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Stabilization of Chimeric BR96-Doxorubicin Immunoconjugate

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Chimeric BR96-doxorubicin conjugate (BR96-DOX) is an immunoconjugate designed to specifically target and kill certain tumor cells. The linker between the chimeric BR96 antibody and DOX is an acid-labile hydrazone group which was designed to undergo lysosomal hydrolysis to release DOX *in vivo*. Stability studies indicated that acid-catalyzed hydrazone hydrolysis was the major degradation route *in vitro*. Even under optimal conditions of pH and temperature, the stability of BR96-DOX in solution was not acceptable for long-term storage. Lyophilization of BR96-DOX in the presence of added sugars, such as lactose or sucrose, and subsequent storage of the lyophile under refrigeration significantly improved the stability. Therefore lyophilization appears to be a viable approach for achieving long-term stabilization of BR96-DOX.

KEY WORDS: chimeric antibody; protein; immunoconjugate; lyophilization; freeze-drying; stability; aggregation.

INTRODUCTION

Chimeric BR96 is an internalizing mouse-human monoclonal IgG_1 antibody specific for the Lewis^y (Le^y) antigen found on the surface of tumor cells. The antitumor agent doxorubicin (DOX) has been conjugated to the antibody via an acid-labile maleimidocaproylhydrazone linker at the 13-keto position of DOX to form chimeric BR96-doxorubicin conjugate (BR96-DOX, Fig. 1). The hydrazone linker was designed so that DOX would be released from the antibody in the acidic environment (lysosomes) of the target tumor cells to exert its therapeutic effect (1-3). The linker is attached to the antibody through thioether bonds to reduced disulfide thiol groups. Each mole of antibody is conjugated with 8 moles of doxorubicin.

In a recent paper, Trail et al. reported successful cures of human tumors in mice using BR96-DOX at equivalent DOX doses below the effective dose for free DOX (4). For these studies, the conjugate was formulated in a sodium phosphate buffer and stored frozen at −70°C until use. However, further development of the product required an expanded knowledge of the degradation pathways, factors affecting the stability, and ultimately a stable formulation for clinical studies. A solution frozen at −70° has numerous disadvantages related to large-scale production, shipping, and storage. The stability and physicochemical properties of BR96-DOX have been investigated and are reported here. These results and suitable options for stabilization of BR96-DOX are discussed.

MATERIALS AND METHODS

Materials

BR96-DOX at a concentration of ca. 10 mg/mL in 0.01 M sodium phosphate buffer/0.1 M NaCl at pH 7.5 was produced by Bristol-Myers Squibb Co. and stored at −70°C. Numerous lots of BR96-DOX were used for these studies. Polypropylene Falcon tubes and Type I flint glass vials were used for storage and stability studies. Acrodisc filter units with a 0.2 μ pore size (Gelman) were used to filter conjugate solutions. No apparent loss of conjugate due to binding to any of these surfaces was observed. Centricon 30 microconcentrator units were obtained from Amicon. Gel-filtration standards were obtained from Bio-Rad. Immulon II microtitre plates were obtained from Dynatech, bovine serum albumin, Fraction V, was obtained from Sigma, goat anti-human IgG horseradish peroxidase conjugate (GAH-HRP), 0.4 mg/mL, was obtained from Southern Biotechnology Associates, and Chromogen Substrate and Buffered Substrate were obtained from Genetic Systems. Qualified Lewis^y antigen covalently coupled to human serum albumin (Le^y-HSA), 5.0 mg/mL in Tris Buffered Saline, pH 7.4, was obtained from Alberta Research Council and stored at −90°C. Milli-Q processed water (Millipore) or Barnstead glass-distilled water was used for all studies. All other chemicals were analytical or HPLC grade.

Instrumentation

pH measurements were performed using an Orion Model 601A pH meter. Buffer exchange procedures were carried out at 10°C using an IEC Centra MP4R refrigerated centrifuge. Other instrumentation included a VirTis Unitop 200 lyophilizer and an Olympus BH-2 microscope with a Polaroid camera.

Chromatography

Size exclusion chromatography (SEC) was performed using a Waters HPLC system comprised of a 600E pump, 717 controlled-temperature autosampler, 490E multiwavelength UV/vis detector at 280 and 495 nm, and Millenium 2010 Data System. A TSK G3000SWXL (30 cm × 7.8 mm i.d.) column was used with a mobile phase containing 0.2 M KH_2PO_4 in 0.9% NaCl (pH adjusted to 6.8 with KOH) at a flow rate of 1.0 mL/min. The injection volume was 10-15 μL. Peaks were quantitated using peak height and area, and variability was ±3.6%. The elution volume of BR96-DOX was in the linear portion of a plot of log (molecular weight) versus elution volume of gel filtration standards. Representative chromatograms of bulk and partially degraded BR96-DOX are shown in Fig. 2.

For reversed-phase HPLC, the system consisted of a Varian Vista 5500 pump, Spectroflow 757 UV Absorbance Detector at 254 nm, Waters 712 WISP Autosampler and Waters Millenium 2010 Data System. A Jones Apex Octadecyl column (15 cm × 4 mm i.d., 5 μ particle size) with an Ad-

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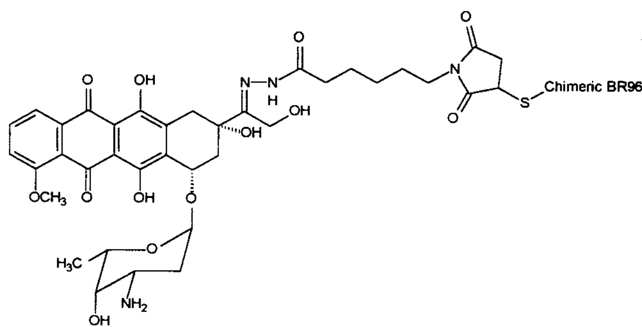

Fig. 1. Structure of chimeric BR96-doxorubicin conjugate (BR96-DOX).

sorbosphere C18 (5 μ, 10 mm) cartridge (Alltech) was used with a mobile phase of 60% methanol/40% 25 mM ammonium phosphate at a flow rate of 1.5 mL/min. The injection volume was 15 μL and peaks were quantitated using peak area. Variability was ±3%. A representative chromatogram of free DOX in a conjugate sample is shown in Fig. 2 (inset).

Buffer Exchange Procedure

The BR96-DOX buffer was exchanged with 4 volumes of the desired buffer using Centricon 30 microconcentrator units. The final solution was aseptically filtered through a 0.2 μm Acrodisc sterile filter assembly into a sterile Falcon tube.

Solution Stability of BR96-DOX

The solution stability of BR96-DOX was evaluated in the pH range of 4 to 8. The pH of the bulk solution of BR96-DOX in 0.01 M sodium phosphate buffer/0.1 M NaCl pH 7.5 was adjusted to the desired value using either 1 N HCl or 1 N NaOH. No adverse effects due to the addition of base or acid were observed. Due to the poor buffer capacity of phosphate at low pH, the pH values were measured at the end of the study to confirm pH control. Approximately 0.25 mL aliquots of these solutions were transferred to 2 mL Type I flint glass vials and sealed with Teflon coated combination seal/stoppers. Sample preparation was carried out aseptically in a biological safety cabinet to prevent microbiological contamination. Samples were stored protected from light at ambient laboratory temperature (ca. 23°C), in a 25°C or 5°C water bath, refrigerated at 2–8°C, or frozen at −15°C. Reference samples from each set were stored in a −70°C freezer. At predetermined time intervals vials were removed and stored at −70°C until analysis.

Size exclusion chromatography (SEC) was used to quantitate the remaining conjugate and the formation of smaller fragments or higher molecular weight species. The percent conjugate remaining was calculated by comparison of peak height for the sample to that of the control sample stored at −70°C. The percentage of dimer and high molecular weight (MW) aggregates were calculated from a ratio of peak area in the sample to the peak area of dimer in the control and normalized to the initial percent. The values reported reflect the absorbance at 495 nm. The starting material contained dimer initially and therefore the sum of percent conjugate remaining and percent dimer will be >100%.

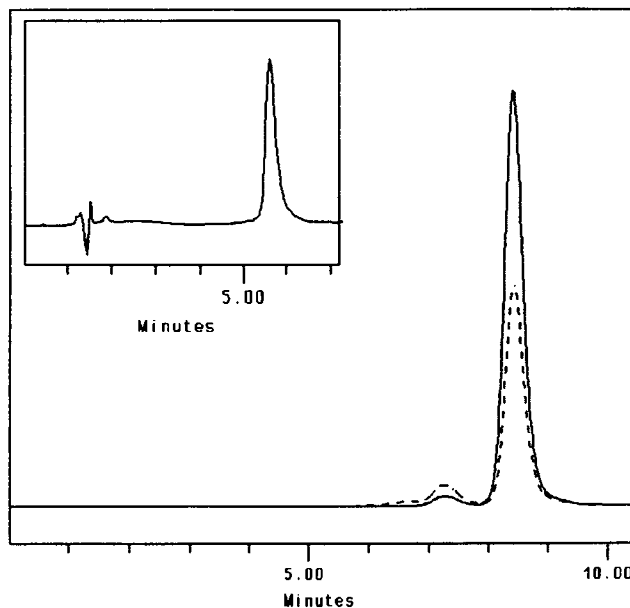

Fig. 2. Representative SEC and RP-HPLC chromatograms. The solid line represents the SEC chromatogram of bulk BR96-DOX (8.4 min) that contains 2.5% dimer (7.3 min) initially. The overlaid dashed line represents partially degraded conjugate that contains an increased amount of dimer and small amounts of higher molecular weight species (6-6.9 min). The inset is a RP-HPLC chromatogram of DOX for a sample of BR96-DOX that contains 49 μg/mL free DOX.

The concentration of free DOX in solution was determined by RP-HPLC and is reported as μg/mL of DOX · HCl.

The effects of various solution components and added excipients on the stability of BR96-DOX were also evaluated. Bulk conjugate was buffer-exchanged into the appropriate buffer and stability was evaluated at various temperatures.

Effect of Multiple Freezing and Thawing

Samples were frozen by placing them in a freezer at −70°C, thawed at ambient temperature (ca. 23°C) for approximately 15 min, and returned to the freezer. This procedure was repeated up to 5 times, and the samples were then stored at −70°C until analysis by SEC and RP-HPLC.

Effect of Agitation

Solutions of BR96-DOX at concentrations of 10, 20 and 50 mg/mL were shaken at 5°C at 200 rpm on an Eberbach shaker for up to 2 days. Samples were removed periodically, assayed, and compared to a control solution.

Lyophilization Process

Solutions of the conjugate in 0.01 M sodium phosphate buffer/0.3 M NaCl at pH 7.5 containing 4% (w/v) of various sugars and amino acids were filtered through 0.2 μm Acrodisc 13 filter units. Aliquots of 0.25 mL were dispensed into 3 mL Type I flint glass vials which were then partially stoppered with West No. 805 red butyl lyophilization stoppers. After freezing at −40°C for 3 hours, lyophilization was ac-

complished by primary drying at −30°C for 18 hours under full vacuum (40 microns), followed by secondary drying at a shelf temperature of 5°C for 24 hours, and an additional 4 hours at 25°C. The vials were stoppered under nitrogen, removed from the lyophilizer, and sealed with aluminum seals.

Reconstitution and Utility Time

One freshly lyophilized vial of each prototype formulation was reconstituted with 0.25 mL of Sterile Water for Injection and gently agitated to disperse the solid. The pH of each solution was measured. Each solution was analyzed by SEC immediately after reconstitution and after standing at ambient temperature for 8 hours. Aliquots of each sample were frozen at −70°C and analyzed later for free DOX.

Solid State Stability of Lyophile

The solid state stability of prototype lyophilized formulations of BR96-DOX was studied by SEC and RP-HPLC. Vials were stored upright at 2–8°C, 30°C, 40°C and 50°C. Reference vials were stored at −70°C. At various time intervals vials were removed, allowed to equilibrate to room temperature, and reconstituted with 0.25 mL of water as described above. Samples were assayed by SEC and RP-HPLC.

Immunoreactivity by BIAcore

The BIAcore system (BioSpecific Interaction Analysis) was used to assess the immunoreactivity of the conjugate. The surface of a sensor chip was coated covalently with a rabbit anti-human F_c-Igγ specific polyvalent antibody (RAH-F_c-Igγ) which was then used to capture the BR96-DOX F_c region. The ability of the F_c-bound conjugate to bind Le^y antigen conjugated to Human Serum Albumin (Le^y-HSA) was then determined. Samples were diluted in 10 mM HEPES pH 7.4 with 3.4 mM EDTA, 150 mM NaCl and 0.05% BIAcore surfactant P20 (HBS) to a final concentration of either 25 or 250 μg/mL, and 15 μL of each was reacted with the RAH-F_c-Igγ sensor surface at a flow rate of 3 μL/min. The RAH-F_c-Igγ bound BR96-DOX was then reacted with 15 μL of Le^y-HSA, diluted previously to 25 μg/mL in HBS, at a flow rate of 5 μL/min. The immunoreactivity of each sample was compared to a reference standard. The associated error is <10%.

Immunoreactivity by ELISA

Immulon II 96-well plates were coated with Le^y-HSA and incubated overnight at 4°C. The wells were then washed and non-specific sites were blocked with 1% BSA in pH 7.4 phosphate buffered saline (PBS). BR96-DOX was diluted to 40 ng/mL in PTB buffer (PBS containing 1% BSA and 0.5% v/v Tween 20), and a standard curve from 0–15 ng/mL was generated. Stability samples were diluted to an initial concentration of 40 ng/mL in PTB buffer and a series of dilutions were made to ca. 2.5, 7.5 and 12.5 ng/mL. The reference standards and stability samples were assayed in triplicate. Quality control samples were prepared separately from the stock solution and run on each plate. Following this incubation, the solution in each well was removed and each well was then washed. GAH-HRP diluted to 57 ng/mL was then added to each well and the plate incubated at ambient temperature for 60 min in the dark. The plate was subsequently developed by Chromogen substrate. The plates were read on a Molecular Devices UV Max Plate Reader at 450 and 630 nm. The coefficient of variation for the assay is ±15%.

RESULTS AND DISCUSSION

Solution Stability of BR96-DOX

Effects of pH, temperature and buffer

The stability of BR96-DOX as a function of pH at 25°C is illustrated in Fig. 3. The pH of maximal stability is near pH 7.5. Two primary routes of degradation have been identified: hydrolysis of the hydrazone linkage is predominant at all pH values and results in release of free DOX; aggregation to higher MW species (primarily dimer) contributes to loss at pH 7.5 and higher. The shape of the profile suggests specific acid catalysis of the hydrazone hydrolysis, which is supported by the formation of free DOX as the primary degradation product in the acidic pH region. Once released from the conjugate, DOX can also undergo pH-dependent degradation to form primarily doxorubicinone in the acidic region and unknown products in the neutral to basic region (5). However, these processes are significantly slower than the rate of hydrazone hydrolysis under the conditions studied.

The predominant route of degradation of BR96-DOX in solution is hydrolysis to release DOX. As expected, hydrolysis is decreased and free DOX levels lower at 2–8°C (Fig. 4) compared to 25°C (t_{90} values of approximately 2 weeks and 3 days, respectively), but the stability, even under refrigeration, is not sufficient for long-term storage. Surprisingly, freezing at −15°C results in production of significant amounts of free DOX as illustrated in Fig. 4. The extent of hydrazone hydrolysis at this temperature is attributed to in-

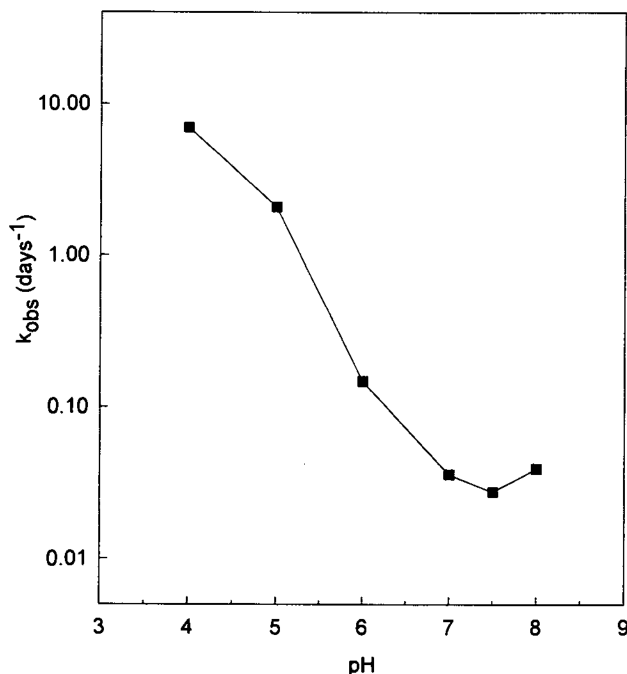

Fig. 3. pH-rate profile for BR96-DOX at 25°C as determined from the rate of loss of BR96-DOX (in 0.01 M sodium phosphate buffer/0.1 M NaCl) by SEC.

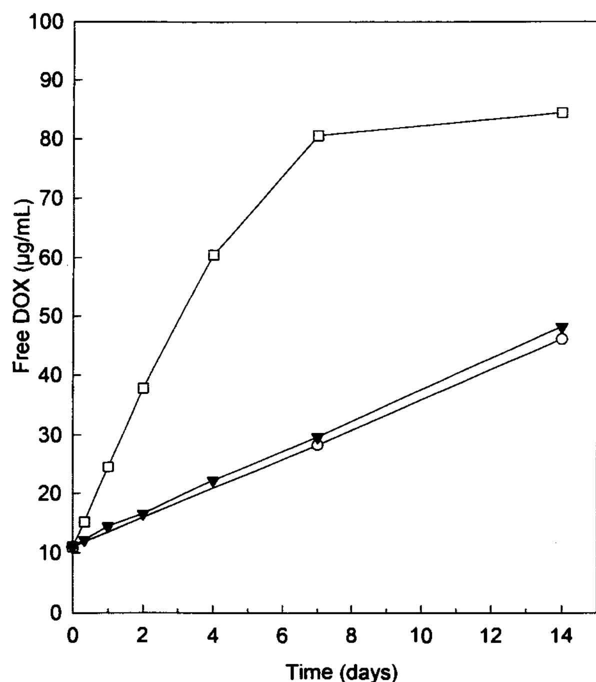

Fig. 4. Release of free DOX (measured by RP-HPLC) from BR96-DOX in 0.01 M sodium phosphate buffer/0.1 M NaCl pH 7.5 as a function of temperature: 25°C (□), 2-8°C (▼), and −15°C (○).

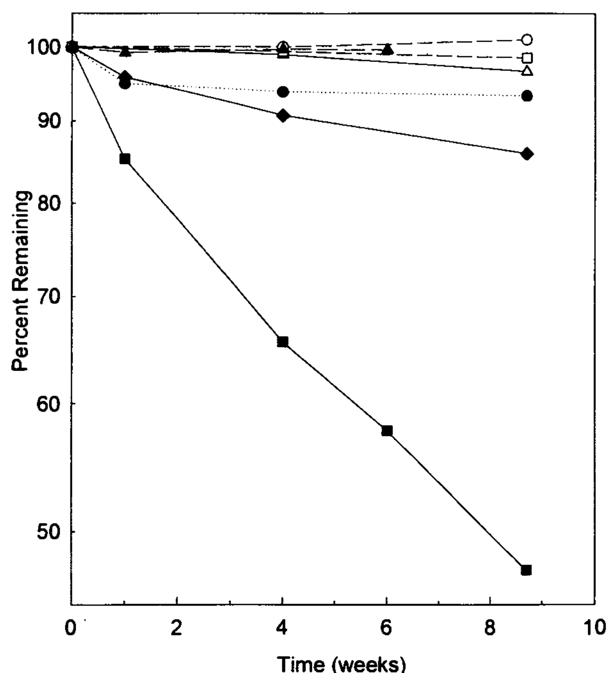

Fig. 5. Percent BR96-DOX remaining (determined by SEC) at −15°C as a function of pH and buffer (0.01 M buffer/0.1 M NaCl): sodium phosphate pH 7.5 (■), sodium phosphate pH 8.0 (●), sodium phosphate pH 8.5 (▲), potassium phosphate pH 7.5 (♦), Tris pH 7.5 (□), Tris pH 8.0 (○), and Tris pH 8.5 (△).

complete freezing of the solution, partial crystallization of Na_2HPO_4 from solution and consequently a drop in pH of the solution (6). Incomplete freezing of the solution at −15°C was confirmed by resistance measurements as a function of temperature, which indicated a freezing point of −43°C (data not shown). The lability of BR96-DOX is increased at low pH, as illustrated in Fig. 3. Raising the initial pH of the buffer or using either potassium phosphate or Tris buffer significantly improved the stability at −15°C (Fig. 5). The changes in pH for the latter two buffers upon freezing are minimal (6). In particular, a buffered solution of conjugate in pH 8 Tris buffer exhibited excellent stability at −15°C over 6–9 months.

A second degradation pathway for BR96-DOX is aggregation, which is manifested primarily as dimerization, although increased temperature and pH favor increased production of high MW aggregates (greater than 300,000 Daltons). Fig. 6 illustrates the formation of dimer after storage of BR96-DOX at ambient temperature at pH 7.5. The level of dimer increased with increasing pH and temperature, but the aggregation products remain soluble and are also capable of releasing DOX *in vitro*. The nature of the aggregation process is not fully understood, but analysis by SDS-PAGE indicates a non-covalent process (data not shown).

Physical Stability

Aggregation of BR96-DOX was examined after stresses of multiple freezing/thawing and agitation. Samples of BR96-DOX at pH 7.5 (250 µL) in Type I flint glass vials were repeatedly frozen at −70°C and thawed at ambient temperature. Small increases in aggregates (0.7–1.0%) and up to a 0.6 µg/mL increase in free DOX were evident after 5 cycles (Table I).

Aliquots (250 µL) of BR96-DOX were shaken at 150 rpm in a shaker bath at 5°C for 24 hours. No visual changes (e.g., foaming) were apparent and SEC data indicated that the dimer level did not increase.

These results indicate that solutions of BR96-DOX are

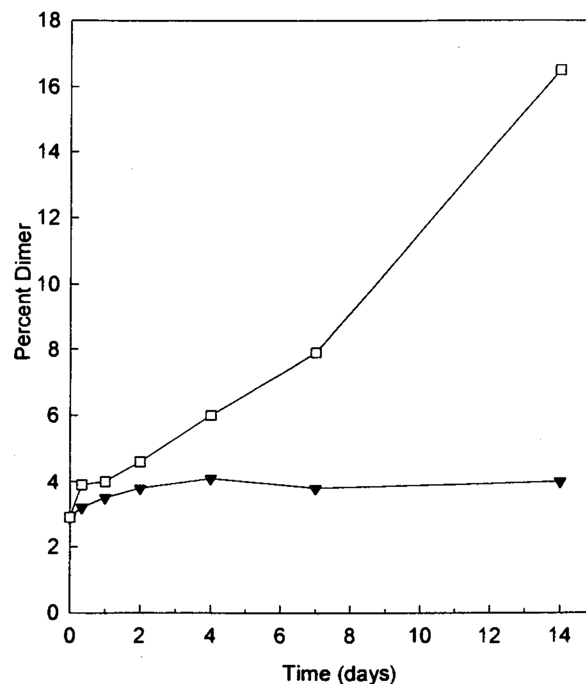

Fig. 6. Formation of dimer from BR96-DOX in 0.01 M sodium phosphate buffer/0.1 M NaCl pH 7.5 as a function of temperature: 25°C (□), and 2–8°C (▼).

TABLE I. Stability of BR96-DOX[a] after Multiple Freezing and Thawing

Number of Cycles	Percent Remaining	Percent Dimer	Free DOX μg/mL
0	100	2.9	11.0
1	100	4.1	12.1
5	99.0	4.1	14.4

[a] 10 mg/mL in 0.01 M sodium phosphate buffer with 0.1 M NaCl at pH 7.5

not adversely affected by multiple freezing and thawing or shaking and should therefore withstand normal handling and shipping.

Immunoreactivity

The ability of the conjugate to bind Le^y antigen is paramount in evaluation of the stability. Because of the insensitivity of SEC to small changes in protein structure, apparently stable solutions of BR96-DOX as measured by SEC could have lost a significant amount of antigen-binding activity. Therefore the immunoreactivity of a series of conjugate solutions which had been stored under refrigeration was assessed using two different methods. The ELISA assay is a direct-binding method that measures the binding of BR96-DOX to Le^y antigen immobilized to HSA. The BIAcore system is capable of rapid characterization of the nature of bimolecular interactions. The system can measure association and dissociation of two or more interacting molecules and provides valuable information about the affinity, specificity, kinetics and multiple binding patterns (7). BIAcore employs surface plasmon resonance (SPR) for detection. The theoretical basis and validity of this method have been documented and are the subjects of several review articles (8).

After storage of a conjugate solution at 2–8°C for one week, SEC indicated no loss of conjugate (Table II). These results were confirmed with ELISA and BIAcore, which indicated no significant changes in binding activity to Le^y. A limiation of the immunoassays, however, is that they do not distinguish between native chimeric BR96 and BR96-DOX. Therefore, degradation of BR96-DOX by hydrolysis would not be evident by these binding assays, which are not stability-indicating methods.

TABLE II. Solution Stability of BR96-DOX[a] at 2–8°C

Time (days)	SEC[b]	ELISA[c]	BIAcore[d]
0	100	97	92
1	97.1	100	100
2	99.1	99	105
3	98.4	96	94
4	100	100	95
7	101	96	105

[a] 10 mg/mL in 0.01 M sodium phosphate buffer with 0.1 M NaCl at pH 7.5
[b] Percent conjugate remaining by SEC
[c] Immunoreactivity (as a percent of the reference standard) by ELISA
[d] Immunoreactivity (as a percent of the reference standard) by BIAcore

Lyophilization

Lyophilization was explored as a means to stabilize BR96-DOX for long-term storage. Since hydrolysis was the primary degradation pathway, minimization of water would theoretically reduce the degradation rate. Although many proteins have been successfully lyophilized, lyophilization can cause potential problems due to conformational changes, chemical degradation, aggregation, or alterations in bioactivity which may be induced by either the freezing or drying process (9–12). The mechanisms underlying loss of protein activity upon lyophilization are not well understood, but vary considerably based on the specific protein. Crystallization or freeze-concentration of solutes (10), pH changes (6), and dehydration (13) have been suggested, but no general mechanism can explain the results. Numerous detailed discussions of these suggested mechanisms have been presented and the reader is referred to the literature.

In order to circumvent the instability as a result of lyophilization, many proteins have been lyophilized in the presence of various "lyoprotectants," such as amino acids or sugars, which confer stability during the lyophilization process and also in the solid state during storage (10). Numerous mechanisms have been suggested for the stabilizing effects of many lyoprotectants, including prevention of crystallization during freezing, an increase of the glass transition temperature (12), direct interaction with the protein (14–16), water ordering (17), and distribution of the excipient throughout a glassy matrix preventing bimolecular interactions (10). However, no general mechanism is applicable and the choice of stabilizing excipients is based on empirical evidence. Usually the best stabilization is achieved with excipients which remain amorphous, but this is not necessarily the only criterion (11).

BR96-DOX was lyophilized in the presence of some sugars and amino acids that have shown potential as lyoprotectants. The excipients used are pharmaceutically acceptable and have a good history of parenteral use. The conditions selected for this exploratory study were based on published literature values for other proteins and were not optimized. Primary drying was carried out at a shelf temperature of −30°C, which is below the collapse temperature of the bulk material (−22°C). No evidence of collapse was observed. Small sample volumes (0.25 mL) were used due to a limited amount of material available. The conjugate lot for the lyophilization studies contained a high level of dimer (ca. 12%) in contrast to subsequent lots which contained significantly less (ca. 2%). However, the objective of the work was to examine the feasibility of lyophilization and the important parameter in this case would be changes in dimer and aggregate levels compared to the control. Scale-up and optimization of the formulation and lyophilization process were subsequently carried out with more representative material and realistic fill volumes (18).

Effects of Lyophilization on Conjugative Properties

Properties of prototype lyophilized formulations of BR96-DOX are summarized in Table III. Formulations con-

taining no excipient (control), mannitol (MAN), glycine (GLY), and glycine:mannitol (GLY:MAN) appeared crystalline and were birefringent. The lactose (LAC) and sucrose (SUC) formulations were amorphous whereas the Dextran 40 (DEX) formulation appeared partially crystalline, possibly due to crystallization of the buffer.

The initial water content of the various formulations as determined from Karl Fischer titration varied from undetected to 6%. As expected, the amorphous LAC and SUC systems retained the highest amount of water upon lyophilization whereas the crystalline systems (GLY, DEX, and MAN) retained significantly less amounts of water (up to 3%).

The various lyophiles were reconstituted with water. The pH values ranged from 6.8–7.3 and all but the control were visually clear. The control was hazy, presumably because of an increase in high MW aggregates (Table III). The LAC, DEX, SUC and GLY:MAN formulations exhibited good recovery in the range of 95% to 101%. The recovery of other formulations (control, MAN and GLY) was in the range of 86% to 90%. The amounts of dimer and high MW aggregate were also determined and compared to those prior to lyophilization. In general, the amount of dimer remained close to the initial value of 12% for all formulations except for the control, MAN and DEX formulations which increased 2% to 3%. The amount of high MW aggregate remained at about 0.5% for most formulations, but a 2% increase was observed for the control, MAN and GLY formulations after lyophilization. The changes in dimer and aggregate levels after lyophilization do not account for the apparent loss of parent in certain formulations. In the control solution, this lack of mass balance could be attributed to precipitation, resulting in a hazy solution. Volume changes upon reconstitution could also account for some of the low recoveries. However, the low recoveries in the GLY and MAN formulations cannot be explained.

The concentration of free DOX formed as a result of hydrolysis during the lyophilization process was measured by RP-HPLC after reconstitution with water. The results are shown in Table III. Comparison of DOX levels before and after lyophilization indicates that minimal hydrolysis (<4% free DOX produced) had occurred during the in-process work-up and the lyophilization process.

Stability of Reconstituted Solutions

The stability of reconstituted solutions of prototype lyophilized formulations at a concentration of 8 to 9 mg/mL is summarized in Table III. All formulations with the exception of DEX retained greater than 91% of the initial concentration after 8 hours at ambient temperature, and the amounts of both dimer and high MW aggregate remained essentially unchanged. As expected, the amount of free DOX increased from 2–3 μg/mL to 4–7 μg/mL. The stability of the conjugate following reconstitution is considered acceptable and is in agreement with the results of solution stability studies discussed previously.

Solid State Stability of Lyophilized Systems

The solid-state stability of prototype lyophilized formulations of BR96-DOX was evaluated at 2–8°, 30°, 40° and 50°C. Representative data after storage for 2 weeks at 40°C and 12 months at 2–8°C are summarized in Table IV. The trends observed at 30° and 50°C were similar to 40°C. The stability studies were performed at higher temperatures with the understanding that proteins may or may not exhibit predictive behavior after thermal stress. Real-time data is always necessary to confirm high temperature data. For BR96-DOX, the 40°C data qualitatively predicted the rank order of stability of the various prototype formulations under more realistic storage conditions (2–8°C).

In general, the percent conjugate remaining decreased

TABLE III. Properties of BR96-DOX Before and After Lyophilization

			Reconstitution		Before lyophilization			After lyophilization				Reconstituted solution/8 hr at ambient temperature			
Excipient	Appearance[a]	% H_2O[b]	Appearance	pH	% D[c]	% Agg[d]	DOX[e]	Rec[f]	% D	% Agg	DOX	% R[g]	% D	% Agg	DOX
Control	crystalline	2.7	hazy	7.3	13	0.5	2.2	88.6	16	2.5	2.4	95.6	16	2.0	6.4
Lactose	amorphous	5.8	clear	7.1	13	0.5	2.5	94.5	12	0.5	2.3	93.7	12	0.5	4.6
Mannitol	crystalline	3.1	clear	7.0	12.5	0.5	2.6	86.7	14	2.7	2.2	92.6	14	2.7	5.1
Dextran 40	partially crystalline	n.d.	clear	7.2	12	0.2	2.6	101	14	0.7	2.4	85.3	14	0.7	5.7
Sucrose	amorphous	5.0	clear	7.1	12	0.2	2.5	95.7	12	0.5	2.3	94.4	13	0.5	4.5
Glycine	crystalline	1.1	clear	6.8	12	0.7	3.4	89.3	12	1.7	3.5	96.7	12.5	1.7	7.5
Gly: mannitol	crystalline	1.6	clear	6.8	12.5	0.2	3.5	96.3	12.5	0.3	2.4	91.6	12	0.3	6.4

[a] Polarized light microscopy
[b] Determined by Karl Fischer titration
[c] Percent dimer by SEC
[d] Percent high molecular weight aggregate by SEC
[e] Free DOX in μg/mL
[f] Percent recovery by SEC compared to bulk conjugate prior to lyophilization
[g] Percent conjugate remaining by SEC compared to the reconstituted solution immediately after reconstitution
n.d. = not detected

TABLE IV. Solid-State Stability of BR96-DOX Lyophiles

	40°C/2 weeks				2–8°C/12 months			
	% R[a]	ΔD[b]	ΔAgg[c]	ΔDOX[d]	% R	ΔD	ΔAgg	ΔDOX
Control	67.8	2.3	0.5	2.3	81.6	−0.2	0.3	3.7
LAC	98.2	1.0	n.c.	3.1	104	−0.9	−0.3	2.0
MAN	76.9	3.5	1.5	8.0	94.2	−1.9	−1.3	2.1
DEX	89.9	4.3	n.c.	1.5	97.7	2.3	n.c.	2.2
SUC	99.8	1.0	n.c.	9.1	99.7	−1.0	−0.3	2.5
GLY	83.7	3.9	1.1	1.8	95.2	2.5	0.6	2.9
GLY:MAN	74.7	7.3	3.1	3.8	89.2	1.5	n.c.	7.8

[a] Percent conjugate remaining by SEC compared to the solution immediately after reconstitution
[b] Change in percent dimer by SEC compared to the solution immediately after reconstitution
[c] Change in percent high molecular weight aggregate be SEC compared to the solution immediately after reconstitution
[d] Change in free DOX in μg/mL compared to the solution immediately after reconstitution
n.c. = no change

with time when exposed to thermal stress. The amounts of dimer, high MW aggregate and DOX formed depended on the specific excipient. As shown in Table IV, the crystalline systems including MAN, GLY:MAN and the control were the least stable at 40°C. After 2 weeks at 40°C, the LAC and SUC systems retained greater than 98% of the initial conjugate value whereas the others varied from 68% to 90%. The loss of conjugate was accompanied primarily by an increase in the dimer level, although this did not quantitatively account for the overall amount degraded. Precipitation of high molecular weight aggregates could possibly account for the losses of parent material, but this process was not quantitated. The soluble high MW aggregate did not change in the LAC, DEX and SUC formulations, but was increased in the others, especially the crystalline MAN and GLY:MAN formulations.

The concentration of free DOX generally increased with time upon storage at elevated temperatures. The highest levels of free DOX measured after 2 weeks at 40°C were in the MAN and SUC formulations. The increase corresponded to ca. 3–4% of the total DOX. The extent of hydrolysis was not large and is not considered a major factor in the solid state degradation of BR96-DOX.

The high temperature storage conditions were used as an indicator of stabilizing effects of the excipients, which were further confirmed under a more realistic storage condition of 2–8°C. As shown in Table IV, after 12 months under refrigeration, all formulations with the exception of the control and crystalline GLY:MAN retained greater than 95% monomer by SEC. After storage at 40°C, the control, GLY:MAN and MAN formulations had significantly greater changes in parent, dimer and aggregate compared to the LAC and SUC formulations (Table IV). These results are qualitatively similar to the results at 2–8°C, where the control and GLY:MAN were least stable and LAC and SUC were most stable. The changes in DOX levels in all formulations were small and therefore were not used as a comparison of stability.

The formulations that remained amorphous (LAC and SUC) achieved the greatest stabilization. Under stress conditions (30–50°C) as well as under refrigeration, these excipients minimized or prevented degradation by aggregation and hydrolysis.

Although the microenvironment of the conjugate is unknown, various factors could contribute to the stabilization by lactose and sucrose. One mechanism which has been proposed for sugars is that the amorphous excipient is distributed throughout the protein phase forming a rigid glassy matrix and decreasing the mobility in the system. As a result, the probability of bimolecular protein-protein interactions in the solid state is diminished and consequently formation of additional amounts of dimer and high MW aggregate is efficiently reduced during processing and storage of the lyophilized solid systems (10). Hellman et al. proposed that sugars can stabilize the native state of a protein through their ability to act as water structure promoters (17). Evidence has also been presented to support the theory that stabilizers can hydrogen-bonding to a protein during the drying phase of lyophilization (15–16). In addition, experiments using FTIR have indicated that storage stability of lyophilized proteins is directly correlated with the degree of native structure retained after lyophilization (19). Further studies with BR96-DOX are necessary in order to elucidate the specific mechanism(s) governing the effects of the various excipients on the stability.

Immunoreactivity

As discussed previously, because of possible alterations of biological activity of BR96-DOX upon lyophilization and storage, the immunoreactivity of lyophilized systems was determined by measuring their ability to bind Le^y-HSA antigen by the BIAcore system. The immunoreactivity of samples of prototype formulations of BR96-DOX after lyophilization and storage at 2–8°C for up to 12 months was determined (data not shown). The results indicate that the immunoreactivity of conjugate samples to Le^y-HSA antigen is retained upon lyophilization. In addition, the stoichiometry of the immunoreaction between the Le^y-HSA antigen and the lyophilized conjugate is similar to that of their corresponding samples prior to lyophilization. This suggests that lyophilization at this small scale of conjugate did not induce any irreversible conformational changes in BR96-DOX.

After storage of the lyophile at 2–8°C for 12 months, the binding affinities of all samples were not different from the

reference standard. This supports the SEC and RP-HPLC results which also indicate good stability of the lyophile after storage at 2–8°C.

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REFERENCES

1. G. R. Braslawsky, K. Kadow, J. Knipe, K. McGoff, M. Edson, T. Kaneko, and R. S. Greenfield. Adriamycin(hydrazone)-antibody conjugates require internalization and intracellular acid hydrolysis for antitumor activity. *Cancer Immunol. Immunother.* 33:367–374 (1991).
2. T. Kaneko, D. Willner, I. Monkovic, J. O. Knipe, G. R. Braslawsky, R. S. Greenfield, and D. M. Vyas. New hydrazone derivatives of adriamycin and their immunoconjugates-a correlation between acid stability and cytotoxicity. *Bioconj. Chem.* 2:133–141 (1991).
3. D. Willner, P. A. Trail, S. J. Hofstead, H. D. King, S. J. Lasch, G. R. Braslawsky, R. S. Greenfield, T. Kaneko, and R. A. Firestone. (6-Maleimidocaproyl)hydrazone of doxorubicin-A new derivative for the preparation of immunoconjugates of doxorubicin. *Bioconj. Chem.* 4:521–527 (1993).
4. P. A. Trail, D. Willner, S. J. Lasch, A. J. Henderson, S. Hofstead, A. M. Casazza, R. A. Firestone, I. Hellström, and K. E. Hellström. Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. *Science* 261:212–215 (1993).
5. J. H. Beijnen, O. A. G. J. van der Houwen, and W. J. M. Underberg. Aspects of the degradation kinetics of dosorubicin in aqueous solution. *Int. J. Pharm.* 32:123–131 (1986).
6. S. S. Larsen. Studies on stability of drugs in frozen systems. VI. *Arch. Pharm. Chemi. Sci. Ed.* 1:41–53 (1973).
7. R. Granzow and R. Reed. Interactions in the fourth dimension. *Biotechnology* 10:390–393 (1992).
8. E. Stenberg, B. Persson, H. Roos and C. Urbaniczky. Quantitative determination of surface concentration of protein with surface plasmon resonance using radiolabelled proteins. *J. Coll. Interfac. Sci.* 143:513–526 (1991).
9. M. J. Pikal. Freeze drying of proteins. Part I: Process design. *BioPharm.* 18–27, September 1990.
10. M. J. Pikal. Freeze drying of proteins. Part II: Formulation selection. *BioPharm,* 26–30, October 1990.
11. M. J. Pikal, K. M. Dellerman, M. L. Roy and R. M. Riggin. The effects of formulation variables on the stability of freeze-dried human growth hormone. *Pharm. Res.* 8:427–436 (1991).
12. F. Franks. Improved freeze-drying. An analysis of the basic scientific principles. *Process Biochem.*, iii–vii, February 1989.
13. M. J. Hageman. The role of moisture in protein stability. *Drug Dev. Ind. Pharm.* 14:2047–2070 (1988).
14. K. Tanaka, T. Takeda, and K. Miyajima. Cryoprotective effects of saccharides on denaturation of catalase by freeze-drying. *Chem. Pharm. Bull.* 39:1091–1094 (1991).
15. J. F. Carpenter and J. H. Crowe. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry* 28:3916–22 (1989).
16. J. F. Carpenter, S. J. Prestrelski, and T. Arakawa. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. I. Enzyme activity and calorimetric studies. *Arch. Biochem. Biophys.* 303:456–64 (1993).
17. K. Hellman, D. S. Miller, and K. A. Cammack. The effect of freeze-drying on the quaternary structure of L-asparaginase from *Erwinia carotovora*. *Biochim. Biophys. Acta.* 749:133–142 (1983).
18. T. T. Chen, K. E. Weston, M. N. Nassar, and S. N. Agharkar. Development of a stable lyophilized formulation for a monoclonal antibody-doxorubicin conjugate. Presented at the 8th Annual Meeting and Exposition of the American Association of Pharmaceutical Scientists, Lake Buena Vista, FL, November 14–18, 1993.
19. S. J. Prestrelski. Enhanced stability through optimization of formulation conditions for lyophilized proteins. Presented at the University of Colorado Protein Stability Conference, Breckenridge, CO, July 17–20, 1994.