Blood Purification

Research Article

Blood Purif
DOI: 10.1159/000534906

Received: June 5, 2023
Accepted: October 26, 2023
Published online: January 23, 2024

In vitro Removal of Protein-Bound Retention Solutes by Extracorporeal Blood Purification Procedures

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Keywords
Adsorbers · Hemoperfusion · Protein-bound retention solutes · Extracorporeal blood purification · Uremic toxins · Liver failure

Abstract
Introduction: When the kidneys or liver fail, toxic metabolites accumulate in the patient's blood, causing cardiovascular and neurotoxic complications and increased mortality. Conventional membrane-based extracorporeal blood purification procedures cannot remove these toxins efficiently. The aim of this in vitro study was to determine whether commercial hemoperfusion adsorbers are suitable for removing protein-bound retention solutes from human plasma and whole blood as well as to compare the removal to conventional hemodialysis. ***Methods:*** For in vitro testing of the removal of protein-bound substances, whole blood and plasma were spiked with uremic retention solutes (homocysteine, hippuric acid, indoxyl sulfate, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid) and the toxins of liver failure (bilirubin, cholic acid, tryptophan, phenol). Subsequently, the protein binding of each retention solute was determined. The adsorption characteristics of the hemoperfusion adsorbers, Jafron HA and Biosky MG, both approved for the adsorption of protein-bound uremic retention solutes and Cytosorb, an adsorber recommended for adsorption of cytokines, were tested by incubating them in spiked whole blood or plasma for 1 h. Subsequently, the adsorption characteristics of the adsorbers were tested in a dynamic system. For this purpose, a 6-h in vitro hemoperfusion treatment was compared with an equally long in vitro hemodialysis treatment. ***Results:*** Hippuric acid, homocysteine, indoxyl sulfate, and tryptophan were most effectively removed by hemodialysis. Bilirubin and cholic acid were removed best by hemoperfusion with Cytosorb. A treatment with Jafron HA and Biosky MG showed similar results for the adsorption of the tested retention solutes and were best for removing phenol. 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid could not be removed with any treatment method. ***Discussion/Conclusion:*** A combination of hemodialysis with hemoperfusion seems promising to improve the removal of some toxic metabolites in extracorporeal therapies. However, some very strongly protein-bound metabolites cannot be removed adequately with the adsorbers tested.

Introduction

Protein-Bound Retention Solutes in Chronic Kidney Disease

Chronic kidney disease (CKD) is a global health problem due to its increasing incidence and prevalence [1]. It is defined as a progressive, irreversible loss of

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kidney function in which retention solutes accumulate in the patient's blood. Left untreated, CKD may progress to end-stage renal disease (ESRD), necessitating renal replacement therapy [2]. The uremic toxins have been divided by the European Uremic Toxin Working Group (EUTox) into three groups, namely small water-soluble compounds, protein-bound compounds, and middle molecules [3, 4]. ESRD is treated by chronic hemodialysis or hemodiafiltration, which removes water-soluble low molar mass molecules and middle molecules [5]. Protein-bound compounds are insufficiently removed by these procedures and accumulate in the patient's blood [3]. This is associated to cardiovascular complications and increased mortality in dialysis patients [6].

Homocysteine (HCY) is a sulfur-containing amino acid with a molecular mass of 135 Da and a risk factor for cardiovascular morbidity and mortality in patients with CKD [7]. Hippuric acid (HA) is an organic carbon acid with a molecular mass of 179 Da. It inhibits the protein binding of drugs and glucose utilization in muscle cells, leading to kidney tubular damage [8]. Indoxyl sulfate (IS) is a small hydrophobic acid with a molecular mass of 213 Da. Decreased renal elimination increases plasma concentrations in patients with ESRD. This leads to a direct toxic effect on the renal proximal tubular cells and vascular changes, resulting in an increase in the rate of progression of kidney failure caused by tubular damage [8]. Furthermore, IS has proinflammatory and pro-oxidant effects by stimulating the production of reactive oxygen species [9]. 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF) is a highly lipophilic urofuranic fatty acid with a molecular mass of 240 Da, whose biological origin has not been fully elucidated. It is the most potent inhibitor of protein binding of drugs and natural metabolites such as bilirubin and thyroxine, which increases their toxic effect [7]. Furthermore, it inhibits various enzymes, such as transferases and clearance enzymes, and can cause neurological disorders.

Protein-Bound Metabolites in Liver Failure

ESRD is a severe disease due to the diverse metabolic functions of the liver. One of the tasks of the liver is the excretion of endogenous and exogenous toxins. When the liver function declines or fails completely, toxic metabolites such as bilirubin, bile acids, aromatic amino acids, and phenolic compounds accumulate in the patient's blood and lead to a mortality rate above 60% without liver transplantation [10]. Severe ESRD can be treated by extracorporeal liver replacement therapies such as single-pass albumin dialysis and fractionated plasma separation and adsorption until the liver regenerates itself or a transplant is available [11].

Bilirubin is the breakdown product of hemoglobin and serves as a marker for liver and blood diseases [12]. High levels of unconjugated bilirubin lead to acute bilirubin encephalopathy, kernicterus, and neurotoxicity [13]. Bile acids are the end products of the cholesterol metabolism in hepatocytes. High concentrations of bile acids have a liver-toxic effect and cause itching and unbearable pruritus [14, 15]. Cholic acid (CA) is one of the major human bile acids and was chosen as a representative of all bile acids in this study. Liver failure also leads to high concentrations of phenolic compounds and a shift of the amino acid profile toward aromatic amino acids such as phenylalanine, tyrosine, and tryptophan (TR). This results in an enhanced brain uptake and leads to a subsequently disturbed neurotransmission [16]. Phenol is one of the metabolites generated during the metabolism of tyrosine, and the liver normally detoxifies it. However, during liver failure, phenol accumulates and can cause damage to the liver.

Protein Binding

Human serum albumin is the main transport protein in human blood. It consists of three homologous alpha helices, each subdivided into two subdomains. The subdomains IIa and IIIa are the most important binding sites for exogenous and endogenous toxins [17]. Protein binding is usually reversible because it involves noncovalent bonds such as electrostatic or hydrophobic interactions, van der Waals forces, or hydrogen bonds. Only the nonprotein-bound fraction of toxins can be removed by diffusive or convective membrane-based approaches. The protein-bound fraction serves to establish chemical balance when the free fraction is metabolized, excreted, or removed through extracorporeal blood purification procedures [18, 19]. The binding of toxic metabolites to albumin depends on the concentration of albumin, the binding constant of the toxin, and the presence of binding competitors such as drugs or other protein-bound toxins.

Albumin and Its Redox Properties

The most important tasks of albumin include maintaining the colloid osmotic pressure, transporting water-insoluble substances in the blood, and the buffer function to maintain a stable blood pH. In addition, albumin is an important antioxidant. Besides the reduced form (human mercaptalbumin, HMA), smaller amounts of reversibly

oxidized albumin (human nonmercaptalbumin-1, HNA-1) and irreversibly oxidized albumin (human nonmercaptalbumin-2, HNA-2) are always found in the blood. The higher the oxidative stress, the more albumin molecules are irreversibly oxidized. Irreversible oxidation not only reduces the antioxidant function of albumin but also impairs its binding capacity and transport function. Increased blood concentrations of toxic metabolites and impaired albumin function are also typical for liver failure [20–22].

Extracorporeal Blood Purification

The removal of endogenous toxins that accumulate in the blood of patients due to organ failure is treated with hemodialysis or hemofiltration procedures. For removing water-soluble toxins and toxins with a low molecular weight (≤6,000 Da), low-flux filters, and toxins with a molecular weight ≤50 kDa, high-flux filters are used [23]. These membrane-based methods do not effectively remove protein-bound substances even when combined with the administration of binding competitors that increase the free fraction of toxins in the blood [24]. Another option to remove protein-bound substances from the patients' blood is hemoperfusion adsorbers. These blood-compatible and highly porous adsorbers are used directly in an extracorporeal circuit and bind toxins to their surface [25, 26].

In this in vitro study, we evaluated clinically approved adsorber materials. Jafron HA is approved for the adsorption of medium- and large-molecular weight toxins and protein-bound uremic toxins such as β2-microglobulin (β2M), tumor necrosis factor α, and interleukin 6 (IL-6). Clinical studies have shown benefits for patients with uremic syndrome [26]. Biosky MG is approved for the adsorption of medium- and large-molecular weight toxins and protein-bound toxins such as parathyroid hormone, β2M, leptin, HCY, interleukin 1β, and IL-6 in patients with terminal kidney failure. However, there is only one publication on the clinical use of this adsorber [27]. Cytosorb is approved for the adsorption of proinflammatory cytokines such as interleukin 1β, IL-6, and tumor necrosis factor α, and it is used to treat patients under acute inflammatory conditions such as sepsis, cardiac surgery, or organ transplantation [28–30].

This study aimed to investigate the protein binding of protein-bound uremic retention solutes and liver failure metabolites. The study also examined the effect of extracorporeal blood treatment on the oxidation state of albumin. Additionally, the study evaluated the adsorption characteristics of hemoperfusion adsorbers approved for the adsorption of cytokines or protein-bound retention solutes concerning their adsorption characteristics of these metabolites in comparison to removal by conventional hemodialysis.

Materials and Methods

Human Blood and Plasma

This study was approved by the Ethics Committee of the University of Continuing Education Krems (EK GZ 13/2015-2018). Fresh human blood was obtained from healthy volunteers after signing an informed consent. To determine protein binding of the toxins and the adsorption kinetics of the hemoperfusion adsorbers, whole blood anticoagulated with 10 IU/mL heparin was used. For the extracorporeal blood purification treatments, heparinized plasma with 10 IU/mL heparin was gained from citrated plasma (Red Cross Austria, Linz, Austria) by hemodialysis using a multiFiltrate classic machine and a multiFiltrate Kit 9 CVVHD 600 (Fresenius Medical Care, Germany) to remove the citrate-calcium complex from plasma. Calcium was substituted by the calcium-containing dialysis solution multiPlus (Fresenius Medical Care, Germany). Finally, the heparin plasma was frozen at −20°C in Compoflex bags (Fresenius Kabi, Austria) until use.

Adsorbers

Jafron HA (Jafron Biomedical Co., China) is a styrene-divinylbenzene copolymer, and Biosky MG (Biosun Medical Technology Co., China) is a polystyrene resin, both approved for the adsorption of metabolites with a middle to high molecular weight and protein-bound uremic retention solutes. Cytosorb (CytoSorbents Corporation, USA) is a polyvinyl-pyrrolidone-coated styrene-divinylbenzene copolymer approved for the adsorption of proinflammatory cytokines. Before use, all adsorber cartridges were rinsed according to the manufacturer's instructions.

Retention Solutes

Uremic retention solutes: β2M was purchased from MP Biomedicals Inc. (Santa Ana, USA). Urea, HCY, HPS, IS, and CMPF were purchased from Sigma-Aldrich (St. Louis, USA). Stock solutions of 5 mg/mL for HCY and 500 mg/mL for urea were prepared in 0.9% saline solution. For β2M, HA, IS, and CMPF, 5 mg/mL stock solutions were prepared in 50% dimethyl sulfoxide (DMSO) in saline.

Toxins of liver failure: Bilirubin, CA, TR, and phenol were purchased from Sigma-Aldrich (St. Louis, USA). Stock solutions with a concentration of 20 mM for CA and TR, 60 mM for bilirubin, and 400 mM for phenol were prepared in 0.3 N NaOH.

Plasma and Blood Preparation

Toxins of liver failure were dissolved in 0.3 N NaOH before addition to plasma or whole blood. After 60 min of incubation, the same amount of 0.3 N HCl was added to restore the physiological pH. The final concentrations of the added substances are shown in Table 1. Uremic retention solutes were solved in 50% DMSO. To spike heparinized plasma, the stock solutions were directly added to reach the concentrations listed in Table 1. Since DMSO causes hemolysis when added to whole blood [31], the

Table 1. List of retention solutes that were added to whole blood and to plasma for the in vitro experiments

Retention solute	Molecular weight (Da)	Physiological concentration	Concentration used in the in vitro experiments
β2M	11,800	<2.0 mg/L	4.5 mg/L
Urea	60	<0.4 mg/L	2.4 g/L
Homocysteine	135	<1.7 mg/L	2 mg/L
Hippuric acid	179	<5.0 mg/L	50 mg/L
Indoxyl sulfate	212	0.6±3.3 mg/L	30 mg/L
CMPF	240	7.7±5.4 mg/L	15 mg/L
Bilirubin	585	<20.6 μM	300 μM
Cholic acid	409	0.6±0.3 μM	100 μM
Tryptophan	204	31–64 μM	100 μM
Phenol	94	–	2 mM

toxins dissolved in DMSO cannot be added directly. Therefore, the plasma was separated by centrifugation (3,500 *g*, 10 min, room temperature) and mixed with the DMSO stock solution. Following a 30-min incubation at room temperature, the plasma was gently mixed back with the blood cells.

Protein Binding

To determine the extent of protein binding of toxins added to blood and plasma for our experiments, the protein-bound fraction was calculated. Tests were carried out in heparinized plasma containing uremic retention solutes or metabolites of liver failure at the concentration listed in Table 1. The plasma was centrifuged (15,000 *g*, 20 min, room temperature) through 30 kDa membranes ($n = 6$), and the concentrations of albumin and toxins were analyzed in the filtrate. Protein-binding percentage was calculated from the concentration in plasma and filtrate. Urea and β2M were added as negative and positive controls.

Adsorption of Protein-Bound Retention Solutes

To determine the adsorption of the hemoperfusion adsorbers, batch tests were carried out in heparinized whole blood and plasma ($n = 6$). Adsorbers were incubated with whole blood or plasma (v/v 1:10) at 37°C on a roller mixer for 60 min. The concentrations of the toxins were analyzed before and after the incubation. The adsorption was calculated as the percentage of the concentration incubated without adsorber for 60 min of each toxin.

Elimination of Protein-Bound Retention Solutes by Hemodialysis

To compare the elimination of protein-bound retention solutes by hemoperfusion with hemodialysis, in vitro dialysis experiments were carried out ($n = 3$). The hemodialysis experiment was performed with a multiFiltrate classic, a multiFiltrate Kit Paed CRRT/SCUF, and a multiBic dialysate containing 1.5 mM calcium and 0.5 mM magnesium (Fresenius Medical Care, Bad Homburg, Germany). Heparinized plasma (330 mL) mixed with the toxins (Table 1) was treated over 6 h with a plasma flow rate of 30 mL/min and a dialysate flow rate of 1,500 mL/h. Samples were drawn at 0, 15, 30, 60, 120, 240, and 360 min, aliquoted and stored at −20°C until analysis. The adsorption was calculated as the percentage of the initial concentration for each toxin.

Adsorption of Protein-Bound Retention Solutes by Hemoperfusion

Since there was no difference in the adsorption capacity between batch tests carried out in whole blood or plasma, hemoperfusion experiments were carried out in plasma with each adsorber ($n = 3$). The pediatric hemoperfusion sets were used in in vitro experiments with 30 mL adsorber cartridges and a pediatric disposable kit (AV-Set Paed R, Fresenius Medical Care, Bad Homburg, Germany). 330 mL heparinized plasma mixed with protein-bound toxins was treated over 6 h with a plasma flow rate of 30 mL/min. Samples were drawn at 0, 15, 30, 60, 120, 240, and 360 min, aliquoted and stored at −20°C until analysis. The adsorption was calculated as the percentage of the initial concentration for each toxin.

Measurement of the Oxidation State of Albumin

To determine the redox state of albumin during hemoperfusion and hemodialysis treatments, the plasma samples were analyzed with an ion exchange chromatography. This method allows simultaneous analysis of HMA, HNA-1, and HNA-2, following the protocol described by Imai et al. [32] with minor adaptations. The HPLC system used was a Shimadzu Prominence and the LabSolutions software Vers. 5.54 (Shimadzu, Japan). Plasma samples were diluted 1:7.5 in physiological saline and 5 μL was injected. The peak area was used for quantification.

Measurement of the Toxins

A cobas c311 analyzer (Roche, Basel, Switzerland) was used with the corresponding test kits from Roche (albumin, urea, β2M, HCY, and bilirubin) and from Trinity Biotech (CA; Bray, Ireland). TR, phenol, HA, IS, and CMPF were analyzed with a reversed-phase HPLC method using a Nucleosil EC 150 × 4.6 mm 100-5 C-18 column (Macherey-Nagel, Düren, Germany), an Onyx Monolithic 18 × 4.6 mm C-18 guard column, and a Shimadzu Prominence HPLC system. For the analysis of TR and phenol, we employed a mobile phase consisting of methanol:water (1:1) with a flow rate of 0.5 mL/min. Plasma samples were prepared as follows: 100 μL plasma sample was mixed with 500 μL iced methanol, incubated for at least 20 min at −20°C, centrifuged, and 20 μL of the supernatant was injected by an autosampler. The peak of TR was detected at UV 280 nm

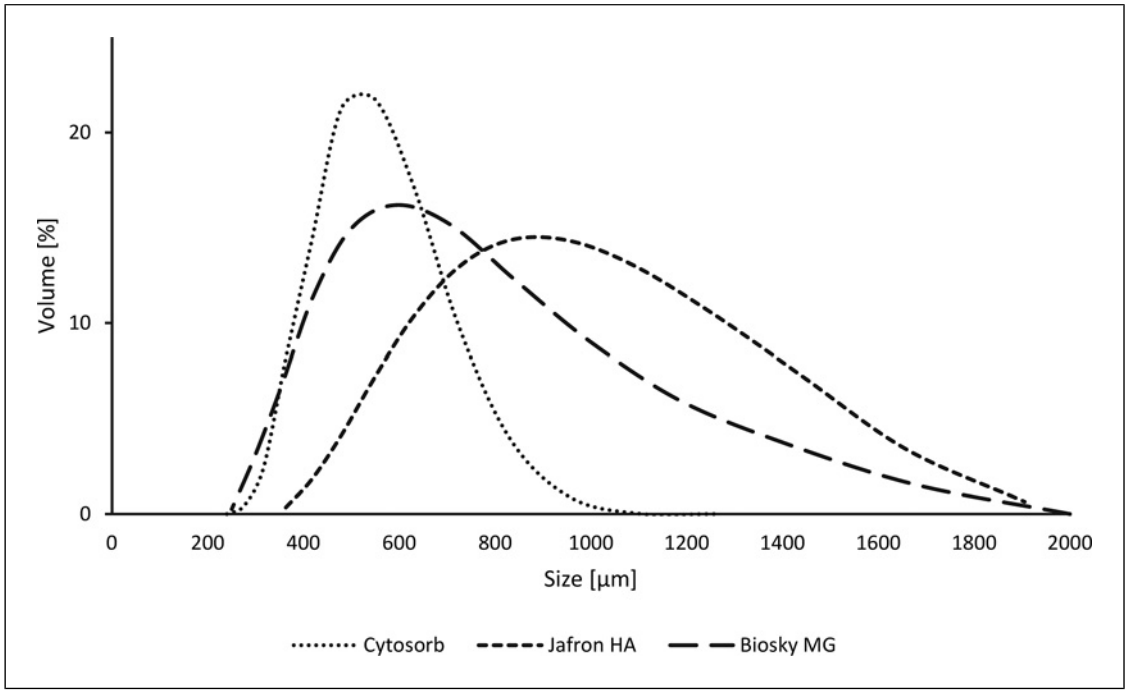

Fig. 1. Volume-size distribution of the hemoperfusion adsorbents Cytosorb, Jafron HA, and Biosky MG.

and the phenol peak was detected at UV 271 nm. For the analysis of HA, IS, and CMPF, we employed the method previously published by Lesaffer et al. [23] with certain adaptations. HA and CMPF were detected at UV 254 nm and IS at excitation 385 and emission at 515 nm. All plasma samples were deproteinized before analysis, mixing 150 μL plasma with 300 μL iced methanol, incubating them at −20°C for 20 min with subsequent centrifugation. An internal standard (naphthalene sulfonic acid) and trichloroacetic acid for acidifying the sample were added to the supernatant before 50 μL of this solution was injected.

Measurement of the Size Distribution of the Hemoperfusion Adsorbers

Particle size distribution was measured using a Mastersizer 2000 with the corresponding software version 5.22 (Malvern Instruments Ltd., Malvern, UK). 0.5 mL of a 50% adsorber suspension in high purity water was prepared and pipetted into the instrument.

Statistics

The experiments for determining the protein binding of retention solutes and the adsorption capacity were conducted in a sixfold setup. The test for normal distribution was carried out with the statistical program SPSS using Shapiro-Wilk test and the statistical significance was tested using a t test for independent samples where a probability of error of $p < 0.05$ was accepted.

The hemodialysis and hemoperfusion treatments were carried out in triplicates. Means and standard deviations were calculated using Microsoft Excel 365.

Results

Volume-Size Distribution of the Hemoperfusion Adsorbers

The distribution curves show the volume of the particles in each size fraction (Fig. 1). Cytosorb is the smallest adsorber with a distribution maximum of 500 μm and a narrow size distribution of 240–1,100 μm. Biosky MG shows a distribution maximum of 600 μm and a relatively broad size distribution ranging from 250 to 1,700 μm. Jafron HA has the largest particles with a distribution maximum of 900 μm and a distribution ranging from 360 to 1,900 μm.

Protein Binding in Plasma

The protein-binding efficiency determined in our study (Table 2) aligns with the literature findings for patient values, demonstrating consistency in the observed protein-binding percentages for all toxins. Notably, a trend is observed where higher levels of protein binding are noted when fewer toxins are present, except for CA.

Adsorption Efficiency of Hemoperfusion Adsorbers for Toxic Metabolites in Whole Blood and Plasma

As shown in Figure 2b, Cytosorb is the most efficient adsorber for removing β2M, HCY, and bilirubin in whole blood and plasma ($p < 0.05$); for CA, it is

Table 2. Protein binding in plasma ($n = 6$) measured after addition of uremic or hepatic toxins and measured after addition of uremic and hepatic toxins compared to published protein-binding values of patients

Retention solute	Binding site	Protein binding, %			References
		uremic OR hepatic toxins	uremic AND hepatic toxins	patient values from Literature	
Homocysteine	Cys 34	75.1	64.1	70–80	[18, 33, 34]
Hippuric acid	Sudlow Site 2	57.3	56.3	> 42	[18, 35]
CMPF	Sudlow Site 1	96.8	85.4	> 95	[18, 35]
Indoxyl sulfate	Sudlow Site 2	84.9	69.8	> 85	[35, 36]
Bilirubin	Sudlow Site 2	100	99.8	> 99	[37]
Cholic acid	Sudlow Site 1	91.6	91.9	> 97	[38, 39]
Tryptophan	Sudlow Site 2	86.9	74.9	> 90	[40, 41]
Phenol	Sudlow Site 1	84.4	70.5	n.a.	[42]

CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid.

significantly more efficient than JF and BS in whole blood but not significantly better than BS in plasma ($p = 0.33$). Jafron HA shows the highest adsorption rate for TR in whole blood and plasma ($p < 0.05$) and for phenol in whole blood, but no significant difference is shown in plasma between JF and BS ($p = 0.31$). Biosky MG is the most efficient adsorbent for HA and IS in whole blood and plasma ($p < 0.05$). All protein-bound metabolites show a higher adsorption efficiency in whole blood than in plasma. The differences between the tested hemoperfusion adsorbents are the same with all metabolites.

The Oxidation State of Albumin during 6 h of Hemoperfusion or Hemodialysis Treatment

Table 3 shows the ratios of the different oxidation states of albumin before and after the 6-h dialysis and hemoperfusion treatments. The difference between the concentrations before and after treatment was not significant ($p > 0.05$). No HNA-2 was detected as the plasma of healthy volunteers was used for the hemoperfusion and hemodialysis treatments. It can therefore be assumed that the free thiol group on cysteine 34 of the albumin molecule was not oxidized and was therefore available as a binding site for HCY and that the antioxidant effect of albumin is not impaired.

Removal of Albumin and Nonprotein-Bound Toxins by Hemodialysis and Hemoperfusion

As shown in Figure 3, albumin and total protein were not removed by hemodialysis but slightly adsorbed by all tested hemoperfusion adsorbers during the first 2 h of treatment. The albumin reduction appears to be caused by protein coating [43], mainly of the outer adsorber surface. Urea was completely removed within 2 h of hemodialysis treatment but not during 6-h hemoperfusion treatment by any of the tested adsorbers. β2M was removed fastest by Cytosorb but also removed by the other adsorbents and hemodialysis over 6 h.

Elimination of Protein-Bound Retention Solutes by Hemodialysis and Hemoperfusion

The results of the hemodialysis and hemoperfusion treatments performed in heparinized plasma are summarized in Figure 4. A slight decrease in toxin concentration after 15 min of treatment resulted from dilution due to the rinsing process of the extracorporeal systems, filters, and adsorbers directly before treatment.

Hemodialysis was able to reduce HCY, which is 70–80% bound to plasma proteins, by 30% within 1 h. A longer treatment time did not lead to any further reduction. The hemoperfusion treatment could only reduce HCY by 7–17%. IS could be dialyzed despite a protein binding of over 85% over the entire treatment time of 6 h. The hemoperfusion treatment reduced the concentration of IS by 11–29%. HA, the uremic toxin with the weakest protein binding (about 46%) was completely removed by hemodialysis within 4 h, whereas the hemoperfusion treatment only showed a removal of 13–30%. CMPF with a protein binding of over 95% could not be removed with either hemodialysis or hemoperfusion treatments.

CA could be reduced to 20% of the initial concentration by hemodialysis despite a protein binding of more than 97% over the treatment period of 6 h. All three adsorbents were able to reduce the CA concentration to

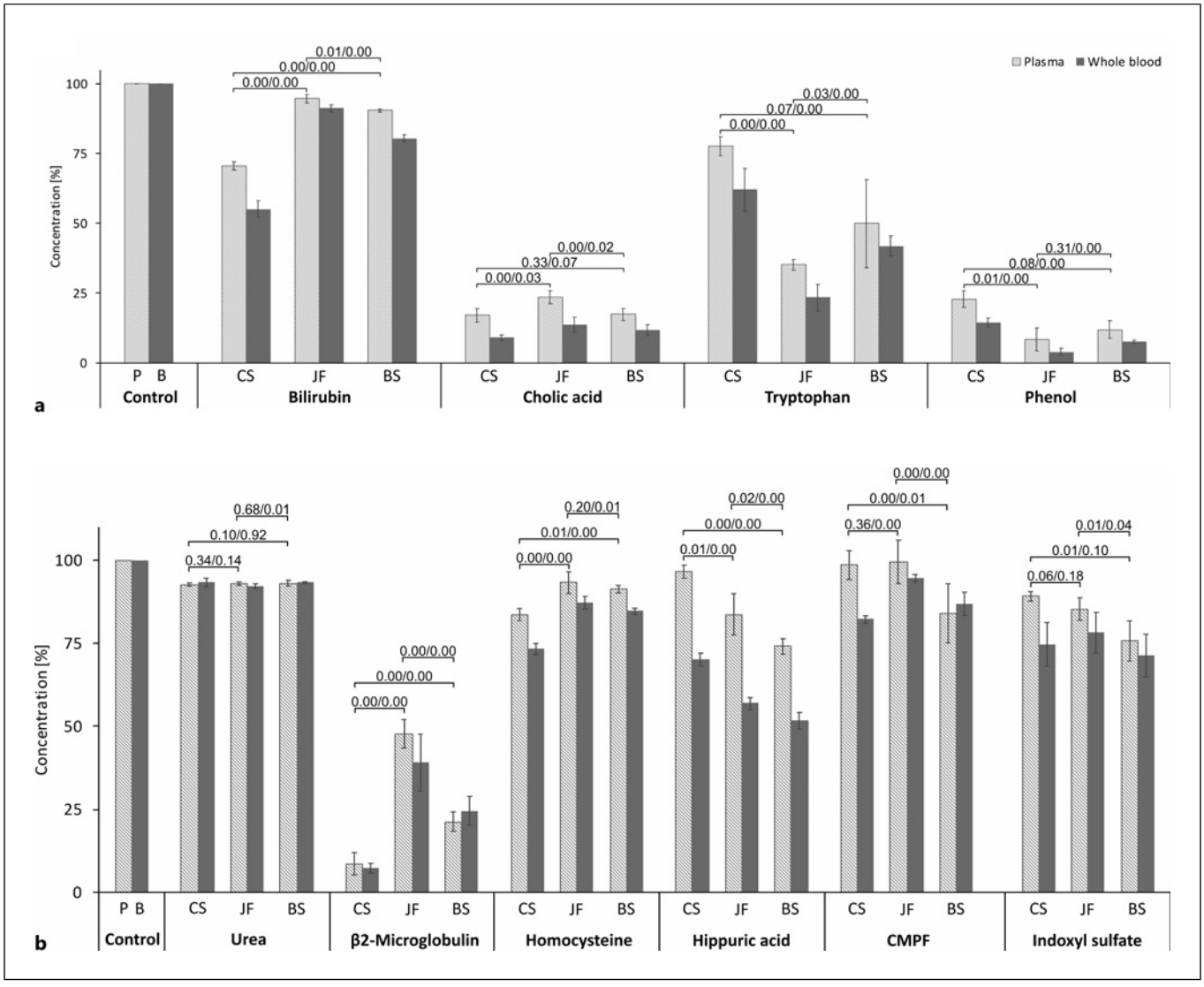

Fig. 2. Adsorption characteristics of the hemoperfusion adsorbers Cytosorb (Cyt), Jafron HA (Jaf), and Biosky MG (Bio) incubated for 1 h in plasma and whole blood at a ratio 1:10, controls are plasma (P) and whole blood (B) incubated without adsorbents. **a** Toxins of liver failure. **b** Uremic retention solutes (n = 6). Percentages were calculated from the untreated plasma or whole blood after 1 h incubation. Data ≤0.05 are considered significant.

Table 3. Oxidation state of albumin during 6 h of hemoperfusion or hemodialysis treatment

		HMA, %	HNA-1, %	HNA-2, %
Before treatment		59.3	40.7	0.0
After 6 h	Dialysis	63.3	36.7	0.0
	Hemoperfusion with Cytosorb	60.1	39.9	0.0
	Hemoperfusion with Jafron HA	59.4	40.6	0.0
	Hemoperfusion with Biosky MG	59.6	40.4	0.0

The numerical values are mean in percent (n = 3). HMA, human mercaptalbumin; HNA-1, human nonmercaptalbumin 1; HNA-2, human nonmercaptalbumin 2.

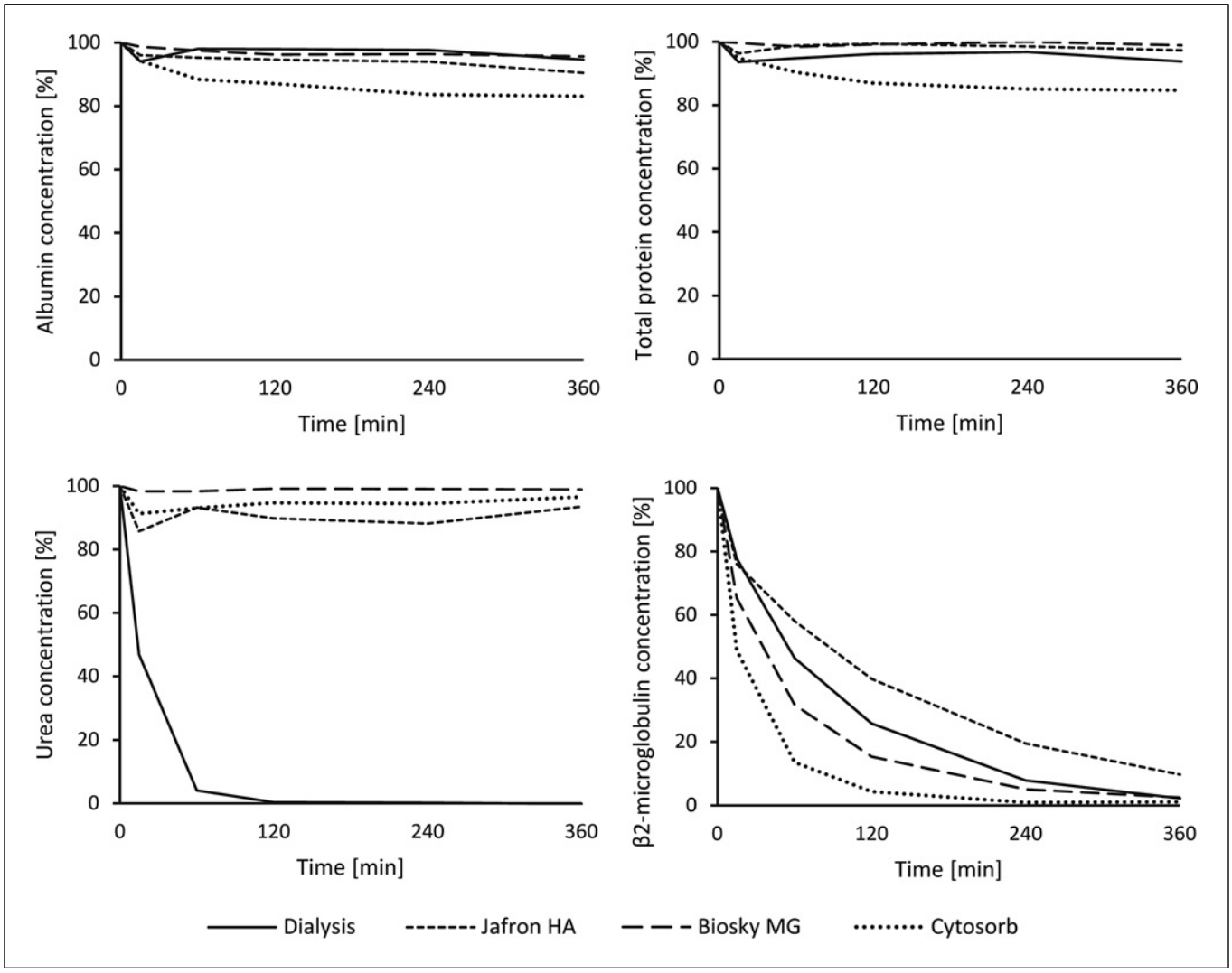

Fig. 3. Concentration in percent of albumin, total protein, urea, and β2M in 330 mL plasma during a 6-h treatment with hemodialysis (FX Paed) or hemoperfusion with 30 mL cartridges of Jafron HA, Biosky MG, or Cytosorb (n = 3).

below 10% of the initial concentration. Bilirubin, the most protein-bound toxin of the liver failure, was reduced by 43% by hemoperfusion treatment with Cytosorb. Treatment with Jafron HA, Biosky MG, and hemodialysis could not reduce the bilirubin concentration. Large differences were found in the elimination of TR. The greatest reduction occurred with hemodialysis treatment (82%), followed by hemoperfusion treatment with Jafron HA (65%), Biosky MG (25%), and Cytosorb (13%). Phenol could be reduced below 20% of the initial concentration by hemodialysis as well as by hemoperfusion treatments with all three adsorbents.

Discussion

Due to the different size distributions of the tested highly porous adsorbers, the external surface area per adsorber volume increases with decreasing adsorber diameter. This means that especially the nonspecific adsorption, as well as the adsorption of larger molecules such as albumin, which cannot penetrate into the pores of the adsorber, is significantly higher with the smaller adsorber Cytosorb. Therefore, choosing the right adsorber is always a trade-off between hemocompatibility and efficiency.

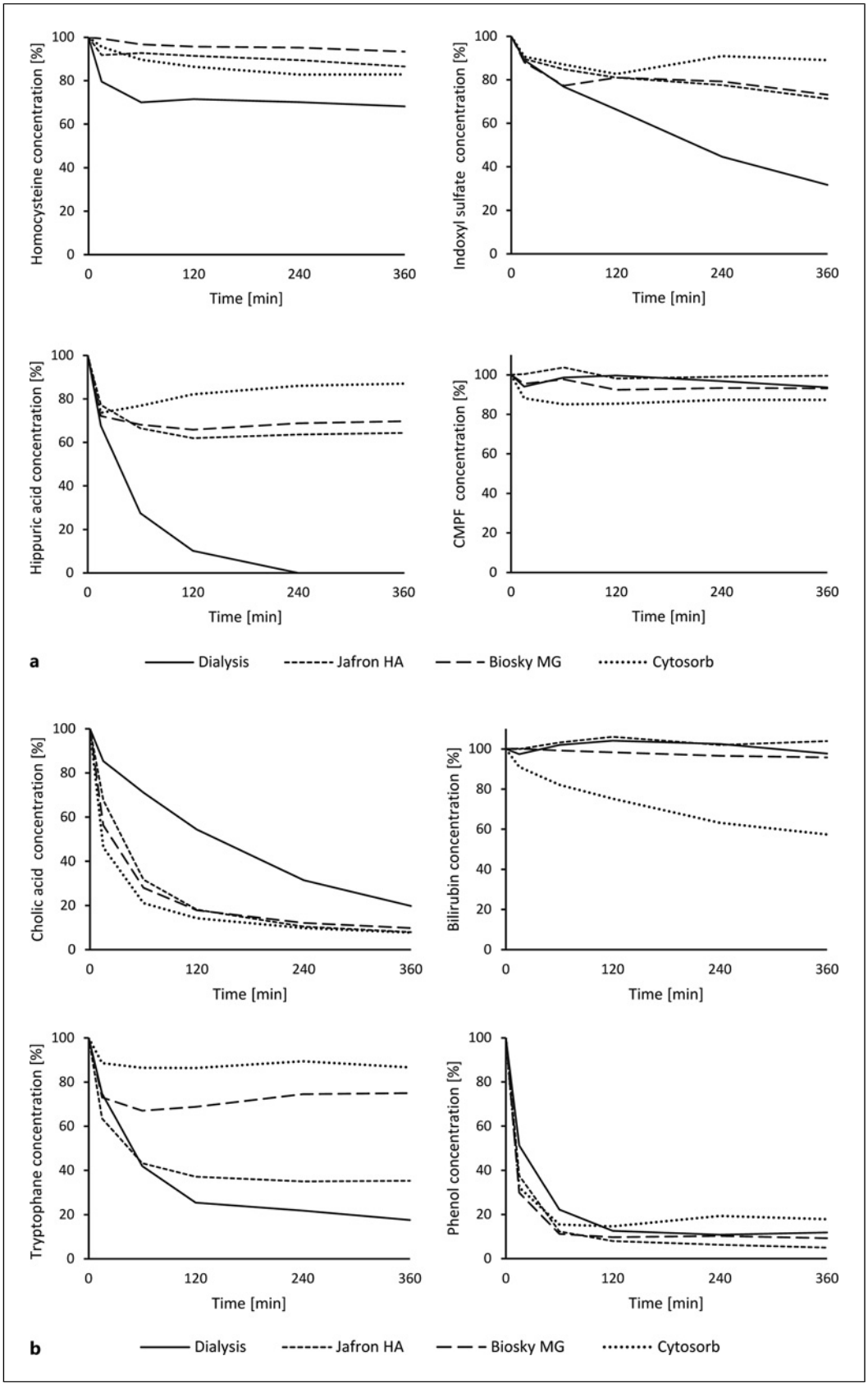

Fig. 4. Concentration in percent of HCY, HA, CMPF, CA, bilirubin, TR, and phenol in 330 mL plasma during a 6-h treatment with hemodialysis (FX Paed) or hemoperfusion with 30 mL cartridges of Jafron HA, Biosky MG, or Cytosorb (n = 3). **a** Uremic retention solutes. **b** Toxins of the liver failure.

The molar ratio between the added toxins and albumin was 5.2, indicating 5.2 toxin molecules per albumin molecule, which led to competition between toxins for binding sites on albumin. CMPF, CA, and phenol bind to the high-affinity binding site Sudlow Site I and HA, IS, bilirubin, and TR bind to the high-affinity binding site Sudlow Site II. HCY, which binds to Cys34, had no competitor in this toxin mixture and, nevertheless, showed a difference in protein binding when other metabolites that bind to albumin were present. A possible explanation for this observation is that HCY binds not only to Cys-34 but also to other binding sites on the albumin molecule, or the presence of the other uremic retention solutes and liver toxins leads to a mild oxidation of albumin, which would mean that HNA-1 is formed. The Cys-34-binding site is blocked. In the presence of bilirubin, which primarily binds to HMA, the binding of HCY is diminished [44]. Since CA does bind not only to albumin but also to lipoproteins [45], sufficient binding sites for CA were always available, and therefore, no competition for binding sites could be observed. In patients with ESRD, the binding of toxins to plasma proteins is reduced due to hypoalbuminemia, accumulation of metabolic end products competing for the binding sites, or a conformational change in the albumin molecule [46]. In these patients, the increased free fraction of the retention solutes can be removed by hemodialysis. However, for the strongly protein-bound retention solutes, a combination of hemodialysis with hemoperfusion might be advantageous for their removal. A clinical study already showed that the treatment with a combined membrane-adsorption system based on hydrophobic and cationic adsorbents had a higher removal rate for protein-bound uremic toxins than a conventional high-flux hemodialysis [47].

Studies where high salt concentrations or ibuprofen were added to the patients' blood to raise the free fraction of protein-bound retention solutes showed promising results, but further aspects such as overdosage and blood cell damage must be considered to ensure a safe treatment [18, 48]. The results (Table 2) show that in the presence of multiple protein-bound toxic metabolites, they compete with each other for binding sites, and therefore, there is a shift in the ratios of bound to free form. It is difficult to predict how severe this shift is because some protein-binding substances have more than one binding site. There may also be some oxidation of the albumin molecule when it has certain binding partners. When removing protein-bound substances, it is important to consider that specific substances exhibit a remarkably high binding affinity to plasma proteins. As a result, these substances can only be effectively eliminated by simultaneously removing the bound protein.

Since aromatic amino acids are present in elevated concentrations in the blood of patients with liver failure, TR was selected in this study as a representative example of aromatic amino acid. TR is not strongly bound to albumin so that conventional dialysis treatment can remove it effectively. The Biosky adsorber, in particular, shows relatively good removal of TR. However, whether this also leads to a positive effect for the patient in the clinic when combined with dialysis would have to be tested in further in vivo studies. The other toxins of liver failure, bilirubin and CA, can also be removed efficiently by Cytosorb.

For the adsorption of HCY and bilirubin, Cytosorb was the most effective adsorber and clearly better than the adsorbers Jafron HA and Biosky MG. For the adsorption of IS and CA, there was no significant difference between the three adsorbers tested. For the adsorption of HA, TR, and phenol, the adsorbers Jafron HA and Biosky MG were significantly better than Cytosorb.

Dialysis removes HA, HCY, and IS better than the adsorbers and the present study provides no rationale for using adsorbers for the removal of these metabolites. CMPF, which has an exceptionally high affinity for albumin, was the only toxin that could not be eliminated by any treatment. HCY with a protein-binding ratio of 70–80% in plasma was not efficiently removed by any treatment because it is known that 20–30% of the HCY molecules form dimers with itself, cysteine, or glutathione [49], so only 1% of HCY is free and can be dialyzed or adsorbed.

Although Cytosorb was initially developed as an adsorber for cytokines, it has recently been increasingly used in extracorporeal liver support. The results of this study confirm that the use of Cytosorb in liver failure is justified. Nevertheless, no data on the removal of protein-bound uremic retention solutes are available to date. Jafron HA showed in two clinical studies an improvement in uremic symptoms and pruritus score as well as a decrease in parathyroid hormone and calcium phosphate [26]. There is only 1 published case study on using Biosky MG in a patient with acute respiratory distress syndrome [27]. Unfortunately, the data available on the clinical benefit of adsorber-based methods are very limited.

Although the use of adsorbers in addition to conventional dialysis treatment seems promising for some substances to be removed, extensive clinical studies are

required to make a clear statement about their benefit. Furthermore, the results of the present in vitro study do not allow a universal recommendation for a specific adsorber. Since the spectrum of protein-bound toxins varies from patient to patient, the decision must be made for each patient in the interest of individualized medicine.

Statement of Ethics

The Ethics Commission of the University for Continuing Education Krems provided a positive votum for the blood donation of healthy volunteers (EK GZ 13/2015-2018) for research before. The included healthy volunteers signed an informed consent before blood donation.

Conflict of Interest Statement

The authors declare that there is no conflict of interest.

Funding Sources

This work was funded by the government of Lower Austria and the IWB/EFRE program, www.efre.gv.at (project ID WST3-F-5030664/007-2018 and WST3-F-5030664/021-2020).

Author Contributions

Conceived and designed the experiments: C.S., J.H., and S.H. Performed the experiments: C.S. and S.H. Measurements with cobas c311, establishment of HPLC methods and measurements, blood donation, and statistical analysis: C.S. Wrote the paper: C.S., S.H., and J.H. Responsible for funding: J.H. All authors participated in the discussion of the data and preparation of the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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