6 ± 0.1
r-hGH/MPB (1.11) (100)	1.11	23.0 ± 0.6	98.5 ± 0.1	1.5 ± 0.2

[a] Storage at −15°C for three months.
[b] Expressed as kdalton ± S.D., where $n = 5$.
[c] Determined by % relative UV peak area at 277 nm ± S.D.

the monomer and dimer were more constant in MPB than in PBS when concentration was varied. This again suggested that MPB does not allow association or dissociation to occur, or to a lesser extent, with varying concentrations, in comparison to other buffers or mobile phases (*e.g.*, RP-HPLC). These MPB results also suggested that the monomer/dimer ratio observed at the lowest concentration studied was probably most representative of the original sample composition before dissolution into any solvent(s). Extrapolation to lower concentrations, if possible under SEC–LALLS requirements, would be expected to show the same monomer/dimer ratios already observed at higher concentrations.

High-resolution SEC. Recombinant human growth hormone (r-hGH/Protropin). A similar workup as for p-hGH was performed on r-hGH. Protropin in PBS (Fig. 7, x and y-coordinates expanded for visual purposes of highlighting the dimer peak) yielded an overall M_w of approximately 22.7 ± 0.5 kdalton with a percent relative UV area for the monomer and dimer of 98.0 ± 0.3% and 2.0 ± 0.1%, respectively. Protropin was not as concentration dependent as previous determinations with p-hGH in the same buffer. The percent monomer/dimer for r-hGH in MPB did not change much with respect to that in PBS when varying concentration. A plot of the ratios of percent relative UV area *versus* injected concentration is shown in order to better visualize the hGH concentration dependence of dimer production in both PBS and MPB (Fig. 8). These percentages of monomer and dimer coincided with previously determined RP-HPLC–LALLS values, again confirming that the RP-HPLC–LALLS dimer peaks were not an artifact of the overall technique. The agreement, not coincidence, also suggested that changes in the monomer/dimer ratios occur prior to injection, as a function of the mobile phase or buffer, rather than on-column under RP-HPLC or SEC [12,13,16,19].

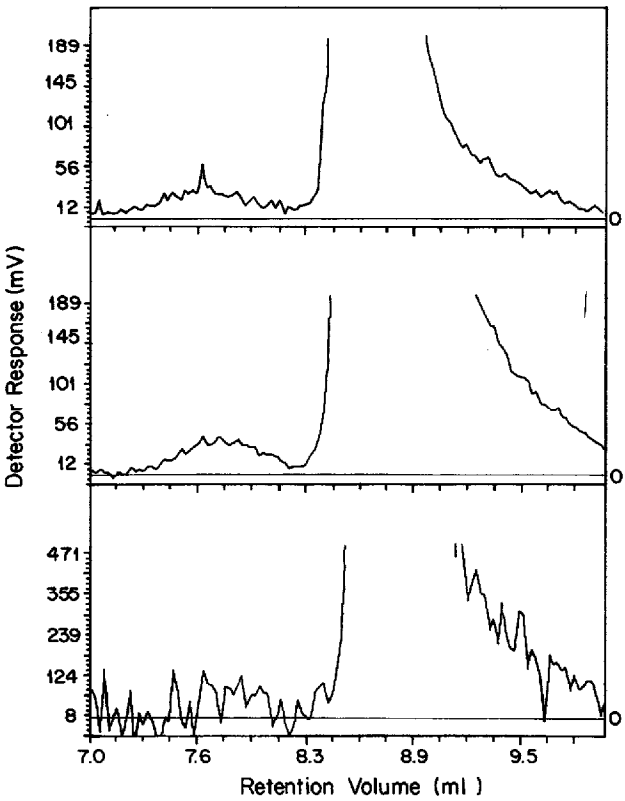

Fig. 7. High-resolution SEC–LALLS–UV–DRI of r-hGH in PBS stored at −15°C for 3 months, 333 μg injected mass, using the TSK-SWXL-2000 column. Chromatographic coordinate expansion. Top: LALLS; middle: UV (0.2 AUFS = 1000 mV); bottom: DRI ($2.0 \cdot 10^{-4}$ RI units full scale = 1000 mV).

CONCLUSIONS

From this study one may conclude that the higher M_w propagation was due, in major part, to pre-column aggregation showing concentration dependence, which may be protein and buffer specific. Pituitary hGH tended to aggregate more readily, since higher order species (*i.e.*, dimer) were already present in the original sample and at higher concentrations than for r-hGH. Recombinant hGH showed signs of similar association, buffer dependent, but of lesser magnitude than the pituitary form.

Proteins, either naturally derived or from recombinant DNA techniques, may form associates during various HPLC assays which LALLS may be able to characterize. These associates may, or may not, be immunogenic and regulatory agencies require evidence of absence in the final product. The results obtained with routine RP-HPLC gradients (solvent change of less than 2%/min), do indicate that it is possible to separate and characterize these proteins in a reasonable amount of time using the RP-HPLC–LALLS technique.

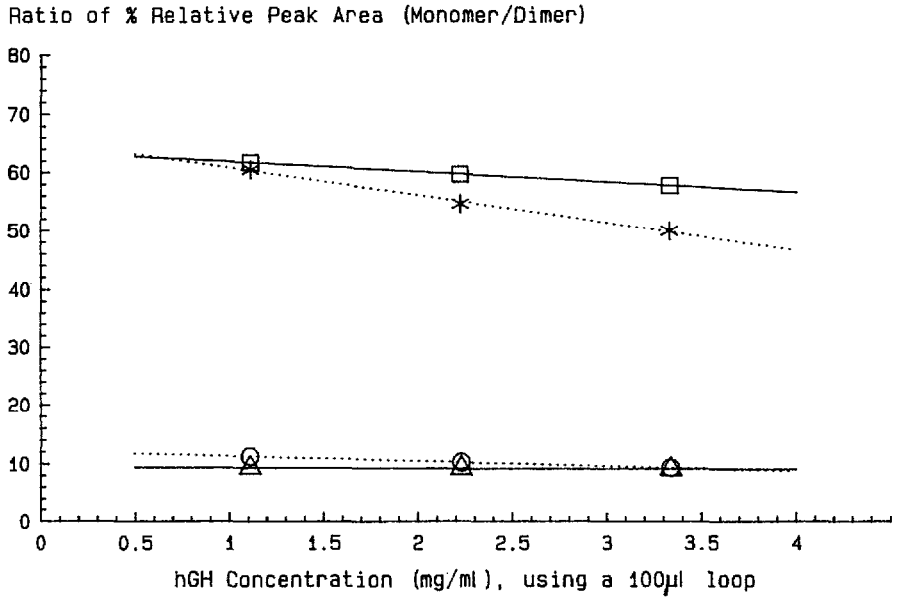

Fig. 8. Ratio of SEC–UV peak area (monomer/dimer) at 277 nm *versus* injected concentration. ∗···∗ = Protropin in PBS; □—□ = protropin in MPB; ○···○ = crescormon in PBS; △—△ = crescormon in MPB. TSK-SWXL-2000 column. SEC–LALLS–UV (277 nm)–DRI (633 nm) system.

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