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Comparative study of protein molecular weights by size-exclusion chromatography and laser-light scattering

Alexis Oliva *, Matías Llabrés, José B. Fariña

Departamento Ingeniería Química y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de La Laguna, 38200, Tenerife, Spain

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Abstract

High-performance size-exclusion chromatography (SEC) based on UV-Vis detection is a relative technique for molecular weight determination whereas procedure based on multi-angle laser light scattering (MALLS) is both rapid and absolute. The two methods using recombinant human growth hormone (rHGH) and β-lactoglobulin samples were compared. A calibration curve for the chromatographic system was generated based on standard proteins and the data were fitted by least squares to a third order polynomial model. The molecular weight from the conventional SEC method for both proteins was higher than the reported values. The molecular weight of rHGH from MALLS was 23.1 ± 0.57 and 21.2 ± 0.80 kDa using differential refractive index (SEC-MALLS/RI) and UV (SEC-MALLS/UV-Vis) detectors as mass detectors. Both values agree, within experimental error with the molecular weight sequence of rHGH, 22.1 kDa. In contrast, the molecular weight from LS for β-lactoglobulin was 22.5 ± 0.55 kDa by SEC-MALLS/RI and 23.0 ± 1.22 kDa by SEC-MALLS/UV-Vis, respectively, values always higher than those supplied by the manufacturer, 18.4 kDa. The reproducibilty of the SEC-MALLS/UV-Vis method versus the SEC-MALLS/RI method was performed using the concordance correlation coefficient. The method′s reproducibility was accepted by assuming a precision of 98% and a 1% loss in precision.

Keywords: Size-exclusion chromatography; Molecular weight; Recombinant human growth hormone; β-Lactoglobulin; Laser-light scattering; Concordance correlation coefficient

1. Introduction

Due to the complexity of proteins, no single analytical method can detect all possible chemical, physical, and immunological changes in the protein structure. Thus, several analytical techniques such as electrophoresis, spectroscopy, chromatography, thermal analysis, immunoassays, and bioassays may be required to completely characterize a protein [1]. The ability to determine molecular weight and size distributions of biopolymers is crucial for understanding proteins and their functions since we often have

* Corresponding author. Tel.: +34-922-318451, fax: +34-922-630095.
E-mail address: amoliva@ull.es (A. Oliva).

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no idea whether the protein exists in solution as monomer, dimers as well as protein molecules self-associate to oligomers for specific purposes [2].

High-performance size-exclusion chromatography (SEC) is probably the most versatile and widely applied method for estimating the molecular weight of a protein in its native form on the basis of its elution volume. However, ordinary SEC techniques based on calibration standards and UV-Vis detection are ineffective and often yield erroneous molecular weight if the standards and the sample have different conformation [3]. Protein elution volume depends not only on the molecular weight, but also on protein shape, its tendency to interact with the matrix column and mobile phase flow variations [2]. Obviously, all these factors could affect the accuracy of the calculated molecular weight.

The development of laser light-scattering detectors and its combination with SE-HPLC has been a great advance in protein characterization because it is possible to determine molecular weight directly, requiring no calibration, being the molecular weight independent of the elution volume [4]. The concentration of each elution fraction as well as the differential refractive index increment, dn/dc, must be known. Thus, if the value of dn/dc or the total mass of eluting solute is known, a light-scattering detector can provide an absolute value of molecular weight when used in series with a concentration detector.

In the present study, the absolute and relative molecular weights of several proteins obtained by SEC were compared.

2. Materials and methods

2.1. Materials

The proteins used β-lactoglobulin, lysozyme, ovalbumin, bovine and human serum albumin, were purchased from Sigma Chemical Co. (St.Louis, MO), and the recombinant human growth hormone was purchased from Novo Nordisk (Bagsvaerd, Denmark).

Samples were prepared by direct dilution with the mobile phase over a range of concentrations of 80–500 μg/ml and analysed the same day. Unless otherwise indicated, all the samples were analysed in triplicate.

2.2. Relative molecular weight determination

The chromatographic system used was a Waters apparatus comprising a pump, 600E Multisolvent Delivery System, 700 Satellite Wisp sample processor, a Shodex C-18 column (8 × 300 mm, Waters) and 490E Programmable Multiwavelenght detector, set at 280 nm. The mobile phase was phosphate-buffered saline (0.12 M NaCl, 0.025 M phosphate, pH 7.0) at a flow rate of 1.0 ml/min. A 25 μl sample of the solution was injected onto the system and data collection and analysis were performed using the Maxima 820 software from Waters. Deionized water prepared with a MIlliQ apparatus (Millipore) was used throughout; all other chemicals and reagents were HPLC grade. All solvents were filtered with 0.45 μm (pore size) filters (Millipore) and degassed.

Lysozyme (14.3 kDa), ovalbumin monomer (44 kDa) and dimer (87 kDa) and, human serum albumin monomer (67 kDa) and dimer (132 kDa) were used to calibrate the SEC system. The calibration curve was generated from these data on three different days. The recombinant human growth hormone (rHGH) and β-lactoglobulin were used as control proteins.

2.3. Absolute molecular weight determination

The same techniques of SEC, but with on-line light-scattering, and refractive index detectors (SEC-MALLS/RI) to determine the absolute molecular weight of several proteins was used. The multiangle laser light-scattering (miniDawn, Wyatt Tech.) detector was placed downstream of the column and upstream of the differential refractive index (DRI) detector, a Waters model 410. To reduce baseline noise a pulse dampener (Alltech Associates, USA) was connected downstream of the pump and two 25 mm high pressure filters with 0.22 and 0.1 μm pores (Millipore) respectively, were used for on-line filtration of the mobile phase.

The column and other chromatographic conditions were identical with those used for SE-HPLC system mentioned earlier. A differential index of refraction (dn/dc) of 0.186 ml/g for the analysed proteins was assumed [2]. Data collection from the miniDawn and the differential refractive-index detectors were controlled by Wyatt Technology's Astra™ program.

The miniDawn detector was calibrated with toluene according to the manufacturer's instructions.Bovine serum albumin monomer in the mobile phase was used for normalization of the three light-scattering detectors (45, 90 and 135°) and to enable proper alignment of the light-scattering and differential refractive index signals, a step necessary for calculation of the molecular weight corresponding to each chromatogram data slice.

The DRI detector was calibrated with sodium chloride standards, operated at room temperature, a sensibility setting of 32 and scale factor of 20 having a calibration constant of 2.99×10^{-4} V/refractive index unit [5]. A 100 μl sample of each solution was injected onto the system and data collection and analysis was performed using Astra software. As alternative, a UV-Vis (490E Programmable Multiwavelenght, Waters) detector at 280 nm was used as mass detector. To obtain the molecular weight directly from the UV-Vis detector, the following equation was required:

$$B_{\mathrm{UV,\ effective}} = \frac{dn/dc}{\varepsilon \cdot l} B_{\mathrm{UV,\ true}} \quad (1)$$

where $B_{\mathrm{UV,effective}}$ was the effective UV-Vis calibration constant; $B_{\mathrm{UV,\ true}}$ was the true UV-Vis calibration constant in AU/V; ε was the extinction coefficient of protein and l was the cell length of your UV-Vis detector in cm [10]. In this case, the extinction coefficient ε of the different proteins must be known. The used values for β-lactoglobulin, ovalbumin, bovine and human serum albumin were 0.851 [6], 0.735 [2], 0.677 [6] and 0.573 [5], respectively. The values for rHGH (0.766) and lysozyme (2.437) were experimentally estimated by UV-Vis spectrophotometry (UV-Vis 1601 model, Shimadzu) at 280 nm.

To calibrate the system and to monitor its performance, a control chart was constructed using a bovine serum albumin standard (0.3 mg/ml) with a nominal molecular weight of 66 kDa. The standard was analysed each working day and the monomer molecular weight was determined. We obtained a mean value for the weight-average molecular weight of 66 kDa with a standard deviation of 622 ($n = 17$), the coefficient of variation was 0.94%. Fig. 1 shows the control chart for the method, indicating the action and warning limits calculated [5].

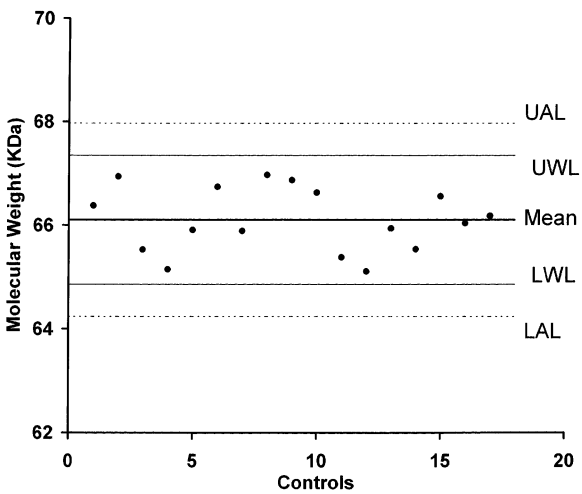

Fig. 1. Control chart for SEC with MALLS detection, constructed using a bovine serum albumin standard (0.3 mg/ml) with nominal molecular weight of 66 kDa. UAL and LAL: upper and lower action limits; UWL and LWL: upper and lower warning limits.

2.4. Concordance correlation coefficient (p_c)

In an instrument, assays or method validation process, the reproducbility properties can be characterized by a concordance correlation coefficient [7]. This index is the correlation between the two readings that fall on the 45° line through the origin. It contains the measurements of accuracy (C_b) and precision (p), normally the Pearson correlation coefficient.

The concordance correlation coefficient (p_c) consist of a measure of precision (p), not correctable, multiplied by a measure of accuracy (C_b), correctable, for example, by calibration ($p_c = p \times C_b$). This bias consist of a scale shift (ratio of two standard deviations, denoted by v)

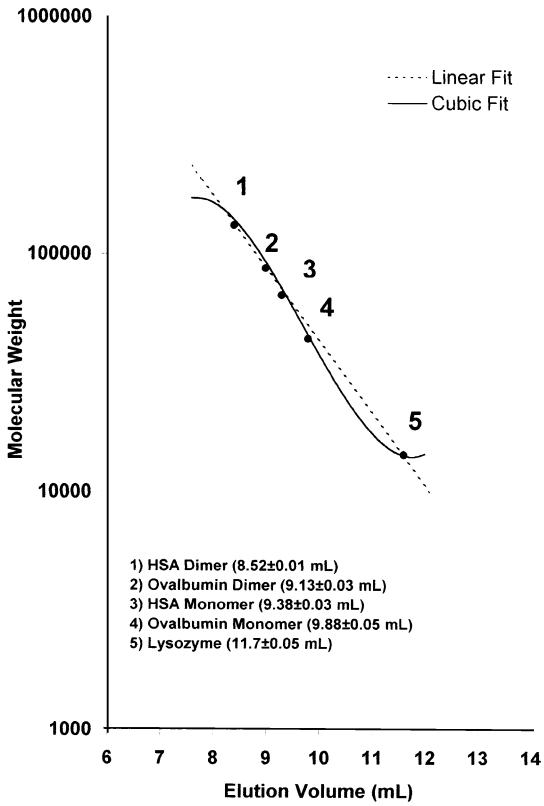

Fig. 2. Calibration curve data molecular weight versus elution volume fitted a third-order polynomial model. The dashed line was a linear model fit. Mean elution volume ± S.D., $n = 3$).

and a location shift (squared difference in means relative to the product of two standard deviations, denoted by u^2).

Then

$$u^2 = (\mu_1 - \mu_2)^2/(\sigma_1\sigma_2);$$

$$v = \sigma_1/\sigma_2;$$

$$C_b = 2[v + (1/v) + u^2]^{-1}.$$

For n independent pairs of samples, it is natural to use the sample counterparts of p_c

$$\hat{p}_c = \frac{2S_{12}}{S_1^2 + S_2^2 + (\bar{Y}_1 - \bar{Y}_2)^2};$$

where

$$\bar{Y}_j = \frac{1}{n}\sum_{i=1}^{n} Y_{ij;} \qquad S_j^2 = \frac{1}{n}\sum_{i=1}^{n}(Y_{ij} - \bar{Y}_i)^2, \; j = 1, 2;$$

And

$$S_{12} = \frac{1}{n}\sum_{i=1}^{n}(Y_{i1} - \bar{Y}_1)(Y_{i2} - \bar{Y}_2).$$

The statistical properties of this estimate can be evaluated using an inverse hyperbolic tangent transformation (Z-transformation):

$$\hat{Z}_c = \tan h^{-1}(\hat{p}_c) = \frac{1}{2}\,\mathrm{Ln}\,\frac{1+\hat{p}_c}{1-\hat{p}_c};$$

yields a distribution asymptotically normal with mean

$$Z_c = \tan h^{-1}(p_c) = \frac{1}{2}\,\mathrm{Ln}\,\frac{1+p_c}{1-p_c};$$

and variance

$$\sigma_z^2 = \frac{1}{n-2}\left\{\frac{(1-p^2)p_c^2}{(1-p_c^2)p^2} + \frac{4p_c^3(1-p_c)u^2}{p(1-p_c^2)^2} - \frac{2p_c^4u^4}{p^2(1-p_c^2)^2}\right\}.$$

The Z-transformation approach was used to calculate the confidence interval for p_c. The p_c allowed us establish the concordance between the absolute molecular weight obtained by laser light scattering in function of the mass detector (RI or UV-Vis detector) used for the studied proteins.

3. Results and discussion

The advantages of SEC over other techniques of molecular weight determination, especially in terms of simplicity of operation and the ability to determine molecular weight averages, were known. However, a calibration procedure must be used, which in practice can present considerable difficulties. The simplest type of calibration is a peak position calibration using suitable standards. The system is usually calibrated with a set of relative standards and the molecular weights are plotted logarithmically against elution volume for constructing column calibration curves.

Fig. 2 shows the calibration curve obtained when the data for the studied proteins were fitted by least squares to a third order polynomial model where the standard error of stimate (s) and

coefficient of determination (r^2) values were better (see Table 1).

As can be seen in Table 1, experimental data fitted quite well the proposed model although the relative molecular weight obtained for the control proteins (rHGH and β-lactoglobulin) varied from the values supplied by the manufacturer, especially in case of β-lactoglobulin. Thus, a mean value of 22.8 ± 0.10 kDa for the rHGH was obtained which was consistent with the value reported in literature 22.1 kDa [8], while the calculated value for β-lactoglobulin 28 ± 0.40 kDa was substantially higher than the supplied value by the manufacturer, 18.4 kDa [9].

According to the molecular weight provided by the manufacturer and found in the literature [8,9], β-lactoglobulin (MW 18.4 kDa) must elute before rHGH (MW 22.1 kDa), in practice it did not, being β-lactoglobulin elution volume of 10.39 ± 0.04 ml ($n = 9$) against 10.68 ± 0.04 ml ($n = 9$) for rHGH.

However, the results seem to suggest a mixed-mode separation mechanism, not a pure size exclusion, but a combination of electrostatic effects, size separation or hydrophobic interaction [10].

A key requirement for the determination of molecular weights by light scattering is the numerical value of dn/dc and the knowledge of the absolute concentration of the sample fraction. For a protein or complex that contains no carbohydrate, dn/dc is constant (≈ 0.186 ml/g) and almost independent of amino acid composition [2].

To validate the SEC-MALLS/RI method, several proteins used in this study were analysed in triplicate on different days. Table 2 summarizes the results for each protein by day and provide their overall means and coefficients of variation (CV). The CV obtained on the same day (intraassay precision) was $< 3\%$ for the weight-average molecular weight, although higher values of number-average molecular weight were obtained, but none exceeded 5%. To complement the information available, one-way analysis of variance to determine if there were differences between days was performed; the results showed that the interassay differences were not significant ($P > 0.05$ always). The interassay precision was always better than 2.5%. These data clearly demonstrate the excellent intraassay and interassay precision of the SEC-MALLS/RI method for protein analysis and routine quality control purposes.

Alternatively, a MALLS detector may be connected in line with UV-Vis absorbance detector, acting as a mass detector (SEC-MALLS/UV-Vis). In this case, the UV-Vis detector is placed just after the column, before the miniDawn. This minimizes the instrumental broadening seen by each detector. To apply this method, the dn/dc and ε values must be known. The most common source of error is the inaccuracy in the value of ε, particularly when various components of the sample may have different absorptivity coefficients. Thus, to determine the proteins absolute molecular weight it was assumed that the dn/dc value was constant and the value of ε did not change with the amino acids protein composition [11]. Table 3 shows the absolute molecular weight of the studied proteins calculated for the SEC-MALLS/UV-Vis method, using the ε values indicated in Materials and Methods. The daily CVs (intraassay precision) were less than 6%, whereas the interassay precision was in the range from 1.07 to 4.41%, except for HSA dimer, with a CV of 6.38%. Fig. 3 shows the chromatogram obtained

Table 1
Results of estimated coefficients, standard error of estimate (s) and coefficient of determination (r^2) corresponding to the calibration curve generated using the molecular weight supplied by the manufacturer

Day	Cubic least-squares fit					
	b_0	b_1	b_2	b_3	r^2	s
1	−17.4	7.65	−0.832	0.0287	0.9996	0.0140
2	−20.9	8.70	−0.936	0.0321	0.9998	0.0100
3	−21.4	8.77	−0.934	0.0318	0.9993	0.0120

Table 2
Results of SEC-MALLS/RI analysis for several proteins

Protein	Day	MW[a]	CV (%)	Mn[b]	CV (%)
β-Lactoglobulin	1	22 000	1.86	21 500	1.71
	2	23 000	1.92	22 000	3.77
	3	22 500	1.04	20 700	3.51
Overall	($n=9$)	22 500	2.45	21 400	3.88
Ovalbumin monomer	1	40 200	0.93	38 400	1.36
	2	40 200	0.80	38 700	1.29
	3	39 700	1.36	35 600	3.38
Overall	($n=9$)	39 800	1.89	37 600	4.26
Ovalbumin dimer	1	87 100	1.84	61 700	1.43
	2	88 400	1.46	64 200	3.48
	3	87 000	0.95	63 500	3.48
Overall	($n=9$)	87 500	1.50	63 100	3.15
HSA monomer[c]	1	66 700	0.24	64 800	0.76
	2	67 100	0.94	62 700	0.80
	3	68 000	0.30	64 800	0.69
Overall	($n=9$)	67 300	0.96	64 100	1.72
HSA dimer	1	132 500	1.74	132 000	2.76
	2	132 300	0.47	129 000	1.01
	3	131 300	1.06	131 000	0.93
Overall	($n=9$)	132 000	1.13	130 700	1.81
Lysozyme	1	14 900	2.30	13 200	1.00
	2	15 000	1.66	14 300	3.81
	3	14 700	1.32	14 300	2.48
Overall	($n=9$)	14 800	2.24	13 900	4.50
rHGH[d]	1	23 100	2.99	19 500	4.74
	2	23 400	1.27	21 000	3.22
	3	22 900	2.87	20 800	3.53
Overall	($n=9$)	23 100	2.48	20 500	4.83

[a] Weight-average molecular weight.
[b] Number-average molecular weight.
[c] Human serum albumin.
[d] Recombinant human growth hormone; unless otherwise indicated, the daily means corresponding to triplicate injections.

by SEC coupled with light-scattering, UV and refractive index detectors.

The obtained values agree quite well for both methods, althought coefficients of variation were higher for the SEC-MALLS/UV-Vis method, for example, in the case of β-lactoglobulin due to the inaccuracy in the ε value used [2]. The observed variability for the ovoalbumin and albumin dimer could be due to differences between their absorptivity coefficients with respect to monomeric species. In this last case, the assumption that both products (monomer and dimer) have the same polypeptide extinction coefficient and dn/dc was made.

When a new method or instrument is developed, it is of interest to evaluate whether the new method can reproduce the results based on a reference method [7]. The concordance correlation coefficient (p_c) can be used to validate the reproducibility of a new method. It is useful and easy to perform. The proposed guidelines for such validation require the specification of allowable losses in precision and accuracy.

A study was conducted to assess the reproducibility of the SEC-MALLS/UV-Vis method, denoted by new method, and to compare it to the SEC-MALLS/RI method, denoted by reference method. The method's reproducibility can be accepted for the studied proteins if the $100(1-\alpha)\%$

Table 3
Weight-average molecular weight and coefficient of variation (in parentheses) corresponding to different proteins determined by MALLS using differential refractive index (SEC-MALLS/RI) or UV-visible absorbance (SEC-MALLS/UV-Vis) detectors

Protein	SEC-MALLS/RI	SEC-MALLS/UV-Vis	Manufacturer's data[a]
Lysozyme	14 850 (2.24)	15 800 (3.20)	14 300
β-Lactoglobulin	22 500 (2.45)	23 000 (4.41)	18 400
rHGH	23 100 (2.48)	21 200 (3.18)	22 120[b]
Ovalbumin Monomer	39 800 (1.89)	43 100 (2.52)	44 000
Ovalbumin Dimer	87 500 (1.50)	85 300 (3.46)	88 000
HSA Monomer[c]	67 300 (0.96)	67 500 (1.46)	67 000
HSA Dimer	132 000 (1.13)	137 400 (6.38)	132 000[b]
BSA Monomer[d]	66 100 (1.11)	65 800 (1.07)	66 000
BSA Dimer	127 000 (2.09)	130 000 (3.25)	132 000

[a] Values are supplied by the manufacturer.
[b] Values found in the literature [2,8].
[c] Human serum albumin.
[d] Bovine serum albumin.

lower confidence limit is greater than or equal to $p_{c.a}$, namely least acceptable p_{ca}., and a $100X\%$ loss in precision [12].

The results are plotted in Fig. 4. The sample concordance correlation coefficient was 0.9968 with the 95% one-tailed lower confidence limit of 0.9950. Also, it was assumed that the SEC-MALLS/UV-Vis method presented a precision of 98% ($P = 0.990$) and a 1% loss in precision ($X = 0.01$) was also determined, this yields a least acceptable p_c of 0.9844. This result shows the reproducibilty of both methods since its 95% lower confidence limit was much greater than the least acceptable [13].

The results from light-scattering detection shows that the rHGH absolute molecular weight obtained was very close to the nominal, 23.1 kDa with 95% confidence intervals of 24.1 and 22 kDa respectively, containing the nominal value 22.1 kDa. In the case of β-lactoglobulin, the molecular weight was 22.5 ± 0.55 kDa, slightly higher than the molecular weight provided by the manufacturer (18.4 kDa), but much lower than the obtained value for the conventional SEC method (Table 4). Considering the different analytical methods used, the SEC/MALLS method gives a reasonable molecular weight for the control proteins, since this method provides a calibration curve for every sample and the molecular weights are determined independently for each elution volume [14].

The model's parameters were reestimated substituting the nominal molecular weight by the molecular weight obtained by SEC-MALLS/RI method (see Table 3). The results showed an improvement in the goodness of fit in the polynomial model being the standard error of estimate lower than 0.0055 and coefficient of determination was higher than 0.9999 obtaining a mean molecular weight of 23.0 ± 0.28 kDa for β-lactoglobulin and 19.0 ± 0.31 kDa for rHGH.

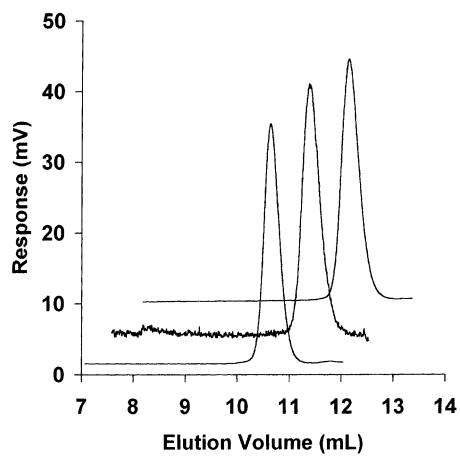

Fig. 3. Chromatograms obtained from a pure recombinant human growth hormone (rHGH) sample in mobile phase. Top trace differential refractive-index detector; middle trace MALLS detector; bottom trace UV-visible absorbance detector.

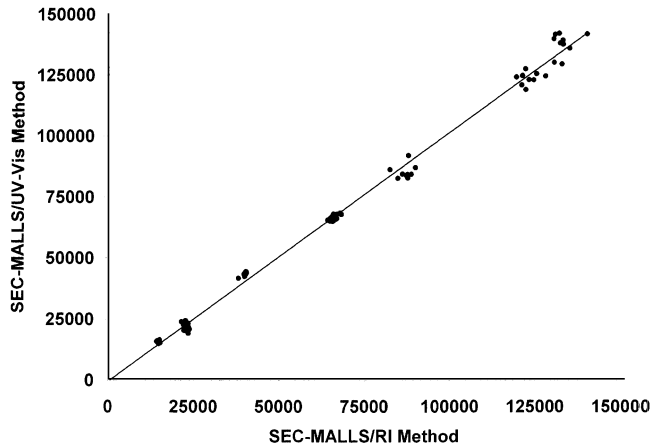

Fig. 4. Reproducibility of the SEC-LS/UV-Vis method versus the SEC-LS/RI method. The concordance correlation coefficient was 0.9968 and its lower 95% confidence interval was 0.9950.

Table 4
Weight-average molecular weight (KDa) obtained for the control proteins using SEC with MALLS detection coupled with refractive index (SEC-MALLS/RI) and UV-visible absorbance (SEC-MALLS/UV-Vis) detectors compared with those obtained by conventional SEC calibration curve using a third-order polynomial model (mean ± S.D., $n = 3$)

Protein	SEC-MALLS/RI	SEC-MALLS/UV-Vis	Conventional SEC		Nominal MW
			A[a]	B[b]	
β-Lactoglobulin	22.5 ± 0.55	23.0 ± 1.22	28.0 ± 0.40	23.0 ± 0.28	18.4
rHGH	23.1 ± 0.57	21.2 ± 0.80	22.8 ± 0.10	19.0 ± 0.31	22.1

[a] The calibration curve was generated using the nominal molecular weight.
[b] The calibration curve was generated using the molecular weight from the SEC-MALLS/RI data.

4. Conclusions

The β-lactoglobulin molecular weight was higher than the value provided by the manufacturer independently of the method used for its determination. These differences between the molecular weights could be due to a mixed-mode separation mechanism since the theoretical elution volume of β-Lactoglobulin should be 11.0 ml instead of the 10.39 ml observed or the molecular weight supplied by the manufacturer was incorrect. At first, the results from light-scattering indicate thah the molecular weight is nearer to 22 kDa than 18.4 kDa.

A MALLS detector coupled with SEC system can provide an absolute molecular weight when used in series with a concentration detector such as refractive index (SEC-MALLS/RI) or UV (SEC-MALLS/UV-Vis) detectors. The reproducibility of both methods was evaluated and verified using the concordance correlation coefficient. This fact allowed us to improve the accuracy of molecular weight determination when there existed uncertainity in the dn/dc value for the studied proteins. The complementary use of the protein ε value (see Eq. (1)) could reduce the error introduced with the approx. dn/dc value.

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