An Albumin–Free Formulation for Escherichia coli –Derived Interferon Beta–1b with Decreased Immunogenicity in Immune Tolerant Mice

Article in Journal of Interferon & Cytokine Research · January 2016
Impact Factor: 2 · DOI: 10.1089/jir.2015.0110

READS
31

6 authors, including:

Mohadeseh Haji Abdolvahab
Utrecht University
6 PUBLICATIONS 8 CITATIONS

Ahmad Fazeli
Tarbiat Modares University
20 PUBLICATIONS 50 CITATIONS

M. Reza Nejadnik
Leiden University
28 PUBLICATIONS 425 CITATIONS

Mohammad reza Fazeli
Tehran University of Medical Sciences
50 PUBLICATIONS 539 CITATIONS

All in-text references underlined in blue are linked to publications on ResearchGate, letting you access and read them immediately.

Available from: Mohadeseh Haji Abdolvahab
Retrieved on: 14 April 2016
Human serum albumin (HSA)-free formulation of *Escherichia coli*-derived human interferon beta (IFN-β-1b) with a high percentage of monomeric protein and low immunogenicity is developed and characterized in the current study. UV spectroscopy, fluorescence spectroscopy, dynamic light scattering, sodium dodecyl sulfate polyacrylamide gel electrophoresis, Western blotting, Micro-Flow Imaging, resonant mass measurement, size exclusion, and reversed-phase high performance liquid chromatographies were applied to assess the effect of excipients on the stability of IFN-β-1b to establish a HSA-free formulation. The antiviral activity of IFN-β-1b was evaluated using human lung carcinoma cell line. Immune tolerant mice to hIFN-β were used to assess the immunogenicity of the HSA-free formulated IFN-β-1b in comparison to Betaferon\textsuperscript{\textregistered} drug product and Avonex\textsuperscript{\textregistered} drug substance as standards through IgG titering of plasma. HSA-free formulated IFN-β-1b, including 200 mM L-arginine, 200 mM trehalose, and 0.1% n-dodecyl β-D-maltoside in 10 mM sodium acetate buffer, pH 7.4, showed the highest biological activity. The stability of IFN-β-1b in the HSA-free formulation was monitored for 3 weeks at 4°C and 37°C with relative humidity of 10% and 75%, respectively. Protein aggregation and immunogenicity in transgenic mice were decreased in the HSA-free formulated IFN-β-1b compared to Betaferon. The stability, biological activity, and immunogenicity of the HSA-free formulation and Betaferon were evaluated. Incubation of formulations at 4°C and 37°C for 3 weeks showed that the HSA-free formulated IFN-β-1b was more stable and less immunogenic in transgenic FVB/N mice. Low immunogenicity and the absence of HSA, which reduces the potential risk of viral infection (eg, HIV and HCV), are promising for clinical studies.

**Introduction**

Interferon beta (IFN-β) is a first-line treatment for relapsing remitting multiple sclerosis. IFN-β-1b (Betaferon\textsuperscript{\textregistered}) derived from *Escherichia Coli* is nonglycosylated and contains a mutation of Cys to Ser at position 17. It was the first to be approved by the FDA in 1994 followed by IFN-β-1a (Avonex\textsuperscript{\textregistered}) in 1996 (Barnard and others 2013). Although, patients treated with these drugs may encounter less relapses and show reduced disability (Schellekens 2002), repeated administration over prolonged periods may result in the formation of neutralizing antibodies that may lead to reduced clinical efficacy (Schellekens 2002; Sorensen 2008; Sorensen and others 2008; Wolbink and others 2009). For Betaferon drug product, the Nab rate is 28%–47% (Paty and Li 1993; Kappos 1998; Giovannoni and others 2002), whereas for Avonex drug product it is 2%–14% (Jacobs and others 2000; Clanet and others 2002; Sominanda and others 2007). The formation of antibodies is thought to be related to protein aggregation and formulation (Barnard and others 2013). To have a successful formulation of therapeutic proteins, understanding of their physicochemical and biological characteristics is needed. The activity of proteins is highly dependent on their conformational structure (Boublik and others 1990). However, the structure of a protein is flexible and sensitive to external conditions such as those used in the expression, purification, formulation, handling, and storage of the drug (Frokjaer and Otzen 2005). Unfavorable conditions may lead to denaturation and formation of soluble and insoluble aggregates, and chemical instabilities including inside
formation, hydrolysis, oxidation, racemization, and deamination. All of these may affect safety and efficacy (Shirley and others 2008).

Several excipients (buffers, sugars, surfactants, amino acids, polyethylene glycols, and polymers) have been added to polypeptide pharmaceutical formulations to increase their stability. The stabilization of polypeptides in pharmaceutical compositions, however, remains largely an area of trial and error (Wang and Hanson 1988; Wang 1999) because the stabilizing effects of these chemical additives differ depending on the protein (Chi and others 2003; Kamerzell and others 2011; Otake and others 2011). The current IFN-β-1b product (Betaferon) is suboptimal. First, it contains high levels of aggregates that cause an immune response (Hermeling and others 2005a; van Beer and others 2012). Second, the IFN-β-1b molecule, which is nonglycosylated, has poor solubility and therefore is formulated with human serum albumin (HSA) as a solubility-enhancing agent (Shirley and others 2008). HSA is obtained from human blood and can potentially be contaminated with human viruses and other pathogenic entities. Moreover, HSA interferes with the ability to accurately measure the stability of the IFN-β-1b in the formulation (Shirley and others 2006).

To overcome these issues, there is a need for a HSA-free formulation of IFN-β-1b with lower levels of aggregates and lower immunogenicity. Previous studies of IFN-β-1b demonstrated benefits of excipients such as arginine, trehalose, and N-dodecyl-β-D-maltoside (DDM) on the stability and solubility of this protein (Isulbishi and others 2005; Kim and others 2009; Rifkin and others 2011; Fazeli and others 2013; Haji Abdolvahab and others 2014a; Fazeli and others 2014). In the current study, the combination of these excipients was examined during storage at 4 and 37°C with relative humidity of 10% and 75%, respectively. The endotoxin level of both IFN-β-1b protein samples was calculated by comparison of their anticytopathic effect (CPE) with that of the 200 mM arginine, 200 mM trehalose, and 0.1% DDM. Antiviral activities of the individual IFN-β-1b protein samples were calculated by comparison of their anticytopathic effect (CPE) with that of the NIBSC IFN-β-1b standard (code: 00/574).

### Materials and Methods

#### Materials

IFN-β-1b (0.78 mg/mL) frozen in 50 mM sodium acetate buffer (pH 5.6) was provided by Zistdaru Danesh Co. Ltd. DDM was obtained from Thermo Fisher Scientific (4870 AA Etten-Leur). L-arginine, D-trehalose, bovine serum albumin (BSA), and polysorbate 20 were from Sigma-Aldrich (Chemie B.V.). Sodium acetate was from Merck (64271 Darmstadt). Betaferon was purchased from Bayer (Groningenweg 1A) and was used within its expiry date. The lyophilized Betaferon powder, which consisted of 15 mg mannitol, 15 mg HSA, and 0.3 mg IFN-β-1b, was reconstituted with 1.2 mL of 0.54% NaCl in a prefilled syringe. The endotoxin level of both IFN-β-1b and Betaferon was quantified and below 0.1 ng/μg (1EU/μg).

#### Methods

**Sample preparation.** IFN-β-1b (0.78 mg/mL) was thawed and formulated with different excipients at different pHs to a concentration of 0.25 mg/mL in 10 mM sodium acetate buffer. The control sample was 0.25 mg/mL IFN-β-1b in 10 mM sodium acetate buffer (pH 5.6). Excipients were chosen based on the effect of each on stability and solubility of IFN-β-1b (Wang and Hanson 1988; van Beers and others 2012). The effects of 3 concentrations of arginine (150, 200, 250 mM), trehalose (150, 200, 250 mM), and DDM (0.01%, 0.1%, 0.5%) were analyzed individually using UV spectroscopy and dynamic light scattering (DLS) at room temperature, and 200 mM arginine, 200 mM trehalose, and 0.1% DDM were selected as the most effective concentrations of these excipients (Supplementary Tables S1 and S2; Supplementary Data are available online at www.liebertpub.com/jir).

In the next step, the combination of 2 excipients was tested and then IFN-β-1b was formulated with all 3 excipients. As the buffer concentration for all samples was relatively low (10 mM sodium acetate buffer), the pH was measured every week during the stability studies.

Furthermore, after determining that 200 mM arginine, 200 mM trehalose, and 0.1% DDM were an appropriate formulation, arginine, trehalose, and DDM were replaced with lysine, sucrose, and polysorbate 20, respectively, to confirm the positive effect of these excipients compared to related excipients. Therefore, 3 additional formulations were analyzed and compared to the 200 mM arginine, 200 mM trehalose, and 0.1% DDM formulation using UV spectroscopy and DLS: the 3 additional formulations are 200 mM lysine, 200 mM trehalose, and 0.1% DDM; 200 mM arginine, 200 mM sucrose, and 0.1% DDM; and 200 mM arginine, 200 mM trehalose, and 0.1% polysorbate (Supplementary Figs. S1 and S2).

#### UV spectroscopy

UV spectra (λ = 250–360 nm) of 250 μg/mL IFN-β-1b were measured at 25°C using a UV-2450 UV/VIS spectrophotometer (Shimadzu Co. Ltd.) with an 8-well quartz cuvette and a 1-cm path length. The corresponding sample buffer was used as the blank.

Protein concentration was calculated based on UV absorption at 280 nm and the IFN-β-1b molar extinction coefficient (1.575 mL mg⁻¹ cm⁻¹). Potential presence of protein aggregates was considered by looking at an increase in OD at 350 nm and a decrease in the ratio of OD280nm/OD226nm (Haji Abdolvahab and others 2014a; van Beers and others 2010a, 2011).

**Dynamic light scattering.** Samples were analyzed using DLS to measure an average diameter (Z-ave) and polydispersity index (PDI) of species, 250 μg/mL IFN-β-1b was measured with a Malvern ALV CGS-3 goniometer (Malvern Instruments) equipped with a HeNe laser source (λ = 632.8 nm, 22 mW output power) at a 90° angle at 25°C. ALV Correlator 3.0 software (ALV) was used to analyze the DLS time correlation.

**Antiviral activity.** Human lung carcinoma (A549) cells infected with EMCV were used to estimate the antiviral activity of IFN-β-1b (24). Antiviral activities of the individual IFN-β-1b protein samples were calculated by comparison of their anticytopathic effect (CPE) with that of the NIBSC IFN-β-1b standard (code: 00/574).

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using an XCell SureLock™
Mini-Cell Electrophoresis System. 2.5 μg of samples with a volume of 10 μL was loaded to each well in a precast gel (Ready Gel, Tris–HCl, 4%–12% NuPAGE, 10-wells × 1.5 mm; Life Technologies). Protein samples were mixed with 4x sample buffer before loading and run under nonreducing condition at room temperature at 70 V for 30 min, followed by a voltage increase to 150 V for 1 h. The electrophoresis buffer was 192 mM glycine, 25 mM Tris (hydroxymethyl) aminomethane, and 0.1% (w/v) SDS. For molecular weight determination, prestained broad range molecular weight markers (Biorad) were run and a Silver Stain Plus kit (Biorad) was used to visualize the protein bands. The gel was scanned with a Bio-Rad GS-800 densitometer using Quantity One software.

Western blotting
SDS-PAGE gels were blotted onto a nitrocellulose membrane with the iBlot Dry Blotting System (Life Technologies) according to the manufacturer’s instructions. The transfer buffer contained 3 mM sodium carbonate, 10 mM sodium hydrogen carbonate, 0.1% SDS (w/v), and 20% (v/v) methanol at pH 10.0. Blots were blocked with 5% (w/v) nonfat milk powder (Elk; Campina Melkboer, The Netherlands) in 0.005% (w/v) polysorbate 20 in PBS overnight at 4°C with constant orbital shaking. After washing with PBS containing 0.05% (w/v) polysorbate 20, blots were incubated with 0.2 μg/mL polyclonal rabbit anti-IFN-β antibody (Acris Antibodies) in 5% (w/v) BSA and 0.005% (w/v) polysorbate 20 in PBS overnight at 4°C with constant orbital shaking. A washing step was then carried out using 0.005% (w/v) polysorbate 20 in PBS. Blots were then incubated with peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG) (Sigma Aldrich), diluted 2,000-fold in PBS containing 5% (w/v) BSA and 0.005% (v/v) polysorbate 20, for 1 h at room temperature with constant orbital shaking. Blots were then washed with 0.005% (w/v) polysorbate 20 in PBS. Protein bands were visualized using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and a Gel Doc Luminescence Imaging system (Bio-Rad Chemdoc XRS). Data were analyzed with the Quantity One software.

Size-exclusion ultra performance liquid chromatography
A Waters ACQUITY UPLC system (Waters) equipped with a sample manager and binary solvent manager was used for sample analysis. The mobile phase consisted of 10% acetonitrile (ACN), 50 mM sodium phosphate buffer, 200 mM arginine monohydrochloride, and 0.1% SDS at pH 7.5. Before measurement, the sensor was flushed for 60 s with Milli-Q water. Subsequently, possible impurities in the system were removed by 2 ‘squeeze’ operations followed by another flushing with Milli-Q water for 60 s. About 150 μL of sample solution was filtered using 0.2-μm filter (GE Healthcare) and was then loaded into the sensor for 45 s. Each measurement consumed about 0.1–0.3 μL of the loaded sample. Each sample was measured 3 times, obtaining values for 500 particles in each run. Particle Lab software version 1.8.510 (Affinity Biosensors) was used to differentiate and quantify protein aggregates from silicon oil.

Fluorescence spectroscopy
The changes in tertiary structure of the HSA-free formulated IFN-β-1b were monitored with a Horiba Fluorolog fluorometer (Horiba Jobin Yvon) and compared with the bulk IFN-β-1b. Excitation was carried out at 295 nm and the fluorescence emission spectra of both IFN-β-1b samples were recorded from 305 to 450 nm in a quartz cuvette with a 1-cm path length at room temperature. The slits were set at 5 nm.

Resonant mass measurement
Resonant mass measurement (RMM) (Affinity Biosensors) equipped with a “micro” format resonant mass sensor (channel cross section 8 × 8 μm², resonant frequency 400 kHz) was used to analyze particles smaller than 5 μm in diameter (Burg and others 2007). Before every measurement, the sensor was flushed with 60 s with Milli-Q water. Subsequently, possible impurities in the system were removed by 2 ‘squeeze’ operations followed by another flushing with Milli-Q water for 60 s. About 150 μL of sample solution was filtered using 0.2-μm filter (GE Healthcare) and was then loaded into the sensor for 45 s. Each measurement consumed about 0.1–0.3 μL of the loaded sample. Each sample was measured 3 times, obtaining values for 500 particles in each run. Particle Lab software version 1.8.510 (Affinity Biosensors) was used to differentiate and quantify protein aggregates from silicon oil.

Microflow imaging
A MFI-5200 (protein sample) equipped with a saline-coated 100-μm flow cell and controlled using the MFI View System Software (MVSS), version 3.1, was used to image micron-sized particles. Milli-Q water was flushed through the system to provide a clean background, and “optimize illumination” procedure was performed before each sample measurement. 0.9 mL samples were drawn from a 1 mL pipette tip at a flow rate of 0.17 mL/min (0.2 μL was used as a prerun volume and 0.7 mL of each sample was monitored). Particle size was calculated as the equivalent circular diameter representing the diameter of a sphere occupying the same projection area as the particle (Sharma and others 2010; Brown 2011). MVAS version 1.3 (Protein-Simple) was used for the data analysis.
Immunogenicity

Animals

FVB/N transgenic mice immune tolerant for hIFN-β (Haji Abdolvahab and others 2014b) were bred at the Central Laboratory Animal Institute (Utrecht University, The Netherlands). The offspring were genotyped using Q-PCR showing the presence or absence of the hIFN-β gene in chromosomal DNA isolated from ear tissue. Both transgenic (PCR-positive) and nontransgenic (PCR-negative) litter mates of 12 weeks of age were used. However, the transgenic mice were genotypically positive for the hIFN-β gene, and they were not confirmed to be phenotypically positive, that is, they actually expressed the human IFN-β protein.

Animal experiments. The animal experiments were approved by the National and Institutional Ethical Committee in agreement with the European guidelines on animal experiments. A group size of 4 was calculated for this experiment (www.cs.uiowa.edu/~rlenth/Power) using power analysis. Food (Hope Farms) and water (acidified) were available ad libitum. Both transgenic (Tg) and nontransgenic (non-Tg) mice were divided into 2 experimental groups, which were injected intraperitoneally (IP) with Avonex drug substance, Betaferon drug product, or the optimized HSA-free formulation of IFN-β-1b (5 μg/injection) on days 1–5, 8–12, and 15–19. Blood was collected before injections on days 1, 8, and 15 through cheek puncture. On day 21, the mice were euthanized using decapitation under isoflurane anesthesia with relative humidity of 10% and 75%, respectively (Table 1).

As stated in Materials and Methods, 250 μg/mL IFN-β-1b was prepared first with only arginine, trehalose, or DDM. Then, the combination of 2 excipients was tested, and then IFN-β-1b was formulated with all 3 excipients. Either arginine or trehalose alone or the combination of these 2 excipients could not maintain the protein in its monomeric and native form at pH ranging from 5.5 to 8.5, and the protein precipitated after 1 day of storage at 4°C and 37°C with relative humidity of 10% and 75%, respectively. DDM, as a surfactant, was essential for the stability of IFN-β-1b in all formulations. Results indicated that in the presence of DDM or the combination of DDM and arginine, the amount of aggregates diminished (Kim and others 2009; Rifkin and others 2011; Haji Abdolvahab and others 2014a; Fazeli and others 2014); however, these 2 excipients could not prevent the formation of IFN-β-1b aggregates during storage at 37°C, and trehalose with its thermostabilizing properties was needed (Fazeli and others 2013). The formulation containing 0.1% DDM, 200 mM arginine, 200 mM trehalose, and 10 mM sodium acetate buffer (pH 7.5) showed the lowest OD at 350 nm and the highest at OD280 nm/OD260 nm, indicating lower amount of aggregates in this solution (Table 1). This formulation was selected and is referred to hereafter as “HSA-free formulated IFN-β-1b.”

Light scattering at high wavelengths was more evident in bulk IFN-β-1b and some solutions, which suggested the presence of large aggregates. These solutions displayed the highest OD350 nm and the lowest OD280 nm/OD260 nm, most likely caused by absorption flattening due to extensive aggregation of the sample (van Beers and others 2010a, 2011; Kuciltro and Middaua 2005). To finalize the HSA-free formulation of IFN-β-1b, each excipient in this formulation was replaced with related ones. Protein precipitations were observed after the substitution of trehalose with sucrose, and DDM with polysorbate after storage for 1 and 2 months at 25°C and 4°C, respectively; however, formulation with 200 mM lysine, 200 mM trehalose, and 0.1% DDM, in which arginine was substituted with lysine, remained stable and few visible particles were detected in this formulation. Therefore, this formulation together with the formulation containing 200 mM arginine, 200 mM trehalose, and 0.1% DDM appeared to yield improved stability, although the latter exhibited slightly lower OD 350 nm and higher OD 280/260 nm after 6 months storage at 4 and 25°C (Supplementary Fig. S1).

Dynamic light scattering. Bulk IFN-β-1b and most excipient-containing samples showed a large Z-ave at 4°C and 37°C, indicating the presence of large aggregates (Table 2). In
AN ALBUMIN-FREE FORMULATION FOR RHIFN-1B

Concording with our observations from UV spectroscopy, the lowest Z-ave was defined for the formulation containing arginine, trehalose, and DDM (pH 7.5) at both 4°C and 37°C. However, the Z-ave of this sample (64 nm) with a corresponding large PDI (0.47) after 3 weeks incubation at 4°C indicated that the solution contained aggregates even though these values were lower than with other excipient combinations (Table 2). Although this sample contained mostly monomeric IFN-β-1b (based on further analytical methods), the strong light scattering because of the presence of aggregates overestimated the size average of the protein solution (van Beers and others 2010a, 2011). The samples formulated with arginine and DDM with or without trehalose (pH 7.5) showed a lower precipitation was observed in bulk IFN-β-1b formulations. However, at 37°C level of particles at 4°C DDM with or without trehalose (pH 7.5) showed a lower light scattering of the gatable material (as also indicated by volume and number average distributions) (Philo 2009; van Beers and others 2010a, 2011). The samples formulated with arginine and DDM with or without trehalose (pH 7.5) showed a lower precipitation was observed in bulk IFN-β-1b formulations. However, at 37°C level of particles at 4°C DDM with or without trehalose (pH 7.5) showed a lower light scattering of the gatable material (as also indicated by volume and number average distributions) (Philo 2009; van Beers and others 2010a, 2011). The light scattering of the aggregates prevents the detection of monomers, which may represent a much larger fraction by weight than the aggregated material (as also indicated by volume and number average distributions) (Philos 1996; Runkel and others 1998; Hawe and Friess 2007). The antiviral activity of Betafeeron drug product or of IFN-β-1b formulated with different combinations of 200 mM arginine, 200 mM trehalose, and 0.1% DDM was evaluated. Activities were determined using the antiviral activity test after the formulated samples of IFN-β-1b had been incubated at 4°C or 25°C for up to 10 weeks. A significant loss in activity was observed for Betafeeron.

Table 1. UV Detection at 350 nm and the Ratio of 280/260 nm of Control (Bulk IFN-β-1b) and IFN-β-1b Formulated with Different Excipients

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>pH</th>
<th>350 nm</th>
<th>280/260 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDM</td>
<td>0.01</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>7.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tre + DDM</td>
<td>0.14</td>
<td>0.14</td>
<td>0.40</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>7.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg + DDM</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>7.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg + Tre + DDM</td>
<td>0.01</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>7.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

— Indicates visible precipitation in the solution.

*0.1% (w/v) DDM was added to the bulk IFN-β-1b.
*200 mM arginine was added to the bulk IFN-β-1b.
*200 mM trehalose was added to the bulk IFN-β-1b.

Formulations with either 200 mM arginine or 200 mM trehalose and combination of these 2 excipients at different pH values precipitated after 1 day of storage at 4°C and 37°C with a relative humidity of 10% and 75%, respectively; therefore, these results are not shown.
drug product, whereas the activity in samples containing arginine and DDM or arginine, DDM, and trehalose showed a smaller loss of activity (Fig. 1 and Supplementary Table S3).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The HSA-free formulated IFN-β-1b was analyzed using SDS-PAGE and compared to Betaferon drug product. SDS-PAGE under nonreducing conditions was applied to assess the relative molecular masses of the IFN-β-1b monomer and any covalent aggregates (Runkel and others 1998; Walker 2002; van Beers and others 2011). The HSA-free formulation contained monomers without any covalent dimer, trimer, or larger cross-linked aggregates, whereas the Betaferon drug product consisted of monomers, dimer, and covalent cross-linked aggregates of IFN-β-1b and/or HSA (Fig. 2A).

The 18.5-kDa band corresponds to the monomeric form of IFN-β-1b (Runkel and others 1998) detected in both samples (Fig. 2A). HSA with the molecular mass of 67 kDa was detected in the Betaferon drug product.

### Table 2. Z-ave (nm) and PDI of Control (Bulk IFN-β-1b) and IFN-β-1b Formulated with Different Excipients

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>pH</th>
<th>Z-Ave (nm)</th>
<th>Temperature</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>7</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDM*</td>
<td>5.5</td>
<td>37</td>
<td>47</td>
<td>103</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>7.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tre* + DDM*</td>
<td>5.5</td>
<td>2504</td>
<td>6432</td>
<td>8980</td>
</tr>
<tr>
<td>6</td>
<td>1081</td>
<td>3572</td>
<td>4490</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>226</td>
<td>320</td>
<td>987</td>
<td>1086</td>
</tr>
<tr>
<td>7.5</td>
<td>279</td>
<td>336</td>
<td>1012</td>
<td>2024</td>
</tr>
<tr>
<td>8</td>
<td>121</td>
<td>183</td>
<td>550</td>
<td>2351</td>
</tr>
<tr>
<td>8.5</td>
<td>157</td>
<td>238</td>
<td>425</td>
<td>3312</td>
</tr>
<tr>
<td>Arg* + DDM*</td>
<td>5.5</td>
<td>111</td>
<td>121</td>
<td>165</td>
</tr>
<tr>
<td>6</td>
<td>112</td>
<td>113</td>
<td>141</td>
<td>165</td>
</tr>
<tr>
<td>7</td>
<td>93</td>
<td>171</td>
<td>186</td>
<td>282</td>
</tr>
<tr>
<td>7.5</td>
<td>60</td>
<td>84</td>
<td>134</td>
<td>157</td>
</tr>
<tr>
<td>8</td>
<td>84</td>
<td>123</td>
<td>241</td>
<td>264</td>
</tr>
<tr>
<td>8.5</td>
<td>115</td>
<td>228</td>
<td>231</td>
<td>374</td>
</tr>
<tr>
<td>Arg* + Tre* + DDM*</td>
<td>5.5</td>
<td>109</td>
<td>115</td>
<td>192</td>
</tr>
<tr>
<td>6</td>
<td>110</td>
<td>118</td>
<td>140</td>
<td>234</td>
</tr>
<tr>
<td>7</td>
<td>63</td>
<td>85</td>
<td>78</td>
<td>124</td>
</tr>
<tr>
<td>7.5</td>
<td>59</td>
<td>71</td>
<td>67</td>
<td>64</td>
</tr>
<tr>
<td>8</td>
<td>86</td>
<td>149</td>
<td>154</td>
<td>160</td>
</tr>
<tr>
<td>8.5</td>
<td>152</td>
<td>164</td>
<td>182</td>
<td>304</td>
</tr>
</tbody>
</table>

—, Indicates visible precipitation in the solution.
*0.1% (w/v) DDM was added to the bulk IFN-β-1b.
*200 mM trehalose was added to the bulk IFN-β-1b.
*200 mM arginine was added to the bulk IFN-β-1b.

Formulations with either 200 mM arginine or 200 mM trehalose and combination of these 2 excipients at different pH values precipitated after 1 day of storage at 4 and 37°C with a relative humidity of 10% and 75%, respectively; therefore, these results are not shown.

PDI, polydispersity index.

**FIG. 1.** Antiviral activity (potency) of different formulations of IFN-β-1b.
covalent cross-linked aggregates observed in this sample could be IFN-β-1b aggregates and/or HSA aggregates. A recent report indicated that Betaferon drug product contains about 15% aggregates (Barnard and others 2013), whereas others indicate levels up to 60% (Runkel and others 1998). The results reported here showed that only monomeric forms were detected using SDS-PAGE when IFN-β-1b was formulated in 200 mM arginine, 200 mM trehalose, and 0.1% DDM, and the larger aggregates that cause an increase in z-average values from DLS may have simply dissolved under conditions used for SDS gel electrophoresis.

Western blotting

The monomeric band of IFN-β-1b in the HSA-free formulation and Betaferon drug product was clearly detected under nonreducing conditions, whereas the dimeric form (37 kDa band), which is present in both samples, was barely detectable in the HSA-free formulation (Fig. 2B). Interestingly, larger aggregates detected using SDS-PAGE were not observed with Western blotting indicating that they were either related to HSA and/or possibly the amount of IFN-β-1b aggregates are too low (less than 0.2% of total protein), to be detected using Western blotting (Gomes 2009) (Fig. 2B). However, the latter is less likely being that Western blotting is generally more sensitive than SDS-PAGE.

In concordance with SDS-PAGE results, no multimeric protein bands were detected in the HSA-free formulation of IFN-β-1b, suggesting that the protein remained in its monomeric form (Fig. 2B).

Size-exclusion ultra performance liquid chromatography

The HSA-free formulated IFN-β-1b was analyzed with a Waters AQUITY BEH 450 SEC UPLC column. The mobile phase contained 0.1% (w/v) DDM to reduce the interaction of the protein with the column matrix, and 200 mM arginine and 50 mM sodium acetate buffer were used to keep the protein stable.

HSA is commonly used in formulations to prevent adsorption (Hawe and Friess 2008). The absence of HSA can cause low protein recovery using this procedure (Hawe and Friess 2008). The use of 0.1% DDM in the mobile phase decreased the adsorption of the protein to the column matrix and also increased the resolution. The HSA-free formulated IFN-β-1b was analyzed using SDS-PAGE before size-exclusion ultra performance liquid chromatography (SE-UPLC) analysis. The results of SE-UPLC showed that the sample contained only monomers and confirmed the results obtained using SDS-PAGE (Figs. 3 and 2A). This observation suggests that the aggregates detected using DLS were likely larger than 0.2 μm and removed during the filtration step before analysis.

Reversed-phase high-performance liquid chromatography

RP-HPLC was used to analyze oxidized or degraded forms of IFN-β-1b. The oxidized form of the protein has been reported to elute before the main peak, followed by other peaks containing oligomerized protein (van Beers and others 2011; Fazeli and others 2014). Oxidized IFN-β-1b was run as a control to qualify the method (data not shown). Neither the HSA-free formulated IFN-β-1b nor the Betaferon drug product showed detectable degradation and oxidation peaks (Fig. 4A, B). Native IFN-β-1b protein peaks eluting at 30–32 min in both samples were as reported previously (Geigert and others 1988; Fazeli and others 2014). Furthermore, Betaferon drug product showed an extra peak eluting at 20–22 min that corresponds to that of HSA (Fig. 4A, B). Because of the gradient elution applied, hydrophilic proteins should elute sooner and hydrophobic molecules should elute later. Therefore, HSA, which is more hydrophilic, is eluted first and then IFN-β-1b, which is known to
FIG. 3. Size-exclusion chromatograms of the HSA-free formulated IFN-β-1b. The size-exclusion ultra performance liquid chromatography peaks were recorded using a UV detector at 280 nm and the emission fluorescence peak using a fluorescence detector at 340 nm with excitation at 295 nm.

FIG. 4. Reversed-phase high performance liquid chromatography analysis of the HSA-free formulated IFN-β-1b and Betaferon drug product. (A) UV absorption chromatogram at 214 nm for Betaferon drug product (upper panel) and the HSA-free formulated IFN-β-1b (lower panel). (B) Fluorescence chromatogram of Betaferon drug product (upper panel) and the HSA-free formulated IFN-β-1b (lower panel). The peak eluting at 20–22 min is HSA, whereas the peak eluting at 30–32 min is IFN-β-1b. An expanded view of the IFN-β-1b peak is shown as an inset in each panel.
be hydrophobic in the absence of the glycan chain attached
to Asn-80, is eluted later (Fig. 4A, B).

Fluorescence spectroscopy. The HSA-free formulated and
bulk IFN-β-1b were excited at a wavelength of 295 nm, and
emission spectra from 305 to 445 nm were recorded (Fig. 5).
The observed intensity provides information on the structure
around the tryptophan residues of IFN-β-1b at positions 22,
79, and 143 (Karpusas and others 1997, 1998; Runkel and
others 1998, 2000). The maximum fluorescence intensity of
the HSA-free formulated IFN-β-1b (at 338 nm) was normal-
ized —, and the fluorescence intensity of another sample was
 calculated relative to this value. Compared to the bulk IFN-β-
1b, HSA-free formulated IFN-β-1b showed a 2-nm blue-shift
and a 44% increase in the intensity of fluorescence emission
due to the absence of aggregates and/or more retention of
tertiary structure. Blue-shift of Trp emission generally means
lower exposure of Trp to the aqueous environment—a more
“folded” structure. Nevertheless, small changes in a fraction
of the protein may not be picked up in the intrinsic fluores-
cence signal (Chen and Barkley 1998; Fan and others 2005;
Qiu and others 2008; Fazeli and others 2013).

Resonant mass measurement

Submicron and micron particles were measured using
RMM. The results showed that the HSA-free formulated IFN-
β-1b contained a low particle concentration (7 × 10⁵ particles/
 mL) with a size range of 0.2–0.4 μm with few larger particles
(Fig. 6). By contrast, Betaferon drug product contained het-
erogeneous particles ranging from 0.2 to larger than 2 μm in
size and, more significantly, in the size range of 0.5–0.8 μm.
The total particle concentration of Betaferon drug product

FIG. 5. Fluorescence emission spectra of the HSA-free
formulated and bulk IFN-β-1b.

FIG. 6. Particle size distribution of the HSA-free formu-
lated IFN-β-1b and Betaferon drug product determined us-
ing resonant mass measurement. Error bars represent
standard deviations from triplicate measurements.

FIG. 7. (A) Total number of particles (size range from 1 to >32 μm) detected using microflow imaging in phosphate-buffer saline (PBS), the HSA-free formulated IFN-β-1b, and Betaferon drug product. (B) Representative images of protein particles observed in phosphate buffer saline, the HSA-free formulated IFN-β-1b, and Betaferon drug product. The protein particles were observed to be heterogeneous in shape, ranging from small circles to large ribbon-like aggregates.
(3.2 × 10^7 particles/mL) is 46 times higher than the number of protein particles in the HSA-free formulated IFN-β-1b (Fig. 6). Increased particle concentration in Betaferon drug product sample might be due to HSA and/or IFN particles.

Microflow imaging

Particles larger than 0.75 μm, which are difficult to measure with other conventional techniques, were detected using microflow imaging (MFI) (Sharma and others 2010). The HSA-free formulated IFN-β-1b showed a lower particle count (1.8 × 10^4 particles/mL) compared to Betaferon drug product (3.8 × 10^5 particles/mL). The buffer control contained 5.7 × 10^3 particles/mL. Results showed that Betaferon drug product has a higher number of particles compared to the HSA-free formulated IFN-β-1b (Fig. 7A). The images of samples are depicted in Fig. 7B. The protein particles in both samples were highly heterogeneous in shape and size, ranging from small circle shape with a size of 5 μm to large ribbon-like aggregates, which were about 40 μm (Fig. 7B).

The presence of other undesired particles like silicone oil, air bubbles, and exogenous contaminants is one of the main complications in protein particle measurement, which could be discriminated and isolated with this technique (Sharma and others 2010).

Immunogenicity

The immunogenicity of therapeutic proteins is influenced greatly by the physicochemical characteristics of the molecule, including the state of aggregation (Chen and Barkley 1998; van Beers and others 2011). To investigate the effect of aggregates on immunogenicity, HSA-free formulated IFN-β-1b and Betaferon drug product were tested in transgenic FVB/N immune tolerant mice.

The level of antibodies in both Tg and non-Tg mice was determined using ELISA and is shown in Fig. 8. In general, all formulations induced high immune responses in the non-Tg animals. IgG titers of Tg mice were significantly lower than non-Tg ones after 14 (\( P = 0.0138 \)) and 21 days (\( P = 0.0038 \)) of treatment. Both Tg and non-Tg mice produced antibody after 1 week of treatment with Betaferon, whereas antibody formation was detected after 2 weeks of treatment for both HSA-free formulated IFN-β-1b and Avonex drug substance in non-Tg and Tg FVB/N mice. Tg mice treated with Betaferon drug product showed more than twice the antibody titer.

FIG. 8. Formation of anti-IFN-β-1b antibodies in (A) Tg and (B) non-Tg FVB/N mice treated with Avonex® drug substance, HSA-free formulated IFN-β-1b, and Betaferon drug product. Mice received 5 μg of the respective IFN-β-5 times per week for 3 weeks. Bars represent the average titer of IgG-positive mice and the corresponding SEM. IgG levels were significantly higher in non-Tg than Tg mice on day 14 (\( P = 0.0138 \)) and day 21 (\( P = 0.0038 \)). Furthermore, \( P \)-value indicates significant lower titers of Tg mice after 21 days treated with the HSA-free formulated IFN-β-1b compared to Betaferon drug product (\( P = 0.0286 \)). \*\( P < 0.05 \); \**\( P < 0.01 \); \***\( P < 0.001 \).
AN ALBUMIN-FREE FORMULATION FOR RHIFN-1B

compared to the HSA-free formulated IFN-β-1b (Fig. 8). Although Avonex drug substance (glycosylated IFN-β-1a) in accordance with previous studies (Brown 2011; Hermeling and others 2005b; van Beers and others 2010b, 2010c; Hermeling and others 2005c, 2006).

Conclusion
In conclusion, the HSA-free formulated IFN-β-1b contained mostly monomeric native protein with a low level of aggregates, which was confirmed using several analytical methods, including UV and fluorescence spectroscopies, DLS, SDS-PAGE, SE-UPLC, MFI, and RMM. Furthermore, this formulation resulted in better antiviral activity and long-term stability. In addition, the lower immunogenicity of the HSA-free formulated IFN-β-1b in the transgenic FVB/N mice and the absence of HSA, which reduces the potential risk of viral and other pathogenic infection, indicate that the novel formulation may be promising for clinical studies.

Acknowledgments
This research was supported financially by the Ministry of Science, Research and Technology of Iran. We thank Zistdaru Danesh Co. Ltd. and Biogen Inc. for kindly providing rhIFN-β-1b drug substance and Avonex drug substance, respectively. We also acknowledge Ahmad S. Sedigh for his valuable assistance with MFI and Liem Andhik Halim for carrying out the RMM measurements. John Carpenter is acknowledged for allowing us to perform RMM in his laboratory. Darren P. Baker from Biogen is acknowledged for critically reviewing this manuscript. We would like to express our gratitude to Professor Wim Jiskoot from Leiden University for allowing us to carry out the MFI analysis in his laboratory, his support, and his comments on this manuscript.

Author Disclosure Statement
No competing financial interests exist.

References


Gomes AV. 2009. Western blotting tips and troubleshooting guide tips for successful Western blots. Gomes Lab/UC Davis.


Address correspondence to: Prof. Haid Schellekens
Department of Pharmaceutics
Utrecht Institute for Pharmaceutical Sciences (UIPS)
Utrecht University
The Netherlands
E-mail: h.schellekens@uu.nl

Received 7 July 2015/Accepted 29 September 2015
SUPPLEMENTARY FIG. S1. UV of different formulations of IFN-β-1b (250 μg/mL) at 350 nm during storage for 6 months at (A) 4°C and (B) 25°C and at 10% and 60% relative humidity, respectively. Furthermore, the ratio of 280/260 nm was calculated for all samples during storage for 6 months at 4°C (C) and 25°C (D) (as participation was observed, the measurement was stopped for that sample).
SUPPLEMENTARY FIG. S2. Z-ave (nm) and polydispersity index of different formulations of IFN-β-1b (250 µg/mL) after storage for 6 months at (A and C) 4°C and (B and D) 25°C at 10% and 60% relative humidity, respectively (as participation was observed, the measurement was stopped for that sample).
Supplementary Table S1. UV Detection at Wavelength of 35 nm and the Ratio of 280/260 nm of Control (Bulk IFN-β-1b) and IFN-β-1b (250 μg/mL) in 10 mM Sodium Acetate Formulated with Different Excipients

<table>
<thead>
<tr>
<th>Excipient</th>
<th>350 nm</th>
<th>280/260 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.33</td>
<td>0.84</td>
</tr>
<tr>
<td>Arginine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 mM</td>
<td>0.20</td>
<td>0.89</td>
</tr>
<tr>
<td>200 mM</td>
<td>0.11</td>
<td>0.92</td>
</tr>
<tr>
<td>250 mM</td>
<td>0.34</td>
<td>0.85</td>
</tr>
<tr>
<td>Trehalose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 mM</td>
<td>0.24</td>
<td>0.83</td>
</tr>
<tr>
<td>200 mM</td>
<td>0.12</td>
<td>0.87</td>
</tr>
<tr>
<td>250 mM</td>
<td>0.23</td>
<td>0.84</td>
</tr>
<tr>
<td>DDM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01%</td>
<td>0.141</td>
<td>1.04</td>
</tr>
<tr>
<td>0.1%</td>
<td><strong>0.001</strong></td>
<td><strong>2.02</strong></td>
</tr>
<tr>
<td>0.5%</td>
<td>0.06</td>
<td>1.89</td>
</tr>
</tbody>
</table>
Supplementary Table S2. Z-AVE (NM) and PDI determined using DLS of control (Bulk IFN-β-1b) and IFN-β-1b (250 µg/mL) in 10 mM sodium acetate formulated with different excipients.

<table>
<thead>
<tr>
<th>Excipient</th>
<th>Z-Ave (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>201</td>
<td>0.62</td>
</tr>
<tr>
<td>Arginine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 mM</td>
<td>1238</td>
<td>0.26</td>
</tr>
<tr>
<td>200 mM</td>
<td>674</td>
<td><strong>0.21</strong></td>
</tr>
<tr>
<td>250 mM</td>
<td>2741</td>
<td>0.23</td>
</tr>
<tr>
<td>Trehalose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 mM</td>
<td>8969</td>
<td>0.2</td>
</tr>
<tr>
<td>200 mM</td>
<td>7681</td>
<td><strong>0.15</strong></td>
</tr>
<tr>
<td>250 mM</td>
<td>9965</td>
<td>0.14</td>
</tr>
<tr>
<td>DDM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01%</td>
<td>152</td>
<td>0.43</td>
</tr>
<tr>
<td>0.1%</td>
<td><strong>41</strong></td>
<td><strong>0.51</strong></td>
</tr>
<tr>
<td>0.5%</td>
<td>95</td>
<td>0.55</td>
</tr>
</tbody>
</table>

DSL, dynamic light scattering; PDI, polydispersity index.
### Supplementary Table S3. The Specific Activity of Different Formulations After 10 Weeks

<table>
<thead>
<tr>
<th>Sample</th>
<th>(Million IU)/(mg of IFN-β-1b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDM + Tre + Arg (4°C)</td>
<td>31.7</td>
</tr>
<tr>
<td>DDM + Arg (4°C)</td>
<td>28.8</td>
</tr>
<tr>
<td>DDM + Tre + Arg (25°C)</td>
<td>24.0</td>
</tr>
<tr>
<td>DDM + Arg (25°C)</td>
<td>22.4</td>
</tr>
<tr>
<td>Betaferon (4°C)</td>
<td>16.0</td>
</tr>
<tr>
<td>Betaferon (25°C)</td>
<td>8.9</td>
</tr>
</tbody>
</table>

All samples have a specific activity of 32 million IU per mg of IFN-β-1b, at zero time.
AUTHOR QUERY FOR JIR-2015-0110-VER9-ABDOLVAHAB_1P

AU1: Please note that gene symbols in any article should be formatted as per the gene nomenclature. Thus, please make sure that gene symbols, if any in this article, are italicized.

AU2: Please review all authors’ surnames for accurate indexing citations.

AU3: Please check the levels of headings in this article.

AU4: The Publisher requests for readability that no paragraph exceeds 15 typeset lines. Please check for long paragraphs and divide where needed.

AU5: Please check the edits to the sentence “Therefore, 3 additional formulations were…”.

AU6: In Ref. “Gomes (2009),” please mention publisher’s location.


AU8: Please mention the postal code.

AU9: “***” is explained in the legend of Figure 8 but not mentioned in Fig. 8. Please check.

AU10: Please mention the significance of bold values in Supplementary Tables S1 and S2.