Effects of High Hydrostatic Pressure on Several Sensitive Therapeutic Molecules and a Soft Nanodispersed Drug Delivery System

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Purpose. According to the development in the last decade of industrial processes using high hydrostatic pressure (HHP) for preservation of several commercial food products, novel sterilization or decontamination processes for pharmaceutical products could be conceivable. The aim of this work is to evaluate the effects of HHP on the integrity of insulin and heparin solutions, suspension of monoclonal antibodies and Spherulites®.

Methods. High performance liquid chromatography, thin layer chromatography, capillary electrophoresis assays, ELISA tests, laser granulometry and spectrophotometry analyses have been performed to compare HHP treated drugs (in a domain of pressure and temperature ranging respectively from 20 up to 500 MPa and from 20°C up to 37°C) vs. untreated ones.

Results. No difference has been detected except for monoclonal antibodies that are altered above 500 MPa.

Conclusions. The structure integrity of sensitive molecule due to the small energy involved by HHP and the development of industrial plants (intended for the decontamination of food products) confer to this technology the potential of a new method for sterilization of fragile drugs and an original alternative to aseptic processes and sterilizing filtration.

KEY WORDS: high hydrostatic pressure; sterilization; sensitive drugs; drug delivery systems; preservation.

INTRODUCTION

Since the beginning of the 20th century, effects of high pressure on biologic systems (bacteria, virus, and toxins) have been studied and inactivation of micro-organisms by such a technology is now well known (1–6). At the end of the 80's, Japanese works have been involved in the development of HHP in Biosciences (7). Hence, in the 90's, the first industrial applications of HHP have concerned the preservation of food stuff; and in 2000, HHP has been considered by the Food and Drugs Administration (FDA) as a new alternative processing technology for microbial inactivation (8).

HHP processes offer industrial advantages: pressure treatment is not time/mass dependent (contrary to steam sterilization); thus reducing processing time (9). Pressure transmission is not controlled by product size and no edge or thickness effect takes place. In addition, pressure transmission is instantaneous and uniform. Due to the low compressibility of water, the amount of energy needed to pressurize drugs is low and does not promote the formation of new chemical compounds, "radiolytic" by-products, or free-radicals.

Nowadays, several potential applications in pharmaceutical and medical fields have been described (10–12), but it is necessary to evaluate two technical aspects in order to develop sterilization processes for pharmaceutical products: (i) the inocuity of HHP treatments on therapeutic molecules or drug carriers and (ii) the capability of HHP to decontaminate drugs.

The aim of this study concerns the first item. Several suspensions of sensitive molecules (somatostatin, insulin, heparin sodium, monoclonal antibodies) and a nanodispersed system (Spherulites®) were submitted to HHP treatments and analyzed in comparison with untreated samples. Taking into account industrial equipment limitations, all experiments were performed within ranges of pressure and temperature compatible with industrial HHP equipments.

MATERIALS AND METHODS

High Hydrostatic Pressure Equipment

Figure 1 shows a schematic view of the HHP equipment designed by NFM-FRAMATOME. The working pressure and temperature ranges were [0.1 MPa; 800 MPa (8 000 bar)] and [−20°C; +80°C] respectively. Thermodynamic parameters (pressure and temperature), were measured by sensors placed inside and outside the high pressure vessel (effective volume: 3 liters), and all data were computer stored and processed.

Packaging

Before HHP treatment, every sample was packaged in heat sealed polyethylene/polyamide. This PE/PA film had previously been validated for high pressure use (13).

Fragile Molecules and Soft Nanodispersed Drug Delivery System Tested

Several therapeutic molecules were chosen with molecular weight ranging from 1200 g/mol up to 150 000 g/mol.:

- Two peptides supplied by a private laboratory. (a) The first one (peptide A) is somatostatin (molecular weight (Mw): 1096.34 g/mol.), (b) the second one (peptide B) remained confidential (molecular formula: $C_{57}H_{81}N_{13}O_{13}S_3$ and Mw: 1252.55 g/mol.)
- Solutions of a human biogenetic insulin (molecular formula: $C_{257}H_{383}N_{65}O_{77}S_6$, Mw: 5 808 g/mol)
- Solutions of sodium heparin (Mw: higher than 8 000 g/mol)
- Suspensions of monoclonal antibodies anti-influenza A (Mw: 150 000 g/mol)

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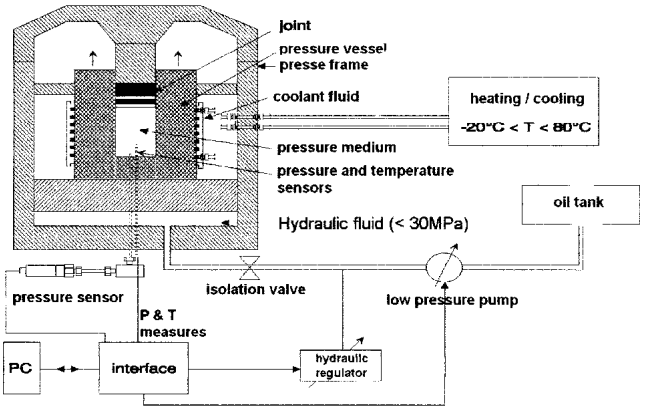

Fig. 1. Schematic view of the high isostatic equipment designed in collaboration with NFM-Framatome and available in the LPCHP.

Effects of high pressure were evaluated on an aqueous suspension containing 40% of microdispersed drug delivery system (Spherulites®) provided by Capsulis (Etypharm group). Spherulites®, multilamellar microvesicles made of surfactants, are able to encapsulate active ingredients (in this work a hydrophilic dye, amaranth). They exhibit an internal structure made of concentric bilayers of amphiphiles alternating with water layers. Spherulites used in this work were characterized by a bimodal size distribution (mean size 1 = 0.29 μm, mean size 2 = 4.38 μm).

Analytical Devices and Methods

ELISA

To carry out ELISA test antibodies were prepared as the follows:

Coating without sucrose: 1) Mix 100 μl of rabbit serum anti-influenza A 1/2000 with a CBC 0.1 M buffer (pH = 9.6); 2) Leave for 12 h at 4°C; 3) Aspirate; 4) Transfer into sugared and saturated wells of ELISA plates, add 250 μl of PBS-T BSA 3%, leave for 1 h at room temperature; and 5) Aspirate, add 250 μl of PBS sucrose and leave for 20 min at room temperature, then dry.

ELISA Test: 1) Take 100 μl of a solution of viral particles in PBS-T, leave for 30 min at 37°C; 2) Wash; 3) Add 100 μl of anti-influenza A monoclonal antibodies 1/1000 in PBS-T, leave for 15 min at 37°C; 4) Wash; 5) Add 100 μl of anti-mouse F(ab)′2 combined 1/5000 in PBS-T, leave for 15 min at 37°C; 6) Wash; 7) Add 100 μl of Sub A/Sub B mixture at room temperature; 8) Stop after 5 min with 50 μl of 2N HCl solution; and 9) Read at 450/692 nm.

HPLC

HPLC was used to quantify peptides A, B and insulin. Peptide A and B were quantified by the supplier using their own method. Insulin was quantified using the following method.

Column: Nucléosil, C18, (5 μm, 125 × 4.6 mm) ; flow: 1 ml/min ; mobile phase: 40% of acetonitrile, 40% of water and 20% of H_3PO_4 buffer solution to reach a final pH of 2.5;

$$\lambda = 214 \text{ nm; injected volume} = 20 \text{ μl.}$$

Capillary Electrophoresis

Heparin analyses have been performed on a Beckman P/ACE MDQ capillary electrophoresis apparatus using the following parameters:

Electrolyte: phosphate buffer 20 mM, pH = 3.5 adjusted with HCl; capillary characteristics: internal diameter 75 μm, length 583 mm; washing: 138 kPa, 2 min, with electrolyte; Injection: 3.5 kPa, 15s; voltage: 30 kV; absorbance: 214 nm, direct mode; temperature: 25°C.

Control of Spherulites®

Suspension of Spherulites® were analyzed before and after HHP treatments. The size distribution was measured using laser granulometry method, the bilayers surfactants were controlled by thin layer chromatography (TLC) and the dye encapsulated rate was calculated from the spectrophotometric assay of amaranth. These analytical methods are routinely used by the supplier.

RESULTS

Peptides A and B

These two peptides are sensitive to heat treatments and must be stored at low temperature (below 4°C). They are routinely sterilized by gamma radiations but they are damaged by this process (a decrease of more than 5% of their purity can be detected).

Peptides A and B were treated at 300, 400, and 500 MPa, during 10 min at 25°C. HPLC assays did not show any differences greater than 1% between samples and references.

Solutions of Insulin and Heparin

Solutions of insulin and heparin were submitted to the same HHP treatments: (i) 400, 500, and 600 MPa, during 10 min, at 25°C, (ii) series of 6 successive cycles of 5 min. of pressurization-depressurization at 500 MPa, 25°C, (iii) 30 successive short cycles of 5 min of low (20 MPa) and high pressure (500 MPa) at 37°C, (iv) solutions of insulin and heparin were also heated at 121°C during 15 min in order to compare the effects of high pressure with conventional sterilization process.

Solution of Insulin

Figure 2 depicts the combination of chromatograms of the untreated insulin solution (reference), the insulin solution after the most efficient HHP treatment (identical chromatograms were obtained with the other high pressure conditions) and the hormone after autoclaving. The largest variation between peaks area for high pressure treated insulin and for the reference was 3% vs. 96% for the conventional heat treatment.

Solution of Heparin

A combination of electropherograms obtained with capillary electrophoresis analyses is depicted in Fig. 3 (untreated heparin solution, heparin solution after the most efficient HHP treatment). No difference between the reference and

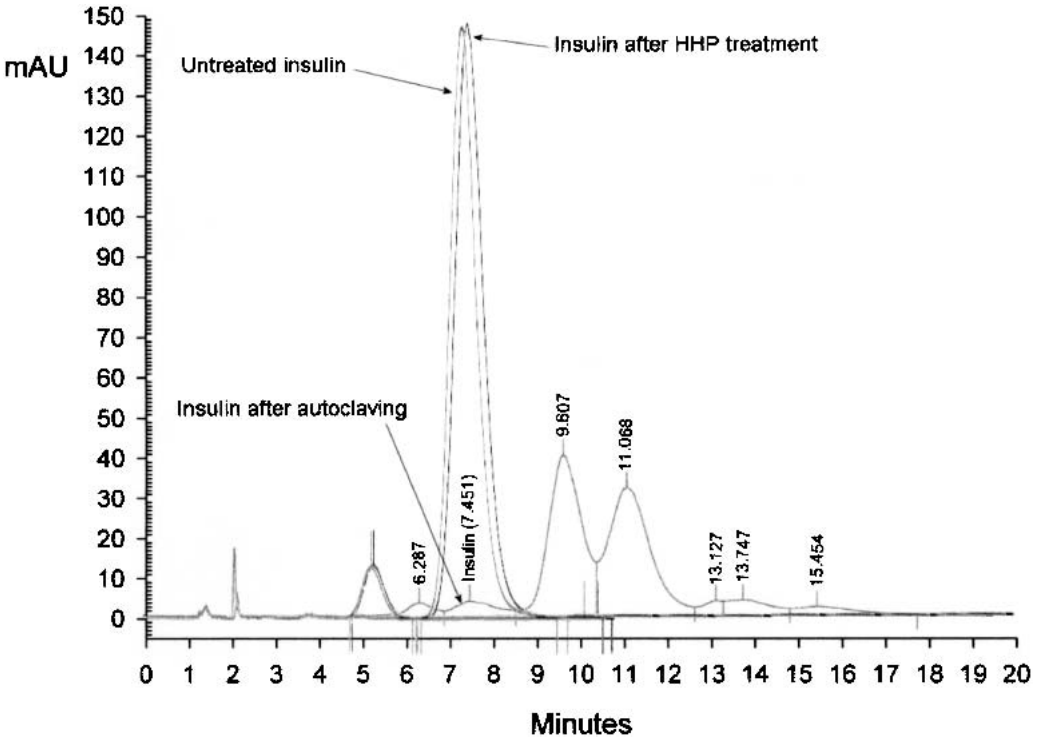

Fig. 2. Combination of chromatograms of the untreated solution of insulin, a solution treated by the most HHP treatment and insulin heated at 121°C and 10 min.

each pressurized solution of heparin was detected by this analytical method whereas heparin seemed to be denatured by heat treatment (see Fig. 3, peaks a, b, and c).

Anti-influenza A Monoclonal Antibodies

Antibodies were treated at 400, 500, and 600 MPa, at 26°C and during 10 min. Their reactivity was evaluated with an ELISA test (Fig. 4). Only for the highest pressure experiment (600 MPa), optical density values (curve Ac 6000) were different than the standard curve (ref 5000–6000). HHP treatments at 400 and 500 MPa did not modify the immunoreactivity of these antibodies whereas at 600 MPa they seemed to be affected by high pressure, they were still able to fix themselves on a "target" but not on a specific one. HHP close to 600 MPa seemed to be the upper limit for these antibodies; beyond such pressure irreversible alteration could be detected. A previous study on aroma had underlined the same phenomenon (14).

Effects of High Pressure on a Soft Nanodispersed Drug Delivery System: the Spherulites®

Aqueous suspensions containing 40% of Spherulites® were treated at 300, 400, and 500 MPa, at 25°C and during 10 min. Analysis of the size distribution and TLC qualitative analysis of the lipids forming the Spherulites® membranes were performed. Concentration of an encapsulated dye (amaranth) was measured by spectrophotometry before and after high pressure treatment to evaluate the influence of high pressure on the encapsulation rate.

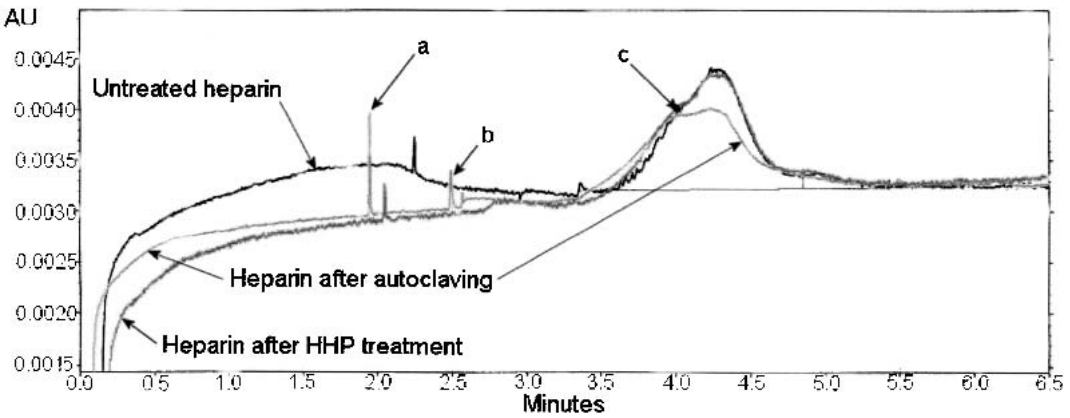

Fig. 3. Combination of electropherograms of the heparin reference solution, after HHP treatment [(20 MPa; 5 min; 37°C); (500 MPa; 5 min; 37°C)] × 30) and the heparin solution after steam sterilization (121°C, 15 min).

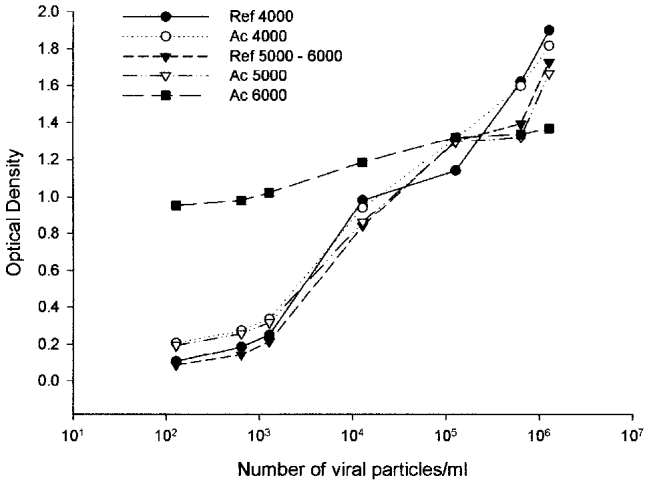

Fig. 4. Graphic representation of the results achieved by the ELISA test on the antibodies treated by high pressure (400, 500, 600 MPa; 10 min; 25°C).

Analysis of the Size Distribution

Laser granulometry analysis showed a bimodal size distribution for each suspension, before and after high pressure treatment. Mean size values of Spherulites® for each mode were: 0.29 μm for mode 1, 4.38 μm for mode 2 and 0.31 μm for mode 1 and 4.75 μm for mode 2, for the reference sample and the treated suspension (500 MPa; 10 min.; 25°C) respectively.

Thin Layer Chromatography Analysis

The lipidic components of the Spherulites® layers were detected by TLC analysis. After high pressure treatments (300, 400, and 500 MPa) integrity of the lipidic components was preserved (each specific spot representing a lipidic component had moved with the same specific migration length as the untreated Sphérulites®).

Dye Encapsulation Rate

After extraction of the Spherulites® from the medium, a spectrophotometric dosage at 522 nm allowed us to measure the amount of amaranth (a red hydrophilic dye) released in the aqueous medium. The rate of encapsulation was consequently calculated with the initial quantity of dye encapsulated in the Spherulites®. No effect of high pressure on the encapsulation rate of amaranth by the Spherulites® was detected.

DISCUSSION

Therapeutic molecules (insulin, heparin, and monoclonal antibodies) and drug carriers (sphérulites®) tested in this work were preserved by HHP treatments in a domain of pressure and temperature ranging respectively from 20 up to 500 MPa and from 20°C up to 37°C. The comparison between HPLC assays of the insulin solution after HHP treatment and after heat treatment underlined the few energy transmitted by high pressure.

The same HHP treatments have previously been developed in order to illustrate the capability of high pressure to inactivate microorganisms. A total inactivation or a reduction higher than 6 $\log_{10}$ was achieved for *Candida albicans*, spores of *Aspergillus niger*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and spores of *Bacillus subtilis*. The last one has recently been proposed by the FDA for validation of HHP processes, with regard to their high resistance to pressure (15).

All these considerations clearly show that HHP could represent an alternative solution for fragile drugs and drug delivery systems (DDS) sterilization, especially when they have a high added value (molecules produced by biotechnologies) and cannot be easily sterilized with conventional technologies. However, it is very difficult to make a general conclusion because each HHP process has to be developed on a case to case basis and several parameters must be taken in account:

(i) Monoclonal antibodies appeared to be altered by pressure greater than 500 MPa. The few energy transmitted by pressure involve fine mechanisms, consequently new arrangements of weak bonds of these quaternary proteins (rupture of electrostatic interactions) can explain the behavior of antibodies after HHP treatment at 600 MPa. Hence, the development of HHP processes in pharmacy cannot be undergone without studying the thermodynamic domain of stability of macromolecules such as proteins, in order to avoid irreversible modification of their structure and consequently of their bioactivity (12,16).

(ii) DDS represent modern galenic forms and the future for development of new therapies (e.g. antitumourous therapies). Our results concerning Spherulites®, previous works on the preservation of liposomes under high pressure (17) (the presence of cholesterol in the phosphatidylcholine bilayer membrane of the liposomes seemed to protect them from high pressure) and a recent work on thermally and hydrolytically labile drug carriers (made with poly(hexadecylcyanoacrylate)) showed that HHP treatments could represent a sterilization process able to preserve sensitive drugs carriers (18). Effects of isostatic pressure on micro- or nanodipersed particles depend on the compressibility of the material (polymers) of which particles are made of. If the particles were isotropic, then the deformation induced by isostatic pressure will be a homothetic reduction of volume that could be more or less reversible (depending on the elasticity of the material) according to the compressibility. If the particles were porous, then a densification may occur, but with a slow and controlled speed of pressurization and depressurization, diffusion of the medium through the pores and inside the particles can be driven. Irregular deformations or crashing could be observed, most of the time due to interactions between particles under pressure; the contact zones between particles being very small, pressure is enhanced at these points.

(iii) Interactions medium or drugs and medium or microorganisms during HHP process have to be taken into account because the composition of the medium can increase or decrease the stability of proteins and the microorganisms resistance to pressure (19,20).

Even if high pressure were already used for microbial inactivation of food stuff, then further microbiology studies are required in order to validate this original technology for decontamination and sterilization of sensitive drugs (biomolecules and DDS) according to Pharmacopoeia standards.

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