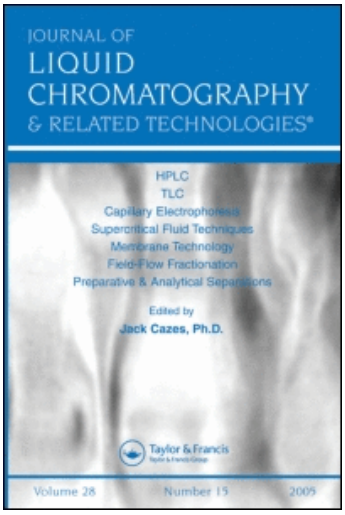

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Elution Behavior of Soluble Intercellular Adhesion Molecule 1 (sICAM-1) During Size Exclusion Chromatography

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ELUTION BEHAVIOR OF SOLUBLE INTERCELLULAR ADHESION MOLECULE 1 (sICAM-1) DURING SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

A soluble recombinant form of intercellular adhesion molecule 1 (sICAM-1) has been analyzed by size exclusion high performance liquid chromatography (HPLC). In the course of the development of an analytical method utilizing this technique an anomalous chromatographic behavior was observed. In its native state, sICAM-1 was found to elute at a higher than expected molecular weight on columns calibrated with commonly used globular protein molecular weight markers. This was rationalized on the basis of known structural features of ICAM-1 as reported in the literature. Addition of sodium dodecylsulfate (SDS) to the mobile phase was found produce an improvement in the estimation of molecular weight. It is suggested that this improvement is a consequence of a tertiary structural rearrangement of the sICAM-1 molecule and, as such, provides further evidence to support the proposed native conformation. The anomalous size exclusion behavior and improvement in molecular weight estimation with SDS was confirmed on several different types of size exclusion columns. The study demonstrates the value of size exclusion HPLC for the rapid characterization of the overall shape of a recombinant protein of pharmaceutical interest and the utility of SDS therein.

INTRODUCTION

Intercellular adhesion molecule 1 (ICAM-1) is a cell surface glycoprotein involved in a wide variety of cellular interactions in the immune system and is also the cellular receptor for the major group of rhinoviruses (1). The natural form of the ICAM-1 molecule is composed of five immunoglobulin-like extracellular domains, a hydrophobic transmembrane domain, and a short cytoplasmic domain. A soluble form of ICAM-1 consisting of only the five extracellular domains has been engineered, expressed in recombinant chinese hamster ovary (CHO) cells, and has been shown to inhibit rhinovirus infection (2). Using this recombinant CHO cell line, large amounts of soluble ICAM-1 have been prepared in our laboratory for pre-clinical investigation. As is done for other biotechnology-derived protein products, purified soluble ICAM-1 was routinely characterized using an array of analytical methods with a heavy emphasis on chromatographic and electrophoretic techniques (3). In this study, the behavior of soluble ICAM-1 during size exclusion HPLC was studied in detail. Our initial work focused on the establishment of a reliable and quantitative HPLC method for the determination of protein concentration in purified soluble ICAM-1 samples. Parameters such as column recovery, detector linearity and reproducibility were examined. Following this, molecular weight calibration curves were constructed using standard globular proteins under both denaturing and non-denaturing mobile phase conditions. These curves were used to estimate the apparent molecular weight of soluble ICAM-1 under each condition. The data suggest that the gross tertiary structure of soluble ICAM-1 plays a significant role in its distribution between intra- and extraporous space in the chromatographic packing. The effect of SDS binding to globular protein standards on size exclusion behavior is also presented.

TABLE I
Protein Molecular Weight Markers Used for Calibration of Size Exclusion Columns

PROTEIN	MOLECULAR WEIGHT	SUPPLIER
Thyroglobulin	669,000	Sigma
ß-Amylase	206,000*	Sigma
Anti-ICAM-1	160,000	Boehringer Ingelheim
Bovine serum albumin	66,000	Sigma
Ovalbumin	44,000	Sigma
Carboxypeptidase	34,600	Sigma
Soybean trypsin inhibitor	21,500	Sigma

*In the SDS-containing mobile phase, the ß-Amylase subunit molecular weight of 57,000 was used (4).

EXPERIMENTAL

Materials

All buffer components and sodium dodecylsulfate were from commercial sources and were reagent grade or better. Water was purified on a Barnstead (Dubuque, IA) NanoPure system.

A large number of commonly used globular protein gel filtration standards were obtained from various commercial suppliers and were examined individually for their suitability. Markers were selected based on three criteria:

1. evenly-spaced distribution of markers which covered the range desired
2. good purity as indicated by size exclusion HPLC and SDS-PAGE
3. well-characterized molecular weight according to literature references.

A consensus set of seven standards were chosen for all studies as shown in Table I.

Soluble ICAM-1 was prepared according to a previously published immunoaffinity purification procedure (1,2). The cell

culture supernatant used as starting material was prepared in-house via large-scale cell culture of a recombinant CHO cell line expressing sICAM-1.

Chromatographic Conditions

The HPLC system consisted of a BioRad (Richmond, CA) AS-48 refrigerated autosampler, a Waters (Milford, MA) Model 590 pump operated at 1 millilter per minute and a Kratos (Ramsey, NJ) Spectroflow 757 variable wavelength UV detector set at 220 nm. The mobile phase employed was either 50 mM sodium phosphate plus 150 mM sodium chloride, pH 7.0 or 23 mM sodium phosphate plus 0.1% sodium dodecylsulfate, pH 6.8. The injection volume was 20 microliters. Data was collected on a Hewlett-Packard HP1000 Lab Automation System utilizing a 18652A A/D converter. Chromatography was performed at room temperature.

Toyo Soda gel filtration columns (TSKGel 3000SW and 4000SW, both 7.5 mm i.d. x 300 mm length) were purchased from Anspec (Ann Arbor, MI). Dupont gel filtration columns (Zorbax GF250 and GF450, both 9.4mm i.d. x 300 mm length) were purchased from MacMod Analytical (Wilmington, DE). The appropriate manufacturers' guard column was also used in each case.

Electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Phast System (Pharmacia-LKB Biotechnology, Piscataway, NJ) using precast Phast system gels. Coomassie Blue staining was performed according to the manufacturer's recommended procedure.

RESULTS

Figure I shows, in cartoon form, the hypothesized structure for soluble ICAM-1 (right). It has been genetically engineered

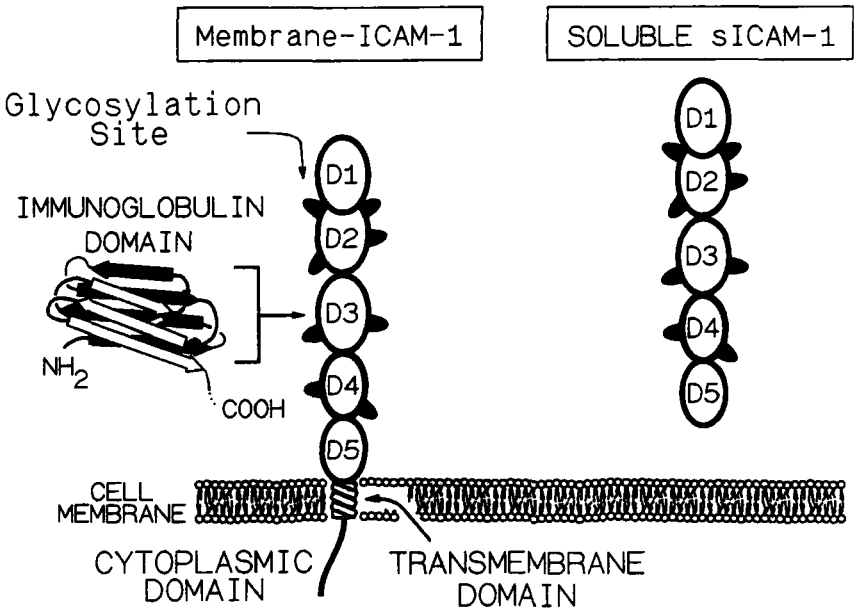

FIGURE I - Idealized ICAM-1 structures. Natural membrane-bound ICAM-1 and genetically engineered soluble ICAM-1 are depicted. Five immunoglobulin-like domains are designated D1 through D5 as shown. The structural features were predicted from analysis of DNA and protein sequence, homology with other characterized proteins, molecular modeling, and derived from electron microscopy, and biochemical analysis (1,6).

from the natural human membrane-bound glycoprotein ICAM-1. The recombinant form is expressed in chinese hamster ovary (CHO) cells which secret the soluble ICAM-1 into the culture medium. Purification is then performed using anti-ICAM-1 immunoaffinity chromatography. Biological activity of the prepared material was confirmed (5). SDS-PAGE was run on the purified product and is shown in Figure II. Based on protein standards run on the same gel, the molecular weight was estimated to be 80,000 +/- 7,000 daltons. This value was also consistent with sequence data for ICAM-1 (6). Protein purity was estimated as > 95% based on scanning densitometry of the sICAM-1 lane.

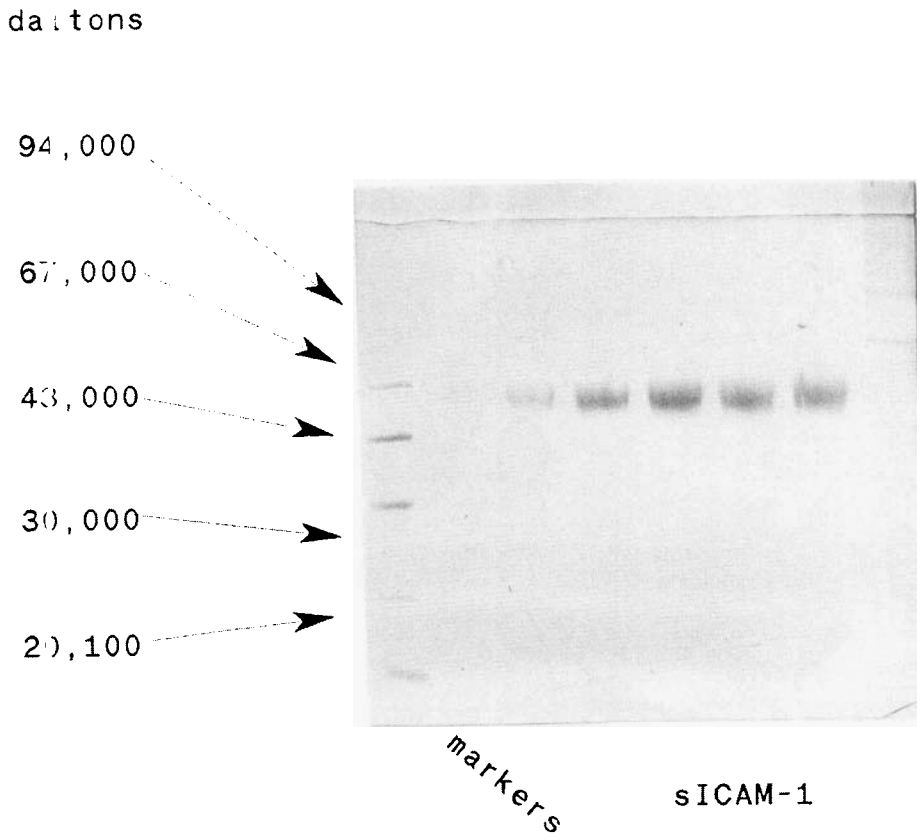

FIGURE II - Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of sICAM-1 used in this study. A molecular weight 80,000 +/- 7,000 was estimated based on standards.

A representative chromatogram of purified soluble ICAM-1 on a TSKGel 3000SW column using a non-denaturing mobile phase is shown in Figure III. A reasonably symmetrical peak was obtained for the main component. The peak eluting at approximately 15 minutes corresponds to the permeation volume of the column and comprises UV-absorbing sample buffer components. A series of dilutions were prepared and analyzed in duplicate using these chromatographic

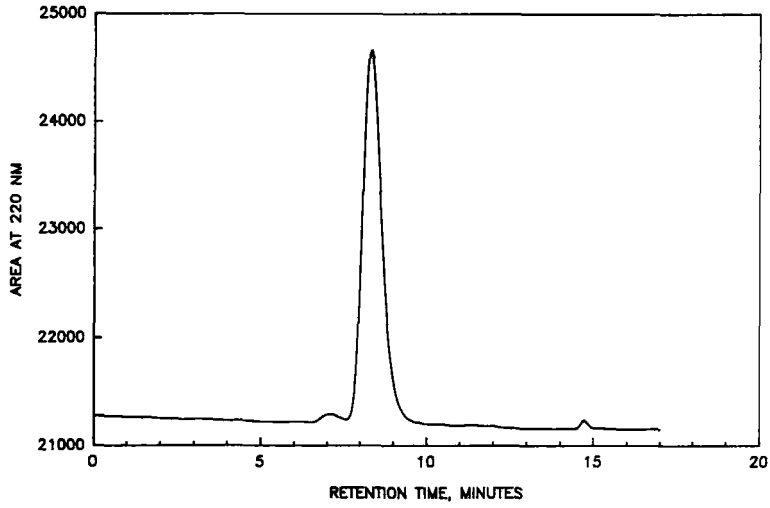

FIGURE III - High performance size exclusion chromatography of sICAM-1 on a TSKGel 3000SW column. Mobile phase contains 50 mM sodium phosphate + 150 mM sodium chloride, pH 7.0. Other HPLC conditions are described in the Experimental section.

conditions. Protein concentration was determined prior to dilution by amino acid analysis. The resulting standard curve, based on peak area of the main component, is shown in Figure IV. As can be seen, good linearity (R >0.999) was obtained. The x-intercept was found to be close to zero, suggesting high column mass recoveries (95% for 1 microgram injection) and reinforcing the identification of the main component as soluble ICAM-1.

Other features of the method of interest for quantitative purposes included a detection limit of approximately 1 µg/mL (5x baseline noise) and an injection reproducibility, based on peak area, of better than 1%. At 220 nm, the phosphate-buffered mobile phase added only 0.75 absorbancy units to the baseline relative to operation at 280 nm. Furthermore, there was no evidence of curvature at the upper end of the calibration plot, which would otherwise suggest saturation of the detector absorbancy range.

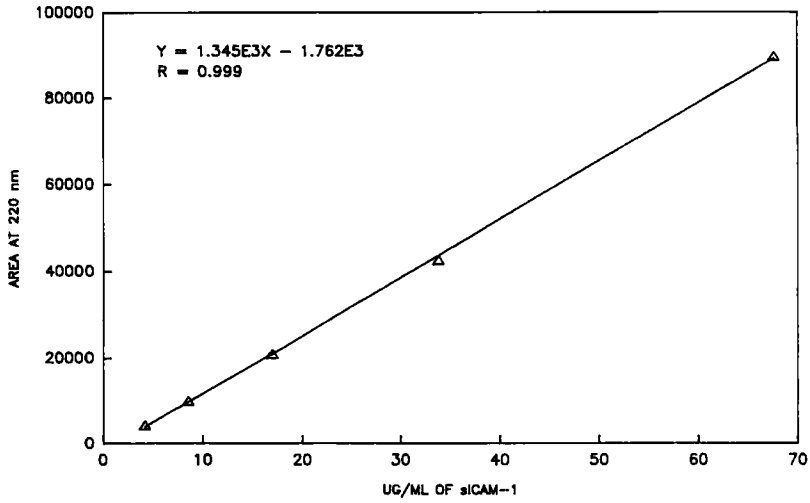

FIGURE IV - Standard curve for sICAM-1 on the TSKGel 3000SW column.

This method has been reliably used in several laboratories in our facility for over one year for quantitation of soluble ICAM-1 concentration in purified preparations.

In order to determine whether the column was behaving in a size exclusion mode, a set of globular protein molecular weight standards were chosen (Table I) as described in the Experimental section. The resulting molecular weight calibration curve is shown in Figure V. As expected, a linear correlation between the logarithm of molecular weight and elution position was found. Using this correlation, the molecular weight of soluble ICAM-1 was calculated to be 213,000 daltons. This deviates significantly from the 80,000 dalton figure estimated from SDS-PAGE (see Figure II) and sequence data (6).

A chromatogram of soluble ICAM-1 obtained with 0.1% SDS in the mobile phase on the same TSKGel 3000SW column is shown in Figure VI. The peak shape and width are comparable to that found under non-denaturing conditions (Figure III). This demonstrates

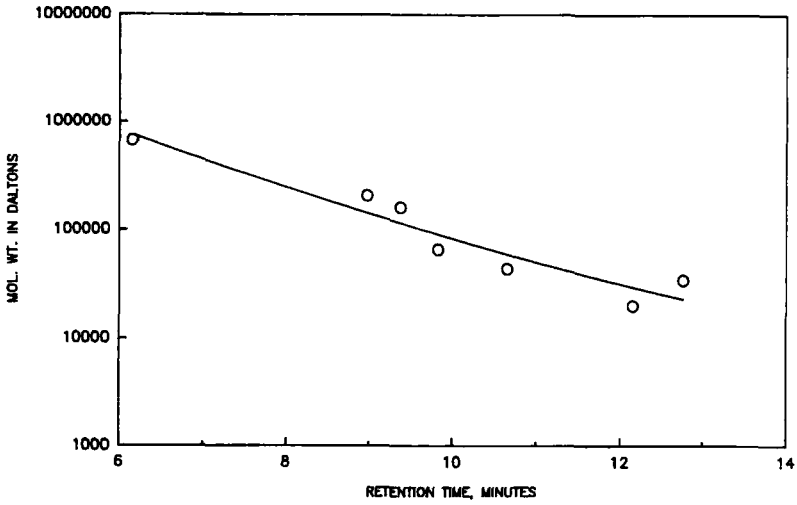

FIGURE V - Molecular weight calibration curve on a TSKGel 3000SW column. Mobile phase is described in Figure III. Globular protein standards used are listed in the Experimental section. A polynomial regression of second order was applied.

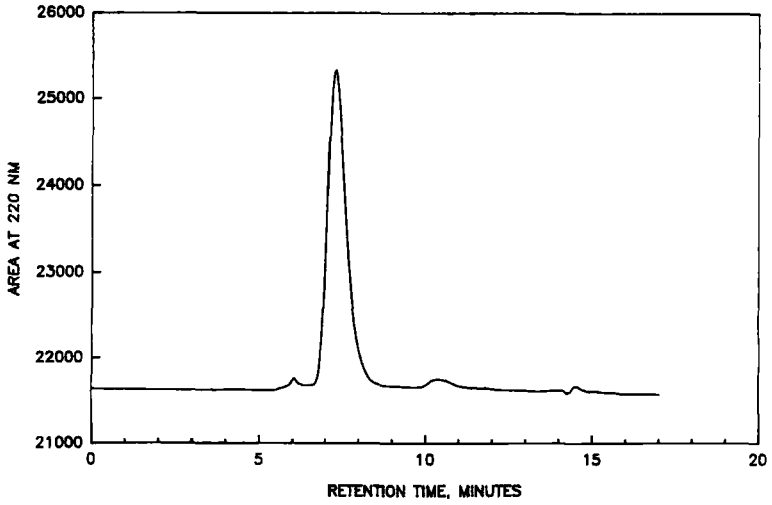

FIGURE VI - High performance size exclusion chromatography of sICAM-1 on a TSKGel 3000SW column. Mobile phase was 23 mM sodium phosphate plus 0.1% sodium dodecylsulfate, pH 6.8. Other HPLC conditions are described in the Experimental section.

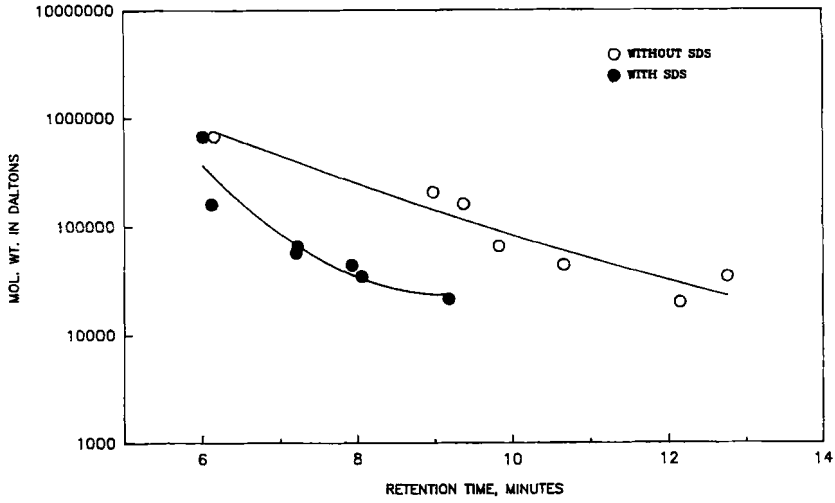

FIGURE VII - Molecular weight calibration curves for the TSKGel 3000SW column in each of two mobile phases. Globular protein standards used are listed in the Experimental section. For each data set, a polynomial regression of second order was applied.

that the elution of soluble ICAM-1 could be studied equally well under denaturing conditions.

Molecular weight standard curves were generated both with and without SDS in the mobile phase on the TSKGel 3000SW column as shown in Figure VII. Inclusion of SDS in the mobile phase was found to produce a drop in apparent molecular weight from 213,000 daltons to 62,000 daltons (Table II); the relative error in the molecular weight determination drops from 166% to 22%. Three other well-known commerically available aqueous size exclusion columns of different pore sizes, particle diameters and surface chemistries (7) were tested and their calibration curves are shown in Figures VIII, IX and X. A complete summary of the molecular weight data is shown in Table II.

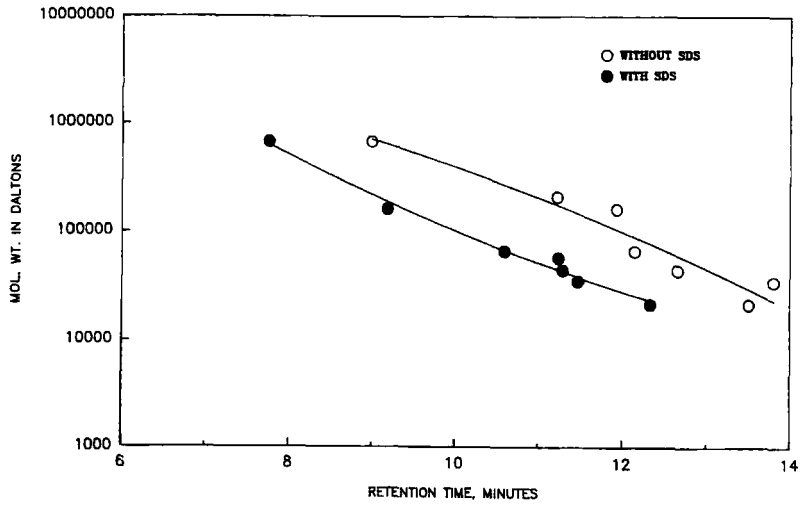

FIGURE VIII - Molecular weight calibration curves for the TSKGel 4000SW column in each of two mobile phases. See Figure VII for other information.

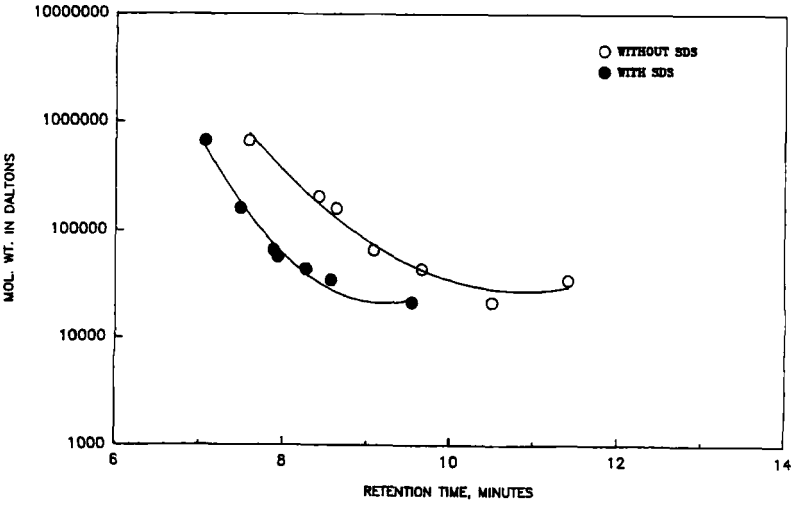

FIGURE IX - Molecular weight calibration curves for the Zorbax GF250 column in each of two mobile phases. See Figure VII for other information.

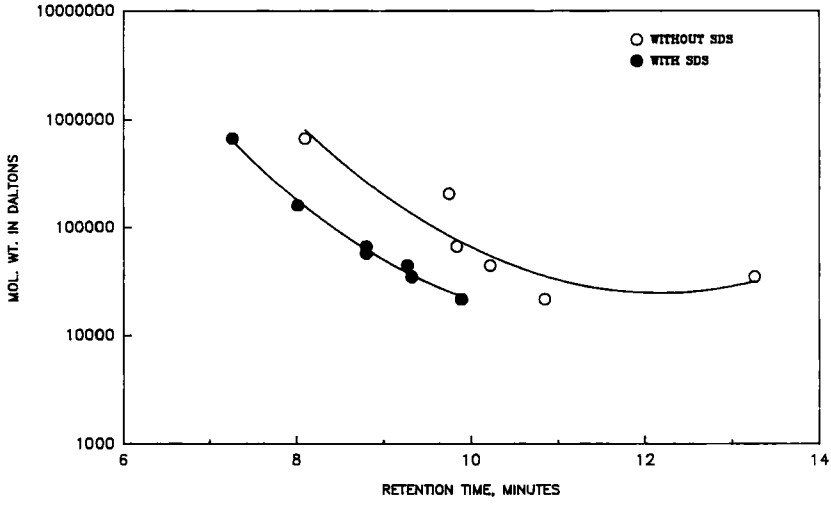

FIGURE X - Molecular weight calibration curves for the Zorbax GF450 column in each of two mobile phases. See Figure VII for other information.

TABLE II
Molecular Weight (MW) of sICAM-1 Calculated from Calibration Curves

Column	WITHOUT SDS		WITH SDS	
	MW	% error*	MW	% error*
TSKGel 3000SW	213,000	166	62,300	22
TSKGel 4000SW	220,000	175	60,100	25
Zorbax GF250	229,000	186	85,000	6
Zorbax GF450	167,000	109	56,800	29

* Relative error with respect to 80,000 daltons as determined by SDS-PAGE (See Figure II).

In each case, the relative error in the estimation of molecular weight drops significantly upon inclusion of 0.1% SDS in the mobile phase. For the four columns studied, the mean relative error drops from 159% to 20%.

DISCUSSION

The anomalously high estimate of molecular weight found using non-denaturing conditions was confirmed independently by several other laboratories in our facility. The source of this deviation was suspected to lie in the known molecular asymmetry of the soluble ICAM-1 molecule. Electron microscopy has been used to show that ICAM-1 is a 19 nm x 2-3 nm hinged rod-like molecule (1), supporting the concept that the arrangement of domains within the protein is extended and unpaired rather than clumped or globular (See Figure I). Such an arrangement would tend to increase the radius of gyration of the molecule relative to a more spheroidal type of configuration. A higher radius of gyration would limit the extent of penetration into the pores of the gel filtration matrix (8,9). The result would be a smaller elution volume and a higher apparent molecular weight.

An attempt was made to destroy the molecular asymmetry of the soluble ICAM-1 molecule by inclusion of a denaturant (SDS) in the size exclusion mobile phase. It was found that the estimation of molecular weight was consistently improved with the use of SDS. This suggested that the SDS mobile phase was inducing denaturation and conformational rearrangement of soluble ICAM-1 to a gross tertiary structure more akin to those of the molecular weight standards used for calibration *under the same conditions*. Thus, the overall effect of the SDS was to dampen specific structural differences between the sample (sICAM-1) and the calibration standards, making the estimation of molecular weight by size exclusion HPLC more accurate. In this respect, SDS serves the same role here as in SDS-PAGE.

For both soluble ICAM-1 and the protein standards, SDS would be expected to bind in a cooperative manner, accompanied by conformational changes in the proteins (10). Such protein-SDS complexes would have higher masses than their uncomplexed native protein counterparts; more restricted access to the pores of the column packing would result. This may be the source of the increased curvature in the high molecular weight region of the calibration plots with SDS, particularly for the smaller pore diameter columns (Figures VII, IX). It should be noted that the use of SDS in size exclusion chromatography has been suggested in a recent compendial monograph on analytical methods for biotechnology-derived products (3).

An alternative explanation for the results presented in this study is that soluble ICAM-1 exists as a dimer, trimer or higher multimer under native conditions and SDS simply serves to break up these multimeric forms into monomeric species. However, two points tend to argue against this possibility. First, there is little evidence from the chromatograms of an equilibrium between monomer and higher multimer. For example, under native conditions, a small monomeric component might be expected to appear after the main component, particularly since dilution of the peak through the column would favor increasing monomer concentration through simple mass action considerations. This is not evident in Figure III. Secondly, in Table II, no common integral multiple of molecular weights exists between different mobile phase conditions among the various columns tested. If the effect of SDS were primarily to break up multimeric forms into monomeric species, a consistent integral might be expected, regardless of the column used.

In summary, a quantitative size exclusion HPLC method for determination of soluble ICAM-1 concentration in purified samples was established. Under non-denaturing mobile phase conditions, soluble ICAM-1 eluted at an anomalously high molecular weight, possibly due to its extended rod-like shape. A significant improvement in molecular weight estimation was realized by

inclusion of 0.1% SDS in the mobile phase. The changes in apparent molecular weight found upon inclusion of 0.1% SDS in the mobile phase serve to confirm the gross shape of the native soluble ICAM-1 molecule.

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REFERENCES

1. Staunton, D.E., Dustin, M.L., Erickson, H.P., and Springer, T.A., Cell, 61, 243 (1990).

2. Marlin, S.D., Staunton, D.E., Springer, T.A., Stratowa, C., Sommergruber, W., and Merluzzi, V.J., Nature, 344, 70 (1990).

3. *Pharmacopeial Forum*, In-Process Revison, May-June 1990, United States Pharmacopeial Convention, Inc., Rockville, MD, 1990, p. 490.

4. (a) Colman, P.M., and Matthews, B.W., J. Mol. Biol., 60, 163 (1971). (b) Takeda, Y., Hizukuri, S., and Murakami, T., Agr. Biol. Chem., 35, 778 (1971). (c) Kim, J.P., Ann, Y.G., and Shim, W.M., Han'guk Saenghwa Hakhoechi, 18, 290 (1985).

5. Last-Barney, K., Marlin, S.D., Cahill, C., Jejer, R., Fortugno-Erikson, D., O'Neill, M., Hayden, F., and Merluzzi, V.J., Antiviral Research SI, 107 (1991).

6. Staunton, D.E., Marlin, S.D., Stratowa, C., Dustin, M.L., and Springer, T.L., Cell, 52, 925 (1988).

7. Anspach, B., Gierlich, H.U., and Unger, K.K., J. Chromatogr., 443, 45 (1988).

8. Le Maire, M., Aggerbeck, L.P., Monteilhet, C., Andersen, J.P., and Moller, J.V., Anal. Biochem., 154, 525 (1986).

9. Dubin, P.L., Kaplan, J.I., Tian, B.-S., and Mehta, M., J. Chromatogr., 515, 37 (1990).

10. Helenius, A., and Simons, K., Biochim. Biophys. Acta, 415, 29 (1975).