**Abstract**

**Background.** Recombinant hirudin (r-hirudin) is a highly selective thrombin inhibitor used for anticoagulation in heparin-induced thrombocytopenia type II. R-hirudin is increasingly applied to patients with renal failure and on renal replacement therapy. Since kidney function impairment strongly prolongs r-hirudin elimination half-life, severe accumulation and bleeding complications may occur. Data on the r-hirudin permeability and elimination capacity of different haemofilters are limited.

**Methods.** Three haemofilter types were investigated: high-flux polysulphone (Fresenius), AN69 (Hospal), and polyamide (Gambro). We used two *in vitro* haemofiltration models: (i) an open post-dilution haemofiltration model with ultrafiltration and fluid substitution (model 1) simulating hirudin intoxication, and (ii) a closed model with ultrafiltrate reinfusion (model 2) to determine steady-state sieving coefficients (SC). Fresh human heparinized blood (2 IU unfractionated heparin/ml blood) was used. In model 2, SC obtained with human whole blood were compared with isotonic saline.

**Results.** In model 1, r-hirudin levels decreased significantly faster with polysulphone than with AN69 or polyamide (P < 0.05). In accordance with this, in model 2 the observed SC in whole blood were 1.11 ± 0.28 (polysulphone), 0.61 ± 0.15 (AN69) and 0.33 ± 0.13 (polyamide), and clearances were 28 ± 7 (polysulphone), 15 ± 4 (AN69) and 8 ± 3 ml/min (polyamide) (P < 0.001 for all comparisons). The SC in saline were slightly but significantly lower for polysulphone (0.88 ± 0.12), similar for AN69 (0.59 ± 0.1), and significantly improved for polyamide (0.83 ± 0.1).

**Conclusions.** Elimination of r-hirudin by haemofiltration strongly depended on the membrane material. Using human blood, we observed large differences between the three high-flux membranes. The saline experiments suggest a membrane-dependent impact of plasma proteins and pH on hirudin sieving. Our findings have implications for r-hirudin dosage in haemofiltration, for treatment of overdosage, and for future *in vitro* haemofiltration studies.

**Keywords:** elimination; haemofiltration; hirudin; *in vitro* study; overdosage; sieving coefficient

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**Introduction**

In 1997, the recombinant hirudin (r-hirudin) lepirudin (Refludan®) was approved for anticoagulation in patients with the immunological form of heparin-induced thrombocytopenia (HIT type II). Other fields of applications for r-hirudin such as treatment of venous thromboembolism [1], acute coronary syndromes [2] and disseminated intravascular coagulation (DIC) [3] are under investigation. Recently, r-hirudin has received increasing attention as an anticoagulant in renal replacement therapy in chronic haemodialysis patients or critically ill patients with acute renal failure [4–7]. In addition, cardiopulmonary bypass surgery has been successfully performed using r-hirudin anticoagulation [8].

R-hirudin works as a highly selective thrombin inhibitor, forming tight stoichiometric complexes independent of plasma cofactors like antithrombin. It is a small single-chain protein consisting of 65 amino acids (molecular weight 6980 Daltons) that rapidly distributes to the extracellular space (t1/2 alpha 0.1–0.35 h, volume of distribution 9–11 litres) showing no relevant interaction with plasma proteins [9,10]. Since r-hirudin is excreted almost exclusively and largely unchanged by the kidneys, impaired renal function increases the elimination half-life (t1/2 beta) from 1–2 h in normal renal function to 36–75 h in chronic haemodialysis patients, depending on the residual renal function. After bilateral nephrectomy, r-hirudin t1/2 of 160–320 h were reported [7,9,11].
Therefore, in patients with renal insufficiency, dosage adjustments of r-hirudin are difficult and require precise monitoring of r-hirudin levels [4,12]. The clinical situation is further complicated by the fact that in cases of overdosage no antidote is available, and serious bleeding complications may result [13–15]. At present, high-flux haemofiltration appears to be the only suitable option to enhance r-hirudin elimination in vivo [16,17]. Despite these facts, experimental and clinical data on the hirudin permeability of different haemodialysis and haemofiltration membranes are scarce and of limited comparability [7,18–20].

In view of the above, we have performed in vitro studies to determine r-hirudin elimination kinetics, sieving coefficients (SC), and drug clearances for various haemofilters that are widely used in chronic and acute renal failure. The data presented in this paper are essentially new, and substantially extend the earlier preliminary results from our group [21]. In addition to the open haemofiltration model described previously, which simulated hirudin intoxication and resembled clinical haemofiltration with ultrafiltrate loss and fluid substitution, we have now employed a second, closed haemofiltration model, with ultrafiltrate reinfusion, for the determination of the SC under steady-state conditions. In contrast to other groups, we employed for the first time fresh heparinized human whole blood instead of citrated bovine blood to study r-hirudin sieving under physiological conditions. To evaluate the impact of plasma proteins and pH, we compared the r-hirudin SC obtained with whole blood and isotonic saline.

Subjects and methods

Materials

Recombinant hirudin lepirudin (Refludan®) was a gift from Hoechst Marion Roussel (Frankfurt/Main, Germany). Unfractionated porcine sodium heparin was purchased from B. Braun Melsungen (Melsungen, Germany). Standard human plasma (SHP) was from Dade Behring (Marburg, Germany). The bicarbonate substitution fluid SH43-HEP and isotonic saline (NaCl 0.9%) were supplied by B. Braun Schiwa (Glandorf, Germany). The blood transfer bags used as reservoirs were purchased from Biotrans (Dreieich, Germany). Meise Medizintechnik (Schalksmühle, Germany) manufactured the specially adapted sterile single-use tubing systems. All experiments were performed with the Hospal BSM 22 SC, containing two peristaltic pumps.

Blood collection

Blood was collected from apparently healthy volunteers (32±15 years, age range 18–64) who had not taken any medication with known influence on platelets or coagulation for at least 2 weeks. Each donor volunteered for only one experiment. After clean puncture of a forearm vein the first 5 ml of blood were discarded to avoid artificial coagulation activation. Then, 450 ml of whole blood was collected in a blood transfer bag containing 50 ml of a sodium heparin solution (unfractionated heparin) giving a final heparin concentration of 2 IU/ml blood. Within 15 min after blood collection the haemofiltration experiment was started.

Haemofilters

For the haemofiltration model experiments, the following commercially available hollow-fibre haemofilters matched for membrane surface area were used: high-flux polysulphone F50 (surface area 1 m²) and F60 (1.3 m², Fresenius Medical Care), AN69 XT Nephral 200 (1.05 m²) and Nephral 300 (1.3 m², Hospal Cobe) as well as poliamide Polyflux 11S (1.1 m²) and Polyflux 14S (1.4 m², Gambro). All haemofilters were kind gifts from the respective manufacturers/distributors.

In vitro haemofiltration models

Two different haemofiltration model designs were used in this study (Figure 1). In model 1, an open system haemofiltration was conducted in the post-dilution mode using the experimental set-up shown in Figure 1A. This model was intended to simulate hirudin intoxication with initial hirudin levels above 15 μg/ml. The freshly drawn heparinized human whole blood was recirculated from a blood reservoir via a peristaltic pump with a constant flow rate of 110 ml/min passing through an adult-size haemofilter. The ultrafiltrate was collected and replaced with the bicarbonate-buffered substitution fluid SH43-HEP supplemented with 4 mmol/l potassium. The flow rate was adjusted via a second peristaltic pump according to the ultrafiltrate flow rate to avoid haemoconcentration. For technical reasons, in model 1 the smaller haemofilters (F50, Nephral 200, and Polyflux 11S) were employed.

In model 2, a closed system was created by complete reinfusion of the ultrafiltrate according to the set-up shown in Figure 1B. The hirudin levels employed here were in the upper therapeutic range. Again, a flow rate of 110 ml/min was used in the blood compartment. With the use of a second peristaltic pump, a constant ultrafiltrate flow of 25 ml/min, comparable with clinically achieved rates, was maintained throughout the experiment.

To investigate the impact of the presence or absence of plasma proteins and the pH on the hirudin SC, we additionally performed experiments employing model 2 and saline (NaCl 0.9%) instead of heparinized human whole blood in the blood compartment.

Experimental procedures

Prior to all experiments, the systems were filled and rinsed with 2 l of isotonic saline. After rinsing, the saline was carefully replaced by 500 ml of anticoagulated human blood, avoiding blood/air interfaces, and the circulation was started.

In model 1, haemofiltration and ultrafiltrate substitution were commenced without r-hirudin, to allow equilibration of the system and formation of a membrane protein layer. After 10 min running time, ultrafiltration and fluid substitution were temporarily stopped. Then a bolus of 6.25 mg r-hirudin was injected into the post-filter port to achieve a target level of 15–18 μg/ml. After 5 min recirculation, allowing sufficient mixing (as shown in the control experiments), ultrafiltration and substitution were restarted and the first blood sample was taken (defined as time point
Further blood sampling was carried out every 5 min for the next 30 min from the pre-filter port. At the end of the experiments, the system was rinsed again with isotonic saline until the blood conducting system appeared macroscopically clean. The haemofilters, tubing, and connections were checked for clotting.

Control experiments with the three haemofilters (n = 2 each) were performed in model 1, omitting ultrafiltration and fluid substitution after the addition of r-hirudin to exclude relevant adsorption of r-hirudin to the tubing, cellular blood components, and membrane surfaces.

In model 2, the experiments also started with an equilibration period prior to r-hirudin injection. After 10 min a reduced lepirudin bolus (about 1.75 mg) was injected in the post-filter port to achieve levels in the upper therapeutic range (3–4 μg/ml). After another equilibration period of 15 min in order to ensure constant r-hirudin levels in all compartments, the first samples were simultaneously taken from the pre-filter, post-filter, and ultrafiltrate port (defined as time point 0 min). Additional samples were drawn every 6 min for the next 30 min.

**Sample processing**

Blood samples were placed into micro tubes (Sarstedt, Nümbrecht, Germany) containing 1/10 vol 0.106 mol/l trisodium citrate for the coagulation assays or potassium EDTA for the determination of haematocrit and total plasma protein. Ultrafiltrate samples were also collected into micro tubes containing 1/10 vol citrate to avoid a dilution error in the calculations. Platelet-poor plasma was obtained by centrifugation (2000 g, 10 min, room temperature). Plasma and ultrafiltrate samples were stored in small aliquots (Eppendorf Reaktionsgefäße, Eppendorf, Hamburg, Germany) at −80°C until analysis. Plasma protein determinations were made on the day of the experiment. For monitoring of pH, bicarbonate, lactate, and electrolyte levels, blood was collected into self-filling heparinized syringes (Pico 70®, Radiometer Medical, Denmark), stored at 4°C (ice water) and measured within 1 h.

**Laboratory analysis**

Hirudin levels were assayed using a commercially available chromogenic test kit (Hirudin activity assay, Dade Behring, Marburg, Germany), which measures the functionally active free hirudin (measurement range 0–5 μg/ml). The kit reagents contain a heparin inactivator. As shown in pilot experiments, the heparin concentration used (2 IU/ml blood) did not affect the assay performance. Further, the hirudin recovery in spiked samples (0–4 μg/ml) was similar for standard human plasma, protein-free buffered substitution fluid (SH43-HEP) and isotonic saline. The intra-assay coefficient of variation for the hirudin assay calculated from repeated measurements of the same sample was below 5%. Plasma samples with expected hirudin levels exceeding values covered by the standard curve were diluted in autologous donor plasma.

Prothrombin fragments 1 and 2 (F1+2) were determined in blood and ultrafiltrate samples with the Enzygnost® F1+2 micro test kit (ELISA, Dade Behring, Marburg, Germany) to detect coagulation activation caused by the extracorporeal circulation, which could cause hirudin consumption and thereby impair the results.

Total plasma protein concentration was measured using the Biuret method (Roche Reagents) on a Hitachi 747 system (Roche Diagnostics, Mannheim, Germany) and used for calculation of the plasma water drug concentration.

Haematocrits were determined on the Cell-Dyn 4000 haematology system (Abbott Laboratories, Illinois, USA) to detect haemoconcentration or dilution in the course of the experiments as a possible confounder of the results. Blood pH, bicarbonate, lactate and electrolyte levels were analysed on the ABL System 610 (Radiometer Copenhagen, Denmark) to control for comparable experimental conditions.
Comparisons) were considered to be significant. To exclude F1 + 2 elimination via haemofiltration, we repeatedly analysed ultrafiltrate samples and found no detectable F1 + 2. Thus, the chosen heparinization (2 IU/ml blood) effectively prevented thrombin generation during the extracorporeal circulation experiments. The haematocrit as a marker of the fluid balance remained almost unchanged, indicating that no relevant haemoconcentration or haemodilution occurred during the experiments.

The baseline hirudin levels essentially met the targeted range of 15–18 μg/ml, being 14.6 ± 1.4 μg/ml for F50, 16.1 ± 1.2 μg/ml for Nephral 200, and 17.5 ± 1.8 μg/ml for Polyflux 11S. The differences between filters were not statistically significant. Figure 2 depicts the time course of the pre-filter plasma r-hirudin concentrations obtained with model 1 for the three filters F50 (n = 7), Nephral 200 (n = 6), and Polyflux 11S (n = 5). The hirudin levels are expressed as fractions of the respective concentration at baseline (time point 0 min). With high-flux polysulphone the hirudin plasma level decreased most rapidly, whereas with Polyflux the slowest decay was observed. AN69 revealed an intermediate performance. The hirudin decay curves of the three haemofilters were significantly different (P < 0.001 for all comparisons). The time course of the decay curves matched first-order elimination kinetics, which can be mathematically expressed as an exponential function.

During the control experiments (n = 2 for each filter) omitting ultrafiltration, the r-hirudin levels did not decrease with polysulphone and AN69, excluding a relevant adsorption of r-hirudin to the inner circuit surfaces. Although there was a trend to lower hirudin levels after 30 min with polyamide, this did not appear to affect the general findings, since it led, if anything, to an overestimation of the SC.

Results

Model 1

The ultrafiltrate flow rates achieved were similar for F50, Nephral 200, and Polyflux 11S (35.2 ± 1.2, 35.1 ± 1.0 and 36.0 ± 1.2 ml/min) and remained unchanged during the course of the experiments. The values for pH, electrolytes, lactate, and bicarbonate were comparable for all filters (at 30 min pH 7.35 ± 0.05, lactate 2.6 ± 0.12 mmol/l, bicarbonate 37.3 ± 3.4 mmol/l). Lactate and bicarbonate levels were slightly elevated above the normal reference values in humans because the substitution fluid contained bicarbonate and lactate as buffers. Sodium and potassium were within the normal ranges (respectively 140 ± 5 and 3.9 ± 0.2 mmol/l). The concentrations of ionized calcium (Ca^{2+}) were slightly increased (1.42 ± 0.1 mmol/l) because the substitution fluid contained 1.83 mmol/l Ca^{2+}.

Since coagulation activation could substantially affect hirudin levels measured by a functional assay because of hirudin-thrombin complex formation, we determined the prothrombin fragments 1 + 2 (F1 + 2) as a sensitive marker for thrombin generation at time points 5 and 30 min. The F1 + 2 levels in the blood compartment did not change during the experiments. 

Statistical analysis

The results are expressed as means ± SD. Statistical analysis was carried out using SPSS 10.0 software for Windows. Univariate analysis of variance (ANOVA) and post hoc Scheffé contrasts for multiple comparisons were used to compare continuous variables in model 2. To analyse differences between the time courses of the hirudin decay curves obtained for the three haemofilters with model 1, we employed ANOVA for repeated measurements and Scheffé contrasts for multiple comparisons. To explore differences between SC obtained in model 2 with whole blood or saline for the three filter types Student’s t-test for independent groups was used. P-values < 0.05 (adjusted for multiple comparisons) were considered to be significant.

Model 2

Whole blood. Measurement of pH, electrolyte, lactate, and bicarbonate levels after 30 min showed similar experimental conditions for the three filters, with all parameters being in the physiological range (pH 7.38 ± 0.02, sodium 139 ± 1.4 mmol/l, potassium 3.6 ± 0.1 mmol/l, Ca^{2+} 1.24 ± 0.03 mmol/l, lactate 2.0 ± 0.2 mmol/l, and bicarbonate 28.2 ± 0.9 mmol/l). As there was no relevant thrombin generation during the experiments of model 1, the determination of F1 + 2 was omitted in model 2. During the 30 min running time of the experiments, the hirudin levels did not decrease in the two compartments (blood and ultrafiltrate), showing a sufficient mixing during the equilibration period and excluding a relevant loss of hirudin due to adsorption to the inner circuit surface and the membrane structure, as well as due to hirudin consumption. The haematocrit changes were below 5% during each experiment.

The SC obtained for the three membrane types with whole blood in the closed system of model 2 are
The highest SC were seen using high-flux polysulphone (F60, 1.11 ± 0.28), whereas the SC with AN69 (Nephral 300, 0.61 ± 0.15) and polyamide (Polyflux 14S, 0.33 ± 0.13) were significantly lower (P < 0.001 for all differences). The respective clearances calculated from SC and ultrafiltrate flow (25 ml/min for all filters) were 28 ± 7 (F60), 15 ± 4 (Nephral 300), and 8 ± 3 ml/min (Polyflux 14S). The differences were again all statistically significant (P < 0.001).
Saline. To explore the impact of the plasma proteins and the pH value on the SC of r-hirudin, we performed an additional series of haemofiltration experiments comparing the three membrane types (n=4 for each type) using isotonic saline (NaCl 0.9%) instead of human heparinized whole blood. The results are shown in Figure 3 and indicate significant differences between saline (hatched columns) and whole blood (black columns). High-flux polysulphone (F60) again revealed the highest SC (0.88±0.12), which was significantly lower than with whole blood. AN69 (Nephral 300) showed a SC similar to the values obtained in whole blood (0.59±0.1) and significantly lower than high-flux polysulphone and also polyamide (P<0.001). In contrast to this, with polyamide (Polyflux 14S), the SC for r-hirudin was significantly improved (0.83±0.09, P<0.001) compared with whole blood, becoming significantly higher than Nephral 300 (P<0.001).

The calculated clearances were 22±3 for F60, 15±2 for Nephral 300, and 21±2 ml/min for Polyflux 14S (F60 vs Nephral, P<0.001; F60 vs Polyflux, P=NS; and Polyflux vs Nephral, P<0.001). The pH values were comparable between the three filter types in the expected range for isotonic saline (4.9±0.2, range 4.58–5.12).

Discussion

Various membrane types are currently used for intermittent and continuous haemofiltration for chronic renal failure patients or for those in intensive care. However, detailed quantitative data comparing the hirudin permeability of these membranes are limited and are of questionable relevance for haemofiltration in humans [17–20]. With regard to the development of membrane-specific hirudin dosage guidelines and for the choice of the membrane type to treat hirudin overdosage, the characterization of different haemofiltration membranes appears to be of major importance.

Findings from in vitro experiments and animal models revealed that hirudin can pass through high-flux haemofiltration membranes, as expected from its pharmacokinetic features, but no quantitative data on permeability or membrane differences were given [17,18]. Recently, Bucha et al. [19] performed in vitro dialysis studies with bovine blood or saline + albumin and tested a broad range of membrane materials. They compared membrane performance by reporting a so far unpublished permeability factor, but did not use dialysate flow, thereby omitting convective transmembranous forces. Therefore, these data are of limited value in the case of haemofiltration, since solute clearance is mediated primarily by convection. Koster et al. [20] investigated four haemofilter types, different from the ones studied in our work, included in a cardiopulmonary bypass in vitro model, and estimated the membrane permeability by comparing the hirudin level before and after an ultrafiltration volume of 1 litre. Again, these data are of limited relevance for clinical haemofiltration. Additionally, they studied two plasmapheresis filters that were clearly superior to the haemofilters, offering another treatment option for hirudin intoxication. However, an important disadvantage is the need for restoration of coagulation factors by fresh frozen plasma [20].

Haemodiafiltration (HDF) may be another technique for the elimination of hirudin, since Bauersachs et al. [16] reported a case of successful treatment of a hirudin overdosage. However, the quantitative effects of HDF on the kinetics of hirudin compared with conventional haemofiltration or dialysis have to be evaluated.

In view of the above data, we aimed to compare three membrane types widely used for haemofiltration in chronic or acute renal failure and to simulate clinical conditions as closely as possible. We determined SC as a generally accepted and comparable measure of membrane permeability [22,23]. The present paper contains essentially new data confirming and substantially extending earlier preliminary data from our group [21]. To obtain more accurate SC, we now additionally employed a closed model with ultrafiltrate recirculation, offering the advantage of stable hirudin levels in the blood and ultrafiltrate compartment. This prevents a systematic overestimation of SC due to rapidly changing hirudin levels, as was seen in the open haemofiltration model in our earlier work [21], which retrospectively appeared inappropriate for determining SC reliably, but which nevertheless resulted in the same ranking for polysulphone and AN69. With the polyamide filter, furthermore, we now included a third high-flux membrane, which showed a distinctly different behaviour not expected from the so far known sieving characteristics. To our knowledge, we were the first to study hirudin elimination by using heparin-anticoagulated fresh human whole blood, thereby maintaining the physiological blood pH, blood composition, plasma protein concentrations, and electrolyte levels. This approach was chosen to minimize artificial changes of molecular charges, hydration, and protein layer formation, which may all influence the drug–membrane interactions, thereby facilitating extrapolation of the results to the situation in vivo.

In this setting, we observed substantial differences between the investigated membranes which, considering the broad range of the mean SC (0.33–1.11), are highly likely to be of clinical relevance. We observed concordant findings in the two different model designs, which further strengthens our data. High-flux polysulphone appeared to be superior to AN69 and polyamide regarding hirudin permeability. High-flux polysulphone led to a significantly more rapid decay of the hirudin levels in the open system (model 1) compared with the two other membranes, and it also revealed the highest SC in the closed system (model 2). As shown by the control experiments, r-hirudin was not significantly adsorbed to the extracorporeal circuit. Therefore, the observed hirudin level decay has to be fully assigned to elimination from the system.
Furthermore, the hirudin level did not fall over the time course of the experiments with the closed system (model 2), excluding a relevant loss of hirudin due to adsorption also to the inner membrane structure during ultrafiltration. Thus, our data indicate that the differences were caused by membrane-specific material properties interacting with blood components and the molecular properties of r-hirudin. Probably, the transmembranous transport of hirudin was hindered by electrostatic charges, or the membrane plasma protein layer could have substantially reduced the effective membrane pore size and changed the membrane charge.

To further explore the influence of the milieu on hirudin sieving, we compared the three haemofilter membrane types using heparinized human whole blood or isotonic saline, which exhibit essentially different pH values (7.35 ± 0.05 vs 4.9 ± 0.2) and protein contents (50–55 g/L). Whereas AN69 performed similarly under both conditions, polysulphone showed a significantly decreased hirudin sieving coefficient. In contrast to this, the sieving of hirudin through polyamide was substantially improved. This finding shows that, considering the absent protein binding of hirudin, membrane-specific interactions with plasma proteins as well as pH changes, which influence membrane and molecular charges, have major impact on hirudin permeability. Furthermore, these data clearly emphasize the importance of the experimental conditions for the membrane performance.

In conclusion, we demonstrated in this in vitro study, that r-hirudin (lepirudin) is able to be eliminated by high-flux membrane haemofilters. The saline experiments suggest a membrane-dependent impact of plasma proteins and pH on hirudin sieving. The preservation of a physiological plasma protein content and pH is essential to obtain clinically relevant preservation of a physiological plasma protein content.

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