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Elimination of Uremic Toxins during Dialysis assessed with the Optical and Analytical Methods

KAI LAURI

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TALLINN UNIVERSITY OF TECHNOLOGY
School of Science
School of Information Technologies
Department of Health Technologies

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Supervisor: Prof. Ivo Fridolin, PhD
School of Information Technologies
Tallinn University of Technology, Tallinn, Estonia

Co-supervisor: Jana Holmar, PhD
School of Information Technologies
Tallinn University of Technology, Tallinn, Estonia
RWTH Aachen University Hospital
Institute for Molecular Cardiovascular Research
Aachen, Germany

Reviewed by: Professor Emeritus Hiie Hinrikus, DSc
Department of Health Technologies
Tallinn University of Technology, Tallinn, Estonia

Opponents: Dr. Prof. David Goldsmith
Guy's and St Thomas' Hospitals, London, UK
Molecular and Clinical Sciences Research Institute
St George's University, London, UK

Prof. Per Magnusson
Division of Clinical Chemistry (KK)
Department of Biomedical and Clinical Sciences (BKV)
Linköping University, Sweden

Defence of the thesis: June 15, 2020, Tallinn, Estonia

Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology, has not been previously submitted for a doctoral or equivalent academic degree.

Kai Lauri

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Ureemiliste toksiinide elimineerimise hindamine dialüüsravil optiliste ja analüütiliste meetoditega

KAI LAURI

TAL TECH
KIRJASTUS

Contents

List of Publications

The list of author's publications, based on which the thesis has been prepared:

I **Lauri, K.,** Tanner, R., Jerotskaja, J., Luman, M., & Fridolin, I. (2010). HPLC study of uremic fluids related to optical dialysis adequacy monitoring. *Int J Artif Organs*, *33*(2): 96–104 (DOI: 10.1371/journal.pone.0162346)

II **Lauri, K.**, Luman, M., Holmar, J., Tomson, R., Kalle, S., Arund, J., Uhlin, F., Fridolin, I. (2015) Can Removal of Middle Molecular Uremic Retention Solutes be Estimated by UV-absorbance Measurements in Spent Dialysate? *In: IFMBE Proceedings of World Congress on Medical Physics and Biomedical Engineering*, Toronto, Canada, 7–12. June. 2015. IUPESM WC2015 Oral presentation

III **Lauri, K.**, Arund, J., Holmar, J., Tanner, R., Kalle, S., Luman, M., Fridolin, I. (2019) Removal of Urea, β2-Microglobulin, and Indoxyl Sulfate Assessed by Absorbance and Fluorescence in the Spent Dialysate During Hemodialysis. *ASAIO Journal,* August 16, 2019 - Volume Online First - Issue (DOI: 10.1097/MAT.0000000000001058)

Author's Contribution to the Publications

Contribution to the papers listed in this thesis include the following:

I The author participated in the planning of the experiments and checked the handling of the samples to ensure the reliability and unity of study results. Additionally, the author prepared the serum samples before HPLC analysis (purification), performed data processing and statistical analysis, and contributed to the writing of the paper.

II The primary role of the author was analysing the concentrations of uremic toxins in the clinical laboratory using standardised chemistry methods, data processing and statistical analysis, and contributed to the writing of the paper.

III The author was analysing the concentrations of uremic toxins in the clinical laboratory using standardised laboratory methods, processed the data and performed the statistical analysis, and contributed to the writing of the paper.

Other related publications

1. **Lauri, K.**, Tanner, R., Luman, M., Jerotskaja, J., & Fridolin, I. (2006). Optical dialysis adequacy sensor: contribution of chromophores to the ultra violet absorbance in the spent dialysate. *Conference Proceedings : International Conference of the IEEE Engineering in Medicine and Biology Society.* IEEE, New York City, USA, 2008, pp. 807–810. (https://doi.org/10.1109/IEMBS.2006.259803)

2. Jerotskaja, J., **Lauri, K.**, Tanner, R., Luman, M., Fridolin, I. (2007). Optical dialysis adequacy sensor: wavelength dependence of the ultra violet absorbance in the spent dialysate to the removed solutes. *29th Annual International Conference of the IEEE EMBS Cité Internationale;* Lyon, France; August 23–26, 2007.

3. Fridolin, I., Jerotskaja, J., **Lauri, K.**, Scherbakov, A., Luman, M. (2007). Accurate On-Line Estimation of Delivered Dialysis Dose by Dialysis Adequacy Monitor (DIAMON)". *11-th Mediterranean Conference of Medical and Biological Engineering and Computing (MEDICON 2007);* Ljubljana, Slovenia; June 26–30, 2007.

4. **Lauri, K.**, Tanner, R., Arund, J., Fridolin, I. (2009). HPLC study of uremic toxins in dialysate. *The 5th Conference by Nordic Separation Science Society;* Tallinn, Estonia; September 26–29, 2009.

5. Jerotskaja, J., Fridolin, I., **Lauri, K.**, Luman, M. (2009). An enhanced optical method for measuring concentration of uric acid removed during dialysis. *11th International Congress of the Medical Physics and Biomedical Engineering;* Munich, Germany; September 7–12, 2009.

6. Luman, M, Jerotskaja, J., **Lauri, K.**, Fridolin, I. (2009). A multicentre study of an enhanced optical method for measuring concentration of uric acid removed during dialysis". *31st Annual Internaational Conference of the IEEE Engineering in Medicine and Biology Society;* Minneapolis, USA; September 2–6,2009.

7. Arund, J., Tanner, R., **Lauri, K.**, Luman, M., Fridolin, I. (2010). Relative importance of uremic compounds in total UV absorbance of spent dialysate. *10th Baltic Nephrology Conference;* Jurmala, Latvia; October 14–16, 2010.

8. Fridolin, I., Jerotskaja, J., **Lauri, K.**, Uhlin, F., Luman, M. (2010). Lean Body Mass (LBM) Estimation by UV-Absorbance Measurements in the Spent Dialysate. *ERA/EDTA XLVII and II DGfN Congress 2010;* Munich, Germany; June 25–28, 2010.

9. Jerotskaja, J., Uhlin, F., Luman, M., **Lauri, K.**, Tanner, R., Fridolin, I. (2010). Determination of concentration of uric acid removed during dialysis by ultraviolet absorbance. A multi wavelength and processed spetra approach. *10th Baltic Nephrology Conference;* Jurmala, Latvia; October 14-16, 2010.

10. Arund, J., Tanner, R., **Lauri, K.**, Luman, M., Uhlin, F., Fridolin, I. (2010). Contribution of uremic compounds to the total UV absorbance in respect to optical monitoring of dialysis quality. *World Conference on portable-wearable and miniaturized systems for dialysis and ultrafiltration;* Vicenza, Italy; September 30-October 2, 2010.

11. Jerotskaja, J., Uhlin, F., Luman, M., **Lauri, K.**, Fridolin, I. (2010). Improved optical method for measuring concentration of uric acid removed during dialysis. *The 12th Mediterranean Conference on Medical and Biological Engineering and Computing (MEDICON2010),* Chalkidiki, Greece; May 27-30, 2010.

12. Jerotskaja, J., Uhlin, F.; Fridolin, I.; **Lauri, K**.; Luman, M.; Fernström, A. (2010). Optical online monitoring of uric acid removal during dialysis. Blood Purification, 29 (1), 69−74. (10.1159/000264269)

13. Fridolin, I., Jerotskaja, J., **Lauri, K.,** Uhlin, F., & Luman, M. (2010). A New Optical Method for Measuring Creatinine Concentration During Dialysis. *BT - XII Mediterranean Conference on Medical and Biological Engineering and Computing 2010*: May 27 – 30, 2010 Chalkidiki, Greece. In P. D. Bamidis & N. Pallikarakis (Eds.) (pp. 379–382). Berlin, Heidelberg: Springer Berlin Heidelberg. (https://doi.org/10.1007/978-3-642-13039-7_95)

14. Jerotskaja, J., Uhlin, F., **Lauri, K.**, Tanner, R., Luman, M., & Fridolin, I. (2010). Concentration of uric acid removed during dialysis. Estimated by multi wavelength and processed ultra violet absorbance spectra. *Conference Proceedings: 32nd Annual International Conference of the IEEE Engineering in Medicine and Biology Society.* Buenos Aires, Argentina; August 31 – September 4, 2010, 5791–5794. (https://doi.org/10.1109/IEMBS.2010.5627831)

15. **Lauri, K.,** Arund, J., Tanner, R., Jerotskaja, J., Luman, M., Fridolin, I. (2010). Behaviour of uremic toxins and UV absorbance in respect to low and high flux dialyzers. *Estonian Journal of Engineering*, 16(1), 95–106.

16. Tomson R, Fridolin I, Uhlin F, Jerotskaja J, **Lauri K**, Luman M. (2010). Development of the Model for the Optical Multiwavelength Monitoring of Creatinine in the Spent Dialysate. *12th Biennial Baltic Electronics Conference;* Tallinn, Estonia; October 4– 6, 2010.

17. **Lauri, K.,** Arund, J., Holmar, J., Tanner, R., Luman, M., Fridolin, I. (2011). Optical Dialysis Adequacy Monitoring: Small Uremic Toxins and Contribution to UV-Absorbance Studied by HPLC. In: Angelo Carpi, Carlo Donadio and Gianfranco Tramonti (Ed.). Progress in Hemodialysis - From Emergent Biotechnology to Clinical Practice. *InTech - Open Access Publisher* (143−160).

18. Tomson, R., Uhlin, F., Holmar, J., **Lauri, K**., Luman, M., Fridolin, I. (2011). Development of a method for optical monitoring of creatinine in the spent dialysate. *Estonian Journal of Engineering*, *17*(2), 140–150. (https://doi.org/10.3176/eng.2011.2.04)

19. Holmar, J., Fridolin, I., Uhlin, F., **Lauri, K**., Luman, M. (2012). Optical Method for Cardiovascular Risk Marker Uric Acid Removal Assessment during Dialysis. *The Scientific World Journal*, 2012, 8. https://doi.org/10.1100/2012/506486

20. Tomson R, Fridolin I, Uhlin F, Holmar J, **Lauri K**, Luman M. (2012). Development of the Model for the Optical Monitoring of Urea in Spent Dialysate. *13th Biennial Baltic Electronics Conference; Tallinn,* Estonia; October 3–5, 2012.

21. Holmar, J., Uhlin, F., Ferenets, R., **Lauri, K**., Tanner, R., Arund, J., Luman, M., Fridolin, I. (2013). Estimation of removed uremic toxin indoxyl sulphate during hemodialysis by using optical data of the spent dialysate. *Conference Proceedings : 35th Annual International Conference of the IEEE Engineering in Medicine and Biology Society. Osaka, Japan; July 3–7, 2013*, 6707–6710. (https://doi.org/10.1109/EMBC.2013.6611095)

22. Tomson, R., Fridolin, I., Uhlin, F., Holmar, J., **Lauri, K.**, & Luman, M. (2013). Optical measurement of creatinine in spent dialysate. *Clinical Nephrology*, *79*(2), 107–117. (https://doi.org/10.5414/CN107338)

23. Holmar, J., Luman, M., **Lauri, K.**, Fridolin, I. (2016). Total Removed Beta 2-microglobulin and Urea during Different Dialysis Treatment Modalities. *XIII Baltic Nephrology Conference, Jurmala, Latvia, Oct. 13–15, 2016.* Latvian Association of Nephrology, 36−37.

24. Holmar, J., Luman, M., Arund, J., **Lauri, K**., Tomson, R., Tanner, R., Kalle, S., Fridolin, I. (2016). Reduction of Urea and Indoxyl Sulphate Concentration during Different Dialysis Treatment Modalities. *International Journal of Artificial Organs: XLIII Annual Congress of the European Society for Artificial Organs*, Warsaw, Poland, September 14–17, 2016. Wichtig Editore, 379.10.5301/ijao.5000508.

25. Holmar, J., Arund, J., Kalle, S., **Lauri, K.**, Luman, M., Tanner, R., Tomson, R., Fridolin, I. (2017). Removal Estimation of Uremic CVD Marker Phosphate in Dialysis Using Spectrophoto- and Fluorimetrical Signals. In H. Eskola, O. Väisänen, J. Viik, & J. Hyttinen (Eds.), *EMBEC & NBC 2017* (pp. 358–361). Singapore: Springer Singapore.

Introduction

Various methods for the dialysis treatment quality estimation are used routinely – such as concentration measurements of serum urea, creatinine, uric acid and Kt/V. Dialysis treatment is continuously improved, and it is equally important to improve the treatment quality monitoring. The quality estimation should be simple, broad, and part of everyday routine.

A long list of solutes accumulating in patients with uremic syndrome, so-called uremic toxins, has been identified including water-soluble small molecules (SM), middle molecules (MM) and protein-bound (PB) molecules (Vanholder et al., 2003). The well-known small water-soluble uremic compounds are urea, creatinine and uric acid. As small water-soluble compounds are removed easily from blood during a single hemodialysis session (Fagugli et.al., 2002), the protein-bound solutes like indoxyl sulphate are more poorly eliminated. Most known MM beta-2-microglobulin (B2M) is due to its kinetic behaviour an ideal marker for studying the middle molecules' characteristics (EBPG, 2002). Beta-2-microglobulin is a solid and independent indicator of the outcome of hemodialysis patients, and an excellent marker molecule, and should be monitored regularly in dialysis patients (Canaud et al., 2006). Studies about the removal of middle molecules have been published for many years and it is proven that high-flux dialysis membranes have a beneficial effect on removing middle molecules (Kim et al., 2014). Increase in prescription of high-flux hemodialyzers is supported by the EBPG for maximizing removal of middle-sized uremic toxins.

The general aim of the thesis was to assess the removal of different marker molecules with different characteristics and size by different dialysis modalities using the standardized laboratory methods and optical methods. Also, to evaluate the accuracy of the laboratory and optical methods and how middle and protein-bound molecules behave at various modalities compared to low molecular weight solutes. The final aim was to obtain important information for the development of an optical method and to confirm its reliability in comparison with classical laboratory methods. We hypothesised that the optical method is as reliable as laboratory analysis and we can confirm it for tested substances.

The dialysis was studied to see which molecules are eliminated during its course and reduction ratios (RR) [%] of different toxins were estimated. Additionally, the various treatment modalities were studied and compared for estimating removal patterns of the uremic solutes in case of different types of dialysis treatments. Furthermore, the accuracy of the clinical parameters obtained with routine laboratory methods and accuracy of optical methods were compared.

Publication I investigates the HPLC UV-absorbance profiles of the serum and spent dialysate and studies the removal of uremic retention solutes in connection with the on-line UV-absorbance measurements. The main solute responsible for UV absorbance (at 280 nm wavelength) is a small water-soluble low molecular weight, non-protein-bound compound uric acid (UA). Also, RR was estimated and the lowest elimination was identified in the case of indoxyl sulphate (IS) as a protein-bound solute.

Publications II and **III** are focused on the comparison of RRs of different types of molecules during various treatment modalities. The different dialysis modalities, conventional hemodialysis (HD), high flux hemodialysis (HF), and postdilutional online hemodiafiltration (HDF) with different parameter settings were used during the studies. In the studies the accuracies of the analytical and optical methods were compared.

This thesis summarizes the author's work at the Centre for Biomedical Engineering, Department of Health Technologies of Tallinn University of Technology.

Abbreviations

Abs	absorbance
BIAS	bias (accuracy)
BUN	blood urea nitrogen
B2M	beta-2-microglobulin
CR	creatinine
CV	coefficient of variation
CKD	chronic kidney disease
Da	dalton, unit for molecular weight
EBPG	European Best Practice Guidelines
ESRD	end-stage renal disease
EUTox	European Uremic Toxin Work Group
GFR	glomerular filtration rate
HA	hippuric acid
HD	hemodialysis
HDF	hemodiafiltration
HF	high-flux hemodialysis
HPLC	high-performance liquid chromatography
IS	indoxyl sulphate
Kt/V	dialysis dose efficacy parameter
MM	middle molecule
MS	mass spectrometry
MW	molecular weight
NKF KDOQI	National Kidney Foundation guideline
P	phosphate
PB	protein-bound
R	correlation coefficient
RR	reduction ratio
SD	standard deviation
SE	standard error
SM	small molecule
TRS	total removed solute
UA	uric acid
UR	urea
URR	urea reduction ratio
UV	ultraviolet light
V	volume, mL

1. The kidney failure and dialysis therapy

A kidney is 10–12 cm long, bean-shaped organ. Normally humans have two kidneys, and they are located in the middle of the back of the body. Kidneys have many functions, whereas the primary function is to filter blood. About 200 litres of blood is filtered by kidneys every day (www.niddk.nih.gov). The filtered blood flows back into the body and waste gets turned into urine. Kidneys regulate the balance of electrolytes, acid-base status and fluid level in the blood. They will excrete the excess water or, in case of water deficit, can store water. Kidneys help control blood pressure and help to produce red blood cells and enzymes (National Kidney Foundation guideline - NKF KDOQI).

If kidneys are damaged and their function fails, an excessive amount of water and waste products accumulates in the blood and the body. If the damage to kidneys continues and worsens, chronic kidney disease (CKD) might develop. According to the NKF KDOQI (Daugirdas et al., 2015), CKD is divided into 5 stages which are estimated using a patient's glomerular filtration rate (GFR). The normal GFR in adults is approximately 120–130 mL/min/1.73 m^2 and it declines with age. In CKD stage 1 GFR is lower but remains > 90 mL/min. In CKD stages 2 and 3 renal functions are further decreased. Patients in CKD stages 4 and 5 usually require preparation for active treatment to survive. Stage 5 kidney failure is also called end-stage renal disease (ESRD). NKF KDOQI recommends that planning of dialysis begin when patients reach CKD stage 4 where GFR falls below 15 mL/min /1.73 m^2 body surface area or creatinine clearance reaches below 30 mL/min. By this time about 80–90% of kidney functions will have faded. Indications for starting dialysis in CKD are empiric and vary among doctors (Daugirdas et al., 2015). If kidneys' function is declined, they cannot remove enough waste and excess water from the blood. The treatment for the replacement of the kidneys' filtering function is called dialysis.

Two types of dialysis methods are known: hemodialysis and peritoneal dialysis. In hemodialysis, a dialysis machine and special filter – dialyzer – are used to clean (filter) the blood. A special dialysis fluid flows through the filter and blood flows through the filter in the opposite side. Inside the filter is a semipermeable membrane (Parikh et al., 2013).

Different modalities of hemodialysis treatment have been developed based on variations in solute removal techniques (Tattersall et al., 2007). In conventional hemodialysis, diffusive transport across the membrane is taking place due to the difference in concentration between the blood and the dialysis liquid for each particular solute. The rate of diffusion depends on the molecular size and resistance to flow. In hemofiltration, solute removal relies solely on convection. Pressure is used to intensify the flow of water across the semipermeable membrane. The sieving properties of a membrane, i.e. membranes permeability to solutes, are determined by the size of the membranes' pores. This defines the limit up to which solutes can be dragged across the membrane by the fluid flow. Hemofiltration provides high clearance of medium-sized and large molecules. To increase the efficiency, a higher ultrafiltration flow rate must be generated and this requires a higher blood flow rate and sometimes also a larger membrane surface area. The hemodiafiltration procedure combines the principles of hemodialysis and hemofiltration, ensuring an efficient process. That means hemodiafiltration utilizes a combination of diffusive and convective solute transport through a highly permeable dialysis membrane. Several reports suggest that those therapies potentially improve the outcomes for ESRD patients (Schiffl, 2019). Optimal

forms of hemodiafiltration provide urea clearance 10–15% higher than the corresponding diffusive mode. Compared with standard hemodialysis, HDF removes more middle molecular weight solutes. For example, beta-2-microglobulin (B2M) levels may be reduced up to 70% (Tattersall et al., 2013). The variation of dialysis time and frequency has little effect on the removal of small molecular weight solutes (e.g. urea), but it might improve the removal of middle molecules. Hemofiltration and hemodiafiltration are more effective blood purification therapies than hemodialysis (Ledebo et al., 2010), because these therapies require dialysis membranes which are highly permeable to solutes, as well as fluid, and in both cases, large volumes of ultrafiltration are the condition for convective transport. The schematic principles of different modalities are demonstrated in Figure 1.

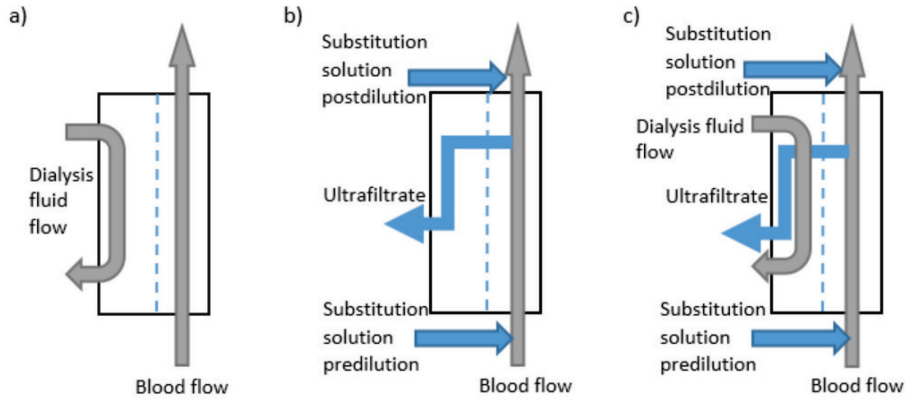

Figure 1. *Schematic principles of different hemodialysis modalities: a) conventional hemodialysis, b) hemofiltration, c) hemodiafiltration*

To support the renal function, dialysis should be delivered at least three times per week, minimum of 4 hours per procedure. The length of dialysis should be adjusted to the needs of each patient. Increasing the treatment session time can improve solute removal, especially of middle molecules such as beta 2-microglobulin, compared to the shorter treatment time. But the effect on increasing the session length on clearance of small water-soluble molecules (e.g. urea) is minimal (Tattersall et al., 2007).

2. Dialysis quality and adequacy

The prevalence of chronic kidney disease (CKD) (stages 1–4) is estimated to be 7.2% in adults over the age of 30 years (Joshi, 2014). Average life expectancy of dialysis patients is 5–10 years after starting the therapy. However, many patients have lived well on dialysis for 20 or more years (NKF-DOQI, 2006). The aim of renal replacement therapy is to prolong the survival of patients with renal diseases. High mortality and morbidity of ESRD patients is still a question despite the resources committed to the treatment. Ongoing improvements in dialysis therapy help improve ESRD patients' life quality. Toxic substances called uremic toxins may accumulate in the body even if the patient receives renal replacement therapy and therefore the assessment of dialysis efficiency is vitally important. Traditionally, dialysis adequacy is assessed by urea clearance quantified as urea reduction ratio (URR) or Kt/V blood. URR is based on blood urea tests. The levels before and after the treatment are measured and removal in percentage is estimated. For URR calculation the following formula is used:

$$URR\ (\%) = 100 \times (1-(\text{Post BUN}/\text{Pre BUN})) \quad (1)$$

where Post BUN is the blood urea nitrogen before the start of the dialysis session and Pre BUN is the blood urea nitrogen after the dialysis session.

The dialysis dose efficacy parameter Kt/V is mathematically related to the URR and can be derived from it (NKF-DOQI, 2006). In a simplified model of urea removal where urea generation is not considered, Kt/V is related to URR as follows:

$$Kt/V = -\ln (1 - URR) \quad (2)$$

where *K* is the dialyzer clearance of urea*, t* is the dialysis time or session length, and *V* is the volume of distribution of urea approximately equal to the patient's total body water. Measuring dialysis adequacy using urea clearance requires careful and precisely timed blood sampling. Because of these challenges, adequacy is usually measured once a month which may not be frequent enough to detect the factors that may reduce the effectiveness of dialysis treatment. It is demonstrated in previous studies that Kt/V still has limitations as a universal predictor of uremic solute elimination (Eloot et al., 2013). The choice of the valid dialysis quality parameter(s) leading to the more physiologically correct concentration of potential uremic toxins in the body fluids is still an unresolved issue (Vanholder et al., 2003).

3. The uremic solutes

In 2003, the European Uremic Toxin Work Group (EUTox) proposed a classification of 90 retention solutes (Vanholder et al., 2003). Over the last decade, this list has been significantly updated with toxins whose behaviour and effects on the human body have been studied (Neirynck et al., 2012; Duranton et al., 2012; Tanaka et al., 2015). Newly identified substances are added to the list on an ongoing basis, providing an increasingly complex picture of their potential toxicity (www.uremic-toxins.org).

The substances that have a damaging effect on biologic functions are called uremic toxins. Uremic toxins differ in their water solubility, protein-binding capacity, molecular weight, the pattern of removal by dialysis, biological properties, and potential to produce clinical symptoms. According to the molecular weight, protein-binding capacity, and the removal pattern by dialysis, three different groups of toxins are proposed by EUTox (Vanholder et al., 2003):

- Small water-soluble molecules (SM) with MW <500 Da, e.g. urea (UR) with MW = 60 Da, creatinine (CR) with MW = 113 Da, uric acid (UA) with MW = 168 Da;
- middle molecules (MM) with MW $\geq$500 Da, e.g. beta-2-microglobulin (B2M) with MW=11 818 Da;
- protein-bound molecules (PB), e.g. indoxyl sulphate (IS) with MW = 251 Da, hippuric acid (HA) with MW = 179 Da.

Several uremic solutes play a role in cardiovascular damage, a major cause of mortality in CKD. Over the past years, especially protein-bound compounds and middle molecules have been identified as some of the main toxins involved in vascular lesions affecting the endothelial cell, leukocyte, platelet, and vascular smooth muscle cell function in CKD (Glorieux et al., 2011). Many of these solutes are difficult to remove by standard dialysis strategies. The removal of protein-bound solutes remains limited because only the free fraction of the solute is available for, mostly diffusive, removal, while removal of the larger middle molecules can be obtained by increasing dialyzer pore size and by applying convective strategies (Glorieux et al., 2016).

3.1. Small free water-soluble solutes

This group consists of small solutes with MW <500 Da. Currently, 68 solutes are listed by EUTox as SM solutes (Vanholder et al., 2003). The most known solutes are creatinine (MW 113 Da), urea (MW 60 Da), and uric acid (MW 168 Da). Creatinine and urea are the most common markers for assessment of renal failure. Moreover, urea has been routinely used to assess the effectiveness of dialysis (Lisowska-Myjak, 2014). The reason is that urea is formed most rapidly of all the substances dissolved in the body and reaches the highest blood concentration. Also, urea production is linked closely to protein metabolism and the kinetic behaviour of urea is well-known (Clark et al., 2019).

The Urea itself is not toxic but the uremic syndrome is related to the retention of urea in the body of patients with renal failure. Accumulated urea has a toxic effect (Glorieux et al., 2015) Due to the water-soluble effect, these solutes are easily removed from the blood during dialysis, even by conventional hemodialysis (Dhondt et al., 2000).

3.2. Middle molecules

Middle molecules are defined as toxins with MW ≥500 Da (Vanholder et al., 2003). One of the best-known molecules in this group is beta-2-microglobulin (B2M), a non-glycosylated polypeptide with a molecular weight of 11 818 Da. The role of B2M in dialysis-related amyloidosis was defined in the 1980s. Later studies provided data about the association between B2M and early-onset atherosclerosis in hemodialysis patients without co-morbidities. The interest in the role of uremic toxins in uremic atherosclerosis and B2M as a marker of cardiovascular and mortality risk has grown (Zumrutdal et al., 2005).

B2M has been used as a marker for describing behaviour and removal of middle molecular weight solutes during dialysis for many years. European Best Practice Guidelines (EBPG) have pointed out that although an ideal marker for middle molecular uremic toxins has not been identified yet, B2M is representative in its kinetic behaviour of other middle molecules (MM) and peptides of similar size and may be used as a marker for such molecules (EBPG 2002).

3.3. Protein-bound solutes

The molecular weight of most members of this group is less than 500 Da (Liabeuf 2011; Vanholder et al., 2003). Well-known protein-bound solutes are indoxyl sulfate (IS), with a molecular weight of 251 Da, and hippuric acid (HA), with a molecular weight of 179 Da (Vanholder et al., 2003). Serum IS level is markedly higher in chronic kidney disease (CKD) patients than the corresponding concentrations in healthy subjects (Shunsuke et al., 2014). IS may induce oxidative stress, dysfunction of endothelium, vascular inflammation and calcification. This molecule has been associated with cardiovascular mortality in CKD patients (Shunsuke et al., 2014; Barreto et al., 2009). The biological effects of hippuric acid (HA) have been less explored, the most described effect is associated with renal tubular damage (Vanholder et al., 2018).

Protein-bound solutes are poorly eliminated by the commonly used dialysis techniques due to their protein-binding capacity. Several studies, conducted to improve the elimination of these components, have shown that the removal of bound solutes can be increased by raising the dialysate flow and dialyzer surface area (Sirich et al., 2012).

4. Methods for uremic solutes' analyses

4.1. Standardized clinical laboratory methods

In the clinical laboratory, the simple and fast biochemical analysis methods are in use for detecting small water-soluble uremic toxins like creatinine (CR), uric acid (UA), and urea (UR). The time of analysis is usually very short; it takes only a few minutes to receive the analysis result. They are direct or indirect methods where the main method principle is enzymatic colorimetric assay. The end-product of the chemical reaction is commonly measured photometrically.

For the quantitative detection of creatinine, the kinetic colorimetric assay is used based on the Jaffe reaction. In alkaline solution, creatinine reacts with alkaline picrate forming an orange-coloured complex. The intensity of the colour formed is proportional to the creatinine concentration in the sample [µmol/L]. The colour intensity is measured photometrically at a wavelength of 505 nm. Reaction equation (Product information: Hitachi 912 autoanalyzer, Roche, Switzerland):

$$\text{creatinine + picric acid} \xrightarrow{alkaline\ solution} \text{creatinine-picric acid complex}$$

For the quantitative detection of uric acid, the enzymatic colorimetric test is used. Enzyme uricase transforms the uric acid in the sample into allantoin, carbon dioxide (CO_2), and hydrogen peroxide (H_2O_2). By the action of peroxidase (POD) and in the presence of phenol-derivative (3,5-dichloro-2-hydroxyl-benzenesulfonic acid - DHBS) and 4-Aminoantipyrine, hydrogen peroxide gives a coloured indicator reaction which can be measured at 520 nm. The absorbance is proportional to the concentration [µmol/L] of uric acid in the sample. Reaction equation (Product information: Hitachi 912 autoanalyzer, Roche, Switzerland):

$$\text{uric acid + 2 } H_2O + O_2 \xrightarrow{uricase} \text{allantoin + } CO_2 + H_2O_2$$

$$2\ H_2O_2 + \text{4-aminophenazone + DHBS} \xrightarrow{POD} \text{quinone-diimine dye + 4 } H_2O$$

The biochemical method for urea detection is based on the determination of urea nitrogen which is an end product of nitrogen metabolism. This method is based on enzymatic reaction using urease and glutamate dehydrogenase. Urea is hydrolysed in the presence of the enzyme urease into ammonia and carbon dioxide. The ammonia reacts with alfa-ketoglutarate and NADH (nicotinamide adenine dinucleotide (NAD) + hydrogen (H)) in the presence of glutamate dehydrogenase. The oxidation reaction of NADH to NAD occurs. The decrease of absorbance due to the consumption of NADH is measured photometrically at a wavelength of 340/410 nm. Product concentration is proportional to the urea concentration [µmol/l] in the sample. Reaction equation (Product information: Siemens Healthcare Diagnostics Inc., Erlangen, Germany):

$$\text{urea + } H_2O \xrightarrow{urease} \text{2 ammonia + } CO_2$$

$$\text{ammonia + NADH + α–ketoglutarate} \xrightarrow{glutamate\ dehydrogenase} \text{glutamate + NAD + } H_2O$$

Measurement of inorganic phosphorus in serum has been accomplished by forming a phosphomolybdate complex and, in turn, reducing it to a molybdenum blue colour complex. Inorganic phosphorus reacts with ammonium molybdate in an acid medium to form a phosphomolybdate complex that absorbs light at 340 nm. The absorbance at this wavelength is directly proportional to the amount [mmol/l] of inorganic phosphorus present in the sample. Reaction equation (Product information: Hitachi 912 autoanalyzer, Roche, Switzerland):

inorganic phosphorus + H_2SO_4 + ammonium molybdate ⟶ unreduced phosphomolybdate complex

For detection of beta-2-microglobulin (B2M) a more sophisticated technology called chemiluminescent enzyme-labelled immunometric assay (CLIA) is used. Because B2M has a complex structure and its molecule is a long amino acid chain, this method is not so simple as the one used for detecting small water-soluble toxins. For B2M detection, a special reagent kit is used which is manufactured by the Siemens (previously developed by DPC, Tarrytown, NY USA) company for the Immulite-type automated analysers. This Immulite B2M kit contains the solid-phase bead which is coated with an affinity-purified murine monoclonal anti-beta-2 antibody. In this reagent kit a serum sample to bind B2M into an antibody sandwich complex is incubated. Then the chemiluminescent substrate, a phosphate ester of adamantly dioxetane, is added. In the presence of a reagent, the alkaline phosphatase produces light which is proportional to the concentration of the B2M in the sample (Product information: Siemens Healthcare Diagnostics Inc., Erlangen, Germany).

Analysis methods used at the clinical laboratory might be affected by interfering factors which influence the accurate determination of the analyte. These kinds of substances might be other drugs in the sample or endogenous compounds, for example hemolysis, lipemia, or high bilirubin level (Kroll et al., 1994; Nikolac, 2014; Mainali et al., 2017). These interferences affect the results of the analysis, delivering increased or decreased concentration values compared to the real ones. Although all manufacturers must assess these interferences before producing the methods, these interferences are not standardised and the influences to the results differ between manufacturers even if the same methodology is used (Nikolac, 2014). It is crucial to know the lowest concentration of the interfering factor that could cause a bias. The bias is expressed by the standard deviation (SD), usually 2 SD. The bias is considered to be significant if it is larger than an analytical error of the instrument.

The parameter of imprecision is described by the intraindividual coefficient of variation (CVw). Allowable bias (total error, TE) for interference is calculated as (Nikolac, 2014):

$$TE = CVw - (1{,}96 \times CVa) - B \quad (3)$$

where *CVa* is the analytical coefficient of variation and *B* is bias.

Therefore, using the clinical chemistry analysis methods, the accuracy should be evaluated and the acceptance criteria for different interferences defined.

4.2. High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is an analytical chemistry technique which can separate and identify the components mixed in a liquid solution. The most commonly used form of HPLC is the reversed-phase HPLC technique. This method is found to be a sensitive, accurate, and reproducible method for qualitative and quantitative analysis of aqueous samples (Vanholder et al., 2001). Furthermore, the method is suitable for the investigation of many non-volatile or thermally unstable compounds commonly found in biological samples because of the use of ambient temperature in reversed-phase columns. The HPLC testing enables the separation of compounds in a mixture sample more efficiently and faster than traditional column chromatography (Lauri et al., 2011).

The typical HPLC system consists of two key segments – a stationary and a mobile phase. The first, stationary phase is a column filled with small solid sorbent particles; in there the separation of different compounds takes place. The second, mobile phase is a flowing liquid (solvent) which transports the compounds from the sample through the stationary phase. Due to the relative affinities of the solvent and stationary phase, the compounds of the mixture travel at different speeds. Thus, separation of compounds in the stationary phase takes place according to slight differences in chemical properties, such as the size of non-polar groups and chemical polarity.

Figure 2 is a simplified block diagram illustrating the main components of a modern HPLC. An HPLC system consists of the following units: pumps, sample-injection, sample separation (column), detection and data-processing.

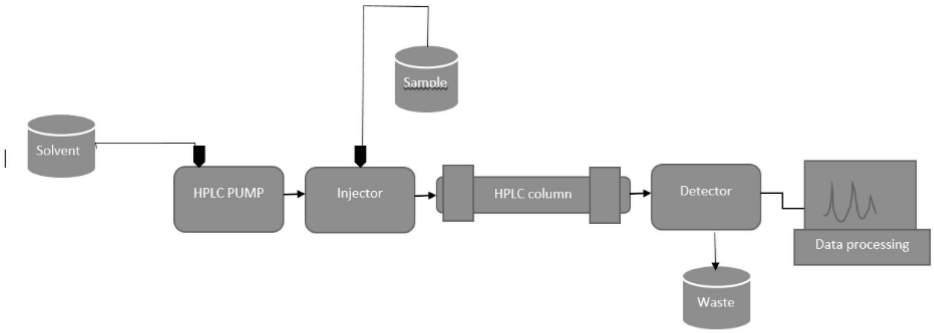

Figure 2. *Schematic reversed-phase HPLC method principle.*

There are several different detection methods used in HPLC, whereas the most popular ones are optical. The most common optical detection method is an ultraviolet – visual light (UV-VIS) absorption detector. In the UV-VIS detector the absorption occurs at a wavelength between 180–800 nm; it can be employed for analysis of a wide range of compounds. UV-VIS spectroscopy is a simple, sensitive, and reliable technique which allows to determine very low concentrations of compounds (Passos et al., 2018).

In modern HPLC systems, fluorescence detectors are used. Those are suitable for detecting the fluorescent compounds or the compounds which exhibit an appropriate fluorescence due to derivatisation. In the fluorescence spectroscopy the intensity of the emission of the sample is measured and that of the emission signal is measured above a low background level. This is 1000 times more sensitive than absorption spectroscopy (Peterson, 1997).

4.3. Optical methods

Two optical techniques have been in use for detecting solutes in biofluids: the ultraviolet (UV) absorbance measurement and fluorescence measurement (Munjanja et al., 2015).

UV spectroscopy is related to the interaction of light with matter. If light interacts with biological fluid or solution, it might be absorbed, scattered, or reflected. Light absorbs in the dialysis fluid. The light absorbed by a chemical compound will produce a distinct spectrum due to the increase in the energy content of the atoms or molecules. The UV spectroscopy unit of measurement is called an Absorbance value (Abs).

The schematic principle of UV spectroscopy is demonstrated in Figure 3.

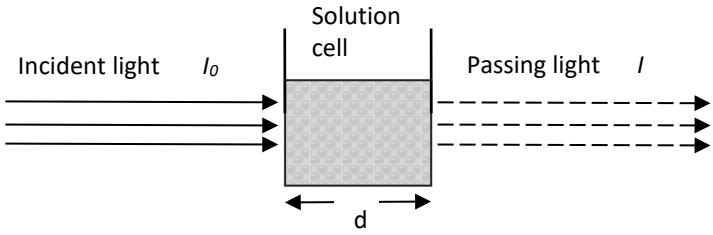

Figure 3. *Schematic UV spectroscopy principle.*

The basic principle of UV spectroscopy follows the Beer-Lambert law (Perkin Elmer, 2000). The principle is that if light is passing through the spectrometer, the incident light intensity decreases with the thickness of the cell (solution) and absorbance of entering light is proportional to the amount of material that absorbs the incident light (i.e., the concentration of the compound). This is expressed through the equation:

$$A = log_{10} \frac{I_0}{I} = \varepsilon C d \quad (4)$$

where:

A – absorbance;

I – the intensity of light passed through a solution;

I_0 – the intensity of incident light;

ε – molecular extinction coefficient, shows the absorption of a 1 molar solution of the test substance at a wavelength of λ if the thickness of the solution is 1 cm;

C – concentration of the solute;

d- the thickness of the solution layer, or optical path length (cuvette thickness).

According to the wavelength (nm), the region of the electromagnetic spectrum is called a UV spectrum – on a wavelength range of 200–350 nm – or VIS spectrum – on a range of 350–700 nm. UV–VIS spectroscopy uses the absorption of electromagnetic radiation in the UV–VIS region – that is the most extensively used spectroscopic technique for measuring chromophores (Munjanja et al., 2015).

Fluorescence spectroscopy analyses fluorescence from a molecule based on its fluorescent properties. Fluorescence spectroscopy is a type of luminescence using a light, UV, VIS, or near-infrared (IR) region. During the process, absorption of light takes place and the subsequently, the molecule is excited, which quickly returns to its grounded state losing a photon (Munjanja et al., 2015). This is more sensitive than UV spectroscopy.

The fluorescence instrument contains three basic items: a source of light radiating an excitation beam (Figure 4), a solution cell and a detector (Perkin Elmer, 2000). A light source is usually the tungsten-halogen lamp or the mercury lamp. A light beam passes through a solution in a cuvette. The light emitted (from an angle) by the sample is measured; both an excitation spectrum (the light that is absorbed by the sample) and/or an emission spectrum (the light emitted by the sample) can be measured. The concentration of the compound in the sample is proportional to the intensity of the emission. A better sensitivity can be achieved if using filters which allow the total range of wavelengths emitted by the sample to be collected.

The schematic principle of fluorescence spectroscopy is demonstrated in Figure 4.

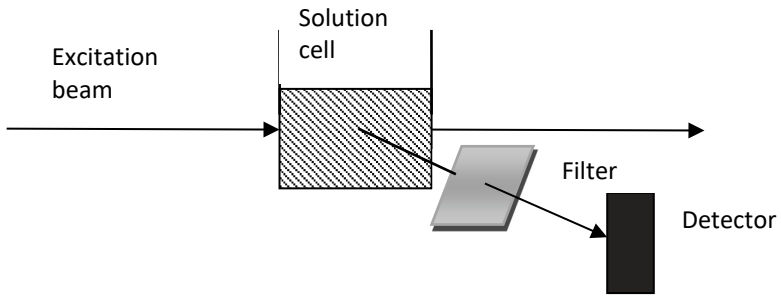

Figure 4. *Schematic fluorescence spectroscopy principle.*

Aims of the study

The thesis aimed to investigate the elimination of uremic toxins with diverse characteristics by different dialysis modalities using standardized laboratory methods and optical measurements.

The specific aims were:

- to identify which uremic solutes contribute to the ultraviolet (UV) absorbance profile in serum and spent dialysate using the high-performance liquid chromatography (HPLC) method;
- to investigate the removal of the small, protein-bound and middle molecules, during various treatment modalities;
- to determine whether the results from optical measurements are comparable to the results from a chemical laboratory and to evaluate the accuracy of the laboratory and optical methods.

5. Experimental studies: Methods

5.1. Clinical studies

In all experiments, patients from North Estonia Medical Centre, Tallinn, Estonia, were included. The studies were performed after the protocols were approved by the Tallinn Medical Research Ethics Committee at the National Institute for Health Development, Estonia. Informed consent was obtained from all participating patients. A summary of patients, dialysis parameters, and dialysate sampling times is presented in Table 1.

Table 1. *Patients, conditions and sampling times*

	Publication I	**Publication II**	**Publication III**
Total number of patients (Male/Female)	10 (7/3)	7 (3/4)	10 (5/5)
Mean age ± SD	62.6 ± 18.6	58.1 ± 8.7	59.1 ± 15.0
Number of sessions	30	28	40
Dialysis machine	Fresenius 4008H	Fresenius 5008	Fresenius 5008
Dialyzers	F8 HPS (HD) F10 HPS (HD) FX 80 (HF)	FX8 (HD) FX1000 (HF, HDF)	FX8 (HD) FX1000 (HF, HDF)
Dialysate flow ml/min	500	500-800	500-800
Blood flow ml/min	245–350	300–350	300–350
Dialysate sampling time	10 min from the beginning and at the end of a session (210 or 240 min)	7 to 10 min from the beginning and at the end of a session (180 or 240 min)	10, 60, 120 min from the beginning and at the end of a session (180 or 240 min) and tank
Blood sampling time	pre-and post-dialysis	pre-and post-dialysis	pre-and post-dialysis
Uremic solutes analysed	CR, UA, Urea, P, IS, HA	Urea, B2M	Urea, B2M, IS

The schematic clinical set-up, sample collection, and analysis are shown in Figure 5.

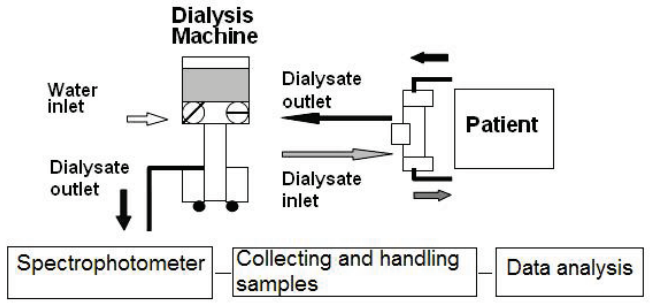

Figure 5. *Clinical set-up and sampling.*

5.2. Optical measurements

All treatments were monitored optically using UV-absorbance registration with the spectrophotometer HR2000 (Ocean Optics, Inc., USA). The device was provided with a specially designed optical cuvette with an optical path length of 5 mm, connected to the fluid outlet of the dialysis machine, all spent dialysate passing through the cuvette during the online experiments. UV-absorbance of the spent dialysate at the selected wavelengths was registered during the dialysis procedures. The absorbance was measured in arbitrary units (a.u.). The sampling frequency was set to two samples per minute. The UV-absorbance values were processed and presented on the computer screen by a PC connected to the spectrophotometer using the Ocean Optics software (OOIBase32, version 2.0.2.2 for Windows).

In Publications II and III the double-beam spectrophotometer (UV-3600 UV-VIS-NIR, Shimadzu Inc, Japan) was used for the determination of UV-absorbance of the spent dialysate samples. Scanning was performed in the UV-range over the wavelengths 190–380 nm. The obtained spectral values were processed and presented via the software UV Probe (Shimadzu Inc., Japan).

Fluorescence analysis (Publication III) was performed over an excitation (EX) wavelength range of 220–500 nm (excitation increment 10 nm) and an emission (EM) wavelength range of 220–500 nm by the spectrofluorophotometer (RF-5301, Shimadzu Inc., Japan). Measurements were performed at room temperature (ca. 22^0 C) within 8 hours after each session. The obtained spectral values were processed and presented via the software Panorama fluorescence (Shimadzu Inc., Japan).

5.3. HPLC analysis

For chromatographic analysis (Publications I and III) the reversed-phase HPLC systems were used as described below.

Publication I: The HPLC system consisted of a quaternary gradient pump unit, a column oven, and a diode array spectrophotometric detector (DAD, all Series 200 instruments from Perkin Elmer, Norwalk, CT, USA), a manual injector from Rheodyne (Rohnert Park, CA, USA), and a Zorbax C8 4.6 x 250 mm column from Du Pont Instruments (Wilmington, DE, USA) with the security guard KJO-4282 from Phenomenex (Torrance, CA, USA). The eluent was mixed with 0.05 M formic acid adjusted to pH 4.0 with

ammonium hydroxide (A), HPLC grade methanol (B) and HPLC-S grade acetonitrile (C), all from Rathburn (Walkerburn, Scotland), with a six-step gradient program as specified in Publication I.

Publication III: The HPLC system consisted of a quaternary gradient pump unit, a thermostated autosampler, a column oven, a diode array spectrophotometric detector (DAD), a fluorescence detector (FLD), all Ultimate 3000 Series instruments from Dionex (Sunnyvale, CA, USA), and two continuous columns of Poroshell 120 C18 4.6 x 150 mm from Agilent Instruments (Wilmington, DE, USA) with the security guard KJO-4282 from Phenomenex (Torrance, CA, USA). The samples were kept at 6°C in the autosampler. The eluent was mixed with 0.05 M formic acid adjusted to pH 4.25 with ammonium hydroxide (A), and an organic solvent mixture of HPLC grade methanol and HPLC-S grade acetonitrile, both from Rathburn (Walkerburn, Scotland) in the ratio of 9:1 with 0.05 M ammonium formate salt (B). A three-step linear gradient elution program was used.

Before the HPLC analysis, the serum samples were purified from proteins by centrifuging with two different Microcon centrifugal filters (Millipore, USA): a YM-3 with cut-off 3 kDa and a YM-100 with cut-off 70 kDa, at room temperature. The dialysate samples were acidified down to pH 4.0.

Every peak in the HPLC chromatograms was characterized by the retention time and the mass spectrum. The chromatographic peaks were identified by comparing the retention time and the mass spectrum of the spent dialysate to the reference compound.

5.4. Clinical laboratory analyses

The concentrations of creatinine (CR), uric acid (UA), urea (UR), and phosphate (P) in Publication I were measured using the Hitachi 912 autoanalyzer (Roche, Switzerland). The coefficient variation (CV) of the methods used for the determination of different solutes in spent dialysate and blood samples were CR 5%, UA 2%, UR 4%, and P 2%.

In publications II and III the urea and beta-2-microglobulin (B2M) concentration measurements in serum and dialysate samples were performed at the clinical chemistry laboratory SYNLAB Eesti OÜ (earlier name Quattromed HTI Laborid), Estonia, using standardized methods. Urea concentrations were measured by ADVIA 1800 (Product information: Siemens Healthcare Diagnostics Inc., Erlangen, Germany) autoanalyzer. B2M concentrations were measured by Immulite 2000 and Immulite 2500 autoanalyzer (Siemens Healthcare Diagnostics Inc., Erlangen, Germany).

The accuracy was 10% for B2M, and 3.2% for UR in dialysate and in blood.

These traditional methods described above need blood, urine, or dialysate sampling which requires extra effort from the medical personnel and can be unpleasant for the dialysis patients. Therefore, sampling is traditionally not performed during every dialysis procedure. Moreover, laboratory analysis requires resources and time. The results of the ongoing procedure arrive when the patient has already left the hospital. By traditional methods modifications in the ongoing treatment are not possible. Therefore, it would be essential to monitor these parameters in real-time to obtain adequate information about the patient's condition and treatment performance during the ongoing dialysis procedure.

5.5. Calculations and statistical analysis

Removal on solutes during dialysis sessions was described by a reduction ratio % (RR) in serum and dialysate.

$$RR = \frac{C_0 - C_t}{C_0} \times 100\ \% \quad (5)$$

where C_0 is a serum sample before and C_t after the dialysis procedure. For spent dialysate, a sample taken after 10 minutes from the start and at the end of the dialysis procedure were utilised as C_0 and C_t, respectively. In the case of optical measurements, C_0 and C_t were replaced by the uremic solute concentration in spent dialysate, calculated from the optical data.

The total removed solute (TRS) of a substance was calculated based on total dialysate collection (TDC) as follows:

$$TRS = C_T \times W_T \quad (6)$$

where C_T is the total substance concentration in the dialysate collection tank and W_T is the weight of the dialysate collection tank [kg].
Achieved results were compared regarding mean values and SD, also by accuracy and standard errors (BIAS±SE) using Excel (version 2007 for Windows). CV was calculated as the ratio of the standard deviation to the mean.

Bias was calculated as follows using RR values based on concentrations from the laboratory as reference:

$$BIAS = \frac{\sum_{i=1}^{N} e_i}{N} \quad (7)$$

where e_i is the residual (difference between laboratory and optically determined RR values for the i-th treatment) and N is the number of observations (Esbensen, 2009)
Standard error (SE) of performance was calculated as follows:

$$SE = \sqrt{\frac{\sum_{i=1}^{N}(e_i - BIAS)^2}{N-1}} \quad (8)$$

where e_i is the difference between the lab and model concentration for the i-th measurement (Esbensen, 2009).

In Publication III a multiple regression analysis was performed for determining the best wavelengths for the B2M IS concentration estimation models using software Statistica 10.0 (Statsoft, Inc. for Windows).

Students' paired t-test was used to compare means and BIAS for different methods and f-test two-sample for variances to compare variances and SE. A p-value lower than 0.05 was considered significant.

6. Results and discussion

In all publications included in the thesis the elimination of uremic toxins during dialysis simultaneously assessed with the different analysis methods has been compared. Standardised clinical laboratory methods, sophisticated high-performance chromatography (HPLC) and optical (UV absorption and fluorescence) measurements were used for the determination of solute removal during dialysis. Dialysis fluid and serum were analysed and the correlation for different uremic toxins (SM, MM, PB) was compared. Additionally, uremic solute removal using various types of dialysis filters and during different dialysis modalities was discussed. The accuracy of determining the uremic solute concentrations using different methodologies was compared.

When some uremic toxins (IS, HA) with a potentially significant impact to the survival of a patient with end-stage renal disease are impossible to detect with standardized clinical laboratory methods, then the knowledge about the blood concentration and dialytic removal could be valuable information for medical doctors. Therefore, our research group has focused on developing novel solutions that can make real-time monitoring of the dialysis available without collecting any blood samples, offering the easiest way to observe the elimination of the different types of uremic toxins from a patient with end-stage renal failure. We have demonstrated that aside standardized clinical laboratory methods, an alternative solution for dialysis adequacy monitoring is available.

The purpose of this dissertation has been to study various analytical methods, to compare their accuracy, and to confirm the reliability of a simple optical method which could be useful in daily medical practice.

6.1. HPLC UV absorption profiles of uremic fluids (Publication I)

The first publication focused on determining concentrations of uremic toxins by high-performance liquid chromatography (HPLC) in serum and spent dialysate. Different HPLC settings were tested and the reliability and performance of the HPLC method was evaluated. Earlier studies have demonstrated that HPLC method is suitable for uric acid (UA) measurements and contributes most to the UV absorption (Donadio et al., 2013), but an experimental work was done to prove that this method is also suitable for identification of creatinine, indoxyl sulphate (IS) and hippuric acid (HA). These three compounds are uremic solutes' chromophores which contribute to UV-absorbance in the spent dialysate (Tomson et al., 2013; Lauri et al., 2010). For this reason, it was possible to identify and measure the concentrations of those uremic solutes by HPLC with an absorbance detector. The dialytic elimination of all compounds was evaluated as a reduction ratio (RR) in both blood serum and spent dialysate. Two different filters, low-flux and high-flux, were used during the dialysis sessions in this study. The membranes showed no difference in the removal of the uremic solutes using chosen dialysis settings as also observed earlier (Lesaffer et al., 2000). The results were promising enough to continue developing the HPLC and optical methods in the future.

The results revealed that absorbance and the number of chromophores from the HPLC profile at a wavelength of 254 nm had higher values compared to the wavelength of 280 nm. All RR results calculated from the concentrations of the uremic solutes were compared to the RR estimated from the total area of HPLC peaks (TA HPLC peaks) at 254 and 280 nm and the on-line UV-absorbance at 280 nm. The best correlation was

between serum UA RR and TA HPLC at 280 nm and UV absorbance at 280 nm (in Publication I, Table II). UA was also the prevalent component of the HPLC UV absorbance profiles. Creatinine had a good correlation with UV absorbance at 280 nm and a relatively high correlation also at 254 nm. In contrast, small protein-bound molecules IS and HA had a low correlation with the UV absorbance. It was concluded that UV absorbance cannot be used to monitor the elimination of these two compounds in the spent dialysate. It is worth noting that neither IS nor HA are detectable by the standardized clinical chemistry methods.

Since urea has no absorbance in the UV spectral range, it is not detectable by the HPLC-UV method and was measured only by using the biochemical analysis method. Therefore, it is important to further develop the optical method so that all different groups of toxins could be determined optically. On the one hand, we want to find a simple method for following the removal of solutes during the dialysis session, but on the other hand, clinical chemistry methods have their disadvantages.

Clinical chemistry methods have various limitation which means methods could be affected by interfering factors. Drugs or abnormalities in the blood sample like hemolysis, lipemia, or high bilirubin level can increase or decrease the concentration of the compound measured (Kroll et al., 1994; Nikolac, 2014; Mainali et al., 2017). These influences affect the colour intensity of the solution resulting from the reaction which is proportional to the concentration. For example, colorimetric measurement is influenced by the high level of haemoglobin, bilirubin, or triglycerides in the serum. Haemoglobin is the protein molecule in red blood cells which carries the oxygen and carbon dioxide between body tissues and the lungs. Destruction of the red blood cells might cause the release of haemoglobin into the blood plasma and makes it reddish. That outcome is called hemolysis and this is higher the more extensive the breakdown of the red cells is. The major causes of hemolysis are wrong sample collection or transport conditions (blood sample shaking, freezing). The visual detection of hemolysis is possible if haemoglobin concentration is higher than 0,2 g/L. The lower concentration is not detectable by eye, but it is detectable by an autoanalyzer as a hemolysis index (Kroll et al., 1994). Hemodialysis might be the cause of hemolysis because this is where the blood is processed mechanically during the procedure and therefore the red blood cells might be damaged. As a result of hemolysis, the colorimetric analysis of the serum does not give correct results (crea, UA). In our study, the hemolysis rate was assessed before the analysis and only non-hemolytic samples were used to avoid inaccurate results.

Bilirubin in serum is the end product of the breakdown of red blood cells in the body. A high level of bilirubin, also known as an icteric sample, transforms the serum green instead light yellow. High bilirubin concentration in the serum/plasma interferes the Jaffe method for creatinine level determination (Kroll, 1994) and bilirubin level higher than 20 mg/dL interferes with the urea determination (Product information: Hitachi 912, Roche, Switzerland).

Additionally, a high level of normal body lipids – triglycerides – might cause the blood plasma colour changes. As fat-like particles, they give white colour to the plasma. Patients with chronic kidney disease (CKD) can often have disorders of lipid metabolism (Bulbul et al., 2018). If the body accumulates the lipoproteins (the proteins which transport lipids like cholesterol and triglycerides) it causes a blood transformation called lipemia (Nikolac, 2014). A lipemic sample might be also caused by inadequate blood collection time (e.g. collected after a meal), but also some diseases, including renal disease, can affect the accumulation of lipoproteins in blood (Mainali et al., 2017). There

are many methods to assess lipemia in the sample. The easiest way to determine the lipemic serum/plasma is visually after the centrifugation of blood. Also, assessing the lipemia degree can be informative for triglycerides' concentration determination. Moreover, the analytical rate of lipemia is expressed by the lipemic index and it is measured by biochemical autoanalyzers. A significant effect to the clinical parameters can occur if the lipemic index is >500 mg/dL (Mainali et al., 2017).

These physiological changes in the human body, being consequences of high bilirubin or high hemoglobin levels in plasma/serum and a redundant volume of triglycerides, might be a significant source of analytical errors in clinical laboratory settings.

In addition to endogenous substances, possible interfering chemical substances, like medications, might influence the measurement of analyte concentrations. A recommendation is given by the uric acid test reagent manufacturer (Product information: Siemens Healthcare Diagnostics Inc., Erlangen, Germany) that N-Acetylcysteine (NAC) or Metamizole administration not be allowed before blood collection. This could cause a false analytical result such as lower uric acid concentration. NAC is used as an antidote of paracetamol intoxication (Benlamkaddem et al., 2018) where hemodialysis is a standard treatment method (Serjeant et al., 2017). Paracetamol is also a common inflammation medicament.

Usually the manufacturers present these limitations in the method sheet and thereby users are informed by these kinds of influences. Not all interferences, especially used medicaments, are possible to assess very thoroughly. Although there may be tens of substances listed by the manufacturer as potentially influential substances, there could be no information about the level of influence. Therefore, it is suggested to evaluate the patient's clinical status, laboratory results, and possible treatment options together as a whole. For a patient, this might mean a longer waiting time for the suitable treatment.

In general, these effects of certain interfering factors must be taken into account. In our study, these kinds of interferences were excluded because the potentially interfering substances used in treatment of the patients were known and performance instructions of the used lab methods were carefully observed during analyses.

The accuracy of clinical laboratory methods is well known. The most common parameter for estimating the precision of the method is the coefficient of variation (CV%). It is the responsibility of each clinical laboratory to determine its intra-laboratory CV of the methods to assess the ability and quality of the analyses. This means, if the analytical CV% of one component is relatively high, i.e. the variability between the results is significant, we cannot fully trust the laboratory results. It is important that the results be reliable to the physician and the accuracy should not change during the time, especially if the patient's treatment quality is evaluated.

The CV values of the toxins studied in this paper were the following: creatinine 5%; UA 2%; urea 4 %. These results were within the manufacturer's stated limits and the laboratory quality can be seen as trustworthy. This means that all the conclusions and comparisons (correlations) made with the UV method can be seen as reliable.

For the toxins like HA and IS, the HPLC method was developed. The HPLC method is reliable, specific and accurate, but it is complex and expensive to use in daily clinical practice. It requires special skills and training of the users. Among others, the pre-treatment of the sample material is sophisticated. For example, proteins must be removed from the serum before the HPLC analysis. One possibility is to use special filters for deproteinization. The other choice is processing the serum at high temperature where the proteins precipitate out and the serum is centrifuged before removing

proteins with an ultracentrifuge to get clear filtrate for HPLC analysis. In the first study, the special filters (Microcon YM-3 and YM-100, Millipore, USA) were used for serum pre-treatment. Such pre-treatment does not make the method user-friendly because it is a time consuming and cumbersome process. Moreover, using improper techniques may cause the loss of important components during pre-processing and therefore the accuracy of the results may be affected. Dialysis fluid also requires pre-treatment. Due to the characteristics of spent dialysate, it is not well preserved at room temperature. To avoid bacterial contamination, the spent dialysate must be pre-treated with formic acid to obtain a pH of at least 4.0. As the samples are exposed to different chemicals when using the HPLC method, this process requires special conditions in the laboratory – a well-ventilated workbench or a laboratory hood.

Publication I also revealed that the removal of HA and IS from the patient's body by dialysis is slower than the removal of small water-soluble substances urea, creatinine, and uric acid, due to the protein-binding nature of HA and IS. This was the reason why the correlation between concentrations of HA and IS and the measured UV absorption in the spent dialysate was worse compared to the water-soluble substances. The paper also confirmed that mainly small water-soluble non-protein-bound molecules contribute to the UV absorption signal.

One of the conclusions of this study was that the expansion of the range of the types of detectors used in the next experimental work is necessary and a solution to enable the detection of middle molecules and protein-bound solutes must be found. All in all, we realized that we need new optical methods and detectors.

6.2. Removal of urea and B2M assessed by UV absorbance (Publication II)

The second publication describes a comparison between the dialysis elimination of a small molecule (SM) urea and a middle molecule (MM) beta-2-microglobulin (B2M) as a further development of an on-line dialysis quality monitoring method. Elimination was assessed by a dialysis adequacy parameter reduction ratio (RR). The serum concentration was estimated using the standardized biochemical methods and spent dialysate was assessed optically by UV spectrophotometry. The dialytic elimination of different toxic compounds by using different dialysis filters and various modalities (HD, HF, HDF) was studied. RR of the toxins was compared to the UV measurements (in Publication II, Fig.1, 2). It was confirmed that the serum urea RR correlates well with RR from the UV absorption at 297 nm at various treatment modalities. However, B2M did not show the same trend (in Publication II, Fig.1). Due to high molecular weight, the removal of B2M is limited and its RR correlation with UV absorbance is poor when using the HD. The results are better using higher convection as during HDF where there was no statistical difference between the eliminations of urea, B2M, and RR using UV measurement ($p<0.05$). Thus, the elimination of B2M is dependent on modality (filter, convection, blood and dialysate flow rate). A measurement error (bias) was also demonstrated that was independent of modality for urea, but not for B2M. The measurement error is significantly reduced for the modalities with higher convection, as HF and especially HDF, compared to HD in case of B2M. The behaviour of the measurement error is shown in the Figure 6.

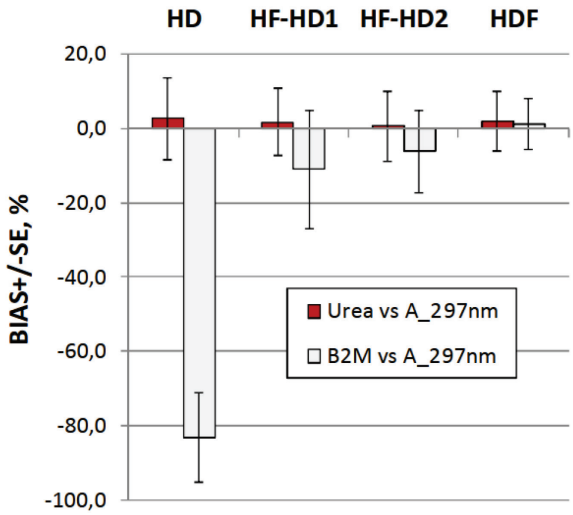

Figure 6. *BIAS±SE values in percentage for various treatment modalities between urea and B2M and UV-absorbance at the wavelength of 297 in spent dialysate (Urea vs A_297nm and B2M vs A_297nm).*

For the clinical personnel, it would be necessary to obtain results quickly and not wait for analysis results to make adjustments towards improving the dialysis treatment. As frequent blood collection from dialysis patients is not suggested due to health reasons, it would be beneficial to have a system that does not require blood collection. Patients with impaired hematopoiesis could be carefully monitored and the effect of different procedures to their health assessed. Moreover, the standardized laboratory method for determining B2M is technically more complex and more expensive than a simple photometric assay for detecting urea which involves a simple enzymatic reaction and optical measurement (at 340/410 nm). Therefore, CV for urea is usually lower than CV for B2M because the methodology used for B2M is based on the sophisticated chemiluminescent immunoassay (CLIA method). A beta-2-microglobulin-specific antibody is used that recognizes a B2M molecule. Furthermore, due to an incubation period of 30 minutes, it will take hours to get the B2M results from the laboratory.

Laboratory methods, such as typical *in vitro* diagnostic methods, have certain disadvantages compared to *in vivo* diagnostics. As described in relation to publication I, the manufacturer performs a series of experiments prior to the use of the method to demonstrate the suitability of the method for *in vitro* diagnostics, examining the effects of various interferences on the assay results. The CLIA method also has these limitations. Heterophilic antibodies in human serum can react with the immunoglobulins included in the reagent used for testing causing incorrect results (Product information: Siemens Immulite Beta-2 Microglobulin, 2015-05-11). Additionally, the serum used for testing should be properly pre-treated – centrifuged – before the analysis and cannot be hemolytic or lipemic. The presence of a high level of haemoglobins (concentration more than 384 mg/dL) or triglycerides (concentration more than 3000 mg/dL) may affect the result of the assay and the accuracy of the concentration of the component being measured (Product information: Siemens Immulite Beta-2 Microglobulin, 2015-05-11).

Accuracy could also be affected by the serum dilution. In case of very high values, where the result is outside the calibration range, dilution is required. For B2M,

the calibration range can be up to 500 ng/mL (Product information: Siemens Immulite Beta-2 Microglobulin, 2015-05-11). Whereas patients with CKD might have very high values of B2M, up to 30,000 ng / mL in some patients, multiple dilutions of the blood serum are required during the assay measurements. Thus, the time it takes to perform an analysis will be longer and the cost higher. Laboratory CV about 10 % between series is higher compared to urea (about 3,2 %). However, despite this variability, the CV was within the allowed range by the manufacturer (Product information: Siemens Healthcare Diagnostics Inc., Erlangen, Germany) and the results of the study are trustworthy.

The experiments described in Publication II were aimed to develop a real-time, reagent-free methodology. The study demonstrated that UV-absorbance can detect the removal of both small molecular weight and middle molecular weight (marker) molecules such as urea and B2M as a reduction ratio. Although this is an indirect method, because originally the chromophores were measured, the elimination of these is similar to the elimination of urea and B2M. The results demonstrated that the estimated RR based on the UV absorption obtained is statistically comparable to the elimination of the urea and B2M assessed by the reference methods, especially at high convection modalities.

In addition to the absorbance, another optical method, fluorescence, enables to detect further uremic toxins and improve the reliability and accuracy of the results obtained.

6.3. Simultaneous removal assessment of marker molecules by UV absorbance and fluorescence (Publication III)

The third paper describes the simultaneous evaluation of the elimination of marker molecules of three different groups of toxins at different modalities, low-flux hemodialysis (HD), high-flux hemodialysis (HF) and hemodiafiltration (HDF). Clinically relevant marker molecules that have also been described in previous studies, were selected, such as small molecule urea, medium molecule B2M, and protein-bound molecule IS. For the determination of urea and B2M, the standardized biochemical laboratory methods were used, just as previously, and for IS the HPLC method was used. In addition to the conventional UV-VIS detector, we also used a fluorescence detector connected to the HPLC which enabled us to identify the IS molecule optically. As in previous publications, optical measurements were performed at the same time as laboratory analyses. In summary, we were able to provide information on all three groups of classical uremic toxins simultaneously. Optical measurements were performed over a wide measuring range, UV absorbance was measured at 190–380 nm and fluorescence analysis was performed over an excitation (EX) wavelength range of 220–500nm (increment 10nm) and emission (EM) wavelength range of 220–500nm (increment 1nm). The optical measurement data were compared to the concentrations obtained by the standardized laboratory methods and the wavelengths at which the results correlate better with the laboratory values were selected. Using multiple linear regression analysis, the models for the calculation of uremic solute concentrations were created based on the optical data (UV absorbance and fluorescence). This statistical method helped select the best wavelengths for predicting the concentrations of substances. For urea one wavelength (UV), 5 wavelengths (UV and Fluo) for B2M and 6 suitable wavelengths (UV and Fluo) for IS were found using this modelling method.

In this study, a strong correlation between the optical measurements and the results obtained by laboratory analysis were demonstrated (in Publication III, Fig. 2). A good linear correlation was found in the dialysate results (in Publication III, Fig. 2, A-C), while the bias increases at higher concentrations. A good correlation was obtained by analysing urea concentration in the spent dialysate by biochemical and optical data modelling, mean ± SD respectively 3.64 ± 2.31 and 3.63 ± 2.06 (Publication III; Supplement). Summary of the statistics for the marker solutes' concentrations in spent dialysate measured chemically (D_lab) and estimated by the models using the optical measurements (D_OPT) is presented in Table 2.

Table 2. *Summary of the statistics for the marker solutes (Urea, IS, B2M) concentrations in spent dialysate measured chemically (D_lab) and estimated by the models using the optical measurements (D_OPT).* [1]*R values are statistically different (p<0.05).*

	Min	Max	Mean	SD	N	R	R^2	BIAS	SE
D_lab_Urea, mmol/L	0.30	12.60	3.64	2.31	246				
D_opt_Urea, mmol/L	0.71	12.27	3.63	2.06	246	0.89	0.79	0.00	1.06
D_lab_IS, µmol/L	0.36	16.96	4.17	2.63	252				
D_opt_IS, µmol/L	0.38	15.44	4.21	2.17	252	0.86[1]	0.74	0.00	1.35
D_lab_B2M, mg/L	0.29	4.07	1.31	0.79	180				
D_opt_B2M, mg/L	0.35	3.68	1.32	0.75	180	0.92[1]	0.85	-0.01	0.30

This has also been presented in earlier studies (Tomson et al., 2012, Uhlin et al., 2005) and can be explained by the physical properties of urea as a water-soluble low molecular weight uremic solute. Urea is very easily removed by dialysis and can be easily measured in a clinical laboratory using standardized methods. Because the CV for urea is relatively low (3.2 %), the experimental results can be considered sufficiently reliable. The clinical chemistry laboratory methods are considered as the "golden standard" because they have been validated carefully before being used. In case the results obtained by the optical method have good compatibility with the lab results, it confirms that the optical method has similar accuracy. This validation will ensure that the novel optical method that has not been validated earlier in clinical conditions has sufficient accuracy for the clinical parameters estimated. Measurement errors arise mainly due to pathophysiological differences in patients, their metabolic characteristics, the dialysis filter properties, differences in dialysis modalities, and the fact that the spent dialysates may have different compositions.

The relative elimination of substances was evaluated by RR in this study in respect to different uremic solutes and dialysis modalities. The highest RR (%) was found for urea for all serum, dialysate and optical measurements. The lowest RR was for IS and was statistically different from urea RR and B2M RR for all modalities ($p < 0.05$). Although IS has a low molecular weight (251 Da) (Vanholder et al., 2003), its elimination from the blood is less effective because IS is partially bound to proteins and blocks them from moving freely through dialysis membranes. However, a much smaller free IS fraction can

be readily removed (Krieter et al., 2019). This is confirmed by the low RR for IS, being lower for serum than for dialysate. Therefore, the optical detection of IS in dialysate would be an important advancement in clinical practice. This study showed that IS elimination assessment is possible with the optical method. Also, the reliability of the optical method was evaluated in this study, presenting that serum and optical measurements have similar accuracy (BIAS < 8.1 %) for all three marker uremic toxins.

As in previous studies, the RR values of B2M behave in a similar way to urea in the high convective treatment modality HDF and in a completely different way in the low convective treatment modality HD. Because of the very low concentration values of B2M in samples collected during HD treatments, it was not possible to estimate RR for B2M (in Publication III, Fig. 3 and Table 3). The average RR (%) values, accuracy (BIAS ± SE) and correlation coefficient (R) between the indicated methods are given in Table 3.

Table 3. *The average RR (%) values, accuracy (BIAS ± SE) and correlation coefficient (R) between the indicated methods. S_lab and D_lab - serum and spent dialysate measured chemically, D_opt - dialysate measured optically.*
[1] No HD treatments were included for B2M. [2] R values are statistically different (p<0.05).

	RR, %			Accuracy (RR)			R (RR)		
	S_lab	D_lab	D_opt	S_lab vs D_lab	S_lab vs D_opt	D_lab vs D_opt	S_lab vs D_lab	S_lab vs D_opt	D_lab vs D_opt
Urea (N=40)	75.3 ± 8.0	73.3 ± 8.8	73.3 ± 7.3	2.0 ± 3.2	1.9 ± 4.0	0.0 ± 4.8	0.932[2]	0.863	0.833[2]
B2M[1] (N=30)	73.1 ± 9.1	72.0 ± 8.8	75.0 ± 6.5	1.1 ± 4.2	-1.8 ± 7.8	2.9 ± 6.8	0.891	0.576	0.650
IS (N=39)	43.5 ± 13.8	55.6 ± 9.5	51.6 ± 11.0	-11.9 ± 8.9	-8.1 ± 9.9	3.3 ± 5.2	0.761	0.702[2]	0.867[2]

In addition to RR, the total removed solute (TRS) value was calculated. The TRS results were also treatment-modality-dependent (in Publication III, Fig. 4), having the lowest values for HD and the highest for HDF. TRS for B2M for the HD treatment could not be calculated because the concentration of B2M in the spent dialysate was below detection limits. No significant differences in TRS values were found for the urea between the modalities (in Publication III, Fig. 4).

Generally, there was a good correspondence between clinical laboratory and optically assessed TRS values and the data displayed strong linear correlation. R was 0.727 for urea, 0.804 for B2M and 0.971 for IS. Accuracy for TRS values as BIAS ± standard error (SE), comparing lab vs. opt, showed no statistical difference for any of the observed uremic solutes (P > 0.05).

In conclusion, RR and TRS values are modality-dependent, and with HDF it is possible to achieve the maximum elimination of different substances. The accuracy of the optical method is not influenced by the dialysis modality (HD, HF and HDF).
Various experiments have shown good correlation and reliability of the optical method in comparison to biochemical standardized methods and HPLC methods. The advantages of the optical method over laboratory methods are as follows: (1) the optical measurements are fast and on-line; (2) it is not necessary to collect serum samples;

(3) a sensor can be connected directly to the dialysate outlet of a dialysis machine without disturbing the treatment.

Optical methods make it possible to monitor the elimination of uremic solutes from the patient's body during each dialysis treatment session. The estimated amount of uremic toxins removed can be presented on a screen of a dialysis on-line monitor. Simple methods that are easy to use and require no special requirements are preferred in everyday clinical practice. The optical on-line monitoring system, under development, will hopefully enable clinicians to evaluate the quality of dialysis treatment daily and make decisions with less effort.

Conclusion

In conclusion, this thesis demonstrated that it is possible to use optical methods for elimination assessment of marker molecules of three different groups of uremic toxins (SM, MM, PB) at different dialysis modalities (HD, HF, HDF). The thesis focuses on the comparison of the results from clinical laboratory and using novel optical methods, which has not been investigated before.

The main results of the thesis are as follows:

- On-line UV absorbance at 280 nm behaves more like a small water-soluble, non-protein-bound solute ($0.873<R>0.890$) than a small protein-bound solute ($0.478<R<0.568$). The main solute responsible for UV absorbance (280 nm) is a compound UA.

- The removal of IS (RR<50%) from a patient's body by conventional hemodialysis using low-flux and high-flux membranes is slower than the removal of small water-soluble substances urea, creatinine, and uric acid, and the protein-bound solute HA (RR>50%). The reduction ratio is similar for small and MM uremic retention solutes in case of dialysis modality with the highest convective transport (HDF) and the agreement is poor when using the HD.

- Spectrophotometric and fluorometric monitoring demonstrated the ability to determine the concentration values for markers of three groups of uremic toxins (SM, MM, PB) in the spent dialysate ($R \geq 0.86$).

- Good agreement between chemically and optically estimated solute removal parameters, RR (mean accuracy as BIAS ≤8.1%) and total removed solute (R from 0.727 for urea to 0.971 for IS), was achieved. The accuracy of the optical method is not influenced by the dialysis modality (HD, HF and HDF).

- All results from the optical method were compared to the carefully validated clinical chemistry laboratory methods, considered as the "golden standard". Laboratory CV values were in the range of acceptable limits (CV<10%), ensuring trustworthy study results. Measurement errors can still arise due to other factors (e.g. pathophysiological differences in patients).

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References

1. Barreto, F.C., Barreto, D.V., Liabeuf, S., et al., (2009). Serum Indoxyl Sulfate Is Associated with Vascular Disease and Mortality in Chronic Kidney Disease Patients. *Clinical Journal of the American Society of Nephrology* 4: 1551–1558.
2. Benlamkaddem, S., Iken, I., Houari, N., Elbouazzaoui, A., Boukatta, B., Sbai, H., et al. (2018). Paracetamol self-poisoning: when oral *N*-acetylcysteine saves life? A case report. *Pan Afr Med J.* 2018; 29:83. doi: 10.11604/pamj.2018.29.83.10595.
3. Bulbul, M.C., Dagel, T., Afsar, B., Ulusu, N.N., Kuwabara, M., Covic, A., Kanbay, M. (2018). Disorders of Lipid Metabolism in Chronic Kidney Disease, *Blood Purif* 2018; 46: 144–152. doi.org/10.1159/000488816.
4. Canaud., B., Morena, M., Cristol, J.P., Krieter, D. (2006). β2-microglobulin, a uremic toxin with a double meaning. *Kidney International*, 69 (8), pp. 1297–1299.
5. Clark, W.R., Dehghani, N.L., Narsimhan, V., Ronco, C. (2019). Uremic Toxins and their Relation to Dialysis Efficacy. Blood Purif 2019; 48: 299–314, doi.org/10.1159/000502331.
6. Daugirdas, J.T. et al. (2015). American KDOQI Clinical Practice Guideline for Hemodialysis Adequacy: 2015 Update. Journal of Kidney Diseases, Volume 66, Issue 5, 884–930.
7. Dhondt, A., Vanholder, R., Van Biesen, W., Lameire, N. (2000). The removal of uremic toxins. *Kidney Int*. 2000; 58: S-47–S-59. doi.org/10.1046/j.1523-1755.2000.07606.x.
8. Donadio, C., Calia, D., Ghimenti, S., et al. (2013). Uric acid is the major determinant of absorbance in spent dialysate allowing spectrophotometric evaluation of dialysis dose. *Journal of Nephrology* 27: 331–337.
9. Dou L., Jourde-Chiche N., Faure V. et al., (2007). The uremic solute indoxyl sulfate induces oxidative stress in endothelial cells. Journal of Thrombosis and Haemostasis 5: 1302–1308.
10. Duranton, F., Cohen, G., De Smet, R., Rodriguez, M., Jankowski, J., Vanholder, R., Argiles, A. (2012). European Uremic Toxin Work Group: Normal and pathologic concentrations of uremic toxins. *J Am Soc Nephrol* 2012; 23: 1258–1270.
11. EBPG (2002). European Best Practice Guidelines: SECTION II. Haemodialysis adequacy II.2 Haemodialysis dose quantification: middle molecules (MM). *Nephrology Dialysis Transplantation* 2002; 17: 21–23.
12. Eloot, S., Van Biesen, W., Glorieux, G., Neirynck, N., Dhondt, A., et al. (2013). Does the Adequacy Parameter Kt/V_{urea} Reflect Uremic Toxin Concentrations in Hemodialysis Patients? *PLOS ONE* 8(11): e76838. doi.org/10.1371/journal.pone.0076838.
13. Esbensen, K.H. (2009). *Multivariate data analysis - in practice*. Oslo, Norway: CAMO.
14. Fagugli, R.M., De Smet, R., Buoncristiani, U., Lameire, N., & Vanholder, R. (2002). Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. *American Journal of Kidney Diseases*, *40*(2), 339–347. DOI:10.1053/ajkd.2002.34518.
15. Glorieux, G., Vanholder, R. (2011). New uremic toxins - which solutes should be removed? *Contrib Nephrol.* 2011; 168: 117–128. doi: 10.1159/000321750.

16. Glorieux, G., Tattersall, J. (2015). Uraemic toxins and new methods to control their accumulation: game changers for the concept of dialysis adequacy. *Clinical kidney journal*, *8*(4), 353–362. https://doi.org/10.1093/ckj/sfv034.
17. Glorieux, G., Krieter, D. (2016). Effects on the Removal of Uremic Toxins. Hemodiafiltration. Springer, Cham. https://doi.org/10.1007/978-3-319-23332-1_14.
18. Joshi, V.D. (2014). Quality of life in end stage renal disease patients. *World Journal of Nephrology*. 2014; 3(4):308–316. doi:10.5527/wjn.v3.i4.308.
19. Kim, H.W., Kim, S.H., Kim, Y.O., et al. (2014). Comparison of the impact of high-flux dialysis on mortality in hemodialysis patients with and without residual renal function. *PLoS One*. 2014; 9(6):e97184. Published 2014 Jun 6. DOI:10.1371/journal.pone.0097184.
20. Krieter, DH., Kerwagen, S., Rüth, M., Lemke, HD., Wanner, C. (2019). Differences in Dialysis Efficacy Have Limited Effects on Protein-Bound Uremic Toxins Plasma Levels over Time. *Toxins* 2019 Jan 16; 11(1). pii: E47. doi: 10.3390/toxins11010047.
21. Kroll, M.H., Elin, R.J. (1994). Interference with clinical laboratory analyses. *Clinical Chemistry* Nov 1994, 40 (11) 1996–2005.
22. Lauri, K., Arund, J., Tanner, R., Jerotskaja, J., Luman, M., Fridolin, I. (2010). *Estonian Journal of Engineering*, 2010, 16, 1, 95–106. DOI: 10.3176/eng.2010.1.09.
23. Lauri, K., Arund, J., Holmar, J., Tanner, R., Luman, M., Fridolin, I. (2011). Optical Dialysis Adequacy Monitoring: Small Uremic Toxins and Contribution to UV-Absorbance Studied by HPLC. In: Angelo Carpi, Carlo Donadio and Gianfranco Tramonti (Ed.). Progress in Hemodialysis - From Emergent Biotechnology to Clinical Practice. *InTech - Open Access Publisher* (143−160).
24. Ledebo, I., Blankestijn, P. (2010). Haemodiafiltration - Optimal efficiency and safety. *NDT plus*. 3. 8–16. 10.1093/ndtplus/sfp149.
25. Lesaffer, G., De Smet, R., Lameire, N., Dhondt, A., Duym, P, Vanholder, R. (2000). Intradialytic removal of protein-bound uraemic toxins: role of solute characteristics and of dialyser membrane. *Nephrol Dial Transplant*. 2000; 15(1): 50–57. DOI: 10.1093/ndt/15.1.50.
26. Liabeuf, S., Drüeke, T.B., Massy, Z.A. (2011). Protein-Bound Uremic Toxins: New Insight from Clinical Studies. *Toxins* 2011, *3*, 911–919.
27. Lisowska-Myjak, B. (2014). Uremic Toxins and Their Effects on Multiple Organ Systems. *Nephron Clin Pract* 2014; 128: 303–311. DOI: 10.1159/000369817.
28. NKF-KDOQI, 2006, *Clinical Paractice Guidlines for Hemodialysis Adequacy, update 2006*, www.kidney.org/professionals/KDOQI/guideline_upHD_PD_VA/index.htm.
29. Neirynck, N., DE Smet, R., Schepers, E., Vanholder, R., & Glorieux, G. (2012). Classification and list of uremic toxins. In T. Niwa (Ed.), *Uremic toxins* (pp. 13–33). Hoboken, NJ, USA: John Wiley & Sons.
30. Nikolac, N. (2014). Lipemia: causes, interference mechanisms, detection and management. *Biochem Med (Zagreb)*. 2014; 24(1): 57–67. Published 2014 Feb 15. doi:10.11613/BM.2014.008.
31. Mainali, S., Davis, S.R., et al. (2017). Frequency and causes of lipemia interference of clinical chemistry laboratory tests. *Practical Laboratory Medicine*, 2017; 8: 1–9.

32. Munjanja, B., Sanganyado, E. (2015). UV-Visible Absorption, Fluorescence, and Chemiluminescence Spectroscopy. *Handbook of Food Analysis*, Edition: 3rd, Chapter: 31, Publisher: CRC Press. 10.1201/b18668-73.
33. Parikh, A., Chawla, L.S. (2013). Continous Renal Replacement Therapy. *Critical Care Medicine E-Book: Principles of Diagnosis and Management in the Adult*, Edition: 4rd, Chapter 18, Elsevier Health Sciences, Oct 16, 2013.
34. Passos, M., Lúcia M., Saraiva, M. (2019). Detection in UV-visible spectrophotometry: Detectors, detection systems, and detection strategies, *Measurement*, Volume 135, 896–904. https://doi.org/10.1016/j.measurement.2018.12.045.
35. Perkin Elmer User assistance (2000). An Introduction to Fluorescence Spectroscopy, Perkin Elmer Ltd., UK.
36. Peterson, W. (1997). The Measurement Of Sensitivity In Fluorescence Spectroscopy, *Photon Technology International, Inc.,* September 22, 1997. www.photonicsonline.com/doc/the-measurement-of-sensitivity-in-fluorescenc-0002.
37. Schiffl, H. (2019). Online hemodiafiltration and mortality risk in end-stage renal disease patients: A critical appraisal of current evidence. *Kidney Res Clin Pract* 2019; 38(2): 159–168.
38. Shunsuke, I., Masayuki, Y. (2014). Protein-Bound Uremic Toxins: New Culprits of Cardiovascular Events in Chronic Kidney Disease Patients. *Toxins* 6, no. 2: 665–678.
39. Serjeant, L., Evans, J., Sampaziotis, F., & Petchey, W. G. (2017). Haemodialysis in acute paracetamol poisoning. *BMJ case reports*, *2017*, bcr2016218667. https://doi.org/10.1136/bcr-2016-218667.
40. Sirich, T.L., Luo, F.J.G., Plummer, N.S., Hostetter, T.H., Meyer; T.W. (2012). Selectively increasing the clearance of protein-bound uremic solutes, *Nephrology Dialysis Transplantation*, Volume 27, Issue 4, 1 April 2012, Pages 1574–1579, https://doi.org/10.1093/ndt/gfr691.
41. Tanaka, H., Sirich, T.L., Plummer, N.S., Weaver, D.S., Meyer, T.W. (2015). An Enlarged Profile of Uremic Solutes. *PLOS ONE* 10(8): e0135657. https://doi.org/10.1371/journal.pone.0135657.
42. Tattersall, J., Martin-Malo, A., Pedrini, L., Basci, A., Canaud, B., Fouque, D., et al. (2007). EBPG guideline on dialysis strategies. *Nephrology Dialysis Transplantation*, *22 (Suppl 2)*, ii5–ii21. DOI: 10.1093/ndt/gfm022
43. Tattersall, J., Ward, R. (2013). EUDIAL group. Online haemodiafiltration: definition, dose quantification and safety revisited. *Nephrol Dial Transplant*. 2013 Mar; 28(3): 542–550. DOI: 10.1093/ndt/gfs530.
44. Tomson, R., Fridolin, I., Uhlin, F., Holmar, J., Lauri, K., Luman, M. (2012). Development of the model for the optical monitoring of urea in spent dialysate, in *Electronics Conference (BEC), 13th Biennial Baltic*. 2012, pp. 179–182.
45. Tomson, R., Fridolin, I., Uhlin, F., Holmar, J., Lauri K., Luman, M. (2013). Optical measurement of creatinine in spent dialysate**.** *Clinical Nephrology* 2013; 79: 107–117. DOI: 10.5414/CN107338.
46. Uhlin, F., Fridolin, I., Lindberg, L., Magnusson, M. (2005). Estimating total urea removal and protein catabolic rate by monitoring UV absorbance in spent dialysate. *Nephrol Dial Transplant* 20: 2458–2464.

47. Uhlin, F., Fridolin, I. (2013). Optical Monitoring of Dialysis Dose. In: Azar A. (eds) Modeling and Control of Dialysis Systems. Studies in Computational Intelligence, vol 405. Springer, Berlin, Heidelberg. DOI: https://doi.org/10.1007/978-3-642-27558-6_3.

48. Vanholder, R., De Smet, R., & Lameire, N. (2001). Protein-bound uremic solutes: The forgotten toxins. *Kidney International*, *59*, S266–S270. DOI: 10.1046/j.1523-1755.2001.59780266.x

49. Vanholder, R., De Smet, R., Glorieux, G., Argilés, A., Baurmeister, U., Brunet, P., et al. (2003). Review on uremic toxins: Classification, concentration, and interindividual variability. *Kidney International*, *63*(5), 1934–1943. DOI: 10.1046/j.1523-1755.2003.00924.x

50. Vanholder, R., Pletinck, A., Schepers, E., Glorieux, G. (2018). Biochemical and Clinical Impact of Organic Uremic Retention Solutes: A Comprehensive Update. *Toxins* 2018, *10*, 33. DOI.org/10.3390/toxins10010033

51. Zumrutdal, A., Sezer, S., Demircan, S., Seydaoglu, G., Ozdemir, FN., Haberal, M. (2005). Cardiac troponin I and beta 2 microglobulin as risk factors for early-onset atherosclerosis in patients on haemodialysis. *Nephrology*. 2005; 10(5): 453–458. DOI: 10.1111/j.1440-1797.2005.00475.x.

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Abstract

Elimination of Uremic Toxins during Dialysis assessed with the Optical and Analytical Methods

The kidneys have many functions, whereas one of the primary functions is to purify the blood from waste products. If kidneys are damaged and their function fails, an excessive amount of water and waste products accumulate in the blood and the body. These waste products can become toxic if not removed from the body. Therefore, these substances are also called uremic toxins. Dialysis treatment is used for the artificial elimination of these toxins. It is important to monitor the elimination of these toxins to ensure the patient's quality of life. So far, a urea-based dialysis efficiency parameter, Kt/V, is mostly assessed. Also, blood levels of other low molecular substances, such as creatinine and uric acid, are measured periodically. However, a long list of uremic toxins which may affect the patient's survival has been identified, including middle molecules (MM) and protein-bound solutes (PBS). Assessment of dialysis quality should be simple and usable in daily practice, but concentrations of all these substances cannot be determined in a clinical laboratory due to missing technology or expensive analytical methods.

The aim of this thesis was to investigate the removal of uremic toxins by different dialysis modalities using optical measurements in spent dialysate and compare the results to analytical laboratory methods. The dialysis efficiency parameters, reduction ratio (RR) and total removed solute (TRS), were used for comparison. It was found that the UV absorbance at a wavelength of 280 nm estimates mainly the elimination of lower molecular water-soluble molecules, such as uric acid (UA), confirming the results reported in earlier studies. In addition, it was found that the elimination of protein-bound solute indoxyl sulphate (IS) was slower than the elimination of the water-soluble molecules. By evaluating the urea elimination rate with UV absorbance at 297 nm, a sufficiently good measurement accuracy was obtained for all modalities. However, RR estimation with UV absorbance for middle molecules (MM) is reliable only using the high convection modality, HDF. The elimination for MM is low with the conventional hemodialysis (HD).

In addition to spectrophotometric method, the fluorometric monitoring was used to investigate the elimination of marker molecules in all three toxin groups (SM, MM, PB). A good agreement between the laboratory and optical results was found by evaluating RR (BIAS ≤ 8.1%) and TRS. The accuracy of the optical method is not affected by different modalities. The results of the optical measurements were compared to the results of standardized laboratory methods (CV<10%) ensuring the reliability of the optical measurement results.

In conclusion, the optical methods and the results presented in this thesis promote further applications for monitoring elimination of uremic toxins and development of innovative technologies for assessing the quality of dialysis.

Lühikokkuvõte

Ureemiliste toksiinide elimineerimise hindamine dialüüsravil optiliste ja analüütiliste meetoditega

Inimese neerud täidavad eri funktsioone, kuid peamine neist on vere puhastamine organismis leiduvatest jääkainetest. Kui neerud on kahjustatud ja nende funktsioon ei toimi, koguneb verre ja kehasse liigne kogus vett ja jääkaineid, mis võivad muutuda toksiliseks, kui neid kehast ei väljutata. Seetõttu nimetatakse neid aineid ka ureemilisteks toksiinideks. Dialüüsravi kasutatakse nende toksiinide kunstlikuks eemaldamiseks. Patsiendi elukvaliteedi tagamiseks on oluline jälgida nende toksiinide eemaldamist. Seni on kasutatud uureal põhinevat dialüüsi efektiivsuse parameetri, Kt/V hindamist. Samuti mõõdetakse perioodiliselt ka teiste madalamolekulaarsete ainete, kreatiniini ja kusihappe sisaldust veres. Kuid on kindlaks tehtud pikk loend ureemilisi toksiine, mis võivad mõjutada patsiendi elulemust. Nende hulka kuuluvad ka keskmise molekulmassiga (MM) ja valkudega seotud (PB) molekulid. Dialüüsi kvaliteedi hindamine peaks olema lihtne ja igapäevases praktikas kasutatav, kuid nende kõigi ainete kontsentratsioone ei saa kliinilises laboris määrata puuduva tehnoloogia või kallite analüüsimeetodite tõttu.

Doktoritöö eesmärk oli uurida eri omaduste ja suurusega molekulide eemaldamist eri dialüüsi modaliteetidega, kasutades heitdialüsaadi optilist mõõtmist, ja võrrelda neid analüütiliste laborimeetoditega saadud tulemustega. Võrdlemiseks kasutati dialüüsi efektiivsuse parameetreid elimineerimise määr (RR) ja kogu elimineeritud aine hulk (TRS). Töö tulemusena leiti, et lainepikkusel 280 nm saab UV-neelduvusega hinnata peamiselt madalamolekulaarsete veeslahustuvate molekulide, näiteks kusihappe (UA), elimineerimist, mis kinnitab varasemates uuringutes esitatud tulemusi. Lisaks leiti, et valkudega seotud indoksüülsulfaadi (IS) elimineerimine oli aeglasem kui veeslahustuvate molekulide puhul. Hinnates uurea elimineerimise kiirust UV-neelduvusega lainepikkusel 297 nm, saadi piisavalt hea mõõtetäpsus kõigi modaalsuste jaoks. Kuid RR hindamine UV-neelduvuse põhjal on usaldusväärne keskmiste molekulide (MM) jaoks ainult kõrge konvektsiooniga modaalsuse (HDF) puhul. MM elimineerimine HD juures on madal.

Lisaks spektrofotomeetrilisele meetodile kasutati ka fluoromeetrilist uuringut, et uurida kõigi kolme toksiinide grupi (SM, MM, PB) markerainete elimineerimist. Hea kokkulangevus laboratoorsete ja optiliste tulemuste vahel leiti hinnates RR-i (BIAS ≤ 8.1%) ja TRS-i. Eri modaliteedid ei mõjuta optilise meetodi täpsust. Optiliselt mõõdetud tulemusi võrreldi standardsete laboratoorsete meetoditega (CV<10%), tagades sellega optiliste mõõtmistulemuste usaldusväärsuse.

Kokkuvõtteks võib öelda, et optilised meetodid ja selles lõputöös esitatud tulemused on eelduseks optiliseks ureemiliste toksiinide kõrvaldamise jälgimiseks ja innovaatiliste tehnoloogiate väljatöötamiseks dialüüsi kvaliteedi hindamiseks.

Appendix

Publication I

Lauri, K., Tanner, R., Jerotskaja, J., Luman, M., & Fridolin, I. (2010). HPLC study of uremic fluids related to optical dialysis adequacy monitoring. *Int J Artif Organs*, *33*(2): 96–104 (DOI: 10.1371/journal.pone.0162346).

Int J Artif Organs 2010; 33 (2): 96-104

Original Article

HPLC study of uremic fluids related to optical dialysis adequacy monitoring

Kai Lauri[1], Risto Tanner[2], Jana Jerotskaja[1], Merike Luman[3], Ivo Fridolin[1]

[1] Department of Biomedical Engineering, Technomedicum, Tallinn University of Technology, Tallinn
[2] Laboratory of Chemical Physics, National Institute of Chemical Physics and Biophysics, Tallinn
[3] Department of Dialysis and Nephrology, North Estonia Medical Centre, Tallinn - Estonia

ABSTRACT: Purpose: *The aim of this study was to investigate uremia-related high-performance liquid chromatography (HPLC) ultraviolet (UV) absorbance profiles of serum and spent dialysate and to study the removal of uremic retention solutes in connection with optical dialysis adequacy monitoring.*
Methods: *10 uremic patients were investigated using online spectrophotometry at a wavelength of 280 nm over the course of 30 hemodialysis treatments. The dialysate and blood samples were taken and analyzed simultaneously using standard biochemical methods and reversed-phase HPLC. Filters with cutoff at 3 kDa and 70 kDa were used for the pre-treatment of the serum. The chromatographic peaks were detected by a UV detector at wavelengths of 254 and 280 nm.*
Results: *This study indicated that the main solute responsible for UV absorbance in the spent dialysate is a low-molecular-weight, water-soluble, non-protein-bound compound uric acid (UA). Three additional uremic retention solutes – creatinine (CR), indoxyl sulphate (IS) and hippuric acid (HA) – were identified from the HPLC profiles. The number of detected HPLC peaks was not significantly different for a serum filtered through the 3 kDa or 70 kDa cutoff filters, and was lower for the spent dialysate, indicating that the molecular weight (MW) of the main UV chromophores in the uremic fluids did not exceed 3 kDa. The reduction ratio (RR) estimated by the total area of HPLC peaks at 254 nm and 280 nm in the serum and by the online UV absorbance at 280 nm was best related to the removal of small water-soluble non-protein bound solutes like urea (UR), CR and UA.*
Conclusions: *The present study contributes new information on the removal of uremic retention solutes during hemodialysis and on the origin of the optical dialysis adequacy monitoring signal. (Int J Artif Organs 2010; 33: 96-104)*

KEY WORDS: Hemodialysis, HPLC, Uremic toxins, Ultraviolet spectrophotometry, Monitoring

INTRODUCTION

Uremic toxicity or the uremic syndrome is described as a clinical picture resulting from impaired renal elimination and accumulation of uremic toxins in the body. To date, a long list of possible uremic toxins have been identified that are believed to be responsible for the multifactorial and cumulative cause of uremic toxicity (1, 2). A deeper understanding about the accumulation and removal mechanisms of the retained solutes during care of renal insufficiency is thus necessary (3, 4). This understanding would be especially informative for predicting the mode of action of uremic toxins and their specific role in complications associated with dialysis or uremia, but also with regard to cardiovascular disease and inflammation (5, 6). It would also allow the methods contributing to the identification, characterization, and evaluation of uremic retention solutes to be assessed in order to ensure dialysis adequacy and quality (7).

Reversed-phase high-performance liquid chromatography (HPLC) is recommended as a sensitive, accurate and reproducible tool applicable to the separation of different compounds in aqueous samples (8). Earlier HPLC studies aimed to examine the behavior of non-protein-bound and

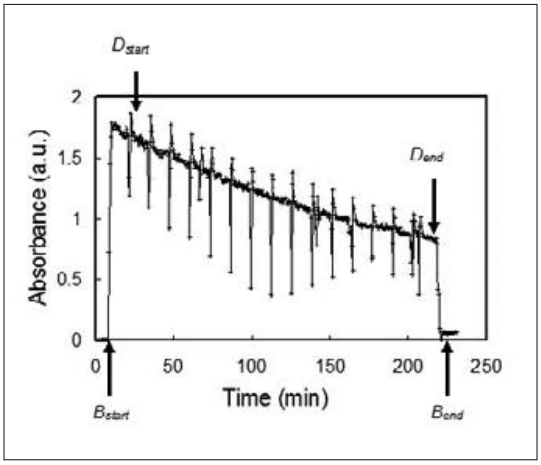

Fig. 1 - *Typical online absorbance curve during a single hemodialysis treatment with UV absorbance at wavelength 280 nm is plotted against time. Time points when the samples were taken for the later analysis are as follows: D_{start} – dialysate sample collected 10 min after the start of hemodialysis, D_{end} – dialysate sample collected at the end of hemodialysis, B_{start} – blood sample collected before dialysis session, B_{end} – blood sample collected at the end of hemodialysis.*

protein-bound uremic solutes (9, 10). The serum is often analyzed by HPLC studies, but the dialysate, which may be a preferred alternative to continuous monitoring of solute removal and adequacy for dialysis, has not been the subject of research to the same extent.

With the aim of continuously monitoring a single hemodialysis session, spectrophotometrical sensors for online monitoring of total UV absorbance or UR in the spent dialysate have recently been introduced (11-14). Findings showing a good correlation between the UV absorbance measured in the spent dialysate and the concentration of several solutes, both in the spent dialysate and in the blood of dialysis patients, have previously been presented (11, 15). UV extinction as an indicator of nucleic acid metabolism has also been discussed (16). The cumulative and integrated UV absorbance measured in the spent dialysate due to the contribution of different chromophore compounds indicates the potential for the technique to be used for estimating the removal of uremic solutes. However, the question of which main chromophores are responsible for the UV absorbance in the spent dialysate according to the classification of uremic solutes remained.

The aim of this study was thus to investigate uremic syndrome-related HPLC UV absorbance profiles of the serum and spent dialysate and to study the removal of uremic retention solutes in connection with optical dialysis adequacy monitoring.

MATERIALS AND METHODS

Clinical study

This study was performed after the protocol was approved by the Tallinn Medical Research Ethics Committee at the National Institute for Health Development, Estonia. Informed consent was obtained from all participating patients.

Ten uremic patients, three females and seven males, mean age 62.6 ± 18.6 years, on chronic three-time weekly hemodialysis were included in the study at the Department of Dialysis and Nephrology, North Estonia Medical Centre. Measurements were performed on collected dialysate and blood samples and online (17). Three different polysulphone dialyzers were used (Fresenius Medical Care, Bad Homburg, Germany): a low-flux (LF) membrane dialyzer F8 HPS (N=14) with an effective membrane area of 1.8 m^2 and an ultrafiltration (UF) coefficient of 18 ml/h x mmHg, LF membrane dialyzer F10 HPS (N=3) with an effective membrane area of 2.2 m^2, a UF coefficient of 21 ml/h x mmHg, and a high-flux (HF) membrane dialyzer FX 80 (N=13) with an effective membrane area of 1.8 m^2, and a UF coefficient of 59 ml/h x mmHg.

The dialysate flow was 500 ml/min and the blood flow varied between 245 to 350 ml/min. The dialysis machine used was the Fresenius 4008H (Fresenius Medical Care).

All treatments were monitored optically by a spectrophotometer (HR2000, Ocean Optics, Inc., Dunedin, FL, USA) which was used to determine UV absorbance. The device was provided with a specially designed optical cuvette connected to the fluid outlet of the dialysis machine which all the spent dialysate passed through during the online experiments. The absorbance was measured in arbitrary units (a.u.). The sampling frequency was set to two samples per minute. The UV absorbance values were processed and presented on the computer screen by a PC connected to the spectrophotometer using Ocean Optics software (OOIBase32, version 2.0.2.2 for Windows).

Figure 1 shows a typical online absorbance curve during

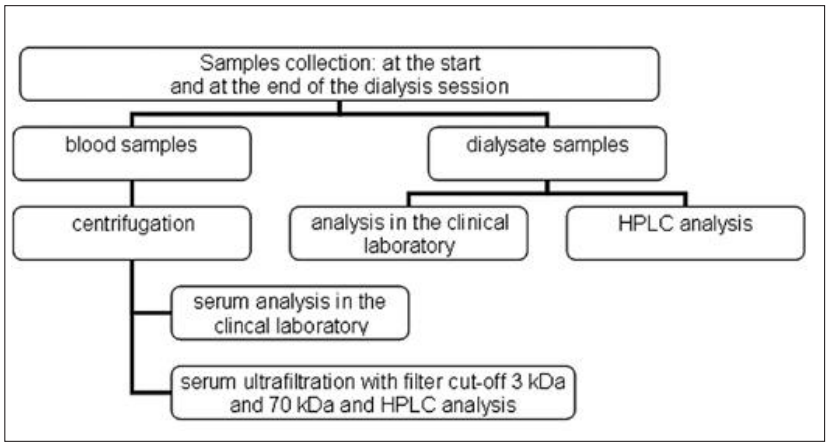

Fig. 2 - *Block diagram of blood and dialysate sample collection and handling.*

a single hemodialysis treatment where UV absorbance at wavelength 280 nm is plotted against time. UV absorbance troughs and peaks during the dialysis correspond to the self-tests in the dialysis machine when the dialyzer is in the by-pass mode.

Blood and dialysate samples were obtained from dialysis patients. Blood samples were drawn before the start of dialysis treatment (B_{start}) and immediately after the treatment (B_{end}) (Fig. 1) using the slow flow/stop pump sampling technique. Blood was sampled into a BD Vacutainer® Glass Serum Tube (red cup, Becton Dickinson, New Jersey, USA) and was allowed to clot. After centrifugation at 3000 rpm the serum was separated from the blood cells.

Dialysate samples were taken 10 minutes after the start of the dialysis session (D_{start}), and immediately before the end of dialysis (D_{end}) (210 or 240 min). Also, pure dialysate used as the reference solution was collected before each dialysis session when the dialysis machine was prepared and the conductivity was stable. Figure 2 presents a block diagram of blood and dialysate sample collection as well as how they were handled.

Biochemical analyses

The serum and the dialysate samples were analyzed immediately at the Clinical Chemistry Laboratory, North Estonia Medical Centre. Creatinine (CR, MW=113.12 Da), uric acid (UA, MW=168.11 Da), urea (UR, MW=60.06 Da) and phosphate (P, MW=96.17 Da) were measured with a Hitachi 912 autoanalyzer (Roche, Rotkreuz, Switzerland). The coefficient variation (CV) of the methods used for the determination of different solutes in dialysate and blood were CR 5%, UA 2%, UR 4%, and P 2%.

Sample preparation for HPLC analysis

Before the HPLC analysis, the serum samples were purified of proteins by centrifuging with Microcon centrifugal filters (Millipore, Billerica, MA, USA) at room temperature. Two different filters were compared during the study: the YM-3 filter (3 kDa cutoff, Yellow Base), and the YM-100 filter (70 kDa cutoff, Blue Base). The dialysate samples were acidified down to pH 4.0 with formic acid for conformation with the pH of the chromatographic eluent used. The sample size of 100 µL for the dialysate and the serum end sample, and 50 µL for the serum start sample, were used for chromatographic separation.

Reversed-phase HPLC study

The HPLC system consisted of a quaternary gradient pump unit, a column oven, and a diode array spectrophotometric detector (DAD, all Series 200 instruments from Perkin Elmer, Norwalk, CT, USA), a manual injector from Rheodyne (Rohnert Park, CA, USA), and a Zorbax C8 4.6 x 250 mm column from Du Pont Instruments (Wilmington, DE, USA) with security guard KJO-4282 from Phenomenex (Torrance, CA, USA). The eluent was mixed with 0.05 M

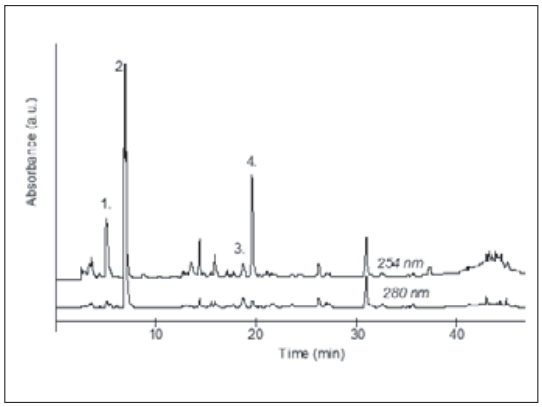

Fig. 3 - *Characteristic HPLC profiles of the serum (filter cutoff: 3 kDa) monitored at the different wavelengths. Blood samples were collected before the dialysis session (B_{start}) and monitored at wavelengths of 254 nm and 280 nm. Detected HPLC peaks- uremic toxins: 1. creatinine (CR), 2. uric acid (UA), 3. indoxyl sulphate (IS), 4. hippuric acid (HA).*

TABLE I - ELUTION PROGRAM USED FOR HPLC SEPARATION OF CONSTITUENTS IN THE UREMIC FLUIDS

Step	Time (MIN)	Buffer (A) %	Methanol (B) %	Acetonitrile (C) %	Gradient
0	0	100	0	0	-
1	30	60	36	4	Linear
2	5	10	81	9	Linear
3	4	10	81	9	No grad
4	1	10	0	90	Linear
5	6	10	0	90	No grad

formic acid adjusted to pH 4.0 with ammonium hydroxide (A), HPLC grade methanol (B) and HPLC-S grade acetonitrile (C), all from Rathburn (Walkerburn, Scotland), with a six step gradient program as specified in Table I.

The total flow rate of 1 ml/min was used continuously and the column temperature was adjusted to 30°C. The UV absorbance was monitored at 280 nm with a measurement interval of 880 ms, spectra registered between 200 nm and 400 nm with a time interval of 1.76 seconds, and data was processed, respectively, by means of Turbochrom WS and Turboscan 200 software from Perkin Elmer (Waltham, MA, USA). Spiking experiments and UV spectra between 200 nm and 400 nm allowed us to identify predominant uremic toxins in four chromatographic peaks identified as CR, UA, IS and HA (Fig. 3).

Data analysis

The reduction ratio (RR) (%) of compounds was defined as a function of pre-dialysis concentration (C_{pre}) and concentration at the end of hemodialysis (C_{post}):

$$RR = \frac{C_{pre} - C_{post}}{C_{pre}} 100\% \quad [1]$$

C_{pre} and C_{post} were replaced by TA $HPLC_{pre}$ and TA $HPLC_{post}$ representing the total area of the HPLC peaks measured at the start, and the end samples for RR from HPLC, respectively.

Results are presented as mean ± standard deviation (SD). Student's t-test was used to compare groups of values while p value less than 0.05 was considered significant. Pearson's correlation coefficient (r) between the UV absorbance from HPLC and online monitoring versus concentration of the substances in the blood was investigated. Samples taken at times coinciding with the self-tests or alarms of the dialysis machine were excluded (3 out of 60 dialysate samples). Some sessions were excluded due to the technical failure of the spectrophotometer (3 out of 30 sessions) and due to laboratory errors (3 out of 30 sessions, 6 out of 30 sessions for P). The data analyses were performed in Microsoft Excel 2003 (for Windows).

RESULTS

Figure 3 presents characteristic HPLC profiles of the serum collected before the dialysis session (B_{start}), measured at the wavelengths of 254 nm and 280 nm. A number of higher prevalent peaks representing chromophore-uremic toxins can be observed. Some HPLC peaks were identified, including 1. CR, 2. UA (the highest contribution), 3. IS, 4. HA. The absorption of most components is higher at 254 nm than at 280 nm.

Figure 4 presents characteristic HPLC profiles of the spent dialysate collected 10 minutes after the start of the hemodialysis (D_{start}) and at the end of the treatment (D_{end}), measured at 280 nm. The decrease in the height of the peaks due to the solute removal is clearly seen.

The number of detected HPLC peaks in the serum and dialysate (mean±SD) measured at 254 nm and 280 nm for

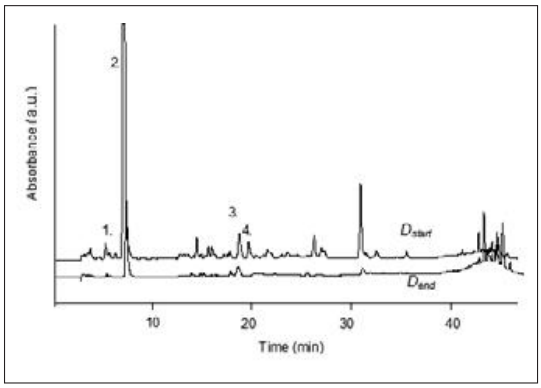

Fig. 4 - *Characteristic HPLC profiles of the spent dialysate monitored at wavelength 280 nm; D_{start} - dialysate sample collected 10 min after the start of hemodialysis, D_{end} - dialysate sample collected at the end of hemodialysis. Detected HPLC peaks-uremic toxins: 1. creatinine (CR), 2. uric acid (UA), 3. indoxyl sulphate (IS), 4. hippuric acid (HA).*

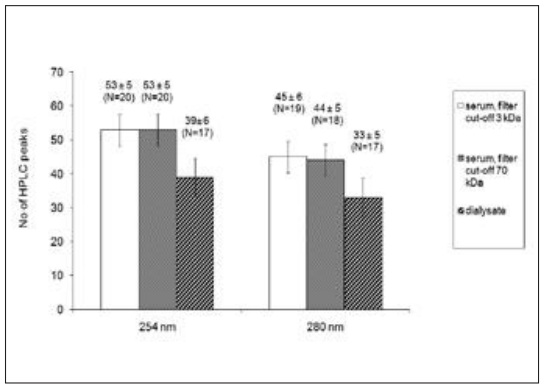

Fig. 5 - *Number of detected HPLC peaks at wavelengths 254 nm and 280 nm in serum (3 kDa and 70 kDa filter cutoffs, respectively) and in dialysate.*

one session per patient is presented in Figure 5. Combining the results from the start and end samples there was no significant difference between the results for serum filtered through the 3 kDa or 70 kDa cutoff filters ($p<0.05$). In comparison with the dialysate, a higher number of peaks can be observed in the serum ($p < 0.05$). A higher number of detected HPLC peaks in the spent dialysate and serum was also observed at 254 nm than at 280 nm ($p<0.05$).

The LF (F8 HPS, F10 HPS) and HF (FX80) membranes showed similar RR for every uremic solute. Combining the results from the LF and HF membranes, three groups were distinguished according to the average RR among the studied solutes (Fig. 6): (i) "high RR" solutes (RR>60%) including the classic marker UR, the small uremic toxin UA and the protein–bound toxin HA; (ii) "medium RR" solutes (50%<RR<60%), including the classic marker CR and the protein-bound solute P, removed with intermediate efficiency; (iii) "low RR" solute (RR<50%), the protein-bound uremic toxin IS. A statistically dissimilar RR was found between the solutes of different groups, and while no statistically different RR was detected between the solutes of the same group ($p<0.05$).

All UV absorbance-based RR values were higher than the "low RR" solute IS. The RR of the TA HPLC at 280 nm was no different from the "high RR" solutes (UR, UA and HA), but was higher than the "medium RR" solutes (P and CR). However, the RR of the TA HPLC at 254 nm and in

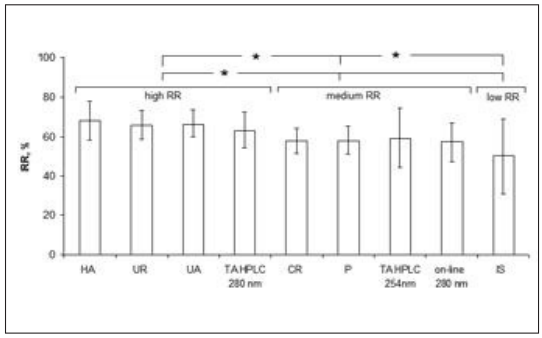

Fig. 6 - *A comparison between the RR of hippuric acid (HA), urea (UR), uric acid (UA), creatinine (CR), phosphate (P), indoxyl sulphate (IS), total area RR of HPLC peaks in the serum (TA HPLC) at 254 nm and 280 nm (3 kDa filter cutoff) and online RR of UV absorbance at 280 nm in the spent dialysate (online 280 nm). Significant differences ($p<0.05$) are marked with an asterisk (*).*

online measurements at 280 nm were comparable with the "medium RR" solutes (P and CR) but were lower than the "high RR" solutes (UR, UA, HA) and the TA HPLC at 280 nm.

The correlation coefficients between the RR from the total area of the HPLC peaks at 254 nm and at 280 nm, from online monitoring at 280 nm, and the RR for the uremic solutes in the serum are shown in Table II. The number of

dialysis sessions included was 25 for UA, 24 for CR, UR and HA, 21 for IS, and 20 for P. If not given separately, the significance level on results in Table II was $p < 0.01$. A high r value was obtained between small water-soluble non-protein-bound molecules (UA, CR and UR) and UV absorbance in the spent dialysate and in the serum. Some differences regarding 254 nm and 280 nm can be observed. A lower correlation was obtained for the small protein-bound molecules HA and IS, and for at least partly protein-bound molecule P.

DISCUSSION

This study investigated uremia-related HPLC UV absorbance profiles of the serum and spent dialysate and studied the removal of uremic retention solutes in connection with optical dialysis adequacy monitoring.

The results indicated that: (i) the main solute responsible for UV absorbance at 280 nm is a low-molecular-weight, water-soluble, non-protein-bound compound UA; (ii) three additional uremic retention solutes – CR, IS and HA – were identified from the HPLC profiles contributing to the UV absorbance; (iii) the number of detected HPLC peaks was not significantly different for the serum filtered with filters with a cutoff at 3 kDa or 70 kDa, and was lower for the spent dialysate, indicating that the MW of the main UV chromophores in the uremic fluids did not exceed 3 kDa; (iv) the intradialytic removal efficiency estimated by the total area of HPLC peaks at 254 nm and 280 nm in the serum and online UV absorbance at 280 nm was best correlated to the removal of small water-soluble non-protein-bound solutes like UR, CR and UA and did not represent similarly fine removal of small protein-bound solutes HA, IS and P; (v) The LF (F8 HPS, F10 HPS) and HF (FX80) membranes showed similar RR for all studied uremic solutes.

Figure 1 shows that UV absorbance decreases during the session as the waste products are removed. This demonstrates the possibility of continuously monitoring a single hemodialysis session and of monitoring deviations in dialyzer performance using the online monitor-based UV absorbance. Moreover, the clinical dialysis adequacy parameters (e.g., Kt/V) can be calculated from the online curve (17).

A number of the higher prevalent peaks on the HPLC profiles of the spent dialysate (Figs. 3 and 4) indicate that there exists a group of compounds, namely, UV chromophores, which are the main cause of cumulative and integrated UV absorbance. Several research groups have investigated HPLC profiles of uremic fluids, both serum fractions (18) and dialysate (19). This indicates that UV absorbance represents a bulk removal of the uremic retention solutes, several of them uremic toxins. Variations in the number of HPLC peaks depending on hemodialysis treatments (19) as well as patients (20) have been demonstrated.

Among the compounds identified on our chromatograms, IS and HA are commonly considered to belong to the group of small protein-bound uremic toxins (1). We compared chromatograms of serum samples filtered through the 3 kDa and the 70 kDa cutoff filters (Fig. 5) and could not find any significant differences between the results, including peaks of both the IS and HA compounds.

TABLE II - PEARSON CORRELATION COEFFICIENTS BETWEEN RR OF SMALL NON-PROTEIN-BOUND AND PROTEIN-BOUND MOLECULES VS. RR OF UV ABSORBANCE

	Small non-protein-bound molecules			Small protein-bound molecules		
	UR	CR	UA	p	HA	IS
TA HPLC 254nm	0.811	0.812	0.755	0.441 (p=0.052)	0.734	0.170 (P=0.487)
TA HPLC 280nm	0.881	0.926	0.933	0.580	0.633	0.273 (P=0.273)
online A 280 nm	0.873	0.888	0.890	0.556 (p=0.017)	0.568	0.478 (P=0.045)

The Pearson correlation coefficients between the RR for small non-protein-bound molecules urea (UR), creatinine (CR), uric acid (UA), small protein-bound molecules phosphate (P), hippuric acid (HA) and indoxylsulphate (IS) in the serum vs. RR UV-absorbance from the total area of the HPLC peaks (TA HPLC) at 254 and 280 nm, and from on-line monitoring at 280 nm (on-line A 280 nm). If not given separately, the significance level on results is P<0.01.

This observation indicates that all main UV chromophores in the serum are comparatively small molecules with MW below 3 kDa. Also, the comparison proves that relatively larger proteins, with MW over 70 kDa, must be involved in binding the UV-absorbing uremic toxins in the serum. The difference in the number of detected HPLC peaks at 254 nm and 280 nm (Figs. 3 and 5) arises due to characteristic absorbing spectra of the UV chromophores. The absorption of many components is higher at the wavelength of 254 nm than at the wavelength of 280 nm. This confirms the results obtained by the spectrophotometrical analysis in this UV region (15).

The LF and HF membranes showed no difference in the removal of the uremic solutes as observed earlier by Lesaffer at al (21). Taking into account both the removal efficiency (Fig. 6) and the correlation analysis (Tab. II), a difference can be observed in the relation of the UV absorbance to small water-soluble non-protein-bound solutes and to small protein-bound solutes.

The removal efficiency was highest (except for HA) and the UV absorbance was best correlated to the removal of the small water-soluble, non-protein-bound solutes UA, CR and UR.

Removal of UA has the highest correlation for the UV absorbance decrease at 280 nm both in the serum and in the spent dialysate, but not at 254 nm in the serum (Tab. II). The outcome can be explained by the higher contribution of UA to UV absorbance compared to other chromophores at 280 nm (Figs. 3 and 4) due to the higher millimolar extinction coefficient for UA (15). However, there are several other strong contributions from other compounds beside UA at 254 nm (e.g., CR), and therefore the correlation is lower.

Removal of CR has the highest correlation with UV absorbance decrease at 280 nm in the serum and in the spent dialysate, and relatively high correlation at 254 nm in the serum (Table II). The reason could be the similar removal of CR and other chromophores absorbing 280 nm, where CR does not contribute significantly to the UV absorbance (Figs. 3 and 4). In contrast, a relatively large contribution of CR to UV absorbance at 254 nm exists, as can be seen from the millimolar extinction coefficients for CR (15).

Removal of UR is strongly related to the RR at 280 nm both in the serum and in the spent dialysate, but less so at 254 nm in the serum. This means that a relatively good correlation between the RR of UV absorbance and a particular solute may be achieved when the removal rate of a non-absorbing solute (such as UR) is similar to that of UV-absorbing substances during hemodialysis (e.g., UA). This is also confirmed by a very good correlation between several small molecular-weight waste products and the UV absorbance (11) and by similar concentration changes during dialysis for several azotemic markers (e.g., UR, CR, UA and pseudouridine) (20). This makes it possible to estimate the delivered dialysis dose in terms of Kt/V by monitoring the UV absorbance in the spent dialysate online (17). The tendency that the reduction of online UV-absorbance is lower than the reduction of UR has been reported earlier (17), since the reduction ratio of UR is correlated to Kt/V (1).

Removal of small protein-bound solutes P, HA and IS seems to be strongly molecule dependent. A higher removal efficiency for HA compared to other protein-bound solutes has been reported earlier (21, 22) and could be due to its lower level of protein binding (21). A different binding of individual uremic retention solutes to serum proteins may modify the percentage of concentration changes of individual solutes in the course of hemodialytic treatment (20), supported by observations of decreased drug/protein interactions in uremic serum (10).

The correlation between the decrease in UV absorbance and the removal of HA, IS and P is lower than a similar correlation in the case of UR, CR and UA. This provides support for the idea that UV absorbance originates mostly from non-protein bound solutes, or chromophores. Similarly, no correlations were observed for the LF membrane between the removal of UR and the removal of HA and IS (22). The lowest correlation was obtained between the decrease of UV absorbance and removal of P and IS. The reason, besides protein binding, may be that these compounds have a distribution volume that is quite different from that of small molecules. For example, since P is partly protein-bound, it is deposited in large amounts in the bone tissue (23).

The difference between the RR of the total area of HPLC peaks and online UV absorbance at 280 nm can be explained by the different number of detectable chromophores in the serum and spent dialysate (Fig. 5). Also, the fact that the serum was collected before the start of dialysis while the dialysate sample was collected 10 minutes after the start may have had some effect. The difference in RR at 254 nm and 280 nm depends at least partly on the different number of detected HPLC peaks at those wavelengths (Figs. 3 and 5) due to the characteristic absorbing spectra of the UV chromophores.

In conclusion, UV absorbance behaves more like small water-soluble, non-protein-bound solutes than small protein-bound solutes. Monitoring the removal of compounds with different properties and elimination characteristics during various dialysis strategies adds knowledge about dialysis treatment. Future research would also benefit from UV absorbance monitoring in order to decrease complications related to dialysis quality and cardiovascular risk factors.

Recommendations for future research

Exact identification of the remaining prevalent peaks on the HPLC UV absorption profiles of uremic fluids should be performed.

A multi-center study to evaluate optical dialysis adequacy monitoring on a larger scale and for different dialysis modalities (HD vs. HDF) would be valuable from the perspective of conventional dialysis adequacy parameters and the removal of uremic retention solutes.

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Address for correspondence:
Kai Lauri
Department of Biomedical Engineering
Technomedicum
Tallinn University of Technology
Ehitajate Rd 5
EST-19086 Tallinn, Estonia
e-mail: kai.lauri@mail.ee

REFERENCES

1. Vanholder R, De Smet R, et al. Review on uremic toxins: classification, concentration, and interindividual variability. Kidney Int 2003; 63: 1934-43.
2. Meert N, Schepers E, De Dmet R, et al. Inconsistency of reported uremic toxin concentrations. Int J Artif Organs 2007; 31: 600-11.
3. Eloot S, Van Biesen W, Dhondt A, et al. Impact of hemodialysis duration on the removal of uremic retention solutes. Kidney Int 2008; 73: 765-70.
4. Eloot S, Torremans A, De Smet R, et al. Complex compartmental behavior of small water-soluble uremic retention solutes: evaluation by direct measurements in plasma and erythrocytes. Am J Kidney Dis 2007; 50: 279-88.
5. Vanholder R, Baurmeister U, Brunet P, et al. A Bench to Bedside View of Uremic Toxins. J Am Soc Nephrol 2008; 19: 863-70.
6. Vanholder R, De Smet R, Glorieux G, Dhondt A. Survival of Hemodialysis Patients and Uremic Toxin Removal. Artif Organs 2003; 27: 218-23.
7. Vanholder R, Laecke SV, Verbeke F, Glorieux G, Van Biesen W. Uraemic toxins and cardiovascular disease: in vitro research versus clinical outcome studies. NDT Plus 2008; 1: 2-10.
8. Vanholder R, De Smet R, Lameire NH. Redesigning the map of uremic toxins. Contrib Nephrol 2001; 133: 42-70.
9. Fagugli RM, De Smet R, Buoncristiani U, Lameire N, Vanholder R. Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. Am J Kidney Dis 2002; 40: 339-47.
10. De Smet R, Vogeleere P, Van Kaer J, Lameire N, Vanholder R. Study by means of high-performance liquid chromatography of solutes that decrease theophylline/protein binding in the serum of uremic patients. J Chromatogr A 1999; 25; 847: 141-53.
11. Fridolin I, Magnusson M, Lindberg LG. Online monitoring of solutes in dialysate using absorption of ultraviolet radiation: technique description. Int J Artif Organs 2002; 25: 748-61.
12. Jensen P, Bak J, Ladefoged S, Andersson-Engels S. Determination of urea, glucose, and phosphate in dialysate with Fourier transform infrared spectroscopy. Spectrochim Acta A Mol Biomol Spectrosc 2004; 60: 899-905.
13. Olesberg JT, Arnold MA, Flanigan MJ. Online Measurement of Urea Concentration in Spent Dialysate during Hemodialysis. Clin Chem 2004; 50: 175-81.
14. Vasilevski AM, Kornilov NV. Monitoring the dialysis liquid during hemodialysis from the extinction spectra in the UV region. J Opt Technol 1999; 66: 692-4.
15. Fridolin I, Lindberg LG. Online monitoring of solutes in di-

alysate using absorption of ultraviolet radiation - wavelength dependence. Med Biol Eng Comput 2003; 41: 263-70.
16. Vasilevsky AM, Konoplyov GA. Peculiar character of dialysate ultraviolet extinction spectra as an indicator of nucleic acid metabolism in humans. J Biomed Opt 2005; 10: doi:10.1117/1.1953268.
17. Uhlin F, Fridolin I, Lindberg LG, Magnusson M. Estimation of delivered dialysis dose by online monitoring of the UV absorbance in the spent dialysate. Am J Kidney Dis 2003; 41: 1026-36.
18. Schoots AC, Peeters JA, Gerlag PG. Effect of hemodialysis on serum concentrations of HPLC-analyzed accumulating solutes in uremia. Nephron 1989; 53: 208-17.
19. Schoots AC, Mikkers FEP, Claessens HA, DeSmet R, VanLandscoot N, Ringoir SMG. Characterization of Uremic "Middle Molecular" Fractions by Gas Chromatography, Mass Spectrometry, Isotachophoresis, and Liquid Chromatogtaphy. Clin Chem 1982; 28: 45-9.
20. Vanholder R, De Smet R, Ringoir SM. Assessment of urea and other uremic markers for quantification of dialysis efficacy. Clin Chem 1992; 38: 1429-36.
21. Lesaffer G, De Smet R, Lameire N, Dhondt A, Duym P, Vanholder R. Intradialytic removal of protein-bound uraemic toxins: role of solute characteristics and of dialyzer membrane. Nephrol Dial Transplant 2000; 15: 50-7.
22. De Smet R, Dhondt A, Galli F, Waterloos MA, Vanholder R. Effect of the super-flux cellulose triacetate dialyzer membrane on the removal of non-protein-bound and protein-bound uraemic solutes. Nephrol Dial Transplant 2007; 22: 2006-12.
23. Hruska KA. Phosphate balance and metabolism. In: Jacobson HR, Striker GE, Klahr S (eds.). The Principles and Practice of Nephrology. Philadelphia: BC Decker, Hamilton, ON; 1991. p 118-24.

Publication II

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Can Removal of Middle Molecular Uremic Retention Solutes be Estimated by UV-absorbance Measurements in Spent Dialysate?

K. Lauri[1], M. Luman[1,2], J. Holmar[1], R. Tomson[1], S. Kalle[1], J. Arund[1], F. Uhlin[1,3], and I. Fridolin[1]

[1] Department of Biomedical Engineering, Technomedicum, Tallinn University of Technology, Ehitajate Rd 5, 19086 Tallinn, Estonia
[2] Centre of Nephrology, North-Estonian Medical Centre, J.Sütiste Rd 19, 13419 Tallinn, Estonia
[3] Department of Nephrology, University Hospital, Linköping, S-581 85 Linköping, Sweden

Abstract— **The objectives of this study were: (1) to compare removal of the middle molecular (MM) and small uremic retention solutes; (2) to investigate if MM removal can be assessed by UV-absorbance at the wavelength of 297 nm during various dialysis treatment modalities.**

Seven uremic patients, four females and three males, mean age 58.1±8.7 years, were included into the study during 28 chronic hemodialysis sessions. A parameter, reduction ratio (RR) in percentage, was calculated for a small uremic retention solute urea, for a MM retention solute beta2-microglobulin (B2M), and for UV-absorbance at the wavelength of 297 during different dialysis modalities: conventional hemodialysis (HD), high flux hemodialysis (HF-HD), and post-dilutional online hemodiafiltration (HDF) with different parameter settings. Achieved results were compared regarding mean values and SD, and by systematic and standard errors (BIAS±SE).

It was found that RR is similar for small and MM uremic retention solutes in case of dialysis modality with the highest convective transport, HDF (78.9±8.1% for urea and 78.1±6.8% for B2M, N=7). Moreover, RR of small uremic retention solutes can be estimated with sufficient accuracy by UV-absorbance at 297 nm in the spent dialysate for all modalities (BIAS±SE: 1.7±4.0%, N=28), and for MM uremic retention solutes only for HDF (BIAS±SE: 1.1±7.1%, N=7). The results should be confirmed by appropriate kinetic modeling in the next studies.

Keywords— **Middle molecules, uremic toxins, beta2-microglobulin, uremic retention solutes, urea, dialysis, UV-absorbance.**

I. Introduction

Beta2-microglobulin (B2M) (MW 11 818 D) is a polypeptide and has been used as a marker for describing behavior and removal of middle molecular weight solutes during dialysis for many years. High level of serum B2M concentrations predict mortality in dialysis patients [1]. European Best Practice Guidelines (EBPG) have pointed out that despite no surrogate molecule has been identified yet with the characteristics of an ideal marker for middle molecular uremic toxins, B2M is representative in its kinetic behavior of other middle molecules (MM) and peptides of similar size, and may be used as a marker for such molecules [2].

An earlier study investigated the possibility of estimating the removal of B2M utilizing on-line UV-absorbance measurements during postdilutional online hemodiafiltration (HDF) [3]. The observed high correlation between B2M and UV-absorbance at the wavelength of 297 nm was utilized when calculating the total removal of B2M. However, the major determinant of absorbance in spent dialysate in this wavelength is a small uremic solute, uric acid (UA) [4]. This allows spectrophotometric evaluation of dialysis dose as earlier studies have shown that UV absorptions at 285 and 297 nm have a close correlation with urea-based dialysis dose estimation [5, 6]. This made it possible to develop a clinically validated online dialysis adequacy monitoring system [7]. The system measures UV-absorption versus time in the spent dialysate and calculates Urea Reduction Ratio (URR) and Kt/V, and is utilized in commercial optical dialysis dose sensors; Adimea (BBraun Avitum, Germany) [8, 9] and Dialysis Dose Monitor (Nikkiso, Japan). Assuming, that kinetic behaviour of small and MM uremic retention solutes during dialysis are different [10], controversy arises regarding assessment of both small and MM uremic retention solutes by UV-absorbance during HDF. Moreover, no results about other dialysis treatment modalities like conventional hemodialysis (HD) and high flux hemodialysis (HF-HD) are available.

The objectives of this study were: (1) to compare removal of the MM and small uremic retention solutes; (2) to investigate if MM removal can be assessed by UV-absorbance at the wavelength of 297 during various treatment modalities (HD, HF-HD and HDF).

II. Subjects

This study was performed after the approval of the protocol by the Tallinn Medical Research Ethics Committee at the National Institute for Health Development in Estonia and conducted in accordance with the Helsinki Declaration. An informed consent was obtained from all participating patients.

Seven uremic patients, four females and three males, mean age 58.1±8.7 years, on chronic hemodialysis were included in the study at Centre of Nephrology, North-Estonian Medical Centre, Tallinn, Estonia.

III. Materials and methods

A. Dialysis treatments

Two different polysulphone dialyzers, FX8 (N=7) and FX1000 (N=21) (Fresenius Medical Care, Germany), with the effective membrane area of 1.4 m2 and 2.3 m2, were used. Four different dialysis (HD, HF-HD1, HF-HD2, HDF) with different parameter settings were used (Table 1).

Table 1 Treatment data.

	HD	HF-HD1	HF-HD2	HDF
Time, min	240	240	240	240
Blood flow, mL/min	300	350	300	350
Dialysate flow, mL/min	500	500	800	800
Dialyzer	FX8	FX1000	FX1000	FX1000
Substitution volume, L	0	0	0	>19

The dialysate flow was 500 and 800 mL/min, the blood flow 300 and 350 mL/min, no substitution fluid was used for HD, HF-HD1, HF-HD2 and was higher than 19 L for HDF. The type of dialysis machine used was Fresenius 5008 (Fresenius Medical Care, Germany).

B. Sampling procedure and laboratory analysis

Blood samples were taken pre-and post-dialysis (C0 and Ct), and spent dialysate samples were taken at 7 minutes in the beginning and at the end of a session (D0 and Dt). Sometimes spent dialysate samples were taken at 10 minutes in the beginning of a session in order to avoid disturbances from self tests of the dialysis machine. Determination of concentrations of B2M and urea was performed at the Clinical Chemistry Laboratory (Quattromed HTI, Estonia) using a standardized methods. The accuracy (%) was 10 for B2M, and 3,2 for urea in dialysate and in blood.

C. UV-absorbance scanning

For the determination of UV-absorbance in the beginning and at the end of a session (A0 and At) in the spent dialysate/ultrafiltrate a double-beam spectrophotometer (UV-3600 UV-VIS-NIR, Shimadzu Inc, Japan) was used. Scanning at the UV-range 190-380 nm was performed by an optical cuvette with an optical path length of 5 mm within 8 hours after each session using pure dialysate as reference.

D. Removal assessment

To assess uremic retention solute removal during a dialysis session the reduction ratio (RR) in percentage was calculated from the solute (urea and B2M) concentrations in blood as:

$$RR = \frac{C0 - Ct}{C0} 100\% \quad (1)$$

In case of UV-absorbance C0 and Ct were replaced by A0 and At, respectively.

E. Statistical analyses

Systematic error was calculated as follows using RR values based on concentrations from the laboratory as reference:

$$BIAS = \frac{\sum_{i=1}^{N} e_i}{N} \quad (2)$$

where e_i is the residual (difference between laboratory and optically determined RR values for the i-th treatment) and N is the number of observations [11].

Standard error was calculated according to [7] as

$$SE = \sqrt{\frac{\sum_{i=1}^{N} (e_i - BIAS)^2}{N}} \quad (3)$$

For the analysis Statistica 10.0 (Statsoft, Inc. for Windows) and Excel (version 2007 for Windows) were used.

Student's paired t-test was used to compare means and BIAS for different methods and F-test two-sample to compare variances and SE, where $p<0.05$ was considered as significant.

IV. Results

Figure 1 shows RR values (mean±SD, N=7) for small (Urea) and MM (B2M) uremic retention solutes in blood, and estimated by UV-absorbance at the wavelength of 297 nm in spent dialysate (A_297nm), during various treatment modalities (HD, HF-HD1, HF-HD2 and HDF). Difference in RR for retention solutes with different molecular size, urea and B2M, is clearly seen. Also, the figure reveals that the RR values for A_297nm resemble more removal of small (Urea) than MM (B2M) uremic retention solutes.

There was no statistical difference between RR values for Urea and from A_297nm ($p<0.05$).

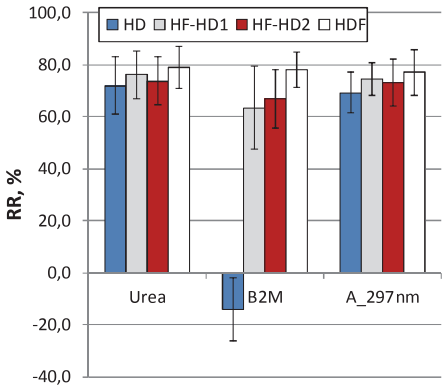

Fig. 1 RR values (mean±SD, N=7) for small (Urea) and MM (B2M) uremic retention solutes, and estimated by UV-absorbance at the wavelength of 297 in spent dialysate (A_297nm), during various treatment modalities (HD, HF-HD1, HF-HD2 and HDF)

More distinct presentation how different treatment modalities influence RR values for small (Urea) and MM (B2M) uremic retention solutes, and estimated by UV-absorbance at the wavelength of 297 in spent dialysate (A_297nm), during various treatment modalities (HD, HF-HD1, HF-HD2 and HDF) can be seen in Figure 2.

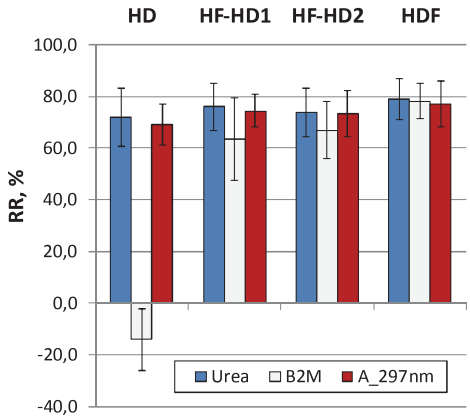

Fig. 2 RR values (mean±SD, N=7) for various treatment modalities (HD, HF-HD1, HF-HD2 and HDF) estimated for small (Urea) and MM (B2M) uremic retention solutes, and by UV-absorbance at the wavelength of 297 in spent dialysate (A_297nm).

Figure 2 indicates that the RR values for MM (B2M) start to approach to the RR values of small (Urea) uremic retention solutes and from A_297nm in case of treatment modalities with higher convective transport (HF-HD and HDF) being totally different for HD treatments. There was no statistical difference between RR values for Urea, B2M and from A_297nm ($p<0.05$) for HDF treatments.

Figure 3 shows BIAS±SE values in percentage for various treatment modalities (HD, HF-HD1, HF-HD2 and HDF) as small (Urea) retention solutes versus UV-absorbance at the wavelength of 297 in spent dialysate (A_297nm), and between MM (B2M) and A_297nm (Urea vs A_297nm and B2M vs A_297nm, respectively).

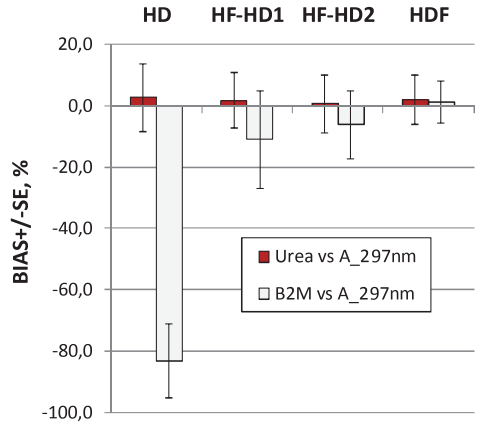

Fig. 3 BIAS±SE values in percentage for various treatment modalities (HD, HF-HD1, HF-HD2 and HDF) between small (Urea) and MM (B2M) uremic retention solutes, and UV-absorbance at the wavelength of 297 in spent dialysate (Urea vs A_297nm and B2M vs A_297nm).

As seen from Figure 3 the BIAS values are largest for HD and decrease for HF-HD and HDF modalities. The trend confirms results from Figure 2, illustrating how RR of MM (B2M) starts approach to RR of small (Urea) uremic retention solutes and RR from A_297nm in case of HF-HD and HDF treatments. There was no statistical difference between BIAS values for Urea versus A_297nm and for B2M versus A_297nm ($p<0.05$) for HDF treatments. The SE values were not different ($p<0.05$).

V. Discussion

Since B2M is used as a marker for MM removal [10], optical system for monitoring this solute would be beneficial. This study investigated removal of small (urea) and

MM (B2M) uremic retention solutes during different treatment modalities (HD, HF-HD, HDF), and whether UV-absorbance at 297 nm in the spent dialysate can be used for estimation of the removal of small and MM uremic retention solutes during dialysis simultaneously. The results indicate that RR for A_297nm is close to RR of small (Urea) uremic retention solutes (Figure 1) supported by the smallest BIAS and RE (Figure 3). The RR values for MM (B2M) start to approach to RR of small (Urea) uremic retention solutes and RR from A_297nm in case of treatment modalities with higher convective transport (HF-HD and HDF) having significant differences and largest BIAS and RE for HD treatments (Figure 2 and 3). The possible explanation is that removal characteristics of small and MM uremic retention solutes are significantly different during HD with prevalent diffusive transport of the uremic solutes, and became similar during dialysis modalities with high convective molecular transport, like HF-HD and HDF, from blood into dialysate. The results, confirming earlier studies [3], should be proofed by appropriate kinetic modeling in the next studies.

VI. Conclusions

It was found that reduction ratio is similar for small and MM uremic retention solutes in case of dialysis modality with the highest convective transport (HDF). Moreover, reduction ratio of small uremic retention solutes can be estimated with sufficient accuracy by UV-absorbance at 297 nm in the spent dialysate for all modalities. The same is valid for MM uremic retention solutes only for HDF.

In the future optical on-line monitoring technique that simultaneously presents reduction ratios of several uremic toxins would also be useful when new dialyzers and dialysis techniques are to be tested and evaluated.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Cheung AK, et al. (2006) Serum beta-2 microglobulin levels predict mortality in dialysis patients: results of the HEMO study. Journal of the American Society of Nephrology 17: 546.
2. EBPG - European Best Practice Guidelines: SECTION II. Haemodialysis adequacy II.2 Haemodialysis dose quantification: middle molecules (MM). (2002) Nephrology Dialysis Transplantation 17: 21-23.
3. Uhlin F, Yngman-Uhlin P, Jerotskaja J, Fridolin I, Fernström A (2009) On−Line Monitoring of Middle Molecule Removal during HemoDiaFiltration - Does It Work? American Society of Nephrology 42th Annual Meeting and Scientific Exposition, vol J Am Soc Nephrol 20 San Diego, California, USA, p pp 461A
4. Arund J, Tanner R, Uhlin F, Fridolin I (2012) Do Only Small Uremic Toxins, Chromophores, Contribute to the Online Dialysis Dose Monitoring by UV Absorbance? Toxins (Basel) 4:849-861
5. Uhlin F, Fridolin I, Lindberg L-G, Magnusson M (2003) Estimation of delivered dialysis dose by on-line monitoring of the UV-absorbance in the spent dialysate. American Journal of Kidney Diseases 41:1026-1036
6. Uhlin F, Fridolin I, Magnusson M, Lindberg LG (2006) Dialysis dose (Kt/V) and clearance variation sensitivity using measurement of ultraviolet-absorbance (on-line), blood urea, dialysate urea and ionic dialysance. Nephrol Dial Transplant. Aug;21:2225-2231
7. Luman M, Jerotskaja J, Lauri K, Fridolin I (2009) Dialysis dose and nutrition assessment by optical on-line dialysis adequacy monitor. Clinical Nephrology 72:303-311
8. Castellarnau A, Werner M, Gunthner R, Jakob M (2010) Real-time Kt/V determination by ultraviolet absorbance in spent dialysate: technique validation. Kidney International 78:920-925
9. Polaschegg HD (2010) Hemodialysis machine technology: a global overview. Expert Review of Medical Devices 7:793-810
10. Vanholder R, et al. (2008) Do we need new indicators of dialysis adequacy based on middle-molecule removal? Nature Clinical Practice Nephrology 4:174-175.
11. K. H. Esbensen (2009) Multivariate data analysis – in practice. CAMO Process AS

Use macro [author address] to enter the address of the corresponding author:

Author: Kai Lauri
Institute: Tallinn University of Technology, Department of Biomedical Engineering, Technomedicum
Street: Ehitajate Rd 5
City: Tallinn
Country: Estonia
Email: kai.lauri@mail.ee

Publication III

Lauri, K., Arund, J., Holmar, J., Tanner, R., Kalle, S., Luman, M., Fridolin, I. (2019) Removal of Urea, β2-Microglobulin, and Indoxyl Sulfate Assessed by Absorbance and Fluorescence in the Spent Dialysate During Hemodialysis. *ASAIO Journal,* August 16, 2019 - Volume Online First - Issue (DOI: 10.1097/MAT.0000000000001058)

Removal of Urea, β2-Microglobulin, and Indoxyl Sulfate Assessed by Absorbance and Fluorescence in the Spent Dialysate During Hemodialysis

Kai Lauri,*† Jürgen Arund,* Jana Holmar,*‡ Risto Tanner,* Sigrid Kalle,* Merike Luman,*§ and Ivo Fridolin*

In this study, simultaneous removal assessment of marker molecules from three uremic toxin groups was performed during different hemodialysis treatment modalities using optical characteristics of spent dialysate. Results from optical measurements were compared with the results from chemical laboratory. Ten chronic dialysis patients, mean age 59 ± 15 years, were included in the study during 40 hemodialysis sessions. Low-flux hemodialysis (HD), high-flux hemodialysis (HF), and postdilutional online hemodiafiltration (HDF) with different settings were used. The reduction ratio (RR) and total removed solute (TRS) of three uremic solutes were determined: small molecular weight urea, middle molecular β2-microglobulin (B2M), and protein-bound indoxyl sulfate (IS). Concentrations of these solutes in the spent dialysate were measured by laboratory (lab) and optical (opt) methods, in the serum by laboratory methods, and calculated RR values in percentage were compared accordingly. Total removed solute was obtained from the total dialysate collection (TDC) using lab and opt methods. The highest RR values were found for urea and B2M, and the lowest for IS. The difference between RR of lab and opt results estimated as mean accuracy (BIAS) was ≤8.1% for all three solutes. Good correspondence between TRS lab *vs.* opt was achieved, resulting in strong linear correlation values R from 0.727 for urea to 0.971 for IS. Accuracy for TRS values as BIAS ± standard error (SE), comparing lab *vs.* opt, showed no statistical difference for any of the observed uremic solutes ($P > 0.05$). The accuracy of the optical method was not influenced by the dialysis modality (HD, HF, and HDF). *ASAIO Journal* XXX; XX:00–00.

Key Words: dialysis, dialysis adequacy, uremic toxins, uremic retention solutes, optical monitoring, ultraviolet absorbance, fluorescence

The uremia or uremic syndrome, a serious complication of renal patients, is characterized by the retention of various solutes because of inadequate kidney function. The classical subdivision of uremic solutes into three physicochemical types as small molecular weight water-soluble compounds (SM), protein-bound solutes (PBS), and middle molecule weight compounds (MM) has been proposed.[1] The most widely used SM marker molecule is urea exploited as a dialysis adequacy parameter Kt/V, and its simplification, Urea Reduction Ratio (URR).[2] However, Kt/V urea insufficiently represents all novel changes in modern dialysis and the complexities of uremia. More holistic assessment of uremic solute removal in dialysis therapy involving a variety of strategic treatment alternatives[3] should comprise kinetic profiling[4] and monitoring of the key molecules of all three groups of solutes in uremic toxicity.

European Renal Best Practice has pointed out that B2M is sufficiently representative in its kinetic behavior of other MM (including peptides of similar size), and may be used as a marker for such molecules.[5,6] A protein-bound uremic solute, indoxyl sulfate (IS), has received attention because of its link to cardiovascular disease and mortality.[7] However, the cumbersome HPLC method hampers its widespread use in daily clinical practice.

Removal of uremic solutes in dialysis is generally estimated by analyzing the blood samples, but an alternative is to analyze spent dialysate by optical methods. Among others, optical monitors have been found to be simple and reliable for monitoring both specific blood[8] and spent dialysate[9] characteristics of renal patients. On-line assessment of urea-based parameters of dialysis quality can be accomplished by monitoring ultraviolet (UV) absorbance of spent dialysate.[10,11] Moreover, the optical monitoring aims to overcome the limitation regarding single-marker–based dialysis adequacy. Earlier research has demonstrated the potential of optical monitoring to estimate the removal of SM,[12] PBS,[13] and MM[14–16] during dialysis. Still, an optical on-line technique that simultaneously can monitor several uremic toxins with different removal patterns when various dialyzers and dialysis modalities are employed has not been evaluated in the clinical settings.

This proof-of-concept study aimed, for the first time, to investigate whether optical dialysis monitoring, based on UV absorbance and fluorescence of spent dialysate, can

From the * Department of Health Technologies, Tallinn University of Technology, Tallinn, Estonia; †SYNLAB Eesti OÜ, Tallinn, Estonia; ‡RWTH Aachen University Hospital, Institute for Molecular Cardiovascular Research (IMCAR), Aachen, Germany; and §Centre of Nephrology, North Estonian Medical Centre, Tallinn, Estonia.

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Correspondence: Kai Lauri, Department of Health Technologies, Tallinn University of Technology, Ehitajate Rd 5, EST-19086 Tallinn, Estonia. Email: kai.lauri@mail.ee.

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simultaneously reveal the removal patterns of marker molecules (urea, β2-microglobulin [B2M], and IS) from all uremic toxins groups during low-flux hemodialysis (HD) and high-flux hemodialysis (HF) and hemodiafiltration (HDF) treatments without any need for blood or dialysate sampling.

Materials and Methods

The study included 10 chronic hemodialysis patients, five males and five females, mean age 59 ± 15 years in the Centre of Nephrology, North Estonian Medical Centre, Estonia. The clinical data of dialysis patients are presented in **Table 1**. The patients were monitored during 40 dialysis procedures in total. The study was performed after the protocol was approved by the Tallinn Medical Research Ethics Committee at the National Institute for Health Development in Estonia (No. 2349) and conducted following the Helsinki Declaration. An informed consent was obtained from all participating patients.

Each patient was observed during four midweek dialysis sessions: one hemodialysis with low-flux dialyzer (HD), two hemodialyses with high-flux dialyzer (HF1 and HF2), and one postdilution HDF treatment with different settings (**Table 2**) to assess accuracy of the optical method for different modalities. The type of dialysis machine was Fresenius 5008 (Fresenius Medical Care, Germany).

No substitution fluid was used for HD, HF1, and HF2. For HDF, the substitution fluid was higher than 19 L. Two different polysulphone dialyzers, FX8 and FX1000 (Fresenius Medical Care) with the effective membrane area of 1.4 and 2.2 m^2, were utilized.

The schematic clinical setup, sample collection, and analysis methods are shown in **Figure 1**. Serum samples were taken before ($C0$) and after dialysis procedure (Ct), dialysate samples were taken 10, 60, 120, and 180 minutes after the start of the session and at the end of the session (240 minutes) from the drain tube of the dialysis machine. After the end of the procedure, dialysate collection tank was weighed, and the sample for TRS calculation was taken from it after careful stirring.

The concentrations of urea and B2M in serum and dialysate samples were determined in the Clinical Chemistry Laboratory (SYNLAB Eesti OÜ, Estonia) using standardized methods. For total IS determination, the serum samples were first deproteinized by heat denaturation. The concentration of IS for all samples was determined in Tallinn University of Technology by the HPLC method as described by Arund *et al.*.[17]

Optical measurements of spent dialysate samples comprised acquisition of UV absorption spectrum and fluorescence spectra. Ultraviolet absorbance spectra were measured over a wavelength range of 190–380 nm by a UVVIS-NIR spectrophotometer (UV-3600, Shimadzu Inc., Japan) using pure dialysate as a reference. Fluorescence analysis was performed over an excitation (EX) wavelength range of 220–500 nm (increment 10 nm) and emission (EM) wavelength range of 220–500 nm (increment 1 nm) by a spectrofluorophotometer (RF-5301, Shimadzu Inc., Japan) on room temperature (ca. 22°C) within 8 hours after each session. Given scanning wavelengths were chosen on the basis of earlier studies.[12–14,18,19] The obtained spectral values were processed and presented by the software Panorama fluorescence and UV Probe (Shimadzu Inc., Japan). Some of incorrect or illogical values of measured concentration or absorption/fluorescence were excluded from the data, for example, if sampling was performed simultaneously with self-tests of the dialysis machine.

Models for uremic solute concentration calculation in spent dialysate from the optical data were created utilizing information from the measured UV absorbance or fluorescence by multiple linear regression (forward stepwise regression method) analysis. The concentration of a substance in dialysate was set as a dependent parameter, and optical characteristics (UV absorbance and fluorescence) of the spent dialysate were set as independent parameters. The algorithms obtained for the calculation of concentrations in spent dialysate (Y) from the optical measurements were in the form of

$$Y = \sum_{i=0}^{N} a_i * A(\lambda_i) + \sum_{j=0}^{M} b_j * F(\lambda_j) \qquad (1)$$

where a and b are the coefficients, $A(\lambda)$ is the UV absorbance value at a certain wavelength [nm], and $F(\lambda)$ is the fluorescence intensity value at certain EX/EM wavelength [nm]. Obtained models for optical estimation of the substance level used up to 6 independent variables (UV absorbance and fluorescence values at certain wavelengths). The wavelengths used in the models were: urea: A(297); IS: A(293 and 303) and F(230/320, 230/428, 250/340, and 270/408); and B2M: A(217, 293, and 301) and F(230/330 and 290/311).

Table 1. Clinical Data of the Chronic Dialysis Patients

Patient	Diagnosis	Age	Gender	Serum Total Protein (g/L)	Dialysis Access
#1	Tubulointerstitial nephritis	65	F	61.8	Native fistula
#2	Hypertension	39	M	68.2	Native fistula
#3	Hypertension	60	M	68.0	Graft
#4	Multiple myeloma	61	M	61.8	Native fistula
#5	Tubulointerstitial nephritis	60	F	65.2	Native fistula
#6	Polycystic kidney disease (ADPCKD)	59	F	70.9	Graft
#7	Hypertension	63	F	60.5	Graft
#8	Hypertension	81	M	53.6	Native fistula
#9	Glomerulonephritis	73	M	59.8	Native fistula
#10	Diabetes, insulin dependent	26	F	61.1	native fistula

#4: Multiple myeloma is in remission, B2MG: 26,440 μg/l; S-Kappa: 255.08 mg/l; S-Lambda: 58.33 mg/l; S-K/S-L: 4.3.
#9: Rapidly progressive glomerulonephritis with crescents (7 years before the study), in remission at time of the study.
ADPCKD, ; F, ; M.

Table 2. Treatment Parameter Settings

	HD	HF1	HF2	HDF
Time, minutes	240	240	240	240
Blood flow, ml/minute	300	350	300	350
Dialysate flow, ml/minute	500	500	800	800
Dialyzer effective membrane area (m^2)	1.4	2.2	2.2	2.2
Substitution volume, L	0	0	0	>19

HD, HF, low- and high-flux hemodialysis; HDF, hemodiafiltration.

To assess uremic retention solute removal during a dialysis session, the RR as a percentage was calculated from the solute concentrations as:

$$RR = \frac{C0 - Ct}{C0} 100\% \qquad (2)$$

where $C0$ is a serum sample before and Ct after dialysis procedure. For spent dialysate, a sample taken after 10 minutes after the start and at the end of dialysis procedure were utilized as $C0$ and Ct, respectively. In the case of optical measurements, $C0$ and Ct were replaced concentrations in spent dialysate calculated from the optical data.

The total removed solute (TRS) of a substance was calculated based on total dialysate collection (TDC) as follows:

$$TRS = C_T * W_T \qquad (3)$$

where C_T is the substance concentration in TDC tank (mg/L) and W_T is the weight of the dialysate collection tank (kg). It was assumed that 1 kg = 1 L of the dialysate. To determine the TRS for urea, B2M and IS by the optical method (opt), corresponding concentrations calculated from the spectral values were utilized.

For the regression analysis, Statistica 10.0 (Statsoft, Inc. for Windows) was used. Mean and BIAS[20] were calculated by Excel (version 2007 for Windows). Student's paired *t*-test was used to compare means and BIAS for different methods, and F-test two-sample for variances to compare variances and SE. For comparing correlations, cocor package was used.[21] $P < 0.05$ was considered significant.

Results

The concentrations of the marker solutes in the spent dialysate are presented in Table 1, Supplemental Digital Content 1, http://links.lww.com/ASAIO/A451, measured by chemical laboratory methods (D_lab) and determined by the models using the optical data (D_opt). The concentrations of B2M in the spent dialysate from HD treatments were below quantification limit, and therefore, the N is smaller for B2M. Concentrations determined by the laboratory and optical methods were not statistically different ($P > 0.05$). **Figure 2** presents the identity and Bland–Altman plots. A strong linear correlation ($r \geq 0.86$) exists between D_lab and D_opt (**Figure 2, A–C**), whereas most residuals are uniformly distributed around the mean (**Figure 2, D–F**). For all solutes, the bias deviation from the identity line seems to change with concentration, increasing with higher values (**Figure 2, A–C**).

Figure 3 shows RR values (mean ± standard deviation [SD]) for urea, B2M and IS in serum (S_lab) and dialysate samples determined by laboratory (D_lab) and optical (D_opt) methods during various treatment modalities (HD, HF1, HF2, and HDF). The difference in RR for marker solutes of different groups is clearly seen, especially for IS being statistically different from urea and B2M over all modalities ($P < 0.05$). Interestingly, the RR values for B2M resemble more urea than IS, except in the case of HD. There was no statistical difference between the RR

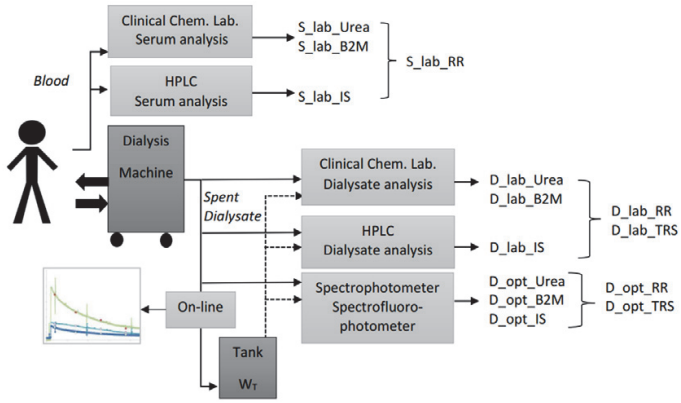

Figure 1. Schematic view of the clinical setup. S_lab_Urea, S_lab_IS, S_lab_B2M and D_lab_Urea, D_lab_IS, D_lab_B2M are urea, indoxyl sulfate, and β2-microglobulin concentrations measured in chemical laboratory in serum and in spent dialysate, respectively. D_opt_B2M are optically estimated concentrations in spent dialysate. S_lab_RR, D_lab_are the reduction ratio (RR) values estimated from solute concentrations in serum and dialysate, and D_opt_RR is RR value estimated from dialysate optical measurements. D_lab_TRS and D_opt_TRS are the total removed solute (TRS) values from the solute concentrations in dialysate measured in chemical laboratory and optically.

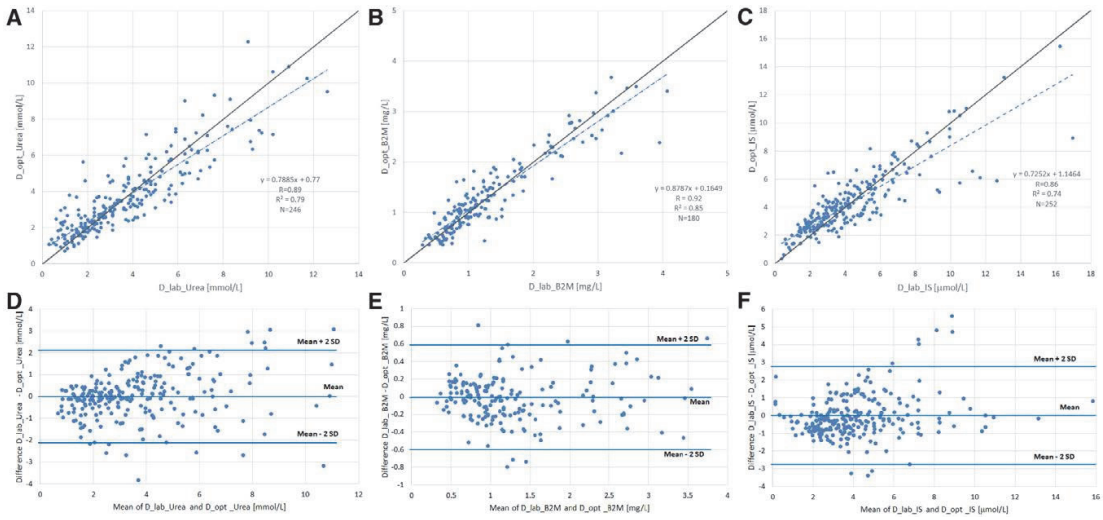

Figure 2. A–C: Identity and Bland-Altman (**D–F**) plots for the concentration values of urea, β2-microglobuline (B2M), and indoxyl sulfate (IS) in the spent dialysate from the laboratory measurements (D_lab) plotted against the values by the optical method (D_opt).

values for uremic solutes determined by laboratory and optical method ($P > 0.05$).

Table 3 summarizes the average RR values of three toxins for all modalities combined. In addition to average values, accuracy as BIAS ± SE and correlation coefficient R between the indicated methods over the whole material are given. The highest average RR values were obtained for urea and B2M, and the lowest for IS.

The average RR values of urea were similar for laboratory and optcal methods and media (serum, dialysate) with a strong linear correlation between the methods. The average RR values of B2M between laboratory methods for serum and spent dialysate were very similar and had a strong correlation, but the link between both media was found to be weaker with the optical method. In the case of RR of IS, there was a strong correlation between laboratory and optical methods of assessing the spent dialysate, but the relation of these methods to the serum-based laboratory method remained weaker.

The effect of various treatment modalities (HD, HF1, HF2, and HDF) on average TRS values determined by laboratory and optical methods (D_lab and D_opt) is shown in **Figure 4**. The lowest average TRS value was obtained with HD compared withto other treatment modalities in the case of urea and IS. The highest average TRS values resulted from HDF for B2M and IS, whereas average TRS values of urea remained almost the same in both HF and HDF.

Table 4 presents the comparison of laboratory and optical methods (D_lab and D_opt) to determine the TRS values of three marker molecules as an average value of all modalities and correlation coefficient R. TRS values by the optical and laboratory methods were not statistically different for any solute ($P = 0.18...0.64$). However, the correlation coefficient for IS is somewhat higher than for urea and B2M indicating a smaller random error in the case of IS for TRS estimated by the optical measurements. The identity plots and the Bland–Altman plots of laboratory and optical methods show good alignment with the identity line for B2M and IS (Figure 1, B and C, Supplemental Digital Content 2, http://links.lww.com/ASAIO/A451), and rather uniform distribution of residuals around the mean value (Figure 1, D–F, Supplemental Digital Content 2, http://links.lww.com/ASAIO/A451).

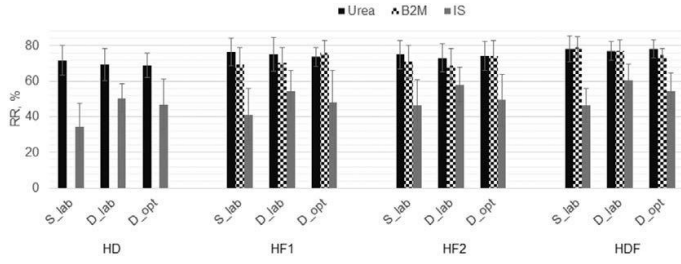

Figure 3. Reduction ratio (RR) values (mean ± standard deviation [SD]) for urea, β2-microglobulin (B2M) and indoxyl sulfate (IS) measured in chemical laboratory in serum (S_lab) and in spent dialysate (D_Lab), and estimated optically in spent dialysate (D_opt) during various treatment modalities (HD: low-flux hemodialysis, HF1, HF2: two hemodialyses with high-flux dialyzer, HDF: hemodiafiltration). No HD treatments were included for B2M.

Table 3. Overall Value for RR and Accuracy (BIAS ± SE) for Different Results and for All Three Toxins

	RR, %			Accuracy (RR)			R (RR)		
	S_lab	D_lab	D_opt	S_lab *vs.* D_lab	S_lab *vs.* D_opt	D_lab *vs.* D_opt	S_lab *vs.* D_lab	S_lab *vs.* D_opt	D_lab *vs.* D_opt
Urea (*N* = 40)	75.3 ± 8.0	73.3 ± 8.8	73.3 ± 7.3	2.0 ± 3.2	1.9 ± 4.0	0.0 ± 4.8	0.932†	0.863	0.833†
B2M* (*N* = 30)	73.1 ± 9.1	72.0 ± 8.8	75.0 ± 6.5	1.1 ± 4.2	−1.8 ± 7.8	2.9 ± 6.8	0.891‡	0.576	0.650
IS (*N* = 39)	43.5 ± 13.8	55.6 ± 9.5	51.6 ± 11.0	−11.9 ± 8.9	−8.1 ± 9.9	3.3 ± 5.2	0.761	0.702†	0.867†

Correlation coefficient R was calculated as a value between the indicated methods.
B2M, β2-microglobulin; D_opt, dialysate measured optically; IS-indoxyl sulfate; RR, reduction ratio; S_lab and D_lab, serum and spent dialysate measured in the chemical laboratory; SE, standard error.
*No HD treatments were included for B2M.
†R values are statistically different from all others.
‡R is significantly different from S_lab *vs.* D_opt and D_lab *vs.* D_opt.

Discussion

The main findings of this study were as follows:

1. For the first time, to our knowledge, simultaneous removal of multiple marker molecules was assessed during HD, HF, and HDF treatments estimated as RR and TRS values using optical characteristics of spent dialysate.
2. Spectrophotometric and fluorometric monitoring demonstrated the ability to determine the concentration values for markers of three groups of uremic toxins in the spent dialysate without any blood or dialysate samples.
3. Reduction ratio calculated from serum measured chemically and spent dialysate derived optically were similar, and the difference estimated as mean accuracy (BIAS) was ≤8.1% for all three marker uremic toxins.
4. Good agreement for TRS between dialysate measured chemically and optically was achieved, resulting in strong linear correlation with R values from 0.727 (urea) to 0.971 (IS).
5. Different dialysis modalities had an impact on the RR and TRS of three marker solutes; however, the modalities did not affect the accuracy of RR and TRS obtained by the optical monitoring method, taking into account that B2M was excluded in the case of HD.

The UV absorbance has been successfully proven to estimate the removal of easily dialyzed uremic solutes with a high RR.[10,22,23] Uric acid (UA) is an SM chromophore with strong correlation between its concentration and UV absorbance at specific wavelengths,[24,25] providing a direct method to determine the levels of UA in spent dialysate. In previous studies, the possibility of monitoring the removal of UA in the spent dialysate[22] and also in serum[26,27] using UV absorbance has been demonstrated. Both urea and UA are small uremic retention solutes and are removed very similarly during dialysis.[20,28] *Via* this connection, the UV absorbance can be utilized to indirectly monitor the removal of urea.[10,29,30] Strong correlation between urea concentrations obtained by optical and laboratory methods presented in previous studies[12,30] and in this study (Table 1 and Figure 2, a and d, Supplemental Digital Content 1 and 3, http://links.lww.com/ASAIO/A451) provides a valid base to this approach.

Considering the optical monitoring of MM uremic solutes, the supportive evidence related to the fluorescence of advanced glycation end products (AGE) and the AGE-modified B2M can be found. The fluorescence spectra of the later with a maximum at EX370/EM445 nm were very similar to that of bovine serum albumin, artificially glycated *in vitro*.[31] Partly fluorescent at EX370/EM440 nm, only a small part of

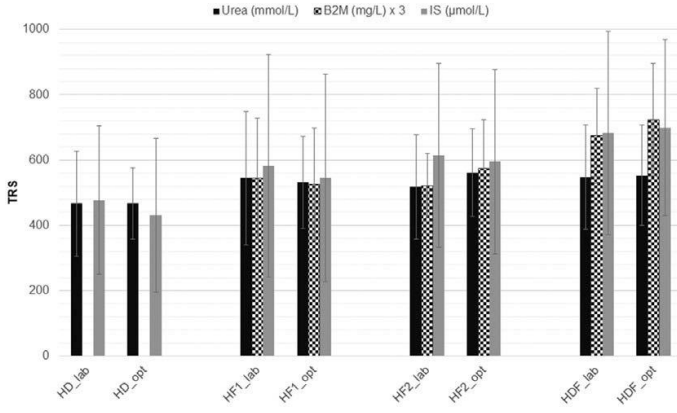

Figure 4. Comparison of different treatment modalities by total removed solute (TRS) values (mean ± standard deviation [SD]) for urea, β2-Microglobulin (B2M) and indoxyl sulfate (IS) measured in chemical laboratory yy (lab), and estimated optically (opt) in spent dialysate, during various treatment modalities (HD: low-flux hemodialysis, HF1, HF2: two hemodialyses with high-flux dialyzer, HDF: hemodiafiltration). No HD treatments were included for B2M.

Table 4. Comparison of Different Methods by TRS Values With Mean, Accuracy (BIAS ± SE), and Correlation Coefficient (R)

	TRS		Accuracy (TRS)	R (TRS)
	D_lab	D_opt	D_lab *vs.* D_opt	D_lab *vs.* D_opt
Urea* mmol (N = 39)	516.8 ± 169.2	525.5 ± 134.7	–8.7 ± 116.7	0.727
B2M† mg (N = 30)	193.3 ± 52.6	202.4 ± 60.3	–9.1 ± 36.1	0.804
IS, µmol (N = 38)	580.2 ± 288.4	566.9 ± 286.9	13.4 ± 69.7	0.971‡

B2M, β2-microglobulin; D_lab, dialysate measured chemical laboratory; D-opt, dialysate measured optically; HD, low-flux hemodialysis; IS, indoxyl sulfate; SE, standard error; TRS, total removed solutes.

*Urea in mg = 60.06 × urea in mmol.

†No HD treatments were included for B2M.

‡R is significantly different from Urea and B2M.

AGEs, covalently bound to proteins, can penetrate the dialysis membrane as digestion products of AGE-modified proteins.[32–35] Fluorescent gel permeation chromatography fractions with molecular weight below 7 kD, between 1.5 and 2.0 kD or of ~5 and ~12 kD[32,36] can be found in serum of dialysis patients, depending on the therapy strategies used. Also, the AGE-modified B2M, the major component of hemodialysis-associated amyloidosis, has the fluorescence maximum at EX360/EM450 comparable to that of AGEs.[31,37] From these observations, it can be concluded that partly the fluorescent AGEs are filtered into the dialysate as a part of the fraction of middle size uremic toxins. The process is evidently promoted by modalities utilizing dialyzers containing membranes with larger pore size and area, as with HF and even more efficiently with convective hemodialysis therapies like HDF. Because the substantial part of AGEs and the AGE-modified B2M can be detected by fluorescence, the optical signal, monitored at appropriate wavelengths during dialysis, seems to offer an alternative for assessment of the removal of MM by dialysis. Strong correlation ($r \geq 0.80$) between laboratory and optical methods to estimate the concentration of the B2M (Table 1 and Figure 2, B, Supplemental Digital Content 1 and 3, http://links.lww.com/ASAIO/A451), RR (**Table 3**), or TRS (**Table 4**, Figure 1, B, Supplemental Digital Content 2, http://links.lww.com/ASAIO/A451) is consistent with previously published results by Holmar *et al.*.[14]

In the case of PBSs, only the free fraction of the blood serum is available to diffuse through the dialysis membrane,[7,38,39] thus making them slowly dialyzed solutes with low RR values. PBS contribute less than 10% to the UV absorbance signal.[22] As a result, the UV absorbance monitoring method lacks potential to estimate the removal of PBS. According to the HPLC studies on the deproteinized uremic serum and spent dialysate samples, from among a group of PBS, IS has a prevalent fluorescence compared with other uremic retention solutes.[17,40–42] Furthermore, the RR of IS was very close to the decrease of the total fluorescence signal of the spent dialysate.[17] Despite that absorbance from certain wavelengths could add precision to the optical model for PBS (IS), the contribution of IS and other indoles to UV absorbance in the spent dialysate is relatively small compared to that of SM uremic toxins.[22] Therefore, using fluorescence of spent dialysate, which has different selectivities than UV absorbance, is well justified for the direct optical monitoring of IS removal. This is supported by the matched concentrations determined by optical and laboratory methods in spent dialysate (Table 1 and Figure 2, C and F, Supplemental Digital Content 1 and 3, http://links.lww.com/ASAIO/A451), and confirms the previously published results.[13] One reason for larger outliers (**Figure 2F**) is related to the uncompensated effects of the UV chromophores to fluorescence of the IS.

Due to protein-bound nature and specific kinetic behavior of PBS in the body, including IS, there is a discrepancy between RR values in the blood and the spent dialysate (**Table 3**), indicating the limited access of total PBS to dialyse. Thus, describing the elimination of PBS by estimating the TRS optically from the spent dialysate would be beneficial. Good agreement between TRS obtained for IS by optical and laboratory methods reported in a previous study[13] and this study (**Table 4**, Figure 1C ans F, Supplemental Digital Content 2, http://links.lww.com/ASAIO/A451) support this approach.

Different treatment modalities (HD, HF, and HDF) had a major impact on the removal of B2M (**Figures 3 and 4**), achieving the maximum RR and TRS for HDF. The RR values for B2M start to approach RR of SM urea in the case of applying treatment modalities with higher convective transport (HF and HDF) (**Figure 3**). TRS for B2M and IS increases for HDF compared with HF modalities but remains at the same level for urea (**Figure 4**), which indicates that further enhancement of the treatment efficiency does not remove higher amounts of small water-soluble compounds. The effect of HF and HDF seems to be moderate for IS RR value (**Figure 3**). Higher removal in HDF *vs.* HF has been demonstrated in prospective trials for urea, phosphate, B2M, factor D, homocysteine, and AGEs.[43] Although the chosen modalities had an impact on the removal of all studied solutes, they did not affect the performance of the dialysate-based optical method.

Among other advantages of dialysate quantification is that TRS gives a direct quantitative estimate of uremic solutes removal. A limitation is that dialysate-based measurements may not reflect total removal in the presence of membrane adsorption. Moreover, deviations may occur due to medications and other uremic solutes that may interfere with the UV-absorbance or fluorescence of spent dialysate during optical online measurements. Usage of complicated apparatus, such as spectrophotometers and spectrofluorometers, for on-line optical monitoring of uremic retention solutes in the spent dialysate during dialysis treatments makes measurements rather cumbersome in the clinical environment and is not suitable for everyday praxis. The latter raises the need for a specially designed compact and robust on-line dialysis monitoring device which will be a task for further development.

Conclusion

This proof-of-concept study demonstrated that optical dialysis monitoring, based on UV absorbance and fluorescence of spent dialysate, can simultaneously reveal removal patterns of urea, B2M, and IS during various dialysis treatment modalities without any blood or dialysate sampling. Good agreement between chemically and optically estimated solute removal parameters, RR and total removed solute, was achieved. Dialysis modality did not affect the accuracy of optical method, taking into account that B2M was excluded from the analysis in the case of dialysis with low-flux dialyzer.

On-line optical monitoring could help to raise the awareness for all parties of renal replacement therapy, offering an easy way to see the course of the ongoing treatment for doctors, nurses, and patients, leading to better treatment planning, performance, and patient compliance.

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References

1. Vanholder R, De Smet R, Glorieux G, *et al*: Review on uremic toxins: Classification, concentration, and interindividual variability. *Kidney Int* 63: 1934–1943, 2003.
2. National Kidney Foundation: KDOQI clinical practice guideline for hemodialysis adequacy: 2015 update. *Am JKidney Dis* 66: 884–930, 2015.
3. Vanholder R, Glorieux G, Eloot S: Once upon a time in dialysis: The last days of Kt/V? *Kidney Int* 88: 460–465, 2015.
4. Eloot S, Schneditz D, Cornelis T, *et al*: Protein-bound uremic toxin profiling as a tool to optimize hemodialysis. *PLoS One* 11: e0147159, 2016.
5. Tattersall J, Martin-Malo A, Pedrini L, *et al*: EBPG guideline on dialysis strategies. *Nephrol Dial Transplant* 22(suppl 2): ii5–ii21, 2007.
6. European Best Practice Guidelines: SECTION II. Haemodialysis adequacy II.2 haemodialysis dose quantification: Middle molecules (MM). *Nephrol Dial Transplant* 17(suppl 7): 21–23, 2002.
7. Vanholder R, Schepers E, Pletinck A, Nagler EV, Glorieux G: The uremic toxicity of indoxyl sulfate and p-cresyl sulfate: A systematic review. *J Am Soc Nephrol* 25: 1897–1907, 2014.
8. Yessayan LT, Yee J, Frinak S, Szamosfalvi B: Online hemoglobin and oxygen saturation sensing during continuous renal replacement therapy with regional citrate anticoagulation. *ASAIO J* 61: 489–495, 2015.
9. Castellarnau A, Werner M, Günthner R, Jakob M: Real-time Kt/V determination by ultraviolet absorbance in spent dialysate: technique validation. *Kidney Int* 78: 920–925, 2010.
10. Uhlin F, Fridolin I, Magnusson M, Lindberg LG: Dialysis dose (Kt/V) and clearance variation sensitivity using measurement of ultraviolet-absorbance (on-line), blood urea, dialysate urea and ionic dialysance. *Nephrol Dial Transplant* 21: 2225–2231, 2006.
11. Luman M, Jerotskaja J, Lauri K, Fridolin I: Dialysis dose and nutrition assessment by optical on-line dialysis adequacy monitor. *Clin Nephrol* 72: 303–311, 2009.
12. Tomson R, Fridolin I, Uhlin F, Holmar J, Lauri K, Luman M: Development of the model for the optical monitoring of urea in spent dialysate, in *Electronics Conference (BEC), 2012 13th Biennial Baltic*. 2012, pp. 179–182.
13. Holmar J, Uhlin F, Ferenets R, *et al*: Estimation of removed uremic toxin indoxyl sulphate during hemodialysis by using optical data of the spent dialysate, in *35th Annual International Conference of the IEEE EMBS*. Osaka, Japan, IEEE, 3–7 July, 2013, pp. 6707–6710.
14. Holmar J, Arund J, Uhlin F, Tanner R, Fridolin I: Beta2-microglobulin measurements in the spent dialysate using fluorescence spectra, in *5th European Conference of the International Federation for Medical and Biological Engineering, Pts 1 and 2*. New York, NY, Springer, 2011, pp. 1035–1038.
15. Uhlin F, Holmar J, Yngman-Uhlin P, Fernström A, Fridolin I: Optical estimation of beta 2 microglobulin during hemodiafiltration—Does it work? *Blood Purif* 40: 113–119, 2015.
16. Lauri K, Luman M, Holmar J, *et al*: Can removal of middle molecular uremic retention solutes be estimated by UV-absorbance measurements in spent dialysate?, in Jaffray DA (ed): *World Congress on Medical Physics and Biomedical Engineering*, June 7–12, 2015, Toronto, Canada, Springer International Publishing, pp. 1297–1300.
17. Arund J, Luman M, Uhlin F, Tanner R, Fridolin I: Is fluorescence valid to monitor removal of protein bound uremic solutes in dialysis? *PLoS One* 11: e0156541, 2016.
18. Fridolin I, Lindberg LG: On-line monitoring of solutes in dialysate using wavelength-dependent absorption of ultraviolet radiation. *Med Biol Eng Comput* 41: 263–270, 2003.
19. Holmar J, Arund J, Uhlin F, Tanner R, Fridolin I: Quantification of indoxyl sulphate in the spent dialysate using fluorescence spectra, in Kim Dremstrup SR, Morten Ølgaard Jensen (eds): 15 Nordic – Baltic Conference on Biomedical Engineering and Medical Physics – NBC15, 14–17 June 2011. Aalborg, Denmark, Springer, pp. 45 – 48.
20. Donadio C, Calia D, Ghimenti S, *et al*: Uric acid is the major determinant of absorbance in spent dialysate allowing spectrophotometric evaluation of dialysis dose. *J Nephrol* 27: 331–337, 2014.
21. Birk Diedenhofen JM: A comprehensive solution for the statistical comparison of correlations. *PLoS One* 10: e0121945, 2015.
22. Arund J, Tanner R, Uhlin F, Fridolin I: Do only small uremic toxins, chromophores, contribute to the online dialysis dose monitoring by UV absorbance? *Toxins (Basel)* 4: 849–861, 2012.
23. Fridolin I, Magnusson M, Lindberg LG: On-line monitoring of solutes in dialysate using absorption of ultraviolet radiation: Technique description. *Int J Artif Organs* 25: 748–761, 2002.
24. Jerotskaja J, Uhlin F, Fridolin I, Lauri K, Luman M, Fernström A: Optical online monitoring of uric acid removal during dialysis. *Blood Purif* 29: 69–74, 2010.
25. Holmar J, Fridolin I, Uhlin F, Lauri K, Luman M: Optical method for cardiovascular risk marker uric acid removal assessment during dialysis. *ScientificWorldJournal* 2012: 506486, 2012.
26. Schoots AC, Homan HR, Gladdines MM, Cramers CA, de Smet R, Ringoir SM: Screening of UV-absorbing solutes in uremic serum by reversed phase HPLC–change of blood levels in different therapies. *Clin Chim Acta* 146: 37–51, 1985.
27. Vanholder RC, De Smet RV, Ringoir SM: Assessment of urea and other uremic markers for quantification of dialysis efficacy. *Clin Chem* 38(8 pt 1): 1429–1436, 1992.
28. Lauri K, Tanner R, Jerotskaja J, Luman M, Fridolin I: HPLC study of uremic fluids related to optical dialysis adequacy monitoring. *Int J Artif Organs* 33: 96–104, 2010.
29. Uhlin F, Fridolin I, Lindberg LG, Magnusson M: Estimation of delivered dialysis dose by on-line monitoring of the ultraviolet absorbance in the spent dialysate. *Am J Kidney Dis* 41: 1026–1036, 2003.
30. Uhlin F, Fridolin I, Lindberg LG, Magnusson M: Estimating total urea removal and protein catabolic rate by monitoring UV absorbance in spent dialysate. *Nephrol Dial Transplant* 20: 2458–2464, 2005.
31. Miyata T, Oda O, Inagi R, *et al*: beta 2-Microglobulin modified with advanced glycation end products is a major component of hemodialysis-associated amyloidosis. *J Clin Invest* 92: 1243–1252, 1993.
32. Münch G, Keis R, Wessels A, *et al*: Determination of advanced glycation end products in serum by fluorescence spectroscopy and competitive ELISA. *Eur J Clin Chem Clin Biochem* 35: 669–677, 1997.
33. Gerdemann A, Wagner Z, Solf A, Bahner U, Heidland A, Vienken J, *et al*: Plasma levels of advanced glycation end products

during haemodialysis, haemodiafiltration and haemofiltration: Potential importance of dialysate quality. *Nephrol Dial Transplant* 17: 1045–1049, 2002.
34. Forbes JM, Cooper ME, Oldfield MD, Thomas MC: Role of advanced glycation end products in diabetic nephropathy. *J Am Soc Nephrol Aug* 14(8 suppl 3): S254–S258, 2003.
35. Schwedler SB, Metzger T, Schinzel R, Wanner C: Advanced glycation end products and mortality in hemodialysis patients. *Kidney Int* 62: 301–310, 2002.
36. Henle T, Deppisch R, Beck W, Hergesell O, Hänsch GM, Ritz E: Advanced glycated end-products (AGE) during haemodialysis treatment: discrepant results with different methodologies reflecting the heterogeneity of AGE compounds. *Nephrol Dial Transplant* 14: 1968–1975, 1999.
37. Kalle S, Kressa H, Tanner R, Holmar J, Fridolin I: Fluorescence of beta-2-microglobulin in the spent dialysate, in Mindedal H, Persson, M (eds): *16th Nordic-Baltic Conference on Biomedical Engineering*. Göteborg, Sweden, Springer, 2014, pp. 59–62.
38. Jourde-Chiche N, Dou L, Cerini C, Dignat-George F, Vanholder R, Brunet P: Protein-bound toxins-update 2009. *Semin Dial* 22: 334–339, 2009.
39. Sirich TL, Luo FJ, Plummer NS, Hostetter TH, Meyer TW: Selectively increasing the clearance of protein-bound uremic solutes. *Nephrol Dial Transplant* 27: 1574–1579, 2012.
40. De Smet R, Vogeleere P, Van Kaer J, Lameire N, Vanholder R: Study by means of high-performance liquid chromatography of solutes that decrease theophylline/protein binding in the serum of uremic patients. *J Chromatogr A* 847: 141–153, 1999.
41. Swan JS, Kragten EY, Veening H: Liquid-chromatographic study of fluorescent materials in uremic fluids. *Clin Chem* 29: 1082–1084, 1983.
42. Barnett AL, Veening H: Liquid-chromatographic study of fluorescent compounds in hemodialysate solutions. *Clin Chem* 31: 127–130, 1985.
43. Canaud B, Grassmann A, Scatizzi L, Marcelli D: Update on clinical evidence supporting hemodiafiltration, in Karkar (ed): *Advances in Hemodiafiltration*, INTECH, 2016, pp. 77–100.

Curriculum vitae

Personal data

Name:	Kai Lauri
Date of birth:	01.08.1972
Place of birth:	Estonia
Citizenship:	Estonia

Contact data

E-mail:	kai.lauri@mail.ee

Education

2006 – ...	Tallinn University of Technology – PhD
2004 – 2006	Tallinn University of Technology, Biomedical Engineering, Faculty of Information Technology, MSc
1990 – 1994	University of Tartu, Faculty of Physics and Chemistry, Chemistry, BSc
1987 – 1990	Põlva High school, Secondary education

Language competence

English	Higher intermediate
Russian	Intermediate
German	Beginner
Finnish	Beginner

Professional employment

2005 – …	SYNLAB Eesti OÜ, Quality Manager
1999 – 2005	The North Estonia Medical Centre, Laboratory Specialist
1994 – 1998	AS Tartu Terminaal, Head of Laboratory

Elulookirjeldus

Isikuandmed

Nimi: Kai Lauri
Sünniaeg: 01.08.1972
Sünnikoht: Eesti
Kodakondsus: Eesti

Kontaktandmed

E-post: kai.lauri@mail.ee

Hariduskäik

2006 – ... Tallinna Tehnikaülikool – PhD
2004 – 2006 Tallinna Tehnikaülikool, Elektroonika ja Biomeditsiinitehnika, MSc
1990 – 1994 Tartu Ülikool, Füüsika-Keemia teaduskond, Keemia, BSc
1987 – 1990 Põlva Keskkool

Keelteoskus

Inglise keel Kõrgem kesktase
Vene keel Kesktase
Saksa keel Algtase
Soome keel Algtase

Teenistuskäik

2005 – ... SYNLAB Eesti OÜ, Kvaliteedijuht
1999 – 2005 Põhja-Eesti Regionaalhaigla SA, Laborispetsialist
1994 – 1998 AS Tartu Terminaal, Laboratooriumi juhataja