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Optical Monitoring of Uremic Metabolites-Fluorophores during Dialysis: the Cases of β_2-microglobulin, Pentosidine, and 4-Pyridoxic Acid

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

Sigrid Kalle

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signature

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Ureemiliste metaboliitide-fluorofooride optiline jälgimine dialüüsi jooksul: β_2-mikroglobuliini, pentosidiini ja 4-püridokshappe näited

SIGRID KALLE

TTÜ KIRJASTUS

Contents

List of Publications

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

I **Kalle, S.,** Tanner, R., Arund, J., Tomson, R., Luman, M., Fridolin, I. (2016). 4-Pyridoxic Acid in the Spent Dialysate: Contribution to Fluorescence and Optical Monitoring, *PLOS ONE*, 11(9) (DOI: 10.1371/journal.pone.0162346)

II **Kalle, S.**, Tanner, R., Arund, J., Tomson, R., Luman, M., Fridolin, I. (2016). Optical Measurement of 4-Pyridoxic Acid in the Spent Dialysate: Algorithm Development, In: *15th Biennial Baltic Electronics Conference*, Laulasmaa, Estonia, October 3–5, 2016, IEEE Proceedings, 115–118 (DOI: 10.1109/BEC.2016.7743742)

III **Kalle, S.**, Tanner, R., Luman, M., Fridolin, I. (2018). Free pentosidine assessment based on fluorescence measurements in spent dialysate, *Blood Purification* (DOI: 10.1159/000493522)

IV **Kalle, S.**, Kressa, H., Tanner, R., Holmar, J., Fridolin, I. (2014). Fluorescence of β_2-microglobulin in the Spent Dialysate, In: *16th Nordic-Baltic Conference on Biomedical Engineering,* Gothenburg, Sweden, October 14–16, 2014, IFMBE Proceedings, 48: 59–62 (DOI: 10.1007/978-3-319-12967-9_16)

Author's Contribution to the Publications

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

I Major role in manuscript writing, participation in sample collection, part of the chromatography and spectrofluorophotometry analysis, chromatography and fluorescence data processing and analysis.

II Major role in manuscript writing, participation in sample collection, part of the chromatography and spectrofluorophotometry analysis, chromatography and fluorescence data processing and analysis.

III Major role in manuscript writing, participation in sample collection, part of the chromatography and spectrofluorophotometry analysis, part of chromatography and fluorescence data processing and analysis.

IV Major role in manuscript writing, part of the chromatography analysis, part of chromatography data processing and analysis

Other related publications

1. **Kalle, S.,** Kressa, H., Tanner, R., Holmar, J., Fridolin, I. (2014). Long wavelength fluorescence of the spent dialysate, In: *12th Conference of Baltic Societies of Nephrology*, Dubingiai, Lithuania, June 12–14, 2014.
2. **Kalle, S**., Kressa, H., Tanner, R., Holmar, J., Fridolin, I. (2014). Fluorescent compounds in spent dialysate, In: *5th meeting „Uremic toxins and Cardiovascular Disease"*, Prague, Czech Republic, June 27–29, 2014.
3. Holmar, J., Luman, M., Arund, J., Lauri, K., Tomson, R., Tanner, R., **Kalle, S**., Fridolin, I. (2016). Reduction of Urea and Indoxyl Sulphate Concentration during Different Dialysis Treatment Modalities. In: *International Journal of Artificial Organs: XLIII Annual Congress of the European Society for Artificial Organs*, Warsaw, Poland, September 14–17, 2016, Wichtig Editore.
4. **Kalle, S.,** Tanner, R., Arund, J., Tomson, R., Luman, M., Fridolin, I. (2016). Could urea in spent dialysate be a marker for monitoring removal of 4-Pyridoxic acid? In: *13th Conference of Baltic Nephrology Conference*, Jurmala, Latvia, October 13–15, 2016, Latvian Association of Nephrology.
5. **Kalle, S**., Tanner, R., Fridolin, I., Luman, M. (2017). Association between skin autofluorescence and fluorescence of spent dialysate. In: *54th ERA-EDTA Congress*, Madrid, Spain, June 3–6, 2017. NDT, 32 (S3): Oxford University Press.
6. Holmar, J., Arund, J., **Kalle, S**., Lauri, K., Luman, M., Tanner, R., Tomson, R., Fridolin, I. (2017). Removal Estimation of Uremic CVD Marker Phosphate in Dialysis Using Spectrophoto-and Fluorimetrical Signals. In: *IFMBE proceedings of the joint conference of the EMBEC and NBC 2017: Joint conference of the EMBEC and NBC*, Tampere, Finland, June 11–15, 2017. Ed. Eskola, H., Väisänen, O., Viik, J., Hyttinen, J. Germany: Springer, 358−361.

Introduction

Chronic kidney disease (CKD) has a substantial global health burden due to its excessive prevalence. In 2013 an estimated one million deaths worldwide were attributable to CKD (Mortality and Causes of Death Collaborators, 2015). In USA, the primary causes of CKD and kidney failure are diabetes (Levey et al., 2012; Saran et al., 2017) and hypertension (Saran et al., 2017). However, in developing countries, the main cause cannot be pinpointed and other effects attribute to the disease (Couser et al., 2011).

The patient may develop kidney (renal) failure, which is most commonly described as loss of kidney function to eliminate waste (Schrier et al., 2004). Thus, a great amount of uremic solutes accumulate in the blood of the patient (Duranton et al., 2012); among these, over one hundred solutes have biologically adverse effects (Glassock, 2008; Vanholder et al., 2008b). Kidney replacement therapy, e.g. dialysis, is applied to remove the accumulated solutes from the blood of the patient. Various marker solutes are used to estimate the adequacy of dialysis therapy. Urea is the most widely used marker substance, as it is the major end-product of protein metabolism (Bankir et al., 1996; Depner, 2001). Blood samples must be taken at the start and the end of the treatment session to assess the adequacy of dialysis procedure. Simple and cost-effective on-line monitoring of the UV-absorption of spent dialysate is used to estimate the removal of uric acid, the main chromophore in spent dialysate, and calculate the conformity of the treatment with medical requirements. However, the fluorescence of spent dialysate appears to offer much more sensitive and selective possibilities for assessing the elimination of some specific and clinically considerable toxins, e.g. indoxyl sulphate from the blood of patients (Arund et al., 2016).

Methods and formulas utilising urea as a marker substance characterise the behaviour of only one class of solutes (Meyer et al., 2011; Vanholder et al., 2018). Many research have demonstrated that urea cannot be used alone, as the kinetic behaviour of urea is different from other molecules (Eloot et al., 2005; De Smet et al., 2007; Vanholder et al., 2008a). Therefore, a traditional single marker like urea is not sufficient for evaluating the dialysis quality (Gotch et al., 1985, 2000; Schoots et al., 1988; Vanholder et al., 1992, 2015; Meyer et al., 2011; Eloot et al., 2012).

Thus, the goal of this doctoral thesis was to develop methods for the assessment of specific metabolites in spent dialysate and to develop algorithms that utilise the fluorescence of spent dialysate to estimate the removal behaviour of the selected uremic solutes. Three clinically essential uremic toxins with known fluorescent properties were selected: AGE-modified β_2-microglobulin (β_2M), pentosidine, and 4-pyridoxic acid (4PA). β_2-microglobulin was selected as a marker of middle size molecules in dialysate and conditional fluorophore (Miyata et al., 1993b; Vanholder et al., 2008a), pentosidine as a recognized fluorescent product of the glycation of proteins (Sell et al., 1989) and 4PA as a fluorescent metabolite of vitamin B6 routinely

used in connection with dialysis therapy (Allman et al., 1989; Descombes et al., 1993, 2000).

The current study provides important evidence on how spent dialysate besides plasma and urine could be used to assess the removal of 4PA **(Publication I)**. The study showed that 4PA is a major fluorescence- contributing peak in the HPLC chromatogram of the spent dialysate. A high correlation between the fluorescence intensity of spent dialysate and the concentration of 4PA in spent dialysate was found. The main contribution of the current study is the developed algorithms (based on the results in **Publication I**) that utilise the fluorescence of spent dialysate to estimate the removal behaviour of the selected uremic solutes (**Publication II**). The dynamics of the removal of specific uremic toxins is described based on the data from the samples collected at the different time points of the process. Two single wavelength models were found to be feasible for the estimation of the concentration of 4PA in spent dialysate through fluorescence measurements (**Publication II**).

In **Publication III**, the marker substance pentosidine was investigated for the estimation of removal of advanced glycation end products (AGEs). Single- and multiwavelength models were developed. It was concluded that the fluorescence multiwavelength model improves the estimation accuracy of the free pentosidine concentration and the estimation of removal ratio (RR) in comparison with the single wavelength model. This preliminary study shows that the optical method for on-line measurements of the concentration of free pentosidine in spent dialysate is feasible but needs further research. This thesis has opened new research perspectives in the field of advanced glycation end product research, especially focused on the optical method for the on-line measurements of the concentration of free pentosidine in spent dialysate (**Publication III**).

It is suggested that β_2M could be used as a marker substance to describe the removal of middle molecular weight solutes (MM) (Vanholder et al., 2008a). This study increased understanding of the possibilities of the fluorescence chromatography to be used in order to measure AGE-modified β2M from the spent dialysate (**Publication IV**). It was found that the contribution of the fluorescence of AGE-modified β_2M in this work was only 0.04% of the overall fluorescence of the spent dialysate at a specific wavelength. Consequently, the usage of the fluorescence of the spent dialysate at the specific wavelength for the assessment of AGE-modified β_2M as the MM marker may be possible only in the case of dialysis patients with severe complications of amyloidosis.

Abbreviations

4PA	4-pyridoxic acid
AGE	advanced glycation end product
β_2M	β_2-microglobulin
B6	vitamin B6
CKD	chronic kidney disease
Da	Dalton, unit for molecular weight
ELISA	enzyme-linked immunosorbent assay
EM	emission
ESI	electrospray ionization
ESRD	end-stage renal disease
EUTox-db	European Uremic Solutes Database
EX	excitation
GFR	glomerular filtration rate
HD	haemodialysis
HDF	haemodiafiltration
HF	haemofiltration
HFBA	heptafluorobutyric acid
HPLC	high performance liquid chromatography
Kt/V	dialysis dose efficacy parameter
MeOH	methanol
MS	mass spectrometry
MW	molecular weight
PD	peritoneal dialysis
PLP	pyridoxal-5'-phosphate
RC	relative contribution
RP	reverse-phase
RR	reduction ratio
SAF	skin autofluorescence
SPE	solid phase extraction
TFA	trifluoroacetic acid
UA	uric acid
UV	ultraviolet light
V	volume, mL

1 The kidney, kidney failure, and dialysis

1.1 Kidneys and kidney failure

Kidneys are bean-shaped paired organs that belong to the urinary system. The main function of kidneys is to excrete the metabolic wastes and other unnecessary compounds in urine. They also regulate blood pressure, sodium (Palmer et al., 2014), body water volume (Danziger et al., 2014), potassium (Subramanya et al., 2014), acid-base balance (Curthoys et al., 2014), and produce hormones.

It is estimated that 1 in every 20 people has chronic kidney disease (CKD) (Teerawattananon et al., 2016). Diabetes (Levey et al., 2012; Saran et al., 2017) and hypertension (Saran et al., 2017) are the primary causes of CKD and kidney failure in developed countries. Patients with diabetes have 3.77 times higher risk of developing end stage renal disease (ESRD) (Rebholz et al., 2015). Additionally, a higher prevalence of ESRD among whites and African Americans correlates with neighbourhood poverty (Volkova et al., 2008).

Kidney (renal) failure is described as a loss of kidney function to eliminate waste, concentrate urine, and maintain fluid balance (Schrier et al., 2004). Depending how kidney failure occurs, it is characterised as acute or chronic. In acute kidney injury (or acute renal failure), the loss of kidney's excretory function happens rapidly (Bellomo et al., 2012). Varied conditions affect the kidney function and structure in CKD. In CKD, the glomerular filtration rate (GFR) has decreased less than 60 mL/min for 3 months or more (National Kidney Foundation, 2002; Vassalotti et al., 2007; Stevens et al., 2009). Chronic kidney disease is classified into five stages according to disease severity, where stage 5 is end-stage renal disease (ESRD) (Couser et al., 2011; Levey et al., 2012). When the kidney function has reached stage 5, kidney replacement therapy is needed.

1.2 Kidney replacement therapy

Kidney replacement therapy is divided into three different possibilities: transplantation, haemodialysis (HD), and peritoneal dialysis (PD). Over 2 million people worldwide receive renal replacement therapy but it is estimated that this represents only 10% who need it (Couser et al., 2011).

Even though the survival of transplant recipients is higher, dialysis is commonly used therapy (Couser et al., 2011). Dialysis is regularly conducted procedure that removes excess water and solutes through a semipermeable membrane. Peritoneal dialysis uses the patient's own peritoneum as a membrane and HD uses artificial membrane. During HD treatment the blood of the patient goes to the dialyser machine where the blood flows in the fibres consisting of semipermeable membrane. Dialysate fluid, which does not contain any waste products, flows on the other side of the membrane. Solutes transfer to the dialysate due to diffusion and/or convection.

Filtered blood goes back to the patient and dialysate fluid is directed to the drain. The simplified principle of haemodialysis is shown in Figure 1.

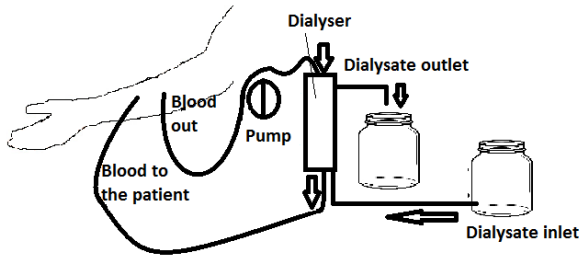

Figure 1. *Simplified principle of haemodialysis.*

2 The uremic solutes and advanced glycation end products

Great amount of uremic solutes accumulate in the blood of the patient due to kidney failure (Duranton et al., 2012). At least 278 uremic solutes have been identified from plasma and plasma ultrafiltrate (Tanaka et al., 2015) and 130 solutes have been added to the European Uremic Solutes Database (EUTox-db) (European Work Group on Uremic toxins) on the basis of convincing criteria confirming their biological adverse effects (Glassock, 2008; Vanholder et al., 2008b) as potential candidates for uremic toxins. A useful and widely accepted categorisation of uremic toxins is based on molecular weight and plasma protein-binding characteristics (Vanholder et al., 2003). In this scheme, uremic toxins are divided into four nonoverlapping categories (Figure 2).

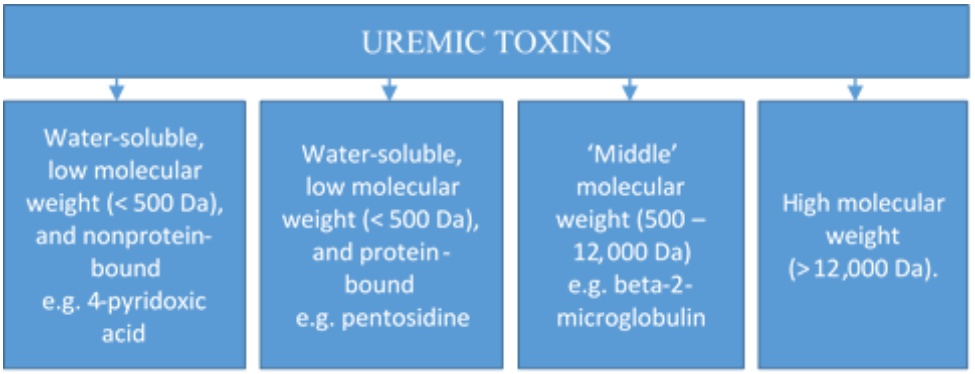

***Figure 2**. Uremic toxin division based on Vanholder et al. (2003).*

2.1 Small free water-soluble uremic solutes

Solutes (MW < 500 Da) of this group are usually easily removed with standard dialysis treatment (Vanholder et al., 2008b; Yamamoto et al., 2016). Exemplary substances in this group include creatinine (MW = 113 Da), asymmetric dimethylarginine (MW = 202 Da), uric acid (UA, MW = 168 Da), xanthine (MW = 152 Da), and urea (MW = 60 Da) (Dhondt et al., 2000). The most studied compound in this group is urea (Yamamoto et al., 2016), which is considered an inert molecule (Vanholder et al., 2015); yet, new studies indicate that urea has toxicity in a clinical setting (Vanholder et al., 2018). Creatinine was found to be responsible for cardiovascular side effects (Weisensee et al., 1993).

Small free water-soluble uremic solutes (e.g., urea, uric acid, and creatinine) have a quick redistribution between cells and body compartments and are thus easily removed with renal replacement therapy. The removal of small free water-soluble uremic toxins depends on treatment time and frequency and the flow rate of blood and dialysate (Basile et al., 2011). Next to conventional HD, daily short haemodialysis, which has six 2-hour dialysis session per week, has shown higher removal ratio (Fagugli et al., 2002). However, treating patients with six-times weekly dialysis instead of three-times weekly dialysis will only cause a small increase in the reduction ratio in different uremic solutes

(Sirich et al., 2018). Haemodiafiltration (HDF) and haemofiltration (HF) are more effective in removing uremic solutes than HD due to an increase of convection. It was found that post-dilution HDF has a better effect on removing small molecules than pre-dilution HDF and HF (Meert et al., 2009).

2.1.1 Vitamin B6 and 4-pyridoxic acid

Vitamin B6 is not an uremic solute by itself but it's catabolite, 4PA, can be recognised as an uremic solute (Tanaka et al., 2015). The modern technology of treatment of uremic patients cannot distinguish solutes depending on their biological toxicity. Various nontoxic solutes, including metabolites vitally needed for functioning of organism, are removed together with toxins (Leblanc et al., 2000; Clase et al., 2013). This restricts acceptable effectiveness and duration of haemodialysis treatment and complicates monitoring of the removal of toxins. Physical principles like conductivity, optical properties etc. readily available for the control of the process cannot distinguish removed solutes depending on their biological toxicity similarly to the dialysis membrane. Therefore, the specific properties of all solutes, including drugs supplemented during the treatment, must be considered for any physical principle planned for monitoring the haemodialysis process. Concerning the fluorescence as the principle of the monitoring, the intensive fluorescence of 4-pyridoxic acid, the main catabolic metabolite of vitamin B6, appears to need attention.

Vitamin B6 (B6) is a term used for a group of interconvertible molecules that contain pyridoxamine, pyridoxal, pyridoxine, and their phosphates (Ueland et al., 2015). It is well known that vitamin deficiency in CKD patients (Corken et al., 2011; Santos, 2015; Obi et al., 2016; Park et al., 2016) and in diabetes patients is common (Iwakawa et al., 2016). Vitamin B6 is known as a necessary cofactor for more than 100 enzymatic reactions mainly in protein and amino acid as well as in energy metabolism (Stover et al., 2015). It is often administered to patients receiving dialysis (Allman et al., 1989; Descombes et al., 1993, 2000). According to literature, the suggested B6 dose range is 10–50 mg/day (Kopple et al., 1981; Descombes et al., 1993; Kasama et al., 1996; Rocco et al., 1997), whereas the Dialysis Outcomes Quality Initiative (DOQI) recommends 10 mg/day (Fouque et al., 2007). Larger doses may potentially cause pyridoxine toxicity (Levine et al., 2002) and neuropathy (Parry et al., 1985). In renal failure, the toxic level of pyridoxine is not known; however, studies have shown that neuropathy could develop after a dose of 200 mg/day (Parry et al., 1985) or even at less than 100 mg/day (Dalton et al., 1987). However, the validity of the latter study is questionable (Institute of Medicine, 1998). After the procedure, a postdialysis supplement of at least 100 to 150 mg/week of pyridoxine hydrochloride (> 15 to 20 mg/day) is suggested to patients receiving regular dialysis treatment (Descombes et al., 1993). Therefore, there is a need for the assessment of the metabolism of B6 from the point of view of applying fluorescence in monitoring the dialysis process.

In B6 studies, pyridoxal-5'-phosphate (PLP) is used as a marker (Leklem, 1990; Percudani et al., 2003); however, the use of other compounds like 4-pyridoxic acid (4PA)

(Leklem, 1990) has also been proposed. Furthermore, plasma 4PA concentration is associated with plasma PLP levels in healthy subjects (Cabo et al., 2014).

4-pyridoxic acid (MW = 183 Da) is the major urinary degradation product of pyridoxal, which is the main active component of B6 (Kelsay et al., 1968; Vrolijk et al., 2017). In a healthy individual, approx. 40–60% of the ingested B6 is oxidised to 4PA (Robinson et al., 1995). It was found that plasma 4PA is sensitive to variations in kidney function (Bates et al., 1999). Therefore, 4PA could potentially be utilised to estimate B6 and overall nutritional status of the dialysis patient (Robinson et al., 1995) and the ratio of 4PA / (PL + PLP) (where PL – pyridoxal PLP – pyridoxal phosphate) could be used as a marker of inflammation and oxidative stress (Bird, 2018).

2.2 Protein-bound uremic toxins

According to the definition referred above, this group is classified as small, reasonably hydrophobic solutes, which in serum are prevalently bound to albumin. Elimination of these compounds by dialysis is strongly dependent on the affinity for albumin on these compounds, which can be characterised by molar proportion of free solute compared to total concentration in serum. Typical protein-bound uremic toxins are e.g. indole derivatives like indoxyl sulphate and indole acetic acid (Vanholder et al., 2001). Many non-toxic metabolites in serum may be protein-bound in the same way as scarcely water-soluble drugs, and therefore possible competition with uremic toxins for binding sites on albumin must be taken into account (e.g. furosemide (Forrey et al., 1974) or ibuprofen (Lockwood et al., 1983)) as quite high molar doses are also used in connection with dialysis therapy.

2.3 Advanced glycation end products (AGEs)

The reaction between sugars and amino acids was first described by Maillard in 1912 and molecular damage to tissue proteins mediated by the reaction have been described in connection with diabetes, aging, and uraemia (Monnier et al., 1992). This heterogenous group of molecules is formed through rearrangement, oxidation, cyclization, and dehydration of glycated proteins (Brownlee, 1994). Glycation is a reaction between an aldehyde group of a sugar and an amino group of a protein. Consequently, a Schiff base will be formed, which will undergo rearrangement and form ketoamines. Oxidative stress and autoxidation of glucose (Stinghen et al., 2016) and direct binding of carbonyl compounds can also create AGEs. *In vivo* studies in mice have shown that carbamylation competes with glycation for protein modification; however, this competitive effect has not been seen on AGE formation (Nicolas et al., 2018).

The general term of AGE defines a large variety of structures in modified proteins (Thomas et al., 2005). Over fifteen AGE-specific structures have been described in the literature. Some of these are well characterised by specific fluorescence like pentosidine, crossline (Obayashi et al., 1996), vesperlysine (Obayashi et al., 1996;

Rondeau et al., 2011), argpyrimidine (Oya et al., 1999); others are not fluorescent, such as carboxymethyllysine, hydroimidazolone (Niwa et al., 1997; Piperi et al., 2012; Arsov et al., 2013), and imidazolone (Niwa et al., 1997). Advanced glycation end-products are often considered to belong to a group of protein-bound uremic toxins due to their removal behaviour. Otherwise, no consensus can be found in earlier literature concerning discrete chemical substances marked by the term 'AGE'. It has been determined by immunosorbent assay that free solutes with a MW of 180–6,000 Da have typical fluorescence of AGEs and that free low molecular weight AGEs with a MW of 2,000–6,000 Da have strong chemical affinity towards collagen (Makita et al., 1994). Another study showed that the main circulating AGEs in serum have a characeristic MW of 1,500–2,000 Da measured by HPLC (Papanastasiou et al., 1994). Advanced glycation end-product specific compounds with a MW more than 30 kDa have also been classified as AGE-modified proteins to be distinguished from AGE peptides (MW 1–30 kDa) as incomplete proteolytic breakdown products of AGE-modified proteins (Deuther-Conrad et al., 2001). Advanced glycation adduct residues are classified into three categories (Thornalley, 2014):

1) glycation adduct residues (MW > 12 kDa);

2) glycation adduct residues of peptides (MW < 12 kDa);

3) glycation free adducts.

In this thesis, the term 'free AGEs' refers to glycated structures that remain intact after the complete enzymatic hydrolysis of proteins down to amino acids.

AGEs and AGE-modified protein fragments are found in collagen (Monnier et al., 1992), haemoglobin (Makita et al., 1992), β_2-microglobulin (Miyata et al., 1993a), lens critalline (Dunn et al., 1989), serum, and urine (Dolhofer-Bliesener et al., 1995; Miyata et al., 1998). The report by Makita et al. (1992) played an important role in introducing the potential effects of AGEs in uremic patients. It was found that AGEs induce different structural modifications, such as cross-linking, binding to a specific membrane receptors etc., which leads to kidney damage as well as damage to other organs and tissues (Busch et al., 2010; Cardenas-Leon et al., 2009; Goh et al., 2008; Stinghen et al., 2016). AGEs may play a role in cardiovascular complications (Yoshida et al., 1998), immune dysregulation (Friedlander et al., 1996a; Stinghen et al., 2016), and inflammatory responses (Sebeková et al., 2001) in uremia. Additionally, AGE accumulation in CKD patients may lead to arterial stiffness, myocardial changes, endothelial dysfunction (Stinghen et al., 2016), and chronic complications (Miyata et al., 1997). It was found that nondiabetic uremic patients who receive dialysis have higher AGE levels than patients with diabetes not receiving dialysis treatment (Monnier et al., 1992; Miyata et al., 1996). This may be due to glucose in the dialysate buffer (Ruth, 2007) and the decline in renal function. A haemodialysis procedure without glucose tends to decrease the levels of protein bound AGEs but not free AGEs (Ramsauer et al., 2017).

The presence of various AGEs are determined from serum regarding different modalities utilised in renal replacement therapies (Gerdemann et al., 2002). Currently

used dialysis methods are ineffective in clearing AGEs from serum (Ateshkadi et al., 1995; Friedlander et al., 1995). Studies have shown that HD decreases the level of AGEs with a MW below 10 kD in serum by 24% (Makita et al., 1991) and HF-HD is more efficient than conventional HD (Makita et al., 1994). To remove more protein-bound uremic solutes, it is recommended to increase the frequency of dialysis per week and/or to make it longer (Fagugli et al., 2002; Eloot et al., 2016) or get a larger dialyser or increase dialysate flow (Sirich et al., 2012). Despite the dialysis mode, AGE serum levels will return to the pre-treatment level within 3 hours (Makita et al., 1994).

The new concept of functional classification of AGEs was developed in connection with researches of pathophysiological mechanisms of toxicity of AGEs (Thornalley, 1998). It is based on the capacity of the interaction of AGE-modified proteins with the AGE-specific receptors (RAGEs) of cell membranes (Thornalley, 1998). Free AGEs are formed by the cellular proteolysis of glycated proteins, direct glycation of amino acids, and the digestion of glycated proteins in food. They have high renal clearance and are the major form in which glycation products are normally excreted from the body in urine and in dialysate in renal replacement therapy. Free AGEs may also contribute vascular disease in uraemia (Thornalley, 2005) and in diabetes (Guerin-Dubourg et al., 2017). The major effect of protein glycation in uraemia seems to be the loss of clearance of free AGEs and their marked increase in plasma (Thornalley et al., 2009). Thus, the assessment of removal of free AGEs by analysis of spent dialysate is valuable for ensuring the better efficiency of dialysis treatment.

2.3.1 Pentosidine

Pentosidine (MW = 378 Da) is formed *in vivo* in proteins by cross-linking arginine and lysine residues by pentose. The reaction can be reproduced in a solution with free amino acids and ribose (Sell et al., 1989). Pentosidine is listed in the uremic toxins database as a protein-bound uremic toxin (Vanholder, 2009). It is part of the AGE group (Suliman et al., 2003) and can be found in urine and plasma (Miyata et al., 1996). Studies showed that in a healthy individual, most of plasma pentosidine is protein bound (Miyata et al., 1996) and only small fraction (approx. 5%) is free circulating pentosidine (Friedlander et al., 1996b; Miyata et al., 1996). It is suggested that free pentosidine is normally filtered by renal glomeruli and after reabsorption in the proximal tubule, it is degraded or modified and then eliminated in the urine (Miyata et al., 1998). If the patient has declined renal function, the concentration of total pentosidine in serum will rise (Miyata et al., 1996; Stein et al., 2001; Vanholder, 2009; Kerkeni et al., 2014). The accumulation of protein-linked pentosidine in serum in rats has been linked to renal tubules injury (Dominguez et al., 2000). Depending on the stage of CKD, there are numerous factors that contribute to the elevation of total plasma pentosidine concentration (Machowska et al., 2016). It was found that HD patients have higher concentrations of total pentosidine compared to PD patients (Machowska et al., 2016). Patients with diabetes and/or ESRD can have over 20 times higher total serum levels of pentosidine (Miyata et al., 1996). Higher total plasma levels of pentosidine are associated with malnutrition (Suliman et al., 2003), inflammation

(Miyata et al., 1996; Suliman et al., 2003; Machowska et al., 2016), low GFR (Machowska et al., 2016), and oxidative stress (Machowska et al., 2016).

Pentosidine has low dialyser clearance, as only unbound solutes diffuse through the dialysis membrane (Sirich et al., 2012). It was found that only free pentosidine is cleared by HD (Miyata et al., 1997; Hohmann et al., 2017); yet, both forms of pentosidine were cleared by PD (Miyata et al., 1997). Therefore, most of the pentosidine in the spent dialysate can be discovered in a free form (Adler, 2017). Free pentosidine has been a highly recognised marker in the assessments of the degree of glycation damages of tissues, including progression rate in diabetic nephropathy (Weiss et al., 1998), for a long time.

2.3.2 AGE-modified β_2-microglobulin (β_2M)

β_2-microgobulin (MW = 11.8 kDa) is a polypeptide found on the surface of nucleated cells and it plays a part in human immune system (Floege et al., 1991). This protein is routinely shed by cells into the blood and is present in most body fluids (Warr, 1985). It is estimated that the average normal serum β_2M production rate is approx. 2.4 mg⁄kg⁄day (Floege et al., 1991). In patients with CKD, β_2M concentration increases due to declined kidney function. The accumulation of AGE-modified β_2M can lead to dialysis-related amyloidosis (Miyata et al., 1993b; Vanholder et al., 2008a). High blood concentrations of β_2M have shown to be indicators of poor outcomes in haemodialysis patients, as β_2M levels can predict mortality in CKD patients (Cheung et al., 2006).

β_2-microglobulin has similar kinetic behaviour as other middle molecules and peptides (Tattersall et al., 2007), and food intake does not affect the levels of β_2M (Vanholder et al., 2008a). Therefore, it was suggested that β_2M should be used as a marker substance to describe the dialysis efficiency in the removal of middle molecular weight solutes.

The clearance of β_2M by HD with low-flux membranes is minimal (Odell et al., 1991; Leypoldt et al., 1997). The clearance with high-flux membrane is better but still limited by diffusion. Therapies based on convection (HDF (Kerr et al., 1992; Locatelli et al., 1996; Ward et al., 2000; Wizemann et al., 2000; Lin et al., 2001) or adsorption (Gejyo et al., 1995; Ronco et al., 2001) are used to raise the efficiency of dialysis.

3 Methods for the analyses of uremic solutes

Different methods have been developed to analyse uremic solutes. Commonly used methods include HPLC, spectrophotometry, spectrofluorometry, electrophoresis, mass spectrometry, and electrochemical and immunochemical methods. Methods described below are used to measure 4PA, β_2M, and AGE products, such as pentosidine.

3.1 Methods for the analysis of small free water-soluble uremic toxins

Small free water-soluble uremic solutes are mainly analysed with chromatographic methods (Duranton et al., 2012), but other methods, such as nuclear magnetic resonance spectroscopy (Jankowski et al., 2003) and immunochemistry (Bayes et al., 2001; McGregor et al., 2001; Fragedaki et al., 2005), are also used. High-pressure liquid chromatography is the most widely used method. It is based on the separation of different molecules through movement in specific separative media consisting of two phases, stationary (porous adsorbent) and mobile phase (liquid phase).

Several methods of 4PA analysis have been described. B6 metabolite, 4PA, is mainly found in urine or human plasma. To determine 4PA from plasma by utilising HPLC, it is necessary to pre-treat the sample to separate 4PA from endogenous substances present in plasma extracts. For this, numerous chemicals, such as sulfosalicylic acid, trichloroacetic acid, perchloric acid or metaphosphoric acid (Bisp et al., 2002), are used. Some methods utilise fluorescence enhancement, which is achieved by adduct formation (Bisp et al., 2002) or reaction with different chemicals (Talwar et al., 2003; Rybak et al., 2004). Bisp et al. (2002) applied C18 column and ion-pair reverse-phase (RP) HPLC with gradient elution of different eluents with low pH and fluorescence detection after post column derivatisation. Another study used RP-HPLC with coulometric electrochemical detection and was able to detect 4PA in nanomolar quantities (Marszałł et al., 2009).

Mass spectrometry (MS) is usually used as coupled to the HPLC system or spectrophotometry (Ahmad et al., 2013). For an example, ultra-performance liquid chromatography-tandem mass spectrometry can be used to analyse small concentrations starting from 0.03 nM (Van der Ham et al., 2012).

These methods, HPLC and MS, are time consuming, require different chemicals and may require pre-treatment of the sample, and qualified personnel. The above-mentioned methods do not provide information about ongoing dialysis procedures and for this reason, optical measurement methods are utilised (Uhlin et al., 2003; Arund et al., 2012, 2016).

3.2 Methods for the analysis of pentosidine and β_2M

The enzyme-linked immunosorbent assay (ELISA) tests measure the degree of enzymatic reaction related to the immune complex. Thanks to quickness and selectiveness, it is the most widely used method for the analyses of large number of

samples. ELISA can be used with various samples, such as plasma, serum, and other biological fluids, to quantify different AGEs and proteins. It can be used to analyse fluorescent (e.g. pentosidine, AGE-modified β_2M) and nonfluorescent [e.g. *N*ε-(Carboxymethyl)lysine] AGEs and AGE protein products. The drawback of the ELISA method is that it might not identify solutes inside of proteins and in some cases, antibodies might not be able to identify the epitope (Suliman et al., 2003). Thus, immunoassay methods might not give the absolute concentration of the analyte (Ashraf et al., 2015).

The most accurate method to analyse pentosidine is HPLC after the full hydrolysis of AGE-modified proteins and peptides (Sell et al., 1989; Miyata et al., 1996; Stein et al., 2001; Suliman et al., 2003; Ni et al., 2009; Ashraf et al., 2015; Hohmann et al., 2017; Lee et al., 2017). For selectivity, different columns can be applied, such as reverse-phase C18 (Sell et al., 1989; Miyata et al., 1996; Stein et al., 2001; Suliman et al., 2003) or a special porous graphite carbon column (Ni et al., 2009). Predominantly mixtures of acetonitrile and water (Sell et al., 1989; Miyata et al., 1996; Stein et al., 2001; Suliman et al., 2003; Ni et al., 2009) are applied for elution with ion-pair modifiers, such as trifluoroacetic acid (TFA) (Ni et al., 2009) or heptafluorobutyric acid (HFBA) (Sell et al., 1989; Suliman et al., 2003). However, HPLC requires expensive equipment and is not suited for regular clinical use.

β_2-microglobulin is generally analysed from serum (Argiles et al., 1992; Miyata et al., 1993b; Liabeuf et al., 2012) or urine (Argiles et al., 1992; Miyata et al., 1993b) by using ELISA (Bjerrum et al., 1986; Miyata et al., 1993b; El-Wakil et al., 2011), immunonephelomentry (Liabeuf et al., 2012) or HPLC. High pressure liquid chromatography analysis is done with different solvent mixtures, such as H_2O/CH_3CN/HCOOH and HCOOH/AcN (Liabeuf et al., 2012) or TFA with water or AcN (Miyata et al., 1994). These measurements are time consuming and require different laboratory solvents.

4 Adequacy of dialysis

The adequacy of dialysis is interpreted as a number of dialyses required to keep the patient as healthy as possible and alive (Mehta et al., 2010). Although the term 'adequacy of dialysis' is widely used, there is no consensus on which parameter or criteria should define the term (Meyer et al., 2011). Several parameters and/or criteria are suggested, such as urea reduction ratio (URR), blood urea nitrogen (BUN), dialysis dose efficacy parameter (Kt/V), serum albumin, serum potassium, serum creatinine, haematocrit level, calcium and phosphorus, dialysis time, frequency, etc. (De Palma et al., 1972; Gotch et al., 1985; Owen et al., 1993; National Kidney Foundation, 2015).

Currently, the adequacy of dialysis is mainly based on blood urea values, as urea is the major end product of protein metabolism (Bankir et al., 1996; Depner, 2001). Parameters, such as URR, BUN and urea based Kt/V, are most widely used (National Kidney Foundation, 2015).

Kt/V is a dimensionless ratio, where K indicates dialyser clearance (mL/min), t indicates time (min), and V indicates the volume of the patient's body fluid (mL). Kt/V can be calculated based on urea concentration in blood samples taken before (C_0) and after (C_t) the dialysis from two blood samples (Gotch et al., 1985; Yanai et al., 1993):

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

The initial Kt/V formula has been modified and some of the outcomes are single-pool Kt/V (Daugirdas, 1995) and equilibrated Kt/V (Daugirdas et al., 1997). The latter takes into account the partial restoration of the concentration of urea in blood on account of the infiltration of urea into blood from other tissues (rebound, Mehta & Fenves, 2010).

It is known that Kt/V characterises the behaviour of only one class of small water-soluble fraction marked by urea (Meyer et al., 2011; Vanholder et al., 2018). However, substances in this group differ from each other and therefore, urea cannot be used as the only marker substance to assess the elimination of small free water-soluble uremic solutes, as the kinetic behaviour may be different from other molecules in the group, such as guanidines (Eloot et al., 2005). Additionally, the removal kinetics of middle molecules is different from urea (De Smet et al., 2007; Vanholder et al., 2008a). This has raised the question if a traditional single marker, such as urea, is adequate enough for evaluating the dialysis quality or whether additional parameters should be included (Gotch et al., 1985, 2000; Schoots et al., 1988; Vanholder et al., 1992, 2015; Meyer et al., 2011; Eloot et al., 2012).

There are currently two approaches used to estimate the adequacy of dialysis: laboratory analysis (indirect method) and/ or real-time monitoring (direct method) (Canaud et al., 1999). The most widely used and accepted reference method is the indirect method. However, the method may give rise to several errors during the process of sampling, storage, and analysis (Beto et al., 1998). Even though guidelines recommend measuring Kt/V monthly (National Kidney Foundation, 2015), only one

third of dialysis centres tend to follow the guideline (Couchoud et al., 2009). Thus, there is a lack of high quality data on most dialysis procedures and this may result in inadequate dialysis.

Other compounds besides urea should be monitored, as the removal of urea does not reflect the complete status of compounds in body fluids. Multicomponent monitoring could be used to detect differences in treatment efficiency and to follow variations in several retained solutes reflecting the clearance status during an ongoing dialysis. The direct methods are based on the measurement of spent dialysate (Lindsay et al., 2001), which eliminates the need to take extra blood samples from patients. The real-time multicomponent monitoring of dialysis ensures better quality of the treatment, which cannot be ensured with solely increasing Kt/V (Eknoyan et al., 2002; Locatelli, 2003). This also tends to underestimate haemodialysis doses in some patients (Spalding et al., 2008). Therefore, a constant Kt/V ratio is not a valid measure for haemodialysis doses (Lowrie, 2008). However, real-time multicomponent monitoring is not widely used due to the lack of accurate, robust, and cost-effective on-line measurement technology regardless of the advantage of real-time monitoring, which lies in the possibility to personalise dialysis treatment and monitor the quality of each dialysis session.

5 Online optical method for uremic solutes assessment

5.1 Optical properties of spent dialysate

The adequacy of dialysis can be assessed by analysing spent dialysate (Uhlin et al., 2003), which is considered to be a weakly scattering media (Tuchin, 2000). In spent dialysate with a low concentration of solutes, light is absorbed, reflected or transmitted and thus, scattering can be ignored (Welch et al., 1995).

Spent dialysate consists of several substances with chromophore and/or fluorophore properties that can be monitored. An analysis of UV-absorbance is used to detect a variety of substances in biofluids. Different wavelengths are used to determine the molecules included in spent dialysate. It appears that the major chromophores in spent dialysate are molecules with a molecular weight of less than 3 kDa (Lauri et al., 2010) and a removal ratio higher than 60% (Arund et al., 2012). Such molecules are UA (Donadio et al., 2014), creatinine, and hippuric acid (Vanholder et al., 2003; Lauri et al., 2006, 2010; Arund et al., 2012). Besides uremic toxins and solutes, different medications, e.g. paracetamol and its metabolites, can also affect the UV-absorbance of spent dialysate (Arund et al., 2012; Tanner et al., 2013).

The main chromophores with substantial contribution to the total absorbance at 280 nm were found to be small water-soluble uremic solutes (Arund et al., 2012). It was found that the main fluorophores in spent dialysate are indoxyl sulphate (Swan et al., 1983; Arund et al., 2016), indole acetic acid, tryptophan, indoxyl glucuronide, indoleacetyl glutamine, 5-hydroxyindoleacetic acid (Arund et al., 2016), and kynurenic acid (Swan et al., 1983).

5.2 UV-absorbance monitoring of small free water-soluble uremic solutes in spent dialysate

An UV-absorbance based optical monitoring method was first proposed almost forty years ago (Gal et al., 1980). Since then, several studies have been conducted and it was determined that the online signal of UV-absorbance can be related to Kt/V (Falkvall et al., 1999; Fridolin et al., 2001), and creatinine quantification (Fridolin et al., 2010; Tomson et al., 2011, 2013) etc.

It was demonstrated that the technique of applying UV-absorbance could be used to monitor the adequacy of dialysis (Fridolin et al., 2002; Uhlin et al., 2003). Several companies, e.g. Adimea and Nikkiso Medical, have introduced this technique and proposed it for clinical practice (Adimea, 2018; Nikkiso Medical, 2018). The advantages of online methods are that they provide information about the adequacy of each dialysis session and the effects of disturbances of the dialysis process are immediately apparent (Uhlin et al., 2006; Daugirdas et al., 2010). The clinical practice guidelines for haemodialysis care lists online methods, such as UV-absorbance measurement of UA to monitor the delivered dose of dialysis, in addition to standard methods (National Kidney

Foundation, 2015). On-line methods are able to estimate the removal of urea (Canaud et al., 1998; Kuhlmann et al., 2001; Fridolin et al., 2002), uric acid, creatinine, potassium (Fridolin et al., 2002), and phosphate (Fridolin et al., 2002; Holmar et al., 2018).

However, as other chromophores may interfere with the UV-absorbance signal (Daugirdas et al., 2010), further knowledge is required on the contributions of different solutes. Several studies have estimated the contributions and interference of different chromophores (Arund et al., 2012; Donadio et al., 2014) and medication (Tanner et al., 2013).

5.3 Principles of fluorescence spectroscopy

Fluorescence is the emission of a photon in certain molecules that occurs when an excited molecule returns to the ground state. A chromophore is a molecule that absorbs light and if it emits a photon, it is called a fluorophore. Not all chromophores are fluorophores, as absorbed energy may be dissipated as thermal or collisional energy, etc. Due to energy dissipation, the energy of emitted photon is lower than the energy of absorbed photon. Emission wavelength is provisionally intrinsic to a fluorophore, but quantum yield (and thereby, emission intensity) is more dependent on the environment (such as temperature, solvent, pH, etc.) In addition, one has to keep in mind that fluorescence intensity, which is linearly proportional to optical density, depends on the wavelength and surrounding environment. (Albani, 2008) Figure 3 depicts an example of a fluorescence emission spectrum of pentosidine at EX320 nm.

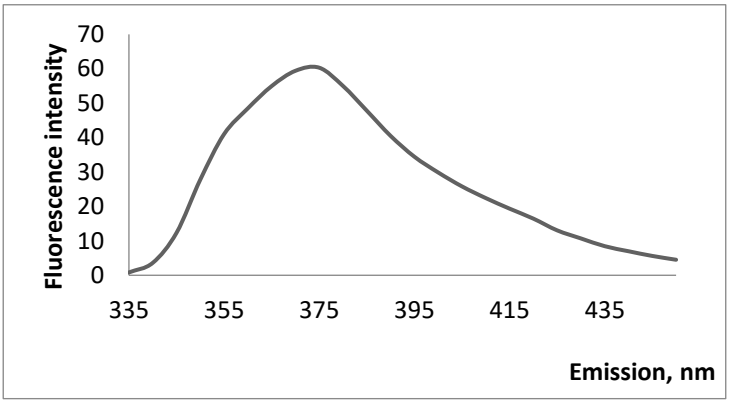

Figure 3. *Emission spectrum of pentosidine at EX320 nm.*

5.3.1 Effects affecting fluorescence

A fluorimeter records the emission beam perpendicularly to the excitation beam and emission is detected in the centre of the cuvette. This means that the beam will travel half the cuvette diameter before emission is excited. In that case, the recorded emission intensity will be underestimated due to the partial absorption of the exiting light on the way to the centre of the cuvette (Albani, 2008). Because of this, the concentration of the fluorescent substance may not be linearly dependent on measurable fluorescence intensity and this effect is known as the primary inner filter

effect (Albani, 2008; Fonin et al., 2014; Chen et al., 2018). The secondary inner filter effect is caused by the reabsorption of the emitted light on the way out from the cuvette (Kubista et al., 1994; Chen et al., 2018). These effects can cause a change in the wavelength of emission maximum and influence measurable fluorescence intensity.

An equation (2) can be used to correct fluorescence intensity (F) by the primary inner filter effect (Albani, 2008; Chen et al., 2018):

$$F_{corr}=F_{rec}*10^{[(OD(em)+OD(ex)]/2} \quad (2)$$

Where F_{corr} – corrected fluorescence intensity, F_{rec} – recorded fluorescence intensity, and OD(em) and OD(ex) represent optical density at emission and excitation, respectively.

It is also proposed to use low concentrations or diluted solutions, as these effects are barely noticed in a low optical density mixture (Chen et al., 2018); however, this still does not solve the problem (Fonin et al., 2014).

In addition to the inner filter effect, measurable emission intensity and quantum yield can be influenced by the quenching of emission by external molecules in the solution. Both the emission intensity and the wavelength maximum position are influenced by the environment comprising the fluorophore and the molecule to which it is bound. In a highly polar environment, the emission maximum is shifted to a higher wavelength and in an apolar medium, the maximum is at a lower wavelength compared to a polar medium. Binding to a protein or a membrane is usually accompanied by the Stokes shift or a change in the quantum yield. When binding to a polar molecule, the emission maximum is shifted to a higher wavelength and when binding to apolar compound, the effect is the opposite. (Albani, 2008)

5.4 Fluorescence and protein-bound uremic solutes

Online UV-absorbance measurements are utilised to measure small water soluble solutes (Fridolin et al., 2003; Jerotskaja et al., 2010; Holmar et al., 2012; Ghanifar et al., 2016). UV-absorbance cannot be used to detect all uremic solutes due to limited selectivity and sensitivity. Therefore, fluorescence spectroscopy is utilised to estimate the removal ratio and concentration of small water-soluble uremic solutes (Holmar et al., 2011a), β_2M (Uhlin et al., 2015), and protein-bound uremic toxins in spent dialysate (Holmar et al., 2011a, 2011b, 2013; Fridolin et al., 2015; Arund et al., 2016). It was found that the major contributors to the fluorescence of spent dialysate are indoxyl sulphate (Swan et al., 1983; Barnett et al., 1985; Arund et al., 2016), tryptophan, indoxyl glucuronide, 5-hydroxy-indole-3-acetic acid, indoleacetic acid, and indoleacetyl glutamine (Arund et al., 2016). The contribution of indoxyl sulphate to the fluorescence of spent dialysate was found to be 35 ± 11% at EX280/EM360 nm (Arund et al., 2016). Studies have shown that fluorescence can be utilised to monitor concentrations and the reduction ratio of indoxyl sulphate (Holmar et al., 2013; Arund et al., 2016).

5.5 Fluorescence and AGEs in ESRD patients and in spent dialysate

There are several AGEs, such as pentosidine, crossline (Obayashi et al., 1996), AGE-modified β_2M (Miyata et al., 1993b), argpyrimidine (Oya et al., 1999), and vesperlysine (Obayashi et al., 1996; Rondeau et al., 2011), that have fluorescence properties. However, none of the above-mentioned AGEs are monitored online optically through fluorescence during dialysis therapy. This shows that there is a research cap in the online monitoring of fluorescent AGEs. Uhlin et al. (2015) studied the possibility of monitoring β_2M through UV-absorbance but the error for RR estimation was high.

AGE accumulation in tissues can be estimated by measuring skin autofluorescence (SAF) (Meerwaldt et al., 2004; Ueno et al., 2008; Ramsauer et al., 2017). In healthy individuals, SAF correlates with age (Duda-Sobczak et al., 2018; Isami et al., 2018), smoking (Isami et al., 2018), consumption of meat or meat products, and waist circumference (Kellow et al., 2017). It was found that SAF is elevated in subjects with type 1 and 2 diabetes (Meerwaldt et al., 2004; Van der Heyden et al., 2016; Osawa et al., 2018). Skin autofluorescence could be used to predict long-term cardiovascular complications and mortality in diabetes and ESRD patients (Meerwaldt et al., 2005, 2007; Lutgers et al., 2006; Kimura et al., 2014; Velayoudom-Cephise et al., 2016), and diabetic neuropathy, retinopathy, and nephropathy (Osawa et al., 2018). The correlation of SAF and the fluorescence of spent dialysate of ESRD patients has not been investigated yet.

Aim of the study

This thesis focuses on the optical monitoring of three uremic toxins – β_2-microglobulin, pentosidine, and 4-pyridoxic acid, since the fluorescence of these solutes in spent dialysate has not been investigated before. The aim of this thesis was to develop methods for the assessment of the content and removal behaviour of selected uremic solutes from the blood of chronic kidney disease patients by means of direct measurement of the fluorescence of spent dialysate.

6 Experimental methods

6.1 Clinical studies

All studies were performed after the acceptance of the protocol by the Tallinn Medical Research Ethics Committee in Estonia with decision No. 2349 (15.03.2011). A written informed consent was acquired from all participating patients. The summary on included patients, dialysis parameters, and dialysate sampling times is presented in Table 1.

Table 1. *Patients, dialysis parameters and sampling times*

Publication	I	II	III	IV
Total number of patients	10	10	40	2
Male/female, mean age ± SD	5/5, 59 ± 15	5/5, 59 ± 15	20/20, 61 ± 16	-
Number of sessions (type)	39 (29 HD / 10 HDF)	40 (30 HD / 10 HDF)	40 (33 HDF / 7 HD)	-
Dialysis machine	Fresenius 5008H	Fresenius 5008H	Fresenius 5008 or 4008H	-
Blood flow, mL/min	300…350	300…350	170…360	-
Dialysate flow, mL/min	500…800	500…800	204…800	-
Session's length, min	240	240	180…247	-
Sampling time, min	7…10, 60, 120, 180, end of dialysis, tank	7…10, 60, 120, 180, end of dialysis	10, end of dialysis	10

6.2 Fluorophore detection by HPLC-MS (publications I, II, and III)

All dialysate samples were acidified down to pH 4.25 with formic acid before the HPLC analysis for the best separation of compounds. The HPLC system contained a thermostated auto sampler, a gradient pump unit, a column oven, a diode array spectrophotometric detector (DAD), and a fluorescence detector RF 2000 (FLD, all Ultimate 3000 Series instruments from Dionex, Sunnyvale, USA). In publication I and II, a Kinetex (C18, 2,6 μm, 100Å, 150*4.6 mm) column (Phenomenex, USA) with a Phenomenex security guard KJO-4282 (Torrance, USA), and in publication III, a Kinetex core-shell column (C18, 2,6 μm, 100Å, 150 × 4.6 mm) with a AJ0-4287 4*3 mm guard (Phenomenex, USA) was used. For mass spectrum analysis, micrOTOF-Q II instrument by Bruker Daltonik GmbH (Billerica, USA) with a ESI source was used. In publication I

and II, the eluent was 0.05 M formic acid adjusted to pH 4.25 with ammonium hydroxide (A) and a mixture of HPLC-grade methanol and acetonitrile in the volume ratio of 9 : 1 (B), both from Rathburn (Walkerburn, Scotland). The five-step gradient elution program used in publication I and II is specified in Table 2.

Table 2. *HPLC gradient program for Publication I and II*

Step	Time (min)	Buffer (A), %	Organic solvent (B), %	Gradient
0	0	100	0	-
1	0	100	0	Linear
2	30	90	10	Linear
3	60	5	95	Nonlinear
4	80	5	95	Linear
5	82	100	0	Linear

In publication III, HPLC grade AcN, (Honeywell, USA) and HFBA (Sigma-Aldrich, USA) solvents were used as a mixture of A: 0.005 M HFBA in MilliQ water, and B: 0.005 M HFBA in AcN. Chromatographic data was processed with Chromeleon 7.1 software by Dionex Thermo Fisher Scientific (Waltham, USA). The three-step program used publication III is specified in Table 3.

Table 3. *HPLC gradient program for Publication III*

Step	Time, min	Buffer (A), %	Organic solvent (B), %	Curve type
0	0	99	1	Linear
1	4	99	1	Linear
2	34	10	90	Nonlinear
3	44	10	90	Linear

The total flow rate of 0.8 mL/min was used in publications I, II, and III. In publication I and II, the column temperature was set to 40 ± 1.0 °C. In publication III, the column temperature was set to 35 ± 1.0 °C. Fluorescence was monitored at the wavelength of EX320/EM430 nm (publication I and II) and EX330/EM373 nm (publication III) with a time interval of 0.5 s. Mass spectra were acquired between 60 and 1700 Da with an acquisition rate of 1 Hz, ESI voltage of 4.5 kV, and a nebulization gas flow of 8.0 L/min, ion source temperature of 200^{o}C, drying gas flow of 1.2 bar, detector voltage of 2.03 kV. HPLC-MS data was processed with Compass HyStar version 3.2 and Compass DataAnalysis version 4.0 SP1 (both Bruker, Billerica, USA).

Full fluorescence spectra of spent dialysates were recorded with RF-5301 spectrofluorophotometer by Shimadzu (Kyoto, Japan) in the range of EX/EM 220–490 nm with an excitation increment of 10 nm. Quarts cuvette of 4 mm optical path length was used and Panorama Fluorescence 1.2 software by Shimadzu was applied in data processing.

6.3 Fluorophore detection by HPLC-MS (publication IV)

Columns Isolute C18(EC) 50 µm, 60 Å, 10 g, 70 ml (Biotage AB, Uppsala, Sweden) were used to concentrate less hydrophilic compounds from the spent dialysate. The concentration procedure consisted of five phases: (1) column washing with 120 ml of methanol (MeOH); (2) washing with 120 ml milliQ water; (3) adding 200 ml of the spent dialysate; (4) washing away salts from sorbent with 120 ml milliQ water; (5) adding 45 ml MeOH to the hydrophobic fraction evaporated to dryness with N_2 flow at 40 °C and redissolved in 1 ml of chromatographic buffer A (NH_4 formiate 0,05 M, pH 4,25) for further separation on the HPLC column. Sedimentation occurred while the concentrate was in +6 °C storage. The sediment was dissolved in 3 ml of 4 : 1 mixture of 6 M urea: 9% sodium chloride. All samples were acidified down to pH 4.25 with formic acid and centrifuged 10 minutes before the HPLC analysis.

The HPLC system contained a thermostated auto sampler, a gradient pump unit, a column oven, a diode array spectrophotometric detector (DAD), and a fluorescence detector RF 2000 (FLD, all Ultimate 3000 Series instruments from Dionex, Sunnyvale, USA), Kinetex (C18, 2,6 µm, 100Å, 150*4.6 mm) column (Phenomenex, USA) with Phenomenex security guard KJO-4282 (Torrance, USA) for the analysis of spent dialysate and Poroshell 120 EC-C18 column (Agilent Technologies, USA) for the analysis of sediment. The eluents were 0.05M ammonium formate (pH 4.25) and acetonitrile (Romil, UK) (Table 4). For mass spectrum analysis, the micrOTOF-Q II instrument by Bruker Daltonik GmbH (Billerica, USA) with ESI source was used. The fluorescence of the sediment was recorded with spectrofluorometer RF-5301PC (Shimadzu, Japan) and the spent dialysate with RF 2000 (Dionex, Sunnyvale, USA). The analysis was performed in the EX/EM range of 220–500 nm. For mass spectra interpretation, MagTran (Amgen Inc, USA) software was used.

In publication IV, a double-beam spectrophotometer (Shimadzu UV-2401 PC, Japan) was used for UV-absorbance spectra recording in the wavelength range of 190–380 nm.

Table 4. *HPLC gradient program for Publication IV*

Step	Time (min)	0.05M ammonium formate (A), %	AcN (B), %
0	0.5	100	0
1	30	75	25
2	30	0	100
3	7	0	100
4	1	0	100
5	2	100	0

6.4 Identification, relative contribution and removal ratio (publications I, II, III, and IV)

The peaks of 4-pyridoxic acid and free pentosidine in the HPLC chromatograms were identified with comparing the retention time, fluorescence spectrum, and mass spectrum data with a pure compound of 4PA (Sigma Aldrich, USA) and pentosidine (Cayman Chemical, USA). HPLC fluorescence data of reference (pentosidine, 4PA) solutions with different known concentrations were used for building up a calibration curve. The concentration of calibration solution of pentosidine was evaluated on the basis of UV-absorbance by using a molecular extinction coefficient 4522 AU/[(mol/l) cm] (Sell et al., 1989). In publication IV, β_2-microglobulin (β_2M) was identified by comparing the fluorescence of an unknown sediment with the fluorescence of a known substance from literature (Miyata et al., 1993b) and finding specific masses in the mass spectrum.

In publications I and II, the relative contribution (RC) of the peak of 4PA in the total fluorescence of the samples was calculated as a ratio of the area of peak of 4PA (A_{PA}) to the total area of all peaks appeared on the chromatogram (A_{total}):

$$RC\ (\%) = (A_{PA}/A_{total})*100 \quad (3)$$

In publication III, the removal ratio (RR) of free pentosidine for a dialysis treatment was calculated based on the start and end concentrations of free pentosidine (C_{start} and C_{end}) in the samples from the dialysis session:

$$RR\ (\%) = 100\ *(C_{start} - C_{end}) / C_{start} \quad (4)$$

Student's t-test was applied to compare the means of RR and RC, and $P \leq 0.05$ was considered significant.

Correlation analysis (Pearson's R) was applied to estimate the amount of linear association between the concentration estimated by HPLC and different optical models and variable groups of RR in publication III. $R > 0.8$ was considered to be a strong correlation. The coefficient of determination (R^2) was calculated to assess the goodness of fit between parameters in publications I and III. In publication III, Bland-Altman analysis was applied to examine the individual differences in RR calculated from concentrations of pentosidine estimated with different regression models.

6.5 Data analysis (publications I, II, III, and IV)

Forward stepwise regression analysis was applied to acquire the best wavelengths for the models for the estimation of concentrations of 4PA or free pentosidine through fluorescence intensity measurements. The concentrations of 4PA or free pentosidine were chosen as dependent variables and the values of fluorescence intensity were set as independent variables. In publication IV, SpektroModel software (Michelis, 2016) was utilised to analyse the data designed to generate regression models.

The algorithms acquired for the calculation of the concentration of 4PA and free pentosidine (C) were as follows:

$$C = a + b_1x_1 + b_2x_2 + \ldots + b_ix_i \quad (5)$$

where a is an intercept, b_i and x_i are a slope and an independent variable for the i-th model parameter.

Developed models were used on the validation set of data.

The independent variables included were fluorescence intensity values at the wavelengths of EX/EM 220–490 nm.

Each model was analysed in terms of standard and systematic error in publications II and III.

Systematic error (*BIAS*) was calculated as follows:

$$BIAS = \frac{\sum_{i=1}^{N}(C_{Lab,i} - C_i)}{N} \quad (6)$$

where $C_{Lab,i}$ is the i-th concentration of the substance in the spent dialysate, calculated based on HPLC results, C_i is the i-th concentration of the substance in the spent dialysate, calculated with the optical model, and *N* is the number of observations (Esbensen, 2009). Standard error (SE) of performance was calculated as follows:

$$SE = \sqrt{\frac{\sum_{i=1}^{N}(e_i - BIAS)^2}{N-1}} \quad (7)$$

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

In publication I and II, StatPlus (AnalystSoft Inc. for Mac) and Excel (version 2011 for Mac) were used. Excel (version 2016 for Mac and Windows) was used in publication I. In publication IV, Excel (version 2011 for Mac) was used. Mass spectra interpretations were done with MagTran (Amgen Inc, USA) software.

7 Results and discussion

7.1 4-Pyridoxic acid in spent dialysate (publication I)

Vitamin deficiency is common in CKD patients. Vitamin B6 deficiency is treated with regular B6 administration (Allman et al., 1989; Descombes et al., 1993, 2000); however, the probable toxicity of greater doses is not considered. It was found that the reduced excretion of pyridoxine and its metabolites are likely to increase the probability of pyridoxine toxicity (Levine et al., 2002); thus, the monitoring of status of B6 would be valuable.

The aim of publication I was to investigate the potential of the analysis of the fluorescence of spent dialysate for the observation of the elimination of 4PA from the blood of dialysis' patients receiving regular vitamin B6 treatment.

Previous studies have shown that spent dialysate could be used instead of blood for diagnostic analyses (Canaud et al., 1998; Kuhlmann et al., 2001; Fridolin et al., 2002; Holmar et al., 2018). The spent dialysate is used to measure urea (Canaud et al., 1998; Kuhlmann et al., 2001; Fridolin et al., 2002), phosphate (Fridolin et al., 2002; Holmar et al., 2018) and other substances, e.g. uric acid, creatinine, calcium, sodium, potassium, glucose, β_2-microglobulin, albumin, vitamin B12 (Fridolin et al., 2002). To our knowledge, the concentration of 4PA has not been analysed from spent dialysate before. Publication I confirms that besides plasma and urine, 4PA could be analysed from the spent dialysate of dialysis patients. Utilising spent dialysate as a medium allows analysing the ongoing dialysis session. It was found that 4PA is a major contributing peak on the HPLC chromatogram analysed at EX320/EM430 nm (Figure 4). 4-pyridoxic acid had 42.2 ± 17.0% of the total fluorescence intensity at the beginning of the dialysis and 47.7 ± 18.0% at the end.

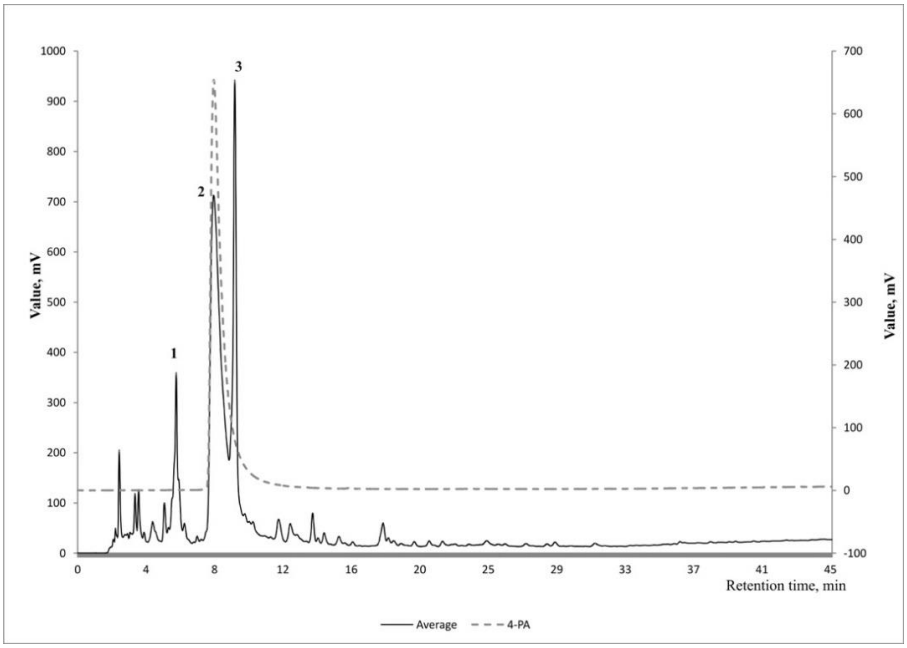

***Figure 4**. An example of an averaged EX320/EM430 nm chromatogram of the spent dialysate (N = 10). Samples were collected 7–10 min after the start of the dialysis. Compound 2 was identified as 4-pyridoxic acid. Raised chromatogram is of a reference 4-pyridoxic acid solution.*

High correlation (R > 0.88) between fluorescence intensity at EX310-330/EM415-500 nm and the concentration of 4PA in the spent dialysate was found. An example of correlation at EX310/EM460 nm is shown in Figure 5.

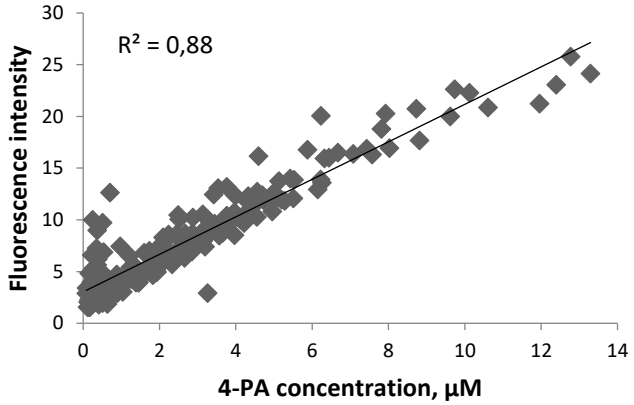

***Figure 5**. An example of correlation between the fluorescence intensity of the spent dialysate and the concentration of 4PA in the spent dialysate at EX310/EM460 nm (P < 0.0001, N = 195).*

7.2 Monitoring of 4-pyridoxic acid (publication II)

4PA is generally measured from plasma or serum with a modified HPLC (e.g. in terms of post-column derivatization) (Busch et al., 2010; Coburn et al., 2002). These methods are laborious, expensive, time consuming, and require blood sampling from the patient. As our previous study (publication I) showed a good correlation between the fluorescence of the spent dialysate and the concentration of 4PA at EX310-330/EM415-500 nm, a regression model was created to find the most suitable wavelength pair for the estimation of concentration. For this reason, the spent dialysate samples from the start, 60 min, 120 min, 180 min, and at the end, were collected. In total, 200 samples of 40 dialysis sessions of 10 patients from Tallinn, Estonia, were included in the study. Regression analysis was created using fluorescence intensity of the spent dialysate and the concentration of 4PA measured with HPLC, which was used as a reference. The best correlation (R = 0.93–0.94) was found at EX310/EM460 nm (Table 5). The wavelength with the best correlation was different from the fluorescence maximum of standard 4PA (publication I). This wavelength shift may be due to other interfering solutes that have fluorescence at the same wavelengths.

Table 5*. Number of cases (N), correlation coefficients (R), systematic (BIAS), and standard errors (SE) of the SW model for the calibration and validation set*

Model	Set	N	R	BIAS (µM)	SE (µM)
EX320/EM430 nm	Calibration	100	0.931	0.00	1.23
	Validation	99	0.853	0.55	1.95
EX310/EM460 nm	Calibration	98	0.944	0.00	0.88
	Validation	97	0.931	0.02	1.02

Pyridoxal-5'-phosphate (PLP) is mainly used to evaluate the status of B6; yet, the concentration of PLP in the plasma of dialysis patients is low (Lindner et al., 2002; Busch et al., 2010), which is the one of the reasons why it is difficult to measure. There is a correlation between the concentration of PLP and 4PA in plasma (Ueland et al., 1992; Mayer et al., 1996; Graham et al., 1997; Busch et al., 2010). The level of 4PA rises in ERSD patients, and as 4PA is easier to measure than PLP, the levels of 4PA could be used as a potential indicator of renal function (Coburn et al., 2002).

The concentration of 4PA correlates with other compounds, e.g. creatinine, and urea (Bor et al., 2003) and homocysteine (Ueland et al., 1992; Mayer et al., 1996; Graham et al., 1997; Busch et al., 2010). Busch et al. (2010) found a correlation between the concentration of plasma 4PA and total pentosidine in a renal transplant and HD patients. It must be noted that those patients did not get vitamin B6 supplementation. In publication I, no correlation between the concentration of 4PA and free pentosidine in the spent dialysate was found (R^2 = 0.07, P < 0.0001, unpublished data) and it was noticed that both compounds had independent fluorescence from each other.

The accuracy of the model in publication II needs improvement at low concentrations, as it underestimates the concentrations of 4PA at lower values. This could be due to the large variance of data.

This SW model, based on an analysis of the fluorescence of the concentration of 4PA in the spent dialysate, allows a quick and safe measurement of 4PA. To estimate the nutritional status of B6 through the assessment of the concentration of 4PA from the spent dialysate, further studies are needed to establish how much pyridoxine is oxidized to 4PA and how much 4PA is removed by dialysis, as 4PA is also removed by the kidneys. In publications I and II, residual renal function of the patient was not taken into account.

Overall, the results indicate that the fluorescence of the spent dialysate can be utilised to estimate the concentration of 4PA.

7.3 Free pentosidine in spent dialysate (publication III)

Our previous study (Kalle et al., 2017) found a weak correlation between the fluorescence of the spent dialysate and SAF; thus, an interesting possibility for the evaluation of the elimination of free pentosidine from CKD patients by the assessment of fluorescence properties of the spent dialysate could be seen as an cost-effective alternative to AGE removal monitoring. For this reason, a regression analysis was applied to estimate the concentration of free pentosidine from the spent dialysate by utilising fluorescence intensity. Average concentrations of free pentosidine assessed with HPLC at the start and at the end of the dialysis session in the spent dialysate were 4.25 ± 3.11 μg/L and 0.94 ± 0.69 μg/L, respectively. The concentrations of free pentosidine estimated with fluorescence-based models were not statistically different ($P > 0.22$) from the concentrations assessed with HPLC.

The highest correlation between the fluorescence of the spent dialysate and the concentration of free pentosidine was found in the wavelength region of EX310-350/EM360–385 nm. The fluorescence wavelength (EX350/EM363 nm) with the highest correlation (R = 0.94–0.97) with a single wavelength (SW) model did not correspond to the maximum wavelength EX330/EM373 nm (Slowik-Zylka et al., 2004) of standard pentosidine. There may be interferences by other fluorophores influencing the correlation maximum. The MW model improved the estimation of the concentration and RR of free pentosidine from the spent dialysate compared to the SW model and had lower standard error values (Table 6).

Table 6*. The number of cases (N), the standard (SE) and systematic (BIAS) errors of the models, correlation coefficients (R), and coefficients of determination (R^2) for the calibration and validation sets.*

	Set	N	BIAS	SE	R	R^2
SW	Calibration	53	0.000	0.702	0.966	0.934
	Validation	18	0.031	1.135	0.943	0.889
MW	Calibration	50	-0.055	0.464	0.985	0.970
	Validation	18	-0.109	0.962	0.962	0.926

The removal ratio (RR) of free pentosidine could be used to assess the adequacy of dialysis. The RR of free pentosidine has been found to be more than 75% (Miyata et al., 1997) and 95% (Hohmann et al., 2017) with HD. In publication III, the RR of free pentosidine from the spent dialysate was over 74% (Table 7).

***Table 7**. The average RR removal ratio of free pentosidine estimated with the SW and MW models (N = 30):*

	RR_Lab Mean±SD, %	**RR_SW Mean±SD, %**	***P*-value* (RR_Lab-RR_SW)**	**RR_MW Mean±SD, %**	***P*-value* (RR_Lab-RR_MW)**
Calibration	75.8 ± 9.9	83.8± 5.9	< 0.0001	74.2 ± 15.2	0.60
Validation	74.2± 10.0	83.1 ± 8.1	0.004	70.1 ± 16.2	0.28

* Calculated with Student's t-test where significance level is 0.05.

This preliminary study shows that the model for the on-line assessment of the concentration of free pentosidine in the spent dialysate is feasible. This model could give supplementary information about the adequacy of an ongoing dialysis session and removal of AGEs that accelerate atherosclerosis (Mallipattu et al., 2014) and may have a connection with the cardiovascular morbidity of patients with CKD (Zoccali et al., 2005). The method displays potential for further research of the peculiarities of the elimination of free pentosidine as AGE and an oxidative stress marker with a dialysis treatment of CKD patients.

7.4 β_2-microglobulin in spent dialysate (publication IV)

β_2-microglobulin is mainly measured with ELISA or HPLC, but a more feasible method would be beneficial. UV-absorbance measurements of β_2M need further validation (Lauri et al., 2015; Uhlin et al., 2015). Some correlation between the concentration of β_2M and the fluorescence of the spent dialysate was found attributable to acidic AGE-modified β_2M (Holmar et al., 2011a). The aim of publication IV was to investigate whether the fluorescence spectroscopy of the spent dialysate could be utilised to assess AGE-modified β_2M. The spent dialysate samples of two patients were selected and concentrated with a SPE column for this study. The typical mass spectrum of a peptide with a neutral mass of 11,728 Da and a fluorescence spectrum coincident with that of amyloid β_2M (Miyata et al., 1993a) was found in the sediment growing in the course of concentration of the SPE eluent. In addition, the same molecular masses evidently belonging to AGE-modified β_2M (11,728 Da) were found on the fluorescence chromatograms of initial spent dialysates. However, the contribution of the fluorescence of AGE-modified β_2M was only 0.04% from the overall fluorescence of the spent dialysate at EX352/EM460 nm.

This study showed that it is possible to detect the fluorescence peak of amyloid B2M on the HPLC chromatogram; however, the contribution may be very low. This means that the usage of the fluorescence of the spent dialysate at the specific wavelength for the assessment of AGE-modified β_2M as the MM marker may be possible only in the case of dialysis patients with severe complications of amyloidosis (Miyata et al., 1993b).

Conclusions

The current chapter summarises the main contributions of this study.

This thesis focuses on the optical monitoring of three uremic solutes – 4-pyridoxic acid, pentosidine, and β2-microglobulin, since the fluorescence of these solutes in spent dialysate has not been investigated before. This dissertation investigates methods for the assessment of the content and removal behaviour of selected uremic solutes from the blood of chronic kidney disease patients by means of direct measurement of the fluorescence of spent dialysate.

The main results of this thesis are as follows:

- The results of this thesis demonstrated that spent dialysate provides a good medium for the monitoring of ongoing dialysis treatment. In addition, the result of the study showed that spent dialysate can be used for a direct spectrofluorometric assessment of the removal of 4PA and free pentosidine from the blood of dialysis patients.
- The highest fluorescence contributing peak on the HPLC chromatogram at EX320/EM430 was 4PA. Mean contribution ± SD at the start of the dialysis was 42.2 ± 17.0% and at the end of dialysis, 47.7 ± 18.0%.
- A high correlation ($R > 0.88$) between the fluorescence intensity of the spent dialysate at EX310-330/EM415–500 nm and the concentration of 4PA concentration in the spent dialysate was found.
- The highest correlation between the fluorescence of the spent dialysate and the concentration of free pentosidine was found in the wavelength region of EX310–350/EM360–385 nm.
- The findings of the study suggest that it is feasible to monitor the elimination of free pentosidine during dialysis by using the fluorescence of the spent dialysate even when the technique evidently assesses several other fluorophores and chromophores in the spent dialysate at the same time. Based on the results from the study the author developed and proposed single-(SW) and multiwavelength (MW) models. The MW algorithm improved the estimation of the concentration of free pentosidine and the estimation accuracy of RR in comparison with the SW algorithm.
- β_2M can be assessed from the spent dialysate. However, the fluorescence intensity of modified β_2M may be low compared to overall fluorescence. This indicates that the usage of the fluorescence of the spent dialysate at the specific wavelength for the assessment of AGE-modified β_2M as the MM marker may be possible only in the case of dialysis patients with severe complications of amyloidosis.

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Abstract

Optical Monitoring of Uremic Metabolites-Fluorophores during Dialysis: the Cases of β_2-microglobulin, Pentosidine, and 4-Pyridoxic Acid

Chronic kidney disease (CKD) is the most prevalent disease. It is estimated that almost one million people die each year because of CKD. A great number of uremic solutes accumulate in the blood of the patient due to the disease. Dialysis is applied to remove the accumulated solutes from the blood of the patient. Different marker solutes are used to estimate the adequacy of applied dialysis therapy. Urea is the most widely used marker substance. However, urea should not be used as the single marker substance, as its kinetic behaviour may be different from other molecules. Furthermore, methods and formulas that utilise urea as a marker substance characterise the behaviour of only one class of uremic solutes.

The aim of this thesis was to develop methods for the assessment of the content and removal behaviour of selected uremic solutes from the blood of chronic kidney disease patients by means of direct measurement of the fluorescence of spent dialysate. The thesis focused on the fluorescence properties of specific uremic solutes in spent dialysate. Three different uremic solutes [β_2-microglobulin (β_2M), pentosidine, and 4-pyridoxic acid (4PA)] were studied. Generally, all these solutes are mainly measured with laborious methods, such as high performance liquid chromatography or enzyme-linked immunosorbent assay. However, these measurement methods require skilled workers, are time consuming, and do not give information about the ongoing dialysis session. In the current study mainly fluorescence measurements were used in order to develop an optical dialysis monitoring method for specific solutes.

The results of this study demonstrated that the spent dialysate could be used instead of blood and urine to measure three selected uremic solutes. The results of the study indicated that 4PA is a major fluorescent contributing peak on the HPLC chromatogram at EX320/EM430 nm. An algorithm was developed to estimate the concentration of 4PA in spent dialysate based on the fluorescence assessment utilising regression analysis.

In addition, the marker substance of pentosidine was studied for the estimation of removal of advanced glycation end products (AGE). Two models were developed and proposed by the author, and it was demonstrated that the optical method for an on-line assessment of the concentration of free pentosidine in spent dialysate is feasible but requires further search. Another substance belonging to the middle molecule group, AGE-modified β_2-microglobulin, was also analysed in the current study. The results of the study revealed that the contribution of the fluorescence of modified β_2M to the overall fluorescence of spent dialysate is very low at EX352/EM460 nm, which means that the patients need to have very high concentrations of acidic β_2M.

Based on the results of the current study it can be concluded that spent dialysate could be used instead of blood and urine to assess three selected uremic solutes. In addition, fluorescence could be used to follow the removal of 4PA and pentosidine during a dialysis session. However, AGE-modified β_2M could be analysed in spent dialysate, but as the contribution tends to be very low, which means that the patients need to have very high concentrations of acidic β_2M for direct β_2M detection.

Lühikokkuvõte

Ureemiliste metaboliitide-fluorofooride optiline jälgimine dialüüsi jooksul: β_2-mikroglobuliini, pentosidiini ja 4-püridokshappe näited

Krooniline neeruhaigus on väga sage haigus, ning on leitud, et igal aastal sureb nimetatud haiguse tõttu ligi miljon inimest. Kroonilise neeruhaiguse tõttu hakkavad patsiendi verre akumuleeruma erinevad ureemilised soluudid. Üks ravimeetod kogunenud soluutide eemaldamiseks on dialüüsravi. Kasutatud dialüüsravi adekvaatsuse hindamiseks kasutatakse erinevaid markeraineid. Kõige sagedamini kasutatud markeraine on uurea. Dialüüsravi adekvaatsuse hindamiseks ei peaks markerainena kasutama vaid uureat, kuna uurea võib käituda kineetiliselt teistmoodi kui mõni muu aine. Seega näitavad vaid uureat kasutavad meetodid ja valemid ureemiliste soluutide käitumist dialüüsravil.

Doktoritöö eesmärk oli metoodika leidmine valitud ainete otseseks tuvastamiseks kulunud dialüsaadis ja mudelite koostamine nende elimineerimise dünaamika hindamiseks kroonilise neerupuudulikkuse patsientidel. Selleks valiti kolm ureemilist soluuti – β_2-mikroglobuliin (β_2M), pentosidiin ja 4-püridokshape (4PA). Nimetatud ainete mõõtmiseks kasutatakse enamasti HPLC- või ELISA-meetodit, mis on aja- ja töömahukad, vajavad koolitatud töötajaid ega anna informatsiooni hetkel toimuva dialüüsravi kohta. Käesolevas töös kasutati mõõtmiste läbiviimiseks peamiselt spektrofluorofotomeetrit, et välja töötada kindlate soluutide optiline jälgimise meetod.

Käesolevas töös kasutati erinevaid meetodeid ja seadmeid, et iseloomustada väljavalitud ureemiliste soluutide fluorestseeruvaid omadusi heitdialüsaadis. Saadud informatsiooni alusel koostati regressioonanalüüs, mille põhjal töötati välja erinevad algoritmid, et hinnata väljavalitud soluutide elimineerimist dialüüsravil.

Töö käigus leiti, et väljavalitud ureemilisi soluutide mõõtmiseks võib lisaks verele ja uriinile kasutada heitdialüsaati. Mõõtmised näitasid, et peamine osa fluorestsentsi signaalist ergastusel 320 nm ja emissioonil 430 nm pärineb 4-püridokshappest. 4PA kontsentratsiooni hindamiseks heitdialüsaadist, mille käigus kasutati fluorestsents-mõõtmisi, loodi regressioonianalüüsi põhjal algoritm.

Glükosüleerimise kaugele arenenud lõppsaaduste (AGE) elimineerimise hindamiseks uuriti markerainena pentosidiini. Töö käigus töötati välja kaks mudelit, mis näitavad, et heitdialüsaadis olevat vaba pentosidiini kontsentratsiooni on võimalik otse ja optiliselt mõõta, kuid edasised uuringud on siiski vajalikud. Samuti uuriti AGE-modifitseeritud β_2-mikroglobuliini. Modifitseeritud β_2M-i panus fluorestsentsi signaali ergastusel 352 nm ja emissioonil 460 nm on väga madal. Seega on kulunud dialüsaadi fluorestsentsi kasutamine modifitseeritud β_2M-i hindamiseks võimalik vaid tõsiste amüloidoosi komplikatsioonidega patsientidel.

Kokkuvõttes saab järeldada, et väljavalitud ureemiliste soluutide mõõtmiseks võib lisaks verele ja uriinile kasutada heitdialüsaati ning 4PA ja vaba pentosidiini

elimineerimist dialüüsravi jooksul saab jälgida, kasutades otse mõõtmiseks fluorestsentsi. Modifitseeritud β_2M-i saab samuti otse mõõta heitdialüsaadis, kuid nimetatud aine osakaal fluorestsentsi signaali teatud lainepikkusel on madal.

Appendix

Publication I

Kalle, S., Tanner, R., Arund, J., Tomson, R., Luman, M., Fridolin, I. (2016). 4-Pyridoxic Acid in the Spent Dialysate: Contribution to Fluorescence and Optical Monitoring, *PLOS ONE*, 11(9) (DOI: 10.1371/journal.pone.0162346)

PLOS ONE

RESEARCH ARTICLE

4-Pyridoxic Acid in the Spent Dialysate: Contribution to Fluorescence and Optical Monitoring

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Abstract

Aim

In this work we estimated the contribution of the fluorescence of 4-pyridoxic acid (4-PA) to the total fluorescence of spent dialysate with the aim of evaluating the on-line monitoring of removal of this vitamin B-6 metabolite from the blood of patients with end-stage renal disease (ESRD).

Methods

Spectrofluorometric analysis of spent dialysate, collected from hemodialysis and hemodiafiltration sessions of 10 patients receiving regularly pyridoxine injections after dialysis treatment, was performed in the range of Ex/Em 220–500 nm. 4-PA in dialysate samples was identified and quantified using HPLC with fluorescent and MS/MS detection.

Results

Averaged HPLC chromatogram of spent dialysate had many peaks in the wavelength region of Ex320/Em430 nm where 4-PA was the highest peak with contribution of 42.2±17.0% at the beginning and 47.7±18.0% in the end of the dialysis. High correlation (R = 0.88–0.95) between 4-PA concentration and fluorescence intensity of spent dialysate was found in the region of Ex310-330/Em415-500 nm, respectively.

Conclusion

4-PA elimination from the blood of ESRD patients can be potentially followed using monitoring of the fluorescence of the spent dialysate during dialysis treatments.

Introduction

Vitamin deficiency is common in chronic kidney disease (CKD) patients. One vitamin that CKD patients are lacking is vitamin B-6 (B6) which is the term for a group of interconvertible molecules containing pyridoxine, pyridoxal, pyridoxamine and their phosphates. The deficiency of B6 has been linked to many pathologies including impaired gluconeogenesis and glucose tolerance [1], metabolism of amino acids [2], regulation of the level of circulating insulin [3,4], diabetes type 1 [5] and type 2 [6]. Many studies have pointed out that lower vitamin B-6 concentrations increase the risk of coronary artery disease [7–9] as well as renal dysfunction [10]. Concerning uremia it is essential to consider metabolic link between urea accumulation in uremic tissues and inactivation of pyridoxal-5'-phosphate (PLP) by carbamoyl phosphate, the very first product of condensation of carbonate with ammonia in the metabolic pathway of urea biosynthesis [11] leading to symptoms of B6 deficiency [12]. The deficit of B6 in dialysis patients is treated by regular B6 administration [13–15] but there is no adequate consideration of potential toxicity of larger doses. Levine *et al*. have found that decreased excretion of pyridoxine and its metabolites might increase the likelihood to pyridoxine toxicity [16] and thus status of pyridoxine monitoring would be beneficial.

The main active form of B6 is pyridoxal-5'-phosphate (PLP). PLP acts as a cofactor for 147 EC-classified enzymes, 64 of which are known to be present in multicellular animals [17]. PLP has been widely employed as a status indicator of vitamin B-6 but recently usage of other vitamers and 4-pyridoxic acid has been proposed [18]. 4-PA is the major urinary catabolite of B6 [19] since it has been estimated that in healthy individuals 40–60% of ingested B6 is oxidized to 4-pyridoxic acid [7,20]. 4-PA can be measured in blood plasma or serum with high sensitivity using HPLC with different modifications such as post-column derivatization [21,22] etc. These methods require blood sampling from the patient and several laborious steps, which are expensive and time consuming.

Our previous studies have shown that UV and fluorescence spectra data, measured directly at the outflow of the spent dialysate from dialysis machine, can be used to calculate contents of different characteristic uremic solutes [23,24]. The benefit of such information is the possibility of on-line monitoring of the dialysis process and rapid correction of the treatment depending on the status of the patient being treated.

The aim of this study was to investigate the potential of measurement of fluorescence in spent dialysate for monitoring of the elimination of 4-PA from the blood of dialysis patients receiving regular B6 treatment. The set aim was achieved.

Materials and Methods

2.1. Ethics

The study was approved by the Tallinn Medical Research Ethics Committee at the National Institute for Health Development, Estonia decision no. 2349. A written informed consent was obtained from all participating patients.

2.2. Patients and samples

39 dialysis sessions of 10 patients (age 59 ± 15 years) were followed. 100 mg B6 was routinely injected to patients after each dialysis session. The dialysis machine used was Fresenius 5008H (Fresenius Medical Care, Germany), dialyzers were FX8 or FX1000, the dialysate and blood flow varied from 500–800 mL/min and 300–350 mL/min, respectively. The dialysate samples were collected 7–10, 60, 120, 180, 240 minutes after the start of the dialysis session from the outlet dialysate line and from tank (145 HD and 50 HDF samples in total). All dialysate

samples were acidified down to pH 4.25 with formic acid before the HPLC analysis for the best chromatographic separation and stable retention times. Full fluorescence spectra of spent dialysates in the range of excitation/emission 220–500 nm and emission with excitation increment 10 nm were recorded with the spectrofluorophotometer RF-5301 by Shimadzu (Kyoto, Japan). The cell with optical path 4 mm was used for measurement and the Panorama Fluorescence 1.2 software by Shimadzu for spectral data processing.

2.3. HPLC and MS system

The HPLC system consisted of a gradient pump unit, a thermostated auto sampler, a column oven, a diode array spectrophotometric detector (DAD) and a fluorescence detector (FLD), all Ultimate 3000 Series instruments from Dionex (Sunnyvale, CA, USA), column of Kinetex C18 100A column (Phenomenex, USA) with a security guard KJO-4282 from Phenomenex (Torrance, CA, USA). The fluorescence was recorded at the wavelength of Ex320/Em430 nm and measurement interval of 0.5 s. Chromatographic data was processed with Chromeleon 7.1 software by Dionex Thermo Scientific (Waltham, USA).

The micrOTOF-Q II instrument by Bruker Daltonik GmbH (Bremen, Germany) with ESI source was used for mass-spectrometric analyses. For the identification of 4-PA both positive and negative ion mode were used. Sample analysis were done with the following parameters: mass range of 60–1700 m/z, 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 and acquisition rate of 1 Hz. Mass calibration was performed with sodium formate solutions from m/z 60 to 1700. For data acquisition software Compass HyStar version 3.2 and for processing Compass DataAnalysis version 4.0 SP1 was used (both Bruker, Billerica, USA).

2.4. Mobile phase

The two-component eluent was used as mixture of A: 0.05 M formic acid adjusted to pH 4.25 with ammonium hydroxide and B: the mixture of methanol and acetonitrile in the volume ratio of 9:1, both HPLC-grade from Rathburn (Walkerburn, Scotland). The five-step linear gradient elution program was used, as specified in Table 1.

2.5. Identification and contribution

Chromatographic peak of the 4-PA was identified by comparing retention time, UV absorption, fluorescence and mass spectra data of an unknown found in the sample with the corresponding characteristics of the reference compound (4-pyridoxic acid, Sigma Aldrich, USA). HPLC fluorescence data of reference 4-PA solution with different known concentrations were

Table 1. HPLC gradient program.

Step	Time (min)	Buffer (A) %	Organic solvent (B) %	Curve type
0	0	100	0	
1	0	100	0	linear
2	30	90	10	linear
3	60	5	95	concave
4	80	5	95	linear
5	82	100	0	linear

The total flow rate of 0.8 mL/min was used with the column temperature of 40°C. The sample volume injected was 20–50 μL.

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used to create a calibration curve. Concentration of 4-PA was calculated on the basis of HPLC fluorescence chromatograms.

The relative contribution (RC) of the 4-PA peak in the total fluorescence of the samples was calculated as a ratio of the area of 4-PA peak (A_{PA}) to the total area of all peaks appeared on the chromatogram (A_{total}): RC (%) = $(A_{PA}/A_{total})^{*}100$

Student's t-test was used to compare Two-Sample dataset, Assuming Unequal Variances, while $p < 0.05$ was considered significant.

Results

3.1. Emission spectra and average HPLC fluorescence chromatogram of spent dialysate

Fig 1 presents the characteristic emission spectrum of spent dialysate compared with the reference spectrum of the 4-PA in the same buffer solution (sodiumbicarbonate/acetate pH 7.6; Fresenius Medical Care AG &Co) (for specific data see Figure A in S1 file). The graph presents quite similar Em spectra shape in the wavelength region 400–500 nm for the total fluorescence signal of spent dialysate and fluorescence of 4-PA.

Fig 2 illustrates an example of averaged HPLC fluorescence chromatograms (Ex320/Em430 nm) of spent dialysate of 9 different patients' collected 7–10 minutes after the start of HD sessions (for specific data see Figure B in S1 File). The peak number 2 was found to coincide with 4-PA on the basis of retention time and mass spectrum.

3.2. Identification of 4-PA

The MS spectrum of the highest peak no 2 (Fig 3A) was compared to the spectrum of the reference substance (Fig 3B) where a good match with 4-PA was found.

3.3. Contribution and concentration of 4-PA

All 39 dialysis sessions followed were used to calculate concentration of 4-PA found chromatographically in spent dialysates. It was found that the average concentration of 4-PA in the beginning of dialysis was 4.20 ± 2.29 μmol/L and in the end 1.71 ± 0.67 μmol/L. Average

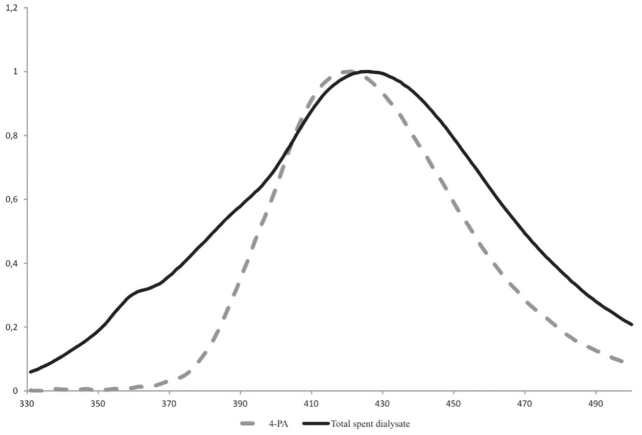

Fig 1. Normalized emission spectra of spent dialysate (solid line) and 4-pyridoxic acid (dotted line) in $NaHCO_3$/acetate buffer pH 7,6. Excitation at 320 nm.

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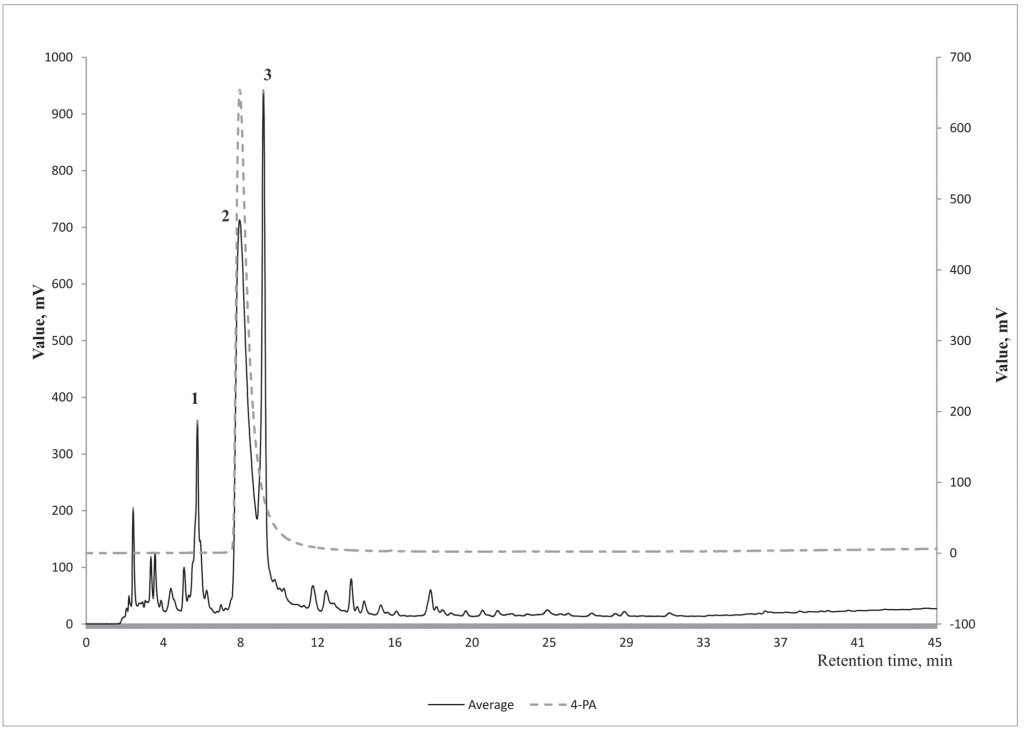

Fig 2. Example of an averaged Ex320/Em430 nm chromatogram of the spent dialysate (N = 10). Samples were collected 7–10 min after the start of the dialysis. Compound 2 was identified as 4-pyridoxic acid. Raised chromatogram is of a reference 4-pyridoxic acid solution (1 μM).

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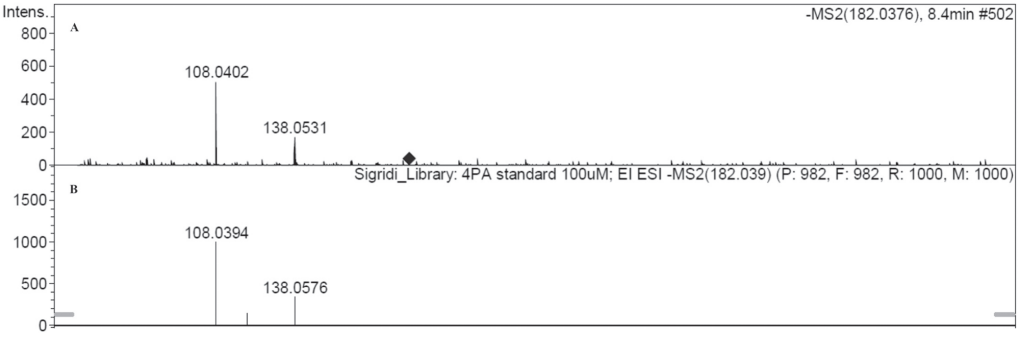

Fig 3. Comparison of mass spectra (negative ionization) of A) biggest fluorescent peak (no 2, Fig 2) and B) 4-pyridoxic acid reference.

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Table 2. Mean contribution (Mean ± SD, N = 10) values in percentage for main fluorescent peaks in the spent dialysate samples (Ex320/Em430 nm).

	Mean contribution ± SD at the start of the dialysis	Mean contribution ± SD at the end of the dialysis
Unknown 1	7.4 ± 2.1	7.2 ± 3.1
4- PA (peak 2)	42.2 ± 17.0	47.7 ± 18.0
Unknown 3	22.4 ± 5.4	19.9 ± 3.1

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contribution of fluorophores in HPLC fluorescence chromatograms of the spent dialysates were calculated using 10 dialysis sessions data. The calculation showed that 4-PA appears to be the main contributor of the fluorescence signal (Table 2) at Ex320/Em430 nm (for the dataset see Table A in S1 File).

3.4. Linear correlation

Linear correlation was calculated between concentration of 4-PA found chromatographically in spent dialysate and directly measured fluorescence intensity of the dialysate. High correlation ($R > 0.88$, $N = 195$) was found in the wavelength region Ex310-330/Em415-500 nm (Fig 4). For correlation on a wider scale of Ex220-500/Em220-500 nm see Figure C in S1 File.

More detailed examination of the region with the highest R values revealed that the best correlation was found at the wavelengths Ex310/Em460 nm ($R_{max} = 0.95$, $N = 195$ (Fig 5, for specific data see Figure D in S1 File).

Fig 6 presents the regression equation of 4-PA against fluorescence intensity at wavelengths of highest correlation Ex310/Em460 nm in the spent dialysate (for specific data see Figure E in S1 File).

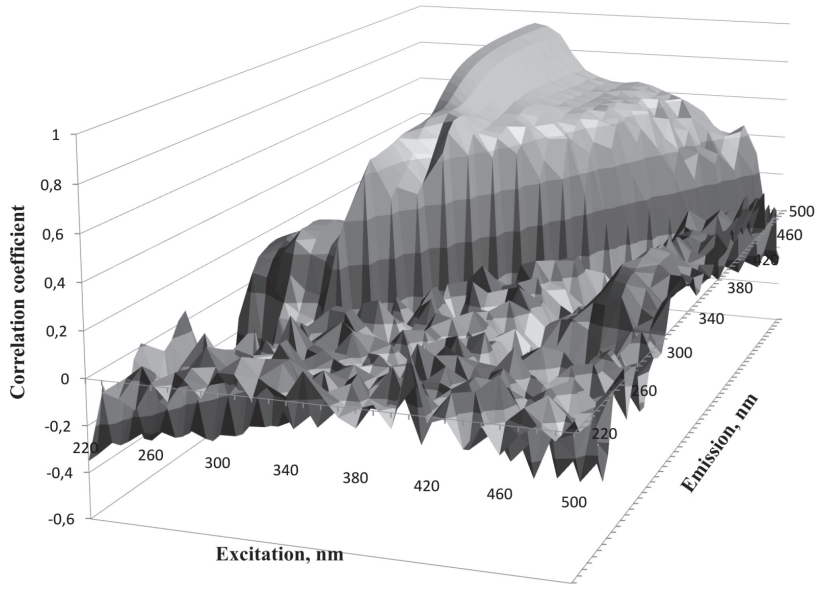

Fig 4. Dependence of the correlation between fluorescence intensity of spent dialysate and 4-pyridoxic acid concentration on the excitation-emission wavelength conditions.

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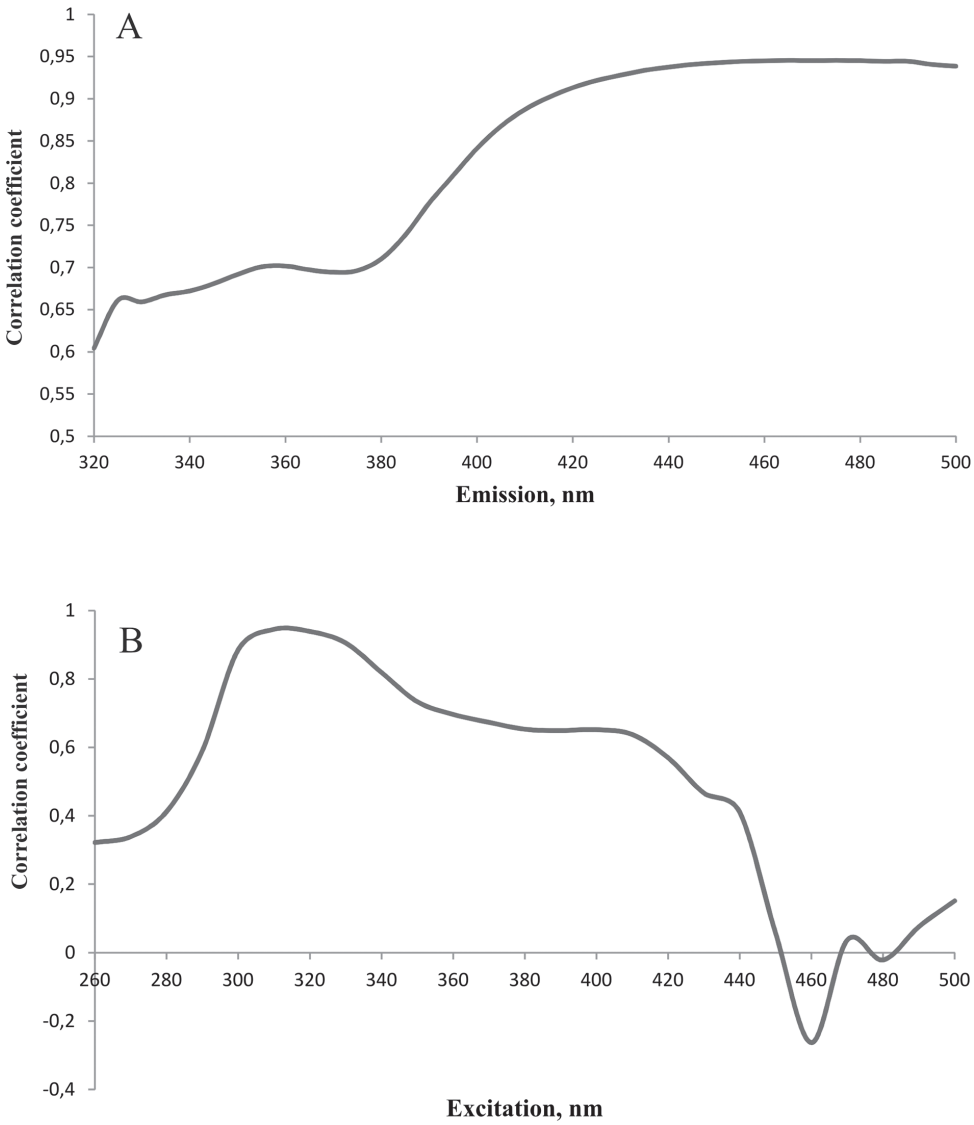

Fig 5. Correlation between fluorescence intensity of spent dialysate and 4-pyridoxic acid concentration at Ex310 nm (A) and at Em460 (B). The best correlation was found with Ex310/Em460 nm (R_{max} value of 0.95, N = 195).

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Discussion

As vitamin B6 is commonly used in the treatment of patients of chronic kidney disease, the monitoring of the vitamin status of the patient is important. Our present study confirms that measurement of fluorescence in spent dialysate may be useful for assessment of elimination of

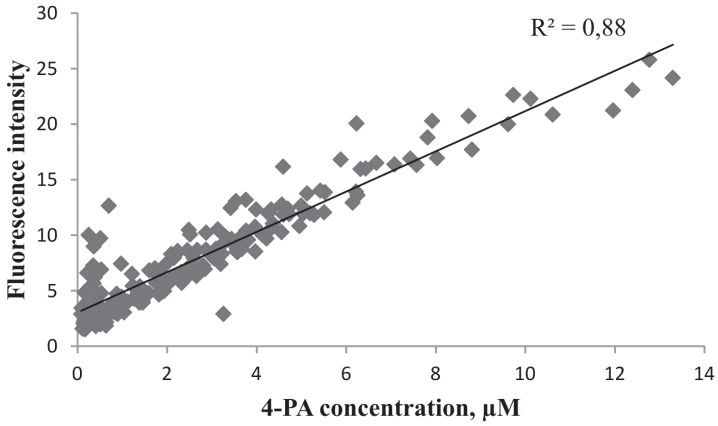

Fig 6. An example of the regression line between concentration of 4-PA in spent dialysate and fluorescence intensity (Ex310/Em460 nm, p <0.0001, N = 195).

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4PA, as the main metabolite of B6, from the blood of dialysis patients receiving regular B6 treatment.

PLP, the main active form of B6 is mostly used to evaluate the status of B6. However plasma PLP levels are low in dialysis patients [21,25] and thus laborious to measure. As plasma 4-PA concentrations are elevated in renal disease it might be used as an indicator of renal function [22]. Moreover 4-PA is not protein bound unlike most of PLP and pyridoxal [26] thus making measurements easier. Another potential benefit of monitoring 4-PA could be the correlation between 4-PA and other compounds like creatinine and urea [27], plasma PLP and homocysteine [21,28–30], whereas higher homocysteine levels are a risk factor for vascular disease [19,22]. Correlation between above mentioned compounds and plasma 4-PA as well as advanced glycation end product pentosidine [21] makes 4-PA potentially a versatile marker for the assessment of multiple aspects of health status of the dialysis patient and not only the B6 catabolism. We studied the possibility to monitor the elimination of 4-PA from the blood of a dialysis patient by fluorescence measurements in the spent dialysate.

To our knowledge, B6 catabolite 4-PA has not been measured in spent dialysate so far. Many 4-PA measurement methods consist of several derivatization steps to enhance fluorescence intensity [19,26]. Cabo *et al.* [26] have done an extensive review on different measuring methods where 4-PA concentration can be measured in down to nanomolar quantities. The drawback of those methods is that they have many preparation steps and use toxic reagents. The results of this study show that 4-PA concentrations in spent dialysate of ESRD patients are high enough (micromolar range) (Fig 6) for direct fluorescence measurements. The pyridoxic acid has been found in different forms in human plasma and urine, as 4-PA and 4/5-pyridoxolactone [31] with different fluorescent characteristics [32]. Contrariwise to these observations we could not find pyridoxolactone on our MS chromatograms of dialysate. Therefore, we cannot connect comparatively long wavelength interval of correlation between emission and 4-PA content in dialysate (Fig 5A) with possibility of lactonization of the 4-PA. Some lactonization has been found to take place also in the time of laboratory manipulations of plasma samples [21]. Correlation between 4-PA and pentosidine content has been found in plasma [21]; it cannot be excluded that some AGE compound with emission up to 460 nm may be involved in

dialysate emission together with 4-PA in dialysate also. Nevertheless quite good correlation between 4-PA concentration and fluorescence of spent dialysate (Figs 4–6) was obtained.

The results of this study indicate that the majority of the fluorescence signal (Ex320/Em430 nm) derives from 4-PA, which is the biggest contributor to the fluorescence signal with 42.2 ±17.0% to the total fluorescence intensity at the beginning of the dialysis and 47.7±18.0% at the end (Table 2, Figs 2 and 3). Also it has to been taken into account that there is inter- and intraindividual variation of 4-PA concentration and fluorescence intensity in the spent dialysate. The most suitable fluorescence regions for 4-PA assessment with sufficiently high correlation ($R>0.88$) appear to be in the wavelength region Ex310-330/Em415-500 nm (Figs 4 and 5) with the highest correlation ($R_{max} = 0.95$, $p<0.0001$) at Ex310/Em460 nm. The maximum correlation wavelength may be different from the 4-PA maximum wavelength of Ex320/Em425nm (Fig 1) due to the different proportion of fluorophores and their change during dialysis.

As 4-PA may be a potentially versatile marker that may help assess multiple aspects of health status of the dialysis patient an easy measurement method would be beneficial for the patient and hospital staff. Our previous study showed that spent dialysate provides a good substitution for blood for diagnostic analyses [33]. Sampling is easier, it does not disturb the patient and the analysis is simpler. This study suggests that 4-PA can be followed by simple direct fluorescence measurement also and provide hospital staff an opportunity for real-time monitoring of 4-PA elimination of the ESRD patient currently treated as well as follow the tendency of changing of the status during long-time dialysis treatment of the particulate patient.

Consequently, on-line fluorescence measurements in the region of Ex310-330/Em415-500 nm could potentially help for assessment of the status of the vitamin B-6 metabolism of ESRD patients during regular dialysis treatment.

The limitations of this study were relatively small data material (only 10 dialysis patients during 40 dialysis sessions were included) and the study did not cover heterogeneity of total dialysis population. The possible role of AGE-s in 4-PA-linked fluorescence as well as the possibility of not-radiative energy transfer (FRET) between fluorophores in dialysate need to be explained for final interpretation of the wavelength shift on the correlation graph (Fig 5A) compared to emission maximum of the 4-PA itself.

Conclusion

The 4-pyridox acid (4-PA) appeared to be the highest contributing peak of the HPLC chromatogram measured in the wavelength of Ex320/Em430 nm. The intensity of the fluorescence in the region Ex310-330/Em415-500 nm has high ($R>0.88$) correlation with 4-PA concentration in spent dialysate. It can be concluded from these observations, that 4-PA elimination from the blood of end stage renal disease patients can be potentially followed using monitoring of the fluorescence of the spent dialysate during regular dialysis treatment.

Supporting Information

S1 File. Includes the following: Figure A: data for normalized emission spectra of spent dialysate and 4-pyridoxic acid at excitation of 320 nm. Figure B: data for an averaged Ex320/Em430 nm chromatogram of the spent dialysate. Table A: data for mean contribution calculations. Figure C: dependence of the correlation between fluorescence intensity of spent dialysate and 4-pyridoxic acid concentration. Figure D: Data for the correlation between fluorescence intensity of spent dialysate and 4-pyridoxic acid concentration at Ex310 nm and at Em460. Figure E: Data of an example of the regression line between concentration of 4-PA in spent dialysate and fluorescence intensity.
(XLSX)

Author Contributions

Conceptualization: IF ML.

Formal analysis: SK.

Investigation: SK RT JA RT IF ML.

Methodology: SK RT JA IF ML.

Resources: RT.

Writing – original draft: SK RT JA RT IF ML.

Writing – review & editing: SK IF RT.

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Publication II

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Optical Measurement of 4-Pyridoxic Acid in the Spent Dialysate: Algorithm Development

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Abstract— **The aim of this study was to develop an algorithm suitable for the estimation of 4-pyridoxic acid (4PA) concentration removed during dialysis through fluorescence measurements in spent dialysate. 4PA is the major urinary catabolite of vitamin B-6 (B6), that is regulary injected to dialysis patients. 4PA could be used to estimate B6 nutritional status of the patients. For the study 10 hemodialysis patients during 40 dialysis sessions were studied. Full fluorescence spectra of spent dialysates were recorded with the spectrofluorophotometer RF-5301 by Shimadzu (Kyoto, Japan) and 4-pyridoxic acid concentrations were measured with HPLC system. Two single wavelength models were developed were correlation coefficients for Ex/Em 320/430 nm were 0.931 and 0.853 and for Ex/Em 310/460 nm were 0.944 and 0.931 for calibration and validation set, respectively. In summary, 4PA concentration can be estimated through fluorescence measurements from spent dialysate.**

Keywords—4-pyridoxic acid; dialysis; model; algorithm

I. INTRODUCTION

The major urinary catabolite of vitamin B-6 (B6) is 4-pyridoxic acid (4-PA) [1]. Vitamin B-6 itself is the term for a group of interconvertible molecules that contain pyridoxine, pyridoxal, pyridoxamine and their phosphates. As vitamin deficiency in chronic kidney disease patients is common, B6 is regularly administered to dialysis patients [2-4]. It has been pointed out that lower B6 concentrations increase the risk of renal dysfunction [5] and coronary artery disease [6-8]. Thus it is important to treat patients with B6 but potential toxicity of larger doses has to be considered as a study found that decreased excretion of B6 metabolites might increase the likelihood to pyridoxine toxicity [9]. In renal failure patients the toxic level of pyridoxine is thus far unknown but it has been shown that pyridoxine neuropathy can develop already after 200 mg/day [10] or even at 100 mg or less [11]. However there have been raised questions about the validity of the study [11] that reporter adverse effects after 100 mg of pyridoxine. Dialysis patients often receive B6 treatment regularly up to 100 mg per session *i.v.* Therefore there is a need for easy and efficient measuring method for assessment of the metabolism of B6 in these patients. Pyridoxal-5'-phosphate (PLP), the main active form of B6 is most frequently analyzed in B6 metabolic studies [12,13]. Recently it has been proposed that other vitaminers and 4PA could be used [13]. In healthy individuals it is expected that 40-60% of ingested B6 is oxidized to 4PA [14,15]. Therefore the urinary excretion of 4PA could potentially be used to estimate B6 nutritional status and after further studies possibly pyridoxine toxicity.

4PA is mainly measured using HPLC with different modifications such as post-column derivatization [16,17] which requires a lot of time, chemicals and blood from the patient. Preliminary studies [18] have shown the possibility that 4PA could be measured and monitored online form spent dialysate using fluorescence intensity, which does not require toxic chemicals and is not laborious.

The aim of this study was to develop an algorithm suitable for the estimation of 4PA concentration removed during dialysis through fluorescence measurements in spent dialysate. The model could potentially be used to help assess B6 nutritional status.

II. SUBJECTS AND METHODS

A. Subjects

The study was approved by the Regional Ethical Review Board, Estonia, and an informed written consent was obtained from all participating patients. Ten patients (age 59 ± 15 years) during 40 dialysis sessions as total were studied. 100 mg B6 was injected to patients after each studied dialysis session. The dialysis machine used was Fresenius 5008H (Fresenius Medical Care, Germany), dialyzers were FX8 or FX1000, the dialysate and blood flow varied from 500 - 800 mL/min and 300 - 350 mL/min, respectively. During each dialysis five samples were collected 7 (or 10), 60, 120, 180, 240 minutes after the start of the dialysis session from the outlet dialysate line (150 HD and 50 HDF samples in total).

B. Sampling and laboratory analysis

4PA concentration was determined by high pressure liquid chromatography (HPLC) measurements. The HPLC system consisted of Ultimate 3000 Series instruments from Dionex (Sunnyvale, CA, USA and column of Kinetex C18 100A column (Phenomenex, USA). Chromatographic data was processed with Chromeleon 7.1 software by Dionex

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Thermo Scientific (Waltham, USA). All dialysate samples were acidified down to pH 4.25 with formic acid before the HPLC analysis as it gave the best separation of compounds. For the chromatographic analysis two-component eluent was used as mixture of A: 0.05 M formic acid adjusted to pH 4.25 with ammonium hydroxide and B: the mixture of methanol and acetonitrile in the volume ratio of 9:1, both HPLC-grade from Rathburn (Walkerburn, Scotland). All the analysis' were done at room temperature.

Full fluorescence spectra of spent dialysates in the range of excitation/emission 220-500 nm and emission with excitation increment 10 nm were recorded with the spectrofluorophotometer RF-5301 by Shimadzu (Kyoto, Japan). The cell with optical path 4 mm was used for measurement and the Panorama Fluorescence 1.2 software by Shimadzu for spectral data processing.

Before the analysis some of the measured values were eliminated from the data. The elimination criteria were illogical or incorrect values of the measured concentration of fluorescence.

C. *Data analysis*

Regression analysis was used to acquire a single wavelength (SW) model for the 4PA concentration assessment through fluorescence intensity measurements. Independent variables included were fluorescence intensity values at the wavelengths Ex/Em 320/430 nm as it is the fluorescence maximum of 4PA, and Ex/Em 310/460 nm as preliminary study found a good linear relationship between fluorescence intensity and 4PA concentration in spent dialysate [18]. Regression analysis was chosen because it was presumed that independent variables are linearly independent, and that 4PA concentration is related to fluorescence intensity. The data was split into calibration set (50% of the data) which was used to develop the algorithm and validation set (50% of the data) which was used to validate the algorithm.

Systematic error (*BIAS*) was calculated as follows:

$$BIAS = \frac{\sum_{i=1}^{N}(c_{average} - c_i)}{N} \quad (1)$$

Where c is the concentration of 4-PA and N is the number of observations [19].

Standard error (SE) was calculated by the following formula [19]:

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

For the analysis StatPlus (AnalystSoft Inc. for Mac) and Excel (version 2011 for Mac) were used. Correlation coefficient (*r*) shows the relationship of the two variables – 4PA concentration measured with HPLC and with the model. *P-value* of <0.05 was considered statistically significant.

III. RESULTS

The scatter plot of 4PA concentration predicted by the SW model on the wavelength Ex/Em 320/430 nm can be seen on figures 1A and 1B, and second model on the wavelength Ex/Em 310/460 nm can be seen on figures 2A and 2B, where A is the calibration set and B validation set. The intensity measurement errors are not graphically represented.

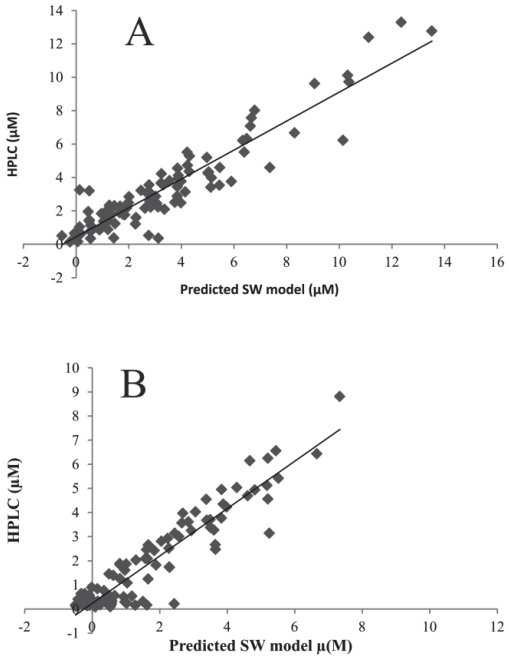
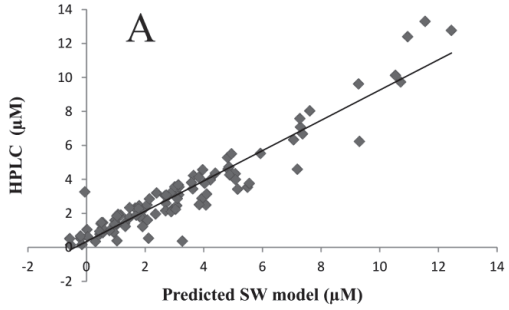


Fig. 1. 4-pyridoxic acid concentration determined by HPLC measurement and by the Ex/Em 320/430 nm model for the calibration (A) and validation (B) set, respectively.



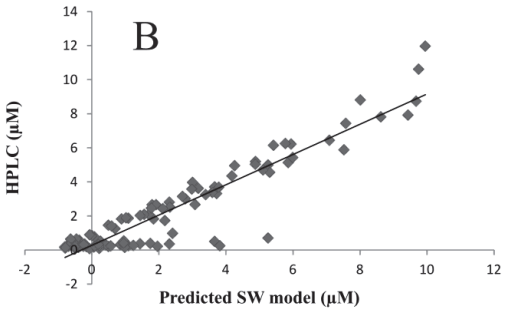


Fig. 2. 4-pyridoxic acid concentration determined by HPLC measurement and by the Ex/Em 310/460 nm model for the calibration (A) and validation (B) set, respectively.

The number of cases (*N*) and correlation coefficients (*r*) of the model can be found in table 1.

TABLE I. NUMBER OF CASES, CORRELATION COEFFICIENTS, COEFFICIENT OF DETERMINATION OF THE SW MODEL FOR THE CALIBRATION AND VALIDATION SET.

Model	Set	*N*	*r*
Ex/Em 320/430 nm	Calibration	100	0.931
	Validation	99	0.853
Ex/Em 310/460 nm	Calibration	98	0.944
	Validation	97	0.931

The systematic and standard errors for the models are presented in table 2. The BIAS values for both validation set were similar (*p-value*=0.0001). The SE values for calibration and validation set for both the models were not statistically different (*p-value*=0.01 and *p-value*=0.04, respectively).

TABLE II. SYSTEMATIC AND STANDARD ERRORS ON THE MODEL FOR THE CALIBRATION AND VALIDATION SET.

Model	Set	*BIAS, µM*	*SE, µM*
Ex/Em 320/430 nm	Calibration	0.00	1.23
	Validation	0.55	1.95
Ex/Em 310/460 nm	Calibration	0.00	0.88
	Validation	0.02	1.02

IV. DISCUSSION

The aim of this study was to develop a single wavelength (SW) algorithm for the optical 4PA concentration estimation in spent dialysate.

As vitamin B6 is regularly administered to dialysis patients, it is important to monitor the vitamin status of the patient. Mostly the main active form of B6, pyridoxal-5'-phosphate, is used to evaluate the status of the vitamin eventhough it is laborious to measure [16]. The benefit of this SW algorithm lies in the possibility to determine the nutritional status of B6 and its metabolites. Additionally this method is quicker and safer as compared to some other methods [16,17] as it does not require toxic chemicals nor derivatization. For determing the nutritional status of pyridoxine through 4PA measurements further studies are needed to determine how much pyridoxine is oxidized to 4PA and removed by dialysis as 4PA is also eliminated by the kidneys. In this study residual renal function of the patient was not taken into account.

A regression analysis was obtained for assessing 4PA concentration through fluorescence measurement. Our unpublished study showed that the wavelength pair of Ex/Em 320/430 nm described the 4PA fluorescence maximum, but Ex/Em 310/460 nm gave the best correlation on 4PA concentration and fluorescence intensity [18]. For Ex/Em 310/460 nm model higher *r* values were obtained (table 1). Although low BIAS values were obtained for calibration set, statistically similar (*p-value* =0.91) BIAS values were achieved for validation set for both models.

Larger variance at low 4PA concentrations can be seen on the scatter plots of 4PA concentration determined by the HPLC measurements and by the SW model (Figs. 1,2). The variance may be conditioned due to other interfering substances that have fluorescence at the same wavelengths. Also the model underestimates 4PA concentrations at lower values, which could be due to the large variance of data. To improve the model a larger calibration and validation set would be needed to test the model.

V. CONCLUCION

The results indicate that it is possible to determine the 4PA concentration in spent dialysate using fluorescence measurements. Although measurement accuracy needs improvement at low concentrations, the method itself gives a fast and good estimation of 4PA concentrations in spent dialysate of dialysis patients without blood and laboratory measurement.

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Publication III

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Methods

Blood Purification

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Free Pentosidine Assessment Based on Fluorescence Measurements in Spent Dialysate

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Keywords
Pentosidine · Spent dialysate · Fluorescence · Optical monitoring · Dialysis

Abstract
The aim of this study was to primarily explore the relationship between free pentosidine and the fluorescence properties of spent dialysate, and also to develop a model to assess the levels of free pentosidine in spent dialysate based on the fluorescence measurements. First, 40 patients (20 females and 20 males) were examined during 40 dialysis sessions. High-pressure liquid chromatography (HPLC) was used to measure the free pentosidine concentrations from the spent dialysate. The full fluorescence spectra of the spent dialysates were recorded and single- and multi-wavelength (MW) models were developed. The average free pentosidine concentrations in the spent dialysate measured by HPLC at the start and end of the dialysis session were (mean ± SD) 4.25 ± 3.11 and 0.94 ± 0.69 μg/L respectively. The removal ratios (RRs) between RR_lab and RR_MW were statistically similar ($p > 0.2$). The concentration of free pentosidine and the RR can therefore be estimated from the spent dialysate when utilising fluorescence measurements.

Introduction

Sell and Monnier first described pentosidine in 1989; it consists of arginine and lysine residues that are cross-linked by pentose [1]. This specific compound is considered to be a uremic toxin and is listed in the protein-bound group of the uremic toxins database [2].

Pentosidine, being a glycoxidation product of proteins, belongs to the advanced glycation end products (AGE) group [3]. Different AGEs tend to accumulate in uremic patients' plasma as a result of oxidative stress and decreased excretion. The accumulation of AGEs in chronic kidney disease (CKD) patients may lead to different complications [4]. In a healthy individual's blood plasma, only 3–4% of the total pentosidine is in the free form [5, 6] but the concentration of free pentosidine will start to increase in patients with declined kidney function [2, 7]. Only a negligible part of the AGE-modified proteins, like amyloid beta-2-microglobulin, can penetrate the dialysis membrane, and therefore, up to 79–84% of the pentosidine in dialysate can be discovered in the free form in CKD patients [8]. The concentration of plasma pentosidine in CKD patients depends on the dialysis modality and on other contributors that increase plasma pentosi-

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Table 1. HPLC gradient programme

Step	Time, min	Buffer A, %	Buffer B, %	Curve type
0	0	99	1	5
1	4	99	1	5
2	34	10	90	8
3	44	10	90	5

dine levels in different stages of CKD [9]. As AGEs are potential uremic toxins [10], monitoring the removal of AGEs during dialysis could be used as an indicator of dialysis therapy. One parameter that could be used to assess the adequacy of dialysis is the pentosidine removal ratio (RR). For RR assessment, the pentosidine levels are needed; however, there is no simple way to measure it. Pentosidine is mainly measured by high-pressure liquid chromatography (HPLC) [11] using reverse-phase C18 column, acetonitrile (AcN)-water eluents [1, 3, 7, 11, 12] combined with ion-pair modifiers heptafluorobutyric acid (HFBA) [1, 3] or trifluoroacetic acid [11]. As an alternative, the porous graphite carbon column has been used [13]. Plasma pentosidine can also be measured with enzyme-linked immunosorbent assay. Both enzyme-linked immunosorbent assay and reverse-phase-HPLC are too laborious to use in clinical practices and do not give adequate information about the ongoing dialysis session.

Real-time optical measurement of spent dialysate, utilising UV-absorbance, has been proposed to monitor uric acid as the marker for small molecule weight water-soluble compound removal, and fluorescence has been used to monitor indoxyl sulfate as the marker for protein-bound solute removal during the dialysis treatment of CKD patients [14, 15]. However, neither of these markers can be utilised to assess the status and removal of AGEs during the dialysis treatment of the CKD patients, as the association of the aforementioned marker substances with AGEs is not yet known. Being the recognised marker of AGEs and easily detectable by fluorescence [1], the free pentosidine in dialysate may be of interest as a possible molecule to fill in this gap in the optical real-time monitoring of dialysis treatment.

The aim of this study was to examine the relationship between free pentosidine and the fluorescence properties of the spent dialysate and to develop an algorithm to assess the concentration of the free pentosidine in the spent dialysate based on fluorescence measurements.

Materials and Methods

Subjects

After the study protocol was approved by the Regional Ethical Review Board, Estonia, 40 patients, aged 61.3 ±16.3 (mean ± SD) years were examined during 40 dialysis sessions. Twenty patients were male and 20 were female. Ten subjects were smokers and 11 had diabetes. The study was carried out in an agreement with the Declaration of Helsinki and approved by the Tallinn Medical Research Ethics Committee at the Estonian National Institute for Health Development, Estonia (decision no. 2349). A written informed consent was obtained from all participating patients.

Thirty-three patients received haemodiafiltration and 7 haemodialysis (HD) treatment. Fresenius 5008 or 4008H (Fresenius Medical Care, Germany) machine was used for the dialysis. Dialysers were FX8, FX100, FX800 and FX1000 and the dialysate and blood flow varied from 204 to 800 and 170 to 360 mL/min respectively. Treatment durations varied from 180 to 247 min.

Sampling and Laboratory Analysis

During each dialysis, 2 samples were collected from the outlet dialysate line – at the start and end of the dialysis session.

HPLC was used to measure the free pentosidine concentrations. The HPLC system consisted of the Ultimate 3000 Series instruments from Dionex (Sunnyvale, CA, USA) and of Kinetex core-shell column C18 2,6 µm 100Å 150*4.6 mm with the guard AJ0-4287 4*3 mm (both Phenomenex, USA). Chromatographic data processing was done with Chromeleon 7.1.2.1478 software by Dionex Thermo Scientific (Waltham, MA, USA). For the analysis, HPLC grade AcN (Honeywell, USA) and HFBA (Sigma-Aldrich, USA) solvents were used. The solvent flow was 0.8 mL/min and the column temperature was 35.0 ± 1.0 °C. All spent dialysate samples were acidified down to pH 4.25 with formic acid before the HPLC analysis. A two-component eluent was used for the chromatographic analysis as mixture of A: 0.050M HFBA in MilliQ water and B: 0.005M HFBA in AcN. The 3-step linear gradient elution program was used (Table 1).

The pH of spent dialysate was lowered to pH 4.25 only for the measurement of the pentosidine concentration (C_{Lab}) with HPLC. All the other measurements were done in the dialysate buffer in order to exclude the effects of different pH on the results of the derived models.

The chromatograms were recorded with the RF 2000 fluorescence detector Dionex (Sunnyvale, CA, USA) using excitation 330 and emission 373 nm. Pentosidine with purity >98% (Cayman Chemical [Ann Arbor, MI, USA]) was used as a reference for building up the calibration curve. The concentration of the calibration solution was estimated on the basis of the UV-absorbance using molecular extinction coefficient of 4522 arbitrary unit (AU)/([mol/L] cm) [1]. Analytical reproducibility of the laboratory measurement method (HPLC) was ±5%, estimated on the basis of double analyses on 24 samples from 12 randomly chosen patients; interpatient variability was ±81%, estimated from the standard deviation of the results from the start of the dialysis samples.

The UV-absorbance spectra of dialysates were recorded with a double-beam spectrophotometer (Shimadzu UV-2401 PC, Japan) over the wavelength range of 190–380 nm using a quarts cuvette

with an optical path length of 4 mm. The full fluorescence spectra of spent dialysates in the range of excitation/emission 220–490 nm, with excitation increment 10 nm, were recorded with the spectrofluorophotometer RF-5301 by Shimadzu (Kyoto, Japan). Measurements were done with an quarts cuvette of 4 mm optical path length and data processing was done with the Panorama Fluorescence 1.2 software by Shimadzu.

Before the analysis, 8 incorrect (e.g., due to self-tests of the HD machine) values, or some pentosidine concentration values, where peak symmetry disturbance on the HPLC chromatogram indicated coelution of any unidentified compound alongside pentosidine, were eliminated from the data.

Data Analysis

Forward stepwise regression analysis was used to acquire a single wavelength (SW) and a multi-wavelength (MW) model for the assessment of the free pentosidine concentration through fluorescence intensity measurements. Included independent variables were fluorescence intensity values at the wavelengths Ex/Em 220–490. The formula for SW model was:

$$a + (b*EX/EM) \quad (1)$$

where a and b are the intercept and slope values derived from the calibration curve at Ex350/Em363 nm.

The formula for MW model was:

$$a + (b_1*EX/EM_1) + (b_2*EX/EM_2) + (b_3*EX/EM_3) \quad (2)$$

where a and b are the intercept and slope values derived from the calibration-validation curve at EX/EM_1 was Ex350/Em363 nm, EX/EM_2 was Ex250/Em321 nm, EX/EM_3 was Ex430/Em463 nm.

It was presumed that the free pentosidine concentration was related to fluorescence intensity and was linearly independent. The data was divided into a calibration set (75% of the data), which was used to develop the algorithm, and a validation set (25% of the data), which was used to validate the algorithm.

Systematic error (*BIAS*) was calculated as follows:

$$\text{BIAS} = \frac{\sum_{i=1}^{N}(C_{\text{Lab},i} - C_i)}{N} \quad (3)$$

where $C_{Lab,i}$ is the i-th concentration of free pentosidine in spent dialysate, calculated based on HPLC results, C_i is the i-th concentration of free pentosidine in spent dialysate, calculated with the optical model, and N is the number of observations [15].

Standard error of performance (SE) was calculated using the following formula [16]:

$$\text{SE} = \sqrt{\frac{\sum_{i=1}^{N}(e_i - \text{BIAS})^2}{N-1}} \quad (4)$$

where e_i is the difference of lab and model concentration for the i-th measurement.

SpektroModel software [17] was used for the analysis of data designed to generate regression models from the spent dialysate fluorescence spectra for predicting concentrations of uremic solutes. Excel (version 2016 for Mac and Windows) was used for the graph generation.

The reduction ratio of free pentosidine for a dialysis treatment was calculated from the start (C_{start}) and end (C_{end}) concentrations of the dialysis session as:

$$\text{RR}(\%) = \frac{C_{start} - C_{end}}{C_{start}} \times 100 \quad (5)$$

RR was calculated based on laboratory values (RR_lab), as well as from the concentrations estimated by the SW model (RR_SW) and MW model (RR_MW). The Student's t test was applied to compare the means for RR_lab, RR_SW and RR_MW and $p < 0.05$ was considered significant. The Bland-Altman analysis [18] was used to examine the individual differences in RR_SW and RR_MW compared to RR_lab values.

Results

An example of the HPLC chromatograms of the spent dialysate of patient #22 and of standard pentosidine can be seen on Figure 1.

Average free pentosidine concentration at the start and at the end of the dialysis sessions are shown in Table 2. The high standard deviation can be explained by the high variation of pentosidine concentration in the spent dialysate of the patients. The reason for it may be different types of nutrition, diagnoses, and so on. Average pentosidine concentrations are not statistically different, when comparing SW and MW model values with lab values (Table 2).

Figure 2 depicts the fluorescence spectrum of standard pentosidine on the surface contour map. The surface contour map shows on the x-axis and y-axis the emission and excitation wavelengths of standard pentosidine, respectively. The emission intensity (AU) is shown with different grey scales. It can be seen that the fluorescence maximum is at the wavelength range of Ex320–330/Em360–385 nm.

Figure 3 shows the correlation coefficient (R) values between the fluorescence of the spent dialysate over the wavelength of Ex220–480/Em230–490 nm and the concentration of free pentosidine (HPLC results). It can be seen that the highest correlation between the fluorescence of the spent dialysate and the free pentosidine concentration is between the wavelength region Ex310–350/Em360–385 nm. The correlation maximum is at Ex350/Em363 nm.

For the SW model, the wavelength pair of the correlation maximum (Ex350/Em363 nm) of the spent dialysate and the concentration of the free pentosidine was chosen. The scatter and Bland-Altman plots of the free pentosi-

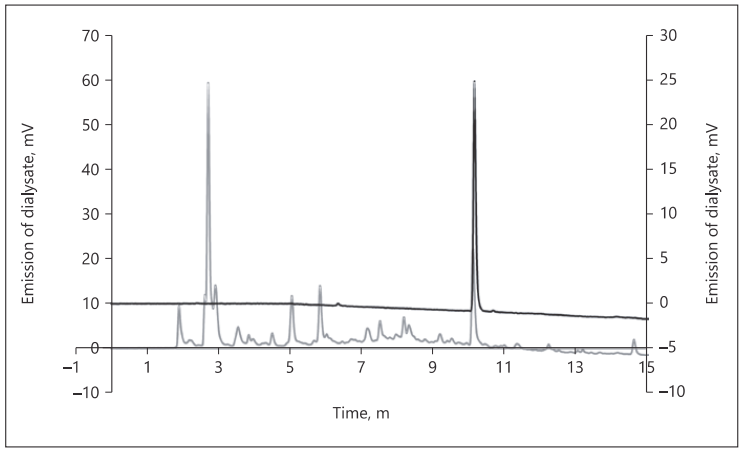

Fig. 1. Fluorescence chromatogram (Ex330/Em373 nm) of the spent dialysate (patient #22, grey line) compared to the chromatogram of standard pentosidine (black raised line).

Table 2. Average free pentosidine concentrations (mg/L) of the dialysis sessions (n = 36)

		Lab, mean ± SD	SW, mean ± SD	p value* (Lab-SW)	MW, mean ± SD	p value* (Lab-MW)
Calibration	Start	4.25±3.11	4.47±2.65	0.81	3.94±3.24	0.98
	End	0.94±0.69	0.76±0.75	0.55	1.02±0.69	0.67
Validation	Start	4.50±4.01	4.82±2.85	0.85	4.58±3.27	0.96
	End	1.07±0.85	0.69±0.25	0.22	1.21±0.69	0.72

* Calculated with Student's t test where significance level is 0.05.

dine concentration, predicted by the SW model at the wavelength of the correlation maximum, can be seen in Figure 4a and b. The SW model gave correlation coefficient R of 0.966 and 0.943 for cal and val sets, respectively.

The MW model includes the fluorescence wavelengths Ex350/Em363 nm, Ex250/Em321 nm and Ex430/Em463 nm. Figure 4c and d shows the scatter and Bland-Altman plots of the concentration of free pentosidine predicted by MW model for the calibration and validation set. The MW model gave even a little higher correlation coefficient R of 0.985 and 0.962 for cal and val sets, respectively, than the SW model.

Detailed statistical information of the developed models for calculating free pentosidine in the spent dialysate is presented in Table 3. For the calibration and validation sets, the highest R and R^2 values were obtained with the MW model. However, BIAS was the lowest for the SW model. SE was the lowest for the MW model for both sets. SE values between the SW and MW models were statistically different (p = 0.03) for calibration set but not for the validation set (p = 0.54).

The average RR of free pentosidine for cal and val set can be seen in Table 4. The RRs between RR_lab and RR_MW were statistically similar (p > 0.2 cal and val set). The differences between RR_lab and RR_SW and between RR_lab and RR_MW can be seen from the Bland-Altman plot on Figure 5a and b.

Discussion

The relationship between free pentosidine and the fluorescence properties of the spent dialysate was studied. The SW and MW emission models were explored to evaluate the free pentosidine concentrations in the spent dialysate and the RR during dialysis.

The main results obtained were as follows: (i) it is feasible to monitor the elimination of free pentosidine dur-

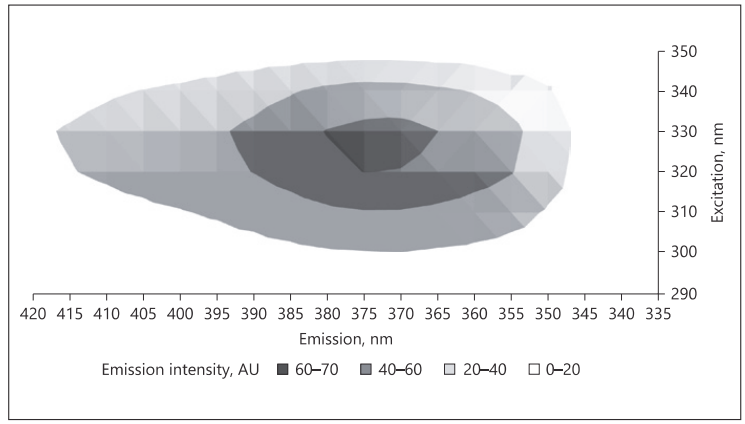

Fig. 2. Map of the fluorescence spectrum values of standard pentosidine in the dialysate buffer. The surface contour map shows on the x-axis and y-axis the emission and excitation wavelengths of standard pentosidine respectively. The emission intensity arbitrary unit (AU) has been shown with different colours. The maximum value of the fluorescence is 66 AU at Ex330/Em370 nm.

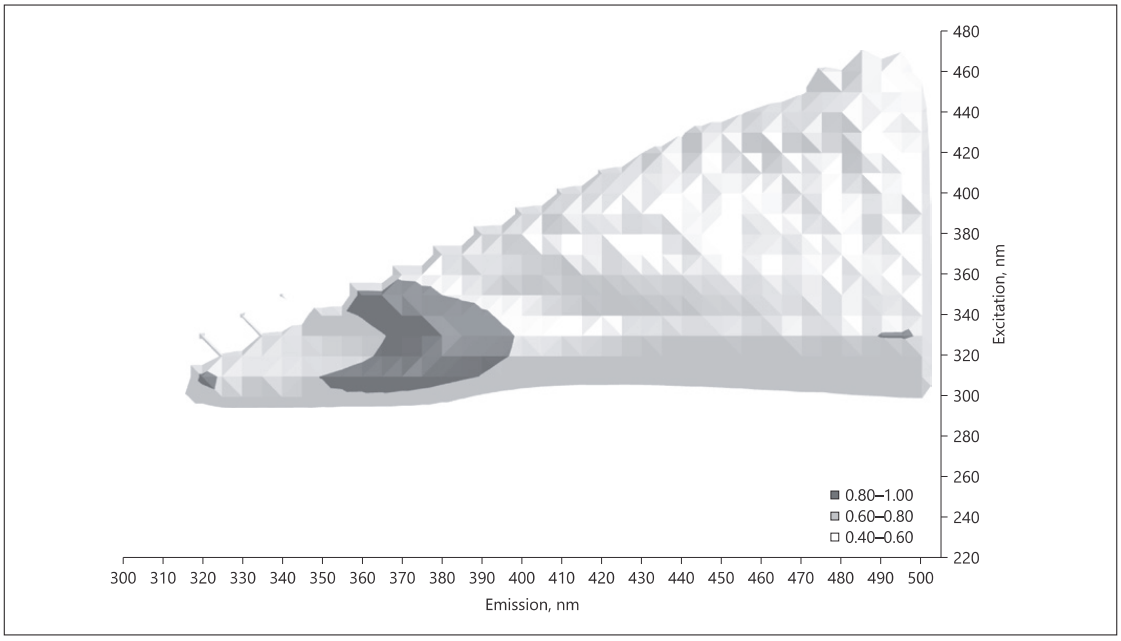

Fig. 3. Map of the correlation coefficient values between the fluorescence of spent dialysate and the concentration of free pentosidine in the dialysate buffer. The correlation maximum is at Ex350/Em363 nm.

ing dialysis by using the fluorescence of the spent dialysate even when the technique evidently measures several other fluorophores and chromophores in spent dialysate at the same time; (ii) the MW algorithm improves the estimation of the free pentosidine concentration and RR estimation accuracy in comparison with the SW algorithm.

Pentosidine is considered to be a uremic toxin [2] associated with inflammation [3, 9, 12], malnutrition [3], oxidative stress [9] and low GFR [9]. Pentosidine belongs

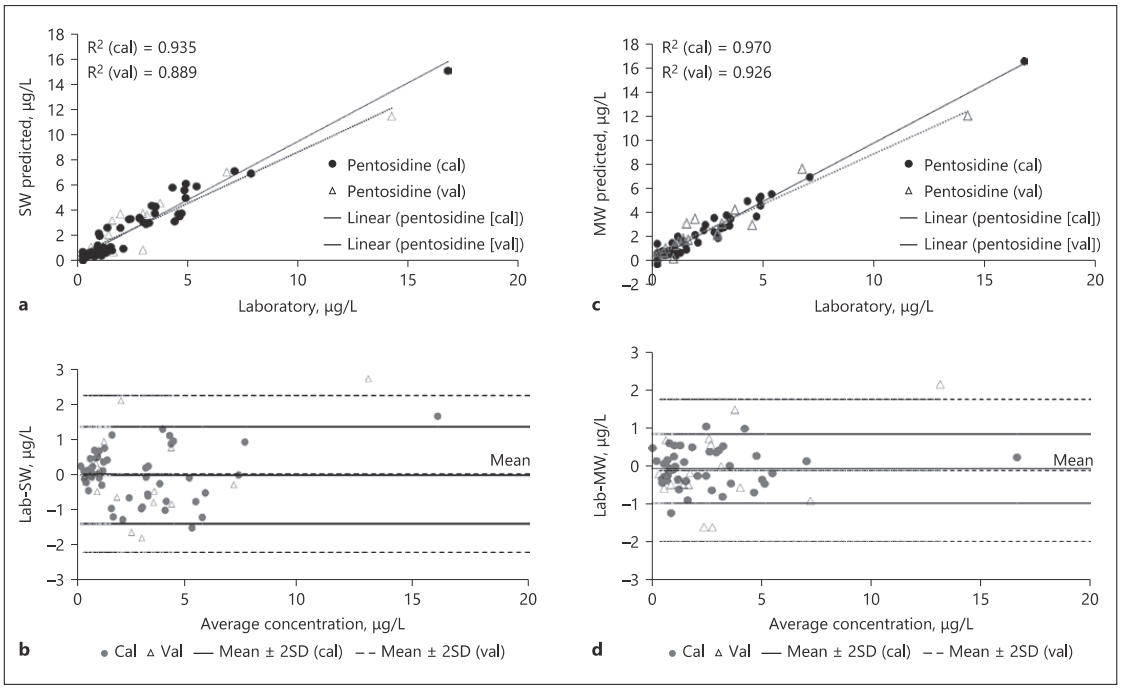

Fig. 4. Free pentosidine concentration determined at the laboratory and predicted by the SW (**a**) and MW (**c**) model, and Bland-Altman plot of the differences between Lab and SW (**b**), Lab and MW (**d**) concentrations. SW, single-wavelength; MW, multi-wavelength.

to the group of AGEs, in which biosynthesis is definitely associated with glycoxidation [19]. As plasma pentosidine correlates strongly with the levels of precursors of carbonyl compounds, it is perceived as a good marker substance for AGE and oxidative stress [12]. Pentosidine appears to be exceptional among other protein-bound uremic toxins and is also different from small water-soluble uremic solutes like uric acid, as pentosidine removal does not reflect the status in body fluids in the same way as it does for small water-soluble uremic solutes [20]. Also, in comparison with some other free AGEs, pentosidine has been found to be barely removable from serum by dialysis [21]. In spent dialysate, the concentration of free pentosidine hardly decreases from the start to the end of a dialysis session in comparison with marked decrease of many other free AGEs [22]. The overall average RR_Lab of free pentosidine 75.3% observed in this work does not confirm these observations. Different studies have also found the reduction ratio of free pentosidine to be more than 75% with HD [5, 23]. The difference of RR with the study by Agalou et al. [22] may be due to our small sample size, different measurement method, and/or dialysis treatment, and/or patient characteristics. The authors conclude from presented contradictions that the elimination of pentosidine by HD treatment of CKD patients deserves further and more profound research.

Recently, a device to measure skin autofluorescence (SAF) was developed to assess the AGE level of patients [24]. In mortality studies on CKD patients, no association between SAF and total plasma pentosidine has been found [9]. Our previous study [25] found a certain correlation between SAF and the fluorescence of spent dialysate. This observation opened an attractive prospect for the assessment of free pentosidine removal from CKD patients by the measurement of the fluorescence properties of dialysates as an easily available alternative to AGE removal. Two different assessment models were compiled for this purpose.

Regression analysis was used to estimate the performance of the SW and MW model for the assessment

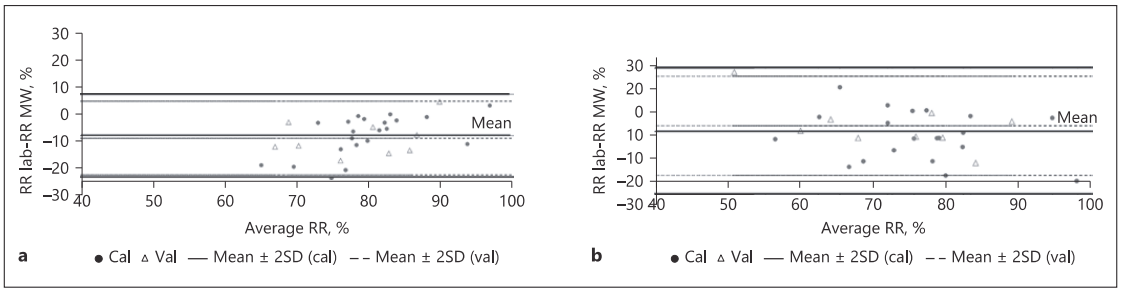

Fig. 5. Bland-Altman plot of the differences between (**a**) RR_lab and RR_SW and (**b**) RR_lab and RR_MW. The mean difference between RR_lab and RR_SW as BIAS ± SE was (–8.0 ± 7.5)% for the cal set and (–8.9 ± 6.8)% for the val set. The mean difference between RR_lab and RR_MW was (1.6 ± 13.6)% for the cal set and (4.1 ± 10.7)% for the val set.

Table 3. The number of cases (*n*), the systematic (BIAS), and standard (SE) errors of the models, correlation coefficients (*R*), and coefficients of determination (R^2) for the calibration and validation sets

	Set	*n*	BIAS	SE	*R*	R^2
SW	Calibration	53	0.000	0.702	0.966	0.934
	Validation	18	0.031	1.135	0.943	0.889
MW	Calibration	50	–0.055	0.464	0.985	0.970
	Validation	18	–0.109	0.962	0.962	0.926

Table 4. The average RR removal ratio of free pentosidine estimated with the SW and MW models (*n* = 30)

	RR_Lab, mean ± SD, %	RR_SW, mean ± SD, %	*p* value* (RR_Lab-RR_SW)	RR_MW, mean ± SD, %	*p* value* (RR_Lab-RR_MW)
Calibration	75.8±9.9	83.8±5.9	<0.0001	74.2±15.2	0.60
Validation	74.2±10.0	83.1±8.1	0.004	70.1±16.2	0.28

* Calculated with Student's *t* test where significance level is 0.05.

of the free pentosidine concentration from the spent dialysate by using fluorescence intensity measurements. Some patient-dependent parameters (blood and dialysate flow, BMI, gender, smoking, UF rate) were included into the model build-up but were discarded as not improving the estimation accuracy. The concentrations estimated with the SW and MW models were not statistically different ($p = 0.95$). However, the highest R^2 value for calibration and validation set was obtained with the MW model (Table 3). Also, the MW model had a lower standard error of performance values for the free pentosidine concentration estimation (Table 3) but higher SE values for the RR estimation (Table 4). This might be due to high inter-patient free pentosidine concentration differences at the start and end of dialysis.

The wavelength of the maximum fluorescence of the standard pentosidine observed in this work (Fig. 2) is in accordance with corresponding data in the literature (Ex330/Em373) [26]. The models include the fluorescence wavelength at the correlation maximum between the free pentosidine concentration and the fluorescence of the spent dialysate (Fig. 3). However, it did not exactly correspond to the fluorescence maximum value of

standard pentosidine (Figs. 2, 3). Evidently, there may appear interferences from other fluorophores, which shift the correlation maximum to a different wavelength pair. More samples should be used to improve and generalise the developed model. Also, additional factors that influence the level of free pentosidine should be included.

In spite of certain limitations, this preliminary study reveals that the MW optical method for the online measurements of the free pentosidine in the spent dialysate is feasible. In the future, online methods and models could be used to assess the dialysis adequacy to give immediate feedback to the clinician. Also, there is no need for blood and dialysate sampling because utilising the online method enables more frequent measurements. This method would give additional information about the adequacy of an ongoing dialysis session and elimination of AGEs, which accelerate atherosclerosis [4] and may have association with cardiovascular morbidity of the patients with CKD [27]. The method displays potential for further research of the peculiarities of the elimination of free pentosidine as an AGE and oxidative stress marker, through dialysis treatment of CKD patients.

Conclusion

This study reveals that the free pentosidine concentration estimation in spent dialysate and the removal assessment from CKD patients during dialysis, when using the measurements of fluorescence of spent dialysate, seems to be feasible and promising. The advantages of the method are that it is easily practicable, does not need blood sampling or chemicals, and is fast. As this study was limited by small data material, future studies are needed to validate and improve this method.

Acknowledgement

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Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

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Publication IV

Kalle, S., Kressa, H., Tanner, R., Holmar, J., Fridolin, I. (2014). Fluorescence of β_2-microglobulin in the Spent Dialysate, In: *16th Nordic-Baltic Conference on Biomedical Engineering, Gothenburg*, Sweden, October 14–16, 2014, IFMBE Proceedings, 48: 59–62 (DOI: 10.1007/978-3-319-12967-9_16)

Fluorescence of Beta-2-microglobulin in the Spent Dialysate

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Abstract— **The aim of the study was to determine if fluorescence chromatography can be used to measure modified beta-2-microgobulin (B2M) from the spent dialysate. Amyloid B2M is the main pathogenic component of dialysis-related amyloidosis. This component is in our sphere of interest being one of the fluorescent advanced glycation end products (AGE). AGEs are potential uremic toxins that can cause amyloidosis and cardiovascular problems in chronic kidney failure patients.**

Two haemodialysis patients with high levels of B2M were selected for this study. Their spent dialysate samples were collected 10 minutes after the start of the dialysis process and less hydrophilic compounds were concentrated using solid phase extraction (SPE) column. Sediment from the concentrate and spent dialysate were analysed with electrospray ionisation mass spectrometer (ESI-MS) MicrOTOF-Q II coupled to high pressure liquid chromatography (HPLC) Dionex UltiMate 3000 RS. The sediment was analysed with Poroshell 120 EC-C18 column and spent dialysate with Kinetex C18 100A column. MagTran was used to interpret mass spectra.

Brown coloured fluorescent sediment of the concentrate was identified as amyloid B2M on the basis of MS and fluorescence spectra. AGE modified B2M was also found from spent dialysate. However the fluorescence intensity was very low compared to overall fluorescence of spent dialysate.

In summary, the study revealed that the fluorescence of AGE modified B2M is possible to detect in spent dialysate. However, the measuring system needs high selectivity and sensitivity for detection due to low contribution of AGE modified B2M to overall fluorescence.

Keywords— **Advanced glycation, beta-2-microgobulin, fluorescence, mass spectrum, dialysate**

I. Introduction

Advanced glycation end products (AGEs) are a heterogeneous group of molecules that accumulate in uremic patients' plasma [1]. AGEs are formed when a carbonyl of a reducing sugar condenses with a reactive amino group in target protein [2]. Free AGEs probably originate mainly from the glycation of proteins and from food. AGEs accumulate as the result of decreased excretion and increased formation from oxidative stress. Accumulation of AGEs may be linked to the increased risk of cardiovascular disease in patients with renal failure [3]. Also, AGEs can predict diabetic microvascular complications [4].

To date, various AGEs have been identified e.g. N(6)-carboxymethyl lysine – the most used marker for AGEs in food analysis [5]. Our interest has captured beta-2-microglobulin (B2M) which can be advanced glycated. B2M is a polypeptide with a molecular weight of 11.8 kDa. It is present on the surface of most nucleated cells and in biological fluids [6]. The normal serum B2M concentration is 1.5–3 mg/l, and its average normal production rate has been estimated to be 2.4 mg/kg/day [7]. In patients with chronic kidney failure, B2M concentration increases as the glomerular filtration rate decreases. The accumulation of AGE modified B2M can lead to different health complications like dialysis-related amyloidosis [2,8].

AGEs are classified as being potential uremic toxins [9], therefore removal of AGEs can be used as an indicator of the dialysis therapy. B2M could be a marker to describe the elimination of middle molecular weight solutes [2] because levels of B2M are not affected by food intake [2]. As a group, AGEs are difficult to measure, but individual compounds have been analyzed. There is no universal method for AGE measurements. B2M is mostly measured via B2M human enzyme-linked immunosorbent assay (ELISA) kit or high pressure liquid chromatography (HPLC). In a health care setting these methods are expensive and the results cannot be used to modify an ongoing dialysis process. An online measuring method would be advantageous. Fluorescence detector is one device that could be used in online measurements.

The aim of the study was to determine if fluorescence chromatography can be used to measure AGE modified B2M from spent dialysate.

II. Materials and Method

This study was performed after approval of the protocol by the Regional Ethical Review Board, Estonia. An informed consent was obtained from all participating patients.

Two haemodialysis patients with high levels of B2M were selected for this study. Their spent dialysate samples were collected from the drain tube of the dialysis machine 10 minutes after the start of the dialysis session. Less hydrophilic compounds were concentrated from spent dialysate using C18 solid phase extraction (SPE) column. The concentration consisted of 5 phases: (1) column was washed

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with 120 ml of methanol (MeOH); (2) MeOH was washed away with 120 ml milliQ water; (3) 200 ml of dialysate was added; (4) 120 ml of milliQ water was used to wash away salts from sorbent; (5) 45 ml MeOH was added and the concentrate was collected. After that the concentrate was put to +6^{o}C storage and sedimentation occured. Sediment of the concentrate was dissolved in 3 ml of 4:1 urea (6 M): NaCl (9%) solvent and centrifuged before analysis.

The sediment and original dialysate were analysed with electrospray ionisation mass spectrometer MicrOTOF-Q II (Bruker Daltoniks, Germany) coupled to HPLC Dionex UltiMate 3000 RS (Dionex, USA). For sediment's fluorescence measurements spectrofluorometer RF-5301PC (Shimadzu, Japan) and for original spent dialysate fluorescence detector RF 2000 (Dionex, USA) was used. Analysis was performed in the excitation (EX) range of 220 – 480 nm and emission (EM) 240 – 500 nm. The sediment was analysed with Poroshell 120 EC-C18 column (Agilent Technologies, USA) and spent dialysate with Kinetex C18 100A column (Phenomenex, USA). Mass spectra interpretations were done with MagTran (Amgen Inc, USA) software.

Chromatographic measurements were conducted with acetonitrile (Romil, UK) and 0.05M ammonium formate (pH 4.25). Samples were adjusted to pH 4.25 and centrifuged for 10 minutes before injection.

III. Results

The fluorescence chromatograph of sediment showed several peaks in the long wavelength region (EX352 nm/ EM460 nm). One of the peaks (Fig. 1) was found to coincide with B2M on the MS chromatograph.

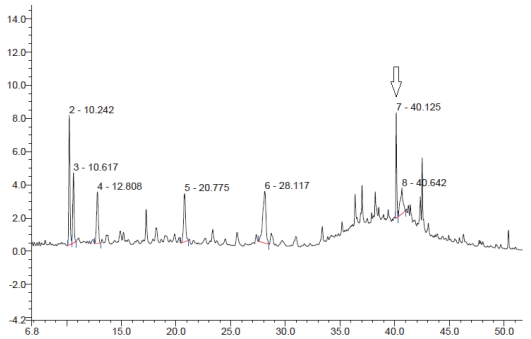

Fig. 1 Fluorescence chromatograph of the sediment. Peak number 7 is the peak coinciding with B2M in the MS chromatograph. EX 352 nm/ EM 460 nm (x-axis is time in minutes, y-axis is fluorescence intensity in mV).

Mass spectrum (MS) of the sediment showed ions with multible charges. The molecule mass was found to be 11772 Da (Fig. 2). Also fluorescence spectra of the sediment and amyloid B2M were compared (Fig. 3).

Analysis of the spent dialysate was done under the same conditions and a polypeptide presence was found (Fig. 4). The data from MS shows several masses close to B2M (Fig. 5).

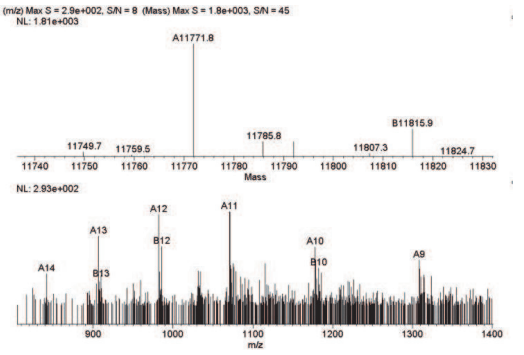

Fig. 2 Sediment's MS spectrum interpertated with MagTran software showing masses belonging to B2M. Charges of ions (down) and deconvulted masses (up).

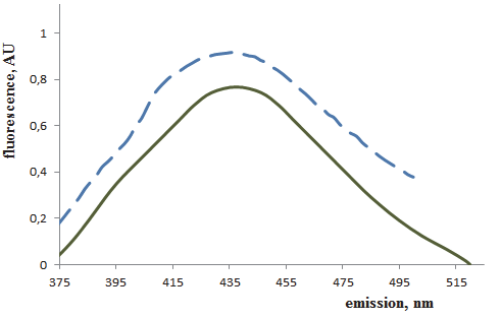

Fig. 3 Sediment's fluorescence spectrum (dashed line) compared with the spectrum of amyloid B2M (solid line) [8]

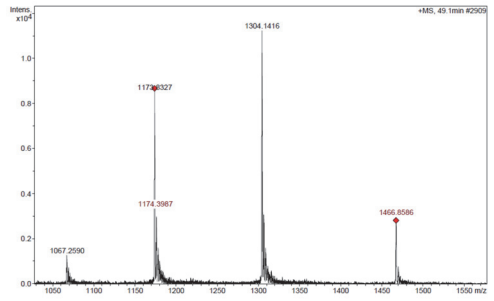

Fig 4 MS of spent dialysate showing a presence of protein.

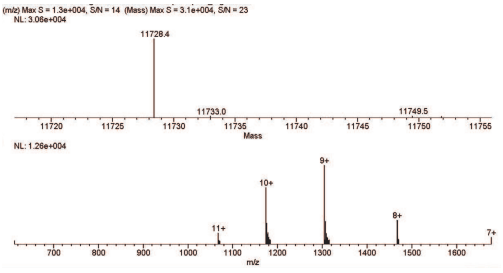

Fig. 5 MS of spent dialysate showing masses belonging to B2M Possible charges of ions (down) and molecule masses (up).

The chromatograph of spent dialysate had many fluorescent peaks in different wavelengths, but we were able to identify B2M peak (Fig. 6).

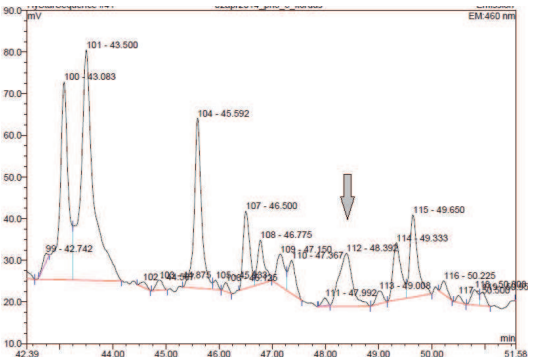

Fig. 6 Fluorescence chromatograph of spent dialysate. Under number 112 is B2M peak (EX 352 nm/ EM 460 nm).

IV. Disccusion

For the present study fluorescence detector was selected because it is easy and cost effective method that could be added to an online monitoring system. Also a good correlation (R=0.95) between B2M concentrations and fluorescence intensity of the spent dialysate was found earlier by our research group [10]. Our latest study, including 10 patient's data, showed again a relatively good R value of 0.74 (unpublished data).

Sedimentation occurred after concentrating two patients' spent dialysate samples with a SPE column. Analysis of the sediment showed that it had fluorescence characteristic to amyloid B2M (figure 3). Unmodified B2M does not have fluorescence but AGE modified compounds have specific fluorescence [8]. Many AGEs have an EX maximum approximately 370 nm and EM maximum around 445 nm [11] but the data varies. For an example, crossline's EM maximum is around 460 nm [12]. Our measurements were done near the maximum EM range of AGEs as seen from figure 3.

Both sediment's and dialysate's corresponding mass spectra showed typical ion masses of a polypeptide. The slight difference in masses can be explained by different degrees of modifications of the protein in amyloid sediment compared with the protein in original dialysate. Finding fluorescent modified B2M from the sediment let us assume that B2M can be measured from the unconcentrated dialysate. From spent dialysate's MS (Fig. 4) molecular masses belonging to B2M (11728 Da) were found (Fig. 5). However, the fluorescence intensity of modified B2M is low (0.04%) compared to overall fluorescence. This indicates that for AGE modified B2M detection a measuring system with high selectivity and sensitivity is necessary.

V. Conclusions

Brown coloured sediment and spent dialysate were analysed with HPLC-MS and fluorescence spectroscopy. The fluorescent sediment of the concentrate was identified as amyloid B2M on the basis of MS and fluorescence spectra. AGE-modified B2M was also measured from spent dialysate. In summary, the study revealed, that the fluorescence of amyloid B2M is possible to detect in spent dialysate, but the contribution to the overall fluorescence in very low. Thus, the measuring system needs high selectivity and sensitivity for detection.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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