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Chromatographic characterization of synthetic peptides: SPf66 malaria vaccine

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Abstract

The development and validation of a quantitative size-exclusion chromatography (SEC) method for SPf66 malaria vaccine was achieved. The results show the reliability of the analytical method for the intended use. SPf66 malaria vaccine characterization was performed using both relative techniques such as the conventional SEC and absolute techniques: mass spectrometry and multi-angle laser-light scattering detection. The relative and absolute molecular masses were in the 4600–18 000 Da range. The results clearly indicate the presence of the monomer and dimer species, whereas the third species could be the trimer or tetramer.

Keywords: Peptides, synthetic; SPf66 malaria vaccine

1. Introduction

During the last few decades, advances in technology have provided vaccines containing whole-cell microorganisms, detoxified toxins, purified chemically defined cell wall polysaccharides, purified protein antigens as well as those obtained by recombinant DNA technology. Synthetic peptides are a new approach representing the protective epitopes of the pathogen [1].

SPf66 vaccine is the first synthetic hybrid-polymer containing amino acid sequences derived from three asexual blood-stage proteins (merozoites) linked by sequences derived from the sporozoites protein of *Plasmodium falciparum* [2].

Various laboratory studies and field trials have shown that this molecule is safe, immunogenic and induces protection [3–9]. Recently, Graves et al. showed the efficacy of SPf66 malaria vaccine against clinical malaria [4]. However, the synthetic peptide can show auto-polymerization reactions once synthesized, consequently the presence of these species could modify its immunogenic capacity since it depends on the proportion of the different species [10–13]. This is the case with SPf66 vaccine, which can show up to three different species when analyzed by chromatography.

Protein characterization requires the use of several techniques; each probe measures a particular struc-

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tural or functional feature [13]. However, a detailed knowledge of their structure and interactions with other molecules is necessary to understand the role of the proteins and their specific purposes. Therefore, molecular mass determination is an important step in understanding proteins and their functions. Several methods have been used, either relative techniques like size-exclusion chromatography (SEC) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) or absolute techniques: mass spectrometry (MS), light-scattering and analytical ultracentrifugation.

The conventional SEC techniques based on calibration standards and UV–Vis detection are ineffective and often yield erroneous molecular masses if the standards and the sample have a different conformation [14]. Protein elution volume depends not only on molecular mass, but also on protein shape, its tendency to interact with the matrix column, and mobile phase flow variations [15]. Obviously, all these factors could affect the accuracy of the calculated molecular mass. Indeed, light-scattering is one of the few absolute methods available for the determination of molecular mass and structure and certainly is applicable over the broadest range of molecular mass of any method. The development of SEC with light-scattering detection offers an alternative technique to investigate the association of macromolecules in solution. As the intensity of the scattered light is detected at different angles, the radius of gyration and the molecular mass can be measured simultaneously, requiring no calibration, both the molecular mass and radius of gyration being independent of the elution volume [16]. An additional benefit of this calculation lies in the molecular mass being that of the dissolved protein solution. This can reveal whether the protein exists as a monomer, dimer, or as a higher aggregation state [17], which would be almost invisible to the differential refractive-index detector. These characteristics make the combination of light-scattering with SEC an easy, accurate and reliable technique [18]. Moreover, SEC with light-scattering detection may be useful in some instances to provide information regarding the conformation of a protein (folded and unfolded) if the molecular mass derived from light-scattering and from elution position are compared. For example, the reduced ribonuclease (unfolded protein) was identical in molecular mass to the native ribonuclease (folded protein), a result very different from that derived from conventional SEC [15].

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS is a widely used method for mass measurement of biomolecules [19–22]. Simplicity, high accuracy (typically $\pm 0.1\%$, and $\pm 0.01\%$ for polypeptides below 10 kDa). Theoretically unlimited mass range and extreme sensitivity (pmol to subpmol range) have made MALDI-TOF-MS an excellent method for routine mass analysis [23]. A MALDI-TOF-MS hydrogen-exchange method for the study of protein folding and conformational changes has been reported [24–28]. The method is based on the fact that the hydrogen/deuterium (H/D) exchange rate of amide protons located on the peptide backbone depends on whether they are participating in intramolecular hydrogen binding and on the extent to which they are shielded from the solvent [29,30]. H/D exchange experiments monitored by MALDI-TOF have also been used to study the conformational stability of Insulin adsorbed on to different solid surfaces [31] and to probe conformational changes in peptides in mixtures of organic solvents and water [23,32].

Each method has advantages and disadvantages. However, both MS and light-scattering methods offer very promising potential for the characterization of proteins and protein aggregates.

The objectives of this study were: first: the development and validation of a quantitative SEC method for SPf66 malaria vaccine, determining its specificity, linearity, accuracy, precision, range, detection and quantitation limits and robustness according to the International Conference on Harmonization Guidelines [33,34], to test the usefulness of the method to detect differences between different peptide samples. Second: the chromatographic characterization of the SPf66 malaria vaccine, determining the relative and absolute molecular mass of the different species and comparing these results with the available mass spectrometry data.

2. Experimental

2.1. Materials

SPf66 malaria vaccine (batch: 15-7) and the peptide 23051 were synthesized at the Instituto de

Inmunología, Hospital San Juan de Dios, Bogotá, Colombia. Bovine serum albumin was purchased from Merck (Darmstadt, Germany), β-lactoglobulin and lysozyme were purchased from Sigma (St. Louis, MO, USA), the recombinant human growth hormone and human insulin were purchased by Novo Nordisk (Bagsvaerd, Denmark).

2.2. SEC analytical method

The chromatographic system used in this study was a Waters apparatus (Waters, Milford, MA, USA) consisting of a pump, a Model 600E Multisolvent delivery system, a 700 Wisp Sample Processor, a 490 programmable multiwavelength detector and a Protein Pak 125 column (300×7.8 mm I.D, Waters) packed with 10 μm particles of 125 Å pore size as stationary phase. The mobile phase was an acetonitrile–water (30:70) mixture with 0.05% trifluoroacetic acid, and a flow-rate of 1.0 ml/min at room temperature and UV detection at 214 nm was used. Deionized water used to prepare the mobile phase was purified by a Millipore Milli-Q system; all other chemicals and reagents were HPLC grade. All solvents were filtered with 0.45-μm pore-size filters (Millipore). The mobile phase was filtered and degassed.

2.3. Relative molecular mass determination

Synthetic peptide (2.0 kDa), human insulin (5.8 kDa), lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), recombinant human growth hormone (22.1 kDa) and bovine serum albumin (66 kDa) were used to calibrate the column for the conventional SEC system, covering the 2000–80 000 molecular mass range specified for this column by the manufacturer. The curve calibration was generated from these data on different days. System suitability and the accuracy of the method were verified by analyzing a lysozyme solution sample as control on each work day.

Samples were prepared by direct dilution with the mobile phase over a range of concentrations of 20–500 μg/ml and analyzed the same day. Unless otherwise indicated all the samples were analyzed in triplicate.

2.4. Absolute molecular mass determination

A miniDawn (Wyatt Technology, Santa Barbara, CA, USA) multi-angle laser-light scattering (MALLS) instrument coupled with the SEC system was used to determine the absolute molecular mass of SPf66 malaria vaccine. The miniDawn was placed downstream of the column and upstream of the differential refractive-index (DRI) detector (Waters Model 410), to avoid the possibility of backpressure on the differential refractive index cell. To reduce baseline noise, a pulse dampener (Alltech Associates, USA) was connected downstream of the pump, to reduce its pulsation, and two 25 mm high-pressure filters with 0.22 and 0.1 μm pores (Millipore) were used for on-line filtration of the mobile phase.

The column and other chromatographic conditions were identical to those used for the SEC method. A differential index of refraction (dn/dc) of 0.186 ml/g was assumed [15]. A computer system running Wyatt Astra software controlled data acquisition and analysis.

The miniDawn 90° detector was calibrated using the intrinsic Rayleigh scattering of a pure solvent such as toluene according to the manufacturer's instructions, and the other detectors were normalized using a protein solution, and the DRI detector was calibrated with sodium chloride standards over a linear concentration range of 1–5 mg/ml, operated at room temperature, a sensibility setting of 32 and scale factor of 20 with a calibration constant of $2.99 \cdot 10^{-4}$ V/refractive index unit [18]. A 100-μl sample of each solution was injected into the system and data collection and analysis was performed using Astra software.

To calibrate the system and monitor its performance, a control chart was constructed using bovine serum albumin standard (0.3 mg/ml) with a nominal molecular mass of 66 kDa. The standard was analysed each working day, and the monomer molecular mass determined. The RSD was <1%. The precision of this calibration method was also pointed out using several proteins in the 14–130 kDa molecular mass range [18].

The results obtained showed an intra- and-inter assay precision lower than 2.5%.

2.5. Mass spectrometry

The samples were prepared using 2,5-dihydroxy-

benzoic acid (DHB) (Sigma) as the matrix for all MALDI experiments. All biomolecules used as a standards were purchased form Sigma. The samples were prepared by mixing a suitable quantity of aqueous analyte solution with an aqueous matrix solution composed of a mixture of acetonitrile–water–trifluoroacetic acid (40:59:1). The amount of analyte placed in the tube was in the range 1–15 pmol.

MALDI-TOF mass spectra were acquired on a Bruker Biflex mass spectrometer equipped with a nitrogen laser with an emission wavelength of 337 nm. Spectra were obtained in the reflector mode at an accelerating voltage of 20 kV. Deflection of the low mass ions was used to enhance the target protein signal. A calibration was performed for each measurement with the ACTH (18–39) fragment and bovine insulin as a standard protein. Each mass determination was averaged from three sample-standard pairs. The accuracy in mass determination obtained for SPf66 malaria vaccine monomer was $\pm$0.9 U. All reported masses are monoisotropic $[M+H]^+$ unless otherwise noted.

3. Results and discussion

3.1. Development of the SEC method

Chromatographic procedures for investigating proteins have developed significantly during the 1980s with numerous advances in the technology of high-performance liquid chromatography (HPLC) methods [35]. Reversed-phase HPLC has become a routine method for the analysis of proteins. Lopez et al. [5] proposed a method for SPf66 malaria vaccine utilizing a C_8 column (Sephasil, 100×4.6 mm, 5 μm, 100 Å, Pharmacia, UK) with a gradient mobile phase, and only the principal peak was detected. These authors used this method to check the reproducibility from different batches and that no intermediary products was generated during the synthesis. We used a reversed-phase C_{18} column (Delta Pack, 100×8 mm, 15 μm, 300 Å, Waters) with an isocratic mobile phase, composed of a buffer (0.2 *M* sodium sulfate anhydrous)–acetonitrile (74:26) mixture, separation into various species was not achieved, obtaining similar behavior to that described by Lopez et al. [5].

SEC provides valuable information on protein size, and the presence of aggregates. Since it is amenable to quantitation, SEC offers a means of gaining precise analytical data on different molecular species in the vaccine, which are resolvable by size. Again, the use of SEC has been greatly expanded with the arrival on the market of different column packing materials that offer various alternatives in the analysis of proteins by SEC [36]. Lopez et al. [5] describe an SEC method using a Superose 12 HR column (300×10 mm, 10 μm, Pharmacia), with a mobile phase composed of an aqueous solution of 0.05 *M* sodium dihydrogenphosphate and 0.15*M* sodium chloride at pH 7.2, to confirm the molecular mass range of several species and that the degree of polymerization of several batches was similar.

As a wide variety of column types are available, where the choice of column depends on the pH at which the analysis is performed, there should be no interaction between the peptide and the surface of the stationary phase.

At this stage, a column (Shodex KW-803, 300×8 mm, 7 μm, Waters) with a mobile phase constituted of 25 m*M* disodium hydrogenphosphate and 120 m*M* sodium chloride adjusted to pH 7.0 with orthophosphoric acid was used. Under these conditions, the different species were not separated since the sample was not stable with respect to the pH of the mobile phase (7.0). Changing the mobile phase and chromatographic conditions optimization did not lead to any improvement in separation and resolution. The following step was to choose different column packing. Protein Pak packing based on a 10 μm diol-bonded silica with different pore sizes was used. A Protein Pak 60 column (300×7.8 mm, Waters) with a pore size of 60 Å was used. The chromatographic conditions were similar to those described in the Experimental section. Three peaks can be resolved perfectly with a very good resolution ($1.5<R_s<2.2$) and selectivity ($1.7<\alpha<3.0$), but a greater analysis time was necessary since the flow-rate was 0.6 ml/min. This column was however appropriate for qualitative analysis of SPf66 malaria vaccine but not for quantitative analysis because a lineal response between the concentration and the total area

of all peaks was not observed. Moreover, the sample mass needed was too high, increasing cost.

In contrast, when a Protein Pak 125 column (300×7.8 mm, Waters) with a pore size 125 Å was used, the separation and quantitation of different species was achieved. In this way, the development and validation of the SEC quantitative method for the in vitro release and stability studies on SPf66 malaria vaccine was achieved.

3.2. Validation of SEC method

3.2.1. Specificity

Fig. 1 shows the chromatogram of the pure SPf66 malaria vaccine sample obtained by the SEC method. Three peaks with elution volume 7.13 ml (peak 1), 7.84 ml (peak 2) and 9.08 ml (peak 3) were detected. With the column used in this study, it is possible to distinguish proteins differing by as little as 15% in molecular mass, with good resolution (R_s=1.4 for peaks 2&3) and selectivity (α=1.2 for peaks 2&3).

3.2.2. Linearity

To validate the analytical method, eight standard solutions were prepared using pure SPf66 malaria vaccine at concentrations of 5–40 μg/ml. Each sample was analyzed four times. To quantify SPf66 peptide, we used the total area of all the peaks (see Fig. 2). The analysis of variance (ANOVA) of the linear regression ''total area of all the peaks versus prepared concentration of peptide'' confirmed the linearity of the method through rejection of the null hypothesis of deviation of the linearity for a significance level of 0.05 (α=0.05); the relative standard deviation (RSD) was 6.73%. The equation of the regression line was:

$$\text{Total area} = (10\,573 \pm 26\,048) + (43\,661 \pm 1032)c; \quad r = 0.992 \quad (n = 32)$$

and the root mean square error (S_{yx}) was 69 752.

The sizes of the individual peaks, as a percentage

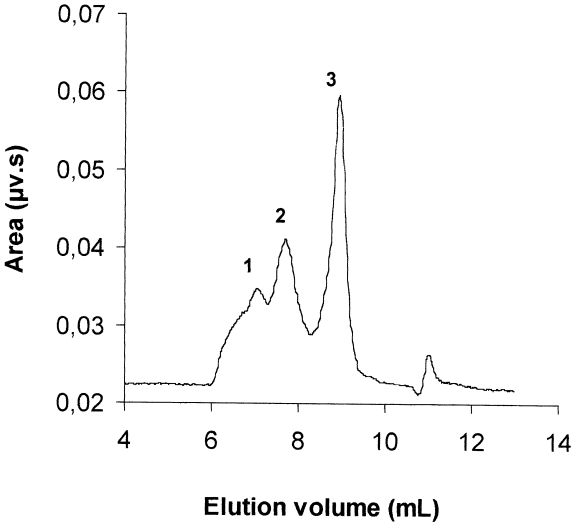

Fig. 1. SEC–HPLC separation of different species of SPf66 peptide on a Protein Pak 125 column with UV–Vis detection at 214 nm. Peaks: 1=trimer or tetramer; 2=dimer; 3=monomer.

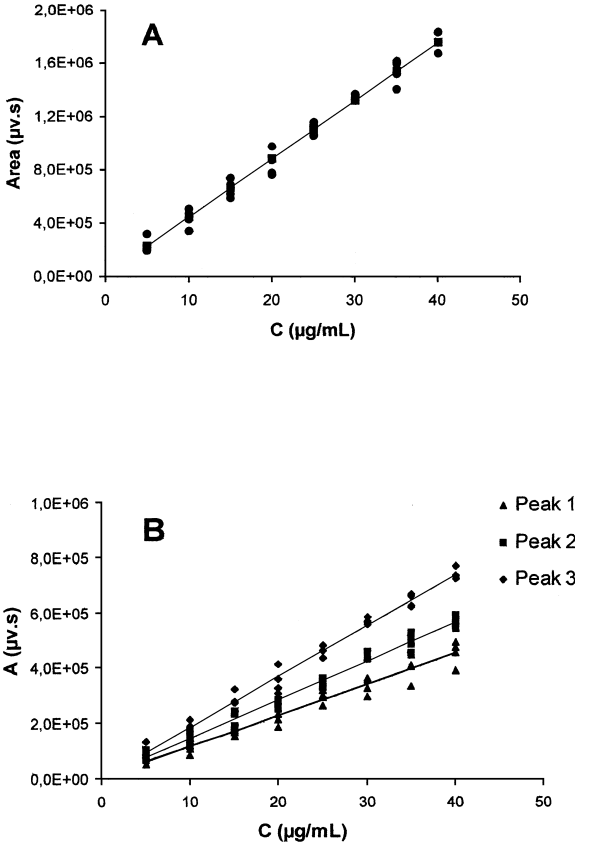

Fig. 2. Regression lines for total area of all the peaks (A) and area each peak (B) versus total concentration of SPf66 peptide.

of the total area, enable estimation of the content of each species, their proportion being constant and independent of the analyzed concentration. The obtained values (mean±SD, $n=32$) were 25.7±1.7% for peak 1, 31.4±1.5% for peak 2, and 41.8±1.3% for peak 3. The method was also validated using the area of each peak versus the prepared concentration of peptide. The linear regression equations were:

$$\text{Area peak 1} = (3116 \pm 11\,406) + (11\,252 \pm 452)c; \quad r = 0.977 \quad (n = 32)$$

$$\text{Area peak 2} = (4869 \pm 8917) + (14\,032 \pm 353)c; \quad r = 0.991 \quad (n = 32)$$

$$\text{Area peak 3} = (2588 \pm 10\,915) + (18\,377 \pm 432)c; \quad r = 0.992 \quad (n = 32)$$

Results from the ANOVA of linear regression for the three peaks confirmed the linearity of the method for a significance level of 0.05 ($\alpha = 0.05$) with RSDs of 11.4, 7.14 and 6.73% for peaks 1, 2 and 3, respectively.

3.2.3. Precision

Expressed as repeatability the SEC system, precision was assessed using a SPf66 peptide solution sample, which was analyzed in triplicate on different days under the same conditions (same analyst, apparatus, identical reagents and short interval of time). The repeatability was $<2.2\%$ for the total area of all the peaks ($n=9$).

3.2.4. Accuracy

System accuracy was expressed as percentage recovery by the assay of a known added amount of pure SPf66 vaccine, the mean value being 100.8% with an RSD of 6.0% ($n=9$).

3.2.5. Detection limit

The detection limit based on the standard deviation of the response and slope [34] was 5.0 μg/ml.

3.2.6. Quantitation limit

The quantitation limit based on the standard deviation of the response and slope [34] was 15.3 μg/ml.

3.2.7. Robustness

A robustness test was performed to examine the effect of operational parameters on the analysis results. The flow-rate (1.0±0.05 ml), injection volume (50.4±1 μl), temperature (20.7±1.6°C), mobile phase composition (30±0.5 for acetonitrile, 70±1 for water, v/v) and column performance over time were determined in order to confirm the method's robustness.

To calibrate the SEC system and monitor its performance, we analyzed an SPf66 solution sample daily as standard. The estimated area for standard concentration was 1 757 013 with an RSD of 6.7%. The upper and lower limits for the control chart were established at ±3SD of this value, taking as standard deviation the value obtained from variance of the analytical method. Fig. 3 shows the control chart for the method, where the total area of all peaks holds between the established limits every time. The chromatographic conditions (e.g., flow-rate, relative mobile phase composition) and column performance, especially the tailing factor and column efficiency were checked. When necessary, corrective action was taken.

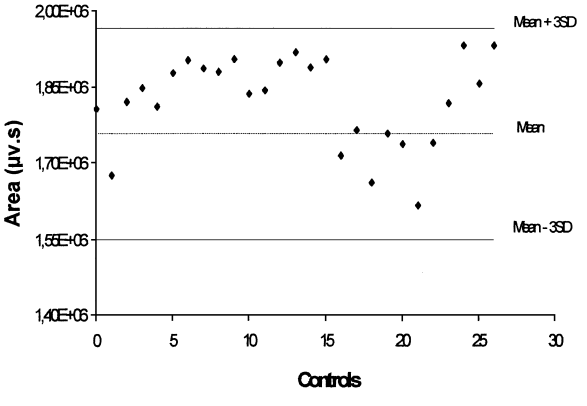

Fig. 3. Control chart for SEC–HPLC method as a function of the estimated area for standard concentration. The upper and lower limits were established at ±3SD of this value, taking as standard deviation the value obtained from variance of the analytical method.

3.3. Intra-batch samples analysis

Three different samples were analyzed in sextuplicate with the proposed method to test its suitability for detection of chromatographic profile differences (referred to three resolved peaks).

Sample 1: 10 mg of recently synthesized peptide, under standard conditions, was weighed out six times and conveniently diluted with mobile phase, previous to analysis.

Sample 2: this was treated identically to sample 1 but after storing peptide for 1 year at 5–7°C in glass vials.

Sample 3: 10 mg of the recently synthesized peptide was weighed out six times, dissolved and stored 24 h at 37°C in a buffer composed of 0.066 *M* disodium hydrogenphosphate dihydrate and 0.066 *M* potassium dihydrogenphosphate, pH 7.4 isotonized with sodium chloride; aliquots of these solutions were conveniently diluted with mobile phase previous to analysis.

Average values for the three peak areas (raw data over 10 000) were (52.90, 63.10, 83.28), (44.05, 55.29, 68.36) and (19.58, 57.15, 83.13) for samples 1, 2 and 3, respectively. Data were analyzed statistically using one-way multivariate analysis of variance (MANOVA) [37]; calculations were carried out using a matrix oriented symbolic calculation language [38]. Null hypothesis of no difference among samples were rejected for $\alpha = 0.05$. The results of multivariate individual contrast were the followings; the difference between the sample 1 (zero time) and sample 2 (after 1 year storage) was statistically significant for peak 1 (difference: 8.84; 95% confidence interval [0.514–17.18]) and peak 3 (difference: 14.93, 95% confidence interval [1.94–27.91]). These differences represent the 15.0 and 17.9% of the initial peak values, suggesting a noticeable and statistically significant alteration of the peptide; the difference for peak 2 was not significant. Only peak 1 shows a difference statistically significant when samples 1 and 3 were compared (difference: 33.32, 95% confidence interval [24.19, 42.44]); but in this difference was equivalent to a 63.0% of the initial peak value.

The obtained results show the usefulness of the proposed SEC method to detect possible variations of monomer or tetramer peak area, due to peptide degradation or any other process, with respect to an initial or predetermined value.

3.4. Comparison of the relative and absolute molecular mass

The conventional SEC is a simple and fast method for estimating the molecular mass of a protein based on its elution volume. However, the column is first calibrated with a test mixture composed of proteins with an exactly known molecular mass and the molecular masses are plotted logarithmically against elution volume to construct a column calibration curve. At this point, variance analysis is used to decide whether it is appropriate to use a quadratic or a cubic equation. Fig. 4 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 estimate ($s = 0.029$) and coefficient of determination ($r^2 = 0.999$) were best according to ANOVA results.

However, the lack of reliability of the conventional SEC method is due, among other factors, to their critical dependence on a constant flow-rate. This fact was pointed out by Knobloch and Shaklee [39], who demonstrated that a 2% error in flow-rate could result in up to a 40% error in molecular mass. So in

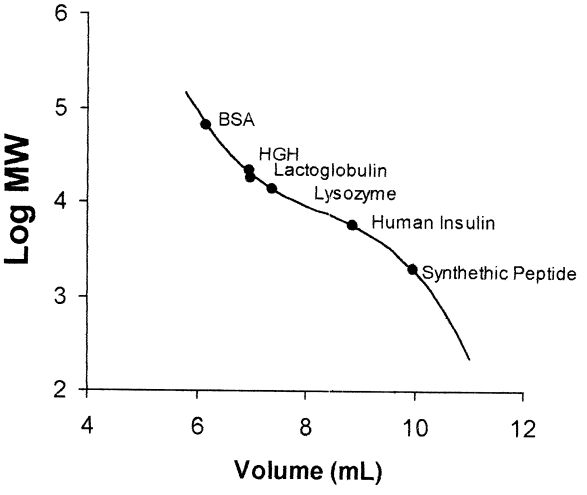

Fig. 4. Conventional SEC method calibration curve, molecular mass (M_r) versus elution volume (V), the resultant equation being fitted to a third-order polynomial model: $\log M_r = 32.1 - 9.78V + 1.15V^2 - 0.05V^3$ with a coefficient of correlation of 0.999.

order to monitor its performance, the relative molecular mass of lysozyme was used as control, obtaining a mean value of 14 060±171 ($n=3$), which varied less than 2.0% with respect to the value reported in the literature, and lower than the 5% used as a criterion to establish the system suitability test. The relative molecular masses calculated for different peaks are summarized in Table 1. At first, this result seems to suggest a clear predominance of monomer, dimer and trimer molecules.

To determine molecular mass by light scattering, the numerical value of dn/dc and the absolute concentration of the sample fraction must be known. A constant value of dn/dc of 0.186 ml/g can be assumed if the protein contains no carbohydrates and is independent of amino acid composition [15]. Thus, the absolute molecular mass of the different species of SPf66 malaria vaccine was 4632±37 Da for peak 3, whereas for peak 2 it was 8642±76 Da, a value close to twice the sequence molecular mass of 4632. A value of 17 953±81 Da was obtained for peak 1, close to four times the sequence weight, although other options cannot be rejected. These results suggest that SPf66 malaria vaccine is a clear mix, composed of the monomer (peak 3) and dimer (peak 2), whereas the peak 1 could be the trimer or tetramer.

Results from MALDI-TOF-MS confirm the molecular mass for the monomer since a principal peak about m/z 4851 Da was detected (see Fig. 5).

Lopez et al. [5] carried out the molecular mass determination of SPf66 in SDS–PAGE, indicating there was a clear predominance of monomeric, dimeric and trimeric molecules, which had a molecular mass ranging from 5 to 15 kDa, although they also detected larger size units corresponding approximately to molecular masses in the 20–25 kDa range. These results seem to confirm our data, indicating

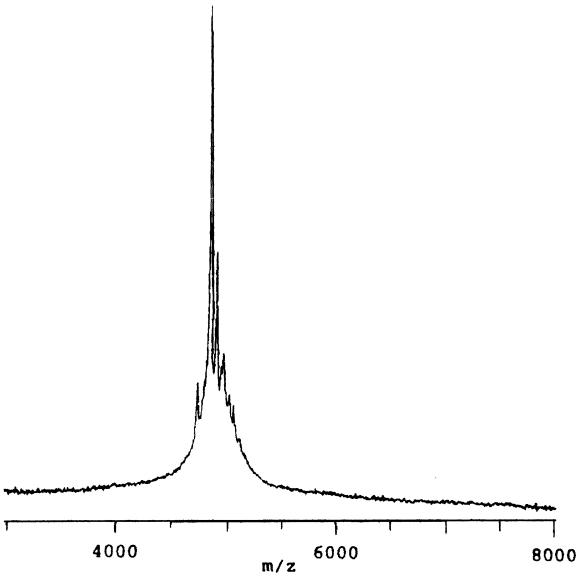

Fig. 5. MALDI-TOF mass spectrum for the monomer of SPf66 peptide obtained using a 1:10 peptide–matrix ratio.

the presence of the monomer and dimer species (peaks 3 and 2), but the identity of peak 1 is not clearly defined. The differences, Δ, between the absolute and relative molecular mass are summarized in Table 2. In this case, if the absolute molecular mass of the monomer from MALLS detection is used as the true value, then the dimer value calculated differs by about 6.7% with respect to the estimated molecular mass, whereas the trimer value varied 22.6% against 3.3% if the tetramer was considered. In contrast, if the molecular mass calculated from only their elution position was used as true value, similar behavior was observed, but the difference between the molecular mass for trimer and tetramer was higher, 12.2 and 17.7%, respectively (see Table 2). This example points out that light-scattering

Table 1
Relative and absolute molecular mass for the different species of SPf66 peptide

Peak	Elution volume (ml)	Relative M_r, conventional SEC	Absolute M_r	
			MALLS	MALDI-TOF-MS
1	7.13	17 413±209	17 953±81	N.D.
2	7.84	10 395±32	8642±76	N.D.
3	9.08	5094±31	4632±32	4850±0.9

N.D.: Not determined.

Table 2
The differences (Δ, in absolute value) between the absolute and relative molecular mass for the different species of SPf66 peptide considering a mixture composed by monomer/dimer/trimer or tetramer molecules

Species	Theoretical M_r [a]	Experimental M_r from conventional SEC	Error (%)	Theoretical M_r [b]	Experimental M_r from MALLS–SEC	Error (%)
Monomer	5094	5094	–	4632	4632	–
Dimer	10 188	10 395	2	9264	8642	6.7
Trimer	15 282	17 413	12.2	13 896	17 953	22.6
Tetramer	20 376	17 413	17.0	18 528	17 953	3.2

[a] Assuming estimated molecular mass from conventional SEC.
[b] Assuming estimated molecular mass from MALLS–SEC.

detection may be a useful tool in some instances to provide information on the molecular mass of predominant species of synthetic peptides.

In the case of SPf66 malaria vaccine, this information could be especially important because the process of auto-polymerization or depolymerization of the peptide due to the degradation process or synthesis modifications could alter its biological activity.

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