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Major Chromophores and Fluorophores in the Spent Dialysate as Cornerstones for Optical Monitoring of Kidney Replacement Therapy

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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 submitted for doctoral or equivalent academic degree.

Jürgen Arund

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LOODUS- JA TÄPPISTEADUSED B211

Peamised kromofoorid ja fluorofoorid heitdialüsaadis neeruasendusravi optilise monitooringu nurgakividena

JÜRGEN ARUND

TTÜ KIRJASTUS

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LIST OF PUBLICATIONS

The current thesis summarizes the author's work at the Department of Biomedical Engineering of the Technomedicum of Tallinn University of Technology and is based on the following publications referred to in the text by their Roman numerals I-IV.

I. **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*, vol 4 (10): 849-861 (DOI: 10.3390/toxins4100849).

II. **Arund J**, Luman M, Uhlin F, Tanner R, Fridolin I, (2016) "Is Fluorescence Valid to Monitor Removal of Protein Bound Uremic Solutes in Dialysis?", PLoS ONE 11(5): e0156541 (DOI: 10.1371/journal.pone.0156541).

III. 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*, vol 16 (1): 95–106 (DOI: 10.3176/eng.2010.1.09)

IV. Tanner R, **Arund J**, Fridolin I, Luman M. (2013) "Paracetamol interference in uric acid levels in uremic patients revealed by monitoring spent dialysate", *ISRN nephrology*, vol 2013, Article ID 515292 (DOI: 10.5402/2013/515292).

V. 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". In: Proceedings of 35th Annual International Conference of the IEEE EMBS: 35th Annual International Conference of the IEEE EMBS, Osaka, Japan, 3 - 7 July, 2013. IEEE, 2013, 6707 – 6710 (DOI: 10.1109/EMBC.2013.6611095).

Author's contribution to the publications

The contribution by the author to the papers included in the thesis is as follows:

- Participation in sample collection – Publications I, II and V;
- All of the chromatography analysis – Publications I, II, IV and V;
- Chromatography data processing and analysis – Publications I – V;
- Major role in manuscript writing – Publications I and II;
- Contributing role in manuscript writing – Publications III – V.

Additionally, the author contributed by equipping a new chromatographic laboratory and setting up the methods with the new chromatographic systems which enabled obtaining the results presented in this thesis.

INTRODUCTION

More than two million people are being kept alive by renal replacement therapy worldwide [1, 2]. Renal replacement therapy is applied to treat chronic kidney disease (CKD), which is a key determinant of the poor health outcomes of major noncommunicable diseases, such as cardiovascular disease and diabetes [2, 3]. Most common for life-sustaining renal replacement for patients with an end-stage kidney disease is dialytic therapy [4]. Dialysis adequacy is a major factor contributing to the morbitity and mortality of the patients with end-stage kidney disease on hemodialysis therapy [5, 6]. Therefore, defining a suitable therapy has been of the crucial interest. For this reason, dialysis dose has been proposed as a parameter to quantify the therapy [5]. Several solutions have been proposed to estimate the dialysis adequacy [7-11], including a method based on UV-light absorbance by the uremic chromophores [12]. Although the chromophoric solutes in the blood serum and urine have been extensively studied [13-22], to our knowledge, no publications have reported the composition of chromophores in the spent dialysate.

For decades, dialysis adequacy has been estimated mainly based on one marker molecule – urea, a non toxic molecule that is easy to remove by dialysis. However, the status of uremia is more complex, involving hundreds of different molecules with different removal properties [23, 24], among these there are toxins that contribute to the patient's morbidity and mortality [25-27]. Although the dialysis adequacy assessment by a classical marker of urea represents the removal of a group of easily dialyzed uremic solutes, it does not cover the removal of a number of uremic toxins with different removal properties, such as protein-bound uremic solutes or middle molecules [23]. Therefore, there is a need for new markers covering the removal of uremic toxins that have different removal patterns from urea [23, 28]. Indoxyl sulfate is one of the most investigated uremic toxins in the last decade, which is proposed as a marker for the group of slowly dialyzed protein-bound uremic solutes (PBS) [25]. As a strong fluorophore, it offers an approach to estimate the removal of indoxyl sulfate via fluorescence at the selected wavelengths of spent dialysate [29].

The strong majority of the uremic solutes in spent dialysate are chromophores and several protein-bound solutes are fluorophores (e.g indoxyl sulfate and p-cresyl sulfate). Proper knowledge about the signal content, i.e. the composition of chromophores and fluorophores in the spent dialysate, serve as a cornerstone for a technique of optical monitoring of dialysis adequacy. Thus, understanding of the contributors to the optical signals of spent dialysate provides a key to the interpretion of the optical signal. Different studies have associated the UV-absorbance and fluorescence in spent dialysate with several uremic solutes by spectrophotometric approach [29-38]. However, more elaborated and precise methods were necessary to identify the composition of major chromophores and fluorophores in spent dialysate, which has led to the studies presented in this thesis.

The general aim of the thesis work was to identify the composition of the main chromophores and fluorophores in the spent dialysate by using chromatographic

separation methods to interpret of the UV-absorbance and fluorescence of spent dialysate, optical approaches proposed for monitoring the dialysis adequacy. Futher aim was to investigate, whether the UV-absorbance can be applied to monitor the removal of easily dialyzed uremic solutes and whether the fluorescence is suitable to assess the removal of slowly dialyzed uremic solutes.

The purpose of the first study (**Publication I**) was to investigate proportions of main chromophores in the spent dialysate combined with their removal ratio by using chromatographic separation. **Publication I** confirmed that the UV-absorbance is suitable to monitor the removal of easily dialyzed uremic solutes. The aim of **Publication II** was to estimate the composition of the main fluorophores in spent dialysate combined with their removal ratios via chromatographic separation. Since a predominant part of the fluorescence signal originates from protein-bound indole derivates, fluorescence monitoring is a promising method to assess the removal of slowly dialyzed uremic toxin of indoxyl sulfate.

The behavior of the UV-absorbance monitoring as compared to different dialyzer membranes (high flux vs low-flux) was studied in **Publication III**, where no statistical differences were found in chromophores contribution to the optical signal. A high contribution to the UV-absorbance by a drug was noticed in Publication I, which raised the question whether drugs may have effect on the optical monitoring of dialysis adequacy, leading to **Publication IV**. The study revealed that a drug of paracetamol may have an effect on the optical monitoring of dialysis adequacy. Thus, caution should be taken on the interpretation of an UV-absorbance signal when paracetamol is administered to a patient during dialysis. **Publication V** introduces an optical method to assess the removal of slowly dialyzed protein-bound toxin indoxyl sulfate by fluorescence measurement at selected wavelengths of spent dialysate.

Approbation

- **Arund J**, Tanner R, Lauri K, Luman M, Uhlin F, Fridolin I. "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.*
- **Arund J**, Tanner R, Lauri K, Luman M, Fridolin I. "Relative importance of uremic compounds in total UV absorbance of spent dialysate". *10th Baltic Nephrology Conference, Jurmala, Latvia, October 14-16, 2010*.
- Holmar J, **Arund J**, Uhlin F, Tanner R, Fridolin I. "Optical Estimation of the Removal Rate of Indoxyl Sulphate in the Spent Dialysate". *4th Meeting Uremic Toxins and Cardiovascular Disease, 20-22 May 2011 - Groningen, The Netherlands*.
- **Arund J**, Tanner, R, Fridolin I, Luman M. "Impact of paracetamol on optical on-line dialysis monitoring and on uricemia. Nephrology, Dialysis". *Nephrology, Dialysis, Transplantation, 27, ii197−ii226, ndt/gfs224, 49th ERA-EDTA Congress, May 24-27, 2012, Paris, France*.
- Tanner R, **Arund J**, Fridolin I, Luman M. "Effect of paracetamol on Uric Acid level and optical dialysis monitoring". *XI Baltic Nephrology Conference; Tartu, Estonia, 20.-22. September 2012*.
- **Arund J**, Tanner R, Fridolin I, Luman M. "Mapping of fluorophores in the spent dialysate: a preliminary study". *8th International Congress on Uremia Research and Toxicity, March 12 - 15 2014, Okinawa, Japan*.
- **Arund J**, Tanner R, Fridolin I, Luman M. "Fluorophores responsible for the fluorescence signal in spent dialysate". *12th Conference of Baltic Societies of Nephrology, June 12-14 2014, Molėtai district, Lithuania*.
- Patent: Fridolin I, Uhlin F, Holmar J, Tanner R, **Arund J,** inventors. Tallinn University Of Technology, 19086, Tallinn, Estonia, assignee. Method and device for determining content of the middle and protein bound uremic toxins in a biological fluid, PCT pat. nr. WO2012000521 patent US9103789 B2. 2015
- **Arund J**, Tanner R, Luman M, Uhlin F, Fridolin I. "On-line fluorescence monitoring of spent dialysate for following dialyzed indoxyl sulfate". *EUTox CME Course «The vessels a target for the uremic toxins"; Marseille, France; 27.-28.03.2015*.

ABBREVIATIONS

BUN – blood urea nitrogen
CE – capillary electrophoresis
CKD – chronic kidney disease
CREA – creatinine
Da – Dalton, unit for molecular weigth
DOQI – Dialysis Outcomes Quality Initiative
eKt/V – equilibrated dialysis dose efficacy parameter
Em – emission
ESI – electrospray ionization
ESRD – end-stage renal disease
Ex – excitation
GSA – guanidinosuccinic acid
GC – gas chromatography
HA – hippuric acid
HD – hemodialysis
HDF – hemodiafiltration
HPLC – high performance liquid chromatography
IS – indoxyl sulfate
K – dialyser clearance
Kt/V – dialysis dose efficacy parameter
LC – liquid chromatography
MG – methylguanidine
MS – mass spectrometry
MW – molecular weigth
PBS – protein bound solutes
pCG – p-cresyl glucuronide
pCS – p-cresyl sulfate
RC – relative contribution
RR – reduction ratio
spKt/V – single pool dialysis dose efficacy parameter
TR – total removed
UA – uric acid
URR – urea reduction ratio
UV – ultraviolet light
V – volume, mL

1 THE KIDNEY, KIDNEY FAILURE AND DIALYSIS

1.1 Kidneys and kidney failure

Kidneys perform excretory, regulatory and metabolic functions in our body. The kidneys are responsible for the regulation of body water volume and osmolality, electrolyte, and acid-base balance, excreating all redundant material. Their endocrine function is the production and secretion of hormones, such as rennin and erythropoietin and activation of vitamin D [39].

Renal (or kidney) failure is characterized by the progressive decline in the capacity of the kidneys to eliminate excessive water and toxic solutes and can be distinguished as acute or chronic failure [39]. While acute kidney failure may be reversible, chronic failure usually is followed by progressive decrease of its performance to remove water and waste. Chronic kidney disease (CKD) is classified into stages 1–5, where stage 1 is a mild loss of kidney function and stage 5 CKD is end-stage renal disease (ESRD) and is identified by glomerular filtration rate less than 15 ml/min or the need for dialysis [2, 40]. CKD is associated with extremely high morbidity and mortality even in its earlier stages [2, 41]. The ultimate goal of treatment for patients with CKD stage 5 is improvement in the quality of life and prolongation of life[42].

1.2 Kidney replacement therapy

Kidney replacement therapy can be provided by three different possibilities: hemodialysis, peritoneal dialysis and transplantation. While the transplantation would be the best solution out of others, this is not always possible due to the absence of a matching donor organ, or due to the condition of the patient. Peritoneal dialysis is done via patients own peritoneum, a thin membrane inside the abdomen. In hemodialysis, the patient's blood is cleaned by using an artificial kidney, a special membrane, allowing the solutes to pass from the blood to the dialysate by a diffusive and/or convective process. Filtered blood is returned to the patient and the spent dialysate with waste material from the body is directed to the drain.

2 DIALYSIS ADEQUACY

In general, adequacy of hemodialysis is referred to as the suitability of kidney replacement treatment which would keep the patient alive and as healthy as possible. Despite the term of dialysis adequacy is widely used in nephrology, there is no consensus in community, which of the criteria or parameters define the adequacy [43]. From the beginning of hemodialysis, various criteria and/or parameters have been proposed to assess the adequacy, such as the blood urea nitrogen (BUN), urea reduction ratio (URR), Kt/V, serum creatinine, serum potassium, serum albumin, calcium and phosphorus, haematocrit level, dialysis time and frequency, blood pressure etc. [5, 44-46]. As urea is the most abundant metabolic end product in uremic sera, it has become the main marker for dialysis adequacy estimation [47, 48]. Today, the adequacy of dialysis is mainly referring to the dialysis dose of Kt/V, based on the urea values of blood sampled before and after the dialysis [5, 42, 48].

To estimate the removal adequacy, the dialysis dose of Kt/V, two possible approaches are common: one is referred to as a “blood-side” approach (called the indirect method), and the other is referred to as a “dialysate-side” approach (called the direct method) [49].

Indirect method, such as BUN concentration measurement, has been commonly accepted as the reference method. It excels in the cost efficiency of laboratory expenses due to easy performance of standard urea measurement methods; however the disadvantages are that the blood sampling is error-prone [50], frequent blood sampling may contribute to anemia, and there is always a variable time gap between the blood-draw until achieving the results from the clinical laboratory. Clinical practice guidelines for hemodialysis adequacy recommend measuring delivered dialysis dose of Kt/V at least monthly [40], while hemodialysis is done more than ten times per month. According to a study published in 2009, more than a third of included dialysis centers disregard this recommendation [51]. The authors of the study indicate that out of 1023 European dialysis centers the study comprised 255 who responded to the questionnaire, raising a doubt that the results of that study may suffer from a positive selection bias [51]. The uncertainty of the delivered dialysis dose may result in inadequate dialysis, in turn, resulting in decreased life quality or even worse - in increased mortality of the CKD patients [52].

Direct methods based on the measurements of the spent dialysate are less common as these methods can be cumbersome due to collection of all spent dialysate for the calculation of the total solute waste, or the lack of accurate, cost-effective, robust and versatile on-line measurement technologies. During one hemodialysis session more than 100L of spent dialysate is normally generated, therefore it is difficult to utilize tank collection routinely for the estimation of the adequacy as the space in dialysis centers is usually limited. On-line methods estimating the removal of water soluble non-bound solutes have been correlated to the removal of a classical marker of urea [12, 53] or measures directly urea in spent dialysate [54], all without the need for frequent blood sampling. As the on-line

methods are designed to be robust, automatic, in-situ and online, these methods benefit from lack of the disadvantages of the blood-side approach and therefore can be easily routinely used to monitor the adequacy of every hemodialysis treatment without extra expenses.

During evolution of hemodialysis adequacy parameters, attention has concentrated on three methods based on blood urea values: BUN, URR and Kt/V. Hemodialysis prescription based on BUN values developed into two blood sample based Kt/V, introduced in 1985 [5]. Later, a simplified treatment prescription of URR was proposed [55]. Kt/V is a dimensionless parameter where K stands for dialyser clearance (mL/min), t stands for time (min) and V is the estimation of the volume of patient's body fluid (mL) and it can be calculated from blood samples taken before (C_0) and after (C_t) the dialysis [5]:

$$Kt/V = - \ln(C_t/C_0) \quad (1)$$

Later, advanced formulas for single-pool Kt/V (spKt/V) [56] and more accurate equilibrated Kt/V (eKt/V) were proposed [57]. URR indicates the decrease of urea concentrations in the blood samples taken before and after the dialysis [55]:

$$URR\ (\%) = 100 * (1 - C_t/C_0) \quad (2)$$

However, the removal kinetics of different uremic molecules, such as protein-bound uremic solutes or middle molecules, has been reported to differ from the classical marker of urea [58-61]. This has raised the question of whether a single marker for the dialysis adequacy is sufficient enough to estimate the quality of the kidney replacement therapy or else multi-marker-based dialysis adequacy parameters should be used [23, 25, 28, 43, 62-64].

3 THE UREMIC SOLUTES AND THEIR MEASUREMENT

Molecular milieu of uremic sera is complex, containing a wide variety of retained uremic solutes. The list of uremic solutes is continuously updated and today there are more than 270 identified compounds [24], which could be classified into three major molecular groups depending on their size or protein-binding [65-67]. If these uremic solutes indicate biological or biochemical negative activity, they are called uremic toxins [68].

The three major groups of uremic solutes can be identified as [66]:

1) free, water soluble, no known protein binding, low MW < 500 Da;
2) uremic solutes, with known or probable protein binding, MW < 500 Da;
3) middle molecules, 500 Da < MW < 60 kDa.

Table 1: Comparison of dialyser clearances (K, mL/min) for groups of uremic solutes

		K (mL/min)
1) Small, non-protein-bound [69] N = 7; low-flux dialyser; Kt/V = 1.77 ± 0.17.	Urea*	261 ± 17
	Creatinine*	264 ± 14
	Min	235 ± 29 (GSA)
	Max	278 ± 15 (MG)
2) Small, protein-bound [61] N = 10; high-flux dialyzers; Kt/V = 1.7 ± 0.3	Urea*	224 ± 20
	Min	21 ± 4 (pCS)
	Max	152 ± 28 (pCG)
Small, protein-bound [70] N = 9; high-flux dialyzers; URR = 84 ± 4 %	Urea*	204 ± 20
	Min	14 ± 5 (IS) 14 ± 6 (pCS)
	Max	121 ± 14 (HA)

*urea and creatinine as a reference
GSA - guanidinosuccinic acid; MG - methylguanidine; pCS – p-cresyl sulfate; pCG – p-cresyl glucuronide; IS – indoxyl sulfate; HA – hippuric acid.

Low molecular weight, non-protein-bound uremic solutes

This group consists of uremic solutes, which are easily removed by any dialysis strategy [68]. These have low molecular weight (MW < 500 Da) and are free water soluble with no known protein binding. Examples are urea (MW = 60 Da), uric acid (UA, MW = 168 Da), and creatinine (CREA, MW = 113 Da). Compounds in this group do not necessarily have a marked toxic activity [68]. Although urea is the most investigated representative of this group of uremic solutes in terms of clinical outcomes, there is lack of indications of its toxicity [23]. The same is true about CREA, as lower rather than higher concentrations have been associated with high death risk [71]. UA is a unique representative in this group because of its limited solubility in water, thus prone to producing urate crystals at high physiologic concentrations [72]. It is a metabolic endproduct of purines and has

been linked with possible toxicity [73-78], though the toxicity is still under a debate [79, 80]. However, the dialyzer clearances K for the different solutes in this class are fairly comparable (Table 1), they can differ from each other in their kinetic behavior during hemodialysis, indicating that urea kinetics does not represent the removal of the other free low MW uremic solutes [69].

The most popular uremic solutes, i.e. urea, CREA and UA, can be measured by simple and robust clinical laboratory methods based on colorimetric detection with or without specific enzymatic reactions (see Section 3.2). The other solutes are mostly measured by chromatographic methods [65]; however, methods based on immunochemistry [81-85] and nuclear magnetic resonance spectroscopy (NMR) [86] have been used.

Protein-bound uremic solutes

Most of the solutes in this group have a low MW with known or probable protein binding, and some may have middle molecule characteristics (e.g. leptin, the cytokines) [68]. The most investigated representatives are the derivatives of phenols (e.g. p-cresyl sulfate – pCS, MW = 188 Da) and indoles (e.g. indoxyl sulfate - IS, MW = 213 Da) [87]. These solutes are difficult to remove by most of the currently available dialysis strategies [19, 61, 68]. For example, the dialyzer clearances for the protein-bound uremic toxin of pCS can be up to tenfold smaller than urea, the representative from the group of free low MW uremic solutes (Table 1) [61, 69, 70]. Low dialyzer clearances of protein-bound solutes occur, as only free, unbound fraction of these solutes is available to the diffusion through the dialysis membrane [70]. To improve the removal of protein-bound uremic solutes, it has been suggested to increase the frequency of the dialysis and/or making the dialysis longer [18, 61], to increase the dialyzer size and dialysate flow [70], or by using online hemodiafiltration (HDF) [88].

Many of the compounds in this group have been reported to contain toxic activity [68]. For example, reviews on toxicity of the most extensively evaluated protein-bound uremic solutes of IS and pCS conclude the IS and/or pCS induced factors contributing to the cardiovascular damage, increased propensity for mortality and cardiovascular events in CKD, and to the progression of CKD [25, 89].

The main methods for the measurement of protein-bound uremic solutes are based on chromatographic separation [65], while few solutes are measured with methods based on immunochemistry [90-94].

Middle molecules

These molecules can only be removed by the dialysis strategies that employ dialyzer membranes containing pores large enough (high flux dialyzers) to allow these molecules to cross the membrane [68]. The most investigated prototype is β2-microglobulin (MW = 11,818 Da), as its concentration has the highest increase in this group of solutes for end-stage kidney disease patients compared to normal

levels [65, 66]. As middle molecules are out of scope of this work, these are not covered in detail.

3.1 Online optical method for uremic solutes assessment

Light and matter

Direct dialysis adequacy is assessed by analyzing the spent dialysate, which can be considered as weakly scattering media [95]. The interactions between light and biofluid involve absorption, scattering and reflection. As the optical path length used in colorimetric detection in the measurements of spent dialysis is typically 10 mm or less, concentration of solutes is relatively low and the solutes have MW less than 50,000 Da (diameter of particles d < 5 nm), then the scattering can be ignored [96]. When the angle of incidence for a ray of light and surface normal of the cuvette is 0-degrees, then the absorption of the light becomes the most important interaction between light and biofluid.

Absorbance and Bouguer-Lambert-Beer law

Absorption is annihilation of photonic energy via interaction with electrons, atoms or molecules and transformation into internal energy of the absorber (e.g. heat) [97]. Fluorescence is the result of the process that occurs in certain molecules, where photonic energy is absorbed by a molecule, which releases a photon while returning to the ground energetic state [98]. Due to energy dissipation, the energy of that photon is lower, thus with higher wavelength. Molecules that absorb light are called chromophores, and molecules that emit light are called fluorophores [99].

Absorbance of a media quantifies how much of the incident light it absorbs and is generally expressed through the Bouguer-Lambert-Beer law (also known as the Lambert-Beer law). The law's derivative form gives absorbance A [arbitrary units] by:

$$A = log_{10} (I_0 / I) , \tag{3}$$

where I_0 is the intensity of received radiation and I is the intensity of transmitted radiation by the media. The relation between absorbance, molar extinction coefficient ε [m^{-1} $(mol/L)^{-1}$], concentration of the absorbing compound C [mol/L] and optical path length d [m] can be written:

$$A = \varepsilon C d \tag{4}$$

In practical spectroscopy, equations (3) and (4) are combined as

$$A = log_{10} (I_0 / I) = \varepsilon C d, \tag{5}$$

allowing calculation of the concentration C of a solute by measuring the change of transmitting light intensities between solutions where the chromophore of interest is present (I) and absent (I_0). If the extinction coefficient ε and the optical path length d are known:

$$C = log_{10} (I_0 / I) / \varepsilon d, \quad (6)$$

The extinction coefficient ε is determined by measuring the reference solutions at different concentrations of a given compound. As the optical path length is constant, the resulting calibration formula includes both ε and d.

Optical monitoring of uremic solutes in the spent dialysate

Based on the UV-light absorption of the spent dialysate, an optical monitoring method for estimating the dialysis efficiency was first proposed by Gal et al. in 1980 [38]. At that time, no specific parametric targets for the dialysis adequacy existed and the method was not adopted in clinical practice. In 1998 the slope of the UV-absorbance online signal of the spent dialysate was related to the dialysate adequacy parameter of Kt/V, which gave to the UV-absorbance signal the value in common clinical practice [100, 101]. Later, the technique was described in detail [12] and a clinical study demonstrated that the UV-absorbance monitor can be a valid tool to estimate the adequacy of the dialysis [102]. The use of light emitting diodes (LEDs) enabled creation of miniature and robust UV-absorbance-based dialysis adequacy assessment monitors [103]. Today, this technology has been taken into routine use in clinical practice [104, 105]. In addition, this online monitoring technology has been proposed to indicate the disturbances in the hemodialysis treatment [106, 107]. Furthermore, it was suggested as a possible method for monitoring the delivered dose in the latest clinical practice guidelines for hemodialysis care [42]. Despite the UV-absorbance online monitoring has become a relevant tool for estimating the dialysis adequacy in clinical practice, there was still lack of knowledge about the contributions and possible interferences to the optical signal of the chromophoric solutes [106]. As a result, the contributions and the relative change during hemodialysis of different chromophores were estimated (Publication I) [108]. A possible intereference to the UV-monitoring by a common medication of paracetamol (acetaminophen) was noticed, leading to the results presented in Publication III [109].

Recently, a novel method for estimating the removal of protein-bound uremic toxin of indoxyl sulfate was proposed, utilizing fluorescence monitoring of spent dialysate (Publication IV) [29, 110]. The prevalent protein-bound uremic solutes of indoles and phenols in biofluids are commonly measured by LC coupled with fluorescence detection [18, 60, 61, 70, 111-114], therefore utilizing fluorescence as a tool for monitoring the removal of protein-bound uremic solutes in the spent dialysate seemed feasible. To prove this hypothesis, the following research estimated the contributions and relative change during the hemodialysis of main fluorophores and the results were concluded in Publication II [115].

3.2 Biochemical methods for uremic toxins analyses

In clinical laboratories, the methods for estimating levels of major uremic solutes are expected to be fast, easy, robust, cost-effective and efficient in terms of analysis quality. The methods have developed from simple chemical reaction based

colorimetric to multi-step enzymatic or immunological selective methods. As the colorimetric and enzymatic methods are or have been the main reference methods in the analysis of uremic fluids, these methods are described below.

Colorimetric methods

Colorimetric methods were the first analytical methods which allowed the determination of the uremic solute levels in biofluids. Though the Jaffe's method is the oldest in the medical laboratories for determining the levels of blood and urine creatinine [116], it is still in common use. Jaffe's reaction is between creatinine and picrate in an alkaline medium, forming a red-orange complex that absorbs light in the range of 480 to 520 nm [117]:

$$\text{Creatinine + picric acid (Yellow)} \xrightarrow[\text{(alkaline medium)}]{\text{NaOH}} \text{Creatinine picrate (Orange)} \quad (7)$$

It is nonspecific and tends to overestimate the creatinine levels, therefore it is recommended to replace this method by more specific measurements, such as methods based on chromatography or enzymatic reactions [118, 119]. An alternative colorimetric method for creatinine is based on the reaction with 3,5-dinitrobenzoic acid [120]. The levels of urea can be colorimetrically determined after the reaction with O-phthalaldehyde [121] or diacetyl monoxime [122]. Methods for determining uric acid involve reaction with phosphotungstate acid [123, 124]. As all these methods lack on the specificity to the compound of interest and suffer from interference of other compounds while there are highly selective and robust methods available, these methods are not recommended in the use of medical diagnosics [125].

Enzymatic determination method

Enzymatic reactions offer highly selective determination of the concentrations of the major uremic solutes, such as urea, creatinine and uric acid. Enzymatic measurements of urea are based on urease, where a reaction product ammonium can be measured by various methods involving conductivity [126, 127], potentiometry [128, 129] or colorimetry [130-132].

$$\text{Urea} + 2H_2O \xrightarrow{\text{Urease}} NH_3 + CO_2 \quad (8)$$

Uric acid is measured via allantoin, a reaction product with uricase, with colorimetric or fluorometric detecion [125, 133, 134].

$$\text{Uric acid} + O_2 + 2H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O \quad (9)$$

Enzymatic determination of creatinine can be done via creatinine deiminase [135]:

$$\text{Creatinine} + 2H_2O + H^+ \xrightarrow{\text{Creatinine deiminase}} \text{N-Methylhydantoin} + NH4^+ \quad (10)$$

$$NH4^{+} + \alpha\text{-Ketoglutarate} + NADPH^{+} \xrightarrow{GIDH} \text{Glutamate} + 2H_2O\ NADP^{+} \quad (11)$$

or creatininase [136-138].

$$\text{Creatinine} + H_2O \xrightarrow{\text{Creatininase}} \text{Creatine} \quad (12)$$

$$\text{Creatine} + H_2O \xrightarrow{\text{Creatinase}} \text{Sarcosine} + \text{Urea} \quad (13)$$

$$\text{Sarcosine} + O_2 + H_2O \xrightarrow{\text{Sarcosine oxidase}} \text{Clycine} + \text{Formaldehyde} + H_2O_2 \quad (14)$$

$$H_2O_2 + \text{4-aminoantipyrine/HTIB} \xrightarrow{POD} H_2O + \text{dye} \quad (15)$$

3.3. Analytical laboratory techniques for uremic solutes analyses

Chromatographic methods

To accurately quantify the solute of interest in the complex matrixes of the uremic biofluid samples, highly selective analytical methods are assumed. Some approaches utilize specific immunologic assays, but most commonly, the analytical quantification is done via chromatographic separation [65]. Chromatographic separation is based on the analyte's different moving velocity in the given separative media, which consists of two phases, mobile and stationary phase. The mobile phase, called eluent, consists of a flow of a gas (gas chromatography), a liquid (liquid chromatography) or a gas in supercritical liquid state. Stationary phase depends on the type of chromatography - for gas chromatography, it is typically a layer of a liquid film on the inner walls of long capillary; for liquid chromatography, it is a column filled with specific adsorber material. In the separation process, the continuously flowing mobile phase carries the sample through the stationary phase, where the solutes interact with the stationary phase. As the strength of interaction differs for different compounds, the compounds have different moving velocities, thus resulting in the separation of the solutes. After the separation, a wide variety of detection methods, including optical (Figure 1), electrochemical, mass spectrometrical, etc. can be applied. An effective separation usually results in normally distributed molecules of a compound in the eluent, generating a Gaussian-shape detection signal in a time axis, which is called a peak. The height and area of a peak depends on the concentration of the compound. The time a solute spents to travel through the separating system is called a retention time. While the conditions of the separation remain stable and constant, the retention time of any compound is a constant, thus it is an important factor for the identification of a peak as a certain compound.

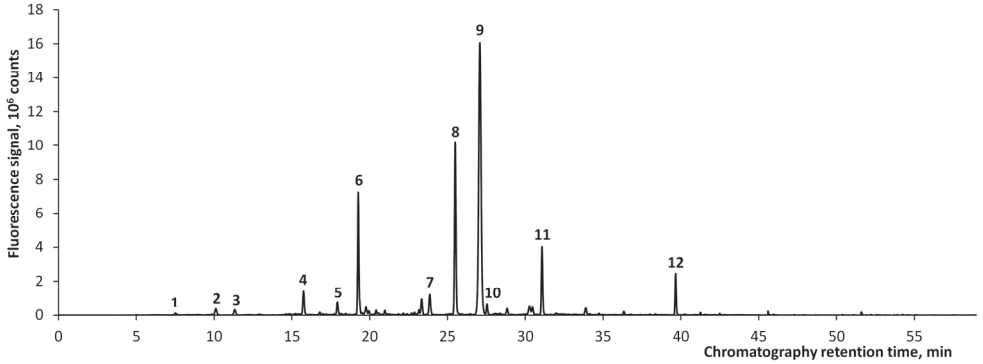

Figure 1 An example of a HPLC chromatogram of a spent dialysate sample monitored by fluorescence detection at Ex: 280 nm and Em: 360 nm.

Gas chromatography

Gas chromatography (GC) is a highly sensitive, accurate, fast and reproduceable method with high separative power for analyzing uremic solutes, which are volatile, thermally stable and not highly polar. As in the native form, the list of suitable uremic solutes for GC separation is rather short, derivatization techniques are used, where a compound suitable for GC is generated [139, 140]. In the research of uremia, the gas chromatography is usually coupled with mass spectrometric detection (GC/MS), which enables very selective detection and quantification. For example, in uremic serum and plasma samples, GC/MS has been used to measure of amino acids [141], alkanals, alkenals [142], polyols [143, 144], phenols [145, 146], and indoles [147, 148].

Liquid chromatography

Liquid chromatography (LC) offers the most flexible separation methods with high accuracy, high sensitive and reproducibility as it can be used to separate a large number of compounds often without preliminary derivatization. Contrary to GC, the separation by LC is slower, as the compounds in the liquid phase at moderate temperatures have lower speeds of interaction than the compounds in a gas phase at high temperatures. This results also in decreased separation efficiency of LC, since compared to GC, a higher level of peak band-broadening occurs. The latest improvements in column particle technology have significantly increased the performance of HPLC separation, as core-shell adsorber particles have been taken into use [149].

The most common detection methods coupled with LC are optical and/or mass spectrometrical [87, 150-153]. Optical detectors are the least expensive detectors for LC, utilizing UV light-absorbance or fluorescence. Concerning uremic toxins, UV-detectors have been used, for example, to measure ribonucleosides [154], purines [108, 155, 156], guanidines [157]; and fluorescence detectors for indoles [18, 60, 61, 70, 111-115], phenols [60, 61, 70, 88, 111-113], with derivatization also for guanidines [158-160] etc. The UV-absorbance detectors are the most

universal, as most of uremic solutes absorb somewhat UV-light, which is also a drawback in detection selectivity. For selective measurement via LC UV-absorbance, orthogonal multidimensional separation techniques or preprocessing of the sample can be applied, e.g. with solid-phase extraction or by using a derivatization method. Fluorescence detection offers high selectivity, however the list of natural fluorophores in the biofluid is limited, therefore often the derivatization with fluorophoric reagent is applied. As optical detectors in LC are not destructive, multiple detectors can be used in a row, by this kind of approach, a variety of compounds can be measured simultaneously [61, 161-163]. In a few cases, methods based on electrochemical detection have been proposed, however these are not common [164-166]. LC coupled with mass spectrometry (LC/MS) offers the most selective detection and is therefore the most valued tool in the research of uremia [150]. However, LC/MS is limited to the compounds that can be ionized and is often less sensitive than LC coupled with fluorometric or electrochemical detection. The higher costs of MS devices and sophisticated data analysis of MS can be listed to the downsides of LC/MS as a tool in large-scale studies.

In modern liquid chromatography studies, high performance analytical solutions are used, named accordingly high performance/pressure liquid chromatography (HPLC) or ultra performance liquid chromatography (UPLC). These methods involve specific columns filled with fine adsorbent particles (≤ 5 μm) where high eluent pressures are applied, in HPLC up to 40 MPa, in UPLC up to 120 MPa).

Capillary electrophoresis

Besides chromatographic separation, capillary electrophoresis (CE) offers an alternative approach to the separation of uremic solutes [167, 168]. As it is a relatively inexpensive system in comparison to LC or GC, it offers an affordable analytical solution for fast, reliable and sensitive separation of small quantities of samples. CE can be coupled with a variety of detectors, including optical and mass spectrometric detectors. However, as the separation is based on the movement of charged compounds in the applied electrical field, the CE suits for compounds that have at least one charged functional group that can form complexes with background electrolytes or can form covalent bond with an additive with charge [169]. CE excels in the separation of proteins, peptides and chiral molecules, however CE is not as universal as LC. Consequently, it is not a main tool in the research of uremia for low MW uremic molecules.

4 EXPERIMENTAL STUDIES: METHODS, RESULTS AND DISCUSSION

4.1 Methods

Clinical studies

All the studies were performed after approval of the protocol by the Regional Ethical Review Board, Linköping, Sweden decision on application no. M153-07 (10.10.2007) and by the Tallinn Medical Research Ethics Committee at the National Institute for Health Development, Estonia decision no. 2349 (15.03.2011). A written informed consent was obtained from all participating patients. The summary on included patients, dialysis parameters and dilaysate sampling times is presented in Table 2:

Table 2. Patients, dialysis parameters and sampling times in accordance with DOQI [42]

Publication	**I**	**II**	**III**	**IV**	**V**
Total number of patients	8	28	10	17	18
- From Tallinn (male/female), mean age ± SD	-	20 (10/10), 59 ± 12	10, 62.6±18.6	Test: 7 (2/5), 65 ± 13; Ref: 10 (8/2), 63 ± 19	10 (6/4), 58 ± 8
- From Linköping, male/female, mean age ± SD	8 (7/1), 73 ± 14	8 (7/1), 73 ± 14	-	-	8 (7/1), 73 ± 14
Number of sessions (type)	48 (24 HD / 24 HDF)	99 (57 HD / 42 HDF)	30 (15 LF-HDF / 15 HF-HDF)	17 (17 HD)	78 (33 HD / 45 HDF)
Dialysis machine	Fresenius 5008H	Fresenius 5008	4008H	Fresenius 4008H	Fresenius 5008
Blood flow, mL/min	280…350	250…350	245…350	245...350	250…350
Dialysate flow, mL/min	500	500	500	500	500
Session's length, min	180...270	180…270	210 or 240	180 or 240	180…270
Sampling time, min	10, end of dialysis	10, end of dialysis	Before and at the end of dialysis	10, end of dialysis	10, end of dialysis, tank

Chromophores detection by HPLC (Publications I and IV)

All dialysate samples were acidified down to pH 4.0 with formic acid before the high performance liquid chromatography (HPLC) analysis for conformation with the pH of the chromatographic eluent used. The HPLC system consisted of a quaternary gradient pump unit, a column oven, and a diode array spectrophotometric detector (all Ultimate 3000 Series instruments from Dionex (Sunnyvale, CA, USA), and Zorbax C18 4.6 × 250 mm column from Agilent Instruments (Wilmington, DE, USA) with a security guard KJO-4282 from Phenomenex (Torrance, CA, USA). For mass spectrum analysis, the micrOTOF-Q II with ESI source by Bruker Corporation was used (Billerica, 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), both from Rathburn (Walkerburn, Scotland). The three-step curve-type gradient elution program was used, as specified in Table 3.

Table 3. HPLC gradient program for Publication I and IV

Step	Time (min)	Buffer (A), %	Methanol (B), %	Acetonitrile (C), %
0	0	100	0	0
1	30	60	36	4
2	5	10	81	9
3	4	10	81	9

The total flow rate of 1 mL/min was used continuously at the column temperature of 30 °C. The UV absorbance was monitored at 210, 254 and 280 nm with a measurement interval of 500 ms. Spectra were registered between 200 and 400 nm with a time interval of 0.50 s, data were processed by Chromeleon 6.8 software (Dionex, USA). Mass spectra were acquired between 100 and 700 Da with a time interval of 1 s, data were processed by DataAnalysis (Bruker Corporation, Billerica, USA).

Every peak in the HPLC chromatograms was characterized by the characteristic absorption spectrum and by the retention time. Peaks were identified by comparing the retention time, absorption spectrum and mass spectrum data of a compound found in the sample with a pure authentic compound.

Chromophores detection by HPLC (Publication III)

Before the HPLC analysis, the serum samples were purified of proteins by centrifuging with the Microcon centrifugal filters (Millipore, USA) at room temperature. 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 × 250 mm column from Du Pont Instruments (Wilmington, DE, USA) with a 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), both from Rathburn

(Walkerburn, Scotland), with a six step gradient program as specified in Table 4. 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 200–400 nm with a time interval of 1.76 s, and data processed respectively by means of Turbochrom WS and Turboscan 200 software from Perkin Elmer. The chromatographic peaks were detected by the UV detector at wavelengths of 254 and 280 nm.

Table 4. HPLC gradient program for Publication III

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

Fluorophores detection by HPLC (Publications II and V)

All dialysate samples were acidified down to pH 4.25 with formic acid before the HPLC analysis for the sake of stable chromatographic retention times. 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 a 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 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 formiate salt (B). The three-step linear gradient elution program was used, as specified in Table 5.

Table 5. HPLC gradient program for Publication II and V

Step	Time (min)	Buffer (A), %	Organic solvent (B), %	Gradient
0	0	99	1	
1	6	99	1	Linear
2	39	10	90	Non-linear
3	15	10	90	No gradient

The total flow rate of 0.6 mL/min was used at the column temperature of 40 °C. All the spent dialysate samples were analyzed by recording 2-dimensional (2D) fluorescence acquisition chromatogram at the Ex: 280 nm and Em: 360 nm with a measurement interval of 0.5 s. For all 2D chromatograms, a blank run was subtracted from the signal. The 3-dimensional (3D) fluorescence acquisition chromatograms were recorded for 10 selected spent dialysate samples taken at the

start of the dialysis, which had the highest common characteristic peak intensities in 2D chromatograms. For 3D chromatograms, each sample was chromatographed two times: one for fluorescence excitation scan and the other for fluorescence Em scan. The fluorescence scanning parameters are given in Table 6 at the measurement interval of 0.5 s. The chromatographic data was processed by Chromeleon 7.1 software by Thermo Scientific (Waltham, USA).

Table 6. Chromatogram recording program

		Excitation	Emission	Samples
2D acquisition		280 nm	360 nm	All
3D acquisition	Excitation scan	220…340 nm	360 nm	10 selected
	Emission scan	280 nm	300…500 nm	10 selected

For mass spectrum analysis, a quadrupole time-of-flight mass spectrometer micrOTOF-Q II with an ESI source was used (Bruker, Billerica, USA). For both negative and positive ion mode, the parameters for sample analysis were as follows: mass range of m/z 50–700; ion source temperature of 200 °C, ESI voltage of 4.5 kV, ESI nebulization gas flow of 8.0 L/min, drying gas flow of 1.2 bar, detector voltage of 2.03 kV, acquisition rate of 1 Hz. Mass calibration was carried out using a sodium formate solution (10 mmol/L) from m/z 50 to 700. Data acquisition was performed using software Compass HyStar version 3.2, and processing of the data was carried out with Compass DataAnalysis version 4.0 SP1 (both Bruker, Billerica, USA).

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 with the reference compound.

In the analysis of the 3D fluorescence chromatograms in Publication II, the fluorescence Em and Ex spectra of the peaks were calculated by summing all the spectra over the respective chromatographic peak. In addition, to simulate Ex and Em spectra of nonfractionated spent dialysate, total fluorescence Ex and Em spectra were calculated on the basis of a typical 3D fluorescence chromatogram as the sum of all time-point spectra over the whole chromatogram up to the chromatographic retention time when the column washing peaks started to elute, the latter system peaks were excluded.

Relative contribution and removal ratio (Publications I, II and III)

The relative contribution (RC) for the i-th chromatographic peak (presumably solute) to the sum of UV absorbances of all peaks for a chromatogram was calculated as a ratio of the area of the i-th peak ($A_{peak\ i}$) to the total area of all peaks appeared on the chromatogram (A_{total}):

$$RC_i\ (\%) = 100\ *(\ A_{peak\ i}\ /\ A_{total}) \quad (16)$$

The contribution values of peaks with similar retention time were averaged separately and depending on the sampling time for all spent dialysate samples from the start and end, also for all of the samples in total. Relative contribution (*RC*) for a specific solute group "j" was calculated similar to RC_i:

$$RC_j\ (\%) = 100\ *(\ A_{group\ j} / A_{total}) \quad (17)$$

The removal ratio (RR) of a specific *i*-th peak (uremic solute) for a dialysis session was defined as a function of the start and end HPLC peak areas ($A_{start\ i}$ and $A_{end\ i}$) of the samples from the dialysis session:

$$RR_i\ (\%) = 100\ *(A_{start\ i} - A_{end\ i}) / A_{start\ i} \quad (18)$$

In Publications I, III and IV, Student's t-test was used to compare the means of RC_i and RR_i, and $p \leq 0.05$ was considered significant. Correlation analysis (Pearsons R) was used to estimate the degree of linear association between the variable groups of RC_i and RR_i in Publication II and variable groups of total removed (TR) IS estimated by HPLC and the optical method in Publication V, $R > 0.8$ was considered as a strong correlation. Coefficient of determination (R^2) was calculated to assess goodness of fit between parameters.

4.2 Chromophores in the spent dialysate (Publication I)

The UV-absorbance measurement of chromophores, whether their native or as a product of derivative reaction in various biofluids is one of the most feasible methods today to measure a wide variety of compounds. It is considered cost-effective and easy to perform with or without separation of the target chromophore from the sample matrix (see Sections 3.2 and 3.3). Although several studies covering the potential use of UV-absorbance-based technology for dialysis adequacy monitoring have been published [30, 33, 35, 36, 100, 170, 171], to our knowledge, no publications has reported the contributions of the main chromophores combined with their removal dynamics. In the research of Publication I, the major chromophores in 96 spent dialysate samples from 48 dialysis sessions for 8 patients were separated and characterized by HPLC UV-absorbance detection.

It was found that chromophores with RR > 60 % contribute more than 90% to the UV absorption at the wavelength of 280 nm, utilized in UV-absorbance-based dialysis adequacy monitoring devices [172, 173]. Among these chromophores there are widely investigated uremic solutes of UA, CREA and HA, while UA was the major contributor to the UV-absorbance. The study also indicated to a possible interference by the chromophoric drug of acetaminophen (paracetamol), which had median contribution of 7% at the wavelength 280nm. Among the group of the chromophores with RR < 60%, the RC to the UV-absorbance was found to be negligible.

The results of Publication I complied with earlier publications where UA was found to be the main chromophore in the spent dialysate [17, 32, 174], also in uremic serum [64, 175, 176]. In earlier publications, the UV-absorbance has been

successfully proven to estimate the removal of easily dialyzed uremic solutes with a high RR [12, 33, 107]. Findings presented in Publication I also agree with earlier publications, where strong relationship between UV-absorbance and UA was reported [34, 36]. This study revealed that the UV-absorbance monitoring method lacks a potential to estimate the removal of slowly dialyzed solutes e.g. PBS, as the compounds with RR less than 60 %, contribute less than 10% to the signal. PBS have low RR values, as only free fraction of the blood serum is available to diffuse through the dialysis membrane [58, 61, 70, 177]. Nevertheless, the results in Publication I confirm that UV-absorbance can be utilized to monitor the removal of easily dialyzed uremic solutes, such as UA or as a surrogate method for urea.

4.3 Fluorophores in the spent dialysate (Publication II)

In the last decade, the research focus of uremic toxicity has been on protein-bound uremic toxins. Some of these, in particular IS and pCS, are known to be linked directly with renal and vascular disease progression and therefore with an increased risk of mortality [25]. Although these solutes are extensively investigated, as yet there are no commonly accepted therapeutic target parameters in use to assess removal adequacy, nor easy, robust and cost-effective methods for measuring markers of PBS in everyday clinical practice. In the publication by Holmar et al., the total removed IS was linked with fluorescence of spent dialysate [29]. To validate the approach based on fluorescence, a HPLC study with a fluorescence detector was carried out and the results were summarized in Publication II. The study included 28 patients from hemodialysis centers from Tallinn, Estonia and Linköping, Sweden, in total, 198 dialysate samples from 99 dialysis sessions. The main fluorophores were separated and characterized by HPLC with a fluorescence detector.

The results revealed that the main fluorophores in the spent dialysate were identified as IS, tryptophan, indole acetic acid, indoleacetyl glutamine, indoxyl glucuronide, and 5-hydroxyindoleacetic acid, whereas one major contributor remained unidentified. More than half of the fluorescence signal at Ex: 280 nm and Em: 360 nm is caused by indolic compounds, while indoxyl sulfate is the most prevalent fluorophore in the spent dialysate. There was no statistical difference between RR of IS and total fluorescence as the sum of peaks at selected wavelengths. Furthermore, there was a strong correlation between these values. The fluorescence Ex and Em spectrogram acquired over the chromatographic separation of 10 selected spent dialysate samples gave additional confirmation that at given wavelengths, the main fluorophores are compounds with indole-like fluorescence spectra. In parallel to dialysate sampling, on-line fluorescence measurements were carried out to illustrate technological possibility for real-time dialysis fluorescence monitoring reflecting removal of the main fluorophores from blood into spent dialysate.

The findings that IS is a main fluorophore in spent dialysate are in agreement with earlier fluorescence studies with HPLC [114, 178]. Also, similar fluorescence

Em spectrum acquired in the spectrogram study has been reported [178, 179]. The strong correlation between the RR of IS and total fluorescence found in this research add supplement confirmation to the conclusion of a study published by Holmar et al., suggesting fluorescence monitoring as a possible method for quantifying the elimination of protein-bound uremic toxins during the dialysis procedure [29]. The findings in Publication II clearly realize the idea to utilize the fluorescence signal at this wavelength region for monitoring the removal of slowly dialyzed uremic toxin IS.

4.4 Effect of different dialyzers on the accuracy of optical monitoring (Publication III)

In kidney replacement therapy by hemodialysis, the dialysis efficiency can be controlled by the type of treatment by choosing between the hemodialysis (HD) or hemodiafiltration (HDF), by the choice of the dialyzer membrane, or by several parameters, such as the flow speed of blood and dialysate. When choosing a dialysis membrane to fit the treatment targets, there are several variables that can be altered, such as the material, size of membrane area, and the dialyzer membrane pore size. As different uremic solutes, chromophores may have different removal by altering the dialysis strategies [14, 19, 70, 88], it raised a question of whether this has an effect on the optical monitoring of dialysis adequacy by UV-absorbance. In the study of Publication III, the removal ratio of 11 different parameters for low flux (LF, pore size 1.8 nm) and high flux (HF, pore size 3.3 nm) dialyzer membranes were compared [17]. The comparison included concentrations of 6 uremic solutes and 2 unknown chromophores in serum samples; the total UV-absorbance of serum as the total area of HPLC peaks; and also the UV-absorbance of the spent dialysate measured by spectrophotometer. 30 dialysis sessions of 10 patients were included to this study.

The most important findings of this study were that as compared to LF and HF dialyzers, the removal efficiencies estimated by the total area of HPLC peaks at 254 and 280 nm in the serum and online UV absorbance at 280 nm in the spent dialysate were statistically not different. This indicates to the similar removal of chromophores with a major contribution to the UV-absorbance as compared to different dialyzer membrane pore sizes. Thus, this study reveals that the dialyzer membrane pore size has an insignificant effect on the dialysis adequacy monitoring by UV-absorbance.

4.5 Effect of drugs on the accuracy of optical monitoring (Publication IV)

The UV-absorbance dialysis adequacy monitor has been accepted by the clinical community to estimate the dialysis dose of Kt/V [42]. It is a rather new method, measuring the sum of a broad variety of different chromophores, therefore the number of studies focused on possible interferences is limited [108, 180, 181]. An earlier publication covering the contributions of the main chromophores revealed

that some drugs, in this case, acetaminophen (paracetamol), may interfere the monitoring of removal of small uremic solutes [108]. To investigate this phenomenon, a study including 17 patients on hemodialysis was carried out with HPLC UV-absorbance detection.

The results showed that acetaminophen and its metabolites are strong chromophores at wavelengths 254 and 280 nm. Surprisingly, when given shortly before or after the start of dialysis, acetaminophen was found to increase the levels of UA in spent dialysate samples. In the reference group of 10 patients on hemodialysis receiving no acetaminophen, the average RR of UA was 78 ± 14% during the dialysis. However, when acetaminophen was consumed during dialysis, the apparent average RR of UA calculated as difference between concentrations in the start and end dialysate samples decreased to 52 ± 9%, with the lowest of 43%. The relationship between the rise of UA level and administration of acetaminophen is unknown. The first notice of such kind of relationship was published already in 1975 [182], however after later re-examination of this phenomenon published in 1979 [183], no known publications have followed. This has raised the question of – whether a patient on hemodialysis benefits from acetaminophen-related increase of UA or it should be avoided. Regardless of that debate, acetaminophen, its metabolites and related increase of UA are strong chromophores and this may affect the accuracy of the UV-absorbance monitoring of dialysis adequacy.

4.6 Possible application: fluorescence study of spent dialysate (Publication V)

Protein-bound uremic toxin IS is mostly measured by HPLC coupled with fluorescence of MS detection [18, 60, 61, 70, 111, 112], which requires specialized laboratory, sophisticated and expensive apparatus and skilled personnel. Fluorescence at the Ex: 280 nm and Em: 360 nm offers selective measurement of indoles [115]. As the HPLC method coupled with fluorescence detection is the most sensitive and selective method employed to measure IS, it raised an interest whether fluorescence at selected wavelengths of spent dialysate can be utilized to estimate total removed IS. For this reason, spent dialysate samples from the tank collection; start and end in total 234 samples of 78 dialysis sessions of 18 patients from Tallinn, Estonia and Linköping, Sweden, were included to the study. To create a regression model, input data included the fluorescence spectra of the spent dialysate and IS concentrations measured by HPLC, while HPLC was handled as reference.

The results showed a strong correlation between the amplitude of fluorescence of the spent dialysate and concentration of IS measured by HPLC. Still more, there is strong relation between the optically estimated total removed (TR) IS and TR IS assessed by HPLC; all estimated from tank collection samples. The mean TR IS in this study was 151.4±87.3 mg per treatment session. This value is relatively close to those reported previously, ranging from 114 ± 50 mg [14] to 240 ± 54 mg [60]. From the past publications, the main parameters for quantifying the removal of

PBS solutes have been RR and TR [14, 15, 19, 20, 58, 60, 70, 88, 113, 184, 185]. The optical monitor utilizing the fluorescence of spent dialysate can provide both of these parameters for IS. Based on these results, it is possible to estimate TR IS using only spectrafluorimetric values of the spent dialysate and the main parameters for quantifying the elimination of IS by the hemodialysis procedure can be used.

CONCLUSIONS

The results of Publications I – V can be divided into two sections, covering separately the chromophores and the fluorophores.

Chromophores (Publications I, III and IV)

- Easily dialyzed chromophores with a removal ratio RR > 60 % (e.g. uric acid) contribute more than 90% to the UV absorbance of the spent dialysate.
- The main identified chromophores in the spent dialysate at wavelengths of 210, 254 and 280 nm are uremic solutes of uric acid, hippuric acid and creatinine.
- The pore size of dialysis membrane (low flux vs high flux) has no effect on dialysis adequacy monitoring by UV-absorbance.
- Interference by a drug of acetaminophen (paracetamol) to the UV-absorbance based dialysis adequacy assessment was determined.
- UV absorbance cannot be utilized to monitor the removal of slowly dialyzed uremic solutes (e.g. indoxyl sulfate).
- UV-absorbance can be utilized for monitoring the removal of easily dialyzed uremic solutes.

Fluorophores (Publications II and V)

- More than 60% of the fluorescence signal of the spent dialysate at wavelengths Ex: 280 nm, Em: 360 nm originates from 5 indolic solutes (indoxyl glucuronide, indoxyl sulfate, 5-hydroxyindoleacetic acid, indoleacetyl glutamine, and indolacetic acid).
- The main contributor to the fluorescence signal at excitation 280 nm and emission 360 nm in the spent dialysate is uremic toxin of indoxyl sulfate.
- The removal ratio of indoxyl sulfate is very close to the removal ratio from the fluorescence at selected wavelengths of spent dialysate (sum of all peaks).
- Fluorescsence signal at a certain wavelength region (e.g. Ex: 280 nm and Em: 360 nm) can be utilized for monitoring the removal of protein-bound solute indoxyl sulfate.

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KOKKUVÕTE

Dialüüsi adekvaatsuse hindamine põhineb tänapäeval ühel markerainel – uureal. Uurea on mittetoksiline ainevahetuse jääkprodukt, mis esindab kergesti eemaldatavate ureemiliste soluutide ainetegruppi. Uurea eemaldamist saab hinnata optiliselt, mõõtes dialüsaadi UV-valguse neelduvust. Uurea aga ei võimalda saada informatsiooni teiste ureemiliste soluutide, näiteks verest raskesti eemaldatavate valkudega seotud ainete, eemaldamise kohta. Seetõttu on välja pakutud uus markeraine, toksiliste omadustega indoksüülsulfaat, valkudega seotud ainete eemaldamise hindamiseks. Indoksüülsulfaadi eemaldamise hindamiseks on välja pakutud fluorestsentsil põhinev meetod.

Kasutades ainete lahutamist vedelikkromatograafiaga, uuriti käesolevasse väitekirja kaasatud uurimistöödes peamiste kromofooride ja fluorofooride osatähtsusi dialüsaadi UV-neelduvuses ja fluorestsentsis. Töö tulemusel avastati dialüsaadi UV-neelduvuse ja fluorestsentsisignaalide interpreteerimiseks uudsed võimalused, mis on kahe erineva eemaldamiskiirusega ainegrupi uue jälgimismeetodi aluseks neeruasendusravil.

Esimeses osas antakse lühike ülevaade neerude rollist inimese kehas, neerufunktsiooni häiretest ning neeruasendusravist. Töö **teises** osas kirjeldatakse dialüüsi adekvaatsuse tausta ja selle arenemist. Kajastatud on ureemiliste soluutide eemaldamise effektiivsust kirjeldavad võimalikud parameetrid. Töö **kolmas** osa vaatleb ureemiliste ühendite sisalduse määramise meetodeid. Kolm alateemat käsitlevad peamisi tavapäraseid ainete kontsentratsioonide määramise viise: kolorimeetrial ja ensümaatilistel reaktsioonidel põhinevad kliinilised meetodid; optilised online meetodid; ning tavapärasemad analüütilised meetodid, peamiselt kromatograafial põhinevad. Töö **neljas** osa esitab autori tehtud eksperimentaalse töö tulemused. Töö hõlmab endas kokkuvõtteid kliinilistest uuringutest Tallinnas, Eestis ja Linköpingis, Rootsis. Kasutati kolme erinevat kromatografeerimismeetodit koos UV-absorptsiooni- ja fluorestsentsdetektoriga.

Töö peamised tulemused on:

- Kergesti dialüüsitavad kromofoorid RR > 60% (näiteks kusihape) moodustavad rohkem kui 90% kogu UV-absorptsiooni signaalist, seetõttu saab UV-neelduvust kasutada kergesti dialüüsitavate ureemiliste soluutide eemaldamise jälgimiseks.
- Dialüüsravis kasutatava membraani poori suurus ei mõjuta dialüüsravi optilist monitoorimist.
- UV-absorbtsiooni ei saa kasutada aeglaselt dialüüsitavate ureemiliste ühendite eemaldamise jälgimiseks.
- Tuvastati ühe ravitoimeaine, paratsetamooli, tekitatud mõju dialüüsi adekvaatsuse hindamises.
- Peamine osa heitdialüsaadi fluorestsentsi signaalist ergastusel 280 nm ja emissioonil 360 nm pärineb valkudega seotud indooli derivaatidelt, millest peamine on indoksüül sulfaat. Seetõttu saab fluorestsentsi kasutada valkudega seotud ureemilise toksiini indoksüülsulfaadi eemaldamise jälgimiseks.

ABSTRACT

The dialysis adequacy is assessed by a single marker molecule of urea, a non-toxic metabolic waste which represents the removal of easily dialyzed uremic solutes. Removal of urea can be optically estimated by UV-absorbance. However, urea does not provide any indication about the removal of other uremic solutes, such as toxic protein-bound solutes, which are slowly dialyzed during hemodialysis. Therefore, a new marker for slowly dialyzed protein-bound uremic solutes, a toxin of indoxyl sulfate, has been proposed. To estimate the removal of a indoxyl sulfate, a novel optical method based on fluorescence has been proposed.

The aim of the thesis work was to study the composition of the main chromophores and fluorophores in the spent dialysate by using chromatographic separation methods for the interpretation of the UV-absorbance and fluorescence of spent dialysate, optical approaches proposed for monitoring the dialysis adequacy for groups of solutes with different removal.

Section I summarizes the role of kidneys, kidney failure and kidney replacement therapy.

Section II describes the dialysis adequacy and its background. Different parameters are in use to quantify the removal of uremic solutes.

Section III reviews the methods used in the assessment of uremic solutes concentrations. Three subtopics cover the main analytical solutions that are commonly used in the quantification of uremic solutes: clinical methods based on colorimetry and enzymatic reactions; online optical methods; and common analytical laboratory techniques, covering mainly the principles and solutions of chromatography.

Section IV presents the results of the author's experimental studies and describes the clinical studies carried out in Tallinn, Estonia and Linköping, Sweden. Three chromatographic methods were used, based on UV-absorbance and fluorescence detection.

The main results of the thesis are:

- Easily dialyzed chromophores with the removal ratio RR > 60 % (e.g. uric acid) contribute more than 90% to the UV absorbance in the spent dialysate, and therefore UV-absorbance can be utilized for monitoring the removal of easily dialyzed uremic solutes.
- Pore size of the dialyzer membrane has no effect on the optical monitoring of dialysis therapy.
- UV absorbance cannot be utilized to monitor the removal of protein-bound uremic solutes (e.g., indoxyl sulfate) due to their negligible contribution.
- Interference by a drug of acetaminophen (paracetamol) to the UV-absorbance based dialysis adequacy assessment was determined.
- Predominant part of the fluorescence signal at excitation 280 nm and emission 360 nm in the spent dialysate originates from protein-bound derivatives of indoles while the uremic toxin of indoxyl sulfate is the main contributor, and for this reason fluorescence could be feasible for monitoring the removal of protein-bound uremic solute of indoxyl sulfate.

APPENDIX 1

PUBLICATIONS

Publication I

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*, vol 4 (10): 849-861 (DOI: 10.3390/toxins4100849).

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Article

Do Only Small Uremic Toxins, Chromophores, Contribute to the Online Dialysis Dose Monitoring by UV Absorbance?

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Abstract: The aim of this work was to evaluate the contributions of the main chromophores to the total UV absorbance of the spent dialysate and to assess removal dynamics of these solutes during optical on-line dialysis dose monitoring. High performance chromatography was used to separate and quantify UV-absorbing solutes in the spent dialysate sampled at the start and at the end of dialysis sessions. Chromatograms were monitored at 210, 254 and 280 nm routinely and full absorption spectra were registered between 200 and 400 nm. Nearly 95% of UV absorbance originates from solutes with high removal ratio, such as uric acid. The contributions of different solute groups vary at different wavelengths and there are dynamical changes in contributions during the single dialysis session. However, large standard deviation of the average contribution values within a series of sessions indicates remarkable differences between individual treatments. A noteworthy contribution of Paracetamol and its metabolites to the total UV absorbance was determined at all three wavelengths. Contribution of slowly dialyzed uremic solutes, such as indoxyl sulfate, was negligible.

Keywords: uremic toxins; hemodialysis; chromophores; retention solutes; absorption; ultraviolet-radiation; liquid-chromatography; dialysis dose; monitoring; spent dialysate

1. Introduction

The search for an easy and robust method for online tracking of a prescribed dialysis dose when dialysis is used as a treatment for patients with kidney failure is a long-term pursuit. Blood samples have been the main source of information concerning the efficiency of dialysis treatment during the history of search for a suitable parameter for dialysis dose description. The Kt/V value based on urea analyses in blood samples has been commonly accepted for the description of a delivered dialysis dose today. However, the method is error-prone in practice [1] and time-consuming, considering the time needed from blood draw until achieving the results. Urea itself does not exhibit toxic properties in concentrations found in the dialysis patients [2], and is not representative for removal of many uremic toxins regarded as groups of protein bound and middle molecules [3].

A principle for a non-invasive dialysis adequacy monitoring method was proposed by Gal *et al*. [4] proposing measuring UV absorbance in the spent dialysate at 254 nm. This method was not widely adopted at the time. A decade later, the principle of conductivity based dialysis monitoring was introduced utilizing the conductivity signal to assess the dialysis dose parameter Kt/V [5,6]. However, the precision of conductivity based Kt/V assessment appeared to be dependent on accurate estimation of total body water [7] and, therefore, not an ideal method for routine use. Also, online urea content monitoring in the spent dialysate has been used (Biostat 1000 Urea Monitor [8,9], Biotrack [10]). The equipment, which is rather cumbersome to handle and involves significant running costs, has not found wider acceptance. The observed relation between the online UV absorbance signal and the parameter Kt/V however, led a step closer to a robust, cheap and reliable way of dialysis monitoring [11]. Use of light emitting diodes made it possible to miniaturize the sensor and minimize the cost of the monitor, without any need for consumables [12].

Earlier studies have shown that UV absorptions at 280, 285 and 297 nm have a close correlation with urea-based dialysis dose estimation [11,13,14]. This made it possible to develop a clinically validated online dialysis adequacy monitoring system [15]. The system measures UV-absorption *versus* time in the spent dialysate at 280 nm and calculates Kt/V.

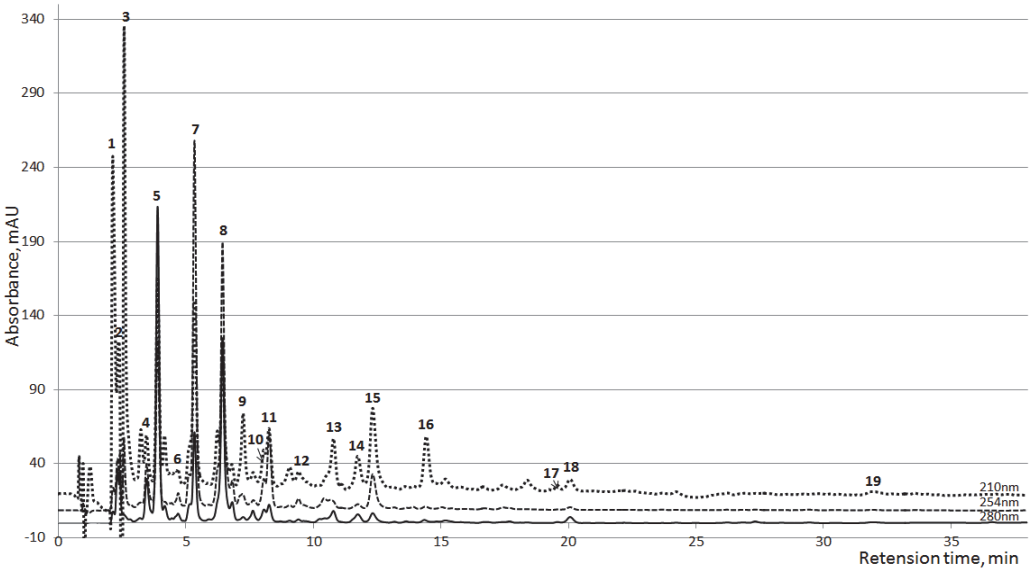
Because the UV method detects a range of solutes, it is sensitive to changes in chromophores' content and the appearance ratio of different UV-absorbing molecules in the spent dialysate. Removal dynamics of different chromophores and contributions to the total UV absorbance are still unknown [16].

The aim of this study was to evaluate the contributions of the main chromophores to the total UV absorbance in the spent dialysate and removal dynamics during optical online dialysis dose monitoring.

2. Results

Thirty clearly resolved peaks of UV absorbing compounds were detected during the HPLC analysis. Seventeen of all peaks had major importance in some samples or prevalent importance in all samples (Figure 1), from these, 10 were identified on the basis of comparisons of the MS-spectra, UV-spectra and the retention time with the corresponding reference substances (Table 1). Identified peaks were grouped considering the widely accepted classification of uremic retention solutes [17]. An additional three peaks (7, 11 and 12, Figure 1), identified as Paracetamol (PAR; *N*-Acetyl-*p*-Aminophenol) and metabolites PAR glucuronide and PAR sulfate, were found from the samples of 33 dialysis sessions out of 48 (including both HD and HDF sessions).

Figure 1. Averaged HPLC chromatograms of the spent dialysate collected 10 min after the start of the dialysis (n = 24) at three wavelengths. 1,2: Unknown; 3: Creatinine; 4: Unknown; 5: Uric acid; 6: Hypoxanthine; 7: PAR Glucoronide; 8–10: Unknown; 11: PAR Sulfate; 12: Paracetamol (PAR); 13: Tryptophan; 14: Indoxyl Sulfate; 15: Hippuric acid; 16–18: Unknown; 19: Indole-3-acetic acid.



Also, five prevalent but unidentified chromatographic peaks were grouped together (Table 1). The group of "All Other Solutes" (AOS) consists of the peaks that had no prevalent signal in the chromatograms or were not clearly identified as separate peaks. This group involves an unknown number of solutes, which separately had very low UV signal, but summed together had noticeable importance in total UV absorbance.

The average relative contributions for all five solute groups at three different wavelengths are illustrated in Figure 2. The group of "Small Molecules" has prevalent contribution at 280 nm. However, at lower wavelengths this group loses dominance in the UV absorbance signal. At 254 nm the group of "5 Prevalent Unidentified Peaks (5PU)" has a significant role in the UV signal, but less significant at 210 and 280 nm. The UV absorbance signal is most complex at 210 nm, where

nearly half of the signal consists of many solutes with low and very low UV absorbance at higher wavelengths.

Figure 3 illustrates the relationship between online UV absorbance of spent dialysate monitored during dialysis session (**I**), HPLC signal (**II**), and the relative contribution of the HPLC peaks to the total UV absorbance (**III**), all signals acquired at 280 nm for a single dialysis treatment. The slope of online UV absorbance signal from the spent dialysate against time enables one to estimate the value of Kt/V (Figure 3I). Method for acquiring the online UV absorbance signal is described in detail elsewhere [18]. Self-tests of the dialysis machine occur as spikes in the signal. Difference in the height of the peaks on the Start and End chromatograms (Figure 3II) demonstrates a concentration decrease in uremic solutes during the dialysis.

Table 1. Grouping of solutes.

Grouping	Compound	Peak nr	RT, min	MW	Class
Small Molecules (**SM**)	Creatinine (Cr) *	3	2.5	113	Guanidines
	Uric acid (UA) *	5	4.0	168	Purines
	Hypoxanthine *	6	4.4	136	Purines
Protein-Bound Solutes (**PBS**)	Tryptophan (Trp)	13	11.4	204	Indoles
	Indoxyl Sulfate (IS) *	14	12.4	251	Indoles
	Hippuric acid (HA) *	15	13.0	179	Hippurates
	Indole-3-acetic acid (I3AA) *	19	31.8	175	Indoles
5 Prevalent Unidentified Peaks (**5PU**)	Unknown	1	2.4		
	Unknown	4	3.6		
	Unknown	8	6.8		
	Unknown	10	8.4		
	Unknown	18	20.7		
Paracetamol and its metabolites (**Par**)	Paracetamol Glucoronide	7	5.5	327	Glucuronides
	Paracetamol Sulfate	11	8.7	231	
	Paracetamol (PAR)	12	11.1	151	Acetanilides
All Other Solutes (**AOS**)	Unknown	2	2.3		
	Unknown	9	7.2		
	Unknown	16	14.4		
	Unknown	17	19.5		

* Note: Grouping according to EUTox (European Uremic Toxin Work Group) classification [17]; RT: chromatographic retention time (minutes), MW: molecular weight (g/mol).

Figure 2. Average contribution of groups of chromatographic peaks to the total UV absorbance in the spent dialysate, including start, end and tank collection samples. **SM**: Small molecules; **PBS**: Protein-Bound Solutes; **5PU**: 5 Prevalent Unidentified Peaks; **Par**: Paracetamol and its metabolites; **AOS**: All other Solutes.

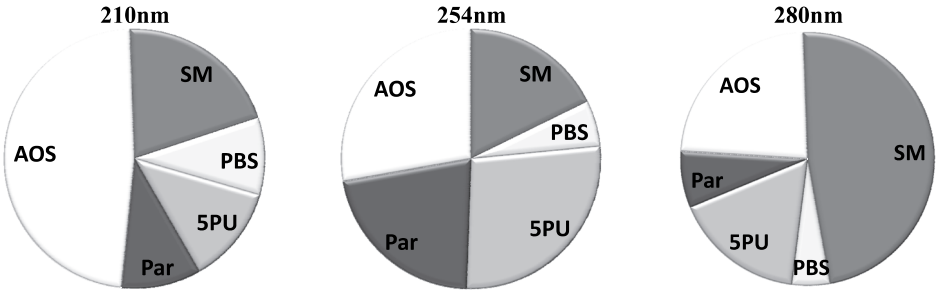
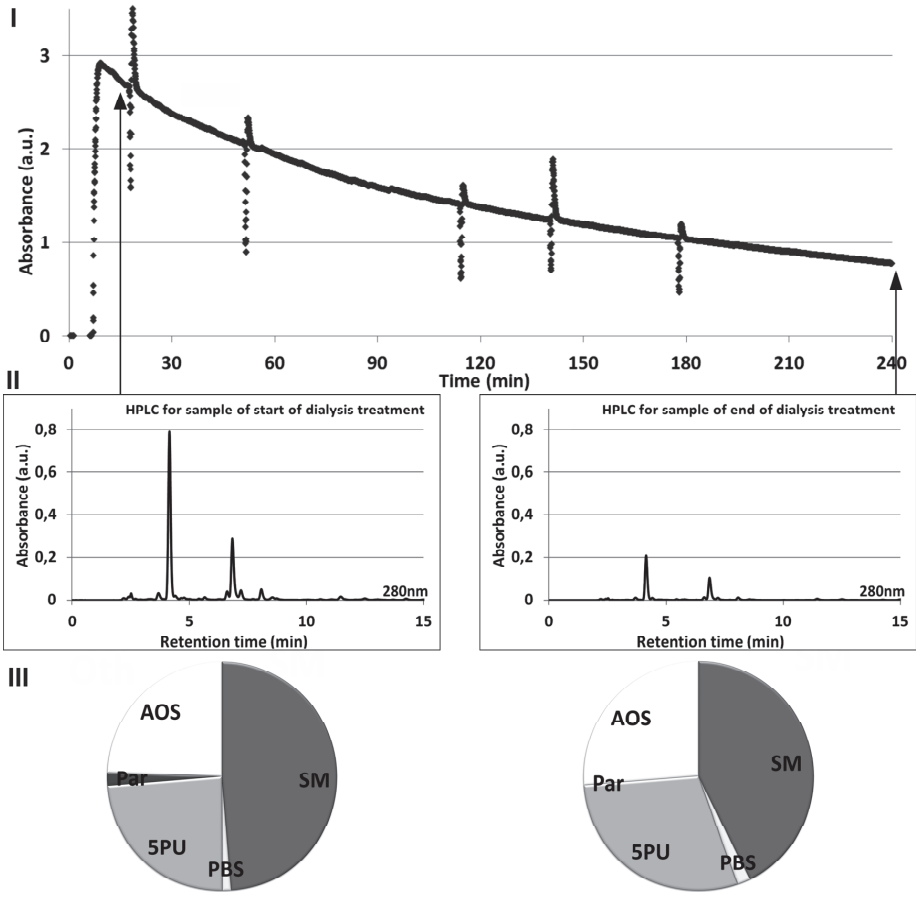


Figure 3: Relationship between online UV absorbance (**I**), HPLC signal (**II**) and the relative contribution of the HPLC peaks to the total UV absorbance at 280 nm (**III**) for a start and end of dialysate samples from a single dialysis treatment.



The average relative contributions for all treatments to the total UV absorbance (Mean ± SD) in percentage for each peak and each solute group are given in Table 2 for start and end samples at three wavelengths. Characteristic dynamics of the contributions from the solutes and solute groups to the total UV absorbance can be distinguished by comparing the data from the start and the end of the dialysis session. Two peaks, identified as Hypoxanthine and I3AA, had very low UV signal value in the chromatograms, and were included into the group of AOS.

As seen from Table 2, the small molecule, Uric Acid (UA) is the main UV absorbing solute in the spent dialysate at 280 nm. During the dialysis, the importance of UA in the UV signal decreases significantly ($p < 0.05$). The decrease of UA contribution is concurrent to the increased contributions from other solute groups, being significant for groups "Protein-Bound Solutes" (PBS) and AOS ($p < 0.05$). As UA is a very important UV absorber in the spent dialysate, the high standard deviation value of UA contribution should be stressed. This indicates high variations between different patients and dialysis sessions (min RC_{UA} = 29%; max RC_{UA} = 75%, RC: relative contribution).

Solute contributions at 254 nm have the highest SD value. The main contribution comes from the group of 5 PU, where the peak Unknown 8 has the highest contribution to the UV signal.

At 210 nm, two solutes (UA and Creatinine) in the "Small Molecules" group are of major importance in the UV signal. Their contributions changed during the dialysis considerably ($p < 0.05$). Changes in the contribution inside the group of PBS were significant. However, no substantial difference in the contribution occurred for the start and end samples for the whole PBS group.

Table 2. Average contributions in percent for each peak and molecule group with a statistical comparison of the start and end samples of the spent dialysate at three wavelengths *).

	210 nm		254 nm		280 nm	
	Start	**End**	**Start**	**End**	**Start**	**End**
Small molecules	24.81 ± 8.02 *	16.36 ± 4.88	18.63 ± 8.86	16.94 ± 7.44	50.07 ± 10.54 *	44.88 ± 9.73
Uric acid	10.30 ± 3.67 *	6.80 ± 2.21	14.83 ± 7.51	12.98 ± 6.04	50.07 ± 10.54 *	44.88 ± 9.73
Creatinine	14.51 ± 5.37 *	9.56 ± 4.77	3.80 ± 1.69	3.96 ± 1.62	0.00	0.00
Protein-Bound Solutes	9.75 ± 3.36 *	9.32 ± 2.61	6.04 ± 3.17	5.42 ± 2.72	3.82 ± 0.97 *	5.87 ± 1.59
Indoxyl Sulfate	1.89 ± 0.79 *	2.57 ± 1.04	0.44 ± 0.38	0.33 ± 0.46	1.42 ± 0.50 *	2.44 ± 1.13
Tryptophan	1.68 ± 0.59 *	2.71 ± 0.73	0.51 ± 0.26	0.52 ± 0.64	1.21 ± 0.29 *	2.41 ± 0.69
Hippuric acid	6.18 ± 3.27 *	4.04 ± 2.18	5.09 ± 2.87	4.57 ± 2.76	1.19 ± 0.62	1.02 ± 0.58
5 Prevalent Unidentified peaks	12.72 ± 4.41	11.55 ± 3.73	26.88 ± 13.50	27.68 ± 12.52	15.80 ± 6.01	17.75 ± 5.18
Unknown1	0.00	0.00	0.00	0.00	0.93 ± 1.71	0.79 ± 1.32
Unknown4	2.55 ± 0.76 *	2.26 ± 0.83	4.21 ± 1.80	4.49 ± 1.81	2.86 ± 0.53 *	3.11 ± 0.48
Unknown8	6.49 ± 4.19	5.66 ± 3.35	18.76 ± 13.61	19.79 ± 13.15	9.16 ± 6.40	10.03 ± 5.86
Unknown10	2.86 ± 1.14 *	2.10 ± 0.90	3.27 ± 1.26 *	2.46 ± 1.38	1.75 ± 0.97 *	1.27 ± 1.10
Unknown18	0.82 ± 0.82	1.53 ± 0.97	0.64 ± 1.39	0.94 ± 1.54	1.10 ± 0.97 *	2.55 ± 1.72
Paracetamol and metabolites	10.01 ± 10.55	8.94 ± 8.80	21.49 ± 22.02	21.18 ± 21.31	7.37 ± 8.16	6.87 ± 7.40
Paracetamol	0.66 ± 0.51	1.08 ± 1.05	0.79 ± 0.78 *	1.40 ± 2.14	0.50 ± 0.52	0.73 ± 1.30
Paracetamol Glucoronide	7.26 ± 8.48	6.23 ± 6.81	16.62 ± 17.59	15.49 ± 15.89	5.59 ± 6.82	5.01 ± 5.73
Paracetamol Sulfate	2.09 ± 2.01	1.63 ± 1.55	4.08 ± 4.29	4.29 ± 4.39	1.28 ± 1.04	1.13 ± 1.13
All Other Solutes	42.72 ± 8.43 *	53.84 ± 6.79	26.97 ± 7.88	28.80 ± 7.93	22.95 ± 3.65 *	24.64 ± 3.71

* Note: Start values with asterisk indicate significant statistical differences ($p < 0.05$) between the values for start and end spent dialysate samples.

An alternative grouping of solutes was done on the basis of solutes' Removal Ratios (RR) during the dialysis (Table 3). RR values for all the detected chromatographic peaks were calculated, and these values were compared with RRs of other chromatographic peaks using Student's *t*-test. Chromatographic peaks the RRs of which were not significantly different were grouped together. Four groups with statistically different RR values were created. Creatinine, which had statistically different RR from both "High RR 1" and "High RR 2" groups, was placed under the latter group in Table 3 as the average RR value was closer to this group. Group of AOS were included to the group "High RR 2" as the RR values were statistically indifferent. The RR value of group of AOS corresponds to the summated change of peaks for the whole group, since it was impossible to evaluate the RR values for single peaks due to low concentration and/or insufficient separation on "end" chromatograms.

Table 3. Grouping of the solutes according to the removal ratio (RR), mean ± SD (%).

High RR 1		**High RR 2**		**Low RR**		**Unstable RR**	
UA	69.0 ± 11.2	Creatinine	63.1 ± 10.3	IS	48.1 ± 13.2	Trp	32.7 ± 23.0
HA	68.4 ± 10.4	Unknown 1	62.1 ± 9.0			PAR.	14.4 ± 64.4
Unknown10	72.7 ± 9.6	Unknown 4	62.3 ± 10.3			Unknown 18	−131.0 ± 309.1
PAR.Gluc	71.9 ± 15.0	Unknown 8	60.9 ± 10.5				
PAR.Sulf	64.4 ± 24.0	All other molecules	63.9 ± 11.1				

As Table 3 shows, the most indicative marker of low RR compounds in terms of UV-monitoring of the dialysis appears to be indoxyl sulfate without any severe rivalry by other common UV-chromophores in the spent dialysate.

Recalculated average relative contributions (Mean ± SD) for alternative grouping based on RR values are given in Table 4. The groups with the highest RR, "High RR 1" and "High RR 2", play a major role both at 280 and 254 nm. Also, they remain prevalent contributors at 210 nm. Both at 280 nm and 254 nm, the "High RR 1" and "High RR 2" groups together are responsible for around 95% of the total UV absorbance (Figure 4). The contribution of the IS as a marker of retention solutes with low RR remains inconsiderable at all wavelengths tested.

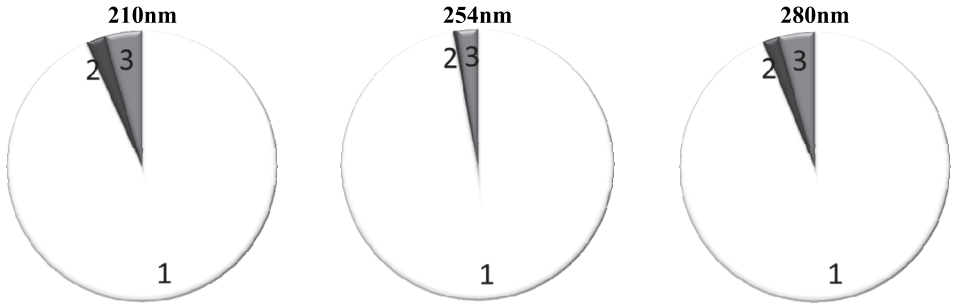
Figure 4 shows the average contribution to the total UV absorbance from the chromophores belonging into different RR based groups when the "High RR 1" and "High RR 2" groups were put together.

Table 4. Average contributions in percent of RR based groups of chromophores to the total UV absorbance in the spent dialysate (Mean ± SD).

		High RR 1	**High RR 2**	**Low RR**	**Unstable RR**
210 nm	Start	28.69 ± 11.28 *	66.26 ± 10.50 *	1.89 ± 0.79 *	3.16 ± 1.23 *
	End	20.79 ± 8.24	71.31 ± 8.24	2.57 ± 1.04	5.32 ± 1.31
254 nm	Start	43.89 ± 18.68	53.74 ± 18.01	0.44 ± 0.38	1.94 ± 1.50 *
	End	39.79 ± 15.84	57.03 ± 16.85	0.33 ± 0.46	2.85 ± 2.46
280 nm	Start	59.88 ± 6.78 *	35.90 ± 7.28	1.42 ± 0.50 *	2.81 ± 1.10 *
	End	53.30 ± 6.23	38.57 ± 6.55	2.44 ± 1.13	5.68 ± 2.01

* Note: Start values with asterisk indicate significant statistical difference ($p < 0.05$) between the values of start and end spent dialysate samples.

Figure 4. Average contribution to the total UV absorbance in percentage from the chromophores belonging to different RR groups, the criterion for the "High RR sum" group inclusion was average RR > 60%: 1: Solutes with high RR; 2: Solutes with low RR; 3: Solutes with unstable RR.



3. Discussion

This study adds an exciting supplement to the current knowledge about the removal dynamics of different chromophores and contributions to the total UV absorbance. The results indicate that: (i) a predominant part (>95%) of the UV absorbance signal in the spent dialysate originates from easily dialyzed uremic solutes with a high removal ratio, like uric acid; (ii) a noteworthy role of Paracetamol and its metabolites in the UV absorbance signal was determined at all three wavelengths; (iii) the contribution of UA changes during the dialysis treatment due to more efficient removal of small water soluble solutes, resulting in an increased contribution in other molecules; (iv) UV absorbance cannot be utilized to monitor the removal of slowly dialyzed uremic solutes (e.g., indoxyl sulfate); (v) an alternative grouping for uremic solutes based on removal ratios is proposed; (vi) a significant part of UV absorbance is caused by unidentified molecules.

Earlier research by Schoots [19] has found that removal ratios for known uremic toxins vary for each solute due to changes in protein binding during the dialysis treatment for protein-bound solutes and changes in clearances for different solutes. It can be concluded from the results presented in this work that a predominant part, roughly 95% of UV-absorbing uremic solutes in the range of absorbance between 254 and 280 nm, evidently do not belong to the group of firmly protein bound substances. The result does not support the earlier conclusion [4] that the UV absorbance at 254 nm should enable one to follow the elimination of accumulated plasma components with particular emphasis on slowly diffusible organic compounds of known or assumed toxicity. This study and several other earlier studies [19–21] have indicated that a major part of the UV signal originates from the small toxic solute of UA, which enables online monitoring of UA [22]. The small water soluble molecule, UA, has the most important role at the wavelength 280 nm where it is responsible for a major part of the total UV absorbance: the mean contribution of UA was about half (48%). The average removal ratio of the UA 69% is comparable to that of a traditional marker urea 71% in this study (unpublished result). These observations strongly empower the spreading practice of using online UV-monitoring to evaluate the dialysis process and to calculate a dialysis dose (KtV value) [23]. Furthermore, recent unpublished results from our research group show very good correlation between the UA concentration and the

UV-signal at 300 nm [24]. The current study has described contributions of UA at three lower wavelengths where large variations in contributions may occur, which leads to a need for a multiwavelength approach.

During the dialysis both the contribution of UA and that of PAR metabolites decrease slightly on account of other chromophores. It can be interpreted as the result of increased relative contribution of protein-bound solutes in the dialysate to the total UV absorbance due to quicker removal of water-soluble fraction, but changes are quite small and with remarkable deviations. The same concerns changes followed at 254 nm, which scarcely can be altered in this study by PAR and metabolites with absorbance maximum is in this region [25]. Those results by our group confirm that also the total UV-absorption of the spent dialysate as a marker for dialysis adequacy assessment marks quite closely the same range of small water-soluble uremic solute removal as urea analysis in the serum of patients and the UA analysis in the spent dialysate. On the other hand, it means that UV-monitoring has also the same deficiencies as the urea analysis and cannot add a substantially new quality to the adequacy assessment in addition to immediacy and handiness already verified.

Since the removal of protein bound solutes is a highly relevant topic in the current dialysis practice [26], an alternative approach was proposed in this work by grouping the peaks-solutes also according to the removal ratios (Table 3). Surprisingly, it turned out that the RR-values of the prevalent majority of UV chromophores are quite comparable with those of the small uremic toxin UA and only the protein bound uremic toxin IS is the single clearly distinguishable UV peak on the chromatograms, which can be used as a marker of a slowly removable fraction for online monitoring of the dialysis process. Unfortunately, the total contribution of IS to the total absorbance is so negligible, from 0.3% at 254 nm up to 2.5% at 280 nm (Figure 4), that it seems impossible to follow the removal of this marker and protein bound solutes in total by means of online UV monitoring of the dialysis. A novel promising method has been proposed lately for monitoring the removal of IS, a known protein-bound solute and uremic toxin, with a low removal ratio, utilizing fluorescence [27].

4. Materials and Methods

4.1. Clinical Study

The study was performed after approval of the protocol by the Regional Ethical Review Board, Linköping, Sweden. A written informed consent was obtained from all participating patients. The study included eight patients, one female and seven male, mean age 77 ± 7 years, being on chronic three-weekly hemodialysis (HD) and high volume post-dilutional online-HDF (ol-HDF) treatment at the Department of Nephrology, Linköping, Sweden. A high-flux dialyzer FX 80 during the HD sessions and FX 800 during ol-HDF was used and the dialysis machine was Fresenius 5008H (all from the Fresenius Medical Care, Germany). The dialysate flow was 500 mL/min, the blood flow varied between 280 and 350 mL/min. The auto subsystem mode for the calculation of the online prepared substitution fluid by the dialysis machine was used, based on total protein and hematocrit. The substitution fluid volume during the ol-HDF sessions varied between 12.2 and 29.7 liters per session (mean 21.9).

Patient treatments were monitored during three consecutive hemodialysis sessions with duration from 180 to 270 min (totally 24 HD and 24 ol-HDF sessions). During the dialysis the following dialysate samples were taken: (1) 10 min after the start of the dialysis session; (2) at the very end of the treatment, and (3) from the dialysate/ultrafiltrate collection tank after careful stirring. Sampling at the moments of self-tests of the dialysis machine was avoided. Pure dialysate was collected as the reference solution before the start of a dialysis session, when the dialysis machine was prepared for startup and the conductivity was stable.

4.2. HPLC Study

All dialysate samples were acidified down to pH 4.0 with formic acid before the high performance liquid chromatography (HPLC) analysis for conformation with the pH of the chromatographic eluent used. The HPLC system consisted of a quaternary gradient pump unit, a column oven, and a diode array spectrophotometric detector (DAD, all Ultimate 3000 Series instruments from Dionex (Sunnyvale, CA, USA), and Zorbax C18 4.6 × 250 mm column from Agilent Instruments (Wilmington, DE, USA) with a 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), both from Rathburn (Walkerburn, Scotland). The three-step linear gradient elution program was used, as specified in Table 5.

Table 5. HPLC gradient program.

Step	Time (min)	Buffer (A) %	Methanol (B) %	Acetonitrile (C) %
0	0	100	0	0
1	30	60	36	4
2	5	10	81	9
3	4	10	81	9

The total flow rate of 1 mL/min was used continuously at the column temperature of 30 °C. The UV absorbance was monitored at 210, 254 and 280 nm with a measurement interval of 500 ms. Spectra were registered between 200 and 400 nm with a time interval of 0.50 s, data was processed by Chromeleon 6.8 software (Dionex, USA).

Every peak in the HPLC chromatograms was characterized by the characteristic absorption spectrum and by the retention time. Peaks were identified by comparing the retention time, absorption spectrum and MS/MS mass spectrum data (micrOTOF-Q II, Bruker, Germany) of a compound found in the sample with a pure authentic compound. The relative contribution (RC) for the i-th chromatographic peak (presumably solute) to the sum of UV absorbances of all peaks for a chromatogram was calculated as a ratio of the area of the i-th peak ($A_{peak\ i}$) to the total area of all peaks appeared on the chromatogram (A_{total}):

$$RC_i(\%) = \frac{A_{peak\ i}}{A_{total}} \cdot 100\% \quad (1)$$

The contribution values of peaks with similar retention time were averaged separately and depending on the sampling time for all spent dialysate samples from the start and end, also for all of

the samples in total. Relative contribution (*RC*) for a specific solute group "j" was calculated analogically to RC_i:

$$\mathrm{RC_j}(\%) = \frac{\mathrm{A_{group\ j}}}{\mathrm{A_{total}}} \cdot 100\% \quad (2)$$

The removal ratio (RR) of a specific *i*-th peak (uremic solute) for a dialysis session was defined as a function of the start and end HPLC peak areas ($A_{start\ i}$ and $A_{end\ i}$) of the samples from the dialysis session:

$$\mathrm{RR_i}(\%) = \frac{\mathrm{A_{start\ i}} - \mathrm{A_{end\ i}}}{\mathrm{A_{start\ i}}} \cdot 100\% \quad (3)$$

Statistical analysis was done with Microsoft Excel 2010 (Microsoft Corporation, USA). Student's *t*-test was used to compare Two-Sample dataset, Assuming Unequal Variances, while $p < 0.05$ was considered significant.

5. Conclusion

The focus of this study was on the contributions of the different chromophores in the UV-absorbance signal of the spent dialysate at different wavelengths. UV signal has been proven to describe elimination of easily dialyzed uremic solutes with a high removal ratio [13,14], fully confirmed by the results published in this article. A predominant part of the UV absorption comes from uremic solutes with a high removal ratio not depending on the wavelength of measurements (Figure 4); among these the small molecule of the uric acid is of major importance. The contribution values have high standard deviation values, which indicate remarkable differences of contributions between different dialysis sessions and different patients. At the same time, significant appearance of Paracetamol and its metabolites was detected in the UV-signal, showing that not all of the major UV-absorbing solutes are uremic toxins. While the UV absorbance signal describes the removal of uremic solutes with a high removal ratio very well, it provides scarce information about other molecules, such as slowly removed uremic solutes like indoxyl sulfate. Therefore, the search for a universal, trustworthy, robust and cheap non-invasive dialysis monitoring method is still ongoing.

Conflict of Interest

The authors declare no conflict of interest.

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PLOS ONE

RESEARCH ARTICLE

Is Fluorescence Valid to Monitor Removal of Protein Bound Uremic Solutes in Dialysis?

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Abstract

The aim of this study was to evaluate the contribution and removal dynamics of the main fluorophores during dialysis by analyzing the spent dialysate samples to prove the hypothesis whether the fluorescence of spent dialysate can be utilized for monitoring removal of any of the protein bound uremic solute. A high performance liquid chromatography system was used to separate and quantify fluorophoric solutes in the spent dialysate sampled at the start and the end of 99 dialysis sessions, including 57 hemodialysis and 42 hemodiafiltration treatments. Fluorescence was acquired at excitation 280 nm and emission 360 nm. The main fluorophores found in samples were identified as indole derivatives: tryptophan, indoxyl glucuronide, indoxyl sulfate, 5-hydroxy-indoleacetic acid, indoleacetyl glutamine, and indoleacetic acid. The highest contribution (35 ± 11%) was found to arise from indoxyl sulfate. Strong correlation between contribution values at the start and end of dialysis (R^2 = 0.90) indicated to the stable contribution during the course of the dialysis. The reduction ratio of indoxyl sulfate was very close to the decrease of the total fluorescence signal of the spent dialysate (49 ± 14% vs 51 ± 13% respectively, P = 0.30, N = 99) and there was strong correlation between these reduction ratio values (R^2 = 0.86). On-line fluorescence measurements were carried out to illustrate the technological possibility for real-time dialysis fluorescence monitoring reflecting the removal of the main fluorophores from blood into spent dialysate.

In summary, since a predominant part of the fluorescence signal at excitation 280 nm and emission 360 nm in the spent dialysate originates from protein bound derivatives of indoles, metabolites of tryptophan and indole, the fluorescence signal at this wavelength region has high potential to be utilized for monitoring the removal of slowly dialyzed uremic toxin indoxyl sulfate.

Introduction

A wide range of uremic solutes retain in the bodies of patients with end-stage chronic kidney disease. According to the EuTOX workgroup classification, these uremic solutes can be divided into three groups: free water-soluble low-molecular-weight solutes (small molecules), protein-

US9103789 B2) and patent application No. EP11740556 that relate to the fluorescence monitoring of removal of protein-bound uremic toxins. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials. ML reported no competing interests.

bound solutes (PBS) and middle molecules (MM) [1]. During dialysis, removal kinetics of these groups of uremic solutes is different. In general, the removal of PBS, for example indoxyl sulfate (IS), and MM is slower than that of representatives from the group of small uremic toxic molecules like uric acid, and classical markers of urea and creatinine [2–4]. Introduction of convective dialysis variants such as hemodiafiltration has improved the elimination of MM but the difficulty consists of PBS [5]. Although the classical markers, urea and creatinine are representative for the removal of the group of small molecules, they give no indication to the other cofactors of mortality of patients with chronic kidney disease [6], such as the role of PBS to the mortality rate [7]. Besides the urea based Kt/V, a dialysis dose parameter for the small water soluble molecules, there are no established markers to assess the dialysis dose for the removal of PBS. Among the PBS, the highest levels of binding to the proteins has been shown for IS, p-cresyl sulfate and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF) [8], all considered as uremic toxins. The most widely studied solute among those protein-bound uremic toxins is IS, a metabolic end product of indole originating from gut flora [9, 10]. The biologic and/or toxic effects of IS leading to cardiovascular mortality and renal impairment have been reported in several studies [11].

The *de facto* most widely used method for PBS determination is optically with a diode array detector or a fluorescence detector by HPLC, since fluorescence offers a highly selective and sensitive analytical method for detection and quantification [12]. However, the HPLC analysis is time-consuming and requires a laboratory with sophisticated analytical chromatography systems and skilled personnel. Thus, it is difficult to monitor levels and removal of PBS in large scale studies and during routine daily dialysis. Novel and robust on-line monitoring methods, similar to those established for urea based dialysis dose optical estimation [13], would be beneficial.

Although the usage of fluorescence has been suggested as a medical diagnostic tool from the beginning of the discovery of the fluorescence phenomenon in uremic fluids [14, 15], fluorescence has not been implemented for the analysis of spent dialysate without the use of HPLC. Lately, a novel optical method was proposed to monitor the concentration and the removal of protein bound uremic solute of IS in the spent dialysate by utilizing fluorescence without the need for HPLC [16]. However, origin of the fluorescence of spent dialysate by means of identification and contribution of single fluorophores to total fluorescence of spent dialysate remain still elusive.

The aim of this study was to investigate the contribution and removal dynamics of the main fluorophores during dialysis by analyzing the spent dialysate samples to prove the hypothesis whether the fluorescence of spent dialysate can be utilized for monitoring removal of any of the protein bound uremic solute.

Materials and Methods

Clinical study

The study was performed after approval of the protocol by the Regional Ethical Review Board, Linköping, Sweden decision on application no. M153-07 (10.10.2007) and by the Tallinn Medical Research Ethics Committee at the National Institute for Health Development, Estonia decision no. 2349 (issued 15.03.2011). A written informed consent was obtained from all participating patients. The study included 20 patients from Tallinn (10 male, 10 female, 59±12 years) and 8 patients from Linköping (7 male, 1 female, 73±14 years). Patients were followed in total 99 sessions from which 57 were hemodialysis (HD) and 42 hemodiafiltration (HDF) sessions. The dialysis machines used were Fresenius 5008 (Fresenius Medical Care, Germany). The dialyzers were FX8, FX10, FX80, FX100, FX800, and FX1000 (Fresenius Medical Care,

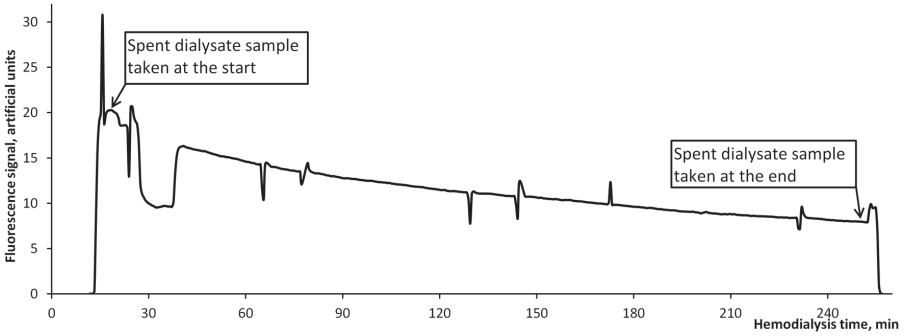

Fig 1. Example of a fluorescence signal of spent dialysate monitored online during a hemodialysis session.

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Germany). The duration of the treatments varied between 180 to 270 minutes, the dialysate flow was 500 ml/min and the blood flow varied between 250–350 ml/min.

During the dialysis, the following dialysate samples were taken: 1) 10 minutes after the start of the dialysis session; and 2) at the end of the treatment (Fig 1). Sampling at the moments of self-tests of the dialysis machine was avoided. Pure dialysate was collected as the reference solution before the start of a dialysis session, when the dialysis machine was prepared for startup and the conductivity was stable.

In parallel to dialysate sampling, on-line fluorescence measurements were carried out during nine selected dialysis sessions (Fig 1) to illustrate technological possibility for real-time dialysis fluorescence monitoring. The clinical set-up was similar to the on-line UV-absorbance measurements as reported earlier [17]. On-line fluorescence measurements were performed by a spectrofluorometer FLD3400RS (Dionex, Sunnyvale, USA) with a fluorimetrical flow cuvette connected to the drain outlet of the dialysis machine. Partial flow of the spent dialysate from the main drain tube was guided into the flow cuvette with a separate pump LC-9A (Shimadzu, Kyoto, Japan), providing a dialysate flow of 2 mL/min. The on-line fluorescence was measured at the excitation (Ex) wavelength of 280 nm and the emission (Em) wavelength of 360 nm with the sampling rate of 12 measurements per minute.

HPLC Study

All dialysate samples were acidified down to pH 4.25 with formic acid before the HPLC analysis for the sake of stable chromatographic retention times. 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 a 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 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 formiate salt (B). The three-step linear gradient elution program was used, as specified in Table 1.

The total flow rate of 0.6 mL/min was used at the column temperature of 40°C. All the spent dialysate samples were analyzed by recording 2-dimensional (2D) fluorescence acquisition

Table 1. HPLC gradient program.

Step	Time (min)	Buffer (A) %	Organic solvent (B) %	Curve type
0	0	99	1	
1	6	99	1	5
2	39	10	90	7
3	15	10	90	5

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chromatogram at the Ex: 280 nm and Em: 360 nm with a measurement interval of 0.5 s. For all 2D chromatograms, a blank run was subtracted from the signal. The 3-dimensional (3D) fluorescence acquisition chromatograms were recorded for 10 selected spent dialysate samples taken at the start of the dialysis, which had the highest common characteristic peak intensities in 2D chromatograms. For 3D chromatograms, each sample was chromatographed two times: one for fluorescence excitation scan and the other for fluorescence Em scan. The fluorescence scanning parameters are given in Table 2 at the measurement interval of 0.5 s. The chromatographic data was processed by Chromeleon 7.1 software by Thermo Scientific (Waltham, USA).

For mass spectrum analysis, a quadrupole time-of-flight mass spectrometer micrOTOF-Q II with an ESI source was used (Bruker, Billerica, USA). For both negative and positive ion mode, the parameters for sample analysis were as follows: mass range of m/z 50–700; ion source temperature of 200°C, ESI voltage of 4.5 kV, ESI nebulization gas flow of 8.0 L/min, drying gas flow of 1.2 bar, detector voltage of 2.03 kV, acquisition rate of 1 Hz. Mass calibration was carried out using a sodium formate solution (10 mmol/L) from m/z 50 to 700. Data acquisition was performed using software Compass HyStar version 3.2, and processing of the data was carried out with Compass DataAnalysis version 4.0 SP1 (both Bruker, Billerica, USA).

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 with the reference compound.

In the analysis of the 3D fluorescence chromatograms, the fluorescence Em and Ex spectra of the peaks were calculated by summing all the spectra over the respective chromatographic peak. In addition, to simulate Ex and Em spectra of nonfractionated spent dialysate, total fluorescence Ex and Em spectra were calculated on the basis of a typical 3D fluorescence chromatogram as the sum of all time-point spectra over the whole chromatogram up to the chromatographic retention time when the column washing peaks started to elute, the latter system peaks were excluded.

The relative contribution (RC) of the i-th chromatographic peak (presumably solute) in relation to the total fluorescence of all peaks on a chromatogram, excluding the system peaks, was calculated as a ratio of the area of the i-th peak ($A_{peak\ i}$) to the total area of all peaks that appeared on the chromatogram (A_{total}):

$$RC_i(\%) = \frac{A_{peak\ i}}{A_{total}} \times 100\%, \quad (1)$$

Table 2. Chromatogram recording program.

		Excitation	Emission	Samples
2D acquisition		280 nm	360 nm	All
3D acquisition	Excitation scan	220. . .340 nm	360 nm	10 selected
	Emission scan	280 nm	300. . .500 nm	10 selected

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The reduction ratio (RR) of a specific i-th peak (uremic solute) for a dialysis session was defined as:

$$RR_i(\%) = \frac{A_{start\ i} - A_{end\ i}}{A_{start\ i}} \times 100\%, \quad (2)$$

where $A_{start\ i}$ and $A_{end\ i}$ are the start and the end HPLC peak areas of the samples from the dialysis session, respectively.

Student's t-test was used to compare the means of RC_i and RR_i, and $p \leq 0.05$ was considered significant. Correlation analysis (Pearsons R) was used for estimation of the degree of linear association between the variable groups of RC_i and RR_i, $R > 0.8$ was considered as strong correlation. Coefficient of determination (R^2) was calculated to assess goodness of fit between parameters.

Results

Fig 1 shows an example of a fluorescence signal of spent dialysate monitored online during a hemodialysis session where fluorescence (Ex: 280 nm, Em: 360 nm) is plotted against the time. The fluorescence drops and peaks during the dialysis correspond to the self-tests in the dialysis machine. The on-line curve reflects elimination of the main fluorophores from blood into spent dialysate.

Fig 2 gives an example of a HPLC chromatogram of a spent dialysate sample taken 10 min after the start of hemodialysis, where the fluorescence was recorded at Ex: 280 nm and Em: 360 nm. In total, 12 clearly resolved chromatographic peaks of fluorophoric compounds were detected in most (82%) of the spent dialysate samples during the HPLC analysis (Fig 2) collected at different time moments in the dialysis. Of these, 5 peaks had a major importance in all samples (peaks no 6, 8, 9, 11 and 12), and 6 of these 12 peaks were identified as Tryptophan (Trp) and their metabolites of indole derivates: Indoxyl glucuronide (IGluc), Indoxyl sulfate (IS), 5-hydroxy-indole-3-acetic acid, Indoleacetyl glutamine (IaG), and Indoleacetic acid (IAA) (Table 3).

The protein-bound uremic toxin IS appeared to be the major contributor to the fluorescence signal (Table 4) in most of the spent dialysate samples. About a third of the fluorescence signal from the spent dialysate originates from IS. The second contributor to the fluorescence signal in the spent dialysate was Trp, which contributes about a fifth to the signal. The mean contribution of each other fluorophoric compound remained below 10%. Compounds Trp,

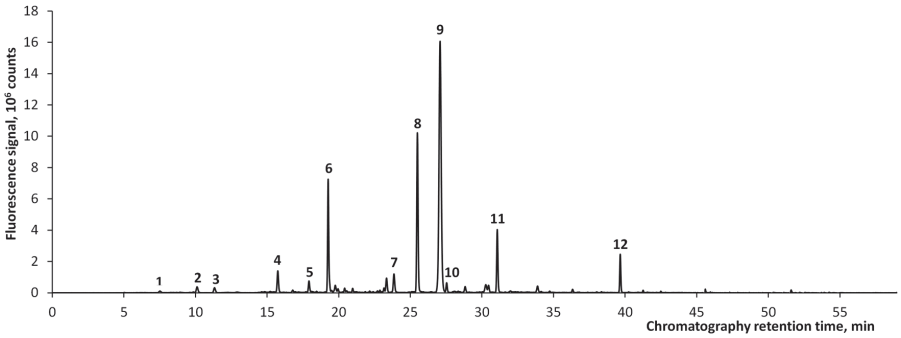

Fig 2. Example of a chromatogram of a spent dialysate sample.

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Table 3. Characteristics of the main chromatographic peaks.

Retention time, min		Peak name	Ion mass, negative mode	Ion mass, positive mode
6	19.54	Unknown 1	303.08; 365.14	367.15; 389.12
7	23.57	Indoxyl glucuronide (IGluc)	308.08	-
8	25.62	Tryptophan (Trp)	203.08	205.10; 227.08
9	27.17	Indoxyl sulfate (IS)	212.00	-
10	27.71	5-hydroxy-indole-3-acetic acid	146.06; 190.05	-
11	31.23	Indoleacetyl glutamine (IaG)	302.12	304.13; 326.11
12	39.91	Indoleacetic acid (IAA)	130.07; 174.06	176.07

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Table 4. Contribution (Mean ± SD, Min, Max, N = 99) in percentage for main fluorescent peaks.

	Mean contribution ± SD	Min	Max
Unknown 1	6 ± 3	2	16
Tryptophan	18 ± 8	3	42
Indoxyl sulfate	35 ± 11	5	57
Indoleacetyl glutamine	2 ± 2	0	8
Indoleacetic acid	4 ± 4	1	31
All Other	31 ± 11	10	90

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Unknown 1, and IaG had significantly different ($p < 0.05$, N = 99) contribution values in the start compared to the contributions in the end of the dialysis. The group of All Other, which consists of the sum of tens of other small peaks, was found to contribute a third to the total fluorescence signal of the spent dialysate in average.

A comparison of the samples, taken from the start and the end of the dialysis session, was made (Fig 3), to clarify whether the contribution of IS to the total fluorescence is stable in the spent dialysate during the course of the dialysis. The analysis revealed no statistical difference (P = 0.30, N = 99) in the contributions of IS in the samples taken from the start and the end of the dialysis session. Also there was a strong correlation ($R^2 = 0.90$, $p < 0.001$) between these values (Fig 3).

Mean contributions were calculated for 4 identified compounds and 1 unknown solute with the largest contribution. The contribution of 2 identified peaks, IGluc and 5-hydroxy indole-3-acetic acid, was below 1% in most of the cases and therefore these compounds are not described separately. All the other peaks with minor importance or with rare appearance were grouped together as "All Other".

The mean reduction ratio (RR) for the IS, as the main contributor of the fluorescence signal, was 49%, which is very close to the mean decrease of the Sum of All Peaks 51% (P = 0.24) (Table 5) There was a strong correlation ($R^2 = 0.86$) between the RR of IS and RR of All Peaks (Fig 4). Trp had very sparse RR with a mean value of 9% and a standard deviation of 24%. It should be noted that during the dialysis, the peak of Trp increased for nearly half of the sessions, resulting in the negative RR values for Trp in these sessions. Peaks of Unknown 1 and IaG had the highest RR values, having close mean values (77 and 79%, respectively), and similar standard deviation, minimal and maximal values. RR of IAA followed with the mean value of 61%, which was higher than the RR of IS (49%, $P < 0.05$). Large difference between the minimum and maximum and mean RR values for the peaks and the groups indicates large variances in the removal of different fluorophores among the patients and the dialysis sessions.

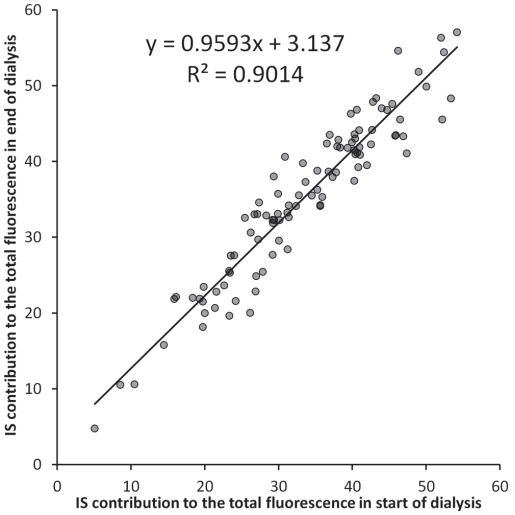

Fig 3. Correlation between the contributions of IS in the Start and End of dialysis.

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Figs 5 and 6 summarize the results of the 3D fluorescence chromatograms (N = 10) with an example of a characteristic spent dialysate sample. In Fig 6 Ex spectra were normalized to their values at 280 nm and Em spectra were normalized to their values at 383 nm. These figures show the similarities between the Ex and Em spectra of the total fluorescence of the spent dialysate and the summary spectra of identified indoles (Trp, IaG, IAA, IS). Fig 5 illustrates the contribution level of the IS and four identified indole derivatives to the total fluorescence signal. The sum contributes to more than half of the total fluorescence. Fig 6 presents very similar Ex and Em spectra shapes for the total fluorescence signal, fluorescence of four identified indoles and IS.

Discussion

Fig 1 presents continuous and on-line fluorescence measurements during a dialysis treatment. This demonstrates first time, by our knowledge, a potential for real-time dialysis monitoring using fluorescence of spent dialysis reflecting removal of the main fluorophores from blood into spent dialysate.

Focus in this study was on the composition of the fluorescence signal measured in the spent dialysate and the estimation of the contribution and removal dynamics of the detected

Table 5. Reduction ratio (Mean ± SD, Min, Max, N = 99) in percentage for fluorescent peaks.

Peak name	Mean RR± STDev	Min	Max
Unknown 1	77 ± 10	31	94
Tryptophan	9 ± 24	-71	71
Indoxyl sulfate	49 ± 14	12	85
Indoleacetyl glutamine	79 ± 12	31	95
Indoleacetic acid	61 ± 13	29	89
All Other	31 ± 11	10	82
Sum of All Peaks	51 ± 13	20	87

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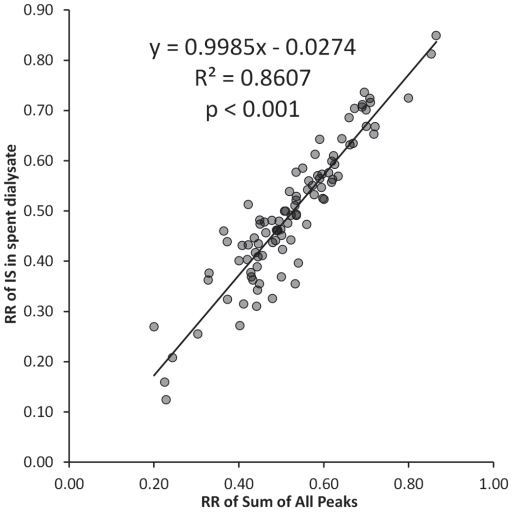

Fig 4. Correlation between the RR of IS and Sum of All Peaks in spent dialysate.

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fluorophores. The results aim to consolidate our approach proposed for monitoring the removal of IS [16]. The study indicates that:

1. a predominant part of the fluorescence signal originates from tryptophan and other indole derivatives (IGluc, IS, 5-hydroxy-indole-3-acetic Acid, IaG, and IAA);
2. the highest contribution (35 ± 11%) was found to arise from IS with strong correlation between contribution values at the start and end of dialysis (R^2 = 0.90);
3. the Reduction Ratio (RR) of IS is very close to the RR from the total fluorescence of spent dialysate (sum of all peaks);

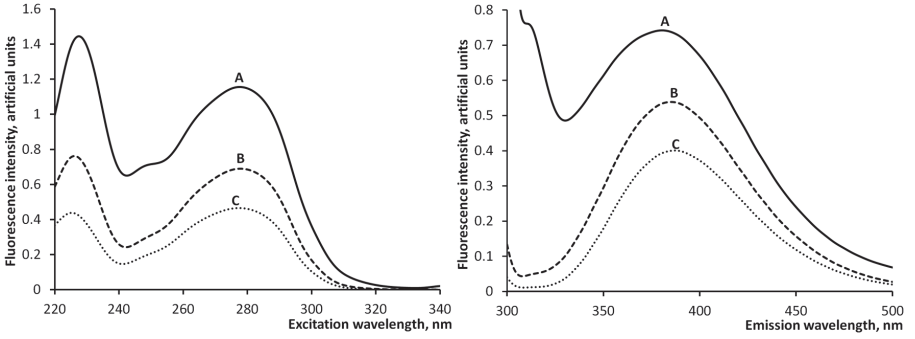

Fig 5. Comparison of Ex and Em scan spectra between the selected peaks and the whole chromatogram. (A)–total fluorescence, (B)–sum of four identified indoles, (C)–IS.

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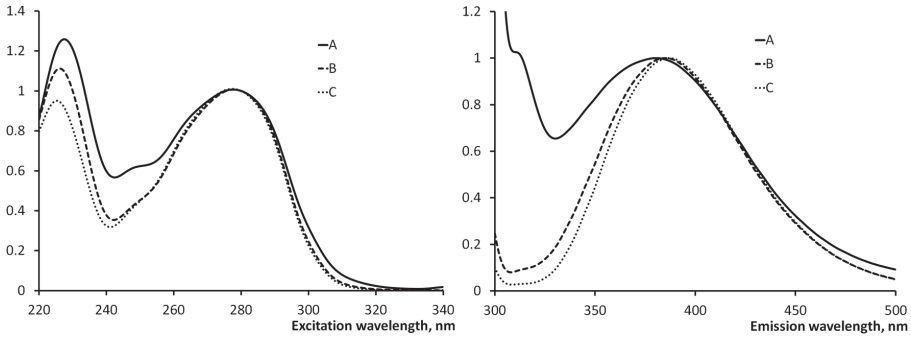

Fig 6. Comparison of normalized Ex and Em spectra between the selected peaks and the whole chromatogram. (A)–total fluorescence, (B)–sum of four identified indoles, (C)–IS.

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4. very similar fluorescence Ex and Em spectra of the spent dialysate samples from the total fluorescence signal, peaks of 4 identified indoles and peak of IS confirm that the foremost part of the fluorescence signal at used wavelengths originates from the indole derivatives;
5. the former leads to a conclusion that the fluorescence signal at a certain wavelength region (e.g. Ex: 280 nm and Em: 360 nm) has high potential to be utilized for monitoring the removal of PBS IS.

The HPLC results demonstrate that the major fluorophores in the spent dialysate at Ex: 280 nm and Em: 360 nm are indole derivates, PB uremic toxin IS being the main contributor in these measuring conditions (Fig 2 and Table 4). This is in agreement with the earlier studies, where IS has been found as a main contributor to the fluorescence in uremic fluids [18, 19]. Other identified fluorophores in the spent dialysate are IGluc, Trp, 5-hydroxy-indole-3-acetic acid, IaG, and IAA. The Unknown 1 fluorophore remained unidentified, however the fluorescence spectra, relative retention times and ESI-MS positive and negative mode mass-spectra (Table 3) indicate to the possible identification as a tryptophan glucoconjucate [20, 21]. Also, the fluorescence Em and Ex spectra scans indicate that the majority of the fluorescence signal originates from solutes with indole-alike fluorescence spectra with Ex maximum at 280 nm and Em maximum between 350 and 400 nm (Figs 5 and 6). The same fluorescence wavelengths for indoles have been published by others [22]. Therefore it can be assumed that the fluorescence of spent dialysate at Ex: 280 nm and Em: 360 nm is related to a patient's metabolic status of Trp, an essential amino acid in tight metabolic relation between the human being and the body and is concurrent with guts microflora.

The contribution of IS in the spent dialysate for the samples taken from the start and the end of the dialysis has no statistical difference ($P = 0.30$) and there is a strong correlation of contribution values between the start and end ($R^2 = 0.90$, $P < 0.001$). This indicates that the contribution of IS to the fluorescence signal does not have significant difference in the start and at the end of dialysis and other fluorophores mutually compensate some differences in the removal rates (Table 4). This is also supported by the very similar RR of IS and the total fluorescence signal in the spent dialysate (Table 5) and strong correlation ($R^2 = 0.86$) between these RR values (Fig 4).

The online optical monitoring technology for estimating the dialysis adequacy by the absorbance of UV-light in the spent dialysate was developed more than a decade ago [23]. It has been

successfully implemented in everyday clinical practice by integrating the optical sensor into the dialysis apparatus [24]. Lately, the phenomenon of UV-absorbance in the spent dialysate was investigated with HPLC, attributing the largest part of the UV-absorbance signal to the small water-soluble uremic toxin of uric acid rather than to the urea itself [25, 26]. The latter also indicates that the contribution of PBS to the UV-absorbance in the spent dialysate is negligible and the technology based on UV-absorbance monitoring is unsuitable for estimating the removal of PBS. IS has been proposed to serve as a useful indicator for the assessment of the dialysis efficiency (clearance) for PBS beside the urea based dose estimation [27, 28]. Several HPLC methods have been developed and proposed to estimate the removal of IS as a marker for protein-bound uremic toxins [12, 29]. In contrast, the online monitoring of the fluorescence of the spent dialysate assumes no sophisticated HPLC apparatus with specialized laboratory and personnel to estimate the removal of IS. Moreover, fluorescence measurements enable the estimation of the amount of total removed IS [16]. Similarity of the fluorescence spectra of whole dialysate with those of main fluorophores strongly suggests (Fig 6), that monitoring of the fluorescence of the spent dialysate in selected wavelength area characteristic to indole derivatives can be used as a supplementary technology for the assessment of the dialysis efficiency (clearance) of a PBS IS. Although strong positive correlation ($R^2 = 0.84$) between RR of IS and other PBS of p-cresyl sulfate has been demonstrated [30] and similar fluorescence detection has been used for analysis of p-cresyl sulfate [31], the fluorescence at Ex: 280 nm and Em: 360 nm is selective only to the indole derivatives and does not cover p-cresol derivatives with different fluorescence wavelength ranges.

A limitation of this study is that it does not yet cover the behavior of main fluorophores in the spent dialysate between the start and the end of dialysis. As it was confirmed by our preliminary results (S1 Fig), the level of fluorophoric dietary amino acid tryptophan had significant fluctuations in concentration in the spent dialysate when the patients had a meal during the dialysis. This effect can be related to the very low mean RR value of Trp (Table 5). Although statistically the contribution of IS remained unchanged during the dialysis, the fluctuating level of Trp may have some influence on the balance of the contribution from the fluorophores to the total fluorescence and on the monitoring accuracy of the removal of IS by fluorescence. Therefore, the behavior of Trp during the whole dialysis should be investigated in more detail to understand the possible errors in estimating the removal of PBS optically. Moreover, fluorescence intensity and spectrum shape of the real spent dialysate as compared to those in the chromatographic eluent are likely to have certain peculiarities, as the pH, different ion composition and the presence of the organic solvents vary in different solvents [22, 32], not forgetting possible quenching and matrix effects [22]. This study was not designed to cover the entire rather complex picture. However, it seems to be prospective to develop the optical monitoring method into a comprehensive technology of on-line assessment of dialysis adequacy, addressing both small water-soluble uremic toxins and some PBS like IS.

Supporting Information

S1 Fig. Example of fluctuation of the tryptophan concentration in spent dialysate during dialysis.
(TIF)

Author Contributions

Conceived and designed the experiments: IF ML FU. Performed the experiments: JA IF ML FU. Analyzed the data: JA. Contributed reagents/materials/analysis tools: RT. Wrote the paper: JA ML FU RT IF.

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Publication III

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Behaviour of uremic toxins and UV absorbance in respect to low and high flux dialyzers

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Abstract. The aim of this study was to investigate the behaviour of uremic toxins and UV absorbance in respect to low and high flux dialyzers during hemodialysis treatments. Ten uremic patients were investigated using online spectrophotometry, with wavelength of 280 nm, over the course of 30 hemodialysis treatments. The polysulphone dialyzers were used. The taken dialysate and blood samples were analysed using standard biochemical methods and reversed phase HPLC. The chromatographic peaks were detected by a UV detector at wavelengths of 254 and 280 nm. Spiking experiments and UV spectra between 200–400 nm allowed to identify predominant uremic toxins in 5 chromatographic peaks identified as creatinine (CR), uric acid (UA), hypoxanthine (HX), indoxyl sulphate (IS), and hippuric acid (HA). Moreover, two persistent, but non-identified peaks, peak 1 (P1) and peak 2 (P2), were detected. There was no significant difference in the reduction ratio of uremic solutes and the UV absorbance between the low and high flux membranes. The reduction ratios, 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, were closest to the removal of small water-soluble non-protein bound solutes urea, creatinine and uric acid. All studied uremic toxins and UV absorbance showed similar reduction for the low and high flux membranes during hemodialysis.

Key words: uremic toxins, hemodialysis, ultraviolet absorption, chromatography, dialysis membrane.

1. INTRODUCTION

Hemodialysis is a treatment that performs the functions of normal kidneys, i.e. removes uremic toxins. To date, a long list of possible uremic toxins has been identified as believed to be responsible for multifactorial and cumulative cause of

uremic toxicity [1]. These toxins have different molecular weights and some of them are bound to proteins. The target of dialysis therapy is adequate solute removal so that plasma concentrations remain at the most non-toxic levels possible. Most common indices of dialysis efficiency are based on the blood urea measurements. Unfortunately, urea is a small molecule weight solute and relying solely on urea may lead to inappropriately short dialysis during high flux or high efficiency dialysis. Thus, urea, the traditional marker for dialysis quality, should not be the only solute used to model the dialysis therapy [2]. At the same time, there is a need for techniques, which can offer a tool for separate monitoring of several compounds, retained in uremic patients and with potential clinical significance. Online monitoring of solute removal by dialysis via an optical UV absorbance-based device represents a valid alternative to the classical and, to date, expensive online urea monitoring devices [3].

For the removal of uremic compounds, different dialysis membranes are available, which will at least partly determine the efficiency of dialysis therapy [4]. Advocacy of the more efficient dialysis modalities with the high flux (HF) and super-flux (SF) membranes stresses the importance to study the behaviour of non-protein-bound and protein-bound uremic toxins in respect to their removal characteristics with different membranes [5]. In this study, the multidimensional effect, how low flux (LF) and HF dialysis membranes can be implicated in the removal of different uremic compounds and measurements of the UV absorbance, was examined. Assuming that the dialysate may be a preferred alternative for continuous monitoring of solute removal and adequacy of dialysis, the HPLC analysis of both serum and dialysate samples would be valuable as a source of information.

The aim of this study was to investigate the behaviour of non-protein-bound and protein-bound uremic toxins and UV absorbance in respect to low and high flux dialyzers during hemodialysis treatments.

2. SUBJECTS AND METHODS

2.1. Subjects and clinical study

This study was performed after the approval of the protocol by the Tallinn Medical Research Ethics Committee at the National Institute for Health Development, Estonia. An informed consent was obtained from all participating patients. Ten patients, mean age 62.6±18.6 years, receiving thrice-weekly hemodialysis, were studied during 30 dialysis sessions (three for each patient). All patients were dialysed with polysulfone membrane dialyzer Fresenius 4008H (Fresenius Medical Care, Germany): (1) 4 patients by low flux dialyzer F8 HPS with an effective membrane area of 1.8 m^2 and an ultrafiltration coefficient of 18 mL/h*mmHg; (2) one patient with a low flux membrane dialyzer F10 HPS with a membrane area of 2.2 m^2, ultrafiltration coefficient of 21 mL/h*mmHg; (3) 5 patients with high flux dialyzer FX 80 with effective membrane area of

1.8 m^2 and ultrafiltration coefficient of 59 mL/h*mmHg. The dialysate flow was 500 mL/min and the blood flow varied from 245 to 350 mL/min. All treatments were monitored optically by a spectrophotometer HR2000 (Ocean Optics, Inc., USA), which was used to determine the UV absorbance with a specially designed optical cuvette, connected to the fluid outlet of the dialysis machine with all spent dialysate passing through during the online experiments. The clinical set-up of the experiments is shown in Fig. 1. The online absorbance curve during a single hemodialysis treatment is also presented. UV absorbance was measured in arbitrary units. The sampling frequency was set to two samples per minute. The obtained UV absorbance values were processed and presented on the computer screen by a PC, connected to the spectrophotometer using the Ocean Optics software (OOIBase32, Ocean Optics, Inc., USA, version 2.0.2.2 for Windows).

2.2. Sampling

Blood and dialysate samples were obtained from dialysis patients. Blood samples were drawn before the start of dialysis (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, Beckton Dickinson) and was allowed to clot. After centrifugation at 3000 rpm the serum was separated from the blood cells.

The 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.

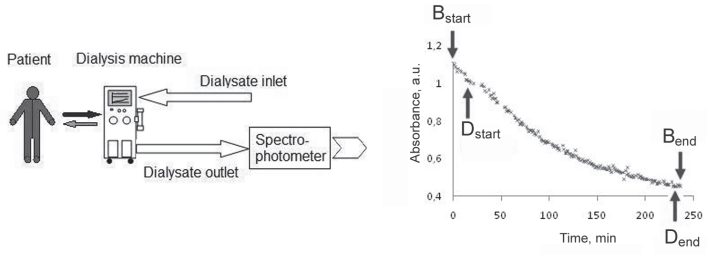

Fig. 1. The clinical set-up of the experiments. Time points when the samples were taken for the later analysis are as follows: B_{start} – blood sample collected before dialysis session, B_{end} – blood sample collected at the end of hemodialysis, D_{start} – dialysate sample collected 10 min after the start of hemodialysis, D_{end} – dialysate sample collected at the end of hemodialysis. The online absorbance measurements after every 30 s are presented by "x".

2.3. Biochemical analyses

The serum and the dialysate samples were analysed immediately at the Clinical Chemistry Laboratory at North Estonia Medical Centre using standardized methods. Urea (UR, MW = 60.06 Da), creatinine (CR, MW = 113.12 Da), and uric acid (UA, MW = 168.11 Da) were measured with a Hitachi 912 autoanalyzer (Roche, Switzerland). The determination of creatinine (μmol/L) in serum based on the Jaffe reaction, the intensity of creatinine complex (with pirate) was measured potentiometrically. Urea (mmol/L) was detected by the kinetic UV assay and uric acid (μmol/L) was detected by the enzymatic colorimetric method. The coefficient variation (CV) of the methods for the determination of different solutes in dialysate and blood were: CR 5%; UA 2%; UR 4%.

2.4. Reversed phase HPLC study

Before the HPLC analysis, the serum samples were purified of proteins by centrifuging with the Microcon centrifugal filters (Millipore, USA) at room temperature. The dialysate samples were acidified down to pH 4.0 with formic acid for conformation with the pH of the chromatographic eluent used.

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 × 250 mm column from Du Pont Instruments (Wilmington, DE, USA) with a 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), both from Rathburn (Walkerburn, Scotland), with a six step gradient program as specified in Table 1.

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 200–400 nm with a time interval of 1.76 s, and data processed respectively by means of Turbochrom WS and Turboscan 200 software from Perkin Elmer. The chromatographic peaks were detected by the UV detector at wavelengths of 254 and 280 nm.

Table 1. Elution program used for HPLC separation of constituents in the dialysate

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

2.5. Data analysis

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

$$RR = \frac{C_{start} - C_{end}}{C_{start}} 100\%. \quad (1)$$

C_{start} and C_{end} 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.

The results are presented as mean ± standard deviation. A non-parametric Mann-Whitney U Test and Student's t-test was used to compare groups of values while $p < 0.05$ was considered significant. Two sessions, inadequate due to the technical failure of the spectrophotometer, were excluded. In the case of HPLC analysis only these peaks were taken into account, where separation from neighbouring peaks was confirmed by comparison of UV spectra with those of the reference standards. The number of omitted cases was: IS (4), HA (4), HX (14), P1 (13), P2 (4), TA HPLC 254 nm (3), TA HPLC 280 nm (2), online 280 nm dialysate (4). The data analyses were performed in Statistica 6.0 (Statsoft, Inc. for Windows).

3. RESULTS

Figure 2 shows the representative HPLC chromatogram of the serum, measured at the wavelength of 254 nm. A number of higher prevalent peaks, representing chromophores-uremic toxins, can be observed. Some HPLC peaks were identified, such as creatinine, uric acid (the highest contribution), hypoxanthine, indoxyl sulphate and hippuric acid. Absorbing spectra of two unknown persistent peaks (P1 and P2) were identified at the retention times (RT) of 15.46 and 15.82 min. Additionally, some unknown peaks between the RT of 21–29 min were detected.

The solute concentrations and the UV absorbance values at the start and end of treatment in the serum and online in the spent dialysate for the LF and HF membranes are presented in Table 2. As Table 2 shows, the solute concentrations and the UV-absorbance values are lower at the end of dialysis (C_{end}) compared to the start concentrations (C_{start}). At the same time, the start and end concentrations and UV absorbance values for different membranes were not statistically different ($p < 0.05$).

Table 3 presents the RR (%) of solutes and the total area of the HPLC UV absorbance peaks at wavelengths of 254 and 280 nm in serum, and for the online UV absorbance at 280 nm in the spent dialysate, for different types of membranes. There was no significant difference between the results for RR of LF and HF membranes ($p < 0.05$).

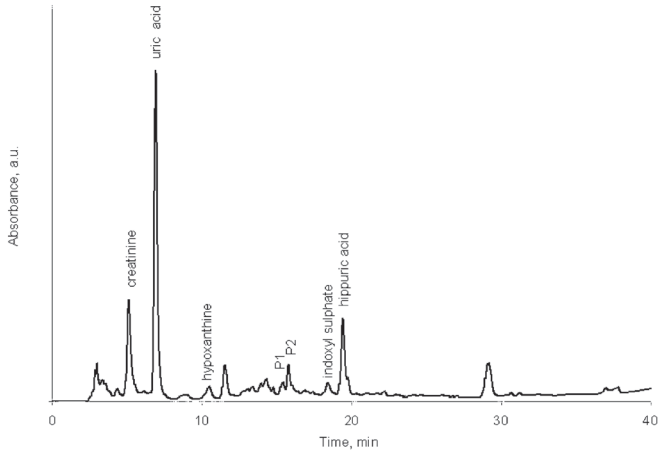

Fig. 2. The representative HPLC chromatogram of the serum, monitored at the wavelength of 254 nm; identified peaks are presented.

Table 2. The start (C_{start}) and end (C_{end}) concentrations of solutes and the total area of the HPLC UV absorbance peaks at the wavelengths of 254 and 280 nm ("TA HPLC 254 nm" and "TA HPLC 280 nm", respectively) in the serum, and the RR of online UV absorbance at 280 nm in the spent dialysate ("Online 280 nm dialysate") for HF and LF membranes; *N* denotes the number of cases

	LF		HF	
	C_{start}	C_{end}	C_{start}	C_{end}
Urea, mmol/L	21.0 ± 4.07 (*N* = 15)	7.03 ± 2.49 (*N* = 15)	19.4 ± 4.48 (*N* = 15)	7.11 ± 1.79 (*N* = 15)
Creatinine, μmol/L	735 ± 172 (*N* = 15)	311 ± 106 (*N* = 15)	591 ± 204 (*N* = 15)	252 ± 83 (*N* = 15)
Uric acid, μmol/L	356 ± 98 (*N* = 15)	116 ± 51 (*N* = 15)	381 ± 62 (*N* = 15)	130 ± 30 (*N* = 15)
Indoxyl sulphate, mg/L	6.20 ± 2.36 (*N* = 15)	3.69 ± 1.74 (*N* = 14)	6.83 ± 3.09 (*N* = 15)	3.76 ± 1.26 (*N* = 13)
Hippuric acid, mg/L	29.9 ± 16.4 (*N* = 15)	6.52 ± 3.95 (*N* = 15)	23.7 ± 28.6 (*N* = 15)	9.22 ± 8.10 (*N* = 11)
Hypoxanthine, mg/L	3.00 ± 2.93 (*N* = 11)	1.42 ± 1.28 (*N* = 14)	2.54 ± 2.34 (*N* = 9)	1.82 ± 2.52 (*N* = 12)
P1, a.u. $\times 10^{-2}$	2.92 ± 1.59 (*N* = 14)	0.99 ± 0.39 (*N* = 12)	2.47 ± 0.70 (*N* = 12)	1.30 ± 0.62 (*N* = 9)
P2, a.u. $\times 10^{-2}$	9.62 ± 5.86 (*N* = 15)	3.99 ± 2.16 (*N* = 14)	7.58 ± 1.73 (*N* = 15)	3.63 ± 1.10 (*N* = 12)
TA HPLC 254 nm, a.u. $\times 10^{7}$	4.00 ± 2.65 (*N* = 14)	1.47 ± 0.98 (*N* = 15)	2.52 ± 0.76 (*N* = 14)	1.16 ± 0.56 (*N* = 14)
TA HPLC 280 nm, a.u. $\times 10^{7}$	3.28 ± 1.42 (*N* = 15)	1.12 ± 0.49 (*N* = 15)	2.89 ± 0.60 (*N* = 14)	1.12 ± 0.21 (*N* = 14)
Online 280 nm dialysate, a.u.	1.53 ± 0.38 (*N* = 15)	0.65 ± 0.22 (*N* = 15)	1.44 ± 0.42 (*N* = 13)	0.60 ± 0.16 (*N* = 13)

Table 3. The RR (%) of solutes and the total area of the HPLC UV absorbance peaks at the wavelengths of 254 and 280 nm ("TA HPLC 254 nm" and "TA HPLC 280 nm", respectively) in the serum, and the RR of online UV absorbance at 280 nm in the spent dialysate ("Online 280 nm dialysate") for different types of membranes; *N* denotes the number of cases

	LF	HF
Urea	67.0 ± 8.7 (*N* = 15)	63.2 ± 5.07 (*N* = 15)
Creatinine	58.2 ± 7.7 (*N* = 15)	56.6 ± 5.4 (*N* = 15)
Uric acid	67.7 ± 8.5 (*N* = 15)	65.6 ± 6.7 (*N* = 15)
Indoxyl sulphate	42.1 ± 18.0 (*N* = 13)	47.8 ± 14.0 (*N* = 12)
Hippuric acid	75.1 ± 11.5 (*N* = 15)	68.1 ± 9.4 (*N* = 10)
Hypoxanthine	42.6 ± 16.0 (*N* = 10)	46.1 ± 18.5 (*N* = 8)
P1	62.1 ± 13.0 (*N* = 12)	61.0 ± 5.3 (*N* = 7)
P2	59.2 ± 17.5 (*N* = 13)	51.6 ± 5.9 (*N* = 12)
TA HPLC 254 nm	60.2 ± 12.5 (*N* = 14)	57.2 ± 7.7 (*N* = 13)
TA HPLC 280 nm	65.2 ± 9.6 (*N* = 15)	60.6 ± 7.9 (*N* = 14)
Online 280 nm dialysate	58.1 ± 8.3 (*N* = 15)	57.0 ± 10.4 (*N* = 13)

Figure 3 presents the RR of the uremic solutes and total area of HPLC peaks (at wavelengths of 254 and 280 nm, "TA HPLC 254 nm" and "TA HPLC 280 nm", respectively) in the serum, and the RR of online UV absorbance at 280 nm in the spent dialysate ("Online 280 nm") combining the results from the start and end samples (Table 3). Three main groups were distinguished according to the average reduction ratio among the studied solutes. The highest RR (>70%) had hippuric acid classified as a "High RR" solute. "Medium RR" solute group ($50\% < RR \leq 70\%$) incorporated the small water soluble compounds uric acid, urea, creatinine, and peaks 1 and 2. The "Low RR" solutes' group ($RR \leq 50\%$) included the protein bound solute indoxyl sulphate and the small water soluble compound hypoxanthine. A statistically dissimilar RR was found between the groups. No statistically different RR was between the solutes within the same group ($p < 0.05$), except for the "Medium RR" group, which could be divided into two subgroups: (1) "Medium 1 RR" solute group ($60\% < RR \leq 70\%$) with uric acid and urea; (2) "Medium 2 RR" group ($50\% < RR \leq 60\%$) with creatinine. The solutes of the subgroups had different removal rates.

All UV absorbance based RR values were higher than the "Low RR" solutes IS, HX, and lower than HA. However, P1 was a "centre-medium peak" removed statistically alike as all "Medium RR" solutes/peaks. The "left side bar" of P1,

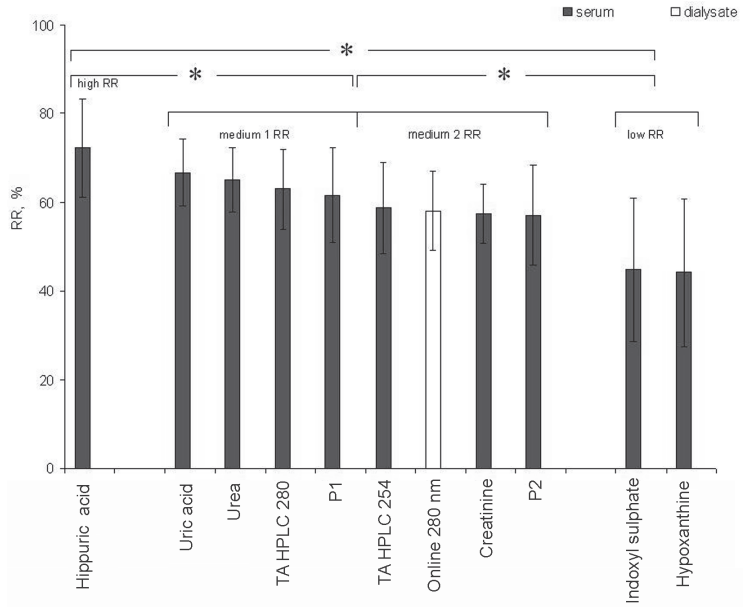

Fig. 3. The RR of solutes and TA HPLC (254 and 280 nm) in the serum and online UV absorbance in the spent dialysate (online 280 nm). Significant differences ($p < 0.05$) are marked with an asterisk (*).

RR of "TA HPLC 280", was similar to UA, UR, "TA HPLC 254", and higher than "Online 280 nm", CR, and P2, whereas the "right side bar" of P1, RR of "TA HPLC 254", was lower than UA, UR, and comparable to all other "Medium RR" solutes/peaks. "Online 280 nm", CR, and P2 were all removed statistically similarly and had lower RR than UA, UR, and "TA HPLC 280".

4. DISCUSSION

The present study investigated behaviour of non-protein-bound and protein-bound uremic toxins and UV absorbance in respect to low and high flux dialyzers during hemodialysis treatment. The results indicated that: (i) the main identified solutes responsible for the UV absorbance at 280 nm are the low molecular weight water-soluble non-protein-bound compounds UA, CR, and the low molecular weight water-soluble protein-bound compounds HA, IS, HX; (ii) two persistent, but non-identified HPLC peaks, P1 and P2, were detected from the HPLC profiles contributing to the UV absorbance, possibly each peak representing a single uremic retention solute; (iii) the LF (F8 HPS, F10 HPS) and HF

(FX80) membranes showed similar start and end concentrations for all studied uremic solutes; (iv) the LF and HF membranes showed comparable RR for all studied uremic solutes, except for P2, having slightly higher RR value for LF membranes ($p = 0.016$); (v) there was no statistical difference between intradialytic start-end values, and removal efficiency for the LF and HF membranes estimated by the total area of HPLC peaks at 254 and 280 nm in the serum and online UV absorbance at 280 nm in the spent dialysate, indicating similar behaviour of the UV absorbance to the uremic toxins.

A number of the higher prevalent peaks on the HPLC profiles of the serum (Fig. 2) indicate that there exists a group of compounds, UV chromophores, which are the main cause of the cumulative and integrated UV absorbance. The results of our study indicate that the main solute, responsible for the UV absorbance, is a low-molecular-weight water-soluble non-protein-bound compound uric acid UA. Four additional uremic retention solutes, creatinine, hypoxanthine, indoxyl sulpate and hippuric acid, were identified from the HPLC profiles contributing to the UV absorbance. Moreover, two persistent, but non-identified HPLC peaks P1 and P2 were identified from the HPLC profiles, contributing to the UV absorbance, possibly each peak representing a single uremic retention solute.

The present randomized trial comparing HD membranes showed no unlike removal of the studied uremic solutes for the LF and HF membranes (Table 3) as presented earlier by Lesaffer et al. [4]. In this study it was found that the cellulose triacetate and polysulphone HF membranes removed similarly classical markers and protein-bound liphophilic solutes as a LF polysulphone membrane. All studied uremic solutes had similar start and end concentrations for different membranes (Table 2). Comparable results were obtained even with the concentrations, corrected by a correction factor, based on the total protein concentration at the start and at the end of dialysis as used by Lesaffer et al. [4]. Furthermore, there was no statistical difference between intradialytic start–end values, and removal efficiency for the LF and HF membranes estimated by the total area of HPLC peaks at 254 and 280 nm in the serum and online UV absorbance at 280 nm in the spent dialysate. This indicates that the UV absorbance is following the behaviour of the UV absorbing compounds – uremic toxins, which are the origin of the total UV absorbance in the serum and in the spent dialysate.

The unaffected removal of the uremic toxins compared to LF and HF membranes can partly be explained by the similar effective blood urea clearance, characterizing the small molecular weight solutes diffusive transport in the dialyzers. The effective blood urea clearance *in vivo* is determined by the dialyzers blood urea clearance *in vitro*, the dialyzer mass transfer area coefficient KoA, and by the blood and dialysate flow rates [6]. There was no statistical difference between the blood and dialysate flow rates, which suppressed the effect of the higher KoA of the HF dialyzer (about 920 mL/min for LF vs 1263 mL/min for HF) yielding almost the same effective blood urea clearances

for both LF (202 ± 25 mL/min) and HF dialyzers (217 ± 25 mL/min). Since the diffusive transport is superior to the convective transport for small non-protein-bound solutes in the dialyzer during conventional hemodialysis, the transport of the studied non-protein-bound solutes in the LF and HF dialyzers was alike regardless of the HF having a higher ultrafiltration coefficient (59 mL/h*mmHg for HF vs 18 and 21 mL/h*mmHg for LF), and slightly higher average total ultrafiltration (UF) (1.95 ± 0.74 l for HF vs 1.57 ± 0.42 l for LF, ns) during the study. Although the mechanism for removal of protein-bound solutes is not well known, diffusion seems to be important [5,7]. This is confirmed by the results from the present study, showing no alteration in the small protein-bound solute removal as observed by LF and HF dialyzers during conventional hemodialysis. Moreover, the RR levels were comparable to those reached by the highly convective hemodiafiltration (HDF) therapies from the study by Meert et al. [7], considering analogous treatment duration, weight loss, and blood and dialysate flow rates in both studies. The removal of low molecular weight proteins (e.g. β2-microglobulin) seems to be dependent on membrane composition and morphology, and the removal of small solutes may be improved by enhancing flow distribution in the dialyser [8].

Some minor effects could arise also from the study design, which benefited from crossover patient dialysing regarding LF and HF membranes. The current study used randomly selected patients with equal sample size for the LF and HF membranes. This caused a slightly higher urea removal rate (URR) value for the LF membrane group (about 4%, but not statistically significant) indicating a somewhat higher delivered dialysis dose for the patients in the LF group. This was primarily due to non-significantly longer dialysis sessions for the patients in the LF group (233 ± 15 min for LF vs 229 ± 17 min for HF) and the dialysis-related difficulties (e.g. non-compliance) in the HF group.

Taking into account the removal efficiency (Fig. 3), a characteristic behaviour can be observed for every uremic toxin group depending on the protein binding. The removal efficiency was highest (except for HA) for the small water-soluble non-protein-bound solutes UA, UR and CR and for the unidentified HPLC peaks P1 and P2, whereas the protein-bound solutes IS and HX had the lowest removal rate. Both HA, being a protein-bound solute, but also the small non-protein-bound compound hypoxanthine, have individual removal patterns, which should be considered when analysing the removal of uremic solutes during hemodialysis. A lower removal of hypoxanthine, compared to urea, is also presented earlier [9]. A slightly higher RR value for LF compared to HF membranes ($p = 0.016$) for P2 was unexpected and the reason must be explained in the future. The total area of HPLC peaks at 254 and 280 nm in the serum and online UV absorbance at 280 nm in the spent dialysate tend to estimate RR levels close to that of the small water-soluble non-protein-bound solutes UA, UR and CR. This can be due to the large contribution of UA and CR to the total UV absorbance at 254 and 280 nm. The variation between the reduction ratio of the total area of HPLC peaks and online UV absorbance at 280 nm can be explained

by a different number of detectable chromophores in the serum and in the spent dialysate and by different sampling times for the serum and the dialysate (the serum was collected before and the dialysate sample 10 min after the start of the dialysis). Due to the characteristic absorbing spectra of the UV-chromophores, the difference in RR at the wavelengths of 254 and 280 nm is seen.

Future studies aim to further identify the remained prevalent peaks on the HPLC UV absorption profiles of uremic fluids, and to investigate the possibility for absolute concentration measurements of the uremic toxins utilizing the UV absorbance.

5. CONCLUSIONS

In conclusion, the present study demonstrated that the studied small water-soluble non-protein-bound solutes UR, CR, UA and HX, and the protein-bound solutes HA and IS showed similar removal efficiency for the LF and HF membranes. Furthermore, the total UV absorbance at 254 and 280 nm seem to estimate removal efficiency levels close to the small water-soluble non-protein-bound solutes.

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Ureemiliste toksiinide ja nende UV-absorptsiooni uurimine vedeliku madala ning kõrge läbilaskevõimega dialüsaatorite korral

Kai Lauri, Jürgen Arund, Risto Tanner, Jana Jerotskaja, Merike Luman ja Ivo Fridolin

Ureemiliste toksiinide elimineerimiseks organismist kasutatakse erinevat tüüpi – madala ja kõrge vedeliku läbilaskevõimega – dialüüsi membraane. Membraani tüübist sõltub osaliselt ka dialüüsiravi efektiivsus. Käesolev uuring näitas, et kasutatud dialüüsravi parameetrite korral elimineerivad madala ja kõrge läbilaskevõimega dialüüsi membraanid sarnaselt nii väikese molekulmassiga vees lahustuvaid valkudega mitte seotud kui ka valkudega seotud aineid. Sarnaselt elimineeritud ureemilistele toksiinidele vähenes ka kõikide kromatograafiliste piikide kogupindala ja UV-absorptsioon.

Publication IV

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Clinical Study

Paracetamol Interference in Uric Acid Levels in Uremic Patients Revealed by Monitoring Spent Dialysate

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The aim of this study was to assess removal dynamics of paracetamol (PAR), as an extraordinary chromophore in spent dialysate, upon the optical monitoring of dialysis of end-stage renal disease patients with inflammation complications. Seven dialysis sessions of different patients were followed to whom PAR was used as a pain reliever or antipyretic. Spent dialysate was sampled hourly and analyzed using HPLC with MS/MS and UV detection. Quantitative calculations were made on the basis of the peak areas on the chromatograms at 280 nm for uric acid (UA) and 254 nm for PAR and its metabolites (PAR-M). Peaks of UA, PAR, PAR-glucuronide, and PAR-sulphate were identified on the basis of specific mass spectra. Removal of PAR was found to be proportional to that of uric acid if intake of the drug by patient occurred half a day before dialysis. But disturbances of the UV-absorbance curves at 280 nm were observed related to rise of UA concentration in spent dialysate when PAR was taken by patients in the course of dialysis. The mechanism of such relation remains unknown. It was concluded that possible benefits and risks of treatment of uremic patients with paracetamol-containing drugs may need to be reassessed.

1. Introduction

Uric acid (UA) is known as a normal final product of human metabolism of purines, essential constituents of nucleic acids, and is normally excreted by kidney with urine [1]. High concentration of UA in serum often associates with severe chronic pathologies, such as arthritis, hypertension, and so forth [2]. Many recommendations have been published for such patients listing purine-rich food products to be avoided as well as some drugs, which are known to be associated with a raise of UA in blood, but paracetamol (PAR), a worldwide used analgesic and antipyretic drug [3], is not usually included into such lists. Paracetamol (PAR) is known as a potent enhancer of the effect of many other drugs [4] and its combination with analgesic drugs as pain reliever, including treatment of acute gout-like arthritis associated with high level of UA in blood [5]. Some observations have been published in the older literature concerning elevated analytical results of UA in serum following PAR administration, but this effect was found to be caused by interference of PAR with phosphotungstate reduction method of UA analysis only and an alternative uricase method did not confirm relationship between UA concentration and PAR administration [6, 7]. Since no strong limitation is known, PAR is dosed sometimes also to patients with renal failure as an analgesic or antipyretic drug.

The online monitoring of total ultraviolet (UV) absorbance in the spent dialysate has empowered itself as a valuable tool for continuous monitoring of a single hemodialysis session with the possibility of customizing the treatment in accordance with the physiological condition of the patient [8, 9]. The wavelength of 280 nm is commonly used in optical dialysis adequacy sensors for this purpose [10] and UA appears to be the main chromophore at this wavelenth among variety of metabolites, eliminated from the blood of end-stage renal disease (ESRD) patients by dialysis [11]. The decline of the absorbance of spent dialysate at 280 nm during dialysis session reflects quite well the removal of all small water-soluble uremic toxins and PAR with its absorbance maximum near 254 nm do not seem to substantially interfere

with optical monitoring of elimination of this group of solutes from the blood of patients with end-stage kidney disease [12]. But when studying dynamics of removal of PAR from the blood of these patients, surprising parallel rise of concentrations of UA together with PAR and its metabolites (PAR+M) in spent dialysate was observed in some cases after giving PAR to patients in the course of a dialysis session. The aim of this study was to evaluate in more details removal dynamics of the paracetamol (PAR), as an extraordinary chromophore in spent dialysate, upon the optical monitoring of dialysis of end-stage renal disease patients with inflammation complications.

2. Subjects and Methods

The study was performed after the approval of the protocol by the Tallinn Medical Research Ethics Committee at the National Institute for Health Development, Estonia. An informed consent was obtained from all participating patients. Only standard therapeutic procedures previously appointed by doctor were used with no alterations in connection with this research. In total 17 patients, receiving thrice-weekly hemodialysis, were followed during a single dialysis session each. PAR had been prescribed to 7 of the patients as an antipyretic or as pain reliever before or during the dialysis session. Five of these patients are females and 2 are males, mean age 65 ± 13 years. 10 patients, 2 female and 8 male, mean age 62.6±18.6 years not receiving PAR were followed as the control group. All patients were dialysed with polysulfone membrane dialyzers (Fresenius Medical Care, Germany) by low flux dialyzers F10 HPS with an effective membrane area of 2.2 m^2, ultrafiltration coefficient 21 mL/h∗mmHg. The dialysis machine used in the study was Fresenius 4008H (Fresenius Medical Care, Germany), duration of sessions 4 hours with one exception (3 hours) concerning the patient number 7. The dialysate flow was 500 mL/min and the blood flow varied from 245 to 350 mL/min depending on the patient but was kept stable during dialysis session. Spent dialysate was sampled hourly or more frequently and the content of main well-known uremic toxins in samples were analyzed using high performance liquid chromatography as described previously [12, 13]. Chromatograms at the wavelengths of 254 and 280 nm were monitored and quantitative calculations were made on the basis of the peak areas on the chromatograms at 280 nm for uric acid (UA) and at 254 nm for PAR and its metabolites (PAR+M). Identification of UV peaks of UA, PAR, PAR-glucuronide, and PAR-sulfate was confirmed on the basis of characteristic mass spectra [14] by means of the MicrOTOF-Q II ESI MS/MS mass spectrometer (Bruker Daltonics, Bremen, Germany) switched online into the postcolumn eluent flow through the flow splitter Model 600-PO10_06 (Analytical Science Instruments, CA, USA). Removal ratio (RR) of UA was calculated in percentage using the formula

$$\mathrm{RR} = 100 * \left(1 - \frac{A_{\mathrm{end}}}{A_{\mathrm{start}}}\right), \quad (1)$$

where A indicates peak areas of UA on the chromatograms of the first (10 min, "start") and the last (180 or 240 min, "end") dialysate samples of the same dialysis session. Two-sample t-test was used for evaluation of differences between groups assuming unequal variances ($P < 0.05$).

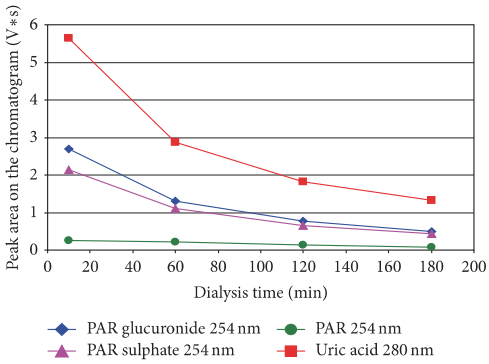

Figure 1: An example of change of concentrations of uric acid and paracetamol (PAR) and PAR metabolites in spent dialysate outflow during the dialysis session for the patient number 7. The patient got paracetamol thrice per day 1 g *per os*, the last dosage 10.5 hours before the dialysis.

3. Results

In the case of the patient receiving PAR overnight before dialysis, normal logarithmic-like decline of content of both UA and PAR+M during the dialysis session was observed (Figure 1). The final removal ratio (RR) of UA appeared to be the same as the average in control group (Table 1). But, unexpectedly, when PAR was given shortly before or after the start of dialysis (6 patients, Table 1), characteristic protuberance of the absorption curve of the UA elimination corresponding to the time could be seen when metabolites of PAR appeared in the spent dialysate (Figure 2). Surprisingly, a sharp increase in concentration of UA parallel to increase of PAR and metabolites in spent dialysate was observed in two cases from total six receiving PAR in the time of dialysis session (Figure 3).

The average removal ratio of UA of those 6 PAR patients 52 ± 9% (Table 1) was found on the basis of t-test to be significantly ($P < 0.05$) lower than the corresponding rate 78 ± 14 of patients not receiving the PAR. The initial concentrations of UA in the spent dialysate appeared to be significantly higher (with the single exception of the patient number 4) in comparison of the corresponding values in the control group (Table 1).

4. Discussion

The typical UV monitoring curve of elimination of small water-soluble uremic toxins by dialysis has been presented, and details of liquid chromatographic analysis of spent dialysate discussed elsewhere by our group in connection with assessment of online dialysis dose monitoring by UV absorbance [10, 12, 13]. The peak of UA is well separated from peaks of PAR and its metabolites on the chromatograms

Table 1: Removal ratio (RR) of uric acid (UA) depending on taking of paracetamol (PAR) in the course of dialysis session by patients with end stage kidney disease.

Patient no.	Dosage of PAR		UA in dialysate		Patient	
	mg	Last dosage time*	mg/L in start sample	RR %	Sex	Age
No. 1	1000	−90**	22.19	66	F	65
No. 2	500	+95	18.11	43	M	47
No. 3	1000	+30	26.73	47	F	78
No. 4	1000	+45	7.93	47	F	76
No. 5	1000	+10	15.32	49	M	76
No. 6	500	+30	12.61	60	F	47
		Mean ± SD	17.15 ± 6.74	52 ± 9		
No. 7	1000	−630***	16.96	77	F	70
Control group	No PAR	Mean ± SD	8.87 ± 2.11	78 ± 14	8 M, 2 F, mean age 62.6 ± 18.6, 10 dialysis sessions in total	

Notes: *Time in minutes before (−) of after (+) the start of dialysis.
**Intravenous dropping 10 mg/min, 1 g total beginning from 90 min before dialysis.
***The last dosage overnight before the dialysis.

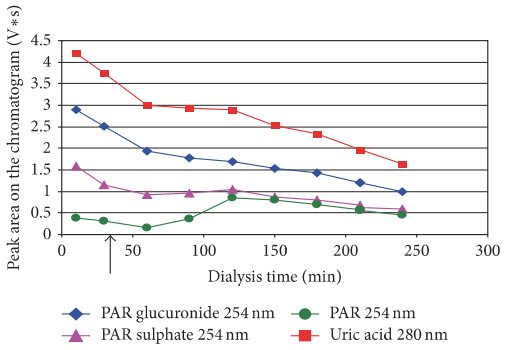

Figure 2: An example of change of concentrations of uric acid and paracetamol (PAR) and PAR metabolites in spent dialysate outflow during the dialysis session for the patient number 6. The patient got paracetamol thrice per day 500 mg *per os*, the first dosage on the day of dialysis 30 min after the start of dialysis session (marked by the arrow).

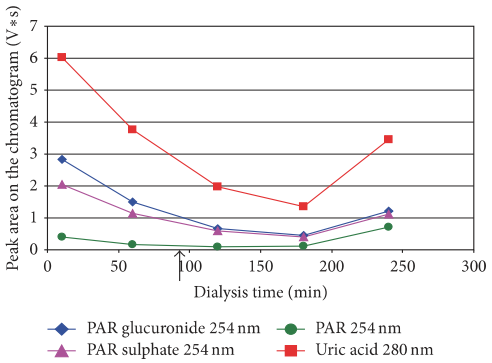

Figure 3: An example of change of concentrations of uric acid and paracetamol (PAR) and PAR metabolites in spent dialysate outflow during the dialysis session for the patient number 2. The patient got paracetamol thrice per day 500 mg *per os*, the first dosage on the day of dialysis 90 min after the start of dialysis session (marked by the arrow).

[12] and any possibility of analytical interference of PAR+ metabolites in HPLC estimation of UA content in spent dialysate seems not to be possible. Consequently, the significant rise of content of UA in spent dialysate after dosage of PAR to ESRD patients in the course of dialysis session seems to really take place at least in some cases of treatments. The chemical mechanism of such relation between PAR and UA in uremic patients remains to be clarified as well as possible good or harm to uremic patients. Uremic patients are under regular medical supervision and rarity of occasions where treatment with PAR has been indicated significantly restricting such kind of research on patients *in vivo*.

UA is not only known as a useless waste product of organism but also has proven to be involved in essential reduction and oxidation reactions in long history of human being development [15]. While UA seems to be an antioxidant in extracellular environment [16] possibly inhibiting the free radical rout of formation of AGEs [17], the UA has adverse prooxidant effect in adipocytes possibly by stimulating NADPH oxidase [18]. However, the harmful effects of high concentrations of UA in blood seems to be strongly prevalent [1], including risk for kidney diseases [19, 20]. Concerning inconsistent activities of UA, Mohandas and Johnson have concluded that "although the concept that uric acid might have a role in kidney disease once suffered a requiem, it has undergone a revival and seems deserving of additional study" [19]. The same can be concluded from our observation concerning treatment of uraemic patients with PAR: this practice "seems deserving of additional study" if PAR may significantly increase UA concentration in blood and cause

additional health risk to uremic patients. An oxidative way of UA degradation concerning myeloperoxidase and hydrogen peroxide has been described in the case of cardiovascular disease [21] and PAR has been found to inhibit this enzymatic system [16]. Does it point on a possible way of direct involvement of PAR in the increase of concentration of UA in uraemic patients? Our observation of parallelism in dynamics of PAR+metabolites and UA in the spent dialysate after giving of PAR to patients with renal failure seems to support such interpretation. Alternatively, we do not know much about influence of PAR upon the distribution of UA between blood, interstitial fluid, and tissue cells. If increase of UA in dialysate after PAR treatment reflects quicker movement of UA from tissues to blood, the drug can be considered as suggested reliever of complaints of hyperuriceamic and dialysis patients.

5. Conclusion

Removal of paracetamol is proportional to that of uric acid and dose not interfere with on-line UV monitoring of removal of small water-soluble uremic toxins at 280 nm, if intake of the drug by patient occurs half a day before dialysis or more. But significant rise of uric acid concentration in the spent dialysate as well as disturbance of the UV-absorbance curve may be caused by intake of paracetamol during the dialysis session in spite of the great difference of absorbance maxima of these solutes. The mechanism of the relation between paracetamol and uric acid in spent dialysate remains unknown. Possible benefits and risks of treatment of uraemic patients with paracetamol-containing drugs may need to be reassessed.

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Publication V

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". In: Proceedings of 35th Annual International Conference of the IEEE EMBS: 35th Annual International Conference of the IEEE EMBS, Osaka, Japan, 3 - 7 July, 2013. IEEE, 2013, 6707 – 6710 (DOI: 10.1109/EMBC.2013.6611095).

35th Annual International Conference of the IEEE EMBS
Osaka, Japan, 3 - 7 July, 2013

Estimation of Removed Uremic Toxin Indoxyl Sulphate during Hemodialysis by Using Optical Data of the Spent Dialysate

Jana Holmar*, Fredrik Uhlin, Rain Ferenets*, Kai Lauri, Risto Tanner, Jürgen Arund, Merike Luman, and Ivo Fridolin*, *Member, IEEE*

Abstract— **The aim of this study was to explore the possibility to determine the amount of total removed Indoxyl Sulphate (TR_IS) during dialysis session, an optical method utilizing absorbance and fluorescence spectral data of the spent dialysate was used. Eight uremic patients from Linköping, Sweden and 10 from Tallinn, Estonia, were studied during dialysis treatments. Dialysate samples were taken during each treatment and analyzed at a laboratory. Fluorescence and absorbance spectra of the spent dialysate were measured with spectrofluorophotometer and spectrophotometer. The spectral values were transformed into IS concentration using multiple linear regression model from the total material noted as optical method (Opt). IS concentration was estimated using high-performance liquid chromatography (HPLC) method as a reference. TR_IS values were calculated. Achieved results were compared regarding mean values and SD and collated with the amount of total removed urea value (TR_Urea) for the same dialysis procedures. Mean TR value±SD (mg) for urea was 28 947±9 241; TR for IS was 151.4±87.3 estimated by HPLC and 149.4±84.9 estimated by Opt. The TR_IS values were not significantly different ($p \leq 0.05$). This study indicates, that it is possible to estimate TR_IS using only spectral values of the spent dialysate and the parameter can be used for quantifying the elimination of protein bound uremic toxins during the dialysis procedure.**

I. Introduction

Uremic toxins can be divided in three large groups: small, water-soluble, not protein bound solutes (molecular weight (MW) < 300D), middle molecules (300<MW<12000 D) and protein bound molecules. Indoxyl Sulphate (IS) (MW 251 D) belongs to protein bound uremic toxins. IS is metabolized by the liver from indole which is produced by the intestinal flora as a metabolite of tryptophan [1, 2]. It has found that serum IS level is markedly higher in chronic kidney disease patients and it accelerates the progression of chronic kidney disease (CKD) [3]. Moreover, studies suggest that IS increases oxygen consumption and aggravates local hypoxia in renal tubular cells and can lead to end stage renal disease (ERSD) [4]. It has discovered that IS may induce oxidative stress, dysfunction of endothelium and affect endothelial wound repair, those conditions may cause cardiovascular disease and higher mortality in CKD patients [5-8]. The present concept of dialysis as the main treatment in case of ERSD, focuses mainly on the removal of small water-soluble compounds [2]. Also the classical ways to estimate dialysis adequacy is based on small water-soluble compounds as urea and creatinine [9]. Monitoring the amount of removed protein bound molecules during dialysis is important and informative. However, due to specific kinetic behavior of some protein bound molecules in the body (including IS), the removal estimation on the basis of blood samples could be misleading [10]. Earlier studies by HPLC have shown that IS can be observed by fluorescence measurements in the plasma as well as in ultrafiltrate [11, 12]. It has been demonstrated that concentration and removal rate (RR) of different uremic solutes can be estimated with optical methods which are using either ultraviolet absorbance [13-15] or fluorescence [16-18] of the spent dialysate. The aim of the study was to enhance current knowledge about using spent dialysate fluorescence and absorbance data to quantify total removed IS (TR_IS) during dialysis.

II. Materials and Methods

The studies were performed after approval of the protocol by the Regional Ethical Review Board, Linköping, Sweden and by the Tallinn Medical Research Ethics Committee at the National Institute for Health Development, Estonia. Informed consent was obtained from all participating patients.

10 patients from Tallinn (6 male, 4 female, 58±8 years) and 8 patients from Linköping (7 male, 1 female, 77±7 years) were included to the study. Patients were followed in total 78 sessions from which 33 were hemodialysis (HD) and 45 hemodiafiltration (HDF) sessions. The dialysis machine used was a Fresenius 5008 (Fresenius Medical Care, Germany). The dialyzers used were FX10, FX80 and FX800 (Fresenius Medical Care, Germany). The duration of the treatments varied between 180 to 270 minutes, the dialysate flow was 500 ml/min and the blood flow varied between 250-350 ml/min.

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During dialysis sessions, the following samples from the drain tube of the dialysis machine were taken: 9-25, 60, 120 minutes after the start of the session and at the end of the session (180-270 min.). After the end of the procedure, dialysate collection tank was weighed and one sample (Tank) was taken from it after careful stirring. Linköping's samples were freezed and transported to Tallinn.

Concentration of IS was determined in Tallinn University of Technology utilizing the HPLC instrument UltiMate 3000 (Dionex Corporation). Concentrations of urea were determined in Clinical Chemistry Laboratories in Sweden and Tallinn using standardized methods (ADVIA Urea Nitrogen (UN) method, enzymatic).

Spectrofluorophotometer (SHIMADZU RF-5301, Japan) was used for the fluorescence measurements. Fluorescence analysis was performed over an excitation (EX) wavelength range of 220 - 500 nm (excitation increment 10 nm) and emission (EM) wavelength range of 220-800 nm. The measurement cell with an optical path length of 4 mm was used (Fig. 1).

Ultra violet (UV) absorbance was measured with UV-VIS-NIR spectrophotometers (V-570, JASCO Corp., Japan-used in Sweden and UV-3600, SHIMADZU, Japan – used in Tallinn), 10 mm optical cell was used (Fig. 2). Measurements were performed at the room temperature (ca. 22^{o} C). The obtained spectral values were processed and presented by software Panorama fluorescence and UV Probe, the final data processing was performed in (Microsoft Office Excel 2003).

Linear correlation coefficients (R) were determined on the basis of the fluorescence/absorbance spectral values and IS concentration values using MATLAB (MATLAB 7.0, MathWorks, US) and EXCEL. The best wavelengths for estimating the concentration of IS were found and multiple linear regression model utilizing information from those wavelengths was created using Statistica 9.0 (Statsoft Inc., US). The obtained model was in following form:

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

where a and b are the coefficients, F(λ) is fluorescence intensity value at certain EX/EM wavelength and A(λ) is UV absorbance value at certain wavelength.

The total removed amount of a substance was calculated as follows:

$$TR = C_t * W_t \qquad (2)$$

where C_t is the substance concentration in total dialysate collection tank (mg/l) and W_t is the weight of the dialysate collection tank (kg). It was assumed that 1 kg = 1 liter of the dialysate. For determination of the TR_IS from the optical method (Opt), concentrations calculated from the spectral values were utilized.

For determining differences between TR values from HPLC and optical method Student t-test ($p<0.05$ was considered significant) and Bland-Altman analysis was used.

III. Results

In the Fig. 1 fluorescence spectrum of the spent dialysate in the beginning and at the end of the procedure is given, corresponding absorbance spectra's are shown in Fig. 2.

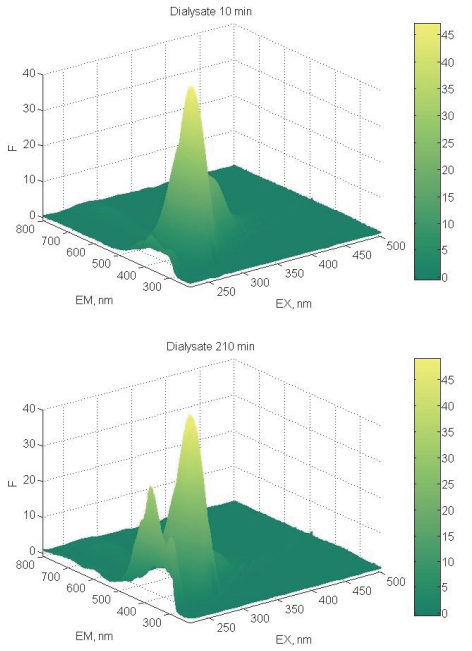


Figure 1. Examples of fluorescence spectra's of spent dialysate, taken at the start and at the end of the dialysis procedure. EX=220-500nm, EM=220-800nm.

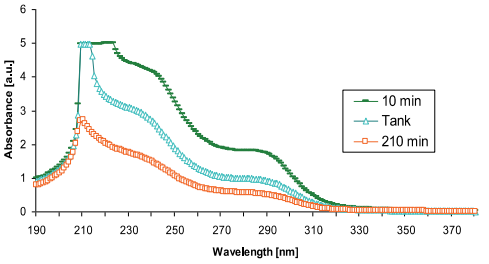


Figure 2. Examples of absorbance spectra's of spent dialysate samples taken at the start and at the end of the dialysis procedure and from dialysate collection tank over a wavelengt range 190-380 nm.

The multiple linear regression model for estimating IS concentration optically (IS_Opt) was created. The goodness of fit of the model is given in the Fig. 3. and mean concentration values of urea and IS estimated with different methods are presented in the Fig. 4.

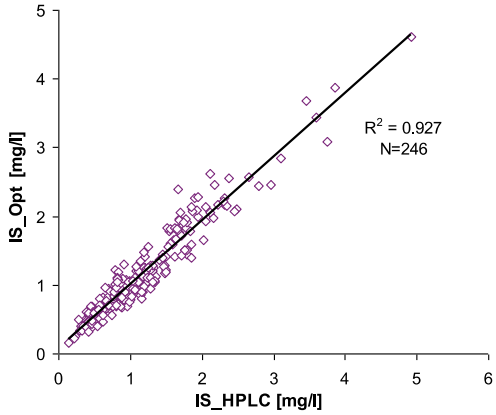
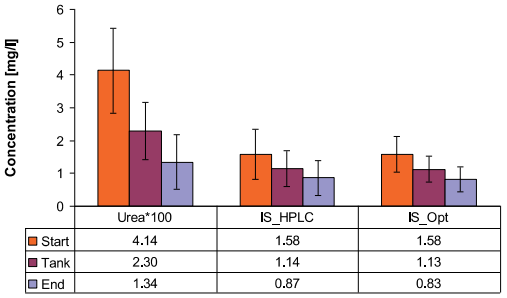


Figure 3. Goodness of fit of the multiple linear regression model for estimating IS concentration.



	Urea*100	IS_HPLC	IS_Opt
Start	4.14	1.58	1.58
Tank	2.30	1.14	1.13
End	1.34	0.87	0.83

Figure 4. Urea and IS concentration values.

The concentration values from the tank were used for calculating the TR value for urea and IS (both HPLC and Opt). The results are presented in the Fig. 5.

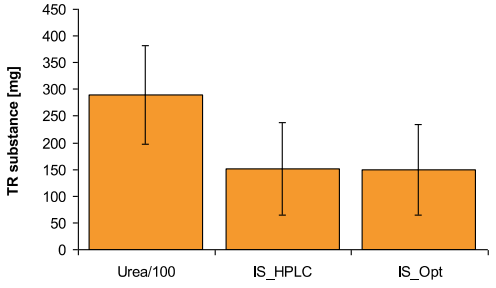


Figure 5. Amount of total removed urea and IS measured with the different methods.

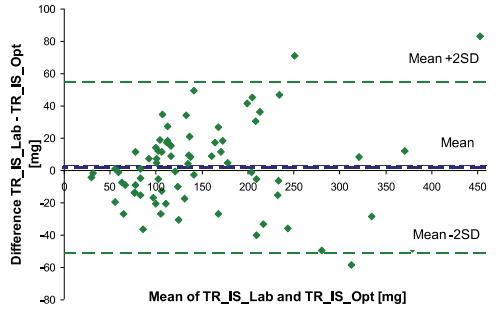


Figure 6. Bland-Altman plot. The difference between TR_IS_HPLC and TR_IS_Opt is plotted against the mean value of TR_IS_HPLC and TR_IS_Opt, (N=74).

Fig. 6 presents the individual differences between TR_IS values estimated by HPLC (reference method) and by the new optical method (Opt), in a Bland-Altman plot.

Neither concentrations nor TR values of IS_Opt were significantly different ($p<0.05$) from the values of the reference method (HPLC).

Table 1 shows a summary of the results regarding the TR values of IS in mean and standard deviation values from the standardized methods (IS_HPLC) and new optical method (IS_Opt), and correlation analysis between two methods.

TABLE I. TOTAL REMOVED IS ESTIMATED BY DIFFERENT METHODS (N=74)

	TR_IS ± SD[mg]	*R*	*R²*
HPLC	151.4±87.3	0.95	0.91
Opt	149.4±84.9		

IV. DISCUSSION

From the Fig. 1 and Fig. 2, some distinctive fluorescence and absorbance maxima at specific regions are clearly seen. The amplitude of the spectra's are proportional to the content of eliminated uremic retention solutes in the spent dialysate being higher/lower in the beginning of the dialysis treatment (10 min) and lower/higher at the end of the dialysis (210 min) at specific regions.

According to the HPLC studies on the heat-deproteinized uremic serum and uremic ultrafiltrate, IS has a prevalent fluorescence compared to other uremic retention solutes [19]. Therefore using fluorescence data in optical model is well justified. It has been confirmed by the HPLC studies of the spent dialysate that UV technique solely is not suitable for monitoring the removal of IS [20]. Although small, IS has specific absorbance spectra in UV region and absorbance values from certain wavelengths adds specificity to the optical model created in this study.

A classical way to estimate dialysis adequacy is to estimate urea reduction ratio (URR) in % [9]. Urea describes removal of small molecules [21] and is not representative for

removal of other molecules such as the protein-bound or the middle molecules groups of uremic toxins [10]. Therefore, describing the elimination of protein bound molecules by estimating the TR_IS optically from the spent dialysate would be beneficial. Moreover, due to specific kinetic behavior of some protein bound molecules in the body, including IS, estimating the TR value on the basis of blood samples, could be misleading [10].

As seen from the Fig. 3, 4 and 5, concentration and removed amount of IS can be estimated optically. From figure 5 it is seen that TR values for urea and IS are rather different. It seems to confirm that a specific parameter is needed for estimation of removed protein bound compounds. On the other hand, there are no guidelines developed yet, how much IS or other protein bound molecules should be eliminated in relation to urea, and perhaps this new method could be useful in this development.

It can be seen from the Fig. 6 that TR values from HPLC are somewhat different compared to TR IS_Opt but the difference is not statistically significant ($p \leq 0.05$).

As seen from the Table 1 estimation of removed IS can be done by using optical information of the spent dialysate.

The clinical aim in the future is to improve an on-line monitoring system including simultaneous monitoring of the removal of markers for different clinically important groups of uremic toxins during hemodialysis. The present technical approach may help to confirm the previous knowledge and broaden the coming information about the uremic toxin, IS, removal during dialysis and a positive impact to the patients according to needs in chronic renal failure therapy [22]. The optical technique for measuring concentration and removal of different uremic toxins may give a useful, rapid and cost-effective tool for clinicians to estimate the effectiveness of dialysis procedure.

V. Conclusion

This study examined whether absorbance and fluorescence spectral data can be used for determination of the amount of removed IS during the dialysis and whether this method could be used for description of removal of IS as a marker of protein bound uremic toxins. It was found that estimation of removal of IS during the dialysis can be done by applying the optical approach developed in this study.

New clinical trials giving access to a larger amount of data of the spent dialysate for creating more specific algorithms will be issue of the next studies.

Acknowledgment

The authors wish to thank all dialysis patients participated in the experiments and clinical- and technical assistance personnel.

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APPENDIX 2

ELULOOKIRJELDUS

1. Isikuandmed

Ees- ja perekonnanimi	Jürgen Arund
Sünniaeg ja -koht	11.07.1984, Põlva, Eesti
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Aadress	Redise 9-15, Laagri, Harjumaa, Eesti
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3. Hariduskäik

Õppeasutus (nimetus lõpetamise ajal)	Lõpetamise aeg	Haridus (eriala/kraad)
Tallinna Tehnikaülikool	2008	Materjalitehnoloogia, bakalaureusekraad
Tallinna Tehnikaülikool	2010	Biomeditsiinitehnoloogia, magistrikraad

4. Keelteoskus (alg-, kesk- või kõrgtase)

Keel	Tase
Eesti	Emakeel, kõrgtase
Inglise	Kõrgtase
Vene	Algtase

5. Täiendusõpe

Õppimise aeg	Täiendusõppe läbiviija nimetus
11.2011-04.2012	2 Rahvusvahelise doktorikooli iBioMEP täiendkursust

6. Teenistuskäik

Töötamise aeg	Tööandja nimetus	Ametikoht
2007-2010	Tallinna Tehinkaülikool	Tehnik
2010-k.a.	Tallinna Tehinkaülikool	Teadur

7. Teadustegevus

Ureemiliste toksiinide ja kardiovaskulaarsete markerite uurimine ning nende dialüüsiravi käigus eemaldamise hindamine reaalajas optiliste meetoditega.

8. Teadustöö põhisuunad

2014- 2019	Biooptilised ja bioelektrilised signaalid meditsiinitehnikas	IUT19-2
2008-2015	Integreeritud elektroonikaüsteemide ja biomeditsiinitehnika tippkeskus – CEBE	TAR8077DB
2011-2014	Uudne optiline meetod ureemiliste toksiinide - alatoitumuse ja kroonilise põletiku ning SVH riski potentsiaalsete markerite, monitooringuks	ETF8621
2007-2010	Uudne optiline multikomponent monitor neerupuudulikkusega patsientide ravi kvaliteedi hindamiseks	ETF6936
2007-2012	Biosignaalide interpreteerimine meditsiinitehnikas	SF0140027s07

Juhendatud lõputööd

I. Elena Vaarmets, magistrikraad, 2012, (juh) Risto Tanner; **Jürgen Arund**, Dialüsaadi vedelikkromatograafilise analüüsi metoodika optimeerimine seoses optilise monitori väljatöötamisega neeruasendusravi jälgimiseks, *Tallinna Tehnikaülikool, TTÜ Tehnomeedikum, Biomeditsiinitehnika Insituut*.

II. Ürgo Saaliste, magistrikraad, 2013, (juh) **Jürgen Arund**, Pöörd-faas kõrgsurve vedelikkromatograafia kromatogrammi baasjoone eemaldamise meetodite võrdlus diood-rivi detektsiooniga saadud kromatogrammide puhul metaboloomikas, *Tallinna Tehnikaülikool, TTÜ Tehnomeedikum, Biomeditsiinitehnika Insituut*.

III. Mari-Ann Pajusoo, magistrikraad, 2013, (juh) **Jürgen Arund**, Peamiste kromofoorsete piikide puhtuse hindamine 2D-kromatograafilise meetodiga

kulunud dialüsaadi proovides, *Tallinna Tehnikaülikool, TTÜ Tehnomeedikum, Biomeditsiinitehnika Insituut*.

IV. Sandra Viira, magistrikraad (teaduskraad), 2016, (juh) **Jürgen Arund**, Proovi ettevalmistamise metoodika optimeerimine ureemilise toksiini indoksüülsulfaadi määramiseks vereseerumis, *Tallinna Tehnikaülikool, TTÜ Tehnomeedikum, Biomeditsiinitehnika Insituut*.

APPENDIX 3

CURRICULUM VITAE

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3. Education

Educational institution	Graduation year	Education (field of study/degree)
Tallinn University of Technology	2008	Technology of Materials, Bachelor degree
Tallinn University of Technology	2010	Biomedical technology, Master degree

4. Language competence/skills (fluent; average, basic skills)

Language	Level
Estonian	fluent, mother-tongue
English	fluent
Russian	basic skills

5. Special Courses

Period	Educational or other organization
11.2011-04.2012	2 graduate courses in International Doctoral Programme – iBioMEP

6. Professional Employment

Period	Organization	Position
2007-2010	Tallinn University of Technology	Technician
2010 -	Tallinn University of Technology	Research scientist

7. Scientific work

Research of uremic toxins and cardiovascular markers and optical on-line estimation of their elimination during dialysis

8. Main areas of scientific work/Current research topics

2014- 2019	Biooptical and bioelectrical signals in Biomedical Engineering	IUT19-2
2008-2015	Centre for Integrated Electronic Systems and Biomedical Engineering – CEBE, Estonian centre of excellence in research	TAR8077DB
2011-2014	A novel optical technology for monitoring of uremic toxins - potential markers for malnutrition–inflammation syndrome and CVD risk	ETF8621
2007-2010	A novel optical multicomponent monitor estimating ESRD patients' treatment quality	ETF6936
2007-2012	Interpretation of Biosignals in Biomedical Engineering	SF0140027s07

Theses supervised

I. Elena Vaarmets, Master's Degree, 2012, (sup) Risto Tanner; **Jürgen Arund**, Optimization of liquid chromatographic method for analysis of spent dialysate in connection with development of optical dialysis adequacy monitor, *Tallinn University of Technology, Technomedicum of TUT, Department of Biomedical Engineering, Chair of Biomedical Engineering*.

II. Ürgo Saaliste, Master's Degree, 2013, (sup) **Jürgen Arund**, Comparison of baseline subtraction methods for reversed-phase high-performance liquid chromatography chromatograms acquired by diode array detection for use in

metabolomics, *Tallinn University of Technology, Technomedicum of TUT, Department of Biomedical Engineering*.

III. Mari-Ann Pajusoo, Master's Degree, 2013, (sup) **Jürgen Arund**, Estimation of purity of main chromophoric peaks in spent dialysate sample chromatograms by two-dimensional chromatographic method, *Tallinn University of Technology, Technomedicum of TUT, Department of Biomedical Engineering*.

IV. Sandra Viira, Master's Degree, 2016, (sup) **Jürgen Arund**, Optimization of a method for sample preparation to assess the uremic toxin of indoxyl sulfate in blood serum, *Tallinn University of Technology, Technomedicum of TUT, Department of Biomedical Engineering*.

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