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Differential hemoglobin A sequestration between hemodialysis modalities

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Abstract: This report evaluates plasma protein patterns, dialysates and protein analysis of used dialysis membranes from the same patient under hemodialysis in three separate modalities, using high-flux membranes in concentration-driven transport (HD), convection-driven hemofiltration (HF) and combined hemodialfiltration (HDF). The plasma protein changes induced by each of the three dialysis modalities showed small differences in proteins identified towards our previous plasma analyses of chronic kidney disease (CKD) patients. The used dialysate peptide concentrations likewise exhibited small differences among the modalities and varied in the same relative order as the plasma changes, with protein losses in the order HD > HDF > HF. The membrane protein deposits allowed quantification of the relative Hb removal ratios as ~1.7 for HD and ~1.2 for HDF vs. ~1.0 for HF. Hence, plasma protein alterations, dialysate peptide contents and membrane Hb deposits all identify HD as the modality with the most extensive filtration results and exemplifies the accessibility of protein analysis of used membrane filters for evaluation of dialysis efficiencies.

Keywords: hemodialysis modalities; hemoglobin; plasma; protein.

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Introduction

Extracorporeal blood purification is the most common form of treatment for end-stage renal disease. However, types of dialysis membrane and dialysis modality differ regarding removal of small and middle molecules, water and in its effects on plasma protein patterns. The HEMO trial, comparing high- and low-dose hemodialysis, showed that increased small molecule removal does not appear to affect outcomes (1). Instead, interest has focused on the use of high-flux filters to remove middle molecules associated with disease in uremia (2), perhaps by removing toxins bound to these molecules (3). Furthermore, introduction of new pumps has meant that different modalities utilizing concentration-driven osmosis (HD), convection-driven hemofiltration (HF) or both combined in on-line hemodialfiltration (HDF) may be used with various filters. As the healthy kidney is an important organ for the metabolism of insulin and other signaling molecules (4), and as uremia is associated with elevated levels of cytokines and adipokines (5), the effect of dialysis on proteins which are normally filtered is of interest, as are the effects of membranes on material removed (6).

In the present study, we show that proteomic analysis of the dialysis-altered plasma, the spent dialysates and the used dialyzer membranes from the same patient treated sequentially with high-flux membranes in HF, HD or HDF shows consistent differences between the modalities and that quantification of the hemoglobin removal can easily evaluate the dialysis differences.

Materials and methods

Dialysate collection

One male hemodialysis patient from the Karolinska University Hospital, aged 32 years and treated with hemodialysis for more than 3 months, three times per week for 3.5 h each time with a commercial dialyser (Gambro Polyflux 210H, Lund, Sweden), was monitored as per approval by the local Ethical Committee (permit number 2006:80-31/4). The dialysis results were evaluated after 3.5 h therapy with (a) HF, (b) HD or (c) HDF, each time using the same filter and Gambro AK200-Ultra S machine (blood flow 300 ml/min, dialysate

flow 500 ml/min, post-dilution substitution set to auto, with a minimum of 6 l exchanged). In each case, therapy was started with 10 min of isolated ultrafiltration to obtain 50 ml of ultrafiltrate for analysis. Venous blood sampling (4 ml into a citrate plasma tube) was performed before the start of therapy and immediately after, concurrent with the handling of the dialysis connections.

Extraction of proteins from filters

Following dialysis, the spent filters were immediately frozen to –20°C. Peptide extraction from the filters was then performed by 40% acetic acid infusion into the emptied filter through the arterial port and collection at the dialysate inlet. A standard dialyzer pump was used (AK95-S, Gambro AB, Lund, Sweden) to infuse the filter at a rate of 50 ml/min, first filling the filter and dispensable tubing and then running the system for 3 min at the same speed. Finally, the system was drained and the fluid (ca 350 ml) recovered.

Immunoaffinity depletion of high-abundant proteins from the plasma

The plasma samples were immunodepleted for albumin, IgG, antitrypsin, IgA, transferrin and haptoglobin on a multiple affinity removal system (MARS) column (Agilent Technologies, Santa Clara, CA, USA), as described (7, 8) by application of 80 µl plasma after dilution in a neutral buffer, pH 7.4 and elution at 0.5 ml/min. The flow through fraction was pooled from 12 runs, buffer-exchanged in a spin concentrator with 10 kDa cutoff (Amicon Ultra, Millipore, Billerica, MA, USA) to 50 mM Tris-HCl, pH 7.4 and then stored at –80°C until analysis. Protein concentrations were measured with a protein assay kit (Novagen, San Diego, CA, USA).

HPLC separation of plasma samples and matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF) mass spectrometry of protein bands

Depleted plasma (300 µg) in 600 µl was loaded onto a C18 column, 4.6×50 mm (Agilent Technologies, Santa Clara, CA, USA) after addition of 0.22 g urea (to a final concentration of 6 M) and 6 µl acetic acid (to 1% final concentration). Proteins were separated at a flow rate of 0.75 ml/min at 80°C with a linear, multi-segmented gradient of eluent A [0.1% trifluoroacetic acid (TFA) in water] and B (0.08% TFA in acetonitrile). Separations monitored at 280 nm gave 58 fractions, which were pooled with identical fractions from additional runs, dried with a centrifugal vacuum concentrator and redissolved in 100 µl water/0.1% TFA.

To the pooled HPLC fractions 7 µl of sodium dodecyl sulfate (SDS) 4× NuPAGE sample buffer (Invitrogen Life Technologies Carlsbad, CA, USA) was added, 15 µl was then applied on 4–12% NUPAGE-gels (Novex Bis Tris 1.0 mm×12 wells, Invitrogen Life Technologies), separated in MES SDS buffer at 200 V for 45 min and stained with Coomassie R-250 over night. Bands were excised manually and digested with trypsin in a Mass PREP robotic protein-handling system (Waters, Milford, CT, USA) after destaining, DTT reduction and iodoacetamide alkylation (7, 8). After extraction and concentration, the material was applied to a MALDI target plate in a 1:1 ratio (v/v) with α-cyano 4-hydroxycinnamic acid (saturated in 60% acetonitrile/0.1% TFA). Mass spectra were obtained with a Voyager DE-PRO MALDI-TOF instrument (Applied Biosystems, Foster City, CA, USA), operated in a positive ion mode. Protein identification used MS-Fit (http://prospector.ucsf.edu) and the SwissProt database, with standard search parameters for monoisotopic peptides, including oxidized methionine and carbamidomethylated cysteine residues, a maximum of one missed cleavage, molecular weight range 1000–300 000 Da and a mass tolerance of 0.1 Da.

HPLC of dialysates

Fifty milliliters of dialysate were desalted and concentrated by OASIS cartridges (Waters) pre-activated with acetonitrile and equilibrated in 0.1% aqueous TFA. Proteins were eluted with 100% acetonitrile/0.1%TFA (1 ml), stored at –80°C for 2 h and dried in a speed vacuum. Protein/peptides (3 mg) were dissolved in 1 ml water/TFA 0.1%, centrifuged for 30 s, loaded onto a Vydac C18, 5 µm, 4.6×250 mm column and fractionated in an ÄKTA system (Amersham Pharmacia Biotech, Uppsala, Sweden), using a gradient of acetonitrile from 0% for 30 min, 0 to 60% for 45 min, 60 to 80% for 10 min and 80 to 100% for 10 min. The effluent was monitored at 214 and 280 nm, and fractions were concentrated under nitrogen to 10 µl volume.

Amino acid analysis of proteins extracted from used dialysis filters

Two-hundred microliter samples of the proteins/peptides from each modality (HF, HD, HDF) were dried, hydrolyzed in 100 µl 6 M HCl/0.5% phenol at 110°C for 24 h in evacuated tubes and analyzed for amino acid compositions with a Biochrom 20 plus instrument (Amersham Pharmacia). Ten microliter aliquots from each modality were also submitted to N-terminal analysis in an Applied Biosystem (Foster City, CA, USA) Prosice cLC 494 sequencer.

Results

Plasma analyses before and after each of three dialysis modalities

Plasma samples from before and after each of the three types of dialysis were collected and compared by high performance liquid chromatography (HPLC) analysis (Figure 1). While there were no discernable differences in peak distribution, we saw higher protein levels following HD and to some extent also after HDF, but only small differences after HF. This overall pattern mirrors the expected efficiency in water removal by the different dialysis modalities in the order HD > HDF > HF.

Plasma from before and after treatment was also compared regarding band patterns upon polyacrylamide gel

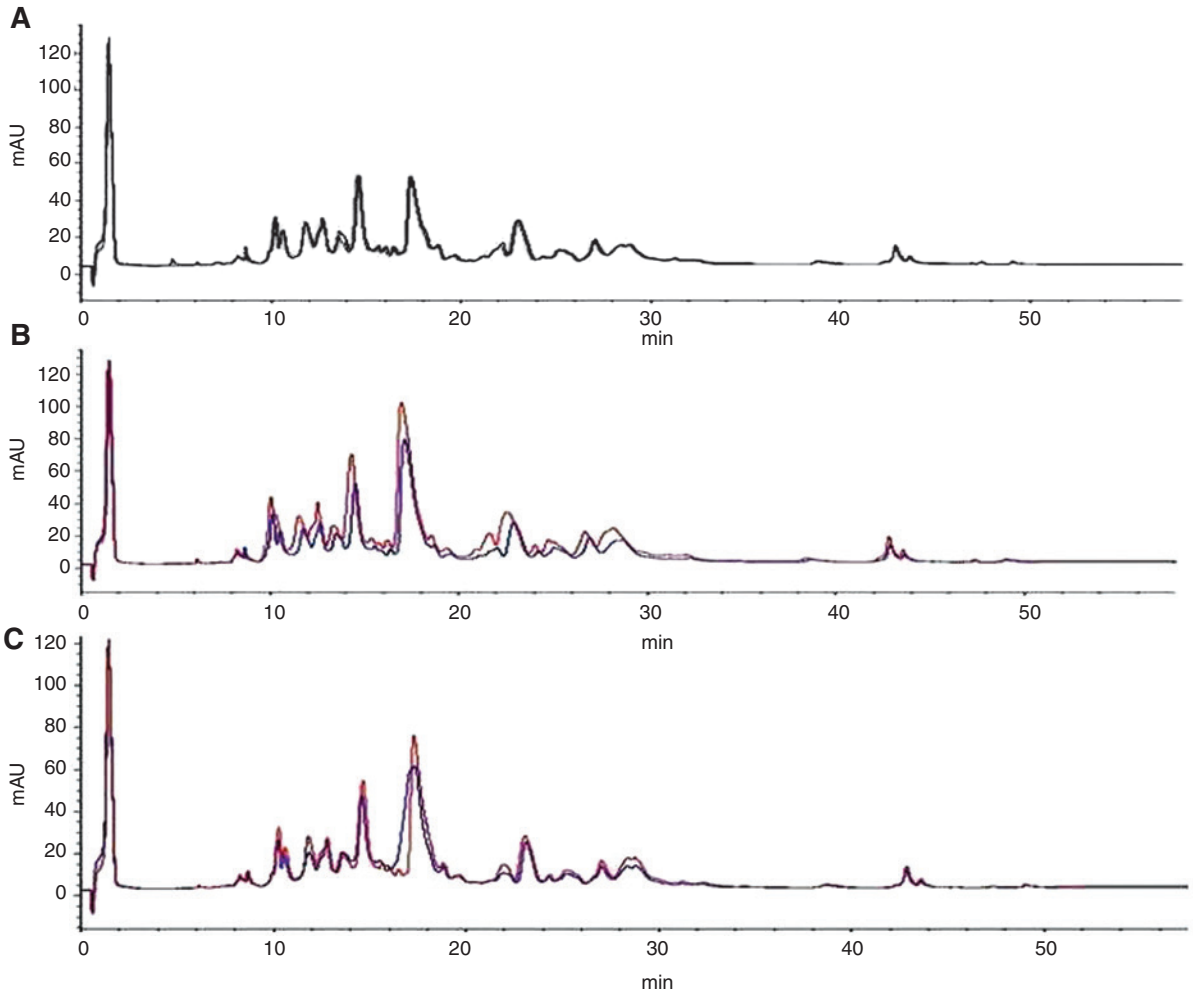

Figure 1: HPLC separation curves from patient plasma before (blue) and after (red) dialysis treatment in three modalities, by hemofiltration (HF) in (A), hemodialysis (HD) in (B) and hemodialfiltration (HDF) in (C). As shown, effects of the separate dialyses differ.

electrophoresis (PAGE) of each of the fractions collected from the HPLC run. A zoom-in on the patterns from fractions 10 to 24, covering the region of the major deviations between the blue and the red curves in Figure 1, is shown in the online supplement (Supplementary Figure S1) for each of the dialysis modalities. Small differences were noticed in individual bands before and after treatment, and those protein bands were cut out, extracted and analyzed by MALDI mass spectrometry for protein identification. The results cover 50 protein spots identified. However, the majority of these identifications (40 proteins) were the same as proteins detected in our previous correlation of protein differences with chronic kidney disease (CKD) (7, 8) and are not reported again. The same correlation may apply to the 10 additional proteins now identified, but as some may also reflect modality correlations, all novel identifications are given in the Supplement (Supplementary Table S1), although difficult to interpret because of protein multiplicity in some fractions.

Dialysate analyses

Analyses by HPLC of the dialysates in each of the three treatment modalities (Supplementary Figure S2) gave peptide contents that also appeared to differ in the relative order HD > HDF > HF. Thus, the efficiency in peptide removal by the three modalities appeared to parallel the order deduced from the plasma patterns above.

Analysis of protein deposits in the used membranes from the three modalities

We evaluated the amount of protein deposited on the membrane in each of the three modalities by: (i) quantitative analysis of total amino acid contents after hydrolysis of the extracted material from the used membranes after each treatment modality; and (ii) N-terminal sequence degradations of the deposited material in each case. In

Table 1: Amino acid compositions (A) and N-terminal sequences (B) of the extracted material from the used membranes in each of the three modalities (HF, HD, HDF).

Residue	Sample			Composition
	HF (mol%)	HD (mol%)	HDF (mol%)	HbA_1 (except G, P, C, W) (mol%)
(A)				
Asx	10.2	9.6	9.8	10.1
Thr	7.2	7.4	7.1	6.5
Ser	8.6	8.2	8.3	6.5
Glx	12.0	10.9	11.5	6.5
Ala	12.9	13.6	13.5	14.6
Val	6.8	6.9	6.8	12.6
Met	1.8	2.2	2.3	1.2
Ile	1.5	1.1	1.6	0.0
Leu	10.2	8.8	9.5	14.6
Tyr	4.4	4.6	4.2	2.4
Phe	6.5	7.8	7.3	6.1
His	5.2	6.5	5.6	7.7
Lys	8.3	7.6	7.9	8.9
Arg	4.5	4.7	4.5	2.4
Sum (nmol)	138.8	228.8	165.6	Sum for 247 residues
(ratios)	1.00	1.65	1.19	(except G, P, C, W)
(B) Amino acid sequence (for 11 cycles):				
1 2 3 4 5 6 7 8 9 10 11				
ValLeuSerProAlaAspLysThrAsnValLys				
ValHisLeuThrProGluGluLysSerAlaVal				

In part B, the two sequences could be read for 11 cycles, with Val at the start position in both cases as deduced from twice the amount of Val in cycle 1 vs. the amounts of residues in cycle 2, and with identical sequences in all three modalities, but greatest purity in HD.

all three modalities, both the α- and β-chain amino acid sequences of hemoglobin were detectable upon monitoring the degradations, corresponding to the two sequences in Table 1B. The amounts were greatest in HD, and again in the order HD > HDL > HF. The total protein compositions (Table 1A) reflected the same pattern and fitted reasonably well the composition of HbA_1 (right-most column, Table 1A), establishing hemoglobin as by far the major component in all three membrane deposits. Only a few amino acids showed deviations between their sums in the deposits and hemoglobin, presumably because they are metabolites present in blood (Z, P, G), or are influenced by slow release (V) or unstability during hydrolysis (C, W). Quantification of the deposited hemoglobin removed was in the ratio ~1.7 for HD and ~1.2 for HDF vs. ~1.0 for HF. Thus, the hemoglobin depository removal (Table 1), the water removal and the dialysate peptide content (Supplementary Figure S2) all appear to follow similar ratios. These data indicate that transmembrane pressure rather than transmembrane flow is the main determinant of protein loss [cf. (9)].

Discussion

This analysis constitutes a screening of the relative effect on circulating proteins of three dialysis modalities in a single patient. Results were evaluated by plasma protein patterns before and after dialysis treatment, dialysate peptide occurrence afterwards and quantitative analysis of the protein deposited on the used membranes. In each compartment, results were similar, suggesting a relative order of efficiency in HD greater than in HDF and in turn greater than HF for the removal of proteins. The dialysate results are limited by the small amounts recovered, and the plasma analyses by the multiplicity of protein differences in CKD [Supplementary Table S1 and (7, 8)], while the membrane deposition analyses are clear and quantitative, suggesting an efficiency ratio of ~1.7 for HD and ~1.2 for HDF vs. ~1.0 for HF (Table 1A). The results establish protein analysis to be a representative indicator of dialysis efficiencies and quantification of the membrane-deposited hemoglobin to be an easily accessible evaluation method.

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Supplemental Material: The online version of this article (DOI: 10.1515/bmc-2017-0006) offers supplementary material, available to authorized users.