Therapeutic protein formulation for sustained delivery: Formulation aspects and stability

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Abstract

The subject of this thesis is the preparation, characterization and optimization of slow release formulations of recombinant protein drugs. The major focus is on recombinant hirudin (rHir), an anticoagulant protein of 65 amino acids, which was shown to precipitate with zinc salts to form Zn-rHir suspensions with prolonged activity in rats (Arvinte, 1994). Both formulation and stability aspects will be addressed. Preliminary data will also be presented on TGF-β3, a transforming growth factor. Here the subject is the validation of a crystallization process.

**Formulation of Zn-rHir suspensions.** At given rHir and zinc salt concentrations, a maximum yield of pelletable rHir (obtained by centrifugation) was reached at pH 7.1. A precipitation curve was obtained when the percentage of pelletable rHir was plotted versus the molar ratio of zinc to rHir. The highest precipitation efficiency of 100% pelletable rHir was observed at a zinc to rHir ratio of 28. Electron microscopic investigations of such Zn-rHir suspensions typically revealed 20 nm-particles as the smallest observed unit. Upon light microscopy, agglomerates of up to 200 μm were obtained and are suggested to consist of agglomerates of the 20 nm-particles. Concerning the efficiency of precipitation, ZnCl₂, ZnI₂, ZnBr₂, and ZnNO₃ resulted in similar sigmoidal precipitation curves and stable Zn-rHir suspensions. The precipitation efficiency of ZnSO₄ was much lower, and aged ZnSO₄-based Zn-rHir suspensions showed an almost rHir-free pellet. These phenomena were concluded to be due to the low concentration (1.5 mM) needed for a ZnSO₄ solution to form basic zinc sulfate as compared to the higher concentrations required with the other zinc salts to form their basic salts (≥ 40 mM) (chapter I).

**Zn-rHir release studies.** To investigate the release of peptides/proteins from formulations such as the Zn-rHir suspensions, an in vitro model was set up. In 5 mL glass vials, the formulations were suspended in agarose gel (2%) and coated with an extra layer of protein-free agarose. The two agarose layers were topped with receiver solution
where the released protein was analyzed at given time intervals. The in vitro model was validated with commercial (fast, intermediate, and slow acting) insulin formulations, and a good correlation with the biological activity in humans was obtained. For rHir formulations, fastest release was observed with aqueous rHir solution, followed by a Zn-rHir suspension with 76% pelletable rHir, and slowest release was found for the Zn-rHir suspension with 100% pelletable rHir. Good discrimination of the release profiles was only observed in HEPES buffer whereas phosphate buffer and serum were unable to discriminate between the various insulin and rHir formulations, indicating an interaction of the formulations with these receiver solutions (chapter II).

Chemical stability of rHir. The chemical degradation of rHir investigated under various conditions is discussed in chapter III. For this purpose, first Asn and Asp related chemical degradation in peptides and proteins is reviewed (chapter III.1). Deamidation at Asn and isomerization at Asp residues occur via a succinimide intermediate. At slightly acidic pH, the rate determining step in the deamidation and isomerization reaction is the hydrolysis of the succinimide ring; at neutral to basic conditions, succinimide formation is rate-limiting. Asn and Asp are most labile when followed by Gly. The effects of buffer species and ionic strength on these degradation reactions are not yet fully clear. A major determinant of Asn and Asp related degradation in peptides and proteins, however, is the higher order structure within the molecules. Rigid higher order structures hinder the degradation in comparison to flexible structures (chapter III.1).

The major degradation products of rHir are the succinimides at Asp\textsuperscript{33}-Gly\textsuperscript{34} (Q5) and Asp\textsuperscript{53}-Gly\textsuperscript{54} (Q4) in acidic solution or after multiple lyophilization (Grossenbacher et al., 1993). To get further information about the degradation kinetics and mechanisms of rHir over the full pH range (pH 1.0 - 9.5), two different capillary electrophoresis (CE) methods were used. One method involved the determination of the degradation kinetics on the basis of rHir itself; the other method involved the quantitation of Q4 and Q5 separately. High rates of rHir degradation were
observed at strong acidic and alkaline pH's, whereas the stability optimum was obtained around neutral pH. At slightly acidic conditions (pH 3 - 5), Q4 and Q5 were the major degradation products whereas at neutral to alkaline pH values, Q4 and Q5 were undetectable. At neutral pH, peaks suggested to represent isomerized and/or deamidated analogs became apparent. At alkaline pH, a strong decrease in the total peak area was observed probably due to the polymerization of rHir via S-S bonds. When degraded at pH 4.0, twofold higher Q4 than Q5 concentrations were observed, which can be explained by Q4 being formed in the highly flexible Gln49-Gln65 C-terminal tail, whereas Q5 forms in the less flexible Gly31-Lys36 loop consisting of only 6 amino acids (chapter III.2).

The degradation of rHir in a Zn-rHir suspension was also investigated by CE. Arrhenius-type studies (at 50, 40, 30, and 25 °C) involving the Zn-rHir suspension and an aqueous rHir solution for comparison resulted in activation energies of 26.3 and 24.9 kcal/mol, respectively. Improved stability of rHir in the Zn-rHir suspensions versus aqueous rHir solution was obtained and documented as the shelf-lives (t90%, 95% confidence limit) of 23 versus 3 days at 25 °C, and of 292 versus 147 days at 4 °C, respectively. Degradation of rHir in the Zn-rHir suspension resulted in markedly lower Q4 levels as compared to Q5, whereas in aqueous rHir solution only slightly lower Q5 than Q4 levels were observed. This observation, coupled with the fact that a typical rHir degradation found in aqueous solution is missing in the Zn-rHir suspension (probably the isoAsp53 analog), led to the proposal that zinc specifically inhibits Q4 (Asp53-Gly54) formation and the subsequent isomerization to isoAsp53 (chapter III.3).

Strategies to stabilize rHir in aqueous solution and in the lyophilized state were also addressed. In solution, high concentrations (15 -50%) of non-reducing sugars and sugar alcohols showed a stabilizing effect or no effect, whereas reducing sugars destabilized rHir, potentially due to the Maillard reaction. In the lyophilized state, trehalose and sucrose stabilized rHir as compared to mannitol, possibly due to an amorphous state of trehalose and sucrose when lyophilized, instead of the crystalline state of mannitol. The pH of the solution prior to freeze-drying was critical.
solution pH ~7 resulted in more stable powders than starting with pH ~4. Improved stability of rHir in the solid state was observed when potassium instead of sodium phosphate-citrate buffer was used for lyophilization. This is suggested to be due to a lower pH shift during freezing of the potassium compared to the sodium buffer. The use of various metal salts as excipients resulted in more stable powders when calcium and magnesium salts were chosen. Both, the formation of Q4 and Q5 were suppressed when CaCl₂ was used as an excipient to lyophilize rHir. This suppression of the Q4 and Q5 formation is suggested to be due to an interaction of the metal ion with the labile Asp³³-Gly³⁴ (Q5) and Asp⁵³-Gly⁵⁴ (Q4) sites (chapter III.4).

TGF-β₃ crystallization. Finally, crystallization of TGF-β₃, a tissue growth factor, was evaluated with respect to its potential to form slow release formulations. A scale up from the hanging drop method (4 μL) to a batch method (2 mL) was performed. Seed concentrations were found to critically influence crystal sizes. A dioxane concentration between 10 and 15% in the medium was required for crystallization. Further progress in the use of TGF-β₃ crystallization for slow release purposes will require biological activity and stability tests with such crystals.

Zusammenfassung


wurde für eine Suspension mit 100% gefälltem rHir ermittelt. Nur in HEPES-Puffer konnten die Freigabekinetiken der verschiedenen rHir- und Insulin-Formulierungen gut differenziert werden. In Phosphatpuffer oder in Serum ergaben sich dagegen einheitliche Freigabeprofile. Dies wurde auf die Bindung von Zn-Ionen an Phosphationen bzw. an Serumproteine zurückgeführt (Kapitel II).


Hauptdegradationsprodukte von rHir in saurer Lösung oder nach mehrmaliger Lyophilisation sind die aus Asp\textsuperscript{33}-Gly\textsuperscript{34} bzw. aus Asp\textsuperscript{53}-Gly\textsuperscript{54} hergeleiteten Succinimide Q5 bzw. Q4 (Grossenbacher et al., 1993). Im Interesse von Kenntnissen über die Degradationskinetik und zu den beteiligten Mechanismen wurde die Degradation von rHir im Bereich von pH 1 bis 9,5 mittels Kapillarelektrophorese (CE) untersucht. Dabei wurde mit zwei verschiedenen CE-Methoden gearbeitet. Eine Methode diente zur direkten Bestimmung von rHir. Mit der zweiten Methode liessen sich Q4 und Q5 einzeln quantifizieren. Hohe Zerfallsraten wurden unter stark sauren und stark alkalischen Bedingungen beobachtet, die höchste Stabilität
im neutralen pH-Bereich. Bei leicht sauren Bedingungen (pH 3 - 5) sind die Hauptdegradationsprodukte Q4 und Q5. Dagegen waren Q4 und Q5 bei neutralen bis alkalischen pH Werten direkt nicht detektierbar. Bei neutralen pH wurden weitere Degradationsprodukte beobachtet, von denen angenommen wurde, dass es sich um isomerisierte und/oder deamidierte Produkte handelte. Bei alkalischem pH erfolgte eine starke Abnahme der totalen Peakflächen, was für eine Polymerisation von rHir via S-S-Brückenbindungen spricht. Bei pH 4.0 führte die Degradation von rHir zu etwa doppelt so hohen Konzentrationen von Q4 als zu Q5. Dies wurde auf die freie Beweglichkeit von Asp<sup>53</sup>-Gly<sup>54</sup> im hoch flexiblen C-terminalen Gln<sup>49</sup>-Gln<sup>65</sup>-Rest zurückgeführt, wohingegen Asp<sup>33</sup>-Gly<sup>34</sup> im Bereich der weniger flexiblen und daher stabileren Gly<sup>31</sup>-Lys<sup>36</sup>-Schleife liegt (Kapitel III.2).

Auch die Degradation von rHir in Zn-rHir-Suspension wurde mittels CE untersucht. Ausgehend von je einem Arrhenius-Diagramm (50, 40, 30 und 25 °C) für Zn-rHir-Suspension und wässrige rHir Lösung wurden praktisch identische Aktivierungsenergien von 26.3 bzw. 24.9 kcal/mol bestimmt. Im Vergleich zur wässrigen Lösung verhielt sich die Zn-rHir-Suspension stabiler. Die Haltbarkeitsfristen (t<sub>90%</sub> mit P = 95%) bei 25 °C betrugen für die Zn-rHir-Suspension 23 bzw. für die Lösung 3 Tage; bei 4 °C waren es 292 bzw. 147 Tage. In Zn-rHir-Suspension wurde nur die Degradation zu Q4 gehemmt, die zu Q5 blieb mehr oder weniger unverändert. In wässriger Lösung wurde eine etwas geringere Bildung von Q5 als von Q4 beobachtet. Dies und der aus der CE abgeleitete Hinweis, dass isoAsp<sup>53</sup>-rHir sich vermutlich nur in Lösung bildet, nicht aber in Zn-rHir-Suspension, führten zur Schlussfolgerung, dass die Fällung mit Zinksalzen die Bildung von Q4 (Asp<sup>53</sup>-Gly<sup>54</sup>) und damit verbunden auch die Isomerisierung zu isoAsp<sup>53</sup> hemmt (Kapitel III.3).


