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(54) Title: STABILIZATION OF FACTOR VIII WITHOUT CALCIUM AS AN EXCIPIENT

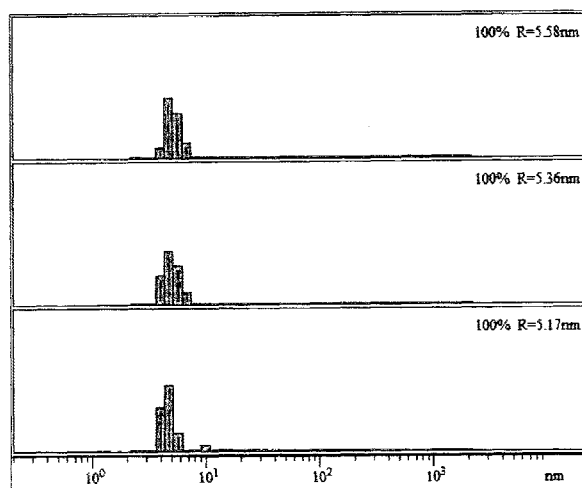


FIGURE 1A

(57) Abstract: The present invention provides a novel albumin-free formulation of recombinant Factor VIII that does not require calcium ions as an added formulation excipient. The absence of calcium chloride in the formulation results in a number of improvements and benefits for a Factor VIII formulation. This invention allows a Factor VIII formulation that can be lyophilized more efficiently as the absence of calcium ions will increase primary glass transition of the amorphous phase. The absence of free calcium ions in the formulation will also improve the stability of the formulation as metal-dependent oxidation reactions will be avoided and the protein molecule will not be subjected to chemical instabilities. Finally, the absence of calcium will further simplify the Factor VIII formulation by reducing the number of excipients in the formulation.

**STABILIZATION OF FACTOR VIII WITHOUT CALCIUM AS AN
EXCIPIENT**

Cross-Reference to Related Applications

[0001] This application is based on and claims priority of 61/973,280 filed April 01, 2014.

FIELD

[0002] This application relates generally to pharmaceutical formulations and particularly to a lyophilized formulation for rFVIII which is stabilized without albumin (albumin-free) and without any addition of divalent cations, such as calcium chloride, to the final formulation.

BACKGROUND

[0003] There are a number of albumin-free rFVIII formulations on the market using stabilization and formulation matrix excipients, such as, sodium chloride, glycine, mannitol, sucrose, trehalose, histidine, and calcium chloride. Calcium chloride is considered an essential component in the formulation because Factor VIII is a large hetero-dimer molecule where the heavy and light chains are held together by a metal ion bridge of divalent cations such as calcium and copper ions. All the current formulations contain calcium chloride, which has been assumed to be essential in order to maintain Factor VIII integrity and stability. The concentration of calcium

chloride in the various formulations varies from 1.7 mM to 5 mM. It has been shown recently the calcium (Ca^{2+}) is necessary for the generation of Factor VIII cofactor activity but that Ca^{2+} did not affect heavy chain-light chain binding affinity. It has been shown that Factor VIII contains two Ca^{2+} binding sites with different affinities and that the cofactor activity of Factor VIII can be reconstituted from heavy chain and light chain only when both chains are preactivated by Ca^{2+} .

BRIEF DESCRIPTION OF THE FIGURES

[0004] Figures 1A and 1B are histograms from Dynamic Light Scattering of F8-E (A) and F8-F (B) formulations where the formulation of F8-E comprises 308 mM NaCl/10 mM histidine, pH 6.5/9 mM sucrose/100 ppm polysorbate 80 and no added CaCl_2 . The formulation of F8-F comprises 308 mM NaCl/10 mM histidine, pH 6.5/9 mM sucrose/100 ppm polysorbate 80 and 1.7 mM CaCl_2 . The histograms show the molecular weight (MW) normalized distribution and the hydrodynamic radius of the protein.

[0005] Figures 2A and 2B are histograms from Dynamic Light Scattering of F8-G (A) and F8-H (B) formulations where the formulation of F8-E comprises 308 mM NaCl/10 mM histidine, pH 7.0/9 mM sucrose/100 ppm polysorbate 80 and no added CaCl_2 . The formulation of F8-F comprises 308 mM NaCl/10 mM

histidine, pH 7.0/9 mM sucrose/100 ppm polysorbate 80 and 1.7 mM CaCl_2 . The histograms show the molecular weight (MW) normalized distribution and the hydrodynamic radius of the protein.

[0006] Figures 3A and 3B are histograms from Dynamic Light Scattering of F8-I and F8-J (A) and F8-K and F8-L (B) formulations where the formulation of F8-I and F8-J comprises 308 mM NaCl/10 mM histidine, 9 mM trehalose/100 ppm polysorbate 80 and no added CaCl_2 at pH 6.5 and 7.0, respectively. The formulations of F8-K and F8-L comprises of 308 mM NaCl/10 mM histidine, pH 7.0/9 mM trehalose/100 ppm polysorbate 80 and 1.7 mM CaCl_2 at pH 6.5 and 7.0, respectively. The histograms show the MW normalized distribution and the hydrodynamic radius of the protein.

[0007] Figure 4 shows the profiles of the tertiary structural conformations of rFVIII in formulations with and without CaCl_2 . F8-E (dotted line) and F8-G (solid line) are formulated without calcium chloride and F8-F (dashed line) and H (large dashed line) are formulated with calcium chloride. Formulations are described in Figures 1 and 2, above. The x-axis is the wavelength and y-axis is the second-derivative values of the absorbance.

[0008] Figure 5 shows the profiles of the tertiary structural conformations of rFVIII in formulations with and

without CaCl_2 . F8-I (solid line) and F8-K (dashed line) are formulated without calcium chloride and F8-J (dotted line) and L (large dashed line) are formulated with calcium chloride. Formulations are described in Figure 3, above. The x-axis is the wavelength and y-axis is the second-derivative values of the absorbance.

[0009] Figure 6 shows similar stability profiles of rFVIII formulations with and without CaCl_2 as a formulation excipient under accelerated storage temperature of 40 °C over 8 weeks of storage. The formulation containing calcium (dashed line) was formulated in 18 mg/ml NaCl, 0.25 mg/ml calcium chloride, 3 mg/ml sucrose and 20 mM histidine at pH 6.5. The formulation without calcium (solid line) was formulated in 18 mg/ml NaCl, 3 mg/ml sucrose and 20 mM histidine at pH 6.5. The x-axis is the storage time/temperature in weeks and y-axis is percent initial potency (at $t=0$).

[0010] Figure 7 shows similar stability profiles of rFVIII formulations with and without CaCl_2 as a formulation excipient under accelerated storage temperature of 40 °C. The formulation containing calcium (solid line) was formulated in 18 mg/ml NaCl, 0.25 mg/ml calcium chloride, 3 mg/ml sucrose and 20 mM histidine at pH 7.0. The formulation without calcium (dashed line) was formulated in

18 mg/ml NaCl, 3 mg/ml sucrose and 20 mM histidine at pH 7.0. The x-axis is the storage time/temperature in weeks and y-axis is percent initial potency (at t=0).

[0011] Figure 8 shows stability of Ca^{+2} free rFVIII formulations during storage at 2-8 °C with no loss of FVIII potency. Empty bar is the Formulation at pH 6.5 and Filled bar is the Formulation at pH 7.0. The x-axis is the storage time/temperature in weeks and y-axis is percent initial potency (at t=0).

[0012] Figure 9 shows the comparative stability of Ca^{+2} free rFVIII formulations with a commercial Factor VIII formulations containing CaCl_2 in its formulation during storage at an accelerated temperature of 40 C. Empty bar is the Ca^{+2} free FVIII formulation at pH 6.5. Filled bar is the Ca^{+2} free FVIII formulation at pH 7.0. The gradient bar is the commercial Factor VIII formulation containing CaCl_2 in its formulation. The x-axis is the storage time/temperature in weeks and y-axis is percent initial potency (at t=0).

DETAILED DESCRIPTION

OBJECTIVES

[0013] The objective that led to this invention was to determine if rFactor VIII could be formulated into a stable lyophilized formulation without any calcium chloride in the

formulation matrix. There are numerous references in the literature and prior art that imply the importance of adding divalent cations, in particular, calcium chloride, as a necessary excipient in the formulation matrix. We wanted to test this hypothesis or assumption and evaluate the structural integrity and stability of rFVIII in formulations with and without calcium chloride. The rFVIII was characterized by a number of analytical tools that measure with high sensitivity the molecular hydrodynamic radius of the protein and tertiary conformation of the protein. The hydrodynamic radius was measured using dynamic light scattering techniques to evaluate any changes in the molecular size of the protein. Any changes in the tertiary conformation of the protein in the presence or absence of added calcium chloride was analyzed by second-derivative UV spectroscopy that detects changes around the micro-environment of the Factor VIII molecule around its aromatic amino acids. To our surprise, no significant differences could be seen in rFVIII formulations with or without calcium chloride. Furthermore, to our surprise, the stability profiles under 2-8 °C and under accelerated temperature conditions of 40 °C showed that both formulations with and without calcium chloride were similar.

[0014] Having a rFVIII formulations without calcium chloride as an excipient provides several useful advantages. First, a simple formulation with one less formulation excipient to analyze and not restrict the choice of excipients that would have the potential of precipitating or forming strong complexes with calcium ions. Second, a calcium-free formulation would have a higher primary glass transition temperature and make lyophilization simpler and faster as the product could be lyophilized at higher shelf temperature during primary drying. Third, the absence of free calcium ions in the formulation is also beneficial for avoiding metal ion assisted oxidation of proteins that would affect the stability of the molecule through chemical instability mechanisms.

SPECIFIC EMBODIMENTS

EXAMPLE 1

[0015] Example 1 shows that there was no difference in the recoveries of factor VIII activity across lyophilization between formulations with and without CaCl_2 .

Table 1. Factor VIII bioactivity (IU/ml) by COATEST: Recovery across freeze-drying.

COATEST	Frz,	Lyo,	FD	Formulation (mg/ml)
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	pre- lyo	T=0	recovery	(NaCl/CaCl ₂ /Sucrose/pH)
F8-E	1211	962	79%	18/-----/3suc/6.5
F8-F	1148	891	78%	18/0.25Ca/3suc/6.5
F8-G	1289	971	75%	18/----- /3suc/7.0
F8-H	1229	999	81%	18/0.25Ca/3suc/7.0

F8-E and F8-G have no CaCl₂ as a formulation excipient.

EXAMPLE 2

[0016] Example 2 shows that there was no difference in the hydrodynamic radius of factor VIII between formulations with and without CaCl₂. Table 2 summarizes the results from formulations made with sucrose and Table 3 summarizes the results from formulations made with trehalose. The representative histograms are shown in Figures 1-3

Table 2. Hydrodynamic radius for F8-E, -F, -G, and -H formulations.

	F8-E	F8-F		F8-G	F8-H
	5.58	5.74		5.10	5.55
	5.36	5.59		5.22	5.32
	5.17	5.40		5.25	5.44
Average	5.37	5.58		5.19	5.44
S.D.	0.21	0.17		0.08	0.12
pH	6.5	6.5		7.0	7.0
CaCl	-	+		-	+

Table 3. Hydrodynamic radius for F8-I, -J, -K, and -L formulations.

	F8-I	F8-J		F8-K	F8-L
	5.59	4.94		4.54	4.67
	5.92	5.03		4.67	5.11
Average	5.76	4.99		4.61	4.89
S.D.	0.23	0.06		0.09	0.31
pH	6.5	6.5		7.0	7.0
CaCl	-	+		-	+

EXAMPLE 3

[0017] Example 3 shows that there are no significant differences in the tertiary conformation of rFVIII in formulations with and without calcium chloride. The tertiary conformation of rFVIII was evaluated by second-derivative spectroscopy and changes in the microenvironments around three aromatic amino acids, phenylalanine, tyrosine, and tryptophan were quantified by their peak positions and relative peak to trough distances expressed as ratios. The 2dUV profiles of rFVIII formulations with and without calcium chloride formulated in sucrose and formulated in trehalose Figures 4 and 5, respectively. The 2dUV parameters are summarized in Tables 4 and 5.

Table 4. 2d UV parameters for F8-E, -F, -G, and -H formulations.

	F8-E	F8-F	F8-G	F8-H
Peak Ratios	F8-220-9	F8-220-11	F8-220-13	F8-220-15
A'/B	1.14	1.19	1.15	1.17
A'/C	3.89	4.10	3.89	4.06
B/C	3.43	3.45	3.37	3.48
Peak Positions				
Phe nm	257.84	257.84	257.87	257.84
Tyr-Trp nm	283.28	283.24	283.34	283.21
Trp nm	290.78	290.78	290.78	290.81
pH	6.5	6.5	7.0	7.0
CaCl ₂	-	9 mM	-	9 mM

Table 5. 2d UV parameters for F8-I, -J, -K, and -L formulations.

	F8-I conc	F8-J conc	F8-K conc	F8-L conc
Peak Ratios				
A'/B	1.08	1.09	1.09	1.10
A'/C	3.68	3.70	3.64	3.63
B/C	3.40	3.40	3.34	3.30
Peak Positions				
Phe nm	257.84	257.84	257.81	257.84

Tyr-Trp nm	283.50	283.56	283.47	283.47
Trp nm	290.81	290.81	290.81	290.81
pH	6.5	6.5	7.0	7.0
CaCl₂	-	9 mM	-	9 mM

EXAMPLE 4

[0018] Example 4 shows the stability profiles of the rFVIII formulated with and without calcium chloride as an added excipient. Formulations were lyophilized and evaluated under accelerated storage temperatures of 40 °C.

[0019] Figures 6 and 7 show stability profiles of rFVIII in sucrose formulations at pH 6.5 and 7.0, respectively. Both formulations with and without calcium chloride show similar losses of FVIII activity over the storage time. Figure 8 shows that there was no significant loss of Factor VIII activity in calcium-free formulations storage at 2-8 °C over an 8 week period. Figure 9 shows the relative stabilities of rFVIII formulations with and without calcium chloride. The formulation with calcium chloride is a commercial Factor VIII formulation.

[0020] The above examples are intended to illustrate the invention and it is thought variations will occur to those skilled in the art. Accordingly, it is intended that the

scope of the invention should be limited only by the claims below.

SUMMARY

[0021] The albumin-free and calcium-free formulation of Factor VIII is a lyophilized formulation that is a pharmaceutically acceptable drug product, which is suitable for treating hemophilia and, therefore, may be used to prepare a medicament to treat such disease. The lyophilized product is stable with respect to its molecular integrity, hydrodynamic radius, cofactor activity, and tertiary conformation. The formulation can be rapidly reconstituted (within 30 seconds) for convenience and patient administration. The formulation comprises of a mixture of salts, amino acids, and sugars, such as sucrose, trehalose, but unlike the prior art, does not have calcium chloride as a formulation excipient. The absence of calcium chloride results in a formulation with a higher primary glass transition of the amorphous phase, thus making the lyophilization process more efficient. The absence of free calcium ions in the formulation is also beneficial as it does not restrict the choice of excipients that would have the potential of precipitating or forming strong complexes with calcium ions. Examples of such excipients are amino acids such as histidine, arginine, and

lysine that are conceivable buffers and stabilizers in Factor VIII formulations. The absence of calcium ions also is beneficial for avoiding metal ion assisted oxidation of proteins that would affect the stability of the molecule through chemical instability mechanisms.

[0022] Preferred formulations are stable, albumin-free and calcium free lyophilized rFVIII preparations, which when reconstituted in water contain up to about 310 mM NaCl, up to about 50 mM Histidine providing a pH of about 6.5 to 7.5, up to about 20 mM of sucrose or trehalose, and about 50-2000 IU recombinant Factor VIII per milliliter, but devoid of divalent cations such as Calcium +2 ion as a formulation excipient. A particularly preferred stable albumin-free and calcium-free lyophilized rFVIII formulation is devoid of divalent cations, such as Ca²⁺, as a formulation excipient, but contains 308 mM NaCl, 20 mM histidine, capable of providing a pH of about 6.5 to 7.5, about 9 mM sucrose or trehalose and about 50 to 2000 IU recombinant FVIII per milliliter. B Domain deleted Factor VIII having a specific activity of between 8000 and 15000 IU per milligram is the preferred form of recombinant Factor VIII.

CLAIMS

1. A stable, albumin-free and calcium free lyophilized rFVIII preparation comprising, when reconstituted in water,
up to about 310 mM NaCl;
up to about 50 mM Histidine, pH 6.5- 7.5;
up to about 20 mM Sucrose or Trehalose;
about 50- 2000 IU rFactor VIII/ml;
with no divalent cations, such as Ca^{2+} , as a formulation excipient.
2. A stable albumin-free and calcium-free lyophilized rFVIII preparation comprising, when reconstituted in water,
About 308 mM NaCl:
About 20 mM Histidine, pH 6.5-7.0:
About 9 mM sucrose or 9 mM trehalose:
About 50- 2000 IU rFactor VIII/ml:
with no divalent cations, such as Ca^{2+} , as a formulation excipient.
3. The preparations of Claim 1 or Claim 2, wherein the rFVIII is B-domain deleted protein that has a specific activity between 8,000 and 15,000 IU/mg.
4. Use of a preparation as defined in any one of claims 1-3 for treating hemophilia.

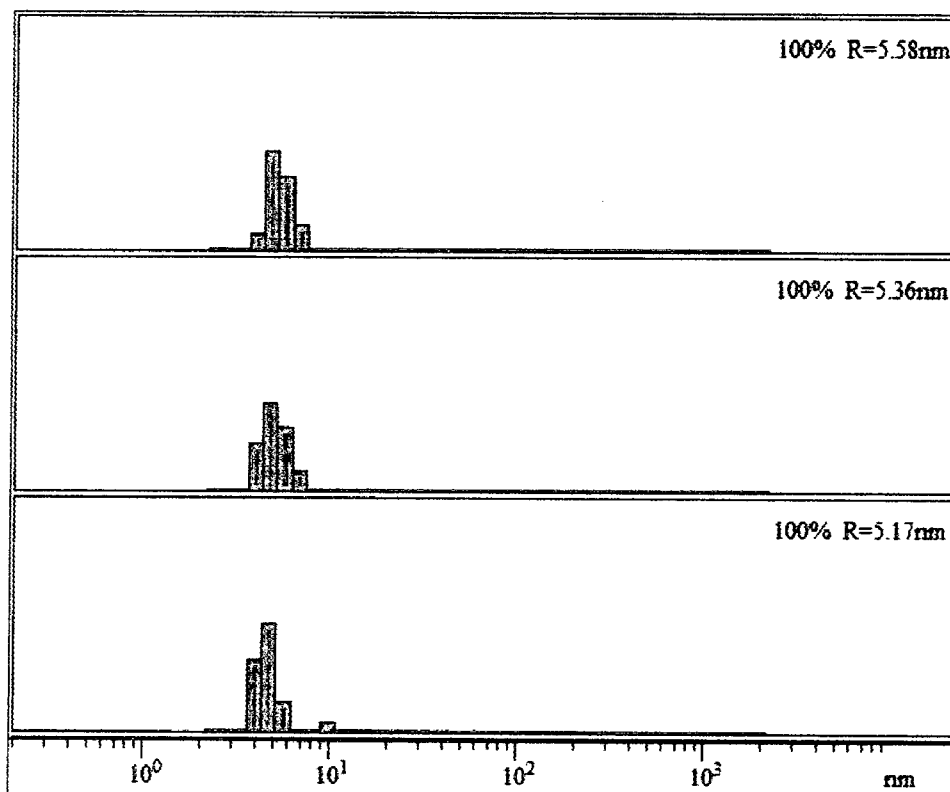


FIGURE 1A

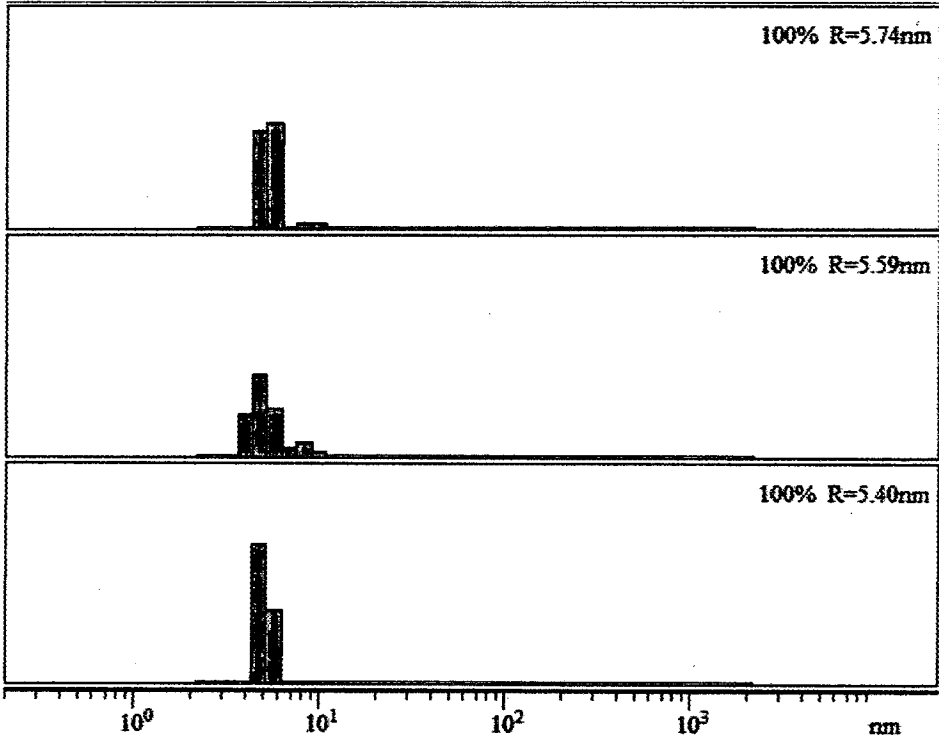


FIGURE 1B

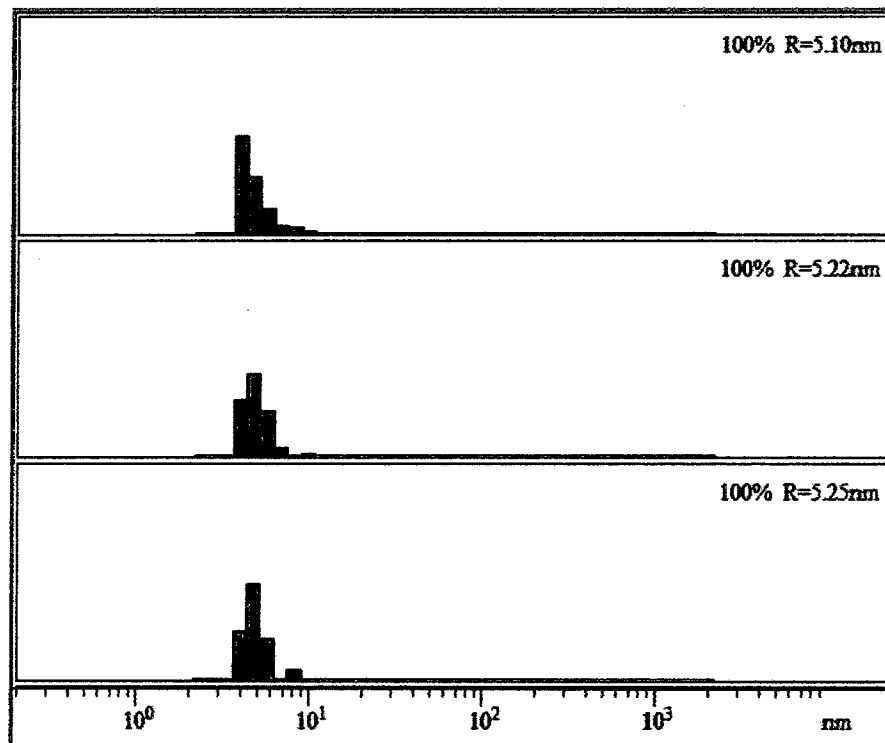


FIGURE 2A

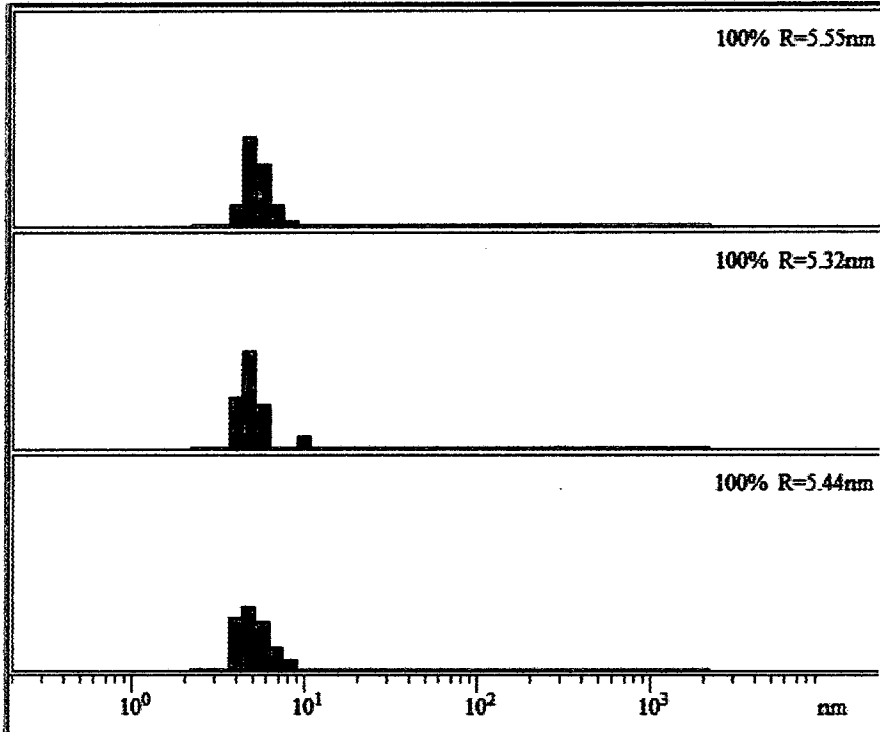


Figure 2B

A

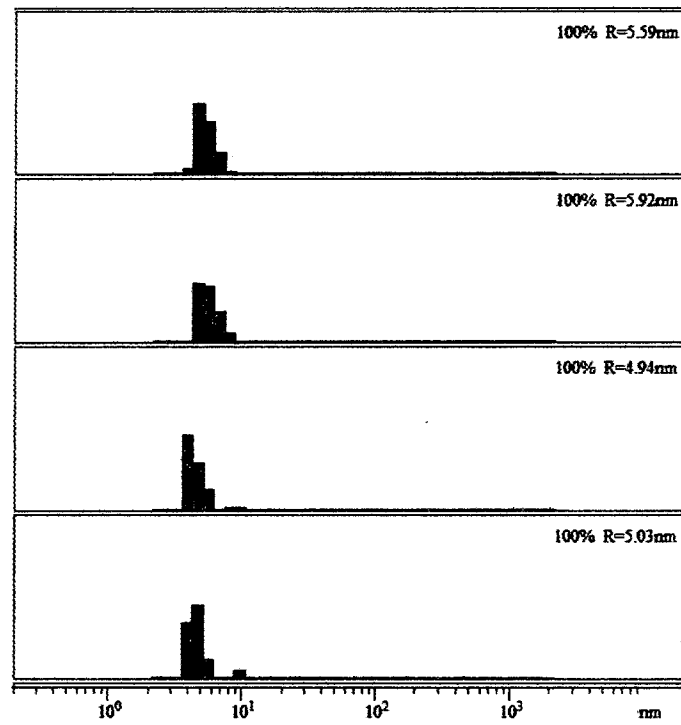


FIGURE 3A

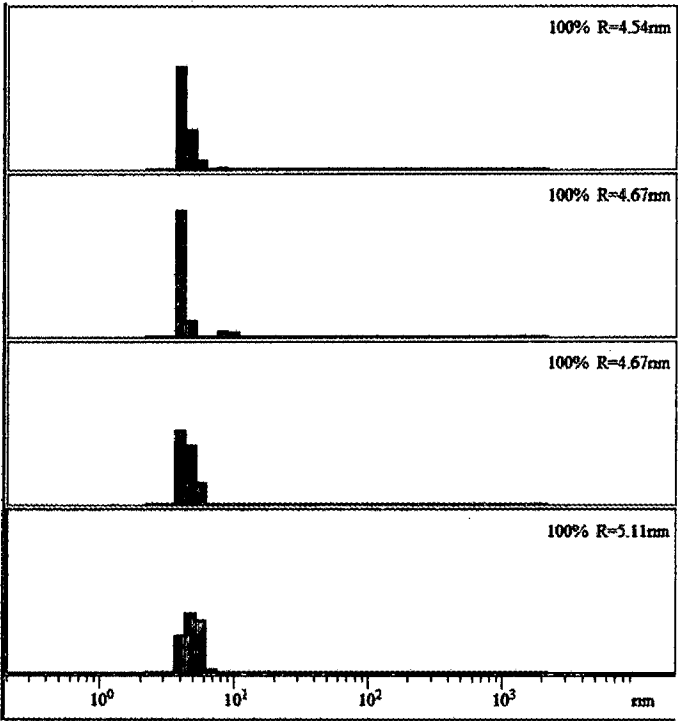


Figure 3B

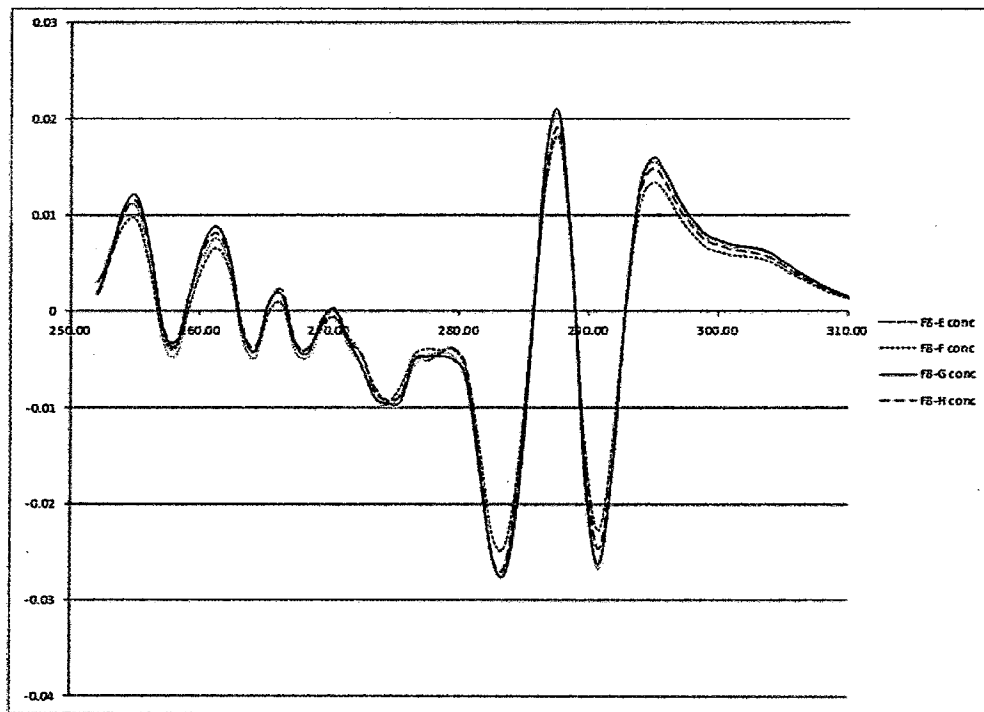


Figure 4

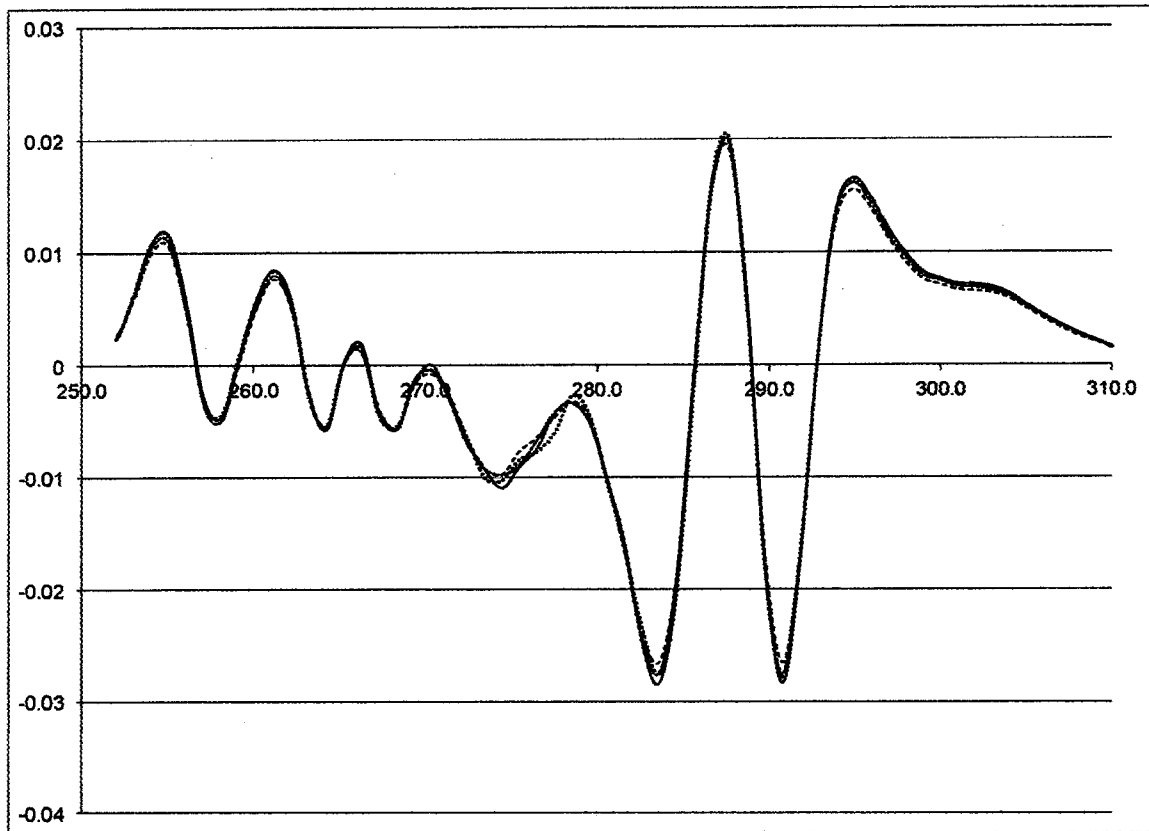


Figure 5

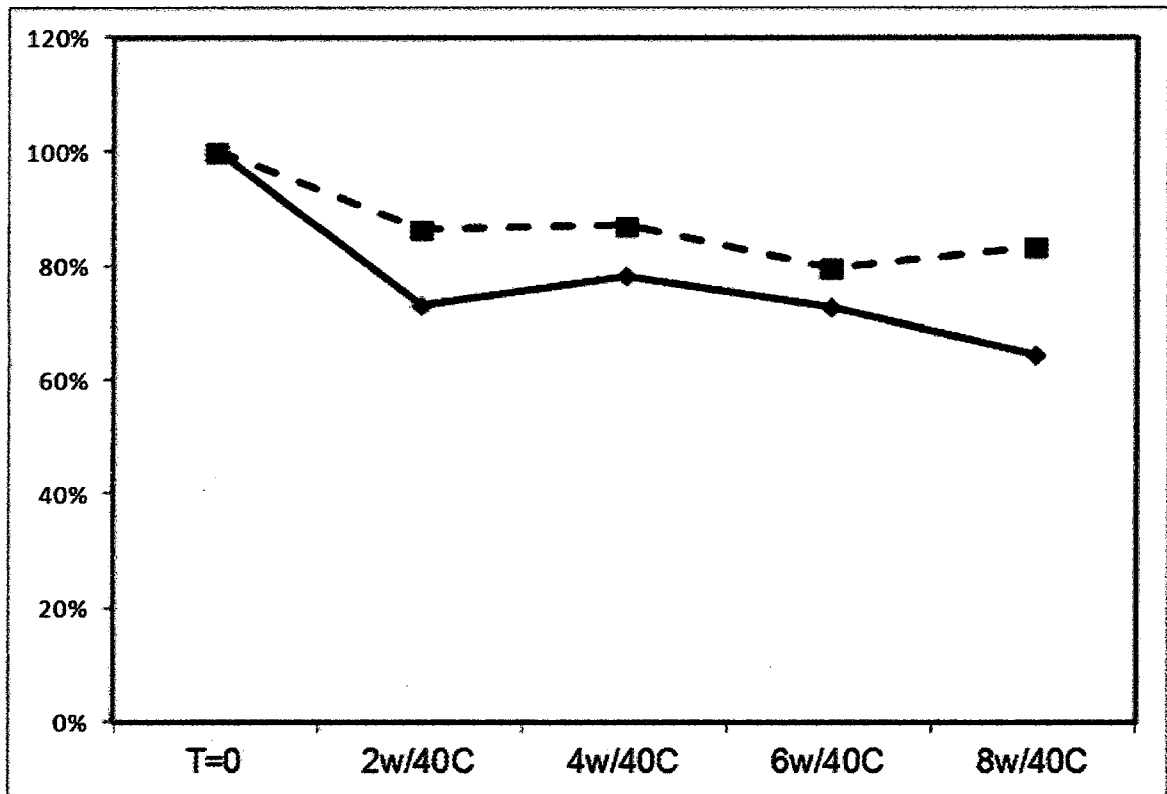


Figure 6

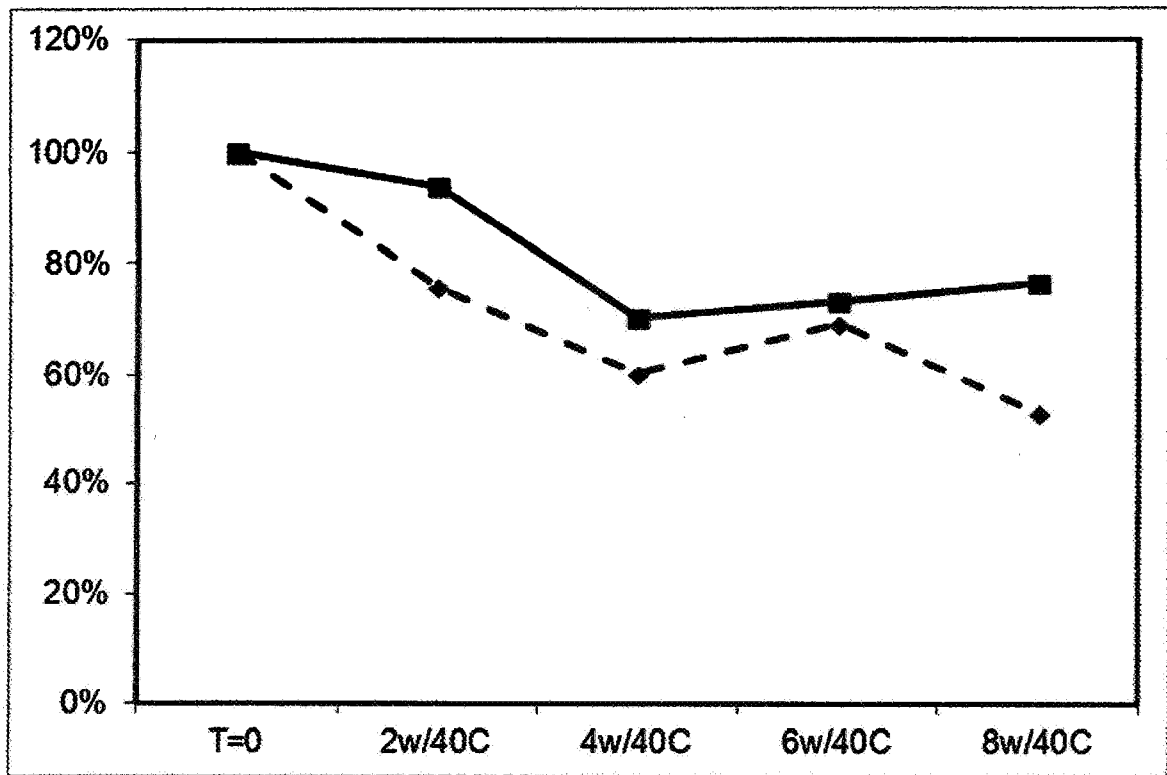


Figure 7

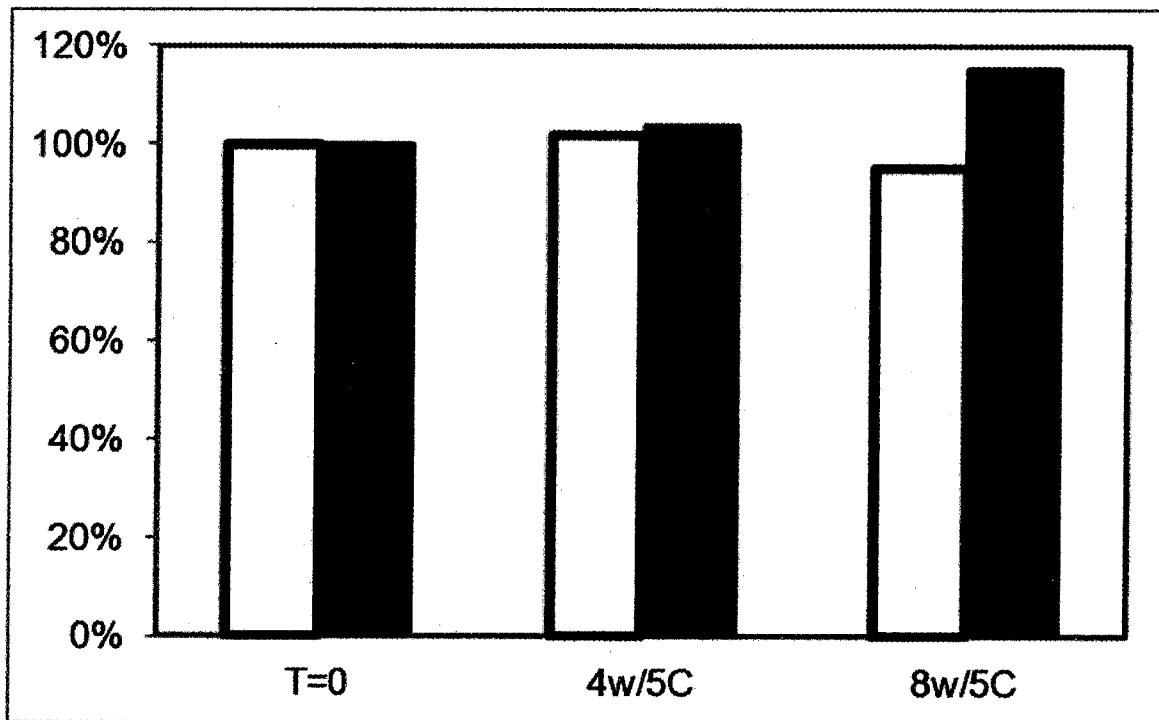


Figure 8

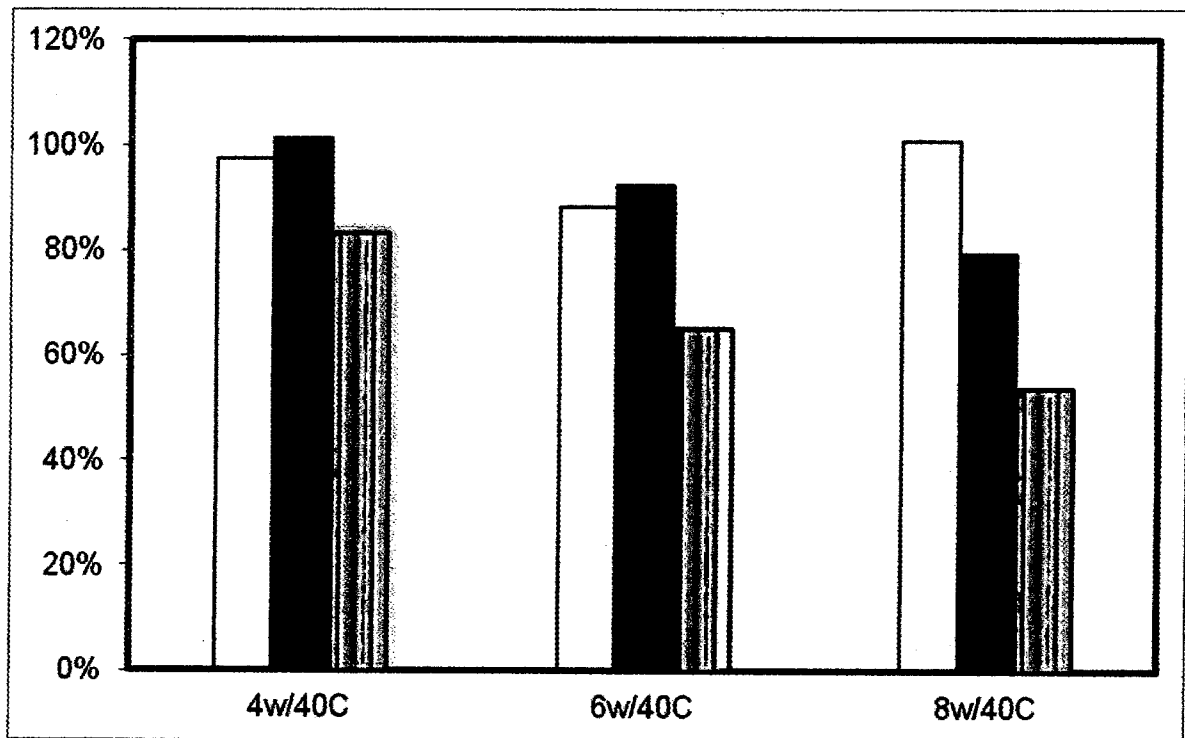


Figure 9