Solution Stability of the Monoclonal Antibody–Vinca Alkaloid Conjugate, KS1/4-DAVLB

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A 3-month solution stability study at 5°C of the monoclonal antibody–vinca alkaloid conjugate KS1/4-DAVLB indicated that phosphate-buffered saline solutions at pH 4.5–5.5 had little tendency to lose vinca by hydrolysis, improved vinca stability, showed acceptable physical stability, and formed minimal amounts of soluble aggregates compared to solutions at pH 6.5–7.4. Hydrolysis and aggregation with concomitant loss of stability were accelerated at 30°C throughout the pH range investigated. As determined by ELISA, the binding properties of KS1/4-DAVLB to tumor antigens were not affected by pH or temperature. A formulation suitable for initial clinical trials in cancer patients is described.

KEY WORDS: monoclonal antibody–drug conjugate; vinca alkaloid; solution stability.

INTRODUCTION

The development of monoclonal antibody (MoAB) technology (1) made it possible to couple cytotoxic drugs, toxins, and radioisotopes to antibodies to investigate site-directed therapy (2,3). Studies have demonstrated tumor localization of MoAB conjugates (2–4).

KS1/4 MoAB (approximately 150,000 Da) is a murine IgG2a monoclonal antibody that recognizes gp40, a 40-kDa glycoprotein cell surface antigen found on the human lung adenocarcinoma cell line P3/UCLA (5). It appears to possess sufficient specificity for lung adenocarcinoma to be of potential diagnostic and therapeutic use (6). Deacetylvinblastine (DAVLB; a derivative of vinblastine) has been chemically linked to KS1/4 antibody to make KS1/4-DAVLB conjugate (6–8). Typically, four to six DAVLB molecules are coupled to each KS1/4 via succinate linkages from the 4-position of DAVLB to the ε-amino groups of lysine residues in KS1/4 (Fig. 1). The resulting conjugate has a therapeutic index greater than that of either DAVLB or KS1/4 alone (6,7).

The objective of this investigation was to develop a solution formulation for initial clinical studies in cancer patients by intravenous infusion. The effects of pH and temperature on the physical and chemical stability of KS1/4-DAVLB in solution were investigated. Excessive hydrolysis of vinca species from the conjugate could increase the toxicity of the preparation. Chemical degradation of the conjugated vinca could result in loss of antitumor activity. Protein degradation in or around the active site of KS1/4 would result in loss of the binding function and antigen specificity. Association of the conjugate to yield soluble aggregates could result in potency loss and possible increases in immunogenicity; insoluble aggregates pose a serious issue for an intravenous preparation. Each of these possible events was investigated. In addition, the qualitative effects of pH and NaCl concentration on KS1/4-DAVLB solubility were examined.

MATERIALS AND METHODS

Stability Program. KS1/4-DAVLB conjugate at approximately 5 mg/ml was prepared in phosphate-buffered saline (PBS) solution at pH 7.4. The solution was divided into four sections; three were adjusted to pH 4.5, 5.5, and 6.5, respectively, with dilute hydrochloric acid and one section remained at pH 7.4. Each section was filtered through a 0.22-μm membrane filter into sterile Type 1 borosilicate glass vials (5 ml), which were closed with halobutyl rubber stoppers. The closures were secured with aluminum seals. After the initial analytical evaluation, samples of each section were stored at −20 (control), 5, and 30°C. Samples were taken after 1.5 and 3.0 months for analysis.

pH, Clarity, and Physical Appearance. Immediately after removal from storage, samples were visually inspected against a light source by three independent observers. The clarity (NTUs) was determined using a turbidimeter (Hach, Model 18900). pH values were measured using a pH meter (USP test method). Samples from these tests were filtered through 0.22-μm Millex-GV (Millipore) filters to remove any precipitate; the filtrates were used for the remaining analyses.

Reversed-Phase High-Performance Liquid Chromatography (HPLC) for Determination of Free Vinca in Conjugate Solution. A Varian (Model 5000) HPLC system equipped with IBM C_{18} (4.6 × 150-mm) column (thermostated at 30°C) was used. A porous silica gel precolumn between pump and injector and a C_{18} bonded pellicular bead guard column (2 to 5 cm) between injector and column were used. The mobile phase for gradient elution was methanol plus a pH 7.4 buffer (diethylamine, 15 ml per liter, adjusted to pH 7.4 with phosphoric acid). The first 5 min of the elution was with 50% methanol. From 5 to 25 min, the gradient was increased linearly at the rate of 2% methanol/minute to 90% methanol. The flow rate was 2 ml/min. The column eluant was monitored by a UV detector at 270 nm. The least-squares calibration curves were established for quantitation of 4-succinoyl-DAVLB (4S-DAVLB) free base and DAVLB free base using their reference standards.

Hydrolysis and Stability of Conjugated Vinca. A portion of each sample was lyophilized and then treated with concentrated HCl for 8 hr at room temperature to cleave the vinca from the antibody as DAVLB. Hydrolyzed samples were analyzed by reversed-phase HPLC as described above. The control samples stored at −20°C were also hydrolyzed and analyzed for comparison.

UV Analysis for Total Protein and Total Vinca Content. The concentrations of the antibody and total vinca (bound plus free, reported as 4S-DAVLB) were calculated from the

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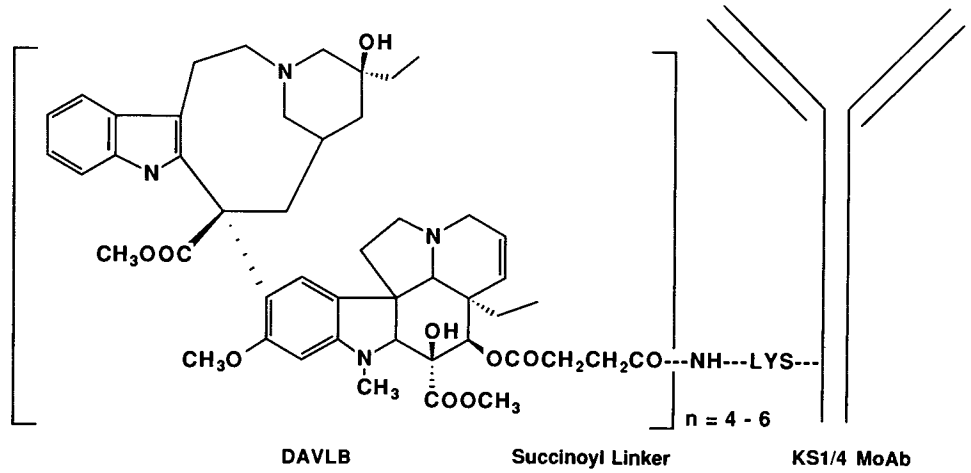

Fig. 1. Structure of KS1/4-DAVLB conjugate.

known absorptivities of KS1/4 and 4S-DAVLB using a least-squares fit for the measured absorbances at 270, 280, 310, and 315 nm (9,10). The approximate maximal wavelengths for the vinca alkaloid and MoAB are 270 and 280 nm, respectively. The average of four replicates for the UV analysis of each lot, assayed shortly after it was manufactured, was used as a reference concentration. The ratio of the total vinca in each sample to the reference value is reported as percentage theory. The ratio of the total vinca at the later time points to the initial data is reported as percentage initial.

Laser Nephelometric Immunoassay for Total IgG in KS1/4-DAVLB (Total Protein Content). An automated system consisting of the laser nephelometer, automatic diluter, transport module (Behring Diagnostics, Model LN), and HP85 computer (Hewlett Packard) was used to quantitate the total mouse IgG. The automatic dilutor made a 0.1 mg/ml solution of KS1/4 reference standard in diluent (3% polyethylene glycol 8000 in pH 7.4 PBS filtered through a 0.2-μm filter). Several dilutions (3.125, 6.25, 12.5, 25.0, 50, and 100 μg/ml) were made. Samples were checked for clarity and the pH was adjusted to 6.8–8.3 prior to dilutions that were expected to fall within the range of the standards. PBS was included as a blank. Fifty microliters of each standard or sample was added to a cuvette. Using the automatic dilutor, 10 μl of anti-mouse IgG antiserum and 190 μl of the diluent were added to cuvettes containing 50 μl of either sample or standard.

The transport module was set to provide a 30-min interval between the addition of reagents; a reading of the light scatter was taken 30 min after the addition of the last reagents. Standards and sample dilutions were analyzed in duplicate. Data analysis was performed using a four-parameter model described by Rodbard *et al.* (11) using nonlinear least-squares curve fitting. The concentration of total mouse IgG in the samples was estimated using a weighted average of the individual dilutions. The weighting factor for each dilution was inversely proportional to the square of the standard error in its estimated potency. Since fetal calf serum may be used in the cell culture process, each lot of goat anti-mouse IgG antibody (ICN Immunobiologicals) was tested for cross-reactivity against bovine albumin and bovine IgG. The antiserum was kept frozen at −20°C and filtered through a 0.2-μm filter prior to use.

Enzyme-Linked Immunosorbent Assay (ELISA) for Antibody Function. Microtiter plates (Nunc Plate II U) were coated with approximately 1×10^5 P3/UCLA cells/well as the antigen source. Plates were dried at 37°C overnight, then covered with parafilm and stored. Before assay, each well was washed three times with a sterile rinsing buffer composed of 1 m*M* sodium phosphate, 154 m*M* sodium chloride, 0.2% Tween 20, and 0.5 m*M* thimerosal (final pH 7.4). Buffer of the same composition containing 0.1% bovine serum albumin (BSA) was used for the dilution of samples. Appropriately diluted samples or standards were added to each well and held for 30 min at room temperature. Each well was then washed with the rinsing buffer followed by the addition of 0.01 unit of horseradish peroxidase-conjugated goat anti-mouse IgG + IgM (Tago 6443) enzyme. The plate was set at room temperature for 30 min, then rinsed with the buffer. A solution of 0.26 m*M* hydrogen peroxide and 2.2 m*M* o-phenylenediamine in 80 m*M* citrate–phosphate buffer (pH 5.0) was added to each well and held for 30 min at room temperature in the dark. The reaction was quenched by adding 4 *N* sulfuric acid to each well, and the absorbance at 490 nm minus the absorbance at 630 nm was measured by a Dynatech MR600 microplate reader. Concentrations were determined by comparison to a standard curve obtained using known concentrations of KS1/4-DAVLB placed on each plate.

Size-Exclusion Chromatography for High Molecular Weight Substances. A Pharmacia FPLC system equipped with a Superose 12 (HR 10/30) column was used at a flow rate of 0.5 ml/min. The mobile phase composition was 50 m*M* K_2HPO_4 and 100 m*M* NaCl, adjusted to pH 10 with 5 *N* NaOH; it was filtered and degassed prior to use. The column eluant was monitored at 280 nm for UV absorbance. Samples were injected at both high (1–2 mg/ml) and low (1/20 dilution) concentrations. The impurities detected at the high level were quantitated versus the main peak detected at the low level (12).

Effect of NaCl and pH on KS1/4-DAVLB Solubility. Precut dialysis bags (Spectra-Por membrane tubing; molecular weight cut off, 12,000–14,000) were soaked for 60 min in distilled water and then thoroughly rinsed (20 min each) with distilled water, 30% ethanol, and again with distilled water. Immediately prior to use, they were soaked in dialyzing

Table I. Free Vinca[a] Content (μg/ml) from Hydrolysis of KS1/4-DAVLB

pH	Initial	5°C 1.5 months	5°C 3.0 months	30°C 1.5 months	30°C 3.0 months
7.4	6.1	9.2	5.6	0.9	0.6
6.5	5.6	8.3	5.8	2.8	1.2
5.5	5.6	6.8	5.8	8.0	4.8
4.5	5.6	6.8	5.9	7.1	5.9

[a] Total of DAVLB-succinate and DAVLB.

buffer for 60 min. Ten-milliliter portions of the KS1/4-DAVLB conjugate (about 5 mg/ml) in PBS, pH 5.2, were dialyzed for 48 hr at 5°C against 4 liters of NaCl (pH 5.2) at concentrations of 0, 2, 4, 6, and 8 mg/ml. The bulk saline solutions were stirred with a magnetic stirring bar and were changed after 24 hr. A blank (10 ml of injectable water) was set up for visual control. After the solvent exchange, 1 ml of each saline solution was titrated in 0.01-ml increments with 0.01 *N* NaOH to pH 10.5. The solution was stirred and equilibrated after each addition (Impulsomat/Set-Point Titration System, Metrohm). Another 1-ml portion of each sample was titrated with 0.01 *N* HCl to pH 3.5. Acidic and basic solutions were then titrated back to pH 10 and pH 3.5, respectively. All of the titrations were performed in duplicate. The presence or absence of precipitated conjugate was recorded at each 0.1 pH unit.

Table II. Stability of KS1/4-DAVLB Conjugate

pH	Reference[a]	Recovered vinca,[b] μg/ml (% total) 3 months/5°C	Recovered vinca,[b] μg/ml (% total) 3 months/30°C
7.4	34 (29)	37 (32)	6 (5)
6.5	31 (27)	34 (29)	13 (11)
5.5	34 (29)	34 (29)	26 (22)
4.5	32 (28)	43 (37)	23 (20)

[a] Pseudoinitial samples, maintained frozen at −20°C until the time of hydrolysis.

[b] DAVLB and DAVLB-succinate (HPLC) after 8-hr hydrolysis in aqueous HCl. Values corrected for initial "free" vinca content.

RESULTS

Hydrolysis of Vinca from KS1/4-DAVLB. After 3 months at 5°C, the total unconjugated or "free" vinca (4S-DAVLB and DAVLB) concentrations at pH 4.5–7.4 were essentially unchanged from initial assays, indicating that at 5°C there is very little hydrolytic release of vinca from KS1/4-DAVLB (see Table I and Fig. 2A). At 30°C, the degree of hydrolysis of vinca from the conjugate was more difficult to estimate due to chemical degradation of the vinca in solution. For example, the degradation of free vinca in the pH 7.4 and 6.5 samples results in total free vinca contents (4S-DAVLB plus DAVLB) that are 11 and 21%, respectively, of the initial values after 3 months at 30°C. At pH 4.5 and 5.5, the free vinca is much more stable; 86% remains at pH 5.5 and there appears to be no or minor degradation at pH 4.5

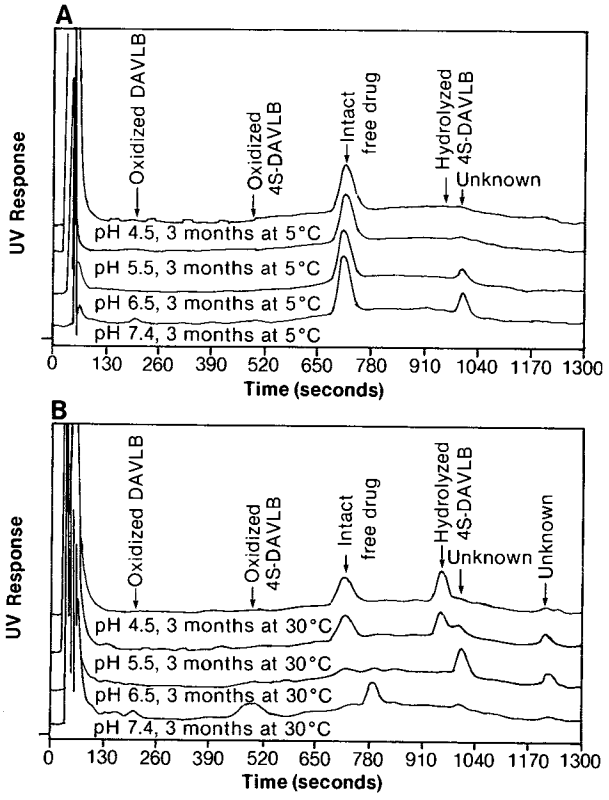

Fig. 2. Free vinca content in KS1/4-DAVLB by RP-HPLC. (A) Storage at 5°C for 3 months; (B) storage at 30°C for 3 months.

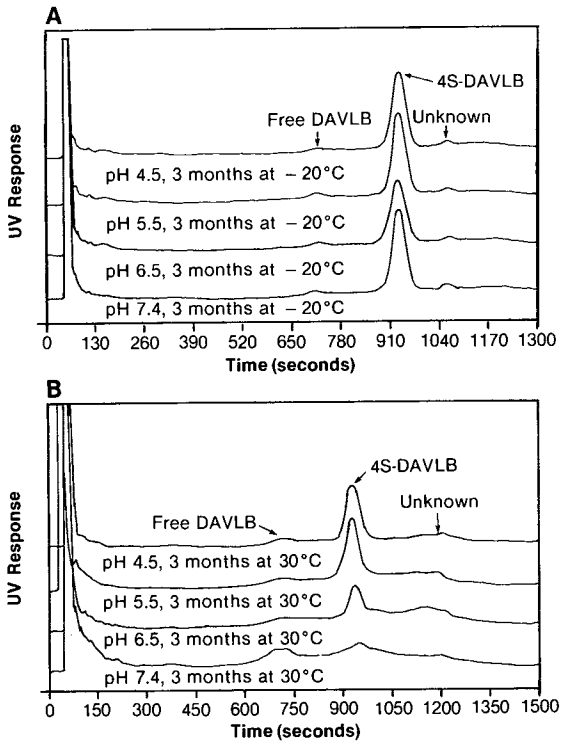

Fig. 3. Vinca integrity determined by RP-HPLC following HCl hydrolysis of aged KS1/4-DAVLB conjugate. (A) Storage at −20°C for 3 months; (B) storage at 30°C for 3 months.

Table III. Total KS1/4-DAVLB Concentration (mg/ml) at Initial, 3 Months at 5°C, and 3 Months at 30°C

pH	Laser nephelometry	UV	ELISA
7.4	4.2–4.0–3.8	4.7–4.5–4.5	3.5–3.6–3.1
6.5	4.2–4.0–3.7	4.9–4.7–4.5	3.5–3.2–3.4
5.5	4.2–4.0–3.9	4.9–4.7–4.7	3.5–3.2–3.3
4.5	4.2–4.1–3.7	4.9–4.7–4.4	3.4–3.2–3.3

after 3 months at 30°C. Examination of RP-HPLC chromatograms (Fig. 2B) shows that mostly 4S-DAVLB and DAVLB are observed at pH 4.5 and that more oxidative and unidentified degradation products are observed as the pH increases. These data demonstrate the importance of pH on the chemical stability of the vinca species; hydrolytic release of vinca from the conjugate and concerns about toxicity of free vinca are less significant.

Stability of Conjugated Vinca. HPLC analysis of the HCl hydrolysates was used to detect degradation of the vinca moiety on the intact conjugate. A low recovery of DAVLB was obtained for all samples; however, examination of the yields and purity of the recovered vinca is probably descriptive of the state of the conjugated species. Similar recoveries (27–37% of the total) of undegraded vincas were obtained from 3-month, 5°C samples compared to "pseudoinitial" samples which had been stored at −20°C (Table II). After 3 months, vinca recoveries from 30°C samples ranged from 5 to 20% of the total, with the lowest recoveries occurring at pH 6.5 and pH 7.4. The vincas recovered for the higher pH samples were highly degraded (Fig. 3). These data suggest that conjugated vinca has similar pH-dependent degradation behavior as free vinca.

Protein Content and Antibody Function. At 5°C, the protein content determined by both UV assay and laser nephelometric immunoassay was essentially unchanged after 3 months (Table III). The protein concentration in the 30°C samples did decrease slightly; however, the decrease did not appear to be pH related. The ELISA data indicate that there is little change in antibody function over 3 months at either 5 or 30°C.

Physical Stability and Protein Aggregation. During these studies, the pH values of all the sample solutions remained constant. After 1.5 months, all samples contained some precipitate regardless of pH. Unambiguously, there was more separated material in 30°C samples. The 1.5-month samples at pH 7.4 and 6.5, at both 5 and 30°C, exhibit a noticeable film deposited on the bottom of the vials, while the film was not observed in pH 4.5 and 5.5 samples. After 3 months, all samples had a thin film at the bottom of the vials and it was more difficult to distinguish 5 and 30°C samples by visual observation. Color variations in the sample were more discernible; at pH 6.5 and 7.4, samples were yellowish, while the solutions stored at pH 4.5 and 5.5 were colorless. Due to the various sized particles in the sample and the presence of the separated film, clarity determination by nephelometry was difficult and misleading. After 3 months of storage, the overall physical appearance of the pH 4.5 and 5.5 samples was much superior to that of the pH 6.5 and 7.4 samples.

Results of size exclusion analysis indicate that at each pH condition, 30°C samples contain significantly more soluble aggregates than 5°C samples (Tables IV and V). The monomer contents of 30°C samples are 12–22% less than for the corresponding 5°C samples. After 3 months at both 5 and 30°C, the pH 7.4 and 6.5 samples have significantly greater concentrations of soluble aggregates (dimer, etc.) than the pH 5.5 and 4.5 samples (Fig. 4). At 5°C, pH 4.5 and 5.5 samples show essentially no decrease in monomer content; size exclusion chromatograms are similar at 1.5 and 3.0 months. Interestingly, the chromatogram from the pH 4.5, 30°C sample exhibited a shift from dimer to higher aggregates, even though the total aggregation was the lowest

Table IV. Aggregation of KS1/4-DAVLB at 5°C[a]

pH	Monomer	Dimer	Higher aggregates	Total aggregates	Other impurities
			Initial		
7.4	92	4.7	2.2	6.9	1.1
6.5	94	3.3	1.9	5.2	1.1
5.5	94	2.9	2.1	5.0	1.0
4.5	94	2.9	2.0	4.8	1.1
			1.5 months		
7.4	90	6.3	2.9	9.2	1.1
6.5	92	4.3	2.5	6.8	1.1
5.5	93	3.0	2.4	5.4	1.2
4.5	93	3.3	2.1	5.4	1.2
			3 months		
7.4	89	6.4	3.5	9.9	1.2
6.5	91	4.6	2.6	7.2	1.5
5.5	92	3.2	2.8	6.0	1.9
4.5	93	3.1	2.2	5.3	1.7

[a] Data by size exclusion; values are percentage of total peak area.

Table V. Aggregation of KS1/4-DAVLB at 30°C[a]

pH	Monomer	Dimer	Higher aggregates	Total aggregates	Other impurities
			Initial		
7.4	92	4.7	2.2	6.9	1.1
6.5	94	3.3	1.9	5.2	1.1
5.5	94	2.9	2.1	5.0	1.0
4.5	94	2.9	2.0	4.8	1.1
			1.5 months		
7.4	77	17	5.7	22	0.6
6.5	78	16	6.2	22	0.3
5.5	86	10	4.1	14	1.0
4.5	89	4.3	5.4	9.7	1.1
			3 months		
7.4	70	17	11	27	2.3
6.5	69	19	10	29	1.8
5.5	80	14	5.1	19	1.1
4.5	80	7.2	11	18	1.1

[a] Data by size exclusion; values are percentage of total peak area.

among the four pH conditions. The gel electrophoretic patterns of the nonreduced material on 8 and 12% polyacrylamide gels were consistent with the SEC results (data not shown).

Effect of pH and NaCl Concentration on the Solubility of KS1/4-DAVLB. The effect of pH and NaCl concentration on the solubility of KS1/4-DAVLB was investigated. The KS1/4-DAVLB solutions were compared after dialysis at pH 5.2 to remove PBS. All were clear and did not differ in appearance from undialyzed samples. Size exclusion chromatography (Superose 12, 0.5 ml/min, UV 280 nm) indicated a total KS1/4-DAVLB concentration of 3.5 mg/ml; the dilution from 4.2 mg/ml was caused by initially dialyzing against a salt gradient. When titrated with NaOH, KS1/4-DAVLB conjugate solution without NaCl became cloudy at pH 6.5. The cloudiness disappeared at pH 8.5. Back titration with HCl regenerated the cloudy appearance in the same pH range. As the salt concentration was increased, the range of pH where precipitation occurs becomes narrower as illustrated in Fig. 5. At a salt concentration of 8 mg/ml, no apparent cloudiness or precipitation was observed over the entire titration range. These data indicate that in the pH 6.5 to 8.5 range, the solubility of KS1/4-DAVLB is very sensitive to ionic strength. This is consistent with results from isoelectric focusing that show multiple bands between *p*I 6.5 and *p*I 7.4 (data not shown).

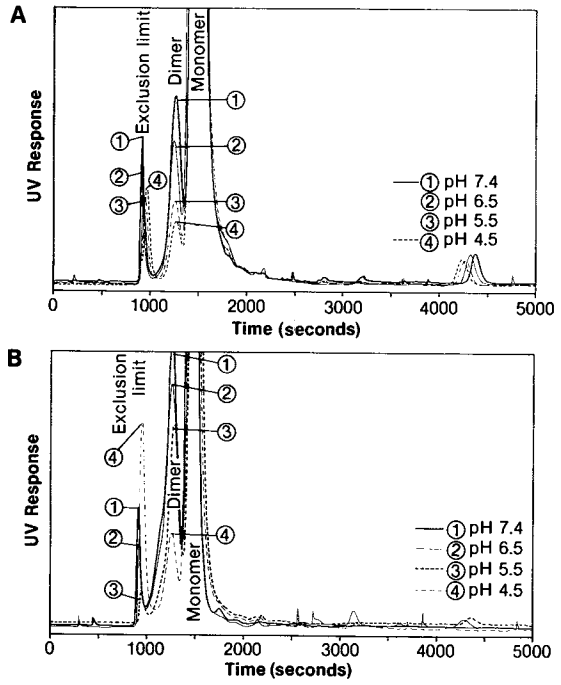

Fig. 4. Protein aggregation by size-exclusion chromatography. (A) Storage at 5°C for 3 months; (B) storage at 30°C for 3 months.

DISCUSSION

The results of this study indicate the following points about the solution stability of KS1/4-DAVLB. (i) Hydrolysis

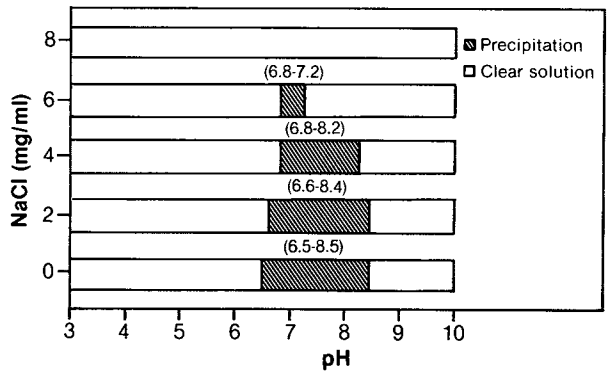

Fig. 5. Effect of NaCl concentration and pH on the precipitation of 3.5 mg/ml KS1/4-DAVLB.

to yield "free" vinca is minor over the pH 4.5–7.4 range, certainly at 5°C. (ii) Conjugated and unconjugated vinca are significantly more stable at pH 4.5–5.5 than at pH 6.5–7.4 and at 5°C rather than 30°C. (iii) No change in antibody function or protein content occurs at pH 4.5–7.4 at 5 or 30°C. (iv) Fewer soluble and insoluble aggregates form at pH 4.5–5.5 than at pH 6.5–7.4 and at 5°C rather than 30°C. (v) The solubility of KS1/4-DAVLB is sharply dependent on ionic strength in the pH 6.5–8.5 range. A formulation consisting of approximately 5 mg/ml KS1/4-DAVLB in sodium chloride (8.8 mg/ml) and monosodium phosphate (1.8 mg/ml) at pH 5.0–5.5 was chosen for initial clinical trials. Even though the data base is limited, we suggest that linker stability (i.e., drug release), formation of soluble and insoluble aggregates, and solubility be investigated as important parameters for formulation development of IgG–drug conjugates. Chemical stability of the drug is dependent upon the specific drug. Stability of antigen binding function cannot be generalized from this study.

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