

Inactivation of Viruses by Incubation with Caprylate

FIELD OF THE INVENTION

The present invention relates to methods for the inactivation of
5 enveloped virus in solutions of therapeutic proteins.

BACKGROUND OF THE INVENTION

Cohn fractionation has been used traditionally in the manufacture of albumin and IgG products and has associated with it some degree of viral
10 inactivation or clearance, which has been substantiated by several studies (Wells et al (1986), Yei et al (1992), Uemura et al (1994) and Johnston (1996). In the manufacture of albumin pasteurisation (liquid heating at 60°C) is included to provide an extra margin of viral safety by inactivating hepatitis viruses and HIV. With the increasing popularity of chromatographic
15 techniques and move away from the Cohn fractionation process (Stoltz, 1993, Yap et al 1993, Veron et al. 1993) there is a need to introduce a second viral inactivation step to add to the pasteurisation step to ensure equivalent safety with the Cohn fractionated albumin product. Currently there are only a few different inactivation/clearance techniques available which could be
20 implemented. Viral filtration separates viruses from proteins on the basis of molecular size, and therefore has the potential to remove hepatitis A and parvo viruses from the smaller of the molecular weight proteins. However large molecular weight proteins (eg IgG and Factor VIII) are limited to filtering through large pore size filters that will not remove the small viruses.
25 Membrane perturbing agents such as solvents and detergents require additional processing (such as a chromatographic step) to remove the toxic agents (Horowitz et al, 1984).

The invention disclosed here describes the use of the fatty acid, caprylate, which is currently included in the albumin solution as part of the
30 formulation and which has been shown in this invention to have virucidal potential at low pH and elevated temperatures.

Pasteurisation of albumin was first introduced as early as 1948 to inactivate primarily hepatitis virus which was being transmitted through the intravenous administration of albumin. Hink and co-workers (1957) added
35 the fatty acids sodium acetyl tryphophante and sodium caprylate as well as sodium chloride to confer stability on the albumin molecule. Boyer et al

(1946) studied the turbidity of albumin at varying concentrations of several different fatty acid salts, including caprylate, at elevated temperatures. Caprylate had a pronounced effect on the stabilisation of albumin with the turbidity being most strongly influenced by the ratio of albumin to salt concentration. The concentration of caprylate used in these studies varied from as low as 8 mM and up to 150 mM. Studies performed by Yu and Finlayson (1984) confirmed this early work showing that 4 mM of caprylate in a 5 %w/v albumin solution was particularly effective in minimising aggregation of albumin during pasteurisation.

Following the early work on the unsaturated alcohols and monoglycerides and their potent virucidal properties (Snipes et al, 1977, Jordan and Seet, 1978), Sands et al (1979) explored the virucidal properties of the fatty acid derivatives. These studies focused on the inactivation of the bacteriophages in aqueous media and showed that the length of the alkyl chain (12 to 14) and the extent and geometry of unsaturation were important parameters in determining antiviral effectiveness. The virucidal potency of the fatty acids (mono-unsaturated) was further explored by Horowitz et al (1989) who demonstrated inactivation of Sindbis virus and vesicular stomatitis virus (VSV) in Factor VIII solutions. In these studies virus kill was dependent on the concentration of fatty acid (up to 0.3%) and on the chain length of acid, above a chain length of 16 a log reduction factor of 4 $\log_{10}$ TCID₅₀ was achieved in most cases. Sodium oleate activity was studied in detail using VSV spiked into albumin, IgG and some clotting factor products. Complete inactivation was demonstrated within 1 hour in IgG and clotting factors whilst albumin and plasma provided some protection to virus. Elevated temperatures up to 37°C and use of low protein concentration (1.5 mg/mL) increased the extent of virus inactivation.

Seng and Lundblad (1990) describe the use of caprylic acid in non-precipitating conditions at a pH at which the protein of interest was stable. The premise in this work was that the non-ionised form of the fatty acid, caprylic acid, interacts with the lipid envelope of the virus or the proteins embedded in the virus and thus causes irreparable damage. The amount of acid formed in the solution was manipulated by lowering the pH and/or increasing the amount of caprylate added. Typically, caprylate was added to achieve a concentration of acid ranging from 0.002 to 0.07% w/w. The influence of temperature on virus inactivation was not examined in

these studies. Concentrations of caprylic acid above 0.07% have been considered above the solubility point and have been used to precipitate proteins from solutions. (Steinbuch and Audran, 1969, Pejaudier et al., 1972, Habeeb 1984).

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SUMMARY OF THE INVENTION

The present inventors have now found that concentrations of caprylic acid above the solubility level (where it exists as an emulsion) can be achieved with advantageous results in the inactivation of enveloped viruses within selected pH and temperature ranges.

Accordingly, the present invention consists in a method of inactivating lipid-enveloped virus in a solution of therapeutic protein, the method comprising incubating the solution at temperature greater than 15°C at a pH in the range of about 4.0 to about 6.0 with caprylate at a concentration of at least about 6mM, the pH and caprylate concentration being selected such that the caprylic acid concentration is greater than 0.07% w/w.

In a preferred embodiment of the present invention the incubation temperature is from 15°C to about 45°C and preferably from about 25°C to about 45°C.

In a further preferred embodiment of the present invention the concentration of caprylate is in the range of about 6mM to about 100mM.

In yet another preferred embodiment of the present invention the pH of the solution is in the range of about pH 4 to about pH 6.

The method of the present invention can be used with any of a large range of therapeutic proteins and is particularly applicable to albumin.

In a particularly preferred embodiment of the present invention the method comprises incubating at 45°C solution comprising 10%(w/w) albumin, 16mM caprylate at a pH of 4.5.

In another preferred embodiment of the present invention the method comprises incubating at 45°C solution comprising 20-25%(w/w) albumin, 30-40mM caprylate at a pH of about 5.2.

In a further aspect the present invention consists in a solution of therapeutic protein prepared according to the method of the first aspect of the present invention.

The present invention also consists in a composition including a therapeutic protein in which the therapeutic protein has been treated according to the method of the first aspect of the present invention.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

10 DETAILED DESCRIPTION OF THE INVENTION

In order that the nature of the present invention may be clearly understood, preferred forms thereof will now be described with reference to the following examples and Figures.

15 FIGURE LEGENDS

Figure 1: The effect of pH and caprylate on Sindbis Virus Inactivation in albumin. (● - 8mM Caprylate, pH6-7, 15°C; ○ - 32mM Caprylate, pH6-7, 15°C; ▼ - 32mM Caprylate, pH4.5, 45°C; ▽ - 8mM Caprylate, pH4.5, 45°C, ■ - 32mM Caprylate, pH6-7, 45°C.)

20 **Figure 2:** Inactivation of BVDV as a result of incubation with caprylate in albumin. (● - pH 4.5 with caprylate at 30°C; ○ - pH 7.0 with caprylate at 30°C; ▼ - pH 4.5 no caprylate at 30°C; ▽ - pH 7.0 no caprylate at 30°C, ■ - pH 4.5 with caprylate at 0°C; □ - pH 7.0 with caprylate at 0°C; ◆ - pH 4.5 no caprylate at 0°C; ◇ - pH 7.0 no caprylate at 0°C.)

25 **Figure 3:** Bovine Viral Diarrhoea Virus Inactivation in albumin treated with 14 mM caprylate at various temperatures. (● - 2-8°C; ○ - 25°C; ▼ - 30°C; ▽ - 40°C, ■ - 40°C; □ - 45°C.)

Figure 4: Sindbis virus Inactivation in albumin treated with various caprylate concentrations. (● - 3mM caprylate; ○ - 8mM caprylate; 30 ▼ - 16mM caprylate; ▽ - 26mM caprylate.)

Figure 5: HAV inactivation in the presence of 12 mM caprylate.

Example 1:

Solutions of 5% w/v and 20% w/v of an albumin preparation
35 containing 8 mM and 32 mM caprylate respectively were pH adjusted to pH 4.5 with 0.5M HCl. Control samples of 5% and 20% were kept aside to

establish the influence of caprylate alone and low pH on the virus kill. The solutions were incubated at 15°C and at 45°C for a maximum period of 24 hours. Sindbis virus (Strain MRM39) was initially spiked into the solutions and samples were removed at time zero, after 12 hours and finally after 24 hours and then assayed for virus titre. The kinetics of virus inactivation are shown in Figure 1.

Figure 1 shows that Sindbis virus was not inactivated (i) without the addition of caprylate, even at pH 4.5 (ii) at 15°C in the presence of caprylate. Greater than 5 logs of virus were inactivated once caprylate is added and the temperature was elevated to 45°C. The kinetics of inactivation were enhanced (virus kill quickest) at the lower pH of 4.5 compared to pH 6.0 and at the highest caprylate concentration of 32 mM.

Example 2:

High titre bovine viral diarrhoea virus, BVDV, was spiked into approximately 10% w/v albumin concentrate. Incubation was performed at 30°C or 0°C with or without caprylate (16mM) and at pH 4.5. Figure 2 shows the importance of both the presence of caprylate at low pH and elevated temperature to inactivate this enveloped virus. Incubation at 0°C in the presence or absence of caprylate did not inactivate this virus.

Example 3:

Albumin (10% w/v) containing approximately 14 mM caprylate was pH adjusted to pH 4.5 with 0.5M HCl. Bovine viral diarrhoea virus, BVDV, (NADL, strain, from ATCC) was spiked into the albumin and samples were then incubated at a range of temperatures from 25 to 45°C. A control sample was kept at 2-8°C. The turbidity of each of the albumin samples after incubation at each of the temperatures was measured to evaluate the stability of the albumin. Aggregate content was also measured by HPLC. The results of this study are illustrated in Figure 3 and Table 1. There was a significant influence of temperature on the rate BVDV inactivation with almost instantaneous inactivation (<0.5 hours) seen at temperatures of 35°C or above. Aggregate content appeared stable up to 35°C but increased above this temperature. Turbidity showed an increasing trend with increasing temperature. Both of these results reflect some onset of stability at the

higher temperatures, however, below 35°C the change in these parameters is not significant.

Table 1

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Temperature	Aggregate (% area)	Turbidity National Turbidity Units
Untreated	0.1	26
0°C	0.0	27
25°C	0.1	27
30°C	0.1	32
35°C	0.1	42
40°C	0.3	48
45°C	0.6	47

Example 4:

Samples of albumin (5%, 10% and 20% w/v) were pH adjusted to pH 4.4 and caprylate was added to achieve a range of caprylate concentrations from 3 mM (20%) to 26 mM. Samples were spiked with Sindbis virus (Strain MRM39) and incubated at 30°C. Samples were removed at several time points up to 10 hours and assayed for Sindbis virus titre. The kinetics of this study are illustrated in Figure 4.

There was a significant trend of increasing rate and extent of Sindbis virus inactivation with increasing caprylate concentration, with no significant kill seen at 3 mM and >4 log reduction in titre seen at ≥16 mM, which occurred within 1 hour.

Example 5:

Samples of albumin were pH adjusted to pH 4.5 and caprylate was added to achieve a final concentration of 12 mM.

Samples were spiked with hepatitis A virus, HAV, (HM175A.2 strain) and incubated at 45°C. Samples were removed after 10 and 24 hours and assayed for HAV titre. The results of this study are shown in Figure 5. Only one log of virus was inactivated after 24 hours providing evidence that this inactivation technique primarily targets enveloped viruses.

As will be recognised by those skilled in this field the present invention discloses conditions for inactivation of viruses in albumin solutions not previously considered feasible and virucidal. The conditions developed by the present inventors include a caprylate concentration of approximately 7.2mM to 40mM which gives a caprylic acid concentration range of 0.1 to 0.50% at pH 4.5, well in excess of 0.07% used by Seng and Lundblad. The use of caprylate to inactivate the enveloped viruses was also found in these studies to be dependent on temperature, with no significant inactivation of Sindbis in an albumin solution seen at 15°C but at 45°C a 4 log reduction factor was achieved for the same concentration of caprylic acid and the same pH. A similar pattern of temperature dependency was seen with bovine viral diarrhoea virus (BVDV) spiked into albumin solutions.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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CLAIMS:-

1. A method of inactivating lipid-enveloped virus in a solution of therapeutic protein, the method comprising incubating the solution at
5 temperature greater than 15°C at a pH in the range of about 4.0 to about 6.0 with caprylate at a concentration of at least about 6mM, the pH and caprylate concentration being selected such that the caprylic acid concentration is greater than 0.07% w/w.
2. A method as claimed in claim 1 in which the incubation temperature
10 is from 15°C to about 45°C.
3. A method as claimed in claim 2 in which the incubation temperature is about 25°C to about 45°C.
4. A method as claimed in any one of claims 1 to 3 in which the concentration of caprylate is in the range of about 6mM to about 100mM.
- 15 5. A method as claimed in any one of claims 1 to 4 in which the pH of the solution is in the range of about pH 4 to about pH 6.
6. A method as claimed in any one of claims 1 to 5 in which the therapeutic protein is albumin.
7. A method as claimed in any one of claims 1 to 6 in which the
20 method comprises incubating at 45°C solution comprising 10%(w/w) albumin, 16mM caprylate at a pH of about 4.5.
8. A method as claimed in any one of claims 1 to 6 in which the method comprises incubating at 45°C solution comprising 20-25%(w/w) albumin, 30-40mM caprylate at a pH of about 5.2.
- 25 9. A composition including a therapeutic protein in which the therapeutic protein has been treated according to the method as claimed in any one of claims 1 to 7.



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