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Research paper

Sustained release of injectable zinc-recombinant hirudin suspensions: development and validation of in vitro release model

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Abstract

In humans, recombinant hirudin (rHir), an anticoagulant protein, has a relatively short half-life (about 1 h). Therefore, a rHir formulation with sustained biological activity was previously proposed to result from complexing zinc salts and rHir (Zn-rHir). The purpose of this paper is to introduce and validate an in vitro release model for subcutaneous Zn-rHir formulations. In glass vials the formulations were suspended in agarose gel (2%) and coated with an extra layer of protein-free agarose. The agarose layers were covered with receiver solution, either buffered solutions (HEPES or PBS, pH 7.4) or human serum. To validate the release model and to demonstrate its potential to discriminate between different formulations, several commercial insulin and Zn-insulin formulations were also tested. The release profiles were evaluated by statistical moment analysis (mean times). Only in HEPES buffer was good discrimination between the investigated insulin formulations observed. The mean times of in vitro release of the insulin formulations and the proposed duration of their biological activities were in correlation. Low discrimination was found in PBS. For rHir, clear discrimination between the investigated rHir formulations was achieved in HEPES buffer, whereas low discrimination was found in PBS or in serum. The developed release model may be a sensitive in vitro test to assure the quality of subcutaneous insulin and rHir formulations, and may also be applicable to assess other slow-release protein and low molecular weight drug injectables. © 1998 Elsevier Science B.V.

Keywords: Recombinant hirudin; Insulin; In vitro release model; Sustained biological activity; Statistical moment analysis

1. Introduction

Hirudin, a 65-amino-acid protein from leech, is a thrombin-specific inhibitor [1] with a strong therapeutic potential in thrombolysis and anticoagulation [2]. Presently recombinant hirudin (rHir) is produced from yeast. In the human body, rHir has a relatively short half-life (about 1 h), requiring frequent injections. Therefore, there is an immediate interest to evaluate the potential of rHir formulations for sustained biological activity. Such formulations have been previously proposed by Arvinte [3] and Gietz et al. [4] to result in Zn-rHir suspensions by complexation of zinc salts and rHir. Moreover, a sustained biological activity of a Zn-rHir suspension after subcutaneous injection was recently demonstrated in the rat [3]. To further evaluate the potential of such Zn-rHir suspensions for sustained release, their in vitro release characteristics will be investigated. For this aim, an in vitro release model has been developed and validated.

For in vitro studies of the release of subcutaneously injected drug formulations, a sufficient representation of a subcutaneous injection environment by appropriate in vitro

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release conditions is required. The release model should be capable to discriminate between formulations of different compositions which have various in vivo release profiles. Graham and Pomeroy [5] presented an in vitro release model for insulin suspensions. The release of insulin was analyzed by means of flow-through ultrafiltration cells. In terms of sensitivity and speed, the authors claimed advantages of their model over the British Pharmacopoeial test (BP 1990) for prolongation of the insulin effect. Another in vitro model was presented by Peschka [6] to study the release kinetics of proteins from liposomes. The liposomes were embedded in an agarose gel, and separated from a receiver buffer solution by an extra layer of a drug-free agarose gel. The amount of protein released was analyzed in the buffer on top of the agarose layer. Spargo et al. [7] developed an implant model to study the release of TGF-β from lipid-based microcylinders. The microcylinders were embedded in agarose gel blocks which were suspended in a receiver buffer at 37°C. The TGF-β released into the buffer was monitored.

The purpose of this paper is to introduce and validate an in vitro release model for subcutaneous Zn-rHir suspensions. The release of the drug from a subcutaneous depot is assumed to be controlled by the blood flow and perfusion of interstitial fluid in subcutaneous tissue. The diffusion process from a subcutaneous slow release formulation into its environment is characterized by flat concentration profiles. This is in contrast to the environment of a drug depot after peroral administration. In this case steady perfusion by fresh intestinal fluid and high blood flow in the absorptive tissue take place, typically leading to steep concentration gradients across the absorption pathway.

The model developed here is derived from previous studies by Peschka [6] on the release of proteins from liposomes. The release studies with Zn-rHir suspensions were performed in buffered solutions and in human serum. As a selective method to analyze rHir in serum, a recently developed fiber optic immunosensor technique was used [8]. In order to validate the release model and to demonstrate its potential to discriminate between various subcutaneous insulin preparations, several commercial Zn-insulin formulations were also tested.

2. Materials and methods

2.1. Materials

The rHir used for the experiments was a gift from Ciba-Geigy (Basel, Switzerland) (CGP 39393/ LOS: 810189).

Commercially available insulin formulations (100 IU/ml) manufactured by Novo Nordisk (Bagsværd, Denmark) were used, i.e. Actrapid® HM and MC (insulin solution; HM, human monocomponent; MC, monocomponent porcine insulin), Semilente® MC (zinc insulin suspension, amorphous), Monotard® HM (zinc insulin suspension, 30% amorphous and 70% crystalline mixed) and Ultratard® HM (zinc insulin suspension, crystalline). The approximate duration of the biological activity of these insulin formulations in humans, as indicated by the manufacturer, are 8, 8, 16, 24, and 28 h, respectively.

Synperonic® PE/F68 was from ICI Surfactants (Cleveland, UK). Monoclonal anti-hirudin antibody (H-mAb 4049-83-12) and fluorescein-labeled sheep polyclonal anti-hirudin antibody (F-H-pAb, recombinant hirudin) were from Ciba-Geigy (Basel, Switzerland). Agarose and all other chemicals of analytical grade were from Fluka (Buchs, Switzerland).

2.2. Zn-rHir suspensions

The method used to precipitate aqueous solutions of rHir was by adding appropriate amount of $ZnCl_2$ solution and shifting the pH to approximately 7 with a NaOH solution. The sample was then quickly shaken by hand for about 15 to 30 s to form a thick white suspension [4]. To determine the rHir content in the pellet, the suspension was centrifuged at $15\,800 \times g$ for 4 min in an Eppendorf centrifuge (Eppendorf-Netheler-Hinz, Hamburg, Germany). The supernatant was diluted with 0.1 N HCl and assayed photometrically at 275 nm (Uvikon 931, Kontron, Milano, Italy). Slightly turbid supernatants were clarified by the addition of 0.1 N HCl. The percentage of rHir in the pellet, $rHir_{pellet}$, was calculated by:

$$\%\,rHir_{pellet} = \frac{rHir_{total} - rHir_{supernatant}}{rHir_{total}} \times 100 \quad (1)$$

where $rHir_{total}$ is the total rHir content of the preparation based on the amount of rHir stock solution used, and $rHir_{supernatant}$ is the rHir content in the supernatant.

2.3. In vitro release method

Release testing of the Zn-rHir suspension and of the various insulin formulations was performed as follows: In screw-topped 5-ml vials, 200 μl of protein formulation corresponding to 4 mg of rHir or 20 IU of insulin, respectively, were suspended in 200 μl of ~60°C warm agarose solution (2%) using polystyrene stirring rods (Hellma, Müllheim, Germany). The agarose solution was made by dissolving agarose at ~80°C in water and cooling it to ~60°C. Right after mixing the protein formulation with the agarose solution, the dispersion was cooled in a water bath (~20°C) resulting in the first gel layer. This protein formulation-containing gel layer was then coated with 200 μl of protein-free agarose (~60°C), which was again rigidified by cooling in a water bath. Finally, the protein-free agarose layer was topped with 3.4 ml receiver solution. Release was conducted on a horizontally rotating shaker (200 min^{-1}; radius of about 1 cm) at 37°C. At given times the bulk solution was withdrawn and replaced by fresh solution. The receiver solutions used were 20 mM 4-(2-hydroxyethyl)-pipera-

zine-1-ethanesulfonic acid buffer, pH 7.4, containing 138 mM NaCl (HEPES), 10 mM sodium phosphate buffer, pH 7.4, containing 128 mM NaCl (PBS), or undiluted human serum. Insulin fibrillation in the receiver solution was prevented by adding 0.5% Synperonic PE/F68 to the buffer [9]. Studies were performed in triplicate. Monitoring of insulin in the buffer was by the Bio-Rad protein microassay, based on the Bradford dye binding procedure [10]. In buffer solutions, rHir was analyzed by fluorescence spectroscopy (λ_{ex} = 274 nm; λ_{em} = 303 nm, FluoroMax, Spex Industries, Edison, USA). In serum, rHir was analyzed by fiber optic biospecific analysis (FOBIA, see below).

2.4. rHir determination in serum by FOBIA

FOBIA (Fiber Optic Biospecific Interaction Analysis), a fully automated analytical tool for performing highly sensitive and selective biological assays, was recently developed at Ciba-Geigy (Basel, Switzerland) as described elsewhere [8]. Briefly, the evanescent wave optic sensor based sensing system uses surface immobilized recognition elements and fluorescently labeled tracers, allowing real-time monitoring of analytes in complex biological matrices without interference from the sample matrix. The sandwich immunoassay using FOBIA for measuring rHir in biological fluids in a physiologically significant concentration range is described in detail elsewhere [11,12]. In this arrangement, a capture monoclonal anti-hirudin antibody was immobilized on the transducer surface. After a 7-min incubation of the sensor with the samples containing rHir, the secondary fluorescein-labeled polyclonal anti-hirudin antibody was added, and a resulting signal indicating binding of the tracer to the surface bound complex was recorded. The signal at 5 min was then read and used for quantification using a standard curve prepared with probes spiked with rHir. The working range of the assay was 0.125 to 16 nmol/l, within 10% CV (LOQ = 1.25 nmol/l rHir for an undiluted sample).

2.5. Calculation of mean times of in vitro data

Total mean times of in vitro release, $MT_{release.vitro}$, were calculated by model-free moment analysis of the cumulative release profiles as a distribution function, according to Eq. (2).

$$MT_{release.vitro} = \frac{\sum_{i=1}^{n} \bar{t}_i \times \Delta M_i}{\sum_{i=1}^{n} \Delta M_i} \quad (2)$$

where $\bar{t}_i$ is the midpoint of the time period t_{i-1} to t_i during which the mass fraction ΔM_i (mg or IU) of the protein has been released [13].

Following the principle of the additivity of mean times [13] the mean time of in vitro release, $MT_{release.vitro}$, may be defined as the sum of the mean time of the dissolution process of Zn-protein formulation at the interface, $MT_{diss.vitro}$, and the mean time of protein diffusion across the agarose layer, $MT_{diff.vitro}$:

$$MT_{release.vitro} = MT_{diss.vitro} + MT_{diff.vitro} \quad (3)$$

$MT_{diff.vitro}$ is equivalent to the $MT_{release.vitro}$ of a protein solution.

3. Results

3.1. Insulin release

Five different insulin formulations were tested for in vitro release, i.e. two aqueous insulin solutions as references (Actrapid HM and Actrapid MC) and three different Zn-insulin suspensions (Semilente MC, amorphous; Monotard HM, 30% amorphous and 70% crystalline; and Ultratard HM, crystalline). The cumulative release profiles of the means of $n = 3$ samples, with HEPES buffer as receiver solution, are presented in Fig. 1. Typically the standard deviation of the data was below ±6%, indicating good reproducibility of the release test. The graph shows the ability of the release model to discriminate between the different commercial insulin formulations used. Whereas the release profiles of the two aqueous insulin solutions were practically identical, the release profiles of the three Zn-insulin suspensions showed a marked sustained release formulation effect, i.e. the higher the content of crystalline Zn-insulin, the lower the release rate. The mean times of in vitro release, $MT_{release.vitro}$, and in vitro dissolution, $MT_{diss.vitro}$, of the various formulations as calculated by Eq. (2) and Eq. (3), and the approximate duration of their biological activity in humans, stated by the manufacturer, are reported in Table 1. The rank orders of both $MT_{release.vitro}$ and $MT_{diss.vitro}$, and of

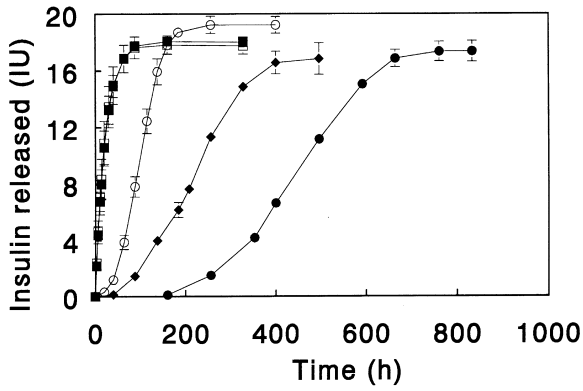

Fig. 1. Cumulative release of different commercially available Zn-insulin formulations are tested in the in vitro release model. As receiver medium HEPES buffer (pH 7.4) is used. Means ± SD; SD partly within the range of the symbols (n = 3). 20 IU of insulin correspond to a 100% release. ■, Actrapid HM (insulin solution); □, Actrapid MC (insulin solution); ○, Semilente MC (Zn-insulin amorphous); ♦, Monotard HM (Zn-insulin, 30% amorphous and 70% crystalline); ●, Ultratard HM (Zn-insulin crystalline).

Table 1

Summary of the total mean times of in vitro release, $MT_{release.vitro}$, and the mean times of in vitro dissolution, $MT_{diss.vitro}$, of insulin, Zn-insulin, rHir, and Zn-rHir formulations, respectively, and the duration of biological activity of insulin formulations in humans

Formulations	$MT_{release.vitro}$; $MT^{b}_{diss.vitro}$ (h) in various isotonic media			Duration of biological activity in humans[a] (h)
	HEPES (pH 7.4)	PBS (pH 7.4)	Human serum	
Insulin formulations				
Actrapid® HM	29; 0	23; 0	–	8
Actrapid® MC	32; 0	27; 0	–	8
Semilente® MC	100; 64	24; <0	–	16
Monotard® HM	216; 187	29; 6	–	24
Ultratard® HM	455; 426	33; 10	–	28
rHir formulations				
rHir solution	23; 0	20; 0	16; 0	–
Zn-rHir suspension 76% pelletable rHir	68; 45	–	–	–
Zn-rHir suspension 100% pelletable rHir	152; 129	26; 5	21; 5	–

[a]Information provided by manufacturer.
[b]$MT_{diss.vitro}$ were calculated according to Eq. (3).

the duration of the biological activities of the formulations in humans were the same.

When using PBS as receiver solution instead of HEPES buffer, only minor differences between the in vitro release profiles of the five insulin formulations tested were ob-served (data not shown). This result is summarized in Table 1, which contains the mean times of in vitro release, $MT_{release.vitro}$, and in vitro dissolution, $MT_{diss.vitro}$, of the insulin formulations tested. The data show that in PBS discrimination by mean times was also low.

3.2. rHir release

rHir release of two different Zn-rHir suspensions in HEPES buffer was analyzed, one with 76% pelletable (or complexed) rHir, and one with 100% pelletable rHir. As a reference, an aqueous rHir solution was tested. The mean release profiles in HEPES buffer are shown in Fig. 2. The observed standard deviations for the rHir formulations were small (SD $<$ 4%; $n = 3$). The release from the formulation containing 76% pelletable and 24% free rHir was faster than the 100% pelleted rHir formulation.

In addition to HEPES buffer, PBS and human serum were also tested as receiver solutions. When releasing into these media, only minor differences between aqueous rHir solution and Zn-rHir suspension (100% pelletable rHir) were observed. Table 1 contains the mean times of release and dissolution for all media tested, and Fig. 3 shows the release profiles in human serum. Compared to the data obtained in HEPES buffer, only small differences between the formulations when tested in PBS (data not shown) or in human serum were observed (see Table 1 and Fig. 3 for human serum data).

4. Discussion

Pharmacopoeial testing conditions, e.g. according to the USP XXII paddle model, do not provide suitable in vitro release test conditions to represent the environment of slow release subcutaneous or intramuscular injections. This is due to the fact that these release tests largely concentrate on perfect or near perfect sink conditions, e.g. by an excess of dissolution medium in combination with stirring, circulation or fresh medium supply. Steep concentration gradients are a characteristic feature of such tests. For subcutaneous or intramuscular administrations, however, flatter concentration gradients are more typical limiting the rates of local dissolution and release. In the release model presented here,

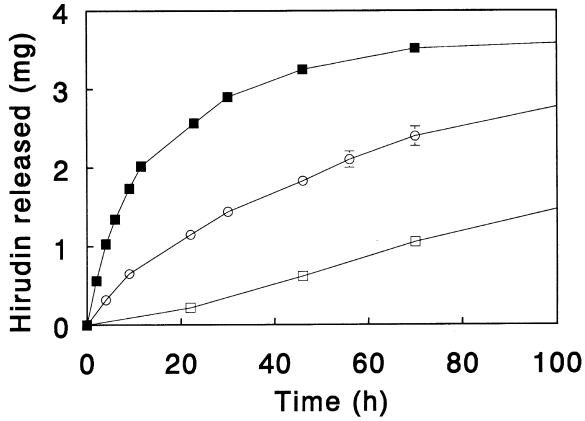

Fig. 2. Cumulative release of different Zn-rHir formulations were tested in the in vitro release model. As receiver medium HEPES buffer (pH 7.4) was used. Means ± SD; SD partly within the range of the symbols ($n = 3$). ■, Aqueous rHir solution; ○, partially complexed Zn-rHir formulation (76% pelletable rHir); □, fully complexed Zn-rHir formulation (100% pelletable rHir).

flat concentration gradients were simulated by embedding the samples into an agarose layer and coating it with an extra agarose layer free of the drug. Thus extended pathways of diffusion were provided, and flat concentration gradients around the samples established. The receiver solution in contact with the agarose was well mixed, limiting the extension of the diffusional pathway and allowing for efficient mixing of the released drug. For the commercially used sustained release insulin preparations, suitable release models have not yet been established. So far quality assurance of insulin formulations still requires biological tests, e.g. the Ph.Eur. 1990 test for prolongation of insulin effect.

4.1. Validation of the model

HEPES buffer was found to be an appropriate receiver solution for the purpose of testing Zn-insulin and Zn-rHir formulations. This buffer discriminates between various insulin and rHir formulations, respectively. Less discrimination was observed for insulin release in PBS and rHir release in both PBS and human serum. We characterized the release model developed for Zn-rHir suspensions by evaluating the release of different commercially available insulin solutions and Zn-insulin suspensions. Two aqueous insulin solutions (Actrapid HM and MC) and three Zn-insulin suspensions were tested. The Zn-insulin formulations contain amorphous (Semilente MC), crystalline (Ultratard HM) and a mixture of 30% amorphous and 70% crystalline (Monotard HM) insulin. The insulin preparations selected correspond to the three typical clinical classifications of insulin, i.e. fast- (solution, amorphous), intermediate- (mixture of amorphous and crystalline) or slow-acting (crystalline) insulin. By means of the in vitro release model a good discrimination of the various insulin formulations was obtained (Fig. 1 and Table 1). The rank orders of the individual release and dissolution parameters, calculated from the experimental release profiles, were the same as the rank order of the biological activities of the insulin and Zn-insulin formulations (clinical product information given by the manufacturer).

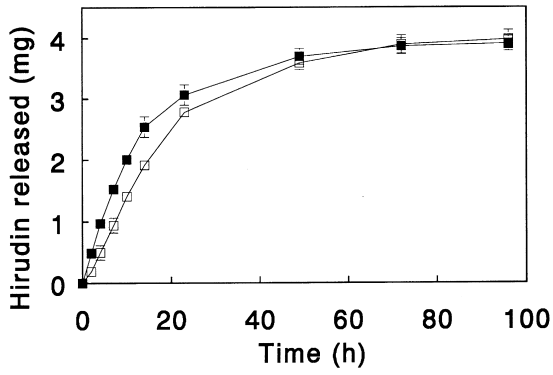

Fig. 3. Cumulative release of (■) aqueous rHir solution, and (□) fully complexed Zn-rHir formulation (100% pelletable rHir) were tested in the in vitro release model. As receiver media, undiluted human serum was used. Means ± SD (n = 2).

We observed a linear correlation between the duration of the biological activities in humans and the logarithms of $MT_{release.vitro}$ and the $MT_{diss.vitro}$, respectively, of these formulations (Fig. 4). The linear correlation indicates the validity of the model to mimic the in vivo release of Zn-insulin preparations. The model may be useful for quality assurance purposes of sustained release insulin formulations. Based on Fig. 4, the experimentally obtained $MT_{release.vitro}$ and $MT_{diss.vitro}$, respectively, may be used to predict the biological activity of Zn-insulin mixtures containing various fractions of soluble, amorphous and crystalline insulin.

4.2. Moment analysis of in vitro release

The release profiles from the agarose layer can be analyzed using the statistical moment analyses method [13]. This method has been used to evaluate pharmacokinetic profiles (e.g. plasma levels after administration of controlled release formulations; [13]). By this method the contributions of various pharmacokinetic events in series are separable from each other. A typical example may be the separation of the effects of a drug release, absorption, distribution and elimination after administration of an oral dosage form. The moment analysis of in vitro release profiles in this study (Eq. (3)) was followed using the same principle.

4.3. Composition of receiver solution

The observation that HEPES buffer allowed clear discrimination between the various insulin or the rHir formulations (Figs. 1 and 2), whereas PBS did not, can be explained by the strong binding of zinc and phosphate ions at neutral pH to form insoluble zinc phosphate. Hence the use of PBS as buffer solution results in an enhanced dissociation of the Zn-insulin and the Zn-rHir complexes by the preferential binding of one of their basic constituents, Zn^{2+}, to phosphate. As a consequence the fraction of free insulin and free rHir will be increased as will the flux of the free proteins out of the agarose into the receiver. It must to be noted that the phosphate concentration of the interstitial liquid, being the typical environment of a subcutaneous depot, is particularly low, similar to serum (approximately 1 mM; [14]), i.e. one order of magnitude lower than in the 10 mM PBS used.

Serum contains little phosphate but considerable fractions of organic acids (5 mM or 5 mval l^{-1}) and negatively charged proteins (2 mM or 16 mval l^{-1}), also able to bind Zn ions. Thus the use of serum as receiver fluid should be equally expected to result in enhanced Zn-rHir dissociation as compared to HEPES buffer. For the same reason, the degree of discrimination between the Zn-rHir formulations obtained with serum is small and similar to PBS. From a kinetic point of view, the effective competition of the serum

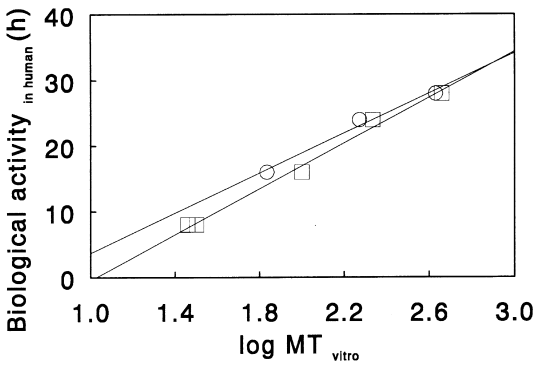

Fig. 4. Correlation between log $MT_{release.vitro}$ (□) ($r = 0.9957$) and log $MT_{diss.vitro}$ (○) ($r = 0.9913$) (Eq. (3)), versus the duration of biological activity in humans for two insulin solutions and three different Zn-insulin preparations.

proteins for Zn ions in the release model may be speculated to be lower than that by phosphate ions. This is most likely because the large molecular size of serum proteins makes them unable to permeate the agarose barrier as efficiently as the much lower molecular weight phosphates or organic acids also present in serum. In summary, the presence of the serum proteins and of its organic acids must be considered to contribute to the high rate of rHir release into this medium, making it unsuitable as receiver fluid.

As an alternative receiver medium interstitial fluid may be considered, having almost the same composition of electrolytes and organic acids as serum, but a much lower concentration of negatively charged proteins (16 mval l^{-1} in serum versus essentially zero in interstitial fluid; [14]). However, the use of this medium for routine release studies is hindered because of its limited availability and high cost. For pharmacopoeial-type studies media containing standardized electrolyte and buffer components are therefore suggested. Here, HEPES buffer was found to represent a feasible alternative to interstitial fluid in this respect.

5. Conclusions

For recombinant proteins the need for slow release formulations is steadily increasing. Quality assurance of such formulations cannot rely only on biological testing. In vitro tests are requested which, after validation by suitable in vitro/in vivo correlation, may replace some of the more costly biological quality assurance methods. The presented release method offers an in vitro alternative for the characterization of a variety of slow release subcutaneous drug formulations of proteins and also low molecular weight drugs.

Acknowledgements

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