Release of uremic retention solutes from protein binding by hypertonic predilution hemodiafiltration: a novel therapeutic approach

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ABSTRACT
Protein-bound uremic retention solutes accumulate in patients suffering from chronic kidney disease and its removal by hemodialysis is hampered. Therefore, we developed a dialysis technique where the protein-bound uremic retention solutes are removed more efficiently under high ionic strength.
Protein-bound uremic solutes like phenylacetic acid, indoxyl sulfate and p-cresyl sulfate was combined with plasma in the presence of increased ionic strength. The protein integrity of proteins and enzymatic activities were analysed. In-vitro dialysis of albumin solution was performed to investigate the clearance of the bound uremic retention solutes. In-vitro hemodiafiltrations of human blood were performed to investigate the influence of increased ionic strength on blood cell survival. The protein-bound fraction of phenylacetic acid, indoxyl sulfate and p-cresyl sulfate was significantly decreased from 59.4±3.4%, 95.7±0.6%, 96.9±1.5% to 36.4±3.7%, 87.8±0.6% and 90.8±1.3% respectively. The percentage of phenylacetic acid, indoxyl sulfate and p-cresyl sulfate released from protein was 23.0±5.7%, 7.9±1.1% and 6.1±0.2%. The clearance during in-vitro dialysis was increased by 13.1±3.6 %, 68.8±15.1 % and 53.6±10.2% respectively. There was no difference in NaCl concentrations at the outlet of the dialyser using isotonic and hypertonic solutions. In conclusion, this study forms the basis for establishing a novel therapeutic approach to remove protein-bound retention solutes.

INTRODUCTION
Patients suffering from chronic kidney disease (CKD) show an increased cardiovascular mortality and morbidity mainly due to the accumulation of protein-bound uremic retention solutes, which strongly contribute to the progression of cardiovascular diseases (CVD) and are poorly cleared by conventional dialysis therapies \(^1\). The kinetic parameters of water-soluble uremic retention solutes are currently used for the evaluation of dialysis adequacy \(^2\-^4\), alt-
hough protein-bound uremic retention solutes contribute to the development and progression of CVD in CKD patients. Dialysis therapies have been optimized for the removal of low-molecular weight water-soluble and unbound uremic retention solutes, but not for protein-bound uremic retention solutes. Protein-bound uremic retention solutes like phenylacetic acid, indoxyl sulfate and p-cresyl sulfate are characterized by its hydrophobic molecular fragments, e.g. aromatic phenyl groups of phenylacetic acid (Figure 1A).

Therefore, there is a strong need to increase the portion of unbound fractions in order to increase the clearance for these uremic toxins. Since protein-bound uremic retention solutes are hydrophobic and charged solutes, the binding is mainly based on both hydrophobic dipole-dipole interactions and hydrophilic acid-base interactions. These uremic retention solutes bind mostly to albumin characterised by two distinct binding sites for solute.

An increase in ionic strength of the surrounding solution might reduce the electrostatic interaction of solute and protein. Thus, increasing the ionic strength by infusing a hypertonic saline might be an appropriate approach; however, this approach is limited by the tolerance of blood cells and plasma proteins. Therefore, we investigated the functional and structural characteristics of randomly chosen proteins, enzymes after incubation in the presence of increased concentration of sodium chloride and the influence of increased ionic strength during in-vitro diafiltration on blood cell survival. These in-vitro studies presented in this papers might be the basis for further application of the technique in a clinical situation.

MATERIALS AND METHODS

MIXTURE OF UREMIC RETENTION SOLUTES WITH HUMAN PLASMA
In this study, we chose concentrations of phenylacetic acid, indoxyl sulfate and p-cresyl sulfate, which are comparable to the mean plasma levels in dialysis patients for in-vitro experiments (phenylacetic acid: 474.6 mg L\(^{-1}\); indoxyl sulfate: 23.1 mg L\(^{-1}\); p-cresyl sulfate 20.1 mg L\(^{-1}\) \(^{15-17}\). NaCl was added to achieve a final concentration up to 0.5 mol L\(^{-1}\). Samples were transferred to centrifugal filters (30 kDa). Half of the plasma-samples were denatured at 95°C; half was kept at room temperature. The samples were centrifuged at 3,000g for 1 hour at 4 °C.

\(^{1}\)H-NMR ANALYSIS OF PLASMA PROTEINS

The structural properties of the three plasma proteins β\(_2\)-microglobulin (β\(_2\)-M), α-chymotrypsinogen (α-CH) and hemoglobin (HB) were determined by using \(^{1}\)H-NMR spectroscopy to analyze the protein integrity (see appendix).

FUNCTIONAL ANALYSIS OF ENZYMES

The enzyme activity of the randomized enzymes lactate dehydrogenase (LDH), superoxide dismutase (SOD) and alkaline phosphatase (AP) was analyzed to test the effect of increased ionic strength on their activity. Enzyme activities were determined by conventional colorimetric enzyme assay kits. Enzyme solution (10 U mL\(^{-1}\)) were incubated in physiological buffer or 0.5 mol L\(^{-1}\) NaCl, 0.01 mol L\(^{-1}\) K\(_2\)HPO\(_4\).

IN-VITRO DIALYSIS OF ISOTONIC AND HYPERTONIC SALINE SOLUTIONS

Human blood was recirculated in a predilution hemodiafiltration setup using isotonic or hypertonic predilution saline. The erythrocyte damage was determined by quantification of free haemoglobin and the standardized index of haemolysis. 2 L of sodium chloride solutions (0.14-0.5 mol L\(^{-1}\) NaCl) were dialyzed for 5 min in a single-pass procedure (blood flow: 200 mL min\(^{-1}\); dialysate flow: 800 mL min\(^{-1}\)). Samples were collected at the sampling ports before and after hemodialyzer.
IN-VITRO DIALYSIS OF ALBUMIN-UREMIC-TOXIN-MIXTURES

10 L of a solution containing 50 g L$^{-1}$ bovine serum albumin, 0.14 mol L$^{-1}$ NaCl and 0.01 mol L$^{-1}$ K$_2$HPO$_4$ (pH value of 7.4) were mixed with the uremic toxins. This solution was stirred overnight, since the kinetics of the adsorption of uremic toxins by plasma proteins is unknown. Therefore, we used the extended incubation time of uremic toxins with the albumin to ensure the equilibration of albumin and toxin. The solution was mixed with NaCl (0.18 mol L$^{-1}$). The solution was dialyzed a single-pass procedure using a hemodialyzer (FX 5, Fresenius Medical Care, Germany). Samples were collected at the sampling ports before and after the hemodialyzer (albumin flow: 200 mL min$^{-1}$; dialysate flow: 500 mL min$^{-1}$). The perchloric acid was precipitated from supernatant by adding 15 mol L$^{-1}$ KOH.

IN-VITRO-HEMODIAFILTRATION OF HUMAN BLOOD

500 mL heparinised blood was circulated for 3 h using a conventional dialysis unit and dialysate (Bibag-5008, Fresenius Medical Care, Germany)(dialysate flow: 800 mL min$^{-1}$; blood flow: 200 mL min$^{-1}$), then infused (flow: 10 mL min$^{-1}$) with isotonic saline (0.14 mol L$^{-1}$) was infused. The infused volume was removed by ultrafiltration. After 1 h of circulation the saline was exchanged with a hypertonic saline (1 mol L$^{-1}$). The free haemoglobin concentration was determined$^{18}$ and the standardized index of the haemolysis (SIH) was calculated:

\[
\text{SIH} = \frac{m_{\text{HB}(t)} \cdot V \cdot \left(1 - \frac{\text{HCT}_{\text{mean}}}{100}\right)}{Q}
\]

where:
- \(m_{\text{HB}(t)}\) = slope of free haemoglobin concentration (mg dL$^{-1}$ min$^{-1}$)
- \(HCT\) = hematocrit (%)

The standardized index of the haemolysis (SIH) was calculated:  

\[ SIH = \frac{m_{\text{HB}(t)} \cdot V \cdot \left(1 - \frac{\text{HCT}_{\text{mean}}}{100}\right)}{Q} \]  

\[ [1] \]

SIH = standardized index of the haemolysis (mg dL$^{-1}$)  
\(m_{\text{HB}(t)}\) = slope \(f_{\text{HB}}(t)\) (mg dL$^{-1}$ min$^{-1}$)  
\(f_{\text{HB}}\) = free haemoglobin (mg dL$^{-1}$)  
HCT = hematocrit (%)
\[ V = \text{blood volume (mL)} \]
\[ Q = \text{blood flow (mL min}^{-1}\text{)} \]

The clearance of uremic toxins were analyzed during an *in-vitro* dialysis of a mixture of an albumin solution and the uremic toxins in the presence of increased ionic strength. Samples were collected at pre- and post-dialyzer and the clearance was calculated:

\[ C_{UT} = Q \times \left(1 - \frac{c_{\text{post}}}{c_{\text{pre}}} \right) \quad [2] \]

- \( C_{UT} \): clearance of uremic retention solute (mL min\(^{-1}\))
- \( Q \): flow of the protein solution (mL min\(^{-1}\))
- \( c_{\text{post}} \): concentration after dialyzer
- \( c_{\text{pre}} \): concentration before dialyzer

**RESULTS**

The influence of increased ionic strength on the protein binding ratio of uremic retention solutes was investigated by using ultrafiltration of phenylacetic acid, indoxyl sulfate and p-cresyl sulfate in plasma. While the unbound fractions of the uremic retention solutes were quantified in the filtrate of native plasma samples. The total amounts of these uremic retention solutes were determined in the heat-denatured samples.

The filtrate concentration of phenylacetic acid, indoxyl sulfate and p-cresyl sulfate were separated and quantified by chromatography. **Figure 1B** shows a representative chromatogram of deproteinized plasma spiked with phenylacetic acid, indoxyl sulfate and p-cresyl sulfate (recovery rate: 94.3 ± 5.2 %). At physiological conditions protein-bound phenylacetic acid, indoxyl sulfate and p-cresyl sulfate was found to be 59.4 % ± 3.4 %, 95.7 % ± 0.6 % and 96.9 % ± 1.5 % respectively. The protein-bound fractions of the uremic retention solutes were decreased by increased sodium chloride concentrations to 36.4 % ± 3.7 %, 87.8 % ± 0.6 % and
90.8 % ± 1.3 % (p<0.05), respectively (Figure 1C). In the presence of increased ionic strength phenylacetic acid, indoxyl sulfate and p-cresyl sulfate are released from their protein binding by 23.0 % ± 5.7 %, 7.9 % ± 1.1 % and 6.1 % ± 0.2 % respectively.

Later, the protein integrity of three representative plasma proteins was investigated. The sodium chloride concentration was reduced after an incubation period of 30 min. The $^1$H-NMR-spectra of $\beta_2$-microglobulin after incubation in the presence of isotonic and hypertonic ionic strength are given in Figures 2A and 2B. The chemical shifts of the protons in the given data showed no significant differences between the native proteins in the controls and protein samples, which had been incubated in the presence of a hypertonic saline. The structural integrity of all three proteins was not influenced by a transient incubation in a hypertonic saline (data not shown).

To analyze the effect of a hypertonic saline on the enzymatic stability of proteins, we determined the enzyme activity in dependence on increased ionic strength. The increased sodium chloride concentrations were reduced by chromatography and the enzyme activities were quantified by using colorimetric activity assays. The relative activities of LDH, SOD and AP were calculated (Figure 2C). The relative enzyme activities of LDH, SOD and AP in the controls and samples after incubation in hypertonic saline were not significantly different (49.1%±0.6% vs. 49.3%±0.2%; 40.0% ± 0.8% vs. 39.4%±1.7%; 47.1%±0.6 % vs. 48.2%±1.2 %; (p > 0.05)).

The individual clearance of phenylacetic acid, indoxyl sulfate and p-cresyl sulfate in the presence of isotonic ionic strength was 82.7 ± 2.1 mL min$^{-1}$, 38.2 ± 4.2 mL min$^{-1}$ and 35.9 ± 1.8 mL min$^{-1}$, respectively (equation [2]). In contrast, in the presence of hypertonic sodium chloride concentration the clearance of phenylacetic acid, indoxyl sulfate and p-cresyl sulfate were
93.5 ± 2.1 mL min\(^{-1}\), 64.8 ± 2.7 mL min\(^{-1}\) and 55.2 ± 3.1 mL min\(^{-1}\) (Figure 3A). Thus the clearance of phenylacetic acid, indoxyl sulfate and p-cresyl sulfate were increased by 13.1 ± 3.6 %, 68.8 ± 15.1 % and 53.6 ± 10.2 % in the presence of increased ionic strength.

Sodium chloride concentrations were analyzed in the post dialyzer samples after a single-pass dialysis. Sodium chloride concentrations were determined by conductivity (Figure 3B). Post dialyzer samples showed no significant differences in sodium chloride concentration between isotonic and hypertonic saline. The conductivity (S m\(^{-1}\)) peak area and the sodium chloride concentration showed a linear correlation (data not shown). The influence of increased ionic strength on blood cells was investigated by determination of haemolysis during in-vitro hemodiafiltration. The "standardized index of hemolysis" (SIH) was calculated from the free hemoglobin concentration (fHB) (equation [1]). fHB and SIH are given in Table 1. The SIH in the absence and in the presence of increased ionic strength (0.16 mol L\(^{-1}\) and 0.18 mol L\(^{-1}\)) were in the range of a conventional hemodiafiltration between 6 mg dL\(^{-1}\) and 13 mg dL\(^{-1}\).

**DISCUSSION**

Although protein-bound uremic retention solutes have a major impact on the morbidity and mortality of CKD patients, the clearance of these solutes by conventional extracorporeal therapies is low until now. Therefore, there is a strong need for the development of therapies with improved removal of these solutes.

The concept of conventional dialysis focuses mainly on the removal of small water-soluble solutes. Currently, applied kinetic parameters of dialysis adequacy are based on the kinetics of water-soluble uremic toxins \(^{19}\). The elimination efficiency of conventional dialysis is based on convective and diffusive transport through the dialysis membrane. Efficiency can be im-
proved by increasing the exclusion limits of the dialysis membranes and an increased concentration difference between blood and dialysate \(^{20}\).

These uremic toxins bind to plasma proteins with a molecular weight greater than the cut-off of dialysis membranes and therefore conventional dialysis therapies do not sufficiently remove protein-bound uremic toxins. Therefore, they can only be removed to a sufficient extent by a significant increase in the exclusion limits of more than 50 kDa. However, membranes with pores of this size would inevitably be permeable for a large part of the plasma proteins and would lead to uncontrolled protein losses because protein binding imposes resistance against transmembrane transfer, dialytic kinetics of protein-bound uremic retention solutes are similar to those of high molecular compounds \(^{19}\) although protein-bound uremic retention solutes exhibit low molecular mass characteristics. Although the molecular weight of these protein-bound solutes is less than 500 Da, these substances should be considered as high MW substances \(^{21}\).

The removal of protein-bound uremic retention solutes by conventional hemodialysis strategies is strongly limited since only the free fraction of the solute is available for diffusion. For example, 30\% of phenylacetic acid \(^{8}\), > 90\% of indoxyl sulfate \(^{22}\) and about 90\% of p-cresyl sulfate \(^{1}\) is protein-bound. The increase in the convective part improves the performance of depuration, but convection only applies to the free fraction and its beneficial effect is limited \(^{23}\). A recent analysis comparing the pre- and post dilution HDF did not demonstrate any difference in protein-bound solute removal \(^{24}\).

Disruption of association with protein and uremic solute is one possibility to significantly improve the removal of protein-bound uremic toxins. Therefore, we developed an approach
for an increased release protein-bound uremic retention solutes from their protein-binding resulting in an increased clearance.

The binding of protein-bound uremic retention solutes to plasma proteins is based on electrostatic interactions. We tested whether the electrostatic interactions can be disturbed by increasing the local ionic strength: The protein-bound portion of uremic retention solutes in plasma was reduced in the presence of increased ionic strength. Increased ionic strength shifts the ratio of unbound and bound portions of these uremic retention solutes to the unbound part.

Three dimensional structure and functional characteristics of proteins are based on electrostatic interactions and van der Waal forces. Therefore, we investigated the protein function and structures under hypertonic saline conditions by $^1$H-NMR-spectroscopy. The chemical shift of the protons in the $^1$H-NMR-spectrum reveals the chemical surroundings of the protons and therefore indicates the structural integrity of the proteins. A transient incubation under hypertonic saline conditions did not significantly influence the $^1$H-NMR of hemoglobin, α-chymotrypsinogen and β2-microglobulin and therefore their 3D structure. The results of $^1$H-NMR-studies were confirmed by enzyme activity studies of representative enzymes. LDH, SOD and AP which were incubated in hypertonic saline conditions showed no significant differences in enzyme activity. Increased ionic strength has no irreversible effect on the enzymatic activity of proteins.

The benefit of hypertonic sodium chloride concentrations during dialysis was evaluated by determining the clearance of representative uremic retention solutes. Clearance of phenylacetic acid, indoxyl sulfate and p-cresyl sulfate were significantly increased in the presence of hypertonic ionic strength. In addition, increased sodium chloride concentrations were reduced to physiological concentrations during a single pass through the dialyzer.
Based on these results, we developed a novel therapeutic approach, which consists of a conventional dialysis combined with an infusion of the hypertonic solution directly in the inlet line to the hemodialyzer. The hypertonic solution increases the local ionic strength at the entrance to the hemodialyzer. In consequence, protein-bound uremic retention solutes are released from their protein binding, which would lead to an increased dialytic removal. The excess sodium chloride is also removed by dialysis. However, since the composition of the dialysate might have an effect on the ion concentration of the blood, attention should be paid to the final ion concentration at the dialyser outlet in future clinical application. The condition described is the compromise of a maximal clearance, low SIH and physiologic ion concentration at the outlet of the dialyser. An increased exposure of the salt would result in a further increased clearance but would cause negative effects on the SIH and the ionic concentration at the outlet of the dialyzer.

The novel dialysis method is principally characterized by a predilution hemodiafiltration technique. In contrast to a conventional predilution hemodiafiltration technique, we suggest the usage of a hypertonic saline as dilution solution. A schematic dialysis circuit for the hypertonic predilution hemodiafiltration approach is given in Figure 3C.

Since blood contains different kinds of blood cells besides plasma proteins, the influence of increased ionic strength on blood cells was investigated by erythrocyte cell damage. The free hemoglobin levels were integrated into the standardized index of hemolysis SIH, which is a quality criterion of the impact of dialysis on the blood-cell survival. The SIH was determined in human blood during in-vitro hemodiafiltration studies in the presence of increased ionic strength. The SIH of these in-vitro hemodiafiltrations were in the range of conventional dialysis treatments. Due to the limitation of the ultrafiltration volume of conventional dialysis ma-
chines to 2 L h⁻¹, the use of 1 mol L⁻¹ sodium chloride stock saline was necessary to achieve effective sodium chloride concentrations from 0.16 mol L⁻¹ to 0.25 mol L⁻¹ at the entrance to the dialyzer. The use of 1 mol L⁻¹ sodium chloride stock saline in the present experiments caused a higher cell damage rate, due to the steep gradient between the ionic strength conditions. An increased ultrafiltration rate would permit the use of less concentrated hypertonic stock saline; the consequence would result in higher effective sodium chloride concentration and simultaneously in lower cell damage rates.

The approach developed within this study focused on modification of therapy of CKD patients; however, the principle of an increased ion-strength can be applied for liver assists therapies, too. For example, acute liver failure patients are treated by extracorporeal liver support adsorption-dialysis devices to remove albumin bound substances²⁵. Implementation of hypertonic predilution hemodiafiltration technique to liver support adsorption-dialysis devices might result in an increased removal in these patients too.

However, further studies will be performed to evaluate the hypertonic predilution hemodiafiltration in an animal-model in order to determine its safety and benefit. Due to its superior clearance of these uremic retention solutes this novel dialysis method could be an alternative therapy in CKD-stage-5d.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Federal Ministry of Education and Research (BMBF; 13920B and 01GR0807), by grant FP7-HEALTH-2009-2.4.5-2 to “SYSKID” from the European Union and by a grant of the Helmholtz Foundation.

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Table 1: Results of *in-vitro* hemodiafiltrations of human blood in the presence of isotonic (0.14 mol L\(^{-1}\)) and hypertonic ionic strength (0.16-0.18 mol L\(^{-1}\)). Data are given as means ± standard error of mean SEM, N=3.

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<th>(m_{\text{Hb(t)}}) (mg dL(^{-1}) min(^{-1}))</th>
<th>SIH (mg dL(^{-1}))</th>
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<td>0.18</td>
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FIGURE LEGENDS

Figure 1

(A) Chemical structures of phenylacetic acid (PAA), indoxyl sulfate (IDS) and p-cresyl sulfate (CRS).

(B) Representative chromatogram of deproteinized human plasma and the uremic retention solutes: phenylacetic acid (PAA), indoxyl sulfate (IDS) and p-cresyl sulfate (CRS).

(C) Protein-bound ratio of phenylacetic acid (PAA), indoxyl sulfate (IDS) and p-cresyl sulfate in the presence of physiological and hypertonic ionic strength, respectively.

Figure 2

(A) $^1$H-NMR-spectrum of $\beta_2$-microglobulin after incubation in an isotonic saline.

(B) $^1$H-NMR-spectrum of $\beta_2$-microglobulin after incubation in a hypertonic saline.

(C) Enzyme activity of lactate dehydrogenase (LDH), superoxide dismutase (SOD) and alkaline phosphatase (AP) after incubation in the presence of physiological and hypertonic ionic strength, respectively. Control samples with known enzyme concentrations of 10 U mL$^{-1}$ were set to 100 % enzyme activity to calculate the relative enzyme activity in the samples.

Figure 3

(A) Clearance of phenylacetic acid (PAA), indoxyl sulfate (IDS) and p-cresyl sulfate (CRS) uremic retention solutes (URS) during an in-vitro dialysis of an albumin solution in the presence of physiological and hypertonic ion strength, respectively.

(B) Removal of excess sodium chloride during an in-vitro dialysis of isotonic and hypertonic saline.
Schematic dialysis circuit for a hypertonic predilution hemodiafiltration approach. A hypertonic saline is infused prior to the dialyzer to the blood. Excess sodium chloride is removed by ultrafiltration via hemodiafiltration. Thus, protein-bound uremic retention solutes are released from their protein binding and are removed more efficiently than in conventional hemodiafiltration.

APPENDIX

CHROMATIGRAPHIC QUANTIFICATION OF UREMIC TOXINS BY REVERSED PHASE

1 mol L\(^{-1}\) triethylammonium acetate (TEAA) was synthesized by adding 70 mL triethylamine and 30 mL acetic acid (100 %) to 100 mL HPLC-water. The solution was stirred and cooled down to ambient temperature. HPLC-grade water was added to a volume of 500 mL. The pH-value was adjusted to 6.5 by adding triethylamine or acetic acid. Phenylacetic acid, indoxyl sulfate and p-cresyl sulfate were separated by gradient elution on a Chromolith\textsuperscript{®} Performance RP-18e (100 x 4.6 mm I.D., Merck, Germany) reversed-phase column in the presence of triethylammonium acetate (40 mmol L\(^{-1}\) pH value of 6.5) as eluent A. Ethanol (100 %) with 0.1 % TFA (pH 6.5) was used as eluent B (26). The gradient was performed as followed: 0-1 min 1 % eluent B, 1-7 min 0-60 % eluent B, 7-12 min 80 % eluent B, 12-17 min 1% eluent B. The mobile phase was pumped at a flow-rate of 2 mL min\(^{-1}\) by a high-pressure gradient pump system (L 6200, Merck-Hitachi, Germany). The sample volume was 50 µL in 100 µL sample buffer (92.5 µL 40 mmol L\(^{-1}\) TEAA, 5.0 µL 1 mol L\(^{-1}\) TEAA, 2.5 µL hydrochloric acid (25 %)). The injection volume was 140 µl. The column temperature was ambient (22 ± 1°C), and UV absorption was measured at 260 nm. The column eluate was monitored with a variable wavelength UV detector (L4250, Merck-Hitachi, Germany). Data were recorded and processed by the Chromeleon Lab System 6.60 (Dionex, Germany).
$^1$H-NMR ANALYSIS OF PLASMA PROTEINS

$\beta_2$-microglobulin, $\alpha$-chymotrypsinogen and hemoglobin (Sigma-Aldrich, Germany) were incubated with increased concentration of sodium chloride solution in an overhead rotator (20 rpm) for 30 min at ambient temperature (22 ± 1 °C). Controls were mixed in isotonic saline using the same conditions. The sodium chloride concentration was reduced by using size exclusion chromatography micro-centrifuge devices (Micro Bio-Spin 30 (Biorad, Germany). 50 µL deuterium oxide (Sigma-Aldrich, Germany) for shimming of the magnetic field and 50 µL deuterium oxide with 0.75 % 3-trimethylsilyl-[2,2,3,3,-2 H$^4$]-propionate (TSP) (Sigma-Aldrich, Germany) as an internal standard was added to 500 µl of each sample and frozen at -20 °C. $^1$H-NMR-analysis was performed using a DRX 600 spectrometer (Bruker, Germany) with a magnetic field strength of 600 MHz. A 90°-impulse of 11.5 µs was used to suppress the signal of water, each sample was measured with 1,024 repeats at 25 °C. $^1$H-NMR-spectra were processed using the MestRe-C software (version 3.4.0, Santiago de Compostela, Spain). The free induction decay was Fourier-transformed, followed by phase and baseline correction. The chemical shift of the reference TSP was set to zero, the amounts of data points were 32 K.

QUANTIFICATION OF SODIUM CHLORIDE CONCENTRATION

The sodium chloride concentration was quantified by conductometry in an isocratic elution using a Chromolith® Performance RP-18e (100 x 4.6 mm I.D., Merck, Germany) reversed-phase column using a isocratic solution of water-ethanol (w/w 90/10 %). The sample volume was 150 µL and the injection volume was 100 µL. The mobile phase was pumped at a constant flow-rate of 2 mL min$^{-1}$ by a high-pressure gradient pump system (L 6200, Merck-Hitachi, Germany). The column temperature was ambient (22 ± 1° C), and conductivity was measured in millivolt (mV). Data were recorded and processed by the Chromeleon Lab System 6.60 (Dionex, Germany).
Figure 1

A

\[
\begin{align*}
\text{PAA} & \quad \text{IDS} & \quad \text{CRS} \\
\end{align*}
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B

UV absorbance (220 nm) (AU)

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retention time (min)

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C

protein-bound fraction (%)

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ion strength

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Figure 2

Böhringer et al. Figure 2

A

rel. resonance intensity (AU)

chem. shift δ (ppm)

B

rel. resonance intensity (AU)

chem. shift δ (ppm)

C

rel. activity of enzyme (AU)

ion strength

LDH  SOD  AP

physiological  hypertonic  physiological  hypertonic  physiological  hypertonic

n.s.
Figure 3

Böhringer et al. Figure 3

A

URS clearance (mL min⁻¹)

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<tr>
<td>hypertonic</td>
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</tbody>
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B

\[ c_{NaCl} \text{ (mol L}^{-1}\text{)} \]

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<tr>
<th></th>
<th>pre dialysis</th>
<th>post dialysis</th>
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</thead>
<tbody>
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<td>isotonic saline</td>
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<tr>
<td>hypertonic saline</td>
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</tbody>
</table>

C

![Diagram of dialysis process]

- Patient
- Dialysate
- Dialyzer
- Blood pump
- Hypertonic saline
- Infused saline