Multicomponent analysis of accumulated solutes in uremia : are the classical markers sufficient to describe uremic solute accumulation?

MULTICOMPONENT ANALYSIS OF

ACCUMULATED SOLUTES IN UREMIA

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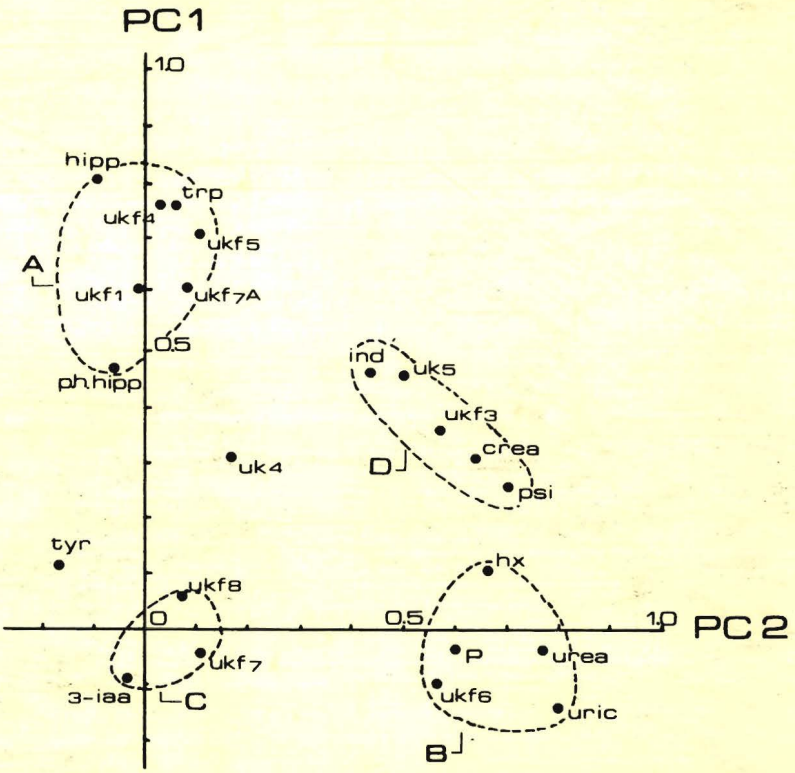

Ad C. SCHOOTS

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by Ad C. SCHOOTS

Eindhoven, February 1988

Front cover: refer to chapter 5.6 .

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Proefschrift

ter verkrijging van de graad van doctor aan de
Technische Universiteit Eindhoven, op gezag
van de rector magnificus, Prof. dr. F.N. Hooge,
voor een commissie aangewezen door het college
van dekanen in het openbaar te verdedigen op
dinsdag 26 april 1988 te 16.00 uur

door

Adriaan Cornelis Schoots

geboren te Terneuzen

Druk: FEBO Enschede

Dit proefschrift is goedgekeurd
door de promotoren:

Prof. dr. ir. C.A.M.G. Cramers
en
Prof. dr. S.M.G. Ringoir

To my parents

VEEL WETEN, WEINIGH

Wat is geleertheit? All te weten dat sy wisten
Die voor ons waeren, en min wisten dan sy misten?
Wien sou die wetenschap dan helpen, en waertoe?
Wat scheelt het, of het veel', of ick alleenigh doe
'T geen maer gedaen en werdt? Maer spreeckt drij niewe woorden,
Drij dingen diemen noijt van and're menschen hoorden;
All die de Wereld soo geringhen gave geeft,
Is 'tmeer als all dat oijt geleert geheeten heeft.

Constantijn Huygens (1596-1687)

(What is learning? To know all that those knew
Who were before us, and knew less than they missed?
Whom would that knowledge help, and for what?
What difference does it make if many, or I myself
Would only do what was already done? But speak three new words,
Three things never heard from other men;
Whoever gives the world that small gift,
T'is more than all that was once called 'learned'.)

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1 GENERAL INTRODUCTION

The human kidney, as a part of the urinary system, plays a keyrole in maintaining homeostasis: a state of equilibrium of the extracellular fluid surrounding tissue cells ("milieu intérieur"). Acute and chronic renal failure may originate from several causes among which are immunological or congenital factors, infectious diseases, injury, hypertension, diabetes mellitus, and poisoning by chemicals and drugs. Initially, damage may occur only to specific functional elements of the nephrons, while with progression of renal disease all functionalities, including the glomerular and tubular system, will be lost.

Advanced impairment of kidney function results in a serious derangement of homeostasis, including accumulation of metabolic waste products, hormonal disregulation, toxic enzyme inhibitions, and disturbed electrolyte- and acid-base balance [Schreiner and Maher,1961; Giovannetti and Berlyne,1975; Bergström and Fürst,1983; Wills,1985]. The gastro-intestinal, cardiovascular, neurological, hematological, immunological and metabolic disorders cause a wide spectrum of symptoms, called *uremia*. The symptoms include anorexia, nausea, vomiting, bone pain, weakness, convulsions, muscle twitching, drowsiness, pruritis, apathy, and reduced libido.

In the early stage of chronic renal failure conservative treatment, comprising dietary measures and drug therapy, is generally applied. However, at reduced renal creatinine clearances as low as 5-10 mL/min, blood concentrations of metabolic waste products do rise to such an extent (creatinine: around 1000 μmol/L, urea: 15 mmol/L) that some form of renal replacement therapy is needed.

Renal replacement therapy may consist of artificial blood purification, or renal transplantation.

Successful transplantation of cadaveric or living donor kidney grafts generally results in complete rehabilitation of the renal patient both clinically and socially. In December 1985, 1694 persons had a functioning graft in The Netherlands, compared to a total of 25,288 in 33 European and Mediterranean countries [EDTA/ERA,1987]. Unfortunately, graft rejection is not an uncommon phenomenon, despite the prolonged postoperative use of immunosuppressive drugs such as cyclosporine, azathioprine, and steroids. Furthermore there is a shortage of donor kidneys, resulting in long waiting lists of renal patients who would benefit from a transplantation. In the Netherlands 659 patients were on such a list in December 1985, and a total of 23,672 persons in the 33 countries included in the EDTA Registry [EDTA/ERA,1987].

Artificial blood purification methods include extracorporeal treatment by use of semi-permeable membranes. Some of the used techniques are: hemodialysis(HD), hemofiltration, hemodiafiltration, and hemoperfusion. Mass transfer in these techniques is based on diffusion, on convection, on a combination of the latter two, and on adsorption respectively. Furthermore, the human peritoneum is used as the semi-permeable membrane in the technique of continuous ambulatory peritoneal dialysis (CAPD). This technique is more "physiological" than hemodialysis because sudden fluctuations in fluid volume and/or electrolytes do not occur. It is used preferentially in pediatric patients, diabetic patients or patients having problems with vascular access for hemodialysis. Cardiovascular complications such as arythmias or myocardial infarct are also indications for CAPD treatment. A problem with CAPD is the repeated occurrence of peritonitis, generally caused by bacterial infection due to nonsterile catheter and dialysate manipulation. Several studies have shown that the peritoneal membrane has a different selectivity for the removal of waste products than do hemodialysis membranes. In this respect the properties of the peritoneum are more alike those of the renal glomerulus.
In this country, a number of 2,025 patients were on hemodialysis, and 440 on CAPD treatment in December 1985. Totals for 33 countries from the EDTA Registry are 75,313 and 7,538. The total costs of hemodialysis treatment of renal patients in the Netherlands, exceed 150,000,000 Dutch guilders (US$ 75,000,000) yearly.

A number of the symptoms have been shown to be reversed, at least partially, by regular dialysis treatment. Therefore it is widely accepted that the accumulating compounds exert toxic effects on biochemical, enzymatically driven, processes. Moreover, specific toxicity very likely results from a combined or synergetic effect of a multitude of accumulated solutes, rather than from the action of a single substance. A variety of identified solutes, accumulating in uremia is given below.

Table#1.1. Some Compounds Accumulating in the Blood of Uremic Patients.

Urea	Creatinine	Uric acid
Phenols	Phenolic acids	Indoles
Indolic acids	Glucuronides	Sulfate(s)
Phosphate	(Methyl)guanidine	Myo-inositol
Aliphatic amines	Aryl acids	Peptides
cyclic-AMP	Glucagon	Ribonuclease
Parathormone	β_2-microglobulin	Lysozyme
Aluminum	Nickel	H_3O^+

Blood levels of the accumulated solutes in uremic, dialyzed, patients are governed by many variables, such as residual renal function, generation rate, catabolic rate, distribution volume, and biological membrane resistances [Sargent and Gotch,1983]. Other variables, related to dialysis treatment are dialyzer properties, dialysis duration and frequency. Important solute properties are molecular mass, hydrated radius, hydrophobicity, hepatic conjugations and protein binding, that are all related to the chemical structure of the compound.

An important feature of "adequate dialysis" is the alleviation of the uremic symptoms, by removing the relevant accumulating toxins as they appear in the individual patient [Schreiner,1975; Gotch and Krueger,1975; Barber et al.,1975; Lowrie et al.,1976; Blagg,1984; Farrell,1986]. Consensus exists about a number of basic conditions of adequate dialysis (e.g. fluid and electrolyte balance). However, adequate dialysis of uremic toxins remains difficult to define as long as no knowledge exists about the toxins or "culprits" [Schreiner,1975] that should be removed preferentially.

In the past fifteen years, two different lines of approach of uremic toxicity, and its reversal by dialysis, have been followed. These approaches were: 1) the square-meter hour hypothesis, introduced by Babb et al.[1971], and 2) urea kinetic modelling advocated by Sargent [1983], Sargent and Gotch [1975], Gotch and Sargent [1985], Lowrie et al.[1976], and Sargent and Lowrie [1982].

In the "square-meter hour hypothesis", later changed to "middle molecule hypothesis", unknown solutes with intermediate molecular mass (and thus high dialyzer mass transfer resistance) were held responsible for uremic polyneuropathy. This was based on observations with patients treated by peritoneal dialysis, who were doing well despite a relative underdialysis with respect to small molecules like urea and creatinine [Scribner,1965]. Later

on, these unknown solutes were thought to be protein breakdown products of a peptidic nature and medium molecular mass [Fürst et al.,1976]. According to this concept, preferential dialysis of the so-called middle molecules could be achieved with more permeable membranes, higher membrane surface area, and extended dialysis time, while blood and dialysate flow rates were expected to have virtually no influence on dialyzer clearance. In 1975 a Dialysis Index was introduced [Babb et al.,1975], which was based on mass balance considerations and properties of a hypothetical middle molecule (Mr 1300). It was shown that even small levels of residual renal function might have considerable influence on the removal of compounds showing low dialyzer clearances.

The concept of urea kinetic modelling is based on the experience that uremic symptoms worsen with increasing daily protein intake, and the observation that urea generation rate is linearly dependent on protein intake. In the National Cooperative Dialysis Study (NCDS) held in the USA, probability of clinical failure was found to depend on amount of dialysis applied, expressed in kt/V, using urea as a marker. Here k represents dialyzer urea clearance, t is dialysis time, and V is the distribution volume [Laird et al., 1983]. A value of $kt/V \geq 0.9$ was found to be adequate on statistical grounds. While urea as such is not particularly toxic, urea kinetic modelling is a rational approach, although, according to Sargent [1983], ".....modelling therapy should not be overly ambitious in its attempt to create an all-inclusive description of uremia".

In order to extend our knowledge of uremic solute accumulation and toxicity, we have developed and applied (bio)chemical analytical techniques. This is reported in the present studies.

In chapter 2 the "middle molecule" discussion is reviewed, mainly from an analytical point of view. The usefulness of the gel filtration technique for separation and isolation of middle molecules is evaluated. Selectivities of gel filtration and hemodialysis are determined for a number of model compounds in order to study whether chemical group contributions are similar in these processes.

Chapter 3 gives an overview of methods developed and used for multicomponent analysis of uremic fluids. A number of criteria are formulated for such methods.

A multicomponent analysis procedure based on high performance liquid chromatography (HPLC) is described in chapter 4. Method development and characteristics are discussed.

Chapter 5 deals with diverse studies on the evaluation of factors governing blood concentrations of the (HPLC-analyzed) accumulating compounds. These concentrations were compared for patients treated by hemodialysis and CAPD. Solute accumulation in relation to residual renal function in dialyzed and non-dialyzed patients is described in a subsequent study. *In vitro* dialyzabilities of the analyzed compounds on artificial kidneys equipped with cuprophan membranes are determined. The change of blood levels during hemodialysis treatment for the various solutes is studied. How the different factors work out in relative blood concentrations in dialyzed and nondialyzed patients is shown in a subsequent section by using the multivariate statistical procedure of principal component analysis. In the last section from this chapter concentrations of HPLC-analyzed solutes and routine clinical biochemistries are related to motor nerve conduction velocities and Hoffmann-reflex latencies in a group of renal patients on hemodialysis therapy. From the results of the studies in chapter 5 an answer will be formulated to the following question: *Are the classical markers sufficient to describe uremic solute accumulation?*

In chapter 6 some related studies based on gas chromatography-mass spectrometry(GC-MS) are presented. Organochlorine pesticides are analyzed in uremic and normal sera to study whether renal patients accumulate these environmental contaminants from dietary intake and dialysate water during hemodialysis. The analysis of carbohydrates (among which the possibly toxic polyols) and organic acids by GC-MS is described in the subsequent section. Finally the identification of these substances by electron-impact and chemical ionization mass spectrometry is reported.

2 UREMIC TOXINS AND THE ELUSIVE MIDDLE MOLECULES

2.1 Testing the Middle Molecule Hypothesis

Both direct and indirect methods of testing the middle molecule hypothesis have been followed. The indirect methods were based on clinical trials with various dialysis strategies applying low dialysate flow rates, large and small surface areas of the dialyzers, and more permeable membranes, in order to cause preferential dialysis of small molecules in one case and "middle molecules" in the other. These studies have led to contradictory results, which have been reviewed [Nolph, 1977; Gotch, 1980]. The notion of middle molecules has generally been associated with substances of intermediate molecular mass. A more careful definition, which does more justice to the original concepts of Babb et al. [1971], would be the following:

Middle molecules are substances that behave in a dialyzer as though their molecular masses were in the range of 300 - 2000 daltons.

In view of the wide application of size-exclusion chromatography, loosely called gel filtration, direct methods of analysis and identification of "middle molecules" have been directed almost exclusively to solutes of intermediate molecular mass.

In the present study the chemical composition of "middle molecular" fractions obtained with Sephadex G-15 gel filtration is evaluated.

2.2 Measurement of Middle Molecules in Uremia

Perhaps chromatography is so often succesful by virtue of the multiplicity of factors brought into play. Where this is true the process, however effective it may be as a separation tool, does not reveal much of the more subtle chemical or physical nature of the solutes.

[L.C. Craig, Arch Biochem Biophys (Suppl.1), 112, 1962]

2.2.1 Overview

Analytical procedures involving separation of solutes of middle molecular mass can be divided roughly in three categories.

1.Separation of middle molecules based on size exclusion chromatography (gel filtration); the purpose of these studies was to separate accumulating uremic serum components according to their molecular mass;

2.Separation or isolation of compounds from a distinct chemical class with expected middle molecular mass (e.g. peptides);

3.Analysis of known biochemically active substances with middle molecular mass (e.g. hormones, glucuronidated drugs, nucleotides). These studies were not necessarily conducted with the aim of analyzing "middle molecules".

An extensive review with many references on "middle molecule" analysis has been published by Contreras et al. [1982].

Using the first approach, numerous investigators have reported on the separation of middle molecule peaks by gel filtration, in some cases followed by ion exchange chromatography. The technique of gel filtration was applied because of the expected fractionation according to molecular size. The application of this technique with uremic fluids was first reported by Dall'Aglio et al.[1972]. Since then many groups have followed this approach and tried to separate, quantitate, and identify the middle molecules.

Fürst et al.[1975, 1976] and Zimmerman et al.[1980] reported on the separation of middle molecules - which they initially claimed to be peptides - by gel filtration, followed by ion exchange chromatography and isotachophoresis. They found various peaks in the ion-exchange chromatogram and designated them "7a", "7b", "7c" etc. Recently, however, they found that the main component of the peak they designated as "7c" was a glucuronide of *o*-hydroxyhippuric acid with a molecular mass of 371, identified by gas chromatography and mass spectrometry [Zimmerman et al.,1981]. No significant correlations were found between

quantitative data from their peaks and uremic symptoms [Asaba et al.,1982]. Moreover, it was observed that the peak areas were influenced equally by dialysis on membranes with different permeability and surface area [Asaba et al.,1979].

Funck-Brentano et al.[1976] and Cueille [1978] used a very similar technique and mentioned the separation of a middle molecule of peptidic nature with a molecular mass between 1100 and 1300 daltons. This substance, designated by them as b_{4-2} was found to be heterogeneous after analysis with paper chromatography, thin-layer chromatography and gas chromatography mass spectrometry. The main component seems to be a glucuronide of which the aglycon is hitherto unknown, and was shown to have a molecular mass of 526 daltons by mass spectrometry [Cueille et al.,1980; Le Moel et al.,1980].

Also other groups have been using gel filtration/ion exchange chromatography [Dzurik et al.,1973; Chapman et al.,1980, and others].

Recently, more "middle molecules" were identified as glucuronides by mass spectrometry and nuclear magnetic resonance spectrometry [Gallice et al., 1985]. One of these solutes was shown to be hydroxybenzoic acid glucuronide, with a molecular mass of 314 daltons, which is very close to the M_r 300-limit. [Monti et al.,1985].

As will be demonstrated in the next chapters [also see Schoots et al.,1982], the middle molecule fractions obtained by gel filtration do not solely, or even mainly, represent substances with middle molecular mass. They appeared to contain many low molecular mass solutes, like carbohydrates, amino acids and UV-absorbing solutes. Furthermore, the fractions proved to contain sodium, chloride, acetate, phosphate and sulfate. The fractions in the middle molecule region of the gel chromatogram were analyzed by isotachophoresis, high-performance liquid chromatography, gas chromatography, mass spectrometry, and routine analytical procedures [Mikkers 1980, Schoots et al.,1982]. Mild mass spectrometric techniques, like field desorption and fast atom bombardment, i.e. techniques that yield molecular mass information, were used to analyze raw gel filtration fractions. A solute with molecular mass below 400 daltons was observed in a fraction with expected molecular mass range of around 1500 daltons, as "predicted" from the molecular mass calibration line.

Obviously, complex mixtures like serum and ultrafiltrate will not be separated by gel filtration techniques according to molecular size only. Therefore fractions obtained by gel filtration of such samples will contain mixtures of solutes with widely varying molecular masses.

Various separation mechanisms are active besides size-exclusion, resulting in an anomalous retention behaviour of relatively low molecular mass solutes, that can be either adsorbed on or excluded from the gel by interactions different from size exclusion. The anomalous interactions, that can also be utilized to obtain a certain desired selectivity, have been

reviewed [Kremmer and Boross, 1979, Barth,1980]. The separation efficiency of gel chromatography is rather poor and thus the "middle molecule" fractions do not contain single substances. A comparison of separation efficiencies obtainable with different chromatographic techniques can be made in terms of theoretical plate number, peak capacity and analysis time (see table#2.1).

Table#2.1. Comparison of Chromatographic Techniques.

TECHNIQUE	COLUMN LENGTH (cm)	EFFICIENCY [a] (plate number)	PEAK CAPACITY [a,b]	ANALYSIS TIME [a] (min)
Gel (e.g. Sephadex)	60	700	5	240
Gel (e.g. TSK)	25	5000	15	25
HPLC (isocratic)	25	20000	65	25
HPLC (gradient)	25	20000	120	45
GC (capillary,isothermal)	5000	100000	150	25

[a]At indicated column lengths; resolution $R_s = 1$; $0 < K_d < 1$ in gel chromatography; $0.5 < k' < 10$ in HPLC and GC; HPLC gradient time 45 min, peak width constant; in all cases N is assumed constant for all peaks.
[b]Calculated according to Giddings [1967], except HPLC gradient. Peak capacity in gradient HPLC was calculated according to [Snyder, 1980]. Gradient analysis, taking 75 min when fully developed, was thought to be halted at 45 min in the calculation.

No further chromatographic analysis following gel filtration, e.g. ion exchange chromatography, will as yet lead to a fractionation according to molecular mass. The same holds for the gel filtration fractions obtained on the newer "high performance" gels of TSK-type [Mabuchi and Nakahashi,1981]. With these gels a reduction of the analysis time can be obtained, but hardly any better separation is achieved.

Different authors have performed a wide variety of *in vitro* toxicity tests on so-called middle molecule fractions. These fractions have been reported to inhibit glucose utilization, phagocytic activity and the activity of various enzymes. In some cases these toxic effects were directly ascribed to the "identified" middle molecules. The subject of *in vitro* toxicity has been reviewed [Navarro et al., 1982]. The *in vitro* tests are very sensitive to variables such as pH, ionic strength, osmolality of the fractions. Therefore utmost care must be taken in the interpretation of the results of these tests on heterogeneous samples, like gel filtration and ion exchange fractions.

A second approach of middle molecule analysis is the search for solutes with a defined chemical structure. Many investigators have focused on peptides. Basic peptides, containing non-amino acid ninhydrin-positive groups like spermidine, amino sugar and guanidine, were

isolated from peritoneal dialysis fluid by gel filtration, ion exchange and paper chromatography [Lutz, 1976]. Peptides of different length and amino acid sequence were isolated and identified by Abiko et al.[1978, 1979] following an elaborate procedure of subsequent ultrafiltration on membranes with different molecular mass cut-off, and chromatography with various Sephadex size exclusion and ion exchange gels. These peptides were identified as a pentapeptide possibly representing a fragment of fibrinogen β-chain and a heptapeptide corresponding to positions 13-19 of β_2 - microglobulin, both peptides inhibiting E-rosette formation *in vitro* [Abiko et al., 1978, 1979]. Furthermore they isolated an acidic tripeptide which was found to inhibit lactate dehydrogenase activity *in vitro*.

Also other authors found ninhydrin-positive compounds and peptide material in uremic serum by combination of bag dialysis, ion exchange chromatography, paper chromatography, and thin-layer electrophoresis [Klein et al., 1978; Menyhart and Gróf, 1981; Ehrlich et al.,1980]. High performance liquid chromatography was used to analyze uremic serum and ultrafiltrate for peptides using fluorescence reaction detection [Mabuchi and Nakahashi,1982,1983]. Besides peptides, also glycopeptides and oligosaccharides were found in hemofiltrate and peritoneal dialysis fluid [Le Moel et al.,1981]. Although the presence of peptides in uremic dialysate and serum has been demonstrated, few quantitative data are available, and their significance as uremic toxins, especially compared to low molecular mass toxicity, is unclear. In order to obtain more data it is necessary to analyze large numbers of samples in relation to dialysis procedures and membranes with different permeabilities. However most of the reported isolation procedures of peptides are very elaborative and seem to be inapplicable to large series of samples.

A third group of analyses of middle molecule-like substances includes the measurement of known biochemically active compounds with the required molecular mass. Especially, different types of hormones have been analyzed in relation to uremia. Gas chromatographic profiling of steroid hormones has been reported [Ludwig et al., 1978]. Hormones of different molecular mass and chemical nature (i.e. steroid and peptide hormones) were measured in uremic serum in relation to a possible deficiency as a result of hemofiltration [Matthaei et al., 1983], by gas chromatography/ mass spectrometry and radio immuno assay.

The analyses of certain peptide hormones and low molecular mass proteins should be mentioned. Although not strictly to be considered "middle molecules" in terms of molecular mass, they do accumulate in uremic blood due to impaired excretion, poor dialyzability and impaired renal degradation. Circulating forms of glucagon (M_r 3500) and related peptides were analyzed in uremic serum [Flanagan et al., 1980]. Low molecular mass proteins were separated simultaneously and efficiently by ion exchange chromatography. Among the

analyzed compounds were β_2-microglobulin, retinol-binding protein and lysozyme [Lindblom et al., 1983]. The possible importance of these substances in uremic intoxication has been described by different authors [Bernier et al., 1968, Maack et al., 1979].
Finally, the analyses of other substances such as nucleotides [De Bari and Bennum,1982], conjugated drugs [Stierlin et al., 1978] and other conjugates [Turner and Wardle, 1978] are mentioned.

2.2.2 Characterization of gel filtration fractions by gas chromatography, mass spectrometry, isotachophoresis and liquid chromatography.

2.2.2.1 Introduction

In order to separate uremic accumulating substances according to molecular mass many authors have applied Sephadex G-15 gel filtration, or combined gel filtration/ion exchange chromatography. As has been pointed out in the overview above, the separation mechanism in gel filtration analysis of complex mixtures is not simply size exclusion. The diverse selectivity effects with the Sephadex gels are already known since the early sixties, and have been described extensively in literature [Eaker and Porath, 1967; Porath, 1979; Kremmer and Boross,1979]. In order to know the composition of the heterogeneous fractions obtained from uremic blood samples, it is therefore necessary to analyze them with more sophisticated techniques that are available today. In the present study we will apply high performance liquid chromatography, capillary gas chromatography - mass spectrometry, and isotachophoresis. Moreover mass spectrometric techniques with soft ionization modes such as field desorption, fast atom bombardment, and chemical ionization will be applied. These techniques are especially suitable to obtain molecular mass information rather than detailed structural information that is generally derived from fragmentation observed under electron impact (EI) conditions.

2.2.2.2 Experimental

Uremic ultrafiltrate samples were obtained during a sequential ultrafiltration and diffusion procedure on uremic patients, who were treated with polyacrilonitrile membrane dialysis. Uremic ultrafiltrates and normal sera were separated by gel filtration yielding 40 5-mL fractions. They were lyophilized and stored at -18°C until further use. Before analysis by different techniques the samples were redissolved in 500 µL of doubly distilled water. From this, 200 µL was used for preparing the GC samples, 100 µL was injected on the liquid chromatograph, and 1-5 µL was needed for isotachophoresis.

Gel filtration. A 60 X 1.6 cm (i.d.) column packed with Sephadex G-15 gel (Pharmacia, Uppsala, Sweden) was used [Ringoir,1978;1980]. Sample volumes applied were 5 mL for uremic ultrafiltrate and 2 mL for normal ultrafiltered serum. The eluent was an aqueous ammonium acetate buffer (30 mmol/L, pH 6.9) at a flow rate of 15 mL/h. After measuring UV-absorbance at 206 and 280 nm, we collected 5-mL fractions.

Gas chromatography was performed as described earlier [Schoots et al., 1979a and 1979b]. After lyophilization and trimethylsilylation the fractions were analyzed on a glass capillary column, coated with SE-30 stationary phase. Detection took place with a flame ionization detector.

Mass spectrometry was used on-line with gas chromatography. For GC-MS conditions used for identification refer to chapter#6. For direct identification of material represented by liquid chromatographic peaks we used a thermal desorption direct insertion probe, operated in the electron impact and chemical ionization mode. The electric current through the platinum wire of the probe, on which the samples were deposited, was programmed from 0 to 2 A within a 2-min period. The wire diameter was 0.15 mm. For chemical ionization a 10/90 by vol mixture of ammonia and methane as the reagent gas was used. The mass spectrometer was a Model 4000 quadrupole GC/MS (Finnigan-MAT, Sunnyvale, CA, USA).

Direct mass spectrometric analysis of gel filtration fractions was done by field desorption (FD) and fast atom bombardment (FAB) as the ionization modes. The FD spectra were recorded on a Model 711 magnetic sector mass spectrometer (Varian MAT) (emitter current:10-20 mA) by Mr. R. Fokkens, at the Dept. of organic chemistry, University of Amsterdam.

The FAB spectra were recorded on a Model Micromass 7070E-HF high resolution mass spectrometer (VG Analytical, Wiesbaden, FRG), by Mr. P. Farrow. Thioglycerol was used as a sample matrix.

Isotachophoresis (ITP). Anionic solutes were separated at pH=3 of the leading electrolytes, as described earlier [Mikkers, 1980, Mikkers et al.,1980]. ITP profiles were obtained by recording UV-absorption at 254 nm and from conductometric signals.

High performance liquid chromatography (HPLC). We used a 25 cm X 4.6 mm. (i.d.) column packed with octadecyl (C18) modified silica (Lichrosorb RP-18, Merck Darmstadt, FRG) with 5 μm particles. The eluent was programmed (gradient elution) from 100% ammonium formate buffer (50 mmol/L, pH 4) to 100% methanol (Rathburn Chemicals Ltd. Walkerburn, Scotland) within 60 min. The apparatus consisted of two Model 6000A pumps, and a Model M660 solvent programmer (Both from Waters Associates Inc., Milford, MA, USA). Absorbance at 254 nm was measured with a Model LC-3, variable wavelength UV

detector (Pye Unicam LTD. Cambridge, UK). The re-analysis of gel filtration fractions was also performed with the present HPLC-procedure, which is described extensively in chapter#4.

2.2.2.3 Results and Discussion

In Fig.#2.1 (see end of this section), a gel filtration profile from a uremic ultrafiltrate is shown. The expected "middle molecular mass" region in the chromatogram was determined from elution volumes of injected molecular mass markers, indicated in the upper region of the figure. On this basis, fractions 12 to 15 should contain the molecules with the anticipated intermediate molecular mass. In Fig.#2.2 (end of this section) a similar gel chromatogram (from another patient's serum) and HPLC profiles of the associated fractions are shown. Here the more recently developed HPLC procedure was used, which will be described in a following chapter. Table#2.2 summarizes a number of solutes that have been analyzed and identified in the adjacent fractions.

Table#2.2. Solutes Found in Adjacent Gel-Filtration Fractions[a].

Solute	M_r	Fr.13	Fr.14	Fr.15	Fr.16	Fr.17	Fr.18
Urea[b]	60					o	O
Creatinine[b]	113				.	O	
Acetate[c]	60	o	O	o	o		
Lactate[c]	90	o	O	o			
Gluconate[c]	196		O				
Aspartic acid[d]	133		O	o			
Phosphate[c,d]	98	o	O				
Serine[d]	105		O	o			
Threonine[d]	119		O				
Sodium (Chloride)[c]	23(35)	o	O	o	o		
Glucose[d]	180		o	o	O		
Fructose[d]	180			O			
Glucitol[d]	182			O	o		
Arabinitol[d]	152			O	o		
Erythritol[d]	122			O	o		
Myo-inositol[d]	180		o	O			

[a]Fractions from gel filtration analysis in Fig.#2.1; o:solute present; O:fraction with maximum concentration. [b]Analyzed conventionally. [c]Analyzed by isotachophoresis. [d]Analyzed by gas chromatography and mass spectrometry.

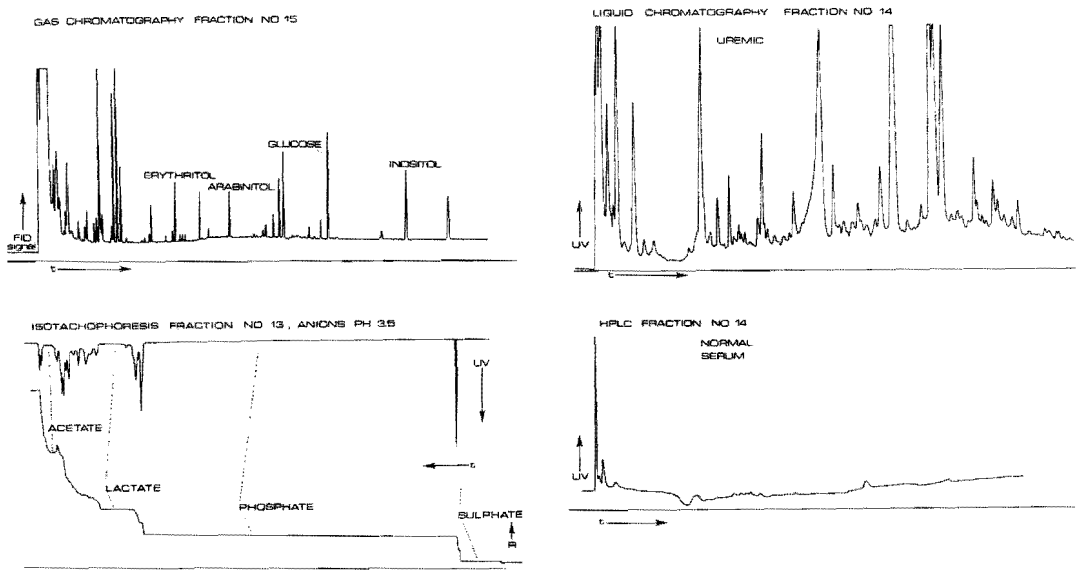


Fig.#2.3. Analysis of "middle molecule" fractions by GC, ITP, and HPLC.

Fig.#2.3 shows the GC, ITP, and HPLC profiles of some of the "MM" fractions. A comparison of the HPLC analyses of fraction 14 from a uremic ultrafiltrate and from a normal serum, reveals the virtual absence of most of the accumulated metabolites in the normal serum fraction. Obviously, many low-molecular-mass solutes elute in the middle molecular mass region. Among them are the acidic compounds acetate, lactate, and phosphate (in fraction 14) and a number of carbohydrates (in fraction 15). The liquid-chromatographic and isotachophoretic profiles [Mikkers,1980; Schoots et al.,1982] of the "middle molecule" fractions revealed the presence of a large number of UV-absorbing solutes and anionic substances. In Fig.#2.1 and Fig.#2.2 the HPLC analyses of some "middle molecule" fractions are presented. It should be noted that peak numbering is not uniform between the two gel chromatograms. Fraction numbers n in the first gel chromatogram correspond to fraction numbers n + 2 in the second gel filtration analysis. The HPLC peaks in the fractions have not yet been identified, although analysis with chemical ionization mass spectrometry (using a thermal desorption probe), and by field desorption MS (the latter in a different gel filtration / HPLC analysis, not shown here) indicated that they are compounds of lower molecular mass (M_r between 200 and 300). Retention times of major peaks in a number of HPLC fractions correspond to those for uric acid, 3-indoleacetic acid and the unknown compounds UK5, UKF3, and UKF8, when compared to HPLC retention times of these solutes in HPLC-analysis of whole serum ultrafiltrate. These gel fractions have elution

volumes much larger than that of the D_2O total permeation marker. This may be explained by adsorption on the gel. Further molecular mass information was obtained by analyzing raw gel fractions by mild mass spectrometric techniques, such as fast atom bombardment (FAB) and field desorption ionization (FD). In the FAB-analyses, in most cases, rather complex spectra were obtained: the solute peaks were immersed in those of the thioglycerol matrix. Nevertheless a relatively "clean" spectrum was obtained for fraction 12 (Fig.#2.4, bottom), which should contain solutes with M_r 1500, as derived from the molecular mass calibration of the gel column. The abundance of the peak at m/z 380 indicates the presence of a compound with this molecular mass. This is confirmed in an independent analysis by field desorption mass spectrometry of a corresponding fraction (Fig.#2.4, top). In both spectra the (M + 23)-ion from Na-attachment (m/z 403) is present. Presumably, the ion at m/z 381 in the FAB spectrum results from protonation of a molecule with M_r 380.

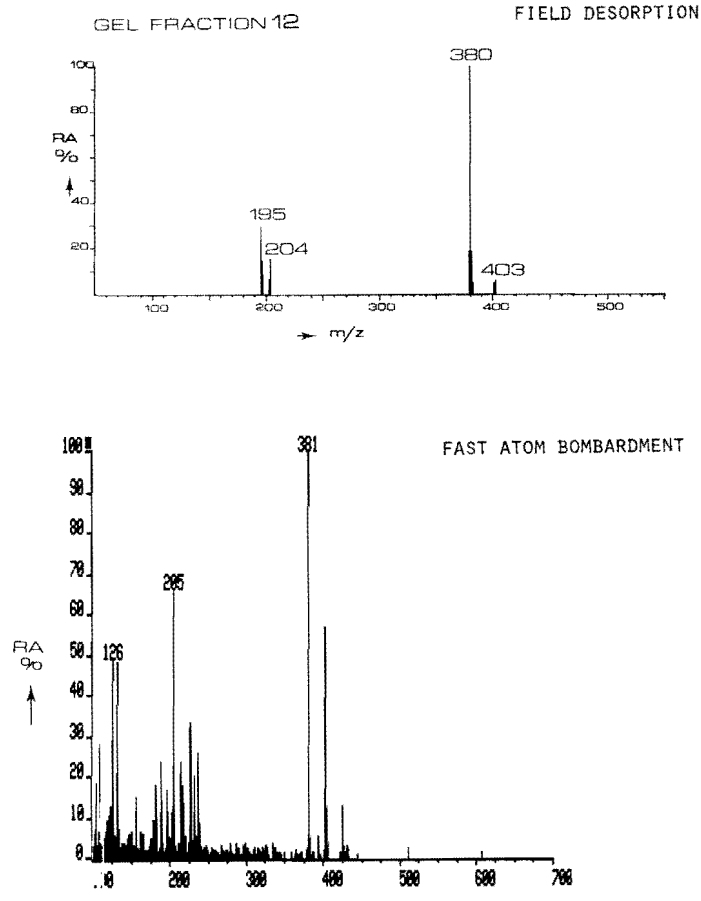


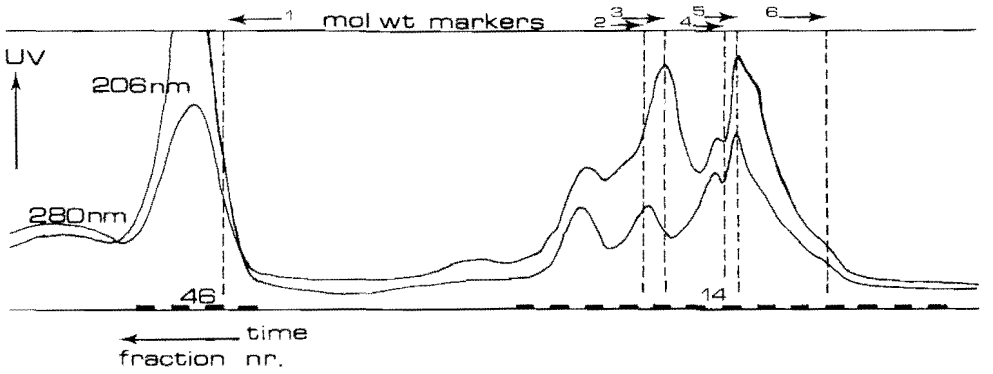
Fig.#2.4. Uremic gel filtration fraction 12 (see Fig.#2.1), analyzed by soft ionization mass spectral techniques: top, field desorption(FD); bottom, fast atom bombardment(FAB).

In the FD experiment the ratio of the abundances of the ions at m/z 380 and m/z 204 decreased with increasing emitter current. This allows for two explanations. First, there are two compounds present, of which that represented by m/z 204 is less volatile, thus desorbing at higher emitter current. A second explanation could be a thermal decomposition of the molecule associated with m/z 380. In the latter case it can be speculated that m/z 380 is the molecular ion of a glucuronide with an aglycon with M_r 204.

Whatever the precise nature of the substance, it is shown that molecules with relatively low M_r (e.g. 380) may elute "anomalously" in fractions that are reserved for middle or high molecular mass compounds, if we go by the column calibration. As will be demonstrated in the study described hereafter, functional groups may work out very differently in the gel chromatographic and dialysis behavior of the molecules.

In conclusion, gel filtration fails as a size-discriminating analytical technique for separation of "middle molecules" from other compounds in complex biological fluids. Obviously, in gel filtration several retention mechanisms are active, rather than size exclusion alone. They may include size exclusion, ion exclusion, ion inclusion, and adsorption. This is not limited to the complex mixtures described here, but is a generally observed phenomenon with the highly crosslinked Sephadex dextran gels [Eaker and Porath,1967; Kremmer and Boross,1979; Barth, 1980].

Different authors have used the UV peak-area of peaks obtained by ion exchange chromatography after gel chromatographic separation as a quantitative measure of so-called middle molecules [Migone et al., 1975, Funck-Brentano et al.,1978, Asaba et al.,1977; Asaba et al.,1979]. Given the proven heterogeneity of the fractions, the quantitation of "middle molecules" by UV peak area is incorrect. Evidently gel filtration / ion exchange chromatography cannot be used as a primary analytical technique for the *quantitation* of "middle molecules". It is doubtful whether this technique is the most rational approach to the *isolation* of molecules with intermediate molecular mass. Dialysis and filtration techniques on inert membranes are more appropriate.



MARKERS:

1. uric acid	168
2. urea	60
3. creatinine	113
4. hypertensine	1029
5. vit. B12	1355
6. dextran blue	2.10^6

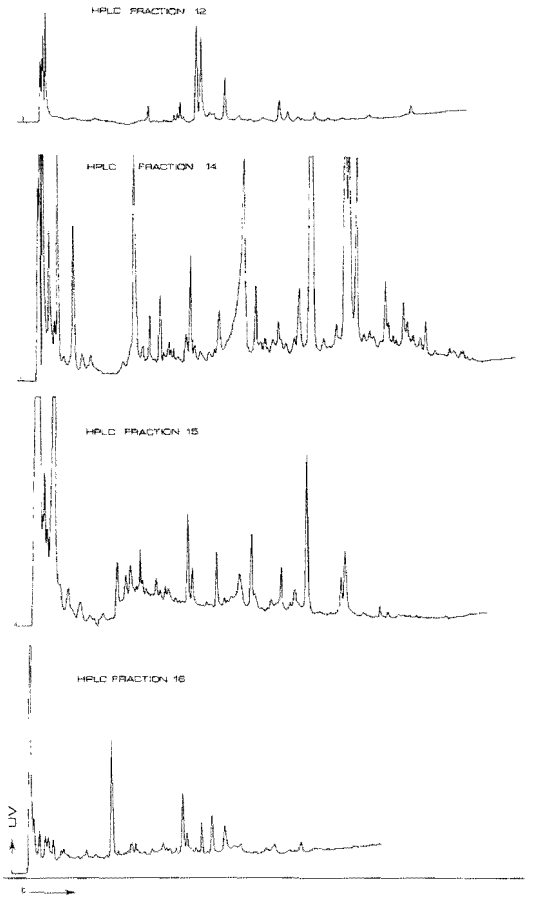


Fig.#2.1. Sephadex G-15 gel filtration analysis of uremic serum ultrafiltrate. Reanalysis of fractions by HPLC (Lichrosorb RP18 column).

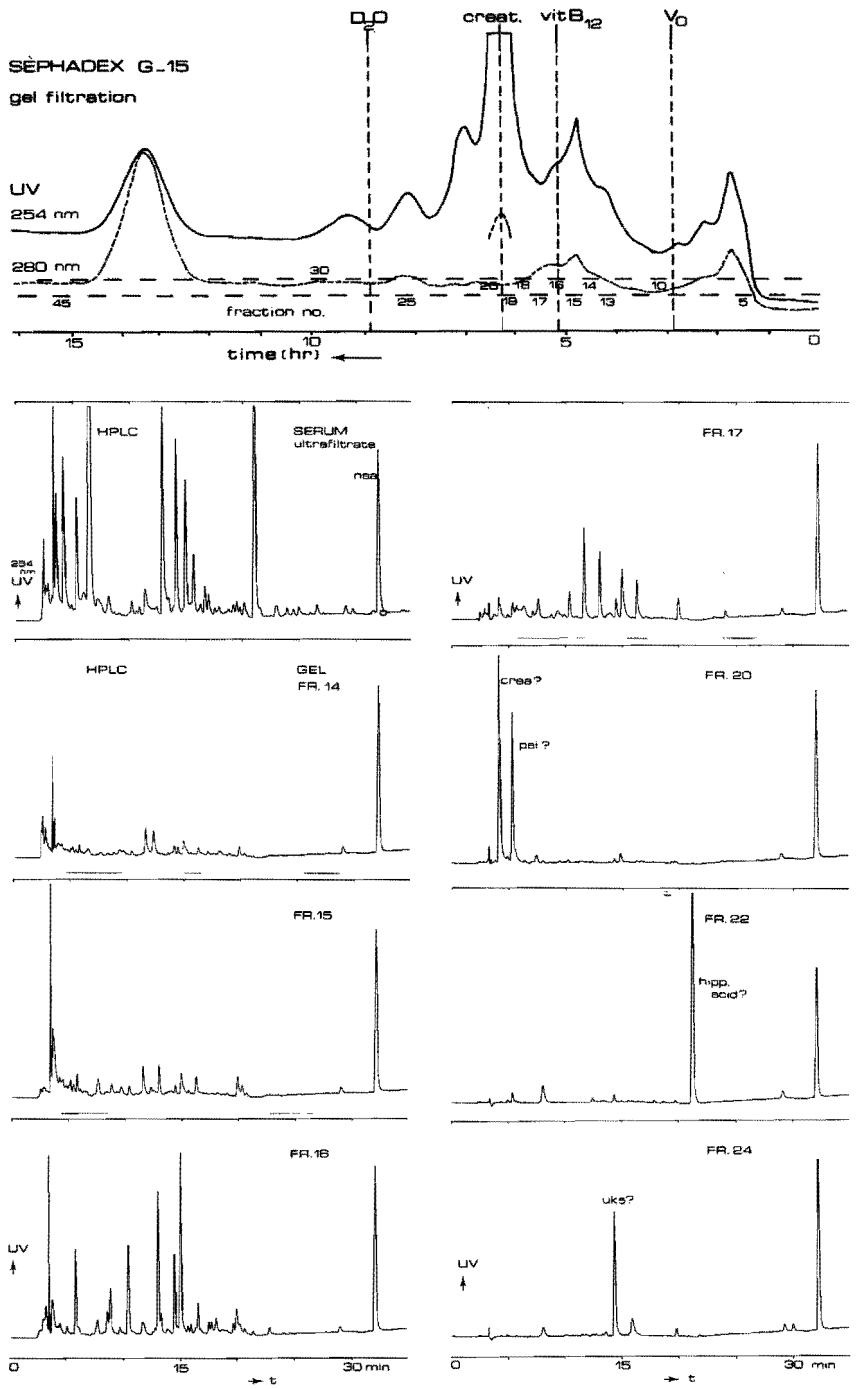


Fig.#2.2. Sephadex G-15 gel filtration analysis of uremic serum ultrafiltrate. Reanalysis of fractions with the HPLC method described in chapter#4 (Ultrasphere Octyl column).

2.2.3 Experiments on the analogy of gel filtration and cuprophan dialysis using model compounds.

2.2.3.1 Introduction

In recent years a number of solutes present in "middle molecule" gel filtration fractions have been identified as glucuronides with rather low molecular masses [Cueille et al.,1980; Zimmerman et al.,1980; Gallice et al.1985, Monti et al., 1985]. An explanation of the anomalous retention of these solutes on Sephadex gels is found in the rejection of these acidic compounds by carboxyl groups present in the gel matrix. It has been reported that MM-peak 7c [Fürst et al., 1976], mainly consisting of *o*-hydroxyhippuric acid glucuronide (M_r 371) [Zimmerman et al., 1980], is dialyzed as if it had a higher molecular mass. A clearance of 36 mL/min on a Gambro Optima (13.5 μm) was reported (the corresponding urea clearance for this dialyzer was 140 mL/min) [Asaba et al., 1979]. It was suggested that the anomalous retention of this acidic compound on gel filtration is also reflected in its diffusive transport through dialyzer membranes [Bergström et al. 1981], i.e. it will not be cleared according to its molecular mass. Such a dialysis behavior is observed with phosphate that has a significantly lower dialyzer clearance than expected from its molecular mass, which is probably due to solvatation.
Alternatively, it has been demonstrated that cuprophan membrane resistances depend linearly (log-log) on solute molecular mass of a wide range of acidic, basic and neutral compounds with different chemical structures in the molecular mass range between 60 and 2000 daltons [Farrell and Babb,1973].

Here we will compare the behavior of commercially available model compounds on gel filtration and on dialysis through cuprophan membranes, in order to study the influence of chemical group contribution of carboxyl, glucoside, and glucuronide moieties on selectivity in these processes.
These model compounds were expected to exhibit adsorption, rejection and size-exclusion behavior in gel filtration, to different proportions.

According to Hammett [1940], in a series of m- and p-substituted benzene derivatives, the logarithms of the substituent effects in one process or reaction are, in general, linearly related to those in another reaction. This phenomenon was interpreted as a linear free energy relationship (LFER). The so-called Hammett-equation representing this observation is:

$$\log\frac{K}{K_0} = \rho \log\frac{K'}{K_0'}$$

where K and K_0 are equilibrium or rate constants of a substituted and an unsubstituted benzene derivative, K'and K_0' are these constants for the corresponding substituted and unsubstituted benzoic acids, and ρ is a proportionality constant corresponding to the reaction involved. The logarithmic term on the right hand side in the equation is called the Hammett substituent constant σ and serves as a reference. If the behavior of the substituted benzenederivatives in one process (A) depends on the nature of the substituent in a similar way as in another process (B), a graph of $\log(K_{s,i}/K_0)_A$ versus $\log(K_{s,i}/K_0)_B$ for various substitutions should be a straight line ($K_{s,i}$ being a rate or equilibrium constant for substitution i), when a single reaction centre (e.g. the aromatic ring) is maintained.

2.2.3.2 Experimental

Materials. The model compounds used were: nitrobenzene (Mr 123), *p*-nitrophenylacetic acid (Mr 181), *p*-nitrophenylglucoside (Mr 301), *p*-nitrophenylglucuronide (Mr 315), and *p*-nitrophenyllactoside (Mr 463), and were purchased from Sigma Chemical Co.(St.Louis, Mo, USA).

Gel filtration. We used a 1.6 cm i.d. column packed with Sephadex G-15, swollen in TRIS/HCl buffer (10 mmol/L, pH 8.6). V_0 (dextran blue) was 18.5 mL and V_t (D_2O) was 40 mL. The flow rate was 14±2 mL/hr, using a peristaltic pump (LKB, Bromma, Sweden). UV-absorbance detection was applied at a wavelength of 254 nm (Jasco Uvidec II, Tokyo, Japan). The column, equipped with a water jacket, was kept at 25 °C.

In vitro dialysis. In the dialysis experiments a Gambro Hemodialysis unit, including a dialysate controller were used. These experiments were carried out in the Dialysis unit, Free Univ. of Amsterdam, with the assistance of Drs. P. De Vries. The test solutes were dissolved in and dialyzed against fresh dialysate solution (obtained from concentrate) at pH 7.4. The volume of the container was 2 L. The test solution was kept at 37 °C. Flow rates of the test solution and dialysate fluid were 200 mL/min and 500 mL/min respectively. A Gambro parallel plate dialyzer (Lundia 3N, 0.8 m^2effective surface area, 10 μm thickness) was used.

Calculations. Chromatographic elution in gel filtration can be expressed in terms of a distribution coefficient K_d as follows:

$$K_d = \frac{V_e - V_0}{V_i}$$

where V_e, V_0, and V_i are peak elution volume, void volume (elution volume of a totally excluded marker, e.g. dextran blue), and the total internal fluid volume of the gel. V_i can be determined from the elution volume of D_2O, although it should be corrected for isotope exchange of hydroxyl hydrogen from the gel [Haglund, 1978]. Then the following holds for Sephadex G-15 gel:

$$K_d = 1.075 \cdot \frac{V_e - V_0}{V_{D_2O} - V_0}$$

Membrane dialyzer clearance (K_B), by definition, equals the amount of solute i removed from the blood side per unit time, M, divided by the blood side inlet concentration (C_{Bi}),

$$K_B = \frac{\dot{M}}{C_{Bi}}$$

The blood side fluid is recycled in a closed loop from a container of volume V. The concentration drop as a result of pure dialysis (no ultrafiltration) is:

$$\ln\frac{C}{C_0} = -\frac{K_B}{V} \cdot t$$

K_B/V is a first order rate constant.

Samples were taken at 10 min time intervals from the recycle container. Clearances were determined by regression of ln C/C_0 versus t/V. The concentrations of the test solutes in the samples were determined by liquid chromatography (HPLC).

2.2.3.3 Results and discussion.

Results of the experiments on gel filtration and dialysis are summarized in Table#2.3 and Fig.#2.5.

Table #2.3. Distribution Coefficients (K_d), Dialyzer Clearances (K_B), and Group Selectivities in Both Processes for the Substituted Nitrobenzenes.

Compound	M_r	K_d	$\ln\left(\frac{K_d}{K_{d,nbz}}\right)$ [a]	K_B [b]	$\ln\left(\frac{K_B}{K_{B,nbz}}\right)$ [a]
Nitrobenzene	123	3.75	-	125	-
p-NO2-Ph.acetic acid	181	0.65	-1.76	95	-0.27
p-NO2-Ph.glucoside	301	2.28	-0.50	75	-0.51
p-NO2-Ph.glucuronide	315	0.49	-2.03	67	-0.62
p-NO2-Ph.lactoside	463	1.85	-0.71	55	-0.82

[a]nbz = nitrobenzene. [b]mL/min.

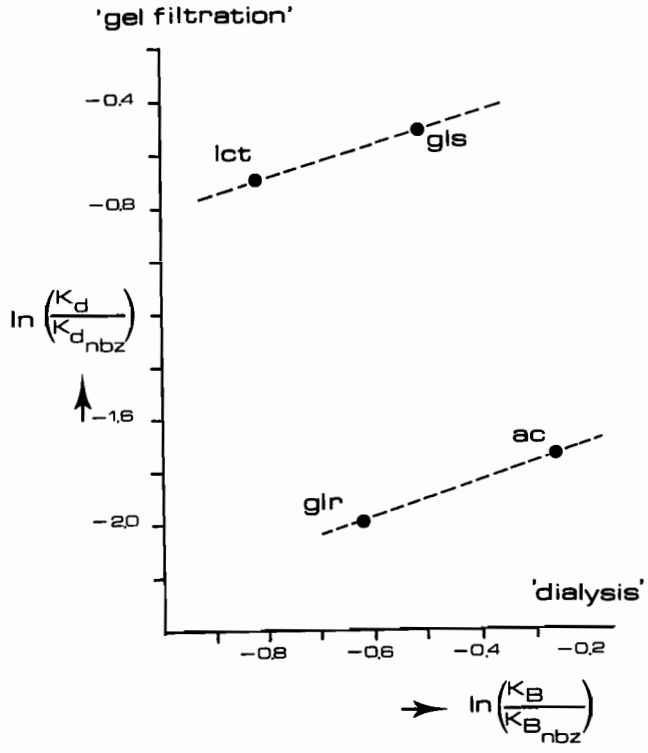


Fig.#2.5. Plot of functional group selectivities in gel filtration versus those in cuprophan dialysis. Lct, gls, glr and ac denote *p*-nitrophenyl -lactoside, -glucoside, -glucuronide, and -acetic acid respectively.

A plot of the group selectivities in gel filtration versus those in dialysis, reveals that there is not one single linear relationship (Fig.#2.5). However, parallel lines may be drawn through the data points of the "acidic" and "neutral" substituents, suggesting a linear relationship within these solute groups.

A general statement that anomalous elution behavior in gel filtration will also be reflected in cuprophan dialysis evidently is not valid. The addition of a carboxylic acid moiety to an aromatic compound changes the reaction center, and rejection becomes the prevailing factor governing retention in gel filtration. This is not observed in dialysis on cuprophan membranes. Retention of the substituted compounds in gel filtration is determined by adsorption, ion exclusion, and size exclusion, the relative importance of which varies with the substitution. From the plots of the group selectivities, in gel filtration and cuprophan dialysis, versus molecular mass (Fig.#2.6), it can be concluded that in dialysis the M_r contribution of the substituent is the major factor determining relative clearances.

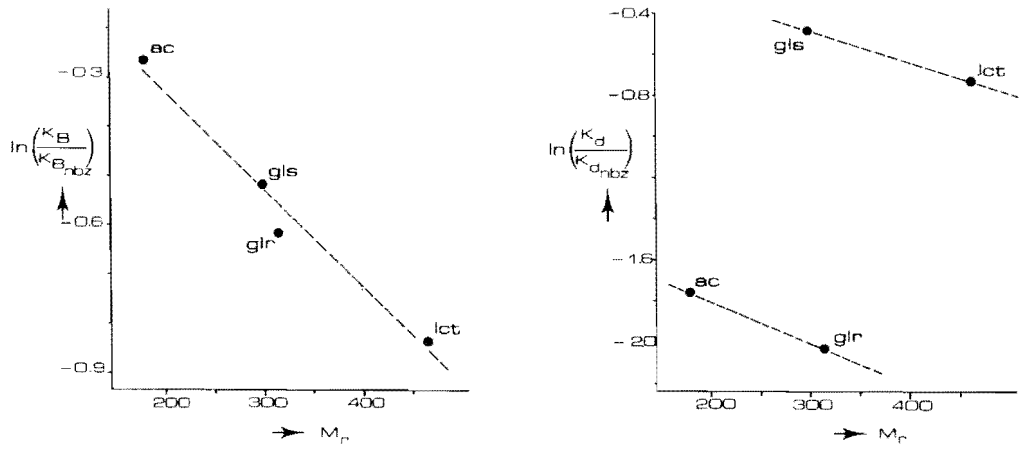


Fig.#2.6. Functional group selectivities as a function of the molecular mass of the substituted nitrobenzenes. left, cuprophan dialysis; right, Sephadex gel filtration. For abbreviations see Fig.#2.5.

In gel filtration an additional "acidic" interaction occurs. The substituted nitrobenzenes behave differently on gel filtration and cuprophan dialysis.

This conclusion may be generalized as follows: *the different interplaying factors governing chromatographic elution in gel filtration do not necessarily work out the same way in membrane dialysis.*

This can be reformulated: Elution volumes of (uremic) serum fractions in Sephadex gel filtration cannot be used to predict dialyzer behavior of the (unknown) solutes in these fractions. Conversely gel filtration elution volumes cannot be predicted from given dialyzer clearances (e.g. those for "middle molecules"). The results of this study support our earlier conclusion that *isolation* of "middle molecules" by gel filtration is not the most rational approach. Dialysis or filtration on inert membranes seems more appropriate.

3 PROFILING OF ACCUMULATING COMPOUNDS IN UREMIC SERUM

OVERVIEW AND CRITERIA

3.1 Introduction

In the present chapter we will discuss the usefulness of the application of "profiling" methods to the study of uremic solute accumulation [Schoots et al., 1984]. A number of criteria will be formulated for such a method, by which uremic compounds of different solute classes can be analyzed simultaneously.

In general linguistic usage the term (metabolic) profiling is used when multiple compounds, both known and unknown, from a certain class of solutes (e.g. nucleosides, fatty acids) are to be analyzed, the interdependencies being of importance. On the other hand "screening" and "multicomponent analysis" refer to procedures designed to yield data on a number of known substances that are not necessarily related, or do not belong to a certain class of compounds (e.g. clinical autoanalyzers) [Holland et al.,1986]. Hereafter sometimes the term "profiling", and in other occasions the term "multicomponent analysis" would be the most appropriate. However, we feel the definitions of the terms are not very dinstinctive, and therefore they will be used interchangeably.

Various analytical procedures have been developed by different authors for the "profiling" of compounds accumulating in uremic blood. Generally chromatographic or electrophoretic methods are applied. In the previous chapter the strategies for middle molecule analysis have been described. Here an overview will be given of the profiling methods that have been developed to analyze lower molecular mass compounds in uremic fluids. They comprise gas chromatography (GC), gas chromatography/mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) and column electrophoresis (EP).

3.2 Overview and criteria

Many solutes accumulate in the body fluids of uremic patients. Many of them were shown to exert some toxic effect in a variety of *in vitro* tests. However the wide range of uremic symptoms could not be attributed to a single compound or class of compounds. Thus the knowledge of the etiology of uremia and the real "culprits" is scarce and scattered. No all-embracing theory of uremic intoxication is available, and with the present doubts around the "middle molecule" hypothesis, the question of the etiology of uremia presently is virtually as topical as it was one century ago. One approach to this problem is to look for interdependencies and relations between the fragments of information that are available from

many studies on individual solutes. This however, seems to be a virtually impossible task, as different patients, treatments and analytical procedures have been used that may not be compared, or are documented inadequately [Lundin et al., 1984]. This may be overcome at least partially when multiple (analytical) data are obtained simultaneously for individual samples. In this case both quantitative information on individual solutes and knowledge of the relative relationships of these solutes is obtained. The application of multicomponent analytical techniques and subsequent multivariate statistical analysis may serve this aim.

Ideally , the analytical data are obtained in connection with well-documented clinical data. There is, however, a lack of consensus on the choice of appropriate and objective clinical "measures" of uremia (Teschan, 1986). Nonetheless multicomponent analytical techniques may be used to study uremic solute accumulation within and among groups of dialyzed and non-dialyzed patients on different therapies. Knowledge of the structure of these patient groups with respect to accumulation of different solutes (and their toxicity) may be a guide to individualized (hemo)dialysis prescription.

The choice of the multicomponent analytical technique depends on the nature of the "target" compounds. In our problem it is desirable to use a technique that facilitates the analysis of both known and unknown substances from different solute classes.
It was our aim to profile a wide range of accumulated components from different solute classes in uremic sera. For this purpose chromatographic methods are very appropriate because both known and unknown compounds can be analyzed.
A number of important requirements for the procedure are:

1. Little preselection of compounds to be measured. This means minimizing sample pretreatment.
2. The method must be "mild" because of the presence of labile molecules.
3. High efficiency in order to maximize peak capacity of the separation process.
4. Adequate detection of which the "selectivity" is "tailored" to the constraint of peak capacity.
5. Sufficient precision to facilitate quantitation.
6. Automation of both analysis and data handling.

In principle modern gas chromatography (GC), high performance liquid chromatography (HPLC), and column electrophoresis (EP) (e.g. isotachophoresis) are suitable for the separation of lower molecular mass compounds. All three techniques may meet the first requirement of minimal sample treatment, although in gas chromatography chemical

derivatization of most compounds from biological fluids is inevitable, which in some cases results in multiple peaks for a single substance. Both HPLC and EP normally work at room temperature which make them suitable to analyze thermolabile compounds. Peak capacity may be sufficient in all three techniques. Suitable universal detection principles in gas chromatography are flame ionization and mass spectrometric detection, and in EP conductivity detection. In HPLC no universal detector is available with the required sensitivity. Nevertheless UV-absorbance detection at 254 nm may be considered semi-universal as many different biological compounds may be detected with this method. The precision of quantitation is comparable for the three techniques. Presently only GC and HPLC can be automated with respect to analysis and data handling. Failure to meet the latter requirement, especially the absence of autosamplers, is a drawback of column EP.

High resolution profiling procedures based on *gas chromatography* have been described. Gas chromatographic separation followed by mass spectrometric identification (GC-MS) of various uremic dialysate and serum constituents have been reported [Masimore et al.,1977; Bultitude and Newham,1975; Schoots et al., 1979; Schoots et al.,1979, see also chapter#6]. More specific classes of solutes, such as steroids [Ludwig et al.,1978], phenolic acids, polyphenols and other compounds [Niwa et al.,1979a,1979b,1980] have been analyzed. A comprehensive review on profiling by gas chromatography-mass spectrometry in clinical medicine and uremia has been published recently [Niwa, 1986].

Profiling of uremic accumulated compounds by *high performance liquid chromatography* (HPLC) has been reported by different authors. UV-absorbing solutes in uremic dialysate and uremic serum [Senftleber et al.,1976; Knudson et al.,1978; Schoots et al.,1982;1985; Brunner and Mann,1984] were analyzed. An attempt was made to characterize the peptidic constituents of uremic body fluids by HPLC with UV and fluorescence detection [Mabuchi and Nakahashi,1983]. Fluorescent substances in serum and urine of uremic patients were studied by Swan et al.[1983], Barnett and Veening[1985], and Williams et al.[1987]. Simultaneous profiling of UV-absorbing and fluorescent solutes in sera of uremic patients on hemodialysis and chronic ambulatory peritoneal dialysis, was reported recently [Schoots et al.,1988]. Guanidino compounds accumulating in uremic fluids were profiled as a class [Yamamoto et al.,1979; Hung,1984]. Some authors separated "middle molecule" gel filtration fractions by HPLC [Schoots et al.,1982; Gallice et al.,1983; Mabuchi and Nakahashi,1983].

Most of the HPLC-profiling studies were of qualitative nature and only a few were demonstrated to be reproducible and applicable routinely for the analysis of large numbers of samples.

Isotachophoretic profiling of uremic compounds using UV-absorption and conductivity detection was reported by Mikkers et al.[1979].

In the following chapter we describe the development and characteristics of a profiling method based on reversed-phase high performance liquid chromatography (HPLC). HPLC gradient elution appears to be the most appropriate for the purpose of profiling. Sample pretreatment is limited to ultrafiltration for the removal of serum proteins. Separation takes place at ambient temperature so thermal decomposition is avoided. The use of column packing materials with particles of small diameter (here 5 μm) enables efficient separation and results in appropriate peak capacity. The combination of semi-universal UV-detection (at 254 nm) and selective fluorescence detection facilitates the quantitation of many solutes expected to accumulate in uremic blood. The precision of HPLC methods is sufficient to facilitate quantitation. HPLC analyses can be automated as autosamplers and computerized data acquisition facilities are commercially available.

4 HPLC PROFILING DEVELOPMENT AND CHARACTERISTICS

4.1 Introduction

In this chapter we will delineate the development of a profiling method based on high performance liquid chromatography (HPLC). Sample pretreatment necessary to determine both non-protein-bound fractions and total concentrations of the analyzed solutes, is described. The chromatographic method was optimized with respect to the phase system and detection selectivity. In the recorded chromatograms unknown peaks were (tentatively) identified by mass spectrometric and enzymatic methods. Quantitation of both known and unknown solutes is described. Further characteristics of the profiles such as precision, accuracy, and interferences of drugs are depicted.

We will discuss the general appearance of the profiles of uremic sera in comparison to those from normal sera. Striking intersolute correlations will be reported and interpreted.

4.2 Sample Route

Sample handling is depicted in Table#4.1.

Table#4.1. Sample Route for Gradient Elution HPLC Profiling.

STEP	DETAILS	REMARKS
Sample	Serum, ultrafiltrate, peritoneal dialysate, 1 mL. Store at -20 °C, or -70 °C (period > 2 months).	Serum and peritoneal dialysate contain proteins that should be removed prior to HPLC analysis either by filtration or by precipitation.
Removal of serum protein by precipitation	add 50 µL of 100 g/L TCA[a] to 1 mL of serum. Ultrasonicate, centrifuge. Use supernatant or apply ultrafiltration as in next step.	To determine total concentrations of protein bound solutes.
Removal of serum protein by ultrafiltration	Amicon Centrifree® UF units, rotated at an angle 60°, 1900x*g*, 20 min. M_r cut-off 25000.	Concentrations of "free" fractions are obtained.
Addition of injection standard	Add 300 µL NSA[b] solution (37 mg/L) to 300 µL filtrate.	For checking of injection integrity.
Autosampler injection	50 µL	Repeated injection of multiple samples.

[a]TCA = trichloroacetic acid; [b]NSA = naphtalenesulfonic acid.

4.3 Operational Conditions

Phase system

We have chosen for octyl-modified silica reversed-phase columns in combination with aqueous ammoniumformate buffer (and methanol as a second solvent) to realize the elution of a wide scope of solutes with different charge and polarity in a single gradient elution analysis. Octyl(C8) was favoured over octadecyl(C18) for its somewhat different selectivity, resulting in better employment of peak capacity. The Ultrasphere Octyl columns (5µm particles, length 25 cm), from a single production batch had an average efficiency of 84000 plates/m (4.9% C.V., n=4) in isocratic test runs at a solvent composition of 60% methanol/40% water, with the test solute anisol. Between-column differences in absolute retention times of test solutes phenol, *p*-cresol, and anisol amounted to 2.5 % C.V., while relative retention times were much better (0.2 % C.V.). The separation column and solvent reservoirs were kept at 25 °C by means of a thermostat bath and a column water jacket.

Ammonium formate buffer at pH 4 was used because its pK value of 3.75 facilitates the retention of weak organic acids, of which many have been detected in uremic fluids. The volatility of this buffer is useful in relation to peak collection and identification, requiring lyophilization of the solvent. Buffering capacity at this ionic strength (0.05 M) was sufficient even with injections of TCA-treated (low pH) samples. Between the buffer pump and the gradient mixer a 15cm Lichroprep RP18 (Merck, Darmstadt, FRG) column was mounted to avoid interference of organic impurities in the buffer during gradient elution on the analytical column.

The value of 1.3 %/min for the gradient slope optimized experimentally, which is equivalent with b=0.1 for the gradient steepness parameter, is in correspondence with literature data on optimal rate of gradient development for maximal peak resolution [Snyder et al.,1978].

Detection

UV-absorbance detection at a wavelength of 254 nm was applied. This technique has been shown to be semi-universal. Many urinary waste metabolites have been detected at this wavelength after separation by anion-exchange chromatography. A number of these substances have subsequently been identified by workers at the Oak Ridge National Laboratory [Burtis and Warren,1968; Mrochek et al.,1971]. They include purine and pyrimidine bases, phenolic acids, amides and various nitrogenous waste metabolites. Some of these substances

have been found in uremic dialysate [Senftleber et al.,1978] and normal sera [Hartwick 1979]. Chapter#4.5 deals with the identification of UV-absorbing and fluorescent solutes in the HPLC chromatograms of ultrafiltered uremic sera.

Many endogenous compounds show native fluorescence emission [Guilbault, 1973]. Especially a number of aromatic and indolic substances such as tryptophan metabolites can be detected by fluorescence at wavelengths of 280 nm excitation and 340 nm emission. As there are indications that neurological disturbances in uremia may be associated with abnormal tryptophan metabolism and abnormal distribution of tryptophan and its metabolites between blood and brain [Tyler,1968; Raskin and Fishman,1976; Holmes and Kahn,1987], we used fluorescence detection in addition to UV-absorbance detection. Excitation at 280 nm and emission at 340 nm (double monochromator) proved to be optimal wavelengths. The specificity of detection at these wavelengths resulted in fluorescence traces showing good resolution, and precision of quantitation. Both UV-absorbance and fluorescence detection are compatible with gradient elution operation. Typical dual-detector chromatograms of ultrafiltered uremic and normal sera are given in Fig.#4.1.

Data acquisition and handling

Dual channel data acquisition of UV-absorbance and fluorescence detector signals was done with an interface for analog-digital conversion and buffering of raw data. Peak detection prior to integration was done with the valley-to-valley option from the Nelson Analytical chromatography software, as this proved to be the most reproducible. The raw data were processed on a microcomputer and quantitative data were loaded in a spreadsheet. For statistical analysis the spreadsheet was exported as a DIF (data interchange format) file and subsequently converted to a SAS data set (SSD file). Statistical analysis was performed with SAS Statistical software on both IBM/PC and VAX 750.

Quantitation

For quantitation we used an internal standard (naphtalene sulfonic acid) both for UV-absorbing and fluorescent compounds. Known substances were quantitated by interpolation on calibration lines obtained for aqueous solutions, and peak heights of unknown compounds were divided by the height of the internal standard. Calibration solutions of all solutes were kept at 4 °C, and those of tyrosine, indoxylsulfate, tryptophan and 3-indoleacetic acid were protected against daylight by means of aluminium foil to prevent photodecomposition. Typical calibration lines for the known solutes are given in the Table#4.2.

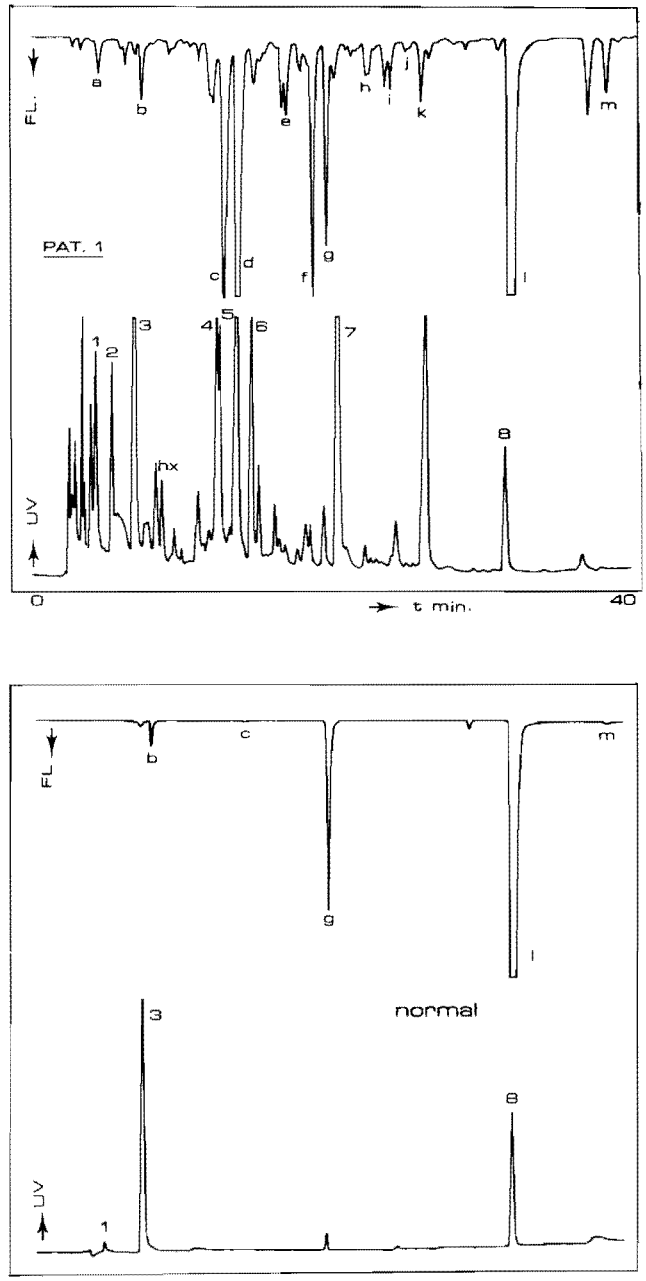


Fig.#4.1. HPLC-profiles from uremic(top) and normal(below) ultrafiltered sera. Peak identifications. UV-trace:1,creatinine; 2,pseudouridine; 3,uric acid; 4 and 5,unknown; 6,*p*-hydroxyhippuric acid; 7,hippuric acid; 8,internal standard. Fluorescence trace:a,UKF1; b,tyrosine; c,UKF3; d,UKF4; e,UKF5; f,indoxylsulfate; g,tryptophan; h,UKF6; i,UKF7A; j,UKF7; k,UKF8; j,UV-standard(=8 in UV-trace); m, 3-indoleacetic acid.

Table#4.2. Calibration Lines of Known Compounds in the HPLC Profiles.

COMPOUND	CALIBRATION LINE[a,b]	r[c] (signif.)	RANGE (μmol/L)
creatinine	Y = 3.37X - 10.67	0.998 (0.0001)	300-2000
pseudouridine	Y = 88.85X - 282.4	0.995 (0.0004)	10-200
uric acid	Y = 19.57X - 2694	0.983 (0.0027)	100-1000
hypoxanthine	Y = 101.49X - 231.5	0.995 (0.0004)	15-75
p-OH-hippuric acid	Y = 170.53X - 175.4	0.998 (0.0023)	20-100
hippuric acid	Y = 22.93X - 475.3	0.998 (0.0001)	100-500
tyrosine	Y = 44.63X - 147.8	0.999 (0.0001)	10-100
indoxylsulfate	Y = 339.3X - 621.2	0.999 (0.0001)	30-120
tryptophan	Y = 591.0X - 148.7	0.999 (0.0001)	15-50
3-Ind.acetic acid	Y = 659.5X - 180.1	0.999 (0.0001)	1-10

[a]Y denotes peak height, X is concentration (μmol/L). [b]All Y-axis intercepts were not significant ($P > 0.05$). [c] = correlation coefficient. signif.= significance of slope.

The operational conditions of the HPLC-profiling procedure are summarized in the following Table.

Table#4.3. Operational Conditions in Gradient Elution HPLC-Profiling.

OPERATION	DETAILS	APPARATUS/MANUFACTURER
Injection	Volume 50 µL, autosampler	Model 500 (Beckman Instr.)
Elution: Column	C8-modified silica 25cmX4.6mm i.d. analytical column (5µm). 3cmX2mm i.d. guard column, (10 µm).	Ultrasphere Octyl, and Ultrapack Octyl (Beckman Instr., Berkeley, CA, USA).
Elution: Solvent	Linear gradient. High pressure mixing. 100% ammonium formate,pH 4, 50 mmol/L, to 60% methanol during 45 min. T=25°C. Deionized water,HPLC-grade methanol.	Two Model 100A double piston pumps. Mixer-volume 0.5 mL. Deionized water from Milli-Q system. (Millipore/Waters, Milford, MA, USA) HPLC grade methanol (Fisons Ltd., Loughborough, UK).
Detection	UV-absorbance at 254 nm. Fluorescence: 280 nm excitation and 340 nm emission.	Model 160 UV-absorbance detector (Beckman Instr.). Model RF-530 Fluorescence detector (Shimadzu, Tokyo, Japan).
Data acquisition peak detection and quantitation	ADC interface (buffer) Valley-to-valley. Sample frequency 1 Hz. Internal standard.	Model 761S data interface (Nelson Analytical, Cupertino, CA, USA). Model 2600 Chromatography software (Nelson Analytical) on IBM PC/XT.
Statistics	descriptive, bivariate and multivariate analysis.	SAS statistical software (SAS Institute Inc., Cary, NC, USA).

4.4 Characteristics of the profiles

Precision

Precision of the peak heights and quantitation was between 3 and 10% (C.V.) for all compounds with repetitive ultrafiltration and injection of an uremic serum sample (n=15). Some of the fluorescent substances show photodecomposition, thus having precision values

at the high end of this range. Precision of retention times and peak heights in analyses of a gradient test mixture were 0.6-1.0% and 1.0-3.6% respectively (10 injections during a 1-month period) [Schoots et al.,1985].

Accuracy

Evidently it is impossible to check the accuracy of unknown compounds in the profiles. Here we have determined the accuracy of the creatinine quantitation for three reasons. First, the creatinine concentration as determined here by HPLC may serve as a reference to literature data. Secondly it is used here as a normalization factor (i.e. in the comparative study of HD and CAPD sera). Third, the creatinine concentration is certified in many human reference sera, so these can be used to compare the certified value with our own HPLC-data. The latter was performed with two certified human reference sera (lot numbers 840704 and no. 840711 respectively) from RIVM (Rijks Instituut voor de Volksgezondheid en Milieuhygiene, Bilthoven, The Netherlands), and two laboratory control sera ("Precinorm", lotno. 155501, and "Precipath", lotno. 151608, Boehringer Mannheim Diagnostica, FRG). The results are shown in the table below.

Table#4.4. Comparison of Creatinine Determinations by HPLC and Reference Methods.

SERUM	REFERENCE METHOD	CREATININE CONC. Ref.method (µmol/L)[a]	CREATININE CONC. HPLC (µmol/L)[b]	DIFFERENCE
RIVM 840711	Modified Jaffé[c]	250 (5)	247	1.2 %
RIVM 840704	Modified Jaffé[c]	71 (2)	65	9 %
Precipath	Enzymatic(UV)	311 - 413	401	within range
Precinorm	Enzymatic(UV)	160 - 212	220	out of range
"	Jaffé[d]	180 - 238	"	within range

[a]Certified values for RIVM sera (95%-interval), and 95% control range for Precipath and Precinorm. [b]Mean value from duplicate measurements. [c]Modified Jaffé-method, Frankonit, KC16, NVKC-RIV method(NVKC=Nederlandse Vereniging voor Klinische Chemie). [d]Jaffé-method with deproteinisation by trichloroacetic acid.

The creatinine determinations by our HPLC method are in good agreement with the certified values of RIVM in two (normal) sera with different certified values. It should be noted that the creatinine concentrations in uremic sera are generally higher (around 1000 µmol/L). To our knowledge, however, no uremic reference sera are available. Care should be taken with the extrapolation of the present results to the concentration range found in uremic sera. It is

seen that our (HPLC)-value of 220 µmol/L is out of the control range of the enzymatic determination specified, and within that of the Jaffé-method. The value of 401 µmol/L determined by us in the Precipath sample by HPLC is within the control range of the enzymatic method. The Precinorm and Precipath samples are not certified reference sera. More importance should be attached to the results with the RIVM sera than with Precipath and Precinorm.

Interference of drugs

Commonly used drugs in the population of uremic patients were: Co-trimoxazol, fenoterol, clonidine, isosorbidedinitrate, cyclandelate, dihydrotachysterol, dimetindeen, thiamin, propanolol, prazosine, nitrazepam, dihydralazine, pyridoxin, cyanocobalamin, metoclopramide, flunitrazepam and cimetidine. Taking into account expected therapeutic serum concentrations in uremic patients and extinction coefficients at 254 nm of the active substances in the drugs, only Co-trimoxazol (trimethoprim and sulfamethoxazol) and neurobion^{R} (thiamin, pyridoxin, and cyanocobalamin) were suspected of interference with the HPLC analysis. This was not checked for fluorescence that has been added later as a detection technique. Injection of methanolic solutions of these drugs, in the expected concentrations, resulted in peaks at retention times t_R=1935 s and t_R=1999 s for Co-trimoxazol and at 848 s for neurobion. Only the latter might interfere with one of the characteristic uremic peaks (UK4 or UK5). A possible interference of drug metabolites could not be estimated as only the native drugs were available.

The profiles are characteristic for uremia

In a series of 33 patients on regular hemodialysis (n=17) and hemodiafiltration (n=16), with residual creatinine clearances between 0 and 8 mL/min, roughly three groups of profiles could be distinguished visually. A group of 5-10 patients showed profiles that approach those of healthy persons (at the used detector range settings), although the creatinine and pseudouridine peaks were significantly higher. These patients all had relatively high residual creatinine clearance (RCC ≥5 mL/min).
The second group, some 20 patients, had HPLC-patterns that seem to be characteristic of uremia, although different levels of accumulation occur between patients (see chapter#5.6, Fig.#5.15). In the profiles of the third group (5 patients) a large number of additional high peaks are found. This group must be considered abnormal within the population of uremic

dialyzed patients. The biochemical origin of these "abnormalities" is unclear. All members of the latter group had negligible residual creatinine clearance (RCC=0). Extreme profiles representative for the first and the third group of patients, are given in Fig.#4.2.

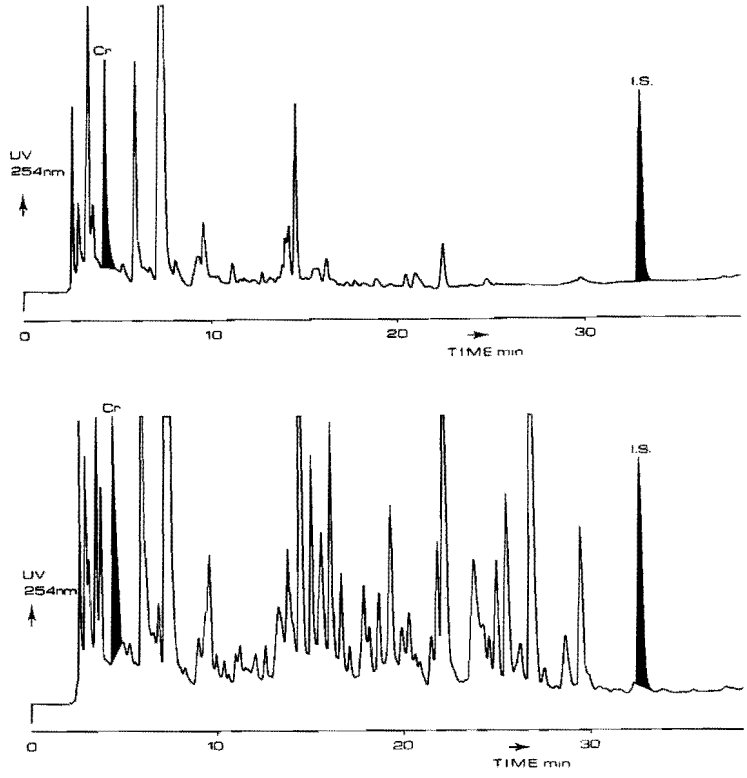


Fig.#4.2. Extreme HPLC-profiles from predialysis sera of two uremic patients; residual creatinine clearance, RCC = 5 mL/min(top) and RCC = 0 mL/min(below).

It may be noted here that the profiles were observed to depend on residual renal function both quantitatively and qualitatively. Especially, peak heights of creatinine, pseudouridine, uric acid and hypoxanthine appeared to vary differently with residual creatinine clearance than did those of hippuric acid, *p*-hydroxyhippuric acid, UKF1 and UKF7A. These findings will be discussed in chapter#5.

No relation of the profiles to type of renal failure could be found in this population of dialyzed patients with various kidney diseases.

Eleven compounds could be identified in the HPLC-profiles: creatinine, pseudouridine, uric acid, hypoxanthine, 2-furoylglycine, *p*-hydroxyhippuric acid, hippuric acid, tyrosine, indoxyl-sulfate, tryptophan, and 3-indoleacetic acid. Identifications will be described later in this chapter.

The HPLC profiles were characteristic for uremia, but also for the individual uremic patients. Furthermore, the HPLC "fingerprints" proved to be relatively constant within patients in the course of several months (see Fig.#4.3). This stability, combined with the short and longterm reproducibility of the automated profiling procedure, facilitates the application of multivariate statistical analysis of quantitative data.

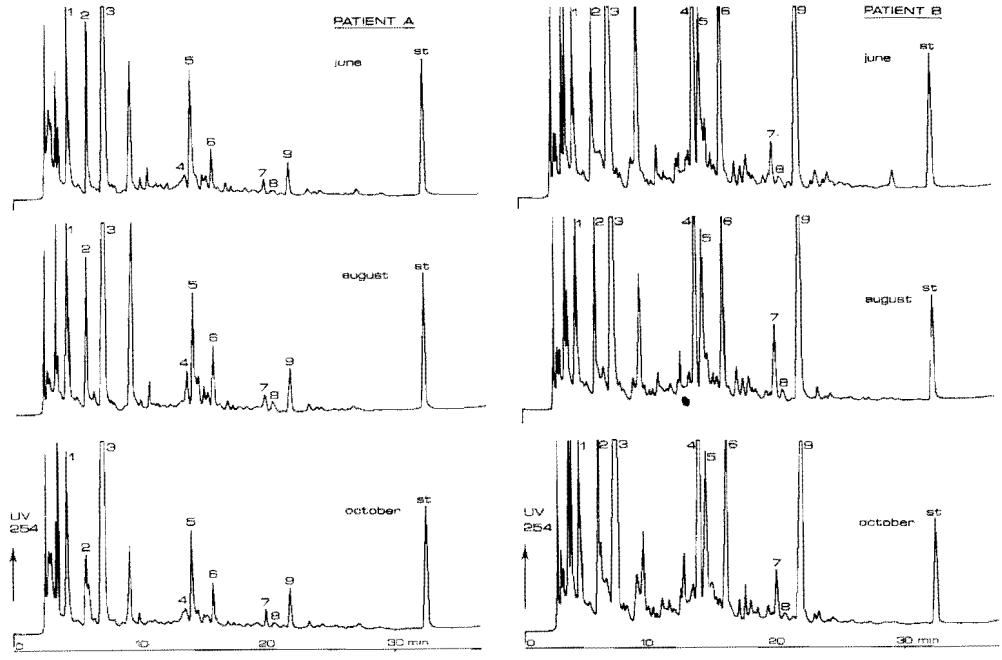


Fig.#4.3. Longitudinal change of serum HPLC-"fingerprints" of two uremic patients on hemodialysis treatment.

Intersolute correlations

A (Spearman rank) correlation matrix of 22 solute concentrations (including the HPLC-analyzed solutes, and urea and phosphate, analyzed conventionally), consists of 231 non-unity correlation coefficients. In this matrix (43 uremic dialyzed patients) there were 21 intersolute correlations significant at $0.01 < P < 0.05$, 15 at $0.001 < P < 0.01$, 4 at $0.0001 < P < 0.001$, and 3 at $P < 0.0001$. A number of striking correlations is tabulated below.

Table#4.5. Some Intersolute Correlations.

SOLUTE PAIR[a]	r[b] n=35-43	SIGNIFICANCE (*P*)	SOLUTE PAIR	r[b]	SIGNIFICANCE (*P*)
Crea - Psi	0.61	0.0001	Urea - Uric	0.63	0.0001
Psi - Uric	0.39	0.009	Urea - Phosph	0.46	0.002
Psi - UKF3	0.75	0.0001	*p*OHH - UKF7A	0.48	0.002
Trp - Ind	0.39	0.009	*p*OHH - Trp	0.43	0.004
Hipp - UKF1	0.49	0.0009	*p*OHH - UKF4	0.45	0.007
Hipp - UK4	0.44	0.004	Trp - UKF4	0.55	0.0005

[a]crea=creatinine, psi=pseudouridine, uric=uric acid, UK and UKF are unknown UV-absorbing and fluorescent solutes, trp=tryptophan, ind=indoxylsulfate, hipp=hippuric acid, phosph=inorganic phosphate, *p*OHH=*p*-hydroxyhippuric acid. [b]r = Spearman rank correlation coefficient.

The correlations of urea, creatinine, pseudouridine and uric acid concentrations are more or less expected as these solutes are all related to dietary (protein) intake. Urea, an end product of protein catabolism, has been shown to directly depend on dietary protein intake, and correlates to phosphate concentrations, because dietary protein is often associated with amounts of phospholipids, which are metabolized to inorganic phosphate. Uric acid is a general product of nucleic acid metabolism, while pseudouridine originates from more specific transfer-RNA turnover. Creatinine is the product of phosphocreatine degradation, the latter being an important energy-storing compound in muscle. The high correlation of pseudouridine and UKF3 is discussed in detail in chapter#5.2. Hippuric acid correlates to the unknown compounds UKF1 and UK4. Tryptophan correlates to UKF4 and indoxylsulfate. The latter is formed from tryptophan by intestinal bacteria. The concentration of *p*-hydroxyhippuric acid is correlated to those of various solutes, among which are tryptophan and the unknowns UKF7A and UKF4. Although not shown in Table#4.5 3-Indoleacetic acid, UKF7, and UKF8 were all correlated mutually, possibly pointing at a metabolic relationship of these fluorescent solutes.
A multivariate approach (Principal Component Analysis) of solute interdependencies is discussed in chapter#5.6.

Cumulative UV-absorbance and Fluorescence as measures of accumulation

Total peak areas in the UV- and fluorescence chromatograms may be considered more general measures of solute accumulation in uremic sera. Hippuric acid, UK4, and UKF4

correlate significantly to both total-UV ($P < 0.007$, 0.03, and 0.0001 respectively) and total-Fluorescence ($P < 0.01$, 0.003, and 0.02 respectively). *p*-Hydroxyhippuric acid, UKF1, and UKF7A correlate significantly to total-UV ($P < 0.03$, 0.04, and 0.006 respectively).

4.5 Identification

4.5.1 Introduction

For the identification of peaks appearing in the HPLC-profiles various sources of information are available. Some of the relevant techniques may be considered as absolute identification (or elucidation) methods, such as mass spectrometry and (certain) enzymatic conversions. Other techniques have only indicative value. Some of the latter, used in the present study, are:

1) Comparison of HPLC retention times of serum peaks with those of appropriate reference solutes by co-injection. GC retention times of isolated and trimethylsilylated HPLC-fractions can be compared with those of similarly treated reference solutes.
2) Comparison of the UV to Fluorescence detector signal ratio with those of reference solutes.
3) Comparing quantitative HPLC-data based on calibration lines obtained with reference solutes, with corresponding data reported in literature (e.g. blood serum or urine "normal" or "uremic" values).
4) Comparing quantitative HPLC-data based on calibration lines for reference solutes, with these data obtained for the same samples determined with other independent techniques (e.g. colorimetric or enzymatic).(see chapter #4.4 on accuracy.

4.5.2 Experimental

Retention times of over sixty UV-absorbing and/or fluorescent urinary compounds have been determined with the HPLC profiling procedure. Solutes eluting at retention times near to those of characteristic peaks in the uremic profiles were co-injected with serum ultrafiltrate samples. Creatinine, L-tyrosine, L-tryptophan, and hippuric acid were obtained from Merck AG (Darmstadt, FRG). Uric acid, hypoxanthine, *o*-hydrohyhippuric acid, pseudouridine and indoxylsulfate were purchased from Sigma Chem. Co. (St. Louis, Mo, USA), and uracil and 3-indoleacetic acid from Fluka AG (Buchs, Switzerland). The *m*- and *p*-hydroxyhippuric

acids were synthesized according to the active ester method described by v.Brussel and v.Summere [1978]: *m*- and *p*-hydroxybenzoic acids (from Sigma Chem. Co.) were converted to their N-hydroxysuccinimide esters using N-hydroxysuccinimide and N,N-dicyclohexyl carbodiimide, and subsequently reacted with sodiumglycinate to form the respective hydroxyhippuric acids. The chemical structure of the products was confirmed by NMR-spectrometry, and purity was checked by HPLC.

Mass Spectrometry. Prior to mass spectrometric analysis, fractions were isolated by semi-preparative HPLC. For this purpose the analytical gradient elution HPLC procedure was "upscaled" to semipreparative level, using a 25cm X 10mm(i.d.) Ultrasphere Octyl column (5 μm particles) instead of the similar analytical column with 4.6mm i.d. In upscaling, the gradient steepness parameter, b [Snyder,1980], was kept close to the value applied in the analytical separation (b=0.1) in order to obtain identical profiles. Thus, in the equation

$$b = \left(\frac{V_m}{F \cdot t_G}\right) \cdot \log\left(\frac{k_A}{k_B}\right)$$

the term $V_m/(F.t_G)$ was kept virtually constant. V_m is the column mobile phase volume, F is the flow rate, and t_G is the gradient time (when fully developed from A to B), and k_A and k_B are the capacity factors of a solute with pure solvent A and pure solvent B as the mobile phase. The choice of F and t_G, had to satisfy the following constraints: column pressure should not exceed 250 bar, and maximum flow rate was 5 mL/min. The final analytical and semipreparative conditions are shown in Table#4.6.

Table#4.6. Operational Parameters in Analytical and Semipreparative Gradient Elution Analyses.

SEPARATION COLUMN	FLOW RATE (ML/MIN)	t_G[a] (MIN)	b[b]	INJECTION VOLUME (μL)
Analytical (4.6 mm i.d.)	1	75	0.09	50
Semipreparative (10 mm i.d.)	2.5	112.5	0.11	1000

[a]gradient time. [b]gradient steepness parameter.

The HPLC apparatus (Ghent University) used for upscaling consisted of: Two Model 2150 HPLC-pumps, a Model 2152 controller, a Model 2238, UVICORD SII UV-absorbance detector, and a Multirac fraction collector (all from LKB, Bromma, Sweden).

HPLC-fractions were lyophilized, dissolved in pyridine, and converted to their trimethylsilyl-derivatives by addition of BSTFA, bis-trimethylsilyl trifluoroacetamide (Pierce, Rockford, USA). GC-MS analyses were performed on a Model HP 5970A mass selective detector coupled to a Model HP 5790A gas chromatograph (Hewlett-Packard, Avondale, PA, USA), equipped with a 40m CPSil5 capillary column (Chrompack, Middelburg, The Netherlands), and on a Model 4000 quadrupole GC-MS system (Finnigan-MAT, Sunnyvale, CA, USA).

Enzymatic conversions. Identification of HPLC peaks by "enzymatic peak shift" has been reported by different authors [Hartwick et al.,1979; Barnett and Veening,1985].
In the present study serum ultrafiltrates were incubated with various enzymes, and the HPLC chromatograms from the sample before and after incubation were compared.
In Table#4.7 the enzymes, corresponding cofactors and reactions used in this study are summarized.

Table#4.7. Enzymatic Conversions Used for Peak Identification.

SUBSTRATE	ENZYME	COFACTOR	PRODUCT
Creatinine	Creatininase		Creatine
Uric Acid	Uricase	O_2	Allantoin
Hypoxanthine, Xanthine	Xanthine Oxidase	O_2	Xanthine, Uric Acid
Indoxylsulfate	Arylsulfatase		3-hydroxy-indole
Tryptophan	Tryptophanase	P5'P[a]	Indole
Nucleosides	Purine Nucleoside Phosphorylase	Phosphate	Purine Bases
β-D-Glucuronides	β-Glucuronidase		Aglycon
Peptides	Aminopeptidase		Amino Acids

[a]P5'P = Pyridoxal-5'-phosphate

4.5.3 Results and Discussion

A number of HPLC peaks was identified by GC-MS analysis of the silylderivatives: *Hippuric acid, p-Hydroxyhippuric acid, Pseudouridine* and tentatively, *2-Furoylglycine.* Mass spectra are given in Fig.#4.4.

The identity of four peaks was established by specific enzymatic conversions: *Creatinine, Uric Acid, Tryptophan,* and *Indoxylsulfate.*

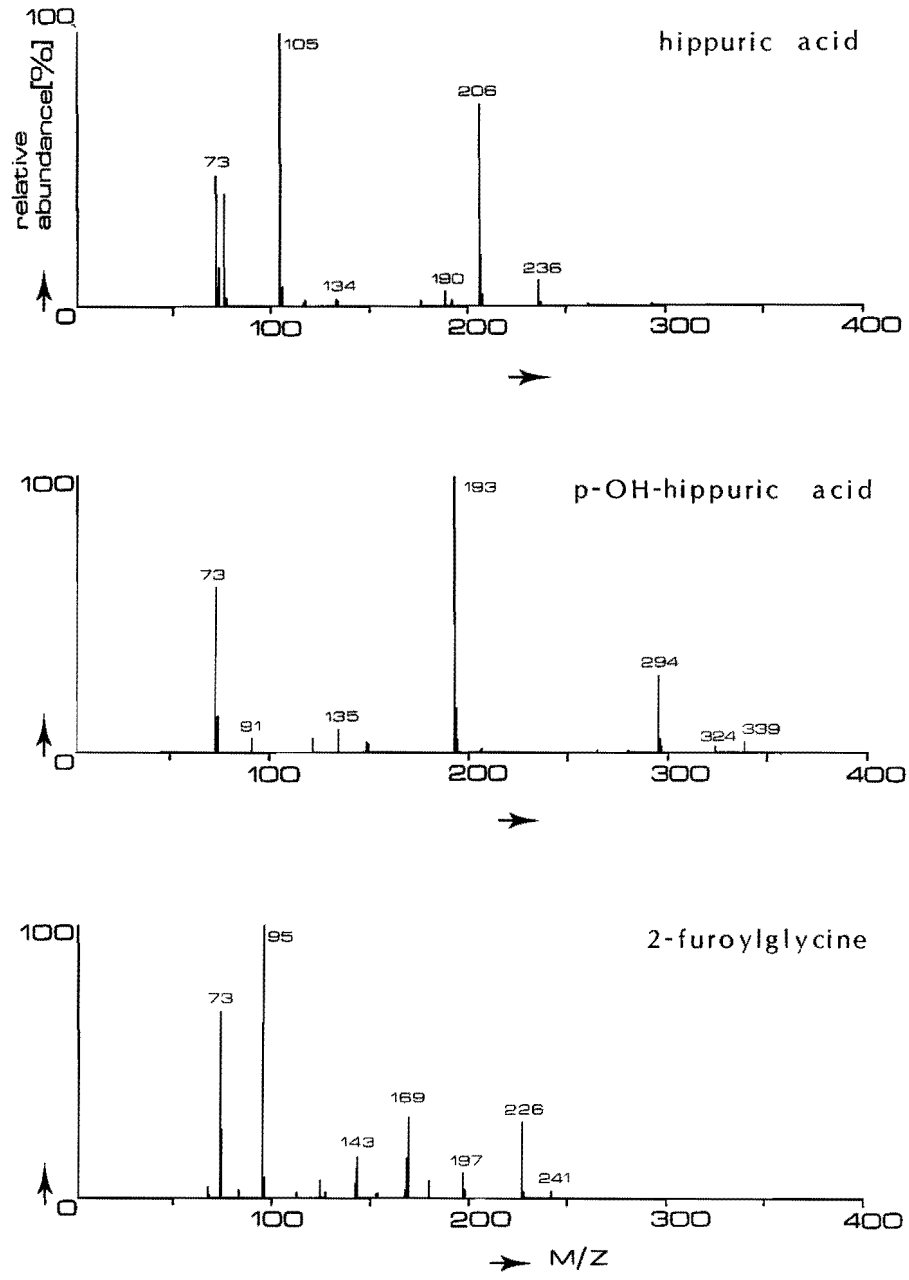


Fig.#4.4. Electron-impact mass spectra of HPLC-peaks identified as hippuric acid, *p*-hydroxyhippuric acid, and (tentatively) furoylglycine. Spectra of trimethylsilylated solutes are shown.

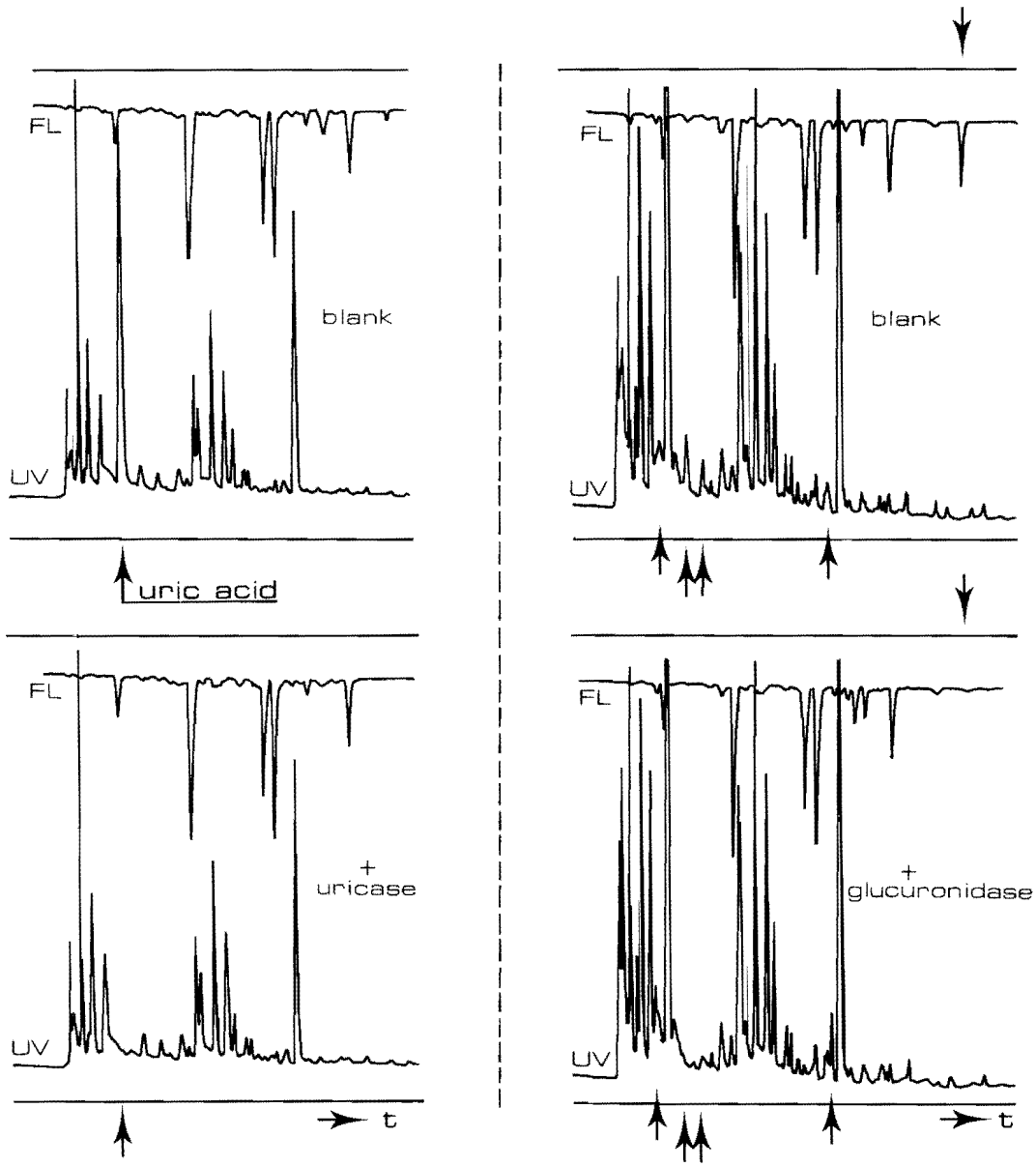


Fig.#4.5. HPLC-patterns of ultrafiltered uremic sera before(top) and after(below) incubation with uricase(left), and glucuronidase(right).

p-Hydroxyhippuric acid. A mass spectrum of the fraction isolated by preparative HPLC was very similar to a reference spectrum of trimethylsilylated hydroxyhippuric acid (EPA/NIH, 1978?). However, as spectra of the meta- and para-substituted acids are very similar, it could not directly be established which of these structures was associated with the HPLC-fraction. *o*-Hydroxyhippuric acid was not a candidate, as it elutes later in the chromatogram. As the m- and p-substituted acids are not available commercially, they were synthesized as described before. Both mass spectra and retention times of the HPLC-peak and pure *p*-hydroxyhippuric acid were identical.

Hippuric acid was identified by GC-MS analysis. Co-injection of pure reference solute confirmed this finding.

2-Furoylglycine was tentatively identified in the fraction of the peak designated UK4, by comparing mass spectrometric results with reference spectra [EPA/NIH]. It is a glycine conjugate of 2-furancarboxylic acid. The occurrence of furancarboxylic acids and furoyl-glycine in uremic plasma has been demonstrated earlier by GC-MS [Liebich et al.,1984]. It has been shown [Mabuchi and Nakahashi,1986; Takeda et al.,1987] that 3-carboxy-4-methyl-5-propyl 2-furanpropionic acid, present in uremic serum can be detected by UV-absorbance at 270 nm. It is to be expected that 2-furoylglycine may be detected by UV-absorbance as well. As the pure compound is presently not available, we were not able to determine its molar absorbance coefficient. It should be noted that the fraction UK4 is not pure, as multiple peaks appeared in the GC-MS analyses. Because these peaks are not yet identified, it could not be established whether they contribute to the UV-signal in HPLC.

Pseudouridine. Previously, the corresponding HPLC-peak was identified tentatively as uracil [Schoots et al.,1985]. However, recently we found that uracil is only a minor "shoulder" on the HPLC-peak that now has been identified as pseudouridine. The pseudouridine structure was elucidated by mass spectrometry, and confirmed by co-injection. This was repeated for the corresponding peak in sera of CAPD patients, that proved to be significantly higher than in patients undergoing hemodialysis (see Chapter#5.2).

Creatinine was identified by enzymatic conversion after incubation of uremic serum ultrafiltrate with creatininase and checking by HPLC-analysis.

Uric acid was similarly identified by use of uricase. HPLC-profiles of uremic serum ultrafiltrate before and after incubation by uricase enzyme are shown in Fig.#4.5 (left).

Tryptophan was converted to indole with the enzyme tryptophanase. Co-injection confirmed the tryptophan as well as the indole structure.

Indoxylsulfate was converted into 3-hydroxy-indole using the arylsulfatase enzyme. Co-injection confirmed the presence of tryptophan and 3-hydroxy-indole in serum samples before and after incubation.

Peptides and Glucuronides. To locate possibly eluting peptidic or glucuronidic compounds, uremic serum ultrafiltrates were incubated with aminopeptidase and β-glucuronidase enzymes respectively. With β-glucuronidase three minor peaks at retention times t_R=7.78 (UV), t_R=9.08 (UV), and t_R=30.32 (fluorescence) disappeared (see Fig.#4.5, right). Aminopeptidase incubation had no effect on the HPLC-profiles and it failed to break the peptidic linkage in hippuric acid and *p*-hydroxyhippuric acid. Xanthine oxidase incubation also had no effect. However, the uremic serum ultrafiltrates demonstrated only very small peaks at the retention times expected for xanthine and hypoxanthine, thus preventing identification by enzymatic peak shift.

3-Indoleacetic acid and *hypoxanthine* were tentatively identified by retention times, by UV/Fluorescence ratio, and by comparing concentration ranges in uremic sera obtained from our calibration lines and those reported in literature.

A number of characteristic "uremic" peaks are not yet identified. The unknown UV-absorbing and fluorescent solutes were designated UK and UKF respectively.

5 EXPLORATIVE STUDIES ON MARKER SOLUTES IN UREMIA

5.1 Introduction

Blood levels of accumulated compounds in uremia are influenced by various parameters such as dialyzer clearance, residual renal function, generation rate, catabolic rate, protein binding and dietary regimen. Knowledge of these parameters for relevant accumulating compounds, facilitates the prediction of their blood levels in individual patients on different therapies. Urea and "middle molecules" have been used for this purpose. In urea kinetic modelling the protein catabolite urea is used as a marker [Sargent and Gotch, 1975]. The "Middle molecules" were modelled using a so-called Dialysis Index based on properties of a hypothetical substance with a molecular mass close to that of vitamin-B_{12} (M_r 1355) [Babb et al.,1975].

Presently the middle molecule hypothesis has been put on ice because clinical studies to substantiate it were contradictory. Furthermore, from an analytical point of view, there is no evidence for toxic concentrations of the anticipated (peptidic?) middle molecules (see chapter#2).

The uremic symptoms have been observed to be positively related to protein intake. Thus it has been proposed that the symptoms are caused by high blood levels of accumulating protein breakdown products. Urea is the end product of protein catabolism, and can be used as a marker of solute accumulation, representing protein breakdown products. Therefore urea kinetic modelling, which was applied in the NCDS (National Cooperative Dialysis Study) is a rational approach.

In the present chapter, it is evaluated whether a single marker, such as urea, is sufficient to decribe uremic solute accumulation. For this purpose the HPLC-profiling technique described in the previous chapter is applied, to study the properties of twenty uremic solutes (urea and phosphate, which are included in the study, were measured conventionally).

It is important to note that apart from the solute properties determined in this explorative study, the question of relative toxicity of different (classes of) solutes remains topical in defining and implementing "adequate (dialysis) treatment", and thus in choosing the proper "marker" solute.

In the first section of this chapter uremic solute retention in renal patients on hemodialysis (HD) and continuous ambulatory peritoneal dialysis (CAPD) is described, starting from the notion that original observations leading to the "middle molecule" hypothesis were clinical findings with patients on peritoneal dialysis. Protein binding of the analyzed solutes was determined in order to compare both "free" and proteinbound fractions in uremic sera in the two patient groups, and to check whether protein binding is a factor that might result in a different weekly clearance of uremic solutes from the blood of patients

treated with HD and CAPD.
These binding data will also be used in combination with dialyzer mass transfer coefficients determined *in vitro*, to estimate the influence of protein binding on clearances of different compounds.

Concentration build-up as a function of residual creatinine clearance will be discussed. It has been shown that residual renal function may have substantial influence on blood levels of poorly dialyzable compounds, because the glomerulus filters these solutes as effectively as urea, which is not true for the dialyzer membranes.

Dialyzability of the analyzed compounds is studied *in vitro* on cuprophan membranes, using uremic ultrafiltrate as a blood substitute. Dialyzer clearances and overall mass transfer coefficients are determined in order to estimate the influence of these factors on solute blood levels in dialyzed patients. Protein binding levels described in the first section of this chapter are used to recalculate the *in vitro* clearances for the proteinbound compounds.

Change of blood levels of different solutes in uremic patients during hemodialysis treatment is studied. Relative contributions of dialyzer clearance, protein binding, and (any) multicompartmental behavior on blood solute concentrations are estimated from these determinations.

How the interplaying factors work out in relative serum concentrations of different solutes in uremic patients, will be shown by means of Principal Component Analysis (PCA) in a subsequent section. The subdivision of the analyzed solutes in different groups (by PCA) will be interpreted, and explained partly from the results in the preceding sections.

Finally, we studied the correlations of the HPLC-profiles and routine clinical biochemistries on one side and neurophysiological indices of uremia on the other.

5.2 HPLC PROFILING OF UREMIC SERUM FROM PATIENTS ON HEMODIALYSIS AND CONTINUOUS AMBULATORY PERITONEAL DIALYSIS (CAPD)

5.2.1 Introduction

During the last ten years, continuous ambulatory peritoneal dialysis (CAPD) has more and more become an alternative to conventional hemodialysis (HD), in the treatment of end-stage renal patients [Boen,1981; Moncrief and Popovich,1985; Popovich et al.,1978]. Although recurrent peritonitis, with the accompanying decreased permeability of the peritoneal membrane, has limited the use of CAPD, this has become the method of choice for treating pediatric patients, and (young) patients awaiting a kidney transplant. Problems of vascular access in hemodialysis, hypertension, and diabetes are also indications for treatment by CAPD.

Homeostasis is better in CAPD treated patients, because sudden fluctuations in fluid volume, electrolytes, and acid-base balance - as are seen in hemodialysis treatment - do not occur. This, combined with the continuous removal of waste metabolites, leads to steady state conditions in CAPD patients. Generally, these patients have higher hematocrit values than do HD patients [Zappacosta et al.,1982].

A controversial property ascribed to CAPD treatment is the supposedly more efficient removal of so-called neurotoxic middle molecules [Scribner, 1965; Babb et al.,1971;1973; Schoots et al.,1984]. In the m^2-hour hypothesis, introduced by Babb and coworkers [1971], these poorly dialyzable solutes are thought to be responsible for neurological disorders in patients undergoing regular dialysis. In kinetic studies [Babb et al.,1973; Nolph et al.,1980] the ratio of predialysis solute concentration in HD to the steady state concentration in CAPD plasma, is higher for solutes with higher molecular mass.

In a comparative study on electrophysiological indices of central nervous system(CNS)-function in patients on hemodialysis and CAPD, the latter group demonstrated more "normal" behavior (Marsh,1986). However, hitherto no evidence has been found that CAPD is more efficient in removing certain solutes that cause neurological symptoms. To measure the differences in solute concentrations in sera of patients treated with CAPD or with HD, the HPLC-profiling technique was used. Because protein binding of the solutes influences their removability by different dialysis procedures, protein binding levels were determined and compared.

5.2.2 Experimental

Patients and sera. Blood was sampled from 15 patients on HD treatment, and 12 patients on CAPD in the dialysis units of the St.Joseph Hospital and the Catharina Hospital, Eindhoven, The Netherlands. For hemodialysis Disscap 160 and Bellco dialyzers equipped with cuprophane membranes were used (Hospal, Meyzieu, France, and Bellco, Mirandola, Italy). Blood flow was 300 mL/min and 200 mL/min respectively; dialysate flow was 500 mL/min. CAPD was performed with four 2-L exchanges within 24 h. HD samples were taken before dialysis. CAPD patients were sampled in the outpatient department, more or less randomly during their daily routine of bag exchange. Table#5.1 summarizes patient characteristics.

Table#5.1. Some Characteristics of the Patient Groups.

	CAPD (n=12)			HD (n=15)		
VARIABLE	MEAN	SD	RANGE	MEAN	SD	RANGE
RCC, mL/min[a]	2.3	2.2	0-7.0	1.7	2.0	0-6.5
Albumin, g/L	32.1	5.4	20.6-38.2	34.0	3.4	30.1-40.9
TTOD, months[b]	9	8	3-25	46[c]	40	6-146
Age, yrs	48	17	18-67	60	10	37-72
Sex ratio, F/M	3/9			9/6		

[a]Residual creatinine clearance. [b]Total time on (any) dialysis. [c]Significantly ($P<0.001$) different from CAPD group.

None of the CAPD patients had symptoms of peritonitis. Two patients volunteered to change the mode of therapy; there was no clinical indication, such as peritonitis, or problems with vascular access. Although the group of CAPD patients were shorter on (any) dialysis treatment, no significant difference was found in residual creatinine clearances between the CAPD and HD groups. All patients were treated for at least three months with the method specified.

Protein binding. Different methods of determining protein binding have been reported in literature. Gel filtration, equilibrium dialysis, and ultrafiltration are the most commonly used techniques, the latter being more practical in terms of laboratory time and convenience [Whitlam and Brown, 1981; Wolfer and Rippon, 1987]. It has been reported that ultrafiltration is theoretically equivalent to equilibrium dialysis [Sophianopoulos et al.,1978]. Protein binding levels can be calculated according to:

$$PBL(\%) = \left(1 - \frac{C_f}{C_t}\right) \cdot 100$$

where C_f is the concentration of free solute, and C_t is the total concentration.

Free concentrations were determined in the filtrate obtained by ultrafiltration of serum samples through Centrifree micropartition units (Amicon Corp., Danvers, MA, USA). Aliquots of 1 mL of serum were ultrafiltered by centrifugation in the units at 22°C, at an angle of 30°, and 1900x*g*.

Total concentrations can be determined by precipitating the proteins by addition of trichloroacetic acid, hydrochloric acid, perchloric acid, acetonitrile, ammonium sulfate or by heat treatment. In this study the TCA precipitation method has been used.

Proteins were precipitated, by adding 50 µL of a 100 g/L solution of trichloroacetic acid (TCA) to 1 mL of serum. After ultrasonication the samples were centrifuged. The supernatant was ultrafiltered analogously, as described for the determination of free fractions. All Centrifree filtrates were analyzed by high performance liquid chromatography, after addition of an internal standard, naphtalene sulfonic acid. Any decomposition of uremic solutes as a result of TCA addition was studied. Decomposition was observed for pseudouridine (3.6%), tyrosine (3.9%), indoxylsulfate (6.7%), peak UKF7A (10%) and 3-indole-acetic acid (10%) with 50 µL of TCA solution added. These values were incorporated in the calculations. Concentrations of all solutes in subsequent ultrafiltrates, collected with 15 minutes time intervals, were constant, indicating that protein binding equilibrium was maintained during ultrafiltration. This was not completely true for tryptophan and indoxylsulfate, which showed a slight increase of ultrafiltrate concentration during ultrafiltration. For these solutes the values obtained by extrapolation to zero ultrafiltration time, were taken.

High performance liquid chromatography (HPLC). HPLC analysis was performed as described in chapter 4.

Gas chromatography-mass spectrometry (GC-MS). HPLC-peak 2 was isolated by repeated collection after HPLC separation, and lyophilized samples were derivatized with bis-trimethylsilyltrifluoroacetamide (BSTFA) (Pierce, Rockford, USA) and injected with a falling needle, solids injector [van den Bergh and Cox,1972]. Gas chromatography-mass spectrometry analysis was performed on a model HP 5970A mass selective detector coupled to a HP 5790A gas chromatograph (both from Hewlett Packard, Avondale, PA, USA), equipped with a CPSil 5 fused silica capillary column (Chrompack, Middelburg, The Netherlands).

Data acquisition, handling and statistics. Chromatographic data were read in SAS data files, and subsequently analyzed with SAS statistical procedures NPAR1WAY, REG and STEPDISC to perform nonparametric Wilcoxon's tests, regression analysis and stepwise discriminant analysis, respectively. With STEPDISC significance levels were 0.05 (significance level to enter, significance level to stay).

5.2.3 Results

The present study was divided into two parts:

A. Blood concentrations of 18 accumulated solutes in two groups of patients, on CAPD (n=12) and HD (n=15), were analyzed and compared.

B. These concentrations were also determined in a longitudinal setting in two patients changing therapy, one from CAPD to HD and the other *vice versa.*

Characteristic HPLC-profiles are presented in Fig.#5.1. UV-absorbance (A) and fluorescence (B) traces are shown that were obtained after injection of ultrafiltered serum from patient R. on hemodialysis (left), and two months later on CAPD treatment (right).

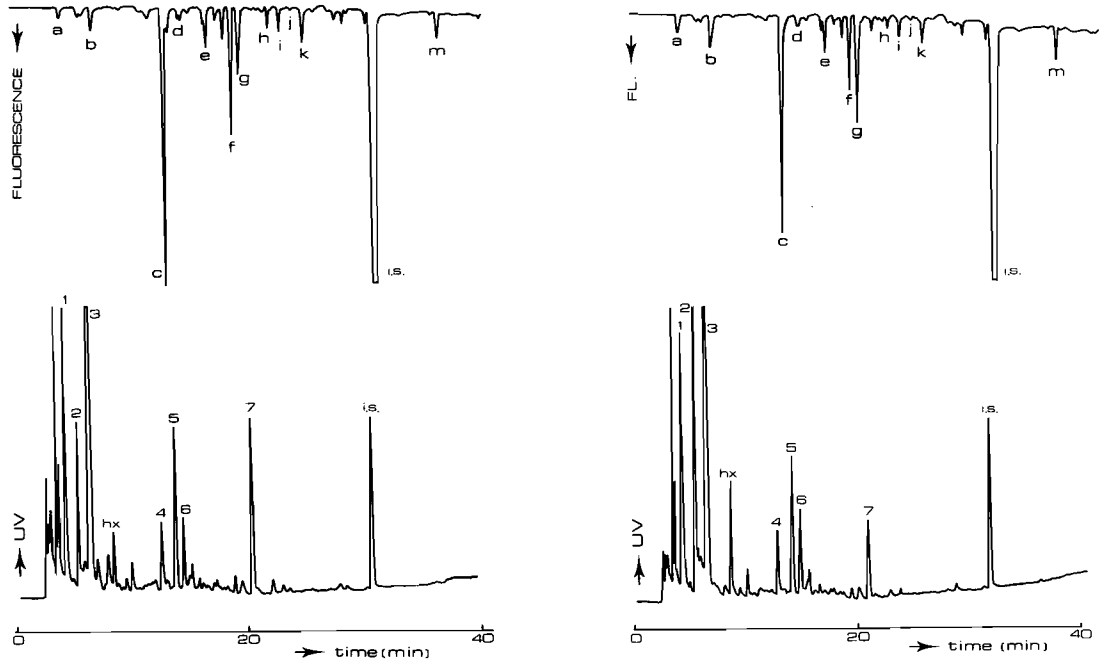


Fig.#5.1. HPLC-profiles of ultrafiltered uremic serum from a patient on hemodialysis treatment(left), and two months later on CAPD(right).

A mass spectrum of the trimethylsilyl derivative of the isolated HPLC peak 2 (see Fig.#5.1) is shown in Fig.#5.2. This spectrum was found to be identical to the spectrum of trimethylsilated pseudouridine standard. The retention times of the isolated HPLC peak and the pseudouridine standard on HPLC (underivatized, also after co-injection), and on GC (derivatized) were identical. The differences between the HPLC profiles in Fig.#5.1 will be discussed below.

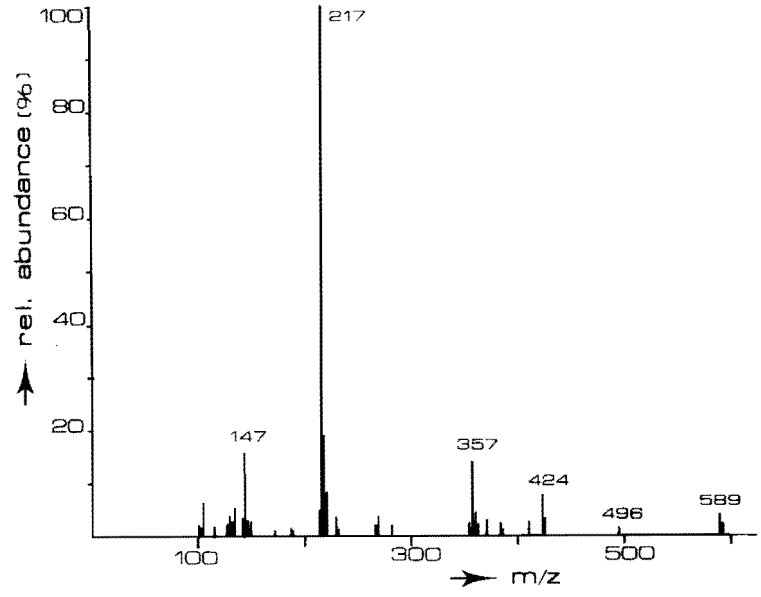


Fig.#5.2. Electron-impact mass spectrum of the HPLC-fraction identified as pseudouridine. The spectrum of the trimethylsilylated solute is shown.

A) Comparing a CAPD and a HD group

The free (non protein bound) fractions and total concentrations of the solutes under study are summarized in Table#5.2 and Table#5.3. The data of the HD group were measured before dialysis.

Table#5.2. "Free Concentrations[a] of Bound and Nonbound Solutes in CAPD and HD Patients.

	CAPD (n=12)			HD (n=15)		
SOLUTE	MEAN	SD	RANGE	MEAN	SD	RANGE
Creatinine	1248	199	757-1511	1469	330	960-2112
Pseudouridine	94.9	40	49.8-170.5	55.9[c,d]	14.4	41.7-83.8
Uric acid	479	75.7	339-564	630[c]	144	455-1057
UK4[b]	0.56	0.43	0.08-1.29	0.46	0.31	0.07-1.00
UK5[b]	1.33	0.49	0.7-2.23	1.31	0.61	0.55-2.62
p-OH-hipp.acid	17.3	11.9	4.3-37.1	22.5	16.1	4.1-63.2
Hippuric acid	208	142	36.7-491	295	242	35.8-894
UKF1[b]	555	456	153-1812	514	329	43-1223
Tyrosine	75.4	29.1	41.4-139	75.2	19.0	40.2-117
UKF3[b]	6368	1682	4050-9756	8795[e]	3401	2982-14761
UKF5[b]	1859	965	734-3309	1276[f]	681	640-2879
Indoxylsulfate	16.4	13.9	4.0-44.5	11.8	7.7	1.7-24.4
Tryptophan	10.7	3.2	5.9-16.1	11.9	3.7	5.9-18.4
UKF6[b]	703	527	152-1640	536	452	75-1926
UKF7A[b]	2.5	1.3	0.9-4.9	2.2	0.8	0.7-3.5
UKF7[b]	332	337	92-1305	333	192	68-828
UKF8[b]	3662	2826	1079-9125	4473	4064	559-16169
3-Ind.acetic acid	2.6	0.8	1.6-4.4	2.8	2.0	0.6-7.2

[a]μmol/L unless indicated otherwise. [b]Arbitrary units, used for unknown solutes. [c-f]Significantly different from CAPD values (by Wilcoxon's test) at: [c]$P<0.001$, [d]$P<0.0001$, [e]$P<0.01$, [f]$P<0.025$; [c-e]Significance of original data; [d,f]Significance after standardization in terms of serum creatinine concentration. All other comparisons not significant ($P>0.05$).

Table#5.3. Total Concentrations[a] of Bound Solutes in Sera of CAPD and HD Patients.

	CAPD (n=12)			HD (n=15)		
SOLUTE	MEAN	SD	RANGE	MEAN	SD	RANGE
UK5[b]	1.45	0.49	0.69-2.23	1.45	0.63	0.57-2.70
p-OH-hipp.acid	19.4	12.8	5.3-39.6	27.4	16.2	4.8-66.4
Hippuric acid	307	184	71.3-687	432	288	78.7-1043
UKF1[b]	706	573	161-2271	681	407	56-1540
Indoxylsulfate	96.7	52.1	6.3-169	94.0	44.3	2.3-152
Tryptophan	27.4	9.2	8.5-43.9	30.7	8.1	20.5-46.1
UKF6[b]	2687	1448	160-4517	2217	1108	228-3921
UKF7A[b]	2.7	1.3	1.1-4.7	2.3	0.8	0.6-3.6
UKF7[b]	509	316	140-1140	754	464	124-1794
UKF8[b]	3891	2875	1167-9575	4939	4216	1313-17015
3-Ind.ac. acid	5.6	2.7	2.8-12.8	8.7[c]	5.1	3.4-22.1

[a]µmol/L unless indicated otherwise. [b]Arbitrary units, used for unknown solutes. [c]Significantly different ($P<0.025$) from CAPD value.

Obviously, comparing the blood concentrations as such, in predialysis sera of HD-treated patients and sera of CAPD patients, is not optimally informative because the HD group is sampled in a worst-case condition with respect to the high predialysis blood concentrations. Predialysis sampling was chosen for the HD patients' sera as this is more reliable than postdialysis where rebound effects and fluid volume changes are influential to blood concentrations. For statistical analysis, all concentrations in the individual HD and CAPD samples were standardized with respect to the corresponding creatinine concentrations:

$$C_i,st = C_i \cdot \frac{1000}{C_{creatinine}}$$

where C_i is concentration of component i, CREA is the corresponding creatinine concentration (in µmol/L), and Ci,st is the "standardized" concentration. Creatinine was taken as a reference as it is more "constant" compared to urea, and as it is widely used as a marker in clinical practice. For the sake of unambiguity, the original concentrations are given in Table#5.2 and Table#5.3. The results of the Wilcoxon's tests are given both for the data, as is, and in terms of the standardized data. The standardized blood concentration of PSI ($C_{psi,st}$) is significantly ($P<0.0001$) higher in the CAPD group than in the HD group, as is true for

UKF5 ($P<0.025$). The standardized concentrations of PSI calculated from the original data by using equation 1, are 76.3 and 38.7 nmol/μmol for CAPD and HD patients, respectively. Standardized blood levels of UKF5 (arbitrary units) were significantly higher in CAPD than in HD patients respectively. Although average concentrations of hippuric acid and *p*-hydroxyhippuric acid tended to be lower in CAPD, this difference was not significant with these numbers of patients and the observed variation between patients. For none of the solutes a significantly different protein binding level was observed between groups (Table#5.4).

Table#5.4. Protein Binding(%) of Uremic Solutes in CAPD and HD Patients' Sera.

	CAPD (n=12)		HD (n=15)[a]	
SOLUTE	MEAN (%)	SD	MEAN (%)	SD
Creatinine	-[b]	-	-	-
Pseudouridine	-	-	-	-
UK5	7	11	11	7
p-OH hipp.acid	14	6	15	8
Hippuric acid	36	8	38	8
UKF1	22	4	25	7
Tyrosine	-	-	-	-
UKF3	-	-	-	-
UKF5	-	-	-	-
Indoxylsulfate	87	8	90	5
Tryptophan	63	14	61	12
UKF6	79	9	74	18
UKF7A	18	8	12	10
UKF7	42	25	55	14
UKF8	8	5	9	5
3-Ind.acetic acid	64	20	71	10

[a]Predialysis. [b]nonsignificant binding. All differences not significant ($P>0.05$).

So far bivariate statistical analysis was performed. To study the differences in the profiles by considering the relation of the solutes one to the other, a multivariate approach was followed using stepwise discriminant analysis [SAS, Inc., 1985]. By stepwise selection at significance levels of $\alpha = 0.05$, only the variables pseudouridine and UKF3 are selected. This means the combination of these two variables, in their mutual relationship, contributes most to the distinction of the HD and CAPD groups; more than pseudouridine alone, as was found by bivariate analysis of the standardized concentrations.

In further analysis of the data the serum concentrations of pseudouridine and UKF3 were found to be significantly correlated in a Spearman rank correlation analysis: in HD, r=0.79 ($P<0.0005$, n=15), and in CAPD, r=0.67 ($P<0.01$, n=12). The group separation by these two variables is visualized in the scatter plot of Fig.#5.3. The separate regression lines through the CAPD and HD data points have significantly different slopes (F-test, $\alpha = 0.005$), possibly pointing at a biochemical or structural relationship of the two solutes. Further indication for this was found during experiments on selective isolation of PSI. Nucleosides (e.g. PSI) are known to be extracted selectively by solid-phase extraction on boronate gel [Gehrke et al.,1978].

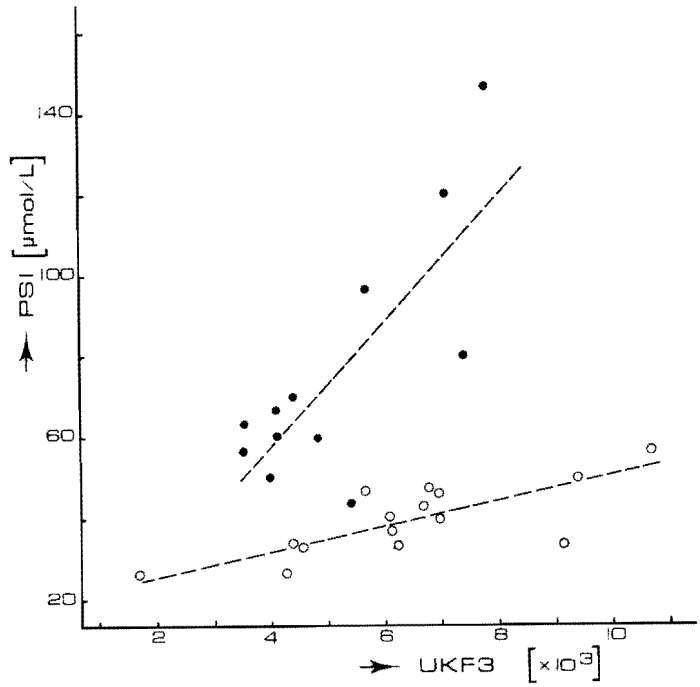


Fig.#5.3. Bivariate scatterplot of serum pseudouridine versus serum UKF3 concentration.

During this procedure not only PSI was extracted, but also UKF3, with approximately equal recovery. This supports the suggestion that UKF3 is a fluorescent nucleoside, carbohydrate, glucuronide, or any solute with a *cis*-diol group. Moreover, it was observed to be present abundantly in normal urine, but has not, until now been identified decisively. As the concentration of UKF5 proved to be higher in CAPD than in HD, this peak deserves further identification.

Effect of changing therapy

Blood serum concentrations of the uremic solutes were followed in two patients, who were changing therapy. One patient (K) volunteered to go from HD to CAPD therapy, and the other (R) changed therapy from CAPD to HD, for reasons of life style. Blood was sampled from both patients during one month before changeover, during the equilibration period, and then for several weeks more. Representative HPLC profiles for the HD and CAPD periods in one patient were given in Fig.#5.1. A very striking observation was the high pseudouridine/creatinine ratio (PSI/CREA) in the CAPD periods of both patients. The UKF3/pseudouridine ratio (UKF3/PSI) changed even more significantly (Fig.#5.4). This is illustrated for patient K (left). A similar, but opposite change of the PSI/CREA ratio was seen in patient R (right).

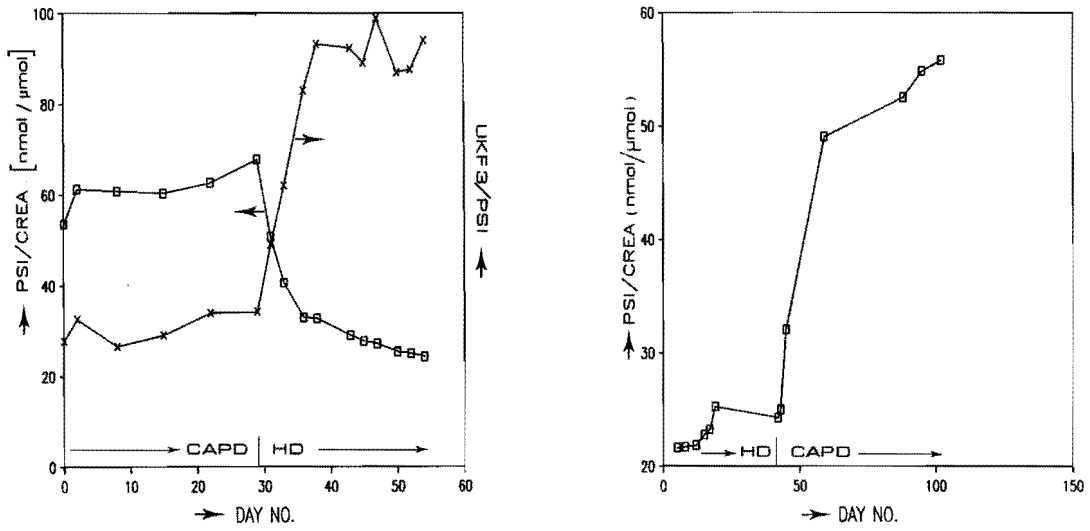


Fig.#5.4. Longitudinal plots of PSI/CREA and UKF3/PSI ratios in one patient changing therapy from CAPD to HD(left) and another patient from HD to CAPD(right).

On the other hand, the hippuric acid concentration in patient R decreased significantly with onset of CAPD treatment (Fig.#5.5), whereas the reverse was not observed for patient K, changing over from CAPD to HD. In both patients the concentration of indoxylsulfate, a tryptophan metabolite formed by intestinal bacteria, and the indoxylsulfate/tryptophan ratio were significantly lower in the CAPD period. These results are summarized in Table#5.5.

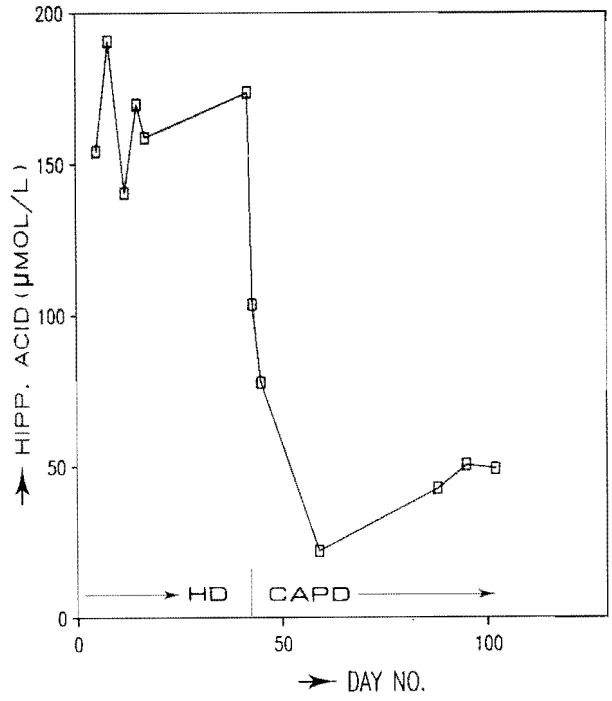


Fig.#5.5. Longitudinal plot of serum hippuric acid in a patient changing therapy from HD to CAPD.

Table#5.5. Significance of Difference in Various Analytes for HD Interval compared to CAPD Interval.

VARIABLE	*P*[a] for patient K (CAPD > HD)	VARIABLE LOWER IN CAPD?	*P*[a] for patient R (HD > CAPD)	VARIABLE LOWER IN CAPD?
Pseudouridine/creatinine	< 0.01	no	< 0.01	no
Indoxylsulfate	< 0.02	yes	< 0.01	yes
Indox.sulfate/tryptophan	< 0.02	yes	< 0.01	yes
UKF3/pseudouridine	< 0.01	yes	< 0.01	yes
Hippuric acid	n.s.	-	< 0.02	yes

[a]Significance of difference by Wilcoxon's test for five samples each taken during the stable HD and the stable CAPD periods. Results for samples from the one-month equilibration period are not included.

5.2.4 Discussion

The most striking difference between HD and CAPD sera demonstrated in the HPLC profiles centers around pseudouridine, a modified nucleoside, and an unknown fluorescent solute UKF3, both of which also occur in normal urine. Pseudouridine has been reported to be elevated in uremia [Asatoor, 1968]. However, the authors did not establish whether the

raised concentrations originated from decreased excretion, increased generation, or both. Pseudouridine is a rare nucleoside found predominantly in transfer RNA (tRNA). It may be present also in messenger RNAs and ribosomal RNAs, but only in much lower proportions [Dirheimer,1983]. Formation of PSI in tRNA takes place at the macromolecular level. Thus far there is no evidence that PSI-phosphates are synthetized prior to use in transcription of tRNA in humans, although in certain bacteria PSI-monophophate-synthetases have been isolated [Heinrikson and Goldwasser,1964]. Therefore, PSI found in normal urine and in uremic serum results predominantly from tRNA turnover. Because the PSI generated, unlike the regular nucleosides, is not phosphated and re-utilized and because it is chemically stable, PSI is excreted unmodified in the urine. Turnover of tRNA occurs during protein synthesis [Schöch et al.,1982]. tRNA plays a keyrole in protein synthesis, although it also has various other regulatory functions [La Rossa and Söll,1978]. High concentrations of urinary tRNA catabolites, such as PSI and methylated nucleosides, are found in patients with different forms of cancer, or various other diseases [Sharma et al.,1983].
In normal healthy adults the urinary PSI/CREA ratio was observed to be higher in women: 26.7 (SD=4.5) vs. 22.4 (SD=2.1) nmol/μmole in men [Sharma et al.,1983]. Although the patient groups studied here are asymmetric with respect to sex, this could not explain the difference, because more females (with higher urinary PSI/CREA ratio) were present in the HD-group, which shows a lower mean value.

It is unlikely that the high concentrations of PSI (Mr 244) found in the sera of CAPD patients should be explained from a less efficient removal relative to creatinine (Mr 113) in these patients. The reverse would be expected, because CAPD clears larger molecules more efficiently than does conventional hemodialysis, when both methods show equivalent urea removal on a weekly basis [Babb et al.,1973; Nolph et al.,1980]. However, there are no literature data on peritoneal membrane permeability for PSI.

Two other possible explanations of the high serum concentrations in CAPD will be discussed here. First, asymptomatic, non-clinical peritonitis accompanied by cell death occurs in all CAPD patients, which could result in generation of PSI [Borek et al.,1977]. Second, protein synthesis may be increased in these patients for various reasons. The possibly more efficient removal of certain unknown uremic toxins that might be inhibiting protein synthesis [Clayton et al., 1965; Goubeaud et al.,1977; Delaporte et al., 1980], and the generally better homeostasis in CAPD, may result in a more anabolic state [Randerson et al.,1981]. On the other hand hemodialysis has been described as a catabolic process [Farrell and Hone,1980], of which the etiology is not yet clearly understood. Or, protein synthesis may be induced by the combination of protein loss, via the peritoneal dialysate, and the availability of essential amino acids, such as tryptophan [Rothschild et al.,1969], as a result of a more free diet.

Although amino acid loss in peritoneal dialysis has been reported [Berlyne et al.,1967], these losses probably do not reduce plasma amino acid concentrations in stable patients, who are eating well [Blumenkrantz,1981].

If the second explanation is valid, perhaps the serum PSI/CREA ratio can serve as an indicator of metabolic and nutritional status in dialyzed patients in addition to methods described by Blumenkrantz et al.[1980]. Urinary PSI/CREA ratio has been proposed as an indicator of nutritional status in "healthy" persons, as an alternative to the determination of nitrogen balance, which is a very complicated procedure [Schöch et al.,1982]. Children exhibit a strong age-dependency of the excretion of creatinine-normalized RNA-catabolites, reflecting age-dependent growth velocity [Schöch et al.,1981]. The PSI/CREA ratio in anabolic as well as catabolic processes was evaluated, and children with "failure to thrive" showed a marked depression of tRNA catabolites [Schöch et al.,1976]. Perhaps, the higher serum PSI/CREA ratio in CAPD patients than in HD patients indicates a better noncatabolic status in CAPD. CAPD treatment reportedly is more beneficial to growth of children than is HD [Balfe,1983; Nolph,1984], probably as a result of better homeostasis and more free diet, despite the risk of undernutrition from treatment-induced anorexia.

It has been reported that urinary PSI excretion is positively related to protein intake [Eisen et al.,1962]. Normal concentrations of PSI in serum have been reported as 2.48 (SD= 0.13) µmol/L (measured by HPLC) [Russo et al.,1982], and 1.72 (SD=0.77) µmol/L (measured by RIA) [Levine et al.,1975]. With a normal serum creatinine value of 80 µmol/L, the normal serum PSI/CREA ratio calculates to 20-30 nmol/mmol, similar to the normal value in urine. Thus the PSI/CREA ratio in HD patients is closer to normal than in patients on CAPD. As healthy individuals ordinarily have neutral or positive nitrogen balance and nonrestricted protein intake, the foregoing invalidates the suggestion that the high PSI/CREA ratio in CAPD is related to protein intake and/or protein synthesis. No hard data were available on differences of diet between the groups of patients. To assess the metabolic state and (or) the influence of diet, careful clinical control of the different variables in a further study is indicated.

Less significant was the observation of the somewhat decreased concentrations of hippuric acid and *p*-hydroxyhippuric acid in CAPD patients compared to HD patients. In a preceding pilot study involving seven patients on CAPD and three on HD, a similar observation was made. In the present study, hippuric acid decreased very significantly with the onset of CAPD for a patient changing therapy. UKF5 blood levels were significantly higher in CAPD. To study further the importance of this finding, the solute causing this peak should be identified.

The initial purpose of this comparative study was to investigate whether blood levels of poorly dialyzable solutes are lower in CAPD patients than in HD patients. This could be a consequence of the "square-meter-hour" hypothesis which was based on observations with patients on peritoneal dialysis. The *in vitro* dialyzabilities of the compounds analyzed with the HPLC-method are discussed in chapter#5.4. Concentrations of most of the solutes, that were shown to have low *in vitro* clearances (as a native solute, or because of protein binding, see chapter#5.2), were not significantly lower in CAPD sera compared to HD sera.

5.2.5 Conclusion

It has been shown that serum pseudouridine concentrations in CAPD patients are significantly higher compared to HD patients. This difference is even more significant, when concentrations are standardized to creatinine. This was found both in multivariate analysis between groups of patients on CAPD and HD, and in a longitudinal setting in two patients changing therapy from CAPD to HD and vice versa. In stepwise discriminant analysis of the HPLC-variables in the CAPD and HD sera, pseudouridine and UKF3 were selected to describe the difference between the patient groups.

Further study is needed to answer the question whether low peritoneal membrane permeability, specific tRNA turnover, asymptomatic peritonitis accompanied by cell death, or another as-yet unrecognized cellular process is the origin of the high pseudouridine concentrations in CAPD.

No significantly lower concentrations of poorly dialyzable solutes (see chapter#5.4 on dialyzer clearances) are found in CAPD. It was observed in this and a previous study that concentrations of hippuric acid and *p*-hydroxyhippuric acid tended to be lower in CAPD.

Protein binding levels are not significantly different between CAPD and HD sera, so this is not expected to be a cause of different dialyzabilities of protein bound solutes in CAPD and HD treated patients.

5.2.6 Acknowledgement

This study was partially supported by a grant from Travenol Ned. B.V., Utrecht, The Netherlands.

5.3 SOLUTE ACCUMULATION AND RESIDUAL RENAL FUNCTION IN NONDIALYZED AND DIALYZED RENAL PATIENTS

5.3.1 Introduction

In the course of progressive renal disease "...plasma concentrations of urea and creatinine show little absolute change until, functionally, the patient has lost one kidney" [De Wardener,1973]. This corresponds to a residual creatinine clearance (RCC) of about 60-70 mL/min. With glomerulonephritis, the predominant type of renal disease, initially only the glomerulus is affected, but in a later stage all functional elements (including the tubular system) are damaged [Pitts,1969].

Generally, at RCCs of only 20 mL/min and lower, the renal damage is such that the highly effective "tubular organic acid secretory mechanism" cannot maintain plasma concentrations of the organic acids (e.g. hippurates, benzoates), and consequently they increase significantly [Porter et al.,1975].

To our knowledge literature data on correlation of uremic accumulant concentrations and residual creatinine clearance in end-stage renal patients on hemodialysis treatment (RCC < 5 mL/min) are available only for a limited number of compounds.

Blood concentrations of accumulated compounds in dialyzed patients depend on different factors, one of which is the degree of residual renal function (see also chapter #5.3). This dependence may be solute specific and is expected to be more pronounced for solutes that have low dialyzer clearances. This may be so because of the continuous activity of remaining nephrons during the off-dialysis periods, and the fact that the glomerulus filters high molecular mass solutes (inulin) as easily as low molecular mass solutes, which is evidently not true for the dialysis membrane. This effect has been treated theoretically by Babb et al.[1972a] and Milutinovic et al.[1974], for the hypothetical "middle molecules".

It has been shown that dialyzed patients were better clinically when they had higher residual creatinine clearances [Ravid et al.,1980].

In the following, we will describe uremic solute accumulation as a function of residual renal function in both dialyzed and non-dialyzed patients.

5.3.2 Experimental

Blood samples were collected from 33 uremic dialyzed patients (predialysis), and from 32 non-dialyzed patients (Renal Division, University Hospital of Ghent). Dialyzed patients were treated by single needle hemodialysis using different types of dialyzers. Serum samples were ultrafiltered using Centrifree ultrafiltration units (Amicon, Danvers, USA) in order to remove serum proteins. The filtrate was diluted 1:1 v/v with a solution of internal standard naphtalene sulfonic acid (37 mg/L), and subsequently analyzed by high performance liquid chromatography (HPLC).

In the present study residual creatinine clearance is employed as an indicator of deteriorating renal function. Although small amounts of creatinine are actively secreted in the tubular lumen by renal tubular cells , and therefore creatinine clearances are somewhat higher than those of inulin, this does not introduce a significant error in its use as a measure of glomerular filtration [Pitts,1969; De Wardener,1973]. Residual creatinine clearance (RCC) is determined by calculating the ratio of the amount of creatinine excreted in the urine per unit time, and creatinine plasma concentration:

$$RCC = \frac{C_{ur} \cdot V_{ur}}{t_{coll} \cdot C_{pl}}$$

where,

C_{ur} = urine creatinine concentration, V_{ur} = urine volume, t_{coll} = urine collection time and C_{pl} = creatinine plasma concentration.

RCCs were 0-5 mL/min and 5-120 mL/min for the dialyzed and nondialyzed patient groups respectively.

Renal diseases of highest incidence in the dialyzed patient group were: glomerulonephritis(33%), polycystic disease(16%), analgetica(16%), pyelonephritis(10%). In the non-dialyzed group these figures were: hypertension(22%), polycystic disease(15%), nephrotic syndrome(10%), analgetica(10%), pyelonephritis(6%), and glomerulonephritis(3%).

5.3.3 Results and Discussion

Pooled dialyzed and non-dialyzed patient groups

Serum concentrations of creatinine, pseudouridine, urea, and phosphate in the pooled (both dialyzed and non-dialyzed) patient groups have been plotted as a function of RCC in Fig.#5.6(for lay-out technical reasons all figures are placed at the end of the current subsection). These solutes, as well as UK5 (not shown) exhibit a significant rise in serum concentrations at RCC=60-70 mL/min.

In similar plots for *p*-hydroxyhippuric acid, hippuric acid, UKF1, and UKF7A (Fig.#5.7) this "sudden" serum concentration increase occurs at RCC=10-20 mL/min. This is also true for indoxylsulfate, UKF4, 3-indoleacetic acid, and UKF8 (curves not shown). These results are in accordance with data reported by Porter for hippuric acid (1975).

Here it may already be noted that the groups of solutes characterized by their RCC-concentration curves ("jump" at 60 and 20 mL/min respectively) largely coincide with the principal components derived in a multivariate analysis of serum concentrations in dialyzed patients (chapter#5.6).

Considering the chemical nature of the different solutes it may be speculated that most compounds showing a significant concentration increase at 60 mL/min and 20 mL/min are excreted mainly by filtration and tubular secretion respectively (Table#5.6). This is evidently not true for inorganic phosphate, which is not secreted, and yet shows a serum concentration increase only at reduced renal function of 25 mL/min (RCC).

Table#5.6. Groups of Solutes Showing Different Types of RCC-Concentration Curves.

GROUP I	GROUP II
Serum concentration increase at 60 mL/min	Serum concentration increase at 20 mL/min
Excretion by "filtration"?	Excretion by "secretion"?
Creatinine Pseudouridine UK5 urea	*p*-hydroxyhippuric acid hippuric acid UKF1 UKF4 indoxylsulfate UKF7A UKF8 3-indoleacetic acid

Dialyzed and non-dialyzed patient groups separately.

Serum concentrations of *p*-hydroxyhippuric acid, hippuric acid, creatinine, and urea are plotted against residual creatinine clearance in Fig.#5.8, for a group of *dialyzed* patients (RCC<5 mL/min). While concentrations of the hippurates continue to increase significantly with decreasing renal function, this is not the case for urea and creatinine. In Table#5.7 correlation coefficients of RCC vs. serum concentrations are given for both dialyzed and non-dialyzed patients.

Table#5.7. Correlation of "Free" Serum Concentrations and RCC.

	NON-DIALYZED (n=30-32)	DIALYZED (n=40-43)
COMPOUND	r^a (*P*)	r^a (*P*)
Urea	-0.72(0.0001)	ns
Phosphate	-0.51(0.003)	ns
Creatinine	-0.74(0.0001)	ns
Pseudouridine	-0.79(0.0001)	ns
Uric acid	-0.47(0.006)	ns
Hypoxanthine	ns	ns
UK4	ns	ns
UK5	-0.55(0.001)	ns
p-OH-hipp.acid	-0.63(0.0001)	-0.57(0.0001)
Hippuric acid	-0.78(0.0001)	-0.60(0.0001)
UKF1	-0.68(0.0001)	-0.30(0.04)
Tyrosine	ns	ns
UKF3	-0.83(0.0001)	ns
UKF4	ns	ns
UKF5	ns	-0.37(0.02)
Indoxylsulfate	-0.73(0.0001)	ns
Tryptophan	-0.40(0.02)	ns
UKF6	-0.50(0.003)	ns
UKF7A	-0.48(0.005)	-0.36(0.02)
UKF7	-0.39(0.03)	ns
UKF8	-0.72(0.0001)	ns
3-Ind.acetic acid	-0.57(0.0005)	ns

[a]Spearman rank correlation coefficient.

While concentrations of almost all compounds are very significantly correlated to RCC in the *nondialyzed* group, this is only true for *p*-hydroxyhippuric acid, hippuric acid, UKF1, UKF5 and UKF7A in the *dialyzed* group. With the exception of UKF5 (with low dialyzer clearance), serum concentrations of these solutes may all be influenced substantially by residual tubular secretory activity as may be derived from their RCC-Concentration plots and their (expected) "organic acid" structure. In normal functioning nephrons, the contribution of tubular secretion to total excretion of organic acids is many times larger than that of glomerular filtration (5 to 6 times for *p*-aminohippurate, PAH) as long as the serum concentration is smaller than 20-30 mg/100mL (1000-1500 µmol/L) for PAH. In that case the organic acid secretory mechanism (which is Tm-limited) is not yet saturated. It has been shown by Smith et al.[1945] that *p*-hydroxyhippuric acid and PAH have identical renal clearances in humans. Therefore, the abovementioned limit of 1000-1500 µmol/L for PAH will conceivably be valid also for *p*-hydroxyhippuric acid (and hippuric acid). The maximum serum concentrations of the latter solutes in dialyzed patients have been shown to be significantly smaller (*p*-hydroxyhippuric acid: 63.2 µmol/L, and hippuric acid: 894 µmol/L; Table#5.2, chapter#5.2.4) than this limit. Therefore, *they will still be excreted mainly by tubular secretion in intact nephrons of the residual renal mass in dialyzed uremic patients.*

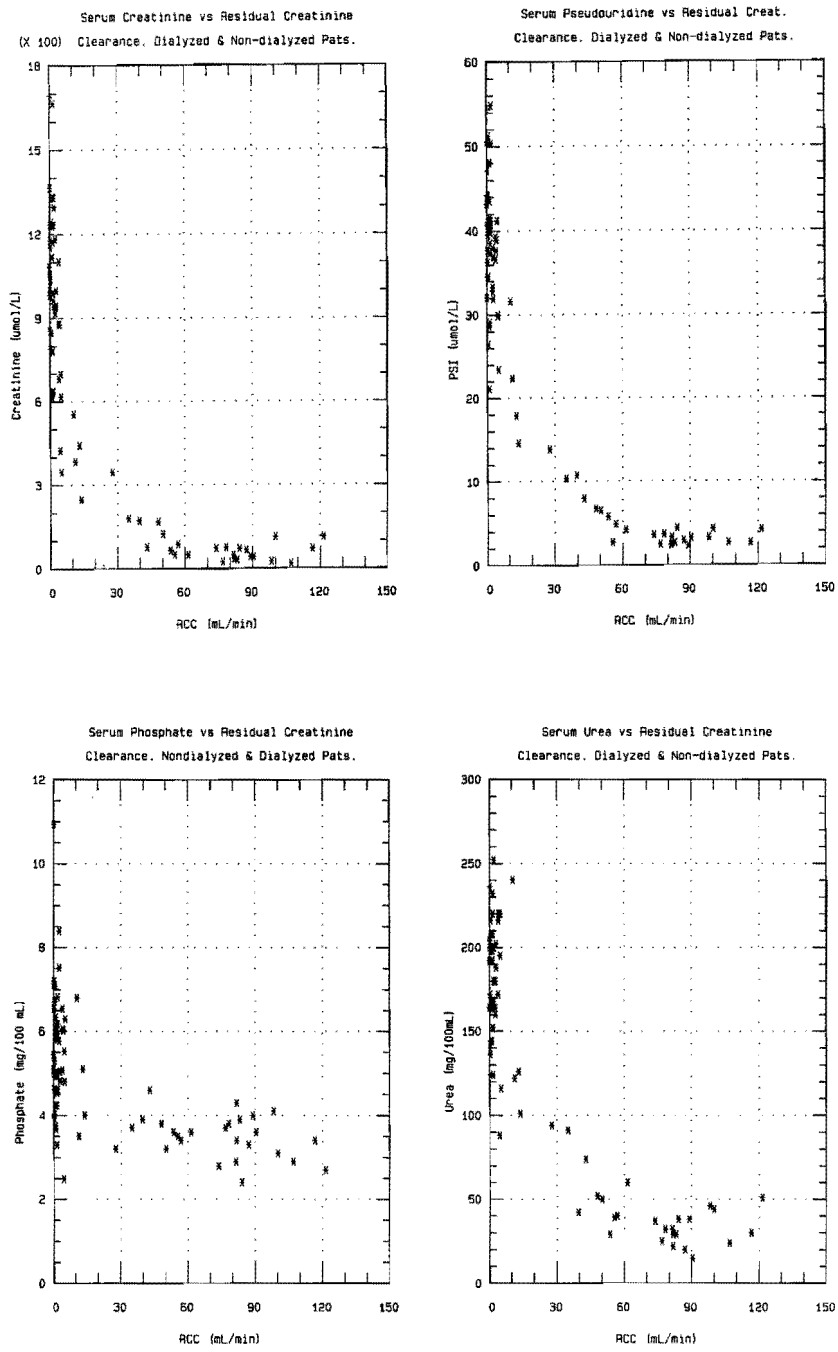


Fig.#5.6. "Free" serum concentrations of creatinine, pseudouridine, urea, and phosphate as a function of residual renal creatinine clearance(RCC) for pooled dialyzed and nondialyzed patients. Note range of x-axis.

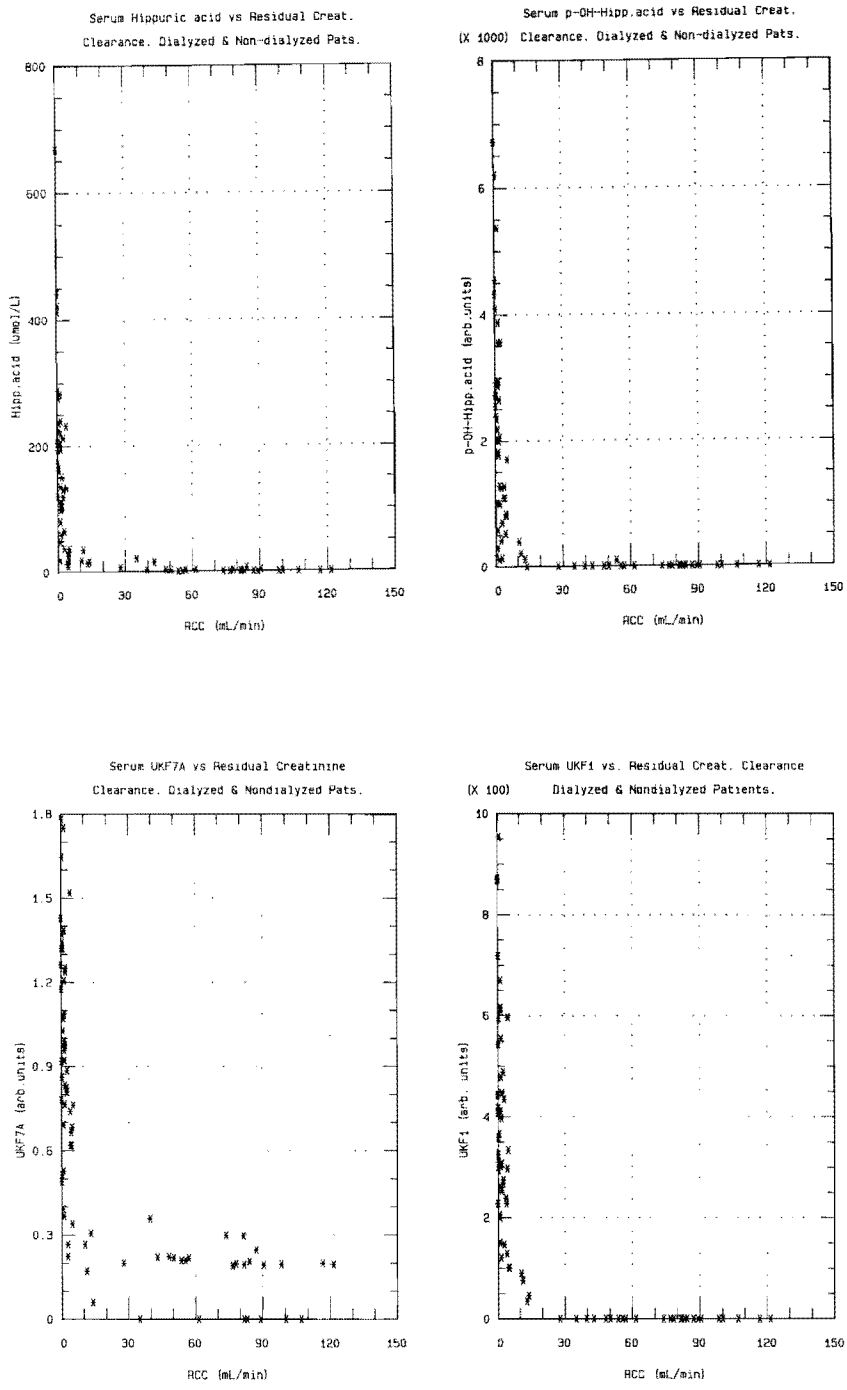


Fig.#5.7. "Free" serum concentrations of hippuric acid, *p*-hydroxyhippuric acid, UKF1, and UKF7A versus residual renal creatinine clearance(RCC) for pooled *dialyzed* and *nondialyzed* patients. Note range of x-axis.

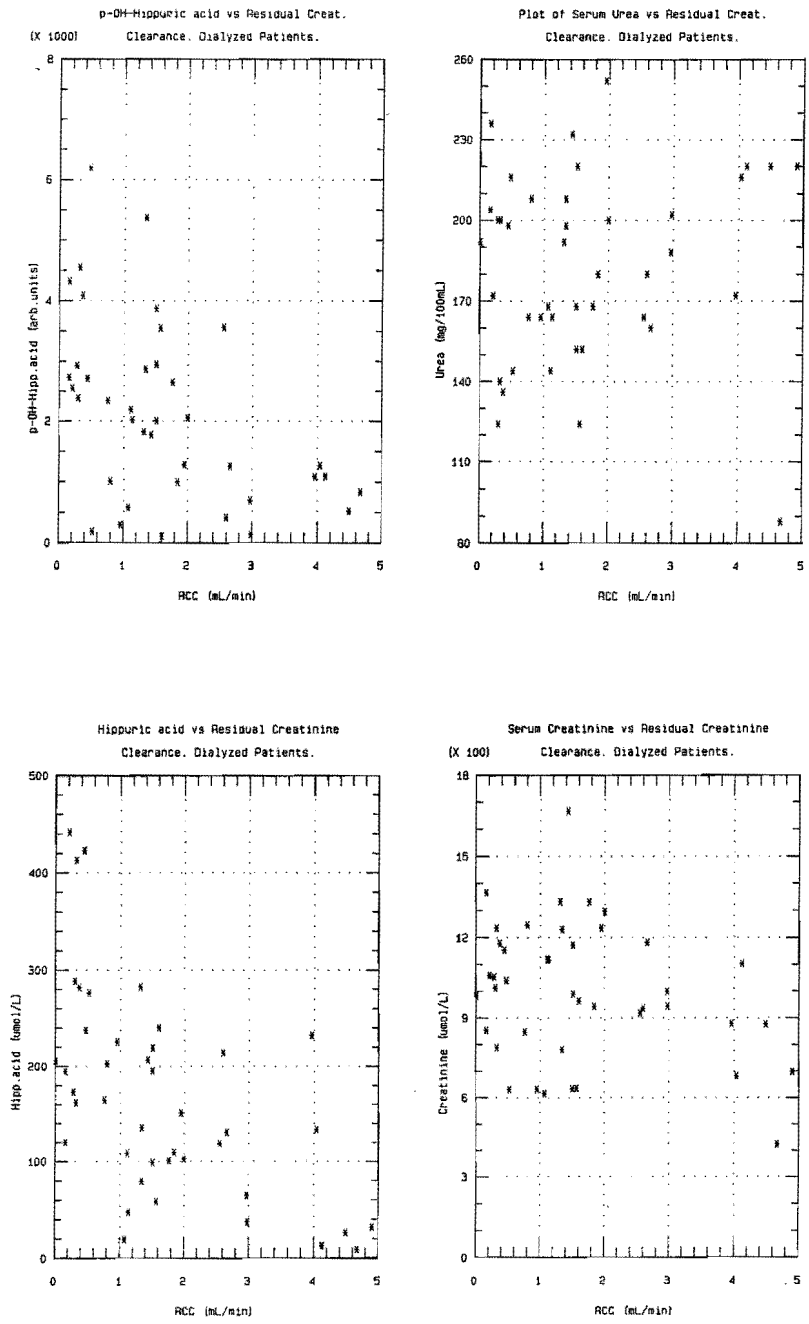


Fig.#5.8. "Free" serum concentrations of *p*-hydroxyhippuric acid, urea, hippuric acid, and creatinine versus residual renal creatinine clearance(RCC) for a group of *dialyzed* patients. Note range of x-axis.

5.3.4 Conclusion

The analyzed solutes could grossly be divided into two groups by the way their serum concentrations increase with deteriorating renal function. Solutes in one group showed a significant serum concentration increase at 60 mL/min residual creatinine clearance, which suggests excretion mainly by filtration in *nondialyzed* renal patients. This group includes creatinine, pseudouridine, UK5 and urea. A second group showed such an increase at only 20 mL/min or less, which suggests their excretion by tubular secretion. This group includes *p*-hydroxyhippuric acid, hippuric acid, UKF1, UKF4, indoxylsulfate, UKF7A, UKF8, and 3-indoleacetic acid. These solutes are (or are expected to be) aromatic and indolic acids. They are excreted by the "organic acid secretory mechanism" of renal tubular cells. Concentrations of almost all analyzed compounds correlated significantly to residual creatinine clearance.

The different behavior of the two groups of solutes was also found in *dialyzed* patients with RCC < 5 mL/min. While concentrations of creatinine, urea, pseudouridine, and UK5 did not correlate to residual creatinine clearance in these patients, the concentrations of the "organic acid-like substances" hippuric acid, *p*-hydroxyhippuric acid, UKF1, and UKF7A did ($P < 0.0001$, 0.0001, 0.04, and 0.02 respectively). The division of solute groups in this study roughly coincides with that found in chapter#5.6, using the multivariate statistical method of Principal Component Analysis.

5.4 DIALYZABILITY OF CHARACTERISTIC UREMIC COMPOUNDS ON CUPROPHAN MEMBRANES

5.4.1 Introduction

In 1971 the square meter-hour hypothesis was introduced by Babb and coworkers [Babb et al.,1971]. In this hypothesis, later changed in the "middle molecule hypothesis" [Babb et al.,1972], solutes with low dialyzer membrane permeabilities were thought to be responsible for symptoms, such as uremic polyneuropathy, observed in dialyzed uremic patients. As uremic symptoms are related to protein intake [Schreiner,1975], it has been speculated that these solutes were protein breakdown products with intermediate molecular masses, such as peptides.

For the last fifteen years the concept of "middle molecules" was associated almost exclusively with certain fractions obtained by gel filtration [Dall'Aglio et al.,1973; Dzúrik et al.,1973; Fürst et al.,1975; Cueille,1978]; a technique that, theoretically, separates substances according to molecular mass. However, it has been shown that charged molecules exhibit anomalous retention behaviour on Sephadex G-15 and G-10 crosslinked dextran gels, due to residual charge from carboxyl groups in the gel, while on the other hand certain compounds do adsorb to the gel [Kremmer and Boross,1979] This has also been shown for uremic metabolites. Uremic substances initially identified as peptides of 1000-1500 molecular mass, more recently proved to be glucuronides with molecular mass below 600 [Zimmerman et al.,1981; Cueille et al.,1980].

From the concept of Babb and Scribner the "middle molecules" could also be defined as those *accumulating solutes that behave in a dialyzer as if they have molecular masses between 300 and 2000 daltons* [Babb et al.,1972; Schoots et al.,1984].

Theoretically, apart from molecular mass, this behaviour may also be influenced by protein binding, electric charge, conformation, ionic strength and pH [Craig, 1970]. The effect of protein binding on dialyzer clearance has been described by Babb and Farrell [Babb et al.,1971; Farrell,1971; Farrell, et al.,1971; Farrell et al.,1972].

Simultaneous measurement of mass transfer of different characteristic uremic solutes in vivo or *in vitro* usually is limited to urea, creatinine, uric acid and phosphate, although a wide range of model solutes, such as dextrans, sugars, glycols, peptides and vitamin B12, have been used for characterization of dialyzer membranes [Colton,1969; Colton et al.,1971; Farrell and Babb,1973; Klein et al.,1979].

In the present study the *in vitro* dialyzabilities of characteristic "real world" uremic solutes, separated by HPLC [Schoots et al.,1985, Schoots et al.,1988], are determined, and the influence of measured values of protein binding will be estimated.

5.4.2 Experimental

Apparatus. A double pump system, model BL760N, a dialysate controller, model Unimat BL 714N, and a bicarbonate module, model BL723, all from Bellco (Mirandola, Italy) were used. Blood substitute was recycled through the system from a container with a volume of 5.5 liter, kept at 37°C using a single needle system. Blood substitute flow was controlled both volumetrically and with an electromagnetic flowprobe, model FF-040T (Nihon Kohden, Tokyo, Japan). Ultrafiltration flow was minimized by regulating the pressures in the blood and dialysate compartment. *In vitro* dialysis experiments were done in the University Hospital of Ghent, Dept. of Nephrology.

Dialyzers. Cuprophane hollow-fiber dialyzers with 0.9 m2 (GF-80-M) and 1.8 m2 (GF-180-M) effective surface area, and membrane thickness 11 microns, both from Gambro Dialysatoren KG (Hechingen, FRG), were used.

Blood substitute and dialysate. Uremic ultrafiltrate was collected from patients (Dept. of Nephrology, Ghent University Hospital), treated with sequential ultrafiltration and dialysis on permeable membranes (AN 69 S-HF, Hospal, Meyzieu, France) and frozen at -18°C until use. Regular dialysis fluid was used, which was prepared from concentrate 714A and 714B (Sterima NV, Bissegem, Belgium). Some data on chemical compositions of blood substitute (uremic ultrafiltrate) and freshly prepared dialysis fluid are given in the following Table#5.8.

Table#5.8. Concentrations of Different Compounds in Uremic Ultrafiltrate and Dialysate Fluid.

PARAMETER	BLOOD SUBSTITUTE[a]	DIALYSATE FLUID[b]
Urea (mg/100 mL)	116-142	0
Creatinine (mg/100 mL)	8.3-11.9	0
Uric acid (mg/100 mL)	5.1-7.3	0
Osmolality (mosmol/kg)	229-312	250-265
pH	7.5-8.4	7.5-7.7

[a]Uremic ultrafiltrate. Ranges indicate use of different pools in the experiments. [b]As freshly prepared from concentrates.

HPLC method. See chapter#4.

Calculations. Under the conditions of negligible ultrafiltration (TMP=0, Q_{uf}=0), and zero dialysate inlet concentration ($C_{D,i}$=0), dialyzer clearance is calculated using the defining relation:

$$K_B = \frac{\dot{M}}{C_{B_i}} = \frac{Q_B(C_{B_i} - C_{B_o})}{C_{B_i}} = \frac{Q_D \cdot C_{D_o}}{C_{B_i}} \qquad (1)$$

where: Q_B = blood flow (mL/min); $C_{B,i}$ = dialyzer blood inlet concentration (μmol/L); $C_{B,o}$ = dialyzer blood outlet concentration (μmol/L), and M = mass transferred per unit time (μmol/min).

Overall mass transfer coefficient (H_0A) is defined by:

$$\dot{M} = H_0A \cdot \overline{\Delta C} \qquad (2)$$

where $\overline{\Delta C}$ is the logarithmic-mean concentration difference for countercurrent flow geometry:

$$\overline{\Delta C} = \frac{(C_{B_i} - C_{D_0}) - (C_{B_0} - C_{D_i})}{\ln \frac{(C_{B_i} - C_{D_0})}{(C_{B_0} - C_{D_i})}} \qquad (3)$$

From equations (1), (2),and (3) an extraction ratio K_B/Q_B was derived by Michaels [1966]:

$$\frac{K_B}{Q_B} = \frac{1 - \exp\left(\frac{H_0A}{Q_B} \cdot \left(1 - \frac{Q_B}{Q_D}\right)\right)}{\frac{Q_B}{Q_D} - \exp\left(\frac{H_0A}{Q_B} \cdot \left(1 - \frac{Q_B}{Q_D}\right)\right)} \qquad (4)$$

After rearrangement the following relation for H_0A is obtained:

$$H_0A = \frac{Q_B}{\left(1 - \frac{Q_B}{Q_D}\right)} \cdot \ln \frac{\left(1 - \frac{K}{Q_D}\right)}{\left(1 - \frac{K}{Q_B}\right)} \qquad (5)$$

Recalculated clearance values, incorporating protein binding, if any, were obtained from [Babb et al.,1971; Farrell et al.,1972]:

$$K = Q_B \cdot \left(\frac{1 - \exp\left(\frac{H_0 A}{\mu(1+\rho) \cdot Q_B} \cdot \left(1 - \mu(1+\rho) \cdot \frac{Q_B}{Q_D} \right) \right)}{\mu(1+\rho)\frac{Q_B}{Q_D} - \exp\left(\frac{H_0 A}{\mu(1+\rho) \cdot Q_B} \cdot \left(1 - \mu(1+\rho)\frac{Q_B}{Q_D} \right) \right)} \right) \quad (6)$$

where:

μ = equilibrium distribution coefficient of free solute

ρ = protein binding coefficient, which is a constant in the linear part of a Langmuir-type adsorption isotherm:

$$\rho = \frac{C_{bound}}{C_{free}} = \frac{PBL}{(100 - PBL)} \quad (7)$$

where PBL = protein binding level (%).

An implicit assumption in using H_0A-values obtained in experiments with Q_B=200 mL/min and Q_D=500 mL/min in equation 6, is a flow independence of H_0A. This is not entirely correct, as will be shown below.

Data evaluation. Polynomial regression lines were plotted using the procedure GPLOT from SAS/GRAPH statistical software [SAS, Inc., 1985b]. CLI designates a confidence interval for an individual predicted value ("prediction interval"). It incorporates both variation in the error term, and variation in the parameter estimates. CLM represents a confidence interval for the expected value of the mean for each observation, and does not account for variation in the error term [SAS, Inc., 1985a].

5.4.3 Results

Clearances and mass transfer coefficients at standard flow rates.

Eighteen compounds characteristic for the uremic state represented in the HPLC chromatograms, were selected in the present study. Urea, which was measured conventionally, was also included in the study. In Table#5.9 the *in vitro* experiments are summarized, and the type of dialyzer, number of dialyzers from one type, surface area, blood and dialysate flow, and sampling times, are given.

Table#5.9. *In vitro* Experiments.

EXP. NO.	DIALYZER TYPE	N	QB (mL/min)	QD (mL/min)	REMARKS time interval, flows
1	0.9 m2 11μ	6	200	500	0, 30, 60 min.
2	1.8 m2 11μ	6	200	500	0, 30, 60 min.
3	0.9 m2 11μ	4	45-300	500	45, 65, 100, 150, 300
4	0.9 m2 11μ	4	200	50-600	50, 100, 150, 250, 600

In experiments 1 and 2 (see Experimental section), eighteen values of K_B for each solute at constant blood and dialysate flow rates (200 mL/min and 500 mL/min respectively) were obtained. These values from sampling times 0, 30 and 60 minutes could be pooled, because S-values in a Friedman test allowed for the decision that there was no difference of clearances of all solutes at these sampling times, in multiplicate measurements in six dialyzers.

Clearances, overall mass transfer coefficients, and overall resistances are given in Table#5.10.

Table#5.10. K_B, H_0A and R_0 Values of UV-absorbing and Fluorescent Compounds.

COMPOUND	K_B (mL/min) Mean (SD) n			H_0A (mL/min) Mean (SD) n			R_0 (min/cm) Mean
	0.9 m²	1.8 m²	Δ %	0.9 m²	1.8 m²	Δ %	0.9 m²
Urea	135(8) 16	148(9) 16	10	273(35) 16	338(54) 16	24	33
Creatinine	118(13) 14	141(9) 16	20	213(46) 14	304(45) 16	43	42
Pseudouridine	90(9) 11	109(15) 8	21	133(21) 11	185(43) 8	39	68
Uric acid	116(10) 16	147(12) 16	27	204(34) 16	336(64) 16	65	44
UK4	92(7) 15	108(14) 16	17	138(16) 15	180(35) 16	30	65
UK5	104(11) 14	118(21) 13	14	169(30) 14	219(67) 13	30	53
p-OH-hipp.acid	83(7) 17	111(6) 16	43	119(15) 17	189(17) 16	59	76
Hippuric acid	90(7) 16	118(6) 14	49	134(15) 16	207(18) 14	55	67
UKF1	70(9) 9	n.a.	33	93(16) 9	n.a.	-	97
UKF3	64(8) 16	88(5) 16	30	83(13) 16	129(10) 16	55	108
UKF4	56(9) 10	73(8) 13	25	70(14) 10	99(15) 13	41	129
UKF5[a]	34(2) 6	n.a.	-		n.a.	-	
Indoxylsulfate	100(5) 16	128(6) 14	57	157(13) 16	243(24) 14	55	57
Tryptophan	n.a.	127(14) 6	-	n.a.	243(50) 6	-	74
UKF6	100(5) 10	n.a.	58	158(13) 10	n.a.	-	57
UKF7A	90(8) 15	117(13) 10	49	134(18) 15	208(41) 10	55	67
UKF7[a]	80(22) 6	n.a.	-		n.a.	-	
UKF8	73(4) 13	108(11) 13	34	98(8) 13	182(37) 13	86	92
3-Ind.ac.acid	89(12) 14	108(14) 15	49	133(27) 14	180(39) 15	35	68

[a]QB=200 mL/min and QD=600 mL/min. n.a. = not available

The urea clearance value of 135 mL/min in the present study with serum ultrafiltrate as the blood substitute, is about 10% lower than that reported by Sigdell [1986] for saline solution, using the same dialyzer. The cause of this discrepancy was not studied here.

The H_0A-values were calculated from the individual values of K_B using equation 5. Both the data from the 0.9 m² and 1.8 m² surface area dialyzers are presented. The number of observations was, in most cases, smaller than eighteen, because some results had to be removed after judgement of the analytical data.

From the H_0A-values at QB=200 mL/min and QD=500 mL/min the curves of K_B versus Q_B, with dialyzer surface area as a third parameter, were calculated from equation 4 and are plotted for UKF3 and UKF4 in comparison with urea in Fig.#5.9. It should be noted that the calculated curves are only approximations as the implicit assumption of flow-independence of H_0A is not entirely justified, as will be discussed hereafter.

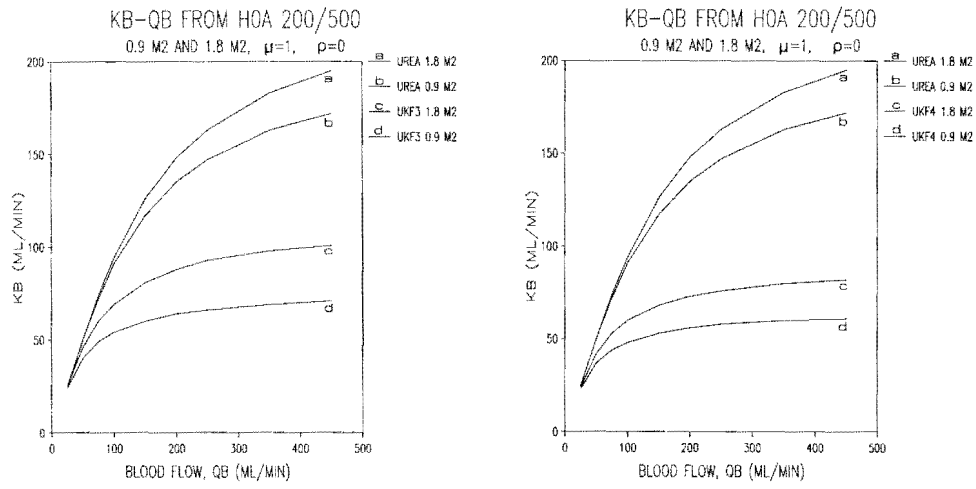


Fig.#5.9. Dialyzer clearance (K_B) versus blood (substitute) flow rate (Q_B), calculated from H_0A-values, for the nonbound solutes UKF3 and UKF4, on 0.9 m² and 1.8 m² surface area membranes. Comparison with values for urea.

Clearances at different flow rates.

K_B-values of the solutes at different Q_B and Q_D were determined in four equivalent dialyzers of 0.9 m² effective surface area. From these data and those from Table#5.10, polynomial regression lines for K_B against Q_B and Q_D with 95% confidence limits (on mean value) were calculated, and plotted for different solutes in comparison with urea in Fig.#5.10 and Fig.#5.11 respectively (These Figures are given at the end of the present section).

Flow-dependence of H_0A.

From the individual K_B values at different flow rates the corresponding H_0A values were calculated using eq.5. Mean values of H_0A at each Q_B or Q_D value are plotted as a function of flows in Fig.#5.12 for urea, creatinine, uric acid and indoxylsulfate. It can be seen that H_0A is not independent of blood and dialysate flow.

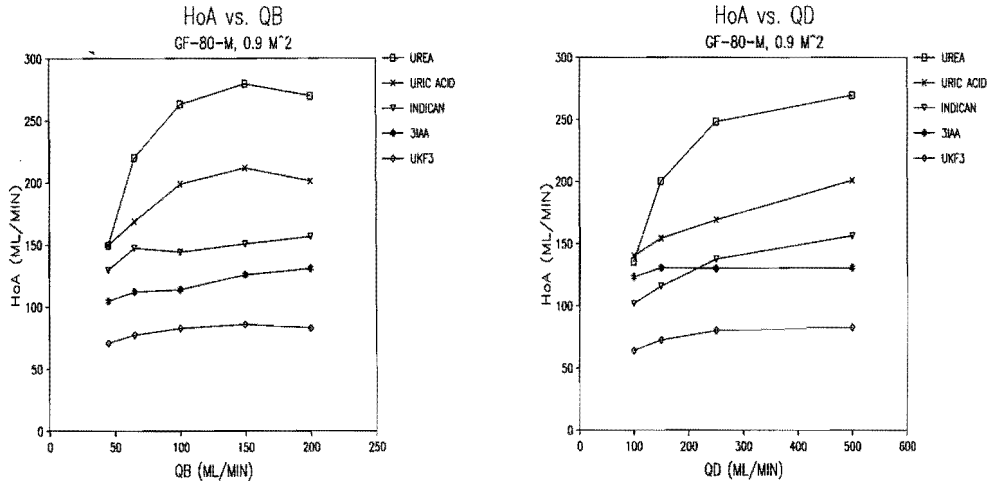


Fig.#5.12. Overall mass transfer coefficient times area product (H_0A) as a function of blood (substitute) flow rate(left) and dialysate flow rate(right), for urea(M_r 60), uric acid(M_r 168), indoxylsulfate (urinary indican)(M_r 251), 3-indoleacetic acid(M_r 175), and UKF3.

The overall mass transfer coefficient times area product (H_0A) not only depends on solute diffusion and distribution coefficients in the membrane, but is also related to the diffusive resistances in the blood and dialysate compartments.

From additivity of resistances in series [Michaels, 1966] it follows:

$$H_0 = \left(\frac{\delta_B}{\overline{D}_B} + \frac{d_M}{k \cdot D_M} + \frac{\delta_D}{\overline{D}_D} \right)^{-1}$$

where:

H_0 (=1/R0) is the overall mass transfer coefficient,

d_B and d_D are apparent blood and dialysate boundary layer thicknesses,

d_M is the membrane thickness,

k is the solute distribution coefficient between membrane and blood, and

D_B, D_M, and D_D are solute diffusion coefficients in the respective phases.

The blood side fluid film resistance d/D has been described in terms of a velocity-dependent log-mean Sherwood film number, $\overline{N}_{Sh}$ for laminar flow in flat ducts [Grimsrud and Babb,1966; Colton et al.,1971].

From the plots in Fig.#5.12 it appears that the magnitude of flow dependence is inversely related to molecular mass. Mass transfer of low molecular mass solutes, with high membrane diffusion coefficients, is apparently limited by fluid film resistances, as expected.

Effect of protein binding.

Clearances, adjusted for protein binding, if any, can be estimated from K_B values in Table#5.10, and protein binding coefficients, using equation 6. The results for a 0.9 m2 surface area dialyzer operated at Q_B=200 and Q_D=500 mL/min are presented in Table#5.11. The columns on apparent molecular mass will be discussed below.

Table#5.11. Recalculated Clearances from Clearance of Native Solutes and Protein Binding Data. Apparent Molecular Masses.

COMPOUND	PBL[a] (%)	ρ	K_B[b] FREE (mL/min)	K_B[c] TOTAL (mL/min)	$M_{r,app}$ FREE	$M_{r,app}$ TOTAL
Urea	0	0	135	141[d]	-	-
Creatinine	0	0	118	123	-	-
Pseudouridine	0	0	90	95	-	-
Uric acid	4[e]	0.03	116	119	-	-
UK4	n.a.	n.a.	92	n.a.	194	n.a.
UK5	11	0.12	104	101	149	158
p-OH-hipp.acid	15	0.18	83	77	-	288
Hippuric acid	38	0.61	90	65	-	418
UKF1	25	0.33	70	58	355	538
UKF3	0	0	64	68	433	-
UKF4	0	0	56	60	581	-
UKF5	0	0	34	36	>1500	-
Indoxylsulfate	90	9.00	100	14	-	>1500
Tryptophan	61	1.56	n.a.	n.a.	n.a.	n.a.
UKF6	74	2.85	100	35	162	>1500
UKF7A	12	0.14	90	86	204	226
UKF7	55	1.22	81	44	257	989
UKF8	9	0.10	73	72	324	334
3-Ind.Ac.acid	71	2.45	89	33	-	>1500

[a]PBL=protein binding level, see chapter#5.2.3 and Schoots et al.[1988].
[b]$K_{B,free}$ from Table#5.10. [c]$K_{B,total}$ calculated from $K_{B,free}$ and ρ, using eqn. 6. [d]different values for $K_{B,free}$ and $K_{B,total}$ for nonbound solutes result from using $\mu = 0.93$. [e]Uric acid protein binding from [Farrell et al.,1971]. n.a.=not available.

The recalculated clearances must be considered approximations, since $\mu = 0.93$ has been used to account for protein volume exclusion; this, however does not account for hematocrit and possible erythrocyte-plasma mass transfer resistance [Colton,1969; Babb et al.,1972].

For solutes that readily permeate the erythrocyte cell membrane, cell water is part of the active, blood side, exchange volume, while for solutes with low cell membrane permeabilities the erythrocytes are not involved and hence μ-values should be lowered by a term of approximately 0.2 reflecting usual hematocrit in dialyzed patients. Because the erythrocyte membrane permeabilities are not known for the analyzed solutes, except for urea and creatinine [Colton,1969], they cannot be incorporated in the equations.

Fig.#5.13 shows the estimated effect of protein binding on the K_B-Q_B curves of UKF1, UKF6, indoxylsulfate and indole-acetic acid. Notice the very flat curves for bound indoxylsulfate, 3-indoleacetic acid and UKF6.

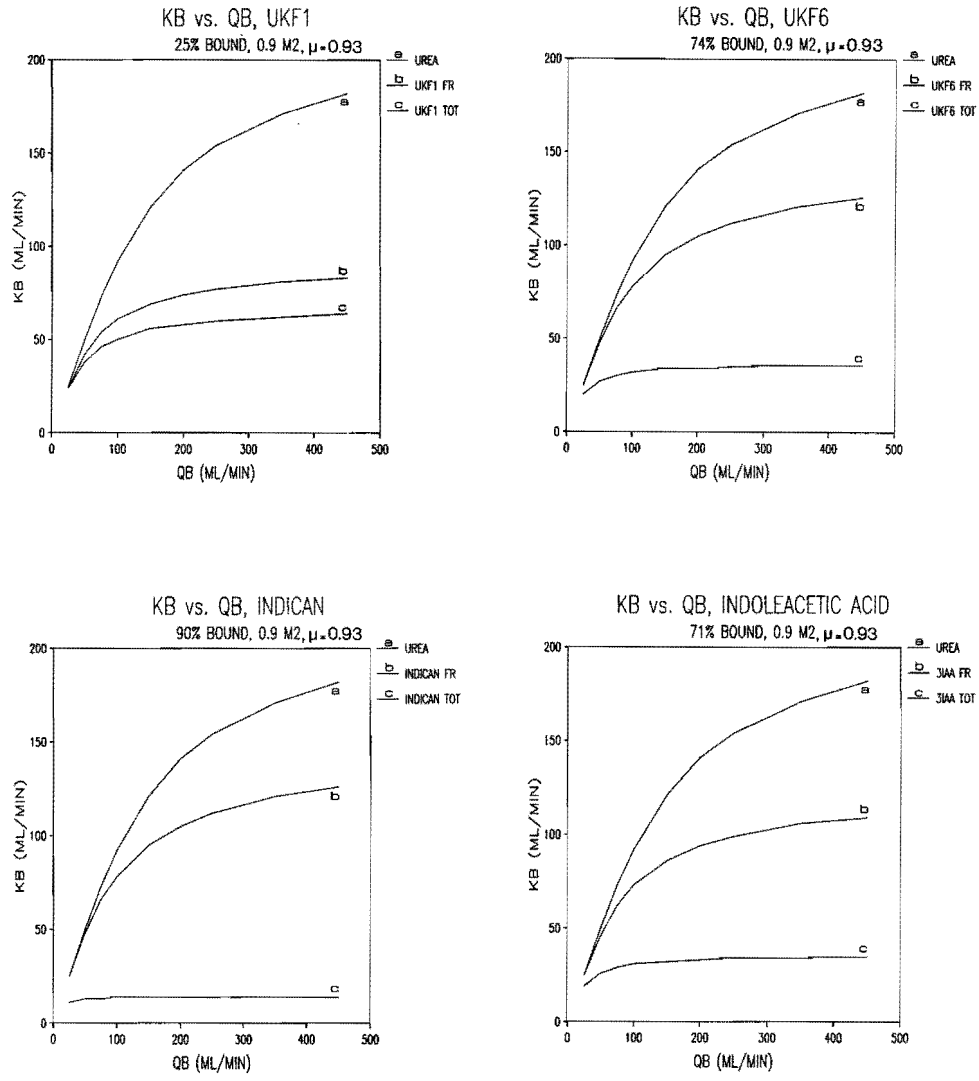


Fig.#5.13. Effect of protein binding on K_B-Q_B curves for UKF1, UKF6, indoxylsulfate, and 3-indoleacetic acid.

Apparent molecular mass from clearance.

Dialyzer behavior of unknown and proteinbound solutes can be characterized by an apparent molecular mass. This property was obtained by interpolation of their measured K_B-values on a regression line of $\log(K_B)$ versus $\log(M_r)$. The line was obtained from the clearance data of known solutes measured in the present study, and on data from the dialyzer manufacturers' documentation (for urea, creatinine, uric acid, and vitamin B_{12}). Fig.#5.14 shows this line with 95% confidence limits (on mean values). Interpolation of bound hippuric acid and bound 3-indoleacetic acid is depicted.

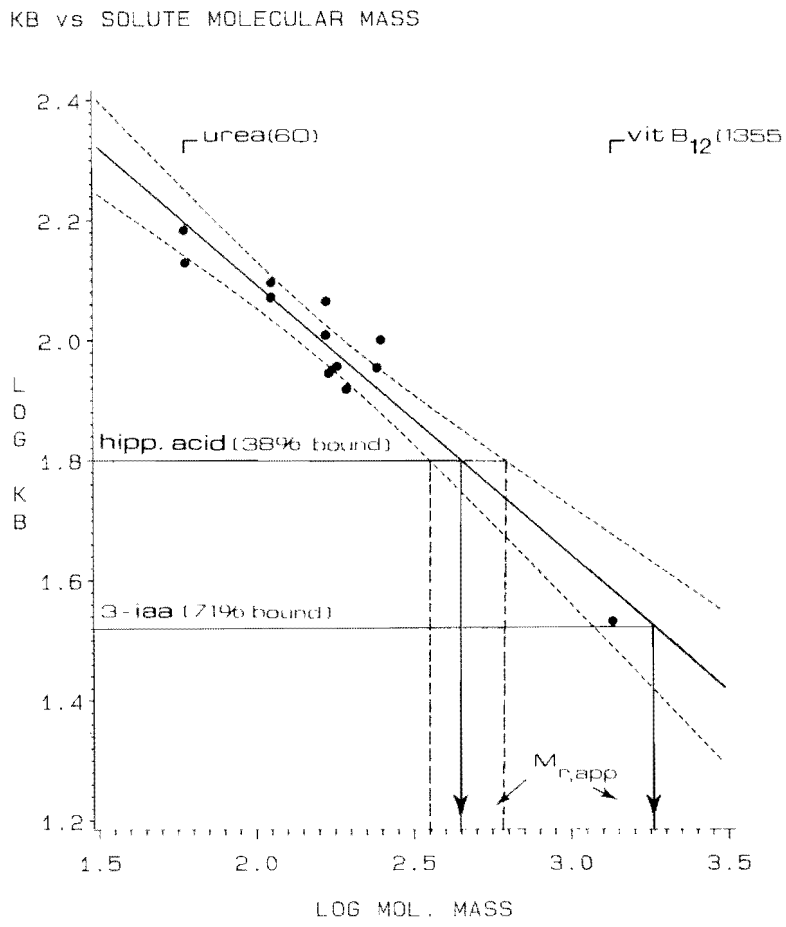


Fig.#5.14. Interpolation of calculated clearances of proteinbound solutes, to obtain the apparent molecular masses.

It should be noted that values of $\log(M_r)$ obtained from $\log(K_B)$ are not really predicted values from the regression model, because M_r has been introduced as the independent variable with "exact" values.

"Confidence" intervals for apparent molecular masses may nevertheless be obtained by drop lines on the x-axis from points of intersection of a horizontal line at a given y-value ($logK_B$) and the dashed confidence curves (on mean values) [Miller,1981]. This is illustrated in Fig.#5.14 for bound hippuric acid ($355<M_{r,app}<603$). The obtained values of apparent M_r have rather wide 95% "confidence limits".

Apparent molecular masses, M_r, are shown in Table#5.11. Those compounds, exhibiting $M_{r,app}$ higher than 300 (the lower limit in the middle molecule definition), have been compiled in Table#5.12 below.

Table#5.12. Native and Proteinbound Compounds with Apparent M_r>300.

COMPOUND *NATIVE:*	APPARENT MOL.MASS
UKF1	355
UKF3	433
UKF4	581
UKF5	>1500
UKF8	324

COMPOUND *BOUND:*	APPARENT MOL.MASS
UKF1	538
Indoxylsulfate	>1500
UKF6	>1500
UKF7	989
3-Ind.ac.acid	>1500
Hippuric acid	418
UKF8	334

Most of them owe their (estimated) apparent "middle molecular" mass ($M_{r,app}$>300) to a significant level of protein binding. Even if they have intermediate or high dialyzer clearances as native solutes.

Five unknown fluorescent compounds have apparent molecular masses above 300 daltons in their native form.

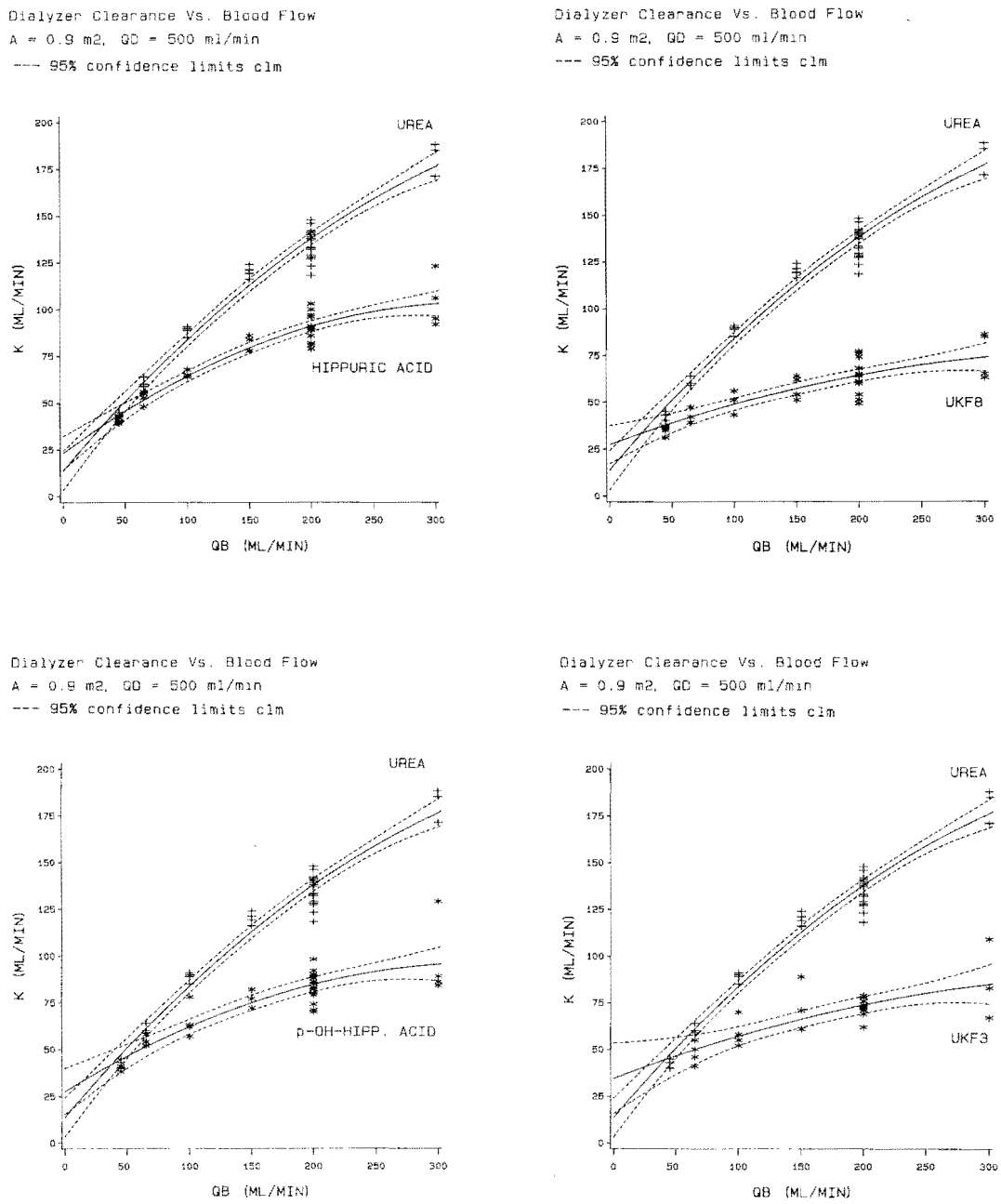


Fig.#5.10. Polynomial regression lines of dialyzer clearance (K_B) versus blood (substitute) flow rate for hippuric acid, UKF8, *p*-hydroxyhippuric acid, and UKF3, in comparison with urea.

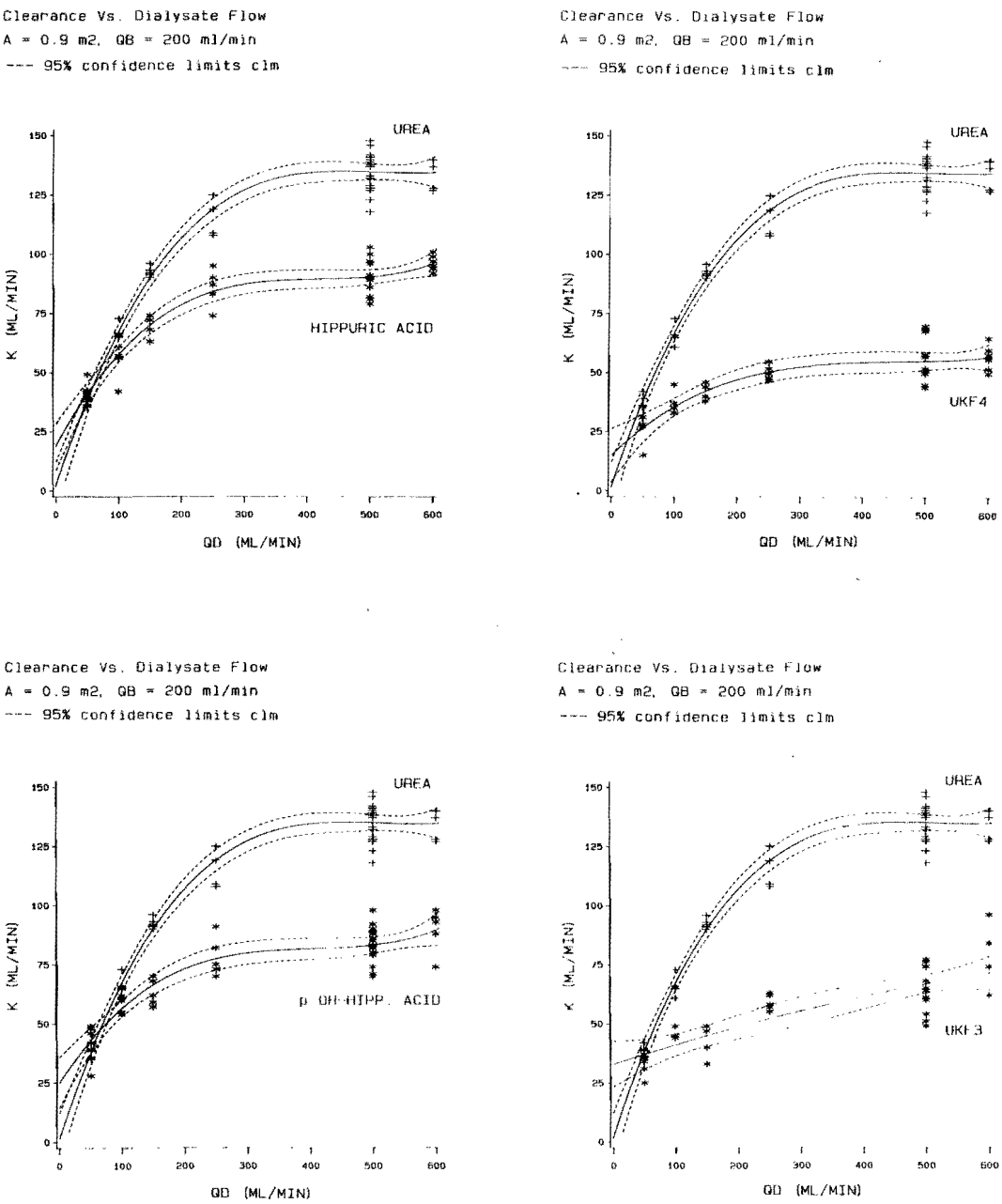


Fig.#5.11. Polynomial regression lines of dialyzer clearance (K_B) versus dialysate flow rate for hippuric acid, UKF4, *p*-hydroxyhippuric acid, and UKF3, in comparison with urea.

5.4.4 Discussion

Urea, Creatinine, Pseudouridine, Uric acid, and UK5 showed to have *in vitro* dialyzer clearances higher than 90 mL/min. The other solutes, including *p*-hydroxyhippuric acid, hippuric acid, UKF1, UKF3, UKF4, UKF5, indoxylsulfate, UKF6, UKF7A, UKF7, UKF8 and 3-indoleacetic acid all showed clearances below 90 mL/min, any protein binding taken into account.

The substances with dialyzer clearances below 90 mL/min mostly have, or are expected to have, aromatic or indolic structure, as may be derived from their fluorescence at the applied HPLC-detection wavelength combination. Some of them are identified, while others remain unknown until now.

Relative differences of the clearances of the compounds on dialyzers with membrane surface areas of 0.9 m^2 and 1.8 m^2, were calculated. Clearances of urea, creatinine, pseudouridine, uric acid, UK4, and UK5, did benefit only to a small extent ($\Delta < 27$ %) from the higher surface area. On the other hand the dialyzer clearances of native *p*-hydroxyhippuric acid, hippuric acid, UKF1, indoxylsulfate, UKF6, UKF7A, UKF8, and 3-indoleacetic acid all increased by more than 30 % (Table#5.10).

The overall mass transfer coefficients proved to be flow-dependent. The magnitude of this flow-dependency was inversely proportional to solute molecular mass, as could be expected.

There were five HPLC-analyzed compounds, showing apparent molecular masses >300 in their native form: UKF1, UKF3, UKF4, UKF5, and UKF8. It has not yet been established whether this dialysis behavior is governed by true molecular mass or by other solute (molecular) properties. Identification of the molecular structures (e.g. by mass spectrometry) is necessary to answer this question. A number of compounds have intermediate apparent molecular masses (>300) due to protein binding. These solutes are: hippuric acid, UKF1, indoxylsulfate, UKF6, UKF7, UKF8, and 3-indoleacetic acid. They are all fluorescent substances, with the exception of hippuric acid. Literally speaking, these solutes are "middle molecules" by the definition given before. Since most of the apparent molecular masses are well below M_r 1000 (except UKF5, and bound UKF6, indoxylsulfate, and 3-indoleacetic acid) it appears more appropriate to designate them as "less removable".

In the perspective of the m^2-hour hypothesis the question rises whether "poorly removable" proteinbound solutes are toxic.

From eqn.6 it can be derived that dialysis strategies with low dialysate flow rate, which have been performed to check for toxicity of solutes of low molecular mass, would result in decreased clearance of protein-bound as well as nonbound low molecular mass compounds, and consequently in increased predialysis blood levels of both [Babb et al.,1971]. It depends

on the result of such a study whether any conclusion can be drawn with respect to the toxicity of proteinbound compounds. A limited number of clinical studies applying low dialysate flow rates (e.g. 100 mL/min) to estimate low molecular mass toxicity have been reported. In none of them a significant deterioration of nerve function or other uremic symptoms was observed [Christopher et al.,1971; Milutinovic et al.,1971], suggesting that neither proteinbound nor nonbound low molecular mass solutes cause neurological symptoms. On the contrary, the patients were doing well and even showed decreased bleeding times. The absence of deterioration in these two studies on a limited number of patients, may also be explained by the suggestion that a decrease in MNCV (motor nerve conduction velocity) takes longer periods on low dialysate flow than were applied.

Various *in vitro* studies have revealed toxic effects of indolic compounds at high concentrations. Although there is no evidence for toxicity of indoles in vivo or *in vitro* at concentrations found in uremic body fluids [Bergström and Fürst,1983], it has been suggested that a combined effect of indolic and aromatic acids may competitively inhibit tubular function measured by para-aminohippurate transport [Bourke et al.,1970]. Aromatic acids have been shown to be toxic *in vitro* at concentrations comparable to those occurring in renal failure [White 1966; Preuss et al.,1966; Orringer et al.,1971; Bourke et al.,1970].
High levels of proteinbound solutes, which may not be toxic per se, may inhibit protein binding of more toxic metabolites, and of drugs. Inhibition of drug protein binding has been found for indoxylsulfate and other indolic tryptophan metabolites [Bowmer and Lindup,1982; McNamara et al.,1981], aromatic acids such as hippuric acid and hydroxyphenolic acids [McNamara et al.,1981; Gulyassy et al.,1986; Vanholder et al.,1988] and a substituted furan-carboxylic acid [Mabuchi and Nakahashi,1986].

For accurate characterization of membrane materials with appropriate marker solutes, solute molal volumes (calculated from atomic volumes, with conformational corrections), or Stokes radii (calculated from the Stokes-Einstein equation with measured or calculated diffusion coefficients) have been proposed as measures of solute dimension [Colton,1969; Colton et al.,1971; Farrell et al.,1973]. It has been shown by Farrell and Babb [1973] that for a whole range of lower molecular mass solutes with different ionic and structural properties, a fairly good correlation exists between molecular masses and true membrane resistances (for cuprophan). Membrane characterization was not the aim of the present study. The purpose was to estimate the dialyzabilities of the different compounds detected in the HPLC-profiles, because dialyzability is one of the factors determining blood levels of accumulated compounds in uremia.

5.4.5 Conclusions

Diffusive mass transfer of UV-absorbing and fluorescent solutes, characteristic for the uremic state was studied *in vitro* with uremic serum ultrafiltrate as a physiological blood substitute. Mass transfer coefficients (h0A) and clearances (KB) of the solutes under study were determined with capillary flow dialyzers, equipped with cuprophane membranes of 0.9 m^2 and 1.8 m^2 effective surface area. Dialyzer clearance values of the free solutes ranged from 34 mL/min (unknown fluorescent peak UKF5) to 135 mL/min (urea), and H_0A values from 38 mL/min (UKF5) to 270 mL/min (urea), at "blood" flow QB=200 mL/min, and dialysate flow QD=500 mL/min in a 0.9 m^2 surface area dialyzer. Clearance values, recalculated for serum protein binding, if any, and protein volume exclusion, ranged between 14 mL/min (indoxylsulfate, 90% bound), to 141 mL/min (urea, 0% bound).

It has been shown that a number of HPLC-analyzed uremic compounds with dialyzer clearances below 90 mL/min, any protein binding taken into account, benefit from dialysis on higher surface area membranes more than do solutes such as urea, creatinine, and uric acid. The concerning compounds have (or are expected to have) aromatic or indolic structure.

Dialyzer behavior of unknown and proteinbound solutes was expressed in terms of an apparent molecular mass ($M_{r,app}$). A number of solutes had apparent molecular masses higher than 300 in their native form, while others did so as a result of protein binding. From the definition these solutes are to be called "middle molecules". The following proteinbound solutes showed $M_{r,app} > 300$: hippuric acid, 3-indoleacetic acid and indoxylsulfate, and the unknown fluorescent solutes UKF1, UKF6, UKF7, and UKF8. Five fluorescent compounds, UKF1, UKF3, UKF4, UKF5, and UKF8, had $M_{r,app} > 300$ in their native, nonbound form.
With the exception of UKF5, all native solutes had (apparent) molecular masses well below M_r 1000. Really low clearances are to be expected only for the proteinbound solutes indoxylsulfate, 3-indoleacetic acid, UKF6 and UKF7.
Protein binding is expected to be the most important factor determining low dialyzer clearances for the analyzed compounds.
In the given context, it is recommendable to speak of "poorly dialyzable molecules" instead of elusive "middle molecules". This seemingly trivial statement may help to go around the Babel-like confusion on "middle molecules".
The significance of the present results will be discussed further in chapter#5.5 and chapter#5.8.

5.5 CHANGE OF BLOOD LEVELS AS A RESULT OF HEMODIALYSIS TREATMENT

5.5.1 Introduction

Blood concentrations of accumulated metabolic waste products are governed by several factors, such as generation rate, residual renal function, dialyzer clearance, and distribution between compartments (distribution volume, intercompartmental resistances). For solutes that demonstrate single pool behavior, such as urea, a mass balance equation may be formulated as follows [Sargent and Gotch,1983]:

$$V \cdot \frac{dC}{dt} = G - (K_R + K_D) \cdot C$$

assuming dV/dt=0. V is distribution volume, C = blood solute concentration, t = time, G = solute generation rate, and K_R and K_D are residual solute clearance and dialyzer clearance respectively. The solution for C is:

$$C = C_0 \cdot e^{-\left(K_R + K_D\right) \cdot t/V} + \frac{G}{(K_R + K_D)}\left(1 - e^{-\left(K_R + K_D\right) \cdot t/V}\right)$$

where $G/(K_R+K_D)$ equals the steady state concentration at large t.
In the case of a two pool model an additional term $-K_1*(C_B-C_C)$ should be added at the right hand side of the above equation. For two-pool and multipool situations the solutions of the differential equations become more complex [Popovich et al., 1975; Frost and Kerr, 1977]. Two different forms of a two-pool model have been described by the latter authors. One in which solute generation takes place in the extracellular fluid (e.g. generation in the liver) accompanied with diffusion into cells with a certain cell wall membrane resistance, and one in which solute generation occurs within cells (i.e. creatinine and methylguanidine). A time-concentration profile of a solute during and after dialysis, in the case of solute generation within cells, shows a sharp drop of extracellular concentration during dialysis and a "rebound" effect right after the discontinuation of treatment.

5.5.2 Experimental

Patients and sera

Blood was sampled from 10 patients on hemodialysis treatment in the Dialysis unit of the St.Joseph Hospital, Eindhoven, The Netherlands. Dialysis was performed with Disscap 160 cuprophan dialyzers (Hospal, Meyzieu, France), a blood flow of 300 mL/min, and a dialysate flow of 500 mL/min, during 4 h. Residual creatinine clearances of the patients were between 0 and 6 mL/min.

5.5.3 Results and Discussion

In the present study the decrease of blood concentrations, as a result of in vivo dialysis with cuprophan membranes, has been determined. The behavior of the 20 compounds will be compared qualitatively, as quantitative data on solute generation rate and cell membrane resistances were not available.

Dialysis Ratio

In Table#5.13 the blood level changes are expressed in terms of the pre to postdialysis concentration ratio (dialysis ratio, D). The mean values for D were obtained from measurements on ten patients.

Table#5.13. Dialysis Ratios for "Free" and "Total" Blood Serum Concentrations.

SOLUTE	D_{free}		D_{total}		D_{free} vs D_{tot}
	MEAN	SD	MEAN	SD	Sign. P[a]
UKF8	3.07	0.65	2.87	0.65	n.s.
Hippuric acid	3.04	0.47	2.52	0.33	P <0.01
UK4	2.97	1.00	2.62	0.69	n.s.[c]
UKF3	2.91	0.49	-	-	-
UKF1	2.83	0.40	3.12	0.36	n.s.
Uric acid	2.81	0.37	-	-	-
p-OH-hipp.acid	2.66	0.69	2.87	0.71	n.s.
Creatinine	2.38	0.35	-	-	-
UKF7A	2.30	0.33	2.41	0.46	n.s.
Pseudouridine	2.00	0.30	-	-	-
UK5	1.93	0.39	2.07	0.48	n.s.
UKF7	1.66	0.43	1.72	0.43	n.s.
Tyrosine	1.46	0.21	-	-	-
UKF6	1.44	0.85	1.56	0.41	P <0.04
3-Ind.ac.acid	1.38	0.46	1.69	0.33	n.s.
Indoxylsulfate	0.99	0.41	1.43	0.19	P <0.003
Tryptophan	0.88	0.24	1.10	0.19	P <0.01

[a]Significance of difference (Wilcoxon) of D_{free} vs D_{total}. [b]n.a.=not available. [c]n.s.=not significant (P>0.05). Data for hypoxanthine, UKF4, and UKF5 not available.

The compounds have been ordered by magnitude of their Dialysis Ratio. Roughly three groups can be distinguished.

UKF7A, pseudouridine and UK5 have D-values comparable to that of creatinine. These solutes also have comparable *in vitro* dialyzer clearances (K_D) as will be shown in chapter#5.4.

Uric acid, UK4, p-OH-hippuric acid, hippuric acid, UKF1, UKF3, and UKF8 have relatively high D-values compared to creatinine. However, in comparison to the first group of solutes (including creatinine), most of these compounds have considerably lower *in vitro*

dialyzer clearances, especially when they are recalculated for protein binding (Table#5.11). This may be explained by a multipool behavior of these compounds in the patient-artificial kidney system.

For a third group of solutes, including tyrosine, UKF6, UKF7, indoxylsulfate, tryptophan and 3-indole-acetic acid, total blood serum concentrations are only decreased to a small extent by hemodialysis treatment, $1 < D < 1.7$, which corresponds to a percentual concentration drop between 0 and 40%). Except for tyrosine, this poor removability can be explained by low dialyzer clearances resulting from protein binding (see chapter#5.2, and below).

It is interesting to note that D-values for "free" indoxylsulfate and "free" tryptophan are smaller than, or equal to, unity, implicating higher blood concentrations of the free solutes postdialysis. This effect is illustrated in Fig.#5.14a. The pre- and postdialysis HPLC-analyses are shown for *ultrafiltered* serum of a dialyzed patient. The peak heights represent concentration of nonbound solutes.

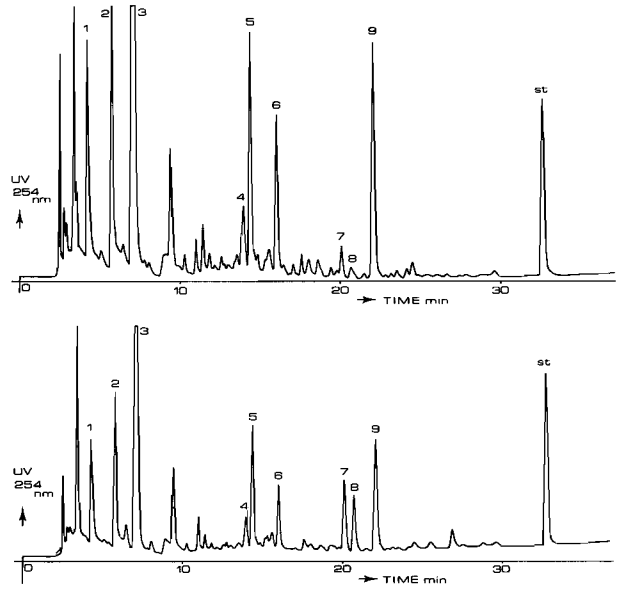


Fig.#5.14a. HPLC-analyses of ultrafiltered serum before and after hemodialysis treatment. Peak identifications: 1,creatinine; 2,pseudouridine; 3,uric acid; 4 and 5,unknown; 6,*p*-hydroxyhippuric acid; 7,indoxylsulfate; 8,tryptophan; 9,hippuric acid; st,internal standard (Only UV-detection was applied here).

The high "free" concentrations for indoxylsulfate and tryptophan after dialysis, may seem peculiar at first glance. Probably this is the result of decreased protein binding during dialysis, as a result of whole body heparinization [Farrell et al.,1972]. It has been suggested that heparin releases the enzyme lipoprotein lipase from tissue, which promotes the hydrolyzation of triglycerides to form free fatty acids (FFA), which on their turn act as

competitors to protein binding of tryptophan, indoxylsulfate and possibly other compounds. The phenomenon of reduced binding postdialysis evidently is not found with hippuric acid (38% pre-, and 49% postdialysis). Perhaps hippuric acid binds to serum proteins at other binding sites than those of the abovementioned indolic compounds, for which there is no competition by free fatty acids.

Change of protein binding during dialysis in vivo

In order to estimate the influence of protein binding on the Dialysis Ratio, the pre- and postdialysis levels of binding were studied in 15 patients on hemodialysis treatment (Dialysis conditions are described in the "Experimental" section; for methodology of determining protein binding refer to chapter#5.2).

Pre- and postdialysis binding levels and significances of difference are presented in Table#5.14. From the compounds that demonstrate protein-binding, hippuric acid and UKF8 have higher binding levels postdialysis, while the other solutes have decreased, or unaltered binding levels in the postdialysis sera. Normally, it is expected that protein binding increases during dialysis as a result of the removal of protein binding inhibitors. A decrease can be explained by lipolytic activity induced by heparinization.

Table#5.14. Differences of Protein Binding Levels of Various Solutes in Pre- and Postdialysis Uremic Sera.

COMPOUND	PROTEIN BINDING (%)		
	Pre-dialysis mean (SD)	Postdialysis mean (SD)	Significance($P<$) of change[a]
UK5	11(7)	5(8)	0.006 (d)
p-OH-hipp.acid	15(8)	8(8)	0.002 (d)
Hippuric acid	38(8)	49(8)	0.001 (i)
UKF1	25(7)	16(3)	0.002 (d)
Indoxylsulfate	90(5)	84(7)	0.002 (d)
Tryptophan	61(12)	49(18)	0.03 (d)
UKF6	74(18)	73(10)	n.s.
UKF7A	12(10)	16(8)	n.s.
UKF7	55(14)	49(17)	0.04 (d)
UKF8	9(5)	15(7)	0.001 (i)
3-Ind.ac.acid	71(10)	64(13)	0.02 (d)

[a](d)=decrease, (i)=increase of binding during dialysis. Significance in a paired Wilcoxon's test. n.s. not significant ($P>0.05$).

The observed changes for tryptophan and hippuric acid protein binding during dialysis are in accordance with those reported by Farrell et al. [1972;1978]. However, our data for tryptophan and 3-indoleacetic acid are in contradiction with those reported by Saito et al.[1980](Table#5.15).

Table#5.15. Comparison of Protein Binding Levels of Tryptophan, Hippuric acid and 3-Indoleacetic acid in Pre- and Postdialysis Sera in This Study and in Literature.

COMPOUND	PROTEIN BINDING[a](%) Mean(SD)				
	This study		Literature		
	Predialysis	Postdialysis	Predialysis	Postdialysis	Reference
Tryptophan[b]	61(12)	49(18)	59(10) 34(-)	37(14) 58(-)	Farrell, 1972 Saito, 1980
3-Ind.ac.acid	71(10)	64(13)	44(-)	64(-)	Saito, 1980
Hippuric acid	38(8)	49(8)	37(12)	48(15)	Farrell, 1978

[a]Binding studies were performed at 37 °C by Farrell, and at 22 °C in the present study. Temperature was not reported by Saito. pH was (adjusted to) 7.4 with Farrell and Saito, and left as is in the present study. [b]Normal values for tryptophan: 78% [Farrell et al.,1972]; 79% (37°C) [Hijikata et al.,1981] and 93.4% [Saito et al.,1980].

It has been suggested [Gulyassy, personal communication] that the observed low binding levels in postdialysis sera originate from continued post-heparin lipolytic activity *in vitro* after sample collection. Pearce and Brown [1983] have proposed a method of heat inactivation of this lipolysis. However the *in vitro* lipolytic activity cannot explain the difference between Saitos' and our data as sample treatments were equivalent in that no heat inactivation was performed in both studies. A possible difference may be duration of sample storage which was several weeks (at -25 °C) in the present study, and was not reported by Saito et al. Opposite temperature dependencies of tryptophan protein binding (of 20°C and 37°C) were reported by Hijikata et al. [1981] and Opienska-Blauth et al.[1963].

The effect of dialysis-induced change of protein binding, on solute clearance, is illustrated in Table#5.16. We have calculated the clearances from protein-binding data and overall mass transfer coefficients determined *in vitro*, using equation#6 in chapter #5.4.2.

Table#5.16. Influence of Protein Binding Level on Dialyzer Clearance During Hemodialysis.

COMPOUND (M_r)	H_0A^a (mL/min)	Start of Dialysis			End of Dialysis		
		PBL(%)[b]	ρ[c]	K_B[d] (mL/min)	PBL(%)	ρ	K_B (mL/min)
Indoxylsulfate(251)	158	90	9.00	14	84	5.25	22
Hippuric acid(179)	132	38	0.61	65	49	0.96	55

[a]Mass transfer coefficient times area product for free solute on Gambro GF-80-M cuprophane hollow fiber dialyzer: blood side and dialysate side flows were 200 and 500 mL/min respectively. [b]PBL=Protein Binding Level. [c]ρ= protein binding coefficient (see chapter#5.4.2). [d]Dialyzer Clearance: refer to eqn.6, chapter#5.4.2. Corresponding values of $K_{B,urea}$ and $K_{B,vitB12}$ are 135 mL/min (own measurement) and 34 mL/min (manufacturers' documentation) respectively.

5.5.4 Conclusions

Three groups of solutes could be distinguished with respect to Dialysis Ratio. A group with low D-values (1<D<1.7) very probably resulting from protein binding, including indoxylsulfate, tryptophan and 3-indoleacetic acid; another group with 1.9<D<2.4, including creatinine and pseudouridine, and a third group comprising hippuric acid, *p*-hydroxyhippuric acid, uric acid, UKF1 and UKF8 with higher Dialysis Ratios (2.5<D<3.1) which were unexpected, because these solutes are bound to protein and have relatively low mass transfer coefficients on dialyzer membranes (except uric acid). Probably an effect of multipool behavior on Dialysis Ratio more than compensates for the effect of reduced dialyzer clearance due to protein binding and intermediate mass transfer coefficient.

The Dialysis Ratio of the free fraction of bound solutes may be smaller than unity due to heparin-induced change of the protein binding level during dialysis. Changes of protein binding during dialysis result in a 50% increase (for tryptophan) and a 15% decrease (for hippuric acid) of dialyzer clearance.

5.6 PRINCIPAL COMPONENT ANALYSIS OF HPLC-ANALYZED UREMIC COMPOUNDS

5.6.1 Introduction

Chronic dialysis therapy has been successful in the treatment of endstage renal patients as it reverses a number of symptoms at least partially, which suggests the removal of accumulated toxic metabolites. Adequate dialysis that alleviates the uremic symptoms, should be designed to remove the accumulated solutes as they appear in the individual patient. Although consensus exists about a number of basic conditions of adequate dialysis (e.g. concerning fluid and electrolyte balance), the latter remains difficult to define as long as no knowledge exists about the toxins or "culprits" [Schreiner, 1975] that should be removed preferentially. Different solutes have been proposed as "marker" solutes, that may serve in defining and implementing "adequate dialysis". They include urea and "middle molecules".
It can be stated that:

Dialysis tuned to the needs of individual patients requires knowledge of both the identity of the "toxins" and the structure of the population of dialyzed patients relative to these toxins.

In order to study uremic solute accumulation, in earlier publications [Schoots et al.,1984;1985;1988] a profiling approach was proposed using modern analytical techniques and multivariate statistical evaluation of the analytical and clinical data.
In order to explore interdependencies between concentrations of multiple uremic accumulated solutes, and their distribution in different patients, a multivariate statistical analysis is described in the present study. The correlation of uremic serum concentrations of accumulated substances with neurophysiological indices of uremia is described in a following chapter.

Quantitative data on twenty-two compounds accumulating in uremic sera, determined by HPLC (urea and phosphate measured conventionally), have to be analyzed. Correlations or interdependencies between these concentrations in sera of dialyzed and non-dialyzed uremic patients have to be studied. With this number of variables 231 non-unity correlation coefficients can be calculated. It is beyond saying that with such an approach we will only advance with difficulty. Moreover, only bivariate correlations will be obtained, and multivariate relations will remain hidden. Principal Component Analysis (PCA) is a multivariate technique by which the dimensionality of the data can be reduced, thus facilitating the interpretation. The principal components are determined in such a way that as few of them

will explain as much as possible of the variance in the original data. The obtained principal component pattern has to be interpreted from a (bio)chemical point of view. Scores on the original variables can be replaced by scores on the, fewer, new variables, the principal components. With these scores the structure of the uremic patient groups can be analyzed.
To my knowledge this is the first time that a multivariate study in the present form, is reported for this number of uremic compounds from different solute classes.

5.6.2 Experimental

Patients and sera. Blood samples were collected from 33 uremic dialyzed patients (predialysis), and from 32 non-dialyzed patients in the Dept. of Nephrology, Ghent University Hospital. Dialyzed patients were treated by single needle hemodialysis using different types of dialyzers. Serum samples were ultrafiltered using Centrifree ultrafiltration units (Amicon, Danvers, USA) in order to remove serum proteins. The filtrate was diluted 1:1 v/v with a solution of internal standard naphtalene sulfonic acid (37 mg/L), and were subsequently analyzed by high performance liquid chromatography (HPLC). By this procedure the non-proteinbound (free) concentrations of the solutes were determined.

Liquid chromatography (HPLC), was performed as described in chapter 4.

Data acquisition and statistics. Chromatographic data acquisition and handling was performed, using a Model 761S data interface and Model 2600 chromatography software (both from Nelson Analytical, Cupertino, USA). Data were read in SAS data-files and analyzed with SAS statistical software (SAS Institute Inc., Cary, NC, USA). The SAS-procedures FACTOR, and CORR were used for principal component analysis and correlation analysis respectively [SAS, Inc., 1985a]. Total UV-absorbance at 254 nm, and total fluorescence emission (at 280 nm excitation, and 340 nm emission) were obtained by determining total peak areas under the UV- and Fluorescence traces of the HPLC chromatograms.

Principal Component Analysis. In principal component analysis (PCA), a set of measured variables may be transformed into a smaller set of non-correlated variates that describe a large proportion of the variation in the original variable set. In this way a dimension reduction of the original data is obtained.
The variates or principal components are linear combinations of the measured variables, according to:

$$PC_i = \sum_{j=1}^{p} w_{ij} \cdot x_j$$

Where W_{ij} are variable weighting coefficients and x_j are the measured variable scores (j=1,2, ...p; p variables, and i=1,2,...k; k components, where $k \leq p$).

By using a correlation matrix rather than a variance-covariance matrix, the data can be standardized to account for large differences in variance of variables expressed in different scales and units. The principal components are extracted in such a way that PC1, the first principal component, is the linear combination describing the largest amount of variation in the standardized data, and PC2, PC3, etc. comprise linear combinations which describe subsequent largest amounts of remaining variation in that way that PC1, PC2, etc. are all non-correlated. This is done by calculating the eigenvalues λ and corresponding eigenvectors γ of the correlation matrix **R.** The eigenvectors are the respective weighting coefficient vectors as encountered (W_{ij}) in the equation given above. Principal components are extracted under the constraints of $\gamma_{i_1}'\gamma_{i_2} = 0$ and $\gamma_{i_1}'\gamma_{i_1} = 1$. Where γ_{i_1} and γ_{i_2} denote subsequent eigenvectors. The maximum number of extracted components is equal to the number of variables. Total variance of the standardized data is the sum of the diagonal elements of the correlation matrix, that amounts to p, the number of variables. The variance explained by individual principal components equals the corresponding eigenvalue divided by the total variance: λ_i/p. There are several criteria for the number of components to retain. In the present study a discontinuity in a scree plot of principal component eigenvalues as a function of extraction order, is used.

After extraction of PC's, orthogonal rotation of the PC-pattern was performed with the varimax method. This comprises a maximization of the variance in the columns of the principal component matrix. This results in values of the column elements as near as possible to 0, -1, or 1.

5.6.3 Results

Representative chromatograms of ultrafiltered sera are given in Fig.#5.15. These profiles will be discussed below. Besides twenty compounds analyzed by HPLC, phosphate and urea were quantitated conventionally.

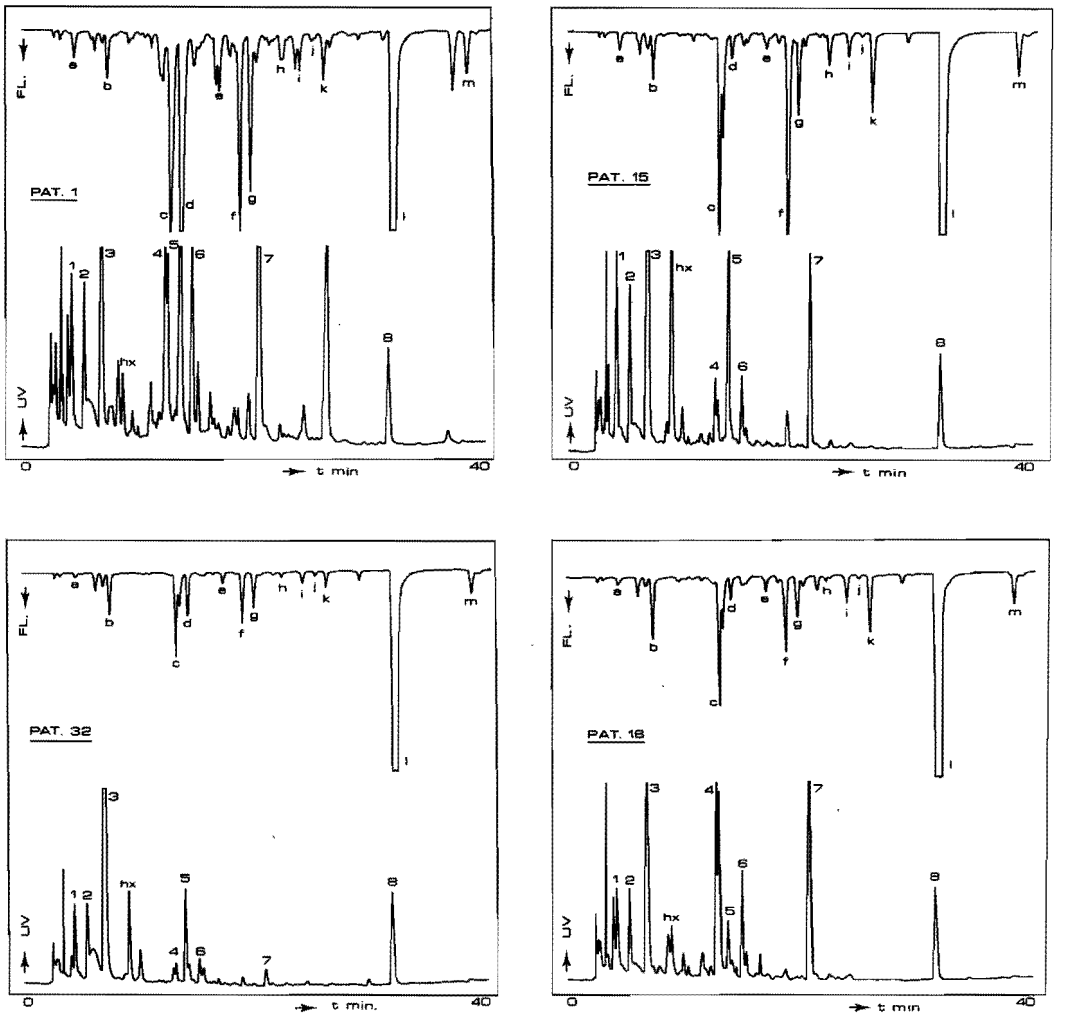


Fig.#5.15. HPLC-profiles of predialysis ultrafiltered sera from four patients on hemodialysis treatment. Peak identifications. UV-trace:1,creatinine; 2,pseudouridine; 3,uric acid; 4 and 5,unknown; 6,*p*-hydroxyhippuric acid; 7,hippuric acid; 8,internal standard. Fluorescence trace:a,UKF1; b,tyrosine; c,UKF3; d,UKF4; e,UKF5; f,indoxylsulfate; g,tryptophan; h,UKF6; i,UKF7A; j,UKF7; k,UKF8; j,UV-standard(=8 in UV-trace); m, 3-indoleacetic acid.

A correlation matrix of the solute concentrations was factored in a principal component analysis, to obtain a smaller number of mutually independent "summary variables", and to visualize correlations and interdependencies between the concentrations of the respective solutes.

Dialyzed uremic patients

After judgement of a scree plot (Fig.#5.16, left) of eigenvalues, three principal components were retained from the data of the hemodialyzed (HD) group. These three

components describe 49 % of total variance in the scaled data (PC1, 22%; PC2, 15%; PC3, 12%). After orthogonal rotation (varimax) a factor pattern was obtained, of which a projection on the PC1-PC2 plane is shown in Fig.#5.17.

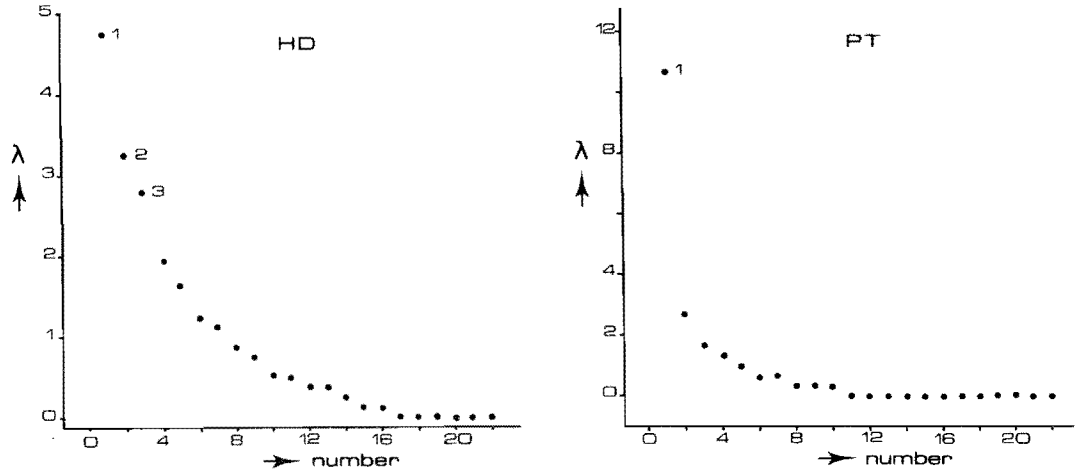


Fig.#5.16. Scree plots of principal component eigenvalues, representing proportion of variance explained by PC's. Left; three PC's retained from analysis of data obtained from sera of a group of *dialyzed* patients. Right; one PC retained from data of a group of *nondialyzed* uremic persons.

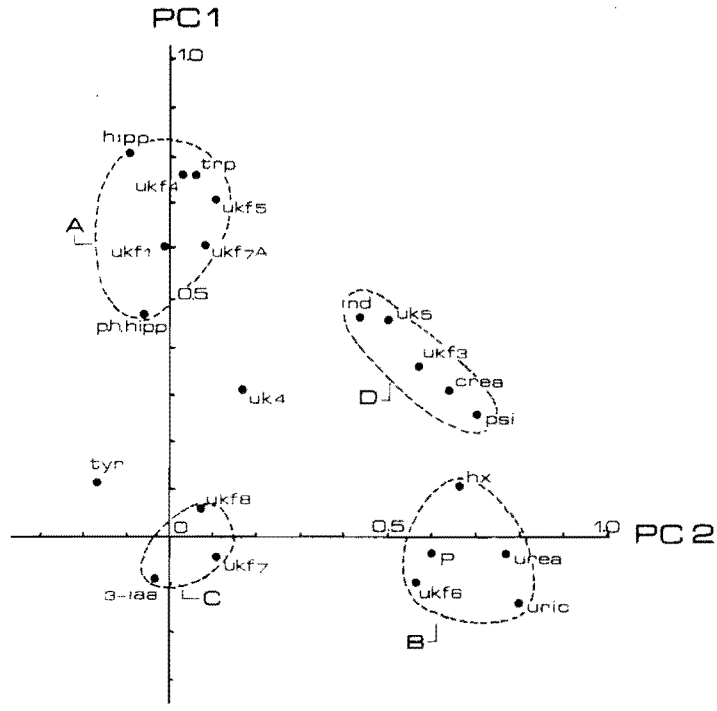


Fig.#5.17. Principal component pattern in the PC1-PC2 plane, showing loadings of 22 uremic compounds on these PC's. Solutes described by PC3 are localized around the origin in this setting. Hipp,hippuric acid; trp,tryptophan; p.h.hipp,*p*-hydroxyhippuric acid; tyr,tyrosine; 3-iaa,3-indoleacetic acid; ind,indoxylsulfate; crea,creatinine; psi,pseudouridine; hx, hypoxanthine; P,phosphate; uric,uric acid.

Variables and their loadings on the principal components are listed in Table#5.17. Four groups of solutes can be distinguished in this table and Fig.#5.17.

Table#5.17. Loadings[a] of Solute Concentrations on Three Principal Components (PC's), for Sera of Dialyzed Patients.

GROUP	COMPOUND	PC1	PC2	PC3	HPLC peak code[b]
A	hippuric acid	0.77	-[c]	-	7
	tryptophan	0.76	-	-	g
	UKF4	0.72	-	-	d
	UKF5	0.72	-	-	e
	UKF1	0.62	-	-	a
	UKF7A	0.62	-	-	i
	p-OH-hipp.acid	0.43	-	-	6
B	uric acid	-	0.79	-	3
	urea	-	0.73	-	conv.[d]
	hypoxanthine	-	0.64	-	hx
	phosphate	-	0.59	-	conv.
	UKF6	-	0.55	-	h
C	UKF7	-	-	0.91	j
	UKF8	-	-	0.91	k
	3-Ind.ac.acid	-	-	0.91	m
D	pseudouridine	0.24	0.68	-	2
	creatinine	0.28	0.63	-	1
	UK5	0.45	0.47	-	5
	UKF3	0.37	0.56	-	c
	indoxylsulfate	0.46	0.42	-	f

[a]The loadings represent the correlation coefficient of each variable and the respective principal components. [b]For HPLC peak codes refer to Fig.#5.15. [c]Dashes represent loadings between - 0.20 and + 0.20; absolute values of the loadings of UK4 (HPLC peak 4 in Fig.#5.15) on all three PCs were smaller than 0.25. [d]conv.=measured conventionally.

Group A has high loadings on PC1 exclusively. Group B is closely related to PC2, group C has very high loadings on PC3, and group D seems to be related to PC1 and to a smaller extent to PC2.

Here it may be noted that PC2 summarizes a number of solutes that have hitherto been used as clinical markers of uremia: urea, uric acid, creatinine, and phosphate.
Furthermore, PC1 comprises a number of aromatic and indolic compounds, such as tryptophan, hippuric acid, *p*-hydroxyhippuric acid, and a number of unknown fluorescent solutes, and correlates well to residual renal function in these dialyzed patients. Furthermore PC1 correlates significantly to the more general measures of accumulation, total UV-absorbance and total fluorescence emission (Table#5.18).

Table#5.18. Correlation[a] Matrix of PC-scores, Total UV-absorbance, Total Fluorescence Emission, and Residual Renal Function for A Group of Dialyzed Uremic Patients (n=33).

	PC1	PC2	PC3	tot. UV	tot. FL	RCC[b]
PC1		0	0	0.67 *P* <0.0001	0.43 *P* <0.02	-0.57 *P* <0.0006
PC2			0	n.s.	n.s.	n.s.
PC3				n.s.	n.s.	n.s.
tot. UV					0.54 *P* <0.001	-0.40 *P* <0.01
tot. FL						n.s.

[a]Pearson correlation. [b]RCC = residual creatinine clearance. [c]n.s.=not significant ($P>0.05$).

Non-dialyzed patients

From the scree plot (Fig.#5.16,right) it was decided that only one PC should be retained after factoring the correlation matrix of the data of nondialyzed (preterminal, PT) uremic patients. This single principal component describes 49 % of the variance in the scaled data. Loadings of the original solute concentrations on this PC are given in Table#5.19. The principal component scores correlate significantly to residual renal function, total UV-absorbance and total fluorescence emission, as was also observed for PC1 in the group of dialyzed patients.

Table#5.19. Loadings[a] of Solute Concentrations on PC1 in a Non-dialyzed Patient Group.

SOLUTE	PC1	SOLUTE	PC1
UKF3	0.97	UKF8	0.82
UKF1	0.97	UKF5	0.79
Pseudouridine	0.97	*p*-OH-hipp.acid	0.78
Urea	0.90	Phosphate	0.73
Hippuric acid	0.89	3-Ind.ac.acid	0.66
Indoxylsulfate	0.87	UKF7A	0.53
UKF6	0.86		

[a]Loadings are correlation coefficients of the variables with PC1.

A correlation matrix is given in Table#5.20.

Table#5.20. Correlation[a] Matrix of Principal Component Score, Total UV-absorbance, Total Fluorescence Emission, and Residual Renal Function, for Non-dialyzed Uremic Patients (n=32).

	PC1	tot. UV	tot. FL	RCC[b]
PC1		0.59 P <0.007	0.53 P <0.02	-0.81 P <0.0001
tot. UV			n.s.[c]	-0.65 P <0.002
tot. FL				n.s.

[a]Pearson correlation. [b]RCC= residual renal function (residual creatinine clearance). [c]n.s. not significant (P>0.05).

Structure of the dialyzed patients group

From the original data and the weights of the variables on the principal components, standardized patient scores on PC1, PC2, and PC3 were calculated.
The data for PC1 and PC2 are given in the bivariate scatter plot in Fig#5.19.

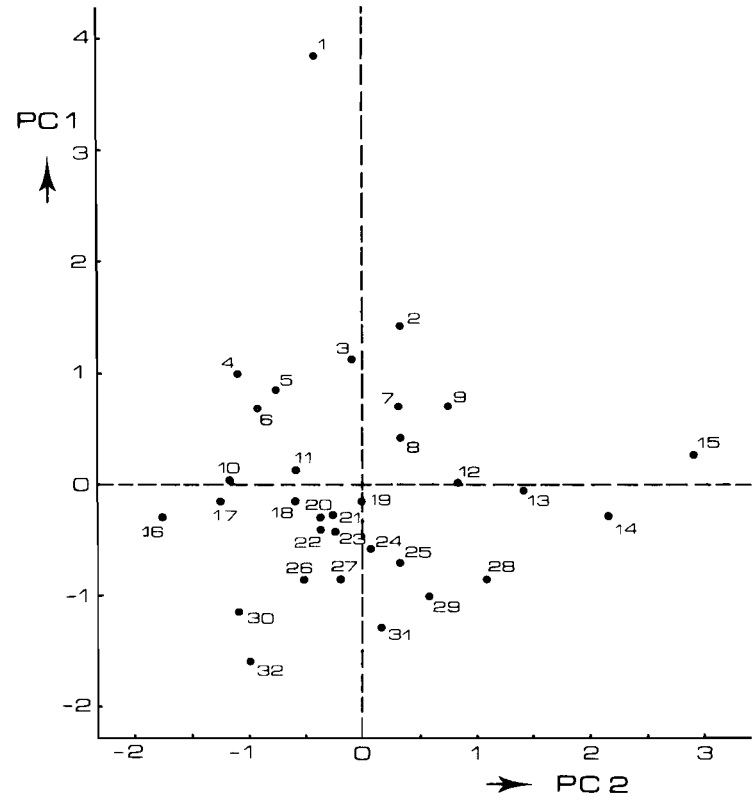


Fig.#5.18. Bivariate scatter plot of patient scores on PC1 and PC2, for 32 dialyzed persons.

In Fig.#5.15 HPLC chromatograms (urea and phosphate concentrations are given in the legend) of sera of patients with "extreme" PC1 and PC2 scores were shown. In patient 1, high concentrations of solutes 6,7, a and d (*p*-hydroxyhippuric acid, hippuric acid, UKF1 and UKF4 respectively), associated to PC1, are found. Only intermediate concentrations of solutes associated with PC2, e.g. peak 1, 2, and 3 (creatinine, uric acid, hypoxanthine respectively), but also urea and phosphate (see figure legends) appear in this patient. Patient 15 with high score on PC2 has intermediate score on PC1. Patient 32 has low scores on both PC1 and PC2, and patient 16 combines low scores on PC2 with an intermediate value for PC1.

It should be noted that no patients have been observed with high score on one PC and low on the other.

5.6.4 Discussion

In the present study the interdependencies of the concentrations of 22 solutes in uremic sera were studied by means of Principal Component Analysis. They include urea, phosphate, creatinine, pseudouridine, uric acid, hypoxanthine, *p*-hydroxyhippuric acid, hippuric acid, tyrosine, indoxylsulfate, tryptophan, and 3-indole acetic acid. Although a number of compounds encountered in the HPLC chromatograms have not yet been identified, they could be quantitated by peak heights. The solutes originate from different metabolic processes. Urea is an end product of protein catabolism, phosphate is related to protein intake or more in general to diet (possibly as phospholipids) and as such is correlated to urea, pseudouridine is a product of specific tRNA-turnover, uric acid and hypoxanthine are more general products of nucleic acid metabolism, and creatinine is the product of phosphocreatine degradation. Hippuric acid is mainly from dietary origin, as it is formed after glycine-conjugation of benzoic acid, which is widely used as a food preservative, and occurs naturally in different foodstuffs. Moreover it is a product of phenylalanine metabolism. *p*-Hydroxyhippuric acid may be an endogenous compound from tyrosine metabolism.

The first principal component(PC1) in the dialyzed patient group includes serum concentrations of hippuric acid, *p*-OH-hippuric acid, tryptophan, unknown fluorescent solutes UKF4, UKF5, UKF1, and UKF7A, that all have, or are expected to have, aromatic or indolic structure. This may be derived from their fluorescence emission at the applied wavelength combination. A number of them exhibit protein binding [Schoots et al.,1988]. Aromatic compounds exert various toxic effects, especially inhibition of cerebral enzyme action [Hicks et al., 1964], and inhibition of ion transport across cell membranes in various tissues [Pappenheimer et al.,1961; Bourke et al.,1970; Orringer et al.,1971; Porter et al.,1975].

The second principal component, PC2, seems to be related to a number of compounds whose concentrations are not directly correlated to residual renal function in terms of creatinine clearance, and that have been used as uremic markers in the past, among which are phosphate and the nitrogenous waste products urea, uric acid, and creatinine. Urea and phosphate concentrations both do correlate to daily protein intake [Sargent and Lowrie,1982], and were found to be correlated significantly in this study ($r = 0.46$, $P < 0.002$). It is interesting to observe that they are located near to each other in the principal component pattern of Fig.#5.17 and that they are closely linked to PC2. Uric acid and its precursor hypoxanthine both have high loadings on PC2 and not on PC1. Urea is used as a marker in the approach of "urea kinetic modelling" to account for toxicity arising from protein

breakdown products [Sargent,1983; Gotch and Sargent,1985]. Phosphate retention causes high plasma levels of parathyroid hormone (PTH) which has been shown to exert various toxic effects [Cooper et al.,1978; Massry and Goldstein,1979].

The solutes with high correlations to PC3 are 3-indoleacetic acid and two unknown fluorescent solutes. There is no significant correlation between PC3 scores on one side and residual renal function, total UV-absorbance, or total fluorescence emission on the other (Table#5.18). In the non-dialyzed group most of the solutes could be described by a single PC.

The PC in the non-dialyzed group, and PC1 from the dialyzed group, correlated significantly to residual creatinine clearance, and to total UV-absorbance and total fluorescence emission.

The principal components can be interpreted in terms of chemical structure of the associated compounds, the mechanism of their excretion by remaining nephrons, and to a lesser extent by the magnitude of their dialyzer clearances. These properties are summarized in Table#5.21.

Table#5.21. Some Properties of The Uremic Solutes under Study.

COMPOUND	PC[a]	r_{RCC}[b] (P)	PBL (%)[c]	$K_b / K_{b,urea}$[d]	TYPE of RCC-CONC. CURVE[e]
Hippuric acid	1	-0.60 (0.0001)	38	0.48	A
p-OH-hippuric acid	1	-0.57 (0.0001)	15	0.57	A
Tryptophan	1	+[f]	61	0.24	-
UKF4	1	-[f]	0	0.42	A
UKF5	1	-0.37(0.02)	0	0.25	-
UKF1	1	-0.30(0.04)	25	0.43	A
UKF7A	1	-0.36(0.02)	12	0.64	A
Uric acid	2	-	3[g]	0.86	B
Urea	2	-	0	1	B
Hypoxanthine	2	-	n.a.[h]	n.a.	-
Phosphate	2	-	25[i]	n.a.	A
UKF6	2	+[f]	74	0.26	A
UKF7	3	-	55	0.33	A
UKF8	3	-	9	0.53	A
3-Ind. acetic acid	3	-	71	0.24	A
Pseudouridine	2(+ 1)[j]	-	0	0.67	B
Creatinine	2(+ 1)	-	0	0.87	B
UK5	2(+ 1)	-	11	0.75	B
UKF3	2(+ 1)	-	0	0.47	B
Indoxylsulfate	1(+ 2)	-	90	0.10	A

[a]Principal component associated with the compound. [b]r_{RCC} = Correlation to residual creatinine clearance. Spearman rank correlation (linear). [c]PBL = Protein binding level (%). [d]$K_b/K_{b,urea}$ = Dialyzer clearance of the solute (proteinbinding taken into account, if any) relative to urea on 0.9 m2 cuprophan membrane *in vitro* [see chapter#5.4.3]. [e]RCC vs solute concentration - curves: A, bend at 20 mL/min; B, bend at 60 mL/min. [f]+ positive correlation; - correlation not significant ($P > 0.05$). [g]from Farrell et al.[1972]. [h]n.a. not available. [i]Phosphate protein binding from Walser [1960].
[j]PC number with highest correlation first. n.s.=not significant ($P > 0.05$).

The correlation of serum concentrations with residual renal function can to some extent be explained by low dialyzer clearances. It has been shown that plasma levels of solutes with low dialyzer clearances are considerably influenced by any residual glomerular filtration [Ahmad et al.,1979]. *In vitro* dialyzer clearances relative to urea (recalculated for any protein binding and protein volume exclusion) tend to be lower for the solutes associated to PC2 and PC3 compared to PC1-compounds (Table#5.21).

Yet there is another attribute of the compounds that could explain the observed division of solute groups. It concerns the mechanism of excretion in remaining nephrons (see chapter#5.2). This may be glomerular filtration (with partial tubular reabsorption), or a combination of filtration and tubular secretion. It has been shown by Porter et al.[1975] that hippuric acid shows no significant increase in serum concentration until residual creatinine clearance has fallen to 20 mL/min in nondialyzed patients. In the present study this is observed for hippuric acid, *p*-hydroxyhippuric acid, UKF1, UKF4, and UKF7A belonging to PC1. These substances probably are excreted in most part by tubular secretion and follow the "organic acid secretory mechanism" [Pitts,1969]. On the other hand serum concentrations of a number of compounds associated to PC2 increased significantly already at RCC = 60 mL/min (urea, creatinine, pseudouridine, UK5, uric acid). This suggests excretion, at least in most part by filtration. In normal functioning nephrons the contribution of tubular secretion to total excretion of *p*-aminohippuric acid (PAH) is more than 6 times larger than that of glomerular filtration, if the serum concentration is smaller than 1000-1500 µmol/L. Above this value the secretory mechanism will become saturated [Pitts,1969]. It has been shown by Smith et al.[1945] that *p*-hydroxyhippuric acid and PAH have identical renal clearances in humans. Therefore the abovementioned limit of 1000-1500 µmol/L for PAH will conceivably be valid also for *p*-hydroxyhippuric acid (and supposedly hippuric acid). As the serum concentrations of these solutes are considerably smaller (*p*-hydroxyhippuric acid: range 4-63 µmol/L, and hippuric acid: range 36-894 µmol/L, both for the nonbound fraction) than this limit (see chapter#5.2), they will still be excreted mainly by tubular secretion in intact nephrons of the residual renal mass in dialyzed uremic patients. In different studies the inhibitory effect of uremic serum on *p*-aminohippurate and *o*-iodohippurate transport in isolated tubules and kidney slices has been reported [White 1966; Bourke et al.,1970; Orringer et al.,1971]. It was proposed that this effect was exerted by high concentrations of hippurates and indole acetic acids, and not by urea, uric acid and creatinine [Bourke et al.,1970]. These latter solutes are, unlike the organic acids, associated to the second principal component (PC2) in the present study.

As there is evidence for the "toxicity" of both PC1 and PC2 - urea and phosphate representing protein toxicity, bone disease, and PTH toxicity respectively, and the organic acids as inhibitors of ion transport in tissues and of cerebral enzymes - dialysis should be designed to remove compounds from both groups. Therefore it is of importance to know what are the relative PC scores ("summary concentrations") for the groups of solutes, in the individual dialyzed patients. Let us consider the structure of the patient group with respect to the uncorrelated principal components PC1 and PC2 (Fig.#5.18). An adequate amount of dialysis in one patient, that brings down the level of "PC2" within acceptable limits, may not be sufficient to attain acceptable levels of "PC1", as the relative scores on the components may be very different among patients (i.e. patient 1 and patient 15 in Fig.#5.18). As an example we consider patient 1 in Fig.#5.18. When this patient is dialyzed for urea (associated to PC2) according to urea kinetic modelling results, a small amount of dialysis may be enough as the score on PC2 is only of intermediate magnitude. However, this may not be sufficient to decrease serum concentrations of solutes associated to PC1, which are relatively high in this individual, to acceptable values.

Table#5.18 shows that PC1 scores in dialyzed patients are significantly correlated to residual creatinine clearance. We found that dialyzed patients, with negligible residual creatinine clearance, generally have higher values of the ratio of PC1 to PC2 scores than do patients with some remaining renal function, or to put it loosely, have relatively high "organic acid"-concentrations compared to urea and creatinine. There was a significant negative correlation of the PC1/PC2-ratio with RCC ($r = -0.45$, $P < 0.01$).

Principal component analysis on the data of the *nondialyzed* patient group appears to result in one single component (PC1). This single PC summarizes almost all compounds that are associated to three independent principal components in the data of the dialyzed patients. As stated above, compounds such as *p*-hydroxyhippuric acid and hippuric acid, described by PC1 in the dialyzed group, only appeared in the blood of nondialyzed patients when residual renal function decreased below 20 mL/min. This may be part of the answer to the question why additional principal components in *dialyzed* patients (residual renal function of 0 to 5 mL/min), are derived (see also chapter#5.3).

Different groups of uremic accumulated compounds of which the serum concentrations were related, could be distinguished in the dialyzed patients' sera. Choosing a single marker, such as urea, to describe uremic solute retention seems only justified in the case of nondialyzed patients, with residual renal function higher than 20 mL/min.

Sargent, who advocates the otherwise rational approach of "urea kinetic modelling", stated that " ..... the goal of modelling therapy should not be overly ambitious in its attempt to create an all-inclusive description of uremia" [Sargent,1983].

The results suggest that adequate dialysis should imply *distinguishing between accumulated solute groups that differ with respect to their mechanism of excretion in intact nephrons*. In its consequence this would be a different approach of accounting for remaining renal function compared to the approach of a Dialysis Index [Babb et al.,1975], which was proposed to model the so-called middle molecules. In that approach a hypothetical middle molecule of M_r 1300 was employed, the removal of which would benefit from any residual renal glomerular filtration [Ahmad et al.,1979]. The glomerulus filters these substances as easily as low molecular mass solutes, which is not true for the dialyzer membrane. Contrary to the hypothetical nonmeasurable "middle molecule", now one of the analytically well-defined organic acids, or maybe more specifically, the hippurates, may be used as a measure of accumulation of "organic acid-like" substances.

It is an important observation that any residual renal function contributes to the well-being of dialyzed patients, whatever the precise explanation for this phenomenon [Ravid et al.,1980]. It has been reported by Nielsen [1975] that clinical neuropathy is not related to the degree of azotemia, as measured by the conventional "indicators"; the serum concentrations of urea and creatinine, but that it is correlated inversely with residual creatinine clearance. Whether this is a consequence of accumulation of tubularly secreted organic acids in patients with negligible renal function (see also chapter#5.3) remains to be established.

5.6.5 Conclusions

The present study shows that, between patients, concentrations of a group of "organic acid-like" substances vary relatively independent from those of another group, including the classical markers such as urea, creatinine, and uric acid. The relative "concentrations" of these groups are different between patients, and are inversely related to residual creatinine clearance.

It seems worthwhile to introduce a marker of uremic accumulation of residual renal function-related "organic acid-like" compounds in addition to urea or creatinine. This could contribute to the goal of "adequate dialysis" in the light of the proven toxicity of this group of substances. *p*-Hydroxyhippuric acid or hippuric acid might prove to be suitable. A choice should be based on considerations of protein binding, measurability, single or multipool kinetics, variation with diet, and (mode of) endogenous generation.

5.7 CORRELATION OF BIOCHEMICAL AND NEURO-PHYSIOLOGICAL INDICES OF UREMIA

5.7.1 Introduction

In patients with progressive renal insufficiency, a number of neurological disturbances are observed. They include both central nervous system dysfunctioning and peripheral neuropathy [Tyler,1968; Nielsen,1975; Raskin and Fishman,1976; Teschan et al.,1979; Jennekens,1983].

Symptoms associated to neuropathy comprise tingling and prickling toes and fingers, the "burning feet syndrome", pain along the nerves, the "restless leg syndrome", impaired vibratory perception, absent deep reflexes, and muscular weakness [Nielsen,1975; Jennekens,1983].

Clinical neuropathy in terms of increased vibratory perceptive threshold (VPT), and decreased motor nerve conduction velocity (MNCV) was associated only to a subset of these symptoms, and to varying degrees. Affection of the lower extremities is most pronounced. Furthermore symptoms are worse in men than in women, while high age in men is a negative factor. Although early symptoms were shown to be reversed at least partially by hemodialysis treatment, this was not the case with more severely developed neuropathy. The degree of neuropathy was found to be inversely related to residual renal function and not to azotemia, as measured by serum concentrations of urea and creatinine [Nielsen,1973]. Alternatively, a significant negative correlation ($P<0.05$) of serum urea and creatinine concentration on one side and ulnar nerve conduction velocity on the other, was observed by Lowrie et al. [1976].

Presently, it is assumed that the etiology of uremic neuropathy may be twofold:

1) Demyelinisation and axonal degeneration, possibly resulting from Schwann cell dysfunction (e.g. due to inhibition of transketolase activity by uremic toxins).
2) Impairment of ion transport across cell membranes, resulting in decreased transmembrane potential, and slowing of nerve conduction velocity. The cell membrane transport disturbance has been reported for various other tissues, e.g. renal tubular cells, responsible for organic acid secretion [Bourke et al.,1970; Porter et al.,1975]. It may be due to inhibition of Na-K-ATPase activity, as a part of the Na-K-pump, or to competitive inhibition of ion transport.

In both processes a detrimental effect of unknown accumulated uremic toxins may be of importance. This is supported by the finding that nerve conduction velocities rapidly increase after renal transplantation.

In a number of studies serum concentrations of uremic solutes were correlated to nerve conduction velocities ["middle molecules": Babb et al.,1971; Man et al.,1980. Myoinositol: Clements et al.,1973; Blumberg et al.,1978. Methylguanidine: Baker et al.,1971. Urea and creatinine: Lowrie et al.,1976; Nielsen,1973].

In the following, the correlations of uremic serum biochemistries with neurophysiological indices of uremia are studied. The neurophysiological parameters are: motor nerve conduction velocities (MNCV) of the ulnar and peroneal nerves, and Hoffmann-reflex latencies (H-M intervals). Biochemical variables were twenty HPLC-analyzed compounds and sixteen routine clinical biochemistries. Regular correlation analysis, canonical correlation analysis, and principal component analysis will be performed.

5.7.2 Experimental

Patients and sera. Blood samples were collected from 26 uremic patients on regular hemodialysis treatment in the Dialysis Unit of the Free University Hospital, Amsterdam, The Netherlands. Gambro Lundia 4H and 5H cuprophan parallel plate dialyzers were used. Blood flow was 200 mL/min, and dialysate flow was 500 mL/min. None of the patients took neurotoxic drugs (such as cytostatics). Serum samples were stored at -70°C, until use.

Routine Clinical Analyses were performed at the Clinical Laboratory of the Free University of Amsterdam, and included: creatinine, urea, uric acid, phosphate, Ca, Na, K, alkaline phosphatase, SGOT, SGPT, LDH, hemoglobine and bilirubine. These determinations were done in samples taken on the day of neurophysiological examination, and in samples obtained every other week during a two month period around this date (5 samples). PTH and Al were determined halfyearly. The routine clinical analyses will be designated CLIN-variables from here.

Neurophysiological measurements. Maximal motor nerve conduction velocities (MNCV) of the peroneal and ulnar nerves, and Hoffmann reflex latencies (H-M intervals) were measured in the dialysis patients at the Dept. of Clinical Neurophysiology, Free University of Amsterdam. These variables will be called NERVE-variables hereafter. The H-M interval, which was found to be a more reliable measure of polyneuropathy than MNCV [Guihéneuc and Ginet,1973; Visser,1979], was measured at the left and right legs. Normal and pathological values of the neurophysiological parameters are given in Table#5.22 below.

Table#5.22. Normal and Pathological Values of MNCV and H-M intervals.

VARIABLE	NORMAL VALUE	MILD/MODERATE NEUROPATHY	SEVERE NEUROPATHY
Peroneal MNCV (m/s)	38-55	25-38	<25
Ulnar MNCV (m/s)	45-75	30-45	<30
H-M interval (ms)	25-34	>34	-

(From: Delano,1983; Visser et al.,1983).

HPLC-analyses were performed as described in chapter 4. The concentrations of the HPLC-analyzed solutes will be designated HPLC-variables.

Statistical Methods. The following methods have been used: 1) correlation analysis (Spearman) of HPLC-variables (HPLC-set) and routine clinical analyses (CLIN-set) with the neurophysiological variables (NERVE-set). 2) Canonical correlation analysis of NERVE-set, with HPLC-set and CLIN-set separately and combined. 3) Principal component analysis of HPLC-set and subsequent correlation of component scores with NERVE-set. 4) Principal component analysis of combined NERVE and HPLC variables and of combined NERVE and CLIN variables. The procedure of principal component analysis has been described in chapter#5.6. Canonical correlation analysis is used to investigate the correlations between two *sets* of variables instead of correlations between individual variable pairs. The procedure derives a linear combination of variables in each set, the canonical variates, maximizing the correlation between these variates. Subsequently, more canonical variate pairs are derived, that are uncorrelated to the other pairs. The maximum number of canonical correlations is equal to the number of variables in the smallest set. When one of the sets contains only one variable, the procedure becomes identical to multiple regression analysis. In principle, repeated multiple regression analyses of each of the dependent variables (e.g. the neurophysiological indices) with all of the variables in the other set is possible. However, in that case the information concerning the coherence of the dependent variables is lost. For the statistical analyses the SAS-procedures CORR, CANCORR and FACTOR were used [SAS Institute Inc., 1985].

5.7.3 Results

Correlation Analysis

Table#5.23 shows the significant correlations of CLIN and HPLC parameters with neurophysiological measurements (NERVE).

Table#5.23. Significant Correlations of CLIN- and HPLC-variables with Neurophysiological Parameters(NERVE)(n=24-26).

HPLC or CLIN variable[a]	NERVE variable[b]	r[c] (*P*)
LDH (date)[d]	H-M right	r=0.50 (0.01)
LDH (date)	H-M left	r=0.56 (0.005)
LDH (mean)[e]	H-M right	r=0.55 (0.006)
LDH (mean)	H-M left	r=0.54 (0.007)
Calcium (mean)	H-M right	r=0.43 (0.03)
Calcium (mean)	H-M left	r=0.50 (0.01)
SGOT (date)	H-M left	r=0.51 (0.01)
SGOT (mean)	H-M left	r=0.48 (0.02)
Phosphate (mean)	Ulnar MNCV	r=0.46 (0.02)
Hb (mean)	Ulnar MNCV	r=0.47 (0.02)
UKF7A	Peroneal MNCV	r=-0.41 (0.05)
p-OH-hippuric acid	Ulnar MNCV	r=-0.47 (0.02)

[a]For explanation of the terms CLIN, HPLC and NERVE see "Experimental". [b]H-M intervals (ms) and motor nerve conduction velocities (MNCV)(m/s). [c]Spearman rank correlation coefficient. [d]Variable determined in blood sample on day of neurophysiological examination. [e]Mean value of variable, from five measurements around date of neurophysiological examination.

Canonical Correlation Analysis

No significant ($P>0.05$) canonical correlations were found between the NERVE-set on one side, and the CLIN and HPLC-sets separately, as well as combined, on the other. By

stepwise multiple regression (at $P<0.15$), with NERVE1 as the dependent, and the complete HPLC-set as the independent variables, the following HPLC-variables were selected: Tryptophan, UKF4, Hippuric acid, Pseudouridine, UKF8, 3-indoleacetic acid and p-hydroxy-hippuric acid. In a subsequent canonical correlation analysis, using the reduced dimensionality of the HPLC-set, a significant first canonical correlation was found (r=0.96, $P<0.001$). It should be noted that this P-value is misleading, because the variables that were entered in the procedure were preselected. Loadings of the HPLC-variables on the first canonical variate in the HPLC-set (HPLC1) were rather small. Tryptophan and *p*-hydroxyhippuric acid showed the largest correlations, r=0.56 and r=0.35 respectively. Their crossloadings on the first canonical variate of the NERVE-set (NERVE1) had a value of 0.54 and 0.34. In a redundancy analysis it proved that HPLC1 describes 24% of the variance in the NERVE-set, and only 9% of the variance in the HPLC-set. Conversely NERVE1 describes 9% of the variance in the HPLC-set and 27% in the NERVE-set.

Correlation of Principal Components and NERVE-variables

Principal components retained in the HPLC-set did not correlate significantly to any of the variables in the NERVE-set.

Principal Component Analysis of the combined HPLC and NERVE variables, and of the combined CLIN and NERVE variables

In PC-analysis of *HPLC + NERVE* two PC's (F1 and F2) were retained, describing 42% of the variance in the standardized data. After varimax rotation, the PC-pattern summarized in Table#5.24A was obtained. A graphical representation is shown in Fig.#5.19. Hoffmann-reflex-latencies (H-M-intervals, ms) and motor-nerve conduction velocities (MNCVs, m/s) are associated to the same principal component (F2), albeit with correlation coefficients (with F1) of opposite sign. The dashed horizontal line was drawn to emphasize the coherence of the variables with F2.

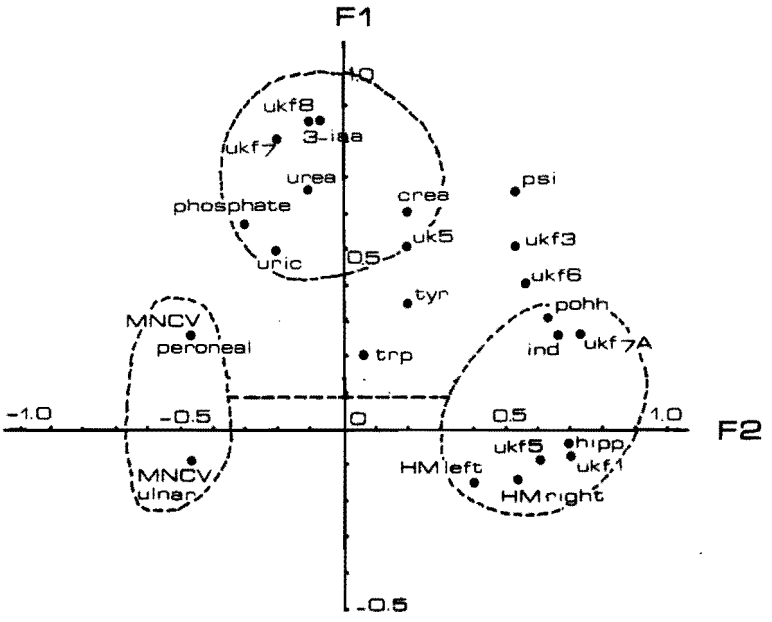


Fig.#5.19. Principal component pattern resulting from analysis of combined HPLC- and neurophysiological variables in a group of dialyzed uremic patients.

PC-analysis of *CLIN + NERVE*, and retention of three PC's (PC1, PC2, and PC3), describing 54% of the variance in the standardized data, resulted in a pattern as given in Table#5.24B.

Table#5.24. Rotated Principal Component Pattern of A) Combined HPLC[a]- and NERVE-variables, and B) Combined CLIN[b]-and NERVE-variables.

A		
VARIABLE	LOADING	
	F1[c]	F2[c]
3-Ind.ac.acid	0.83	-
UKF8	0.83	-
UKF7	0.78	-
Urea	0.67	-
Pseudouridine	0.66	0.52
Creatinine	0.60	-
Phosphate	0.56	-0.33
UKF3	0.52	0.50
UK5	0.51	-
Uric	0.50	-
Tyrosine	0.35	-
Tryptophan	-	-
UKF7A	-	0.73
Hippuric acid	-	0.69
UKF1	-	0.67
Indoxylsulfate	-	0.65
p-OH-hipp.acid	-	0.61
UKF5	-	0.59
UKF6	0.40	0.54
HM-interval R[d]	-	0.50
HM-interval L[d]	-	0.40
Peroneal MNCV	-	-0.49
Ulnar MNCV	-	-0.49

B			
VARIABLE	LOADING		
	PC1	PC2	PC3
HM-interval R[d]	0.90	-	-
HM-interval L[d]	0.86	0.34	-
LDH (mean)[e]	0.62	-	-
Ca (mean)	0.54	-	-0.48
Al (mean)	0.54	-	-
K (mean)	-	-	-
Peroneal MNCV	-0.52	0.47	-
Phosphate(mean)	-0.52	0.36	0.30
Ulnar MNCV	-0.73	-	-
SGOT (mean)	-	0.82	-
Alk.Phosph(mean)	-	0.69	-
SGPT (mean)	-	0.69	0.43
Hb (mean)	-0.34	0.56	-0.38
Uric acid (mean)	-	0.39	-
PTH	-	-0.40	-
Urea (mean)	-	-	0.92
Creatinine(mean)	-	-0.39	0.74
Na (mean)	-	-0.35	-0.70

[a]All HPLC-variables except UK4, Hypoxanthine, and UKF4. [b]All CLIN-variables except bilirubine. [c]Loadings (correlation coefficients) of variables and PC's between -0.30 and +0.30 were omitted. F1 and F2 are the principal components retained in PCA of HPLC+CLIN-variables. [d]R = right; L = left. [e]mean = mean value of multiple determinations around date of neurophysiological examination.

In pattern (A) HM-intervals and MNCV's associate with UKF7A, UKF1, Hippuric acid, *p*-OH-Hippuric acid, and some other solutes, and not with urea, creatinine, uric acid, UK5, and 3-indoleacetic acid.

In pattern (B) the neurophysiological parameters associate with LDH, Ca, and Al, and not with the various enzymes nor with urea(mean), creatinine(mean), and uric acid(mean).

5.7.4 Discussion

A number of correlations of HPLC and CLIN variables with NERVE-variables were significant. LDH correlated very significantly to H-M intervals (left and right). This also holds for calcium and H-M interval (left). *p*-Hydroxyhippuric acid and UKF7A correlated to conduction velocity in ulnar and peroneal nerve respectively.

Moreover in Principal Component Analysis(PCA) (including CLIN and NERVE variables), LDH, Ca, and Al were associated with the MNCV's and HM-intervals, while SGOT, SGPT, Alkaline Phosphatase, PTH, urea(mean), uric acid(mean), and creatinine(mean) were not.

In PCA including HPLC and NERVE variables, the neurophysiological parameters (maximal ulnar and peroneal MNCV, and H-M-intervals left and right) were associated with serum concentrations of UKF7A, Hippuric acid, UKF1, Indoxylsulfate, *p*-hydroxyhippuric acid, and UKF5 (Fig.#5.19). Most of these compounds demonstrated "organic acid"-like RCC-concentration curves, and were found to depend on residual renal function even in dialyzed uremic patients (RCC<5 mL/min) (chapter#5.3). It should be noted that the patient group studied here, is different(another dialysis centre) from that involved in the principal component analysis described in chapter#5.3. The combined concentrations of the compounds, associated to F1, have been shown to inhibit ion transport across cell membranes in renal tubular cells [Bourke et al.,1970], in other tissues [Pappenheimer et al.,1961; Barany,1973], or in brain [Hicks et al.,1964; Raskin and Fishman,1976], by competitive inhibition or by inhibition of Na-K-ATPase. Whether they inhibit ion transport across nerve cell membranes, is not known and may be subject to further study.

The clinical importance of the highly significant correlation of LDH with the HM-intervals is not clear. The alterations in serum enzymes, among which LDH, in chronic renal failure, have been described by Bailey et al. [1970]. These authors reported that the LDH serum levels were higher than normal in 77% of 486 determinations in 76 patients. Only 10% of these cases could be explained by known causes, such as liver disease. The LDH levels were also elevated in the present study.

A role for calcium in uremic polyneuropathy has been proposed [Van Lis,1979; Jennekens,1983]. These authors found calciumcarbonate and calciumphosphate deposits between cell layers of the perineurial myelin sheets.

The issue of calcium and phosphate metabolism, and the involvement of PTH and dihydroxycholecalciferol, is a very complex one, and is beyond the scope of this study.

5.7.5 Conclusions

A number of significant correlations were found between uremic serum biochemistries and neurophysiological indices of uremia. Total serum lactate dehydrogenase (*LDH*), total serum *calcium*, and *SGOT* were correlated positively to the H-M intervals. The HPLC-analyzed compounds *p-hydroxyhippuric acid* and the unknown *UKF7A* were inversely correlated to the maximal motor nerve conduction velocities of N. Ulnaris and N. Peroneus respectively. No significant canonical correlations were found between the set of HPLC-variables and the set of neurophysiological indices, and between the routine clinical biochemistries and the set of neurophysiological measurements. Nevertheless, in principal component analysis, the neurophysiological parameters proved to be associated to a group of HPLC-analyzed *"organic acid-like"* solutes (refer also to chapter#5.3), and not to urea, creatinine and uric acid.

In principal component analysis including the routine clinical biochemistries, the serum levels of *LDH*, *Ca*, and *Al* were associated with the MNCV's and HM-intervals, while SGOT, SGPT, Alkaline Phosphatase, PTH, urea, uric acid, and creatinine, were not.

From the statistical analyses, no conclusions can be drawn concerning cause-effect relationships of accumulated solutes, in sera of uremic patients, and clinical neuropathy. Therefore the clinical importance of these findings should be elucidated in further studies.

5.8 GENERAL CONCLUSIONS ON CHAPTER 5

The accumulation of the HPLC-analyzed uremic solutes was studied in relation to residual renal function in a group of nondialyzed and a group of dialyzed uremic patients. Generally two types of accumulation curves were observed. One showing a "sudden" concentration increase at about 60 mL/min residual creatinine clearance, and another type where this occurred at 20 mL/min or less. While the first type is representative for solutes that are excreted mainly by glomerular filtration, the second in most cases probably reflects a tubular secretory mechanism. The division of solutes with respect to type of accumulation curve, coincides mainly with a grouping found in principal component analysis of characteristic uremic compounds in sera of dialyzed patients. It was shown that most compounds showing a concentration increase at only 20 mL/min were significantly correlated to residual renal function in dialyzed patients with residual creatinine clearances below 5 mL/min. The results suggest that these solutes are still excreted mainly by tubular secretion in nephrons of the remaining renal mass. Therefore residual renal function may prove to be important, not for the remaining glomerular filtration of so-called middle molecules, but for the effective clearance of organic acid-like substances, of which the toxicity has been proven at concentrations found in uremic patients.

The groups of uremic solutes distinguished in principal component analysis could be interpreted in terms of chemical structure and in terms of the mechanism by which they are excreted by remaining nephrons. These solutes also tended to have different *in vitro* dialyzabilities, any protein binding taken into account.

Solutes could be grouped by their concentration decrease during *in vivo* hemodialysis treatment. Compounds from one group had pre-to-post dialysis concentration ratios comparable to that of creatinine. Another group including the "organic acid-like substances", had higher dialysis ratios than creatinine, probably as a result of multicompartmental behavior. A third group of (protein bound) compounds had low dialysis ratios as a result of protein binding.

In a study of the relation between biochemical parameters and neurophysiological measurements it was found that there is a statistically significant correlation between serum lactate dehydrogenase (LDH), serum GOT, and serum calcium on one side and Hoffmann reflex latencies on the other. This was also illustrated in principal component analysis of the neurophysiological and routine biochemical parameters together. Weak correlations were found between peroneal and ulnar nerve conduction velocities and concentrations of *p*-hydroxyhippuric acid and the unknown fluorescent UKF7A. In principal component analysis of the HPLC and neurophysiological variables together, the latter were associated to

a principal component including the same "organic acid-like" HPLC variables, as was found in principal component analysis mentioned above in a patient group from a different dialysis unit. The clinical significance of these findings remains to be established.

In conclusion, from the multidirectional, multicomponent, and multivariate analyses presented here, the possible importance of a group of "organic acid-like" substances emerges. These substances were shown to have the following features:

- they (probably) have aromatic or indolic structure.
- they show intermediate or low dialyzer clearances, mainly due to protein binding.
- their blood concentrations depend significantly on residual renal function.
- probably they are excreted by tubular secretion both in dialyzed and non-dialyzed patients.
- there are indications that they follow multicompartmental behavior in the patient-artificial kidney system.
- the hippurates tended to have (although not statistically significant) lower blood concentrations in CAPD patients than in HD treated patients.
- they are statistically related to the neurophysiological parameters MNCV and Hoffmann-reflex latencies.

It has been reported in literature that these "organic acid-like" substances are toxic at concentrations found in sera of uremic patients, mainly by inhibiting ion transport across cell membranes in renal tubular cells, liver tissue, choroid plexus, and possibly the blood-brain barrier. Therefore they may be of more general significance in the etiology of the multisystem disturbances underlying uremia.

It seems worthwhile to introduce a marker of uremic accumulation of residual renal function-related "organic acid-like" compounds, in addition to urea or creatinine. This could contribute to the goal of "adequate dialysis" in the light of the proven toxicity of this group of substances. *p*-Hydroxyhippuric acid or hippuric acid might prove to be suitable. A choice should be based on considerations of protein binding, measurability, single or multipool kinetics, variation with diet, and (mode of) endogenous generation.

6 RELATED STUDIES USING GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

6.1 HEXACHLOROBENZENE IN SERA OF NON-DIALYZED AND DIALYZED UREMIC PATIENTS

The blood of patients on chronic hemodialysis treatment is exposed to large quantities of dialysate water. Environmental contamination of surface waters by organochlorine pesticides has been reported [Greve,1972; Leoni,1976]. This could result in a gradual increase of body burden of these substances for dialyzed uremic patients. Alternatively pesticides are ingested by diet.

Many dialysis centers use reverse osmosis equipment for dialysate water preparation. Whether the organochlorine pesticides present in the water will be removed efficiently by reverse osmosis (RO), depends on the membranes used. Modern RO-installations generally utilize membranes that are claimed to remove a large part of the organic contaminants with molecular masses exceeding 300 daltons. It is however doubtful whether this is achieved in practice. It has been suggested by Bommer and Ritz [1987] that chloramine and other low molecular mass contaminants (such as aromatic hydrocarbons) will not be removed by RO.

Decreased metabolism, failure to excrete, and inefficient removal by hemodialysis treatment could lead to accumulation of the pesticides. Although these lipophylic compounds are known to accumulate preferentially in adipose tissue, according to Radomski et al.[1971], their blood concentrations are a measure of body burden and exposure. Some data are available on serum concentrations of DDT and its metabolites, and of pentachlorophenol in patients on chronic hemodialysis treatment in the USA [Pearson et al.,1976; Lawton,1971].

We have analyzed the organochlorine pesticides in sera of dialyzed and non-dialyzed uremic patients, and in sera of normal persons [see also Rutten et al.,1988).

Hexane extracts of uremic serum samples were analyzed by gas chromatography (splitless injection) and electron capture detection. Typical chromatograms for a pesticide test mixture and a uremic serum hexane extract are shown in Fig.#6.1. In uremic and normal sera only hexachlorobenzene (HCB) and 1,1-di(4-chlorophenyl)- 2,2-dichloroethene (*p,p'*-DDE, a DDT metabolite) were present consistently in measurable amounts (In Fig.#6.1 concentrations of HCB and *p,p'*-DDE were 17 nmol/L and 29 nmol/L respectively).

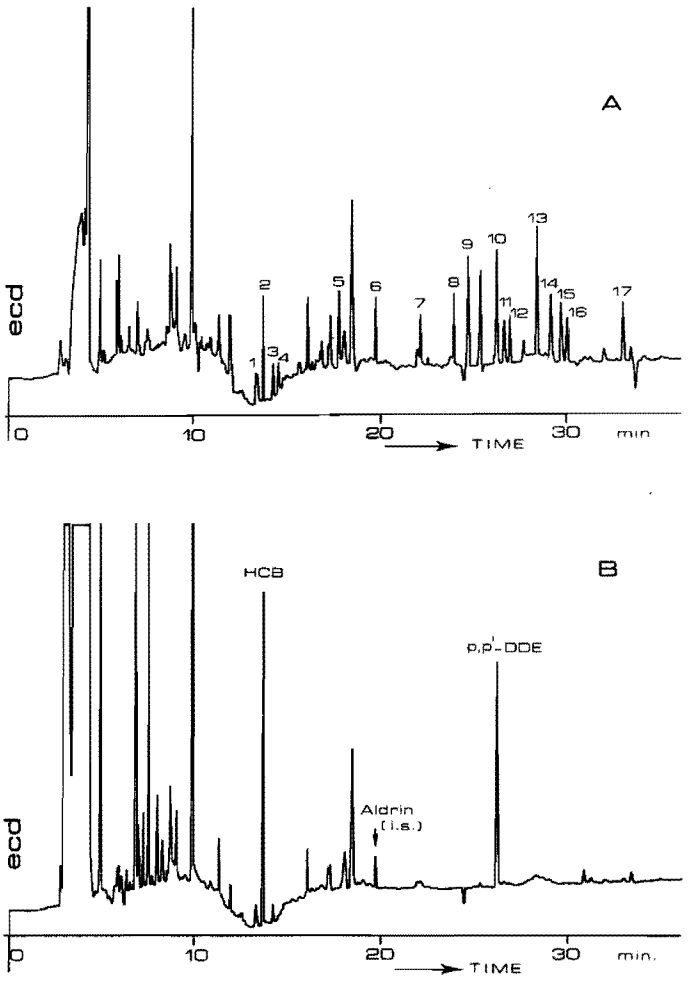


Fig.#6.1. Gas chromatograms of a pesticide test mixture(top), and of uremic serum hexane extract(below), using electron capture detection. Peak identifications: 2,hexachlorobenzene; 6,aldrin; 10,*p,p'*-DDE; 16,*o,p'*-DDT; 17,*p,p'*-DDT.

HCB was identified by GC-MS analysis of a concentrated (100-fold) serum hexane extract. Negative ion chemical ionization (NICI) mass spectra of the peak in uremic serum, of pure HCB and of the background are shown in Fig.#6.2.

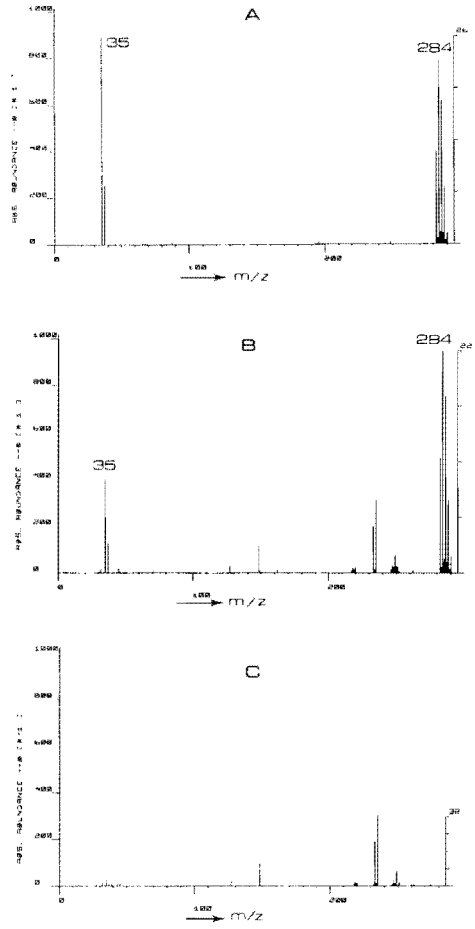


Fig.#6.2. Negative ion chemical ionization mass spectra of pure HCB (A), HCB in a uremic serum extract (B), and background of B (C).

The relative abundances of the peaks in the isotope cluster around m/z 284 strongly indicate the presence of six chlorine atoms, and thus of the occurrence of the hexachlorobenzene structure. No polychlorinated biphenyls (PCB) were found to interfere with the HCB, aldrin, and *p,p'*-DDE peaks at this concentration level, after checking by computer-reconstructed selected ion monitoring at relevant m/z-values.

There was no correlation between pesticide concentrations and residual creatinine clearance or age. Descriptive statistics and tests on difference between patient groups and normals are given in Table#6.1.

Table#6.1. Concentrations of HCB and *p,p'*-DDE in Uremic Patients and Normal Persons.

COMPOUND	CONCENTRATION (nmol/L) in		
	NORMAL PERSONS	NONDIALYZED UREMICS	DIALYZED UREMICS
	Mean (SD) n	Mean (SD) n	Mean (SD) n
HCB	7.7 (1.8) 6	16.2 (6.7) 7	15.5 (11.2) 10
p,p'-DDE	20.1 (10.4) 6	26.4 (31.4) 7	27.0 (38.4) 10

Dialyzed and nondialyzed uremic patients had significantly higher HCB concentrations than normal persons ($P<0.04$, and $P<0.001$ respectively). Concentrations of p,p'-DDE did not differ between these groups, as is true for the comparison of pre- and postdialysis serum concentrations of both HCB and p,p'-DDE.

Discussion

HCB is mainly used as a fungicide in the control of wheat bunt and fungi on other grains. It is used in the manufacture of electrodes, it is an intermediate in dye manufacture, and it is used as a plasticizer for polyvinylchloride [WHO,1979]. Furthermore it is a byproduct in the production of tetrachloroethylene, chlorine, vinylchloride and dimethyl tetrachloro terephtalate. As a result of this diversity of sources, HCB probably is a major contaminant among the organochlorine pesticides.

HCB has a low acute toxicity [Leoni and D'Arca,1976; Gehring and Mac-Dougall,1971]. Chronic toxicity has been reported, including cutaneous porphyria [Schmid,1960; Cam and Nigogosyan,1963; Leoni and D'Arca,1976; Gehring and Mac-Dougall,1971]. In a specific case, Guillan-Barré type polyneuritis was observed in a farmer who had been in contact with HCB [Leoni and D'Arca,1976; Pusic et al.,1967]. In animal experiments with rats and rabbits given oral doses of HCB, neurotoxic symptoms, porphyria and increased liver and kidney weight were observed [De Matteis et al.,1961]. A porphyria cutanea tarda-like syndrome of unknown etiology has been observed in 1 to 2% of hemodialysis patients [Brivet et al.,1978; Poh-Fitzpatrick,1978].

HCB concentrations in whole blood samples as reported in literature vary between 9.1 nmol/L and 105.3 nmol/L, depending on country and region [Morgan and Roan,1970; Warnick,1972; Grosser and Knoll,1973; Richter and Schmid,1976]. Possibly the delicacy of the analytical methods to be used for its determination may also influence the wide concentration range. Most data on blood and plasma concentrations of *p,p'*-DDE originate

from 1970-1974, concern the American population, and vary between 25 nmol/L and 80 nmol/L. The results reported here for serum HCB and *p,p'*-DDE concentrations of healthy persons are in accordance with the above-mentioned data.
For hemodialysis patients no data are available concerning HCB concentrations. In one study [Pearson et al.,1976] average *p,p'*-DDE concentrations in predialysis serum was reported to be 37.4 nmol/L. The results shown here are close to this value.

Conclusions

Only the organochlorine pesticides HCB and *p,p'*-DDE are consistently present in measurable amounts. Serum levels of HCB in both dialyzed and non-dialyzed uremic patients were found to be significantly higher than in non-uremic persons. The fact that HCB is also elevated in nondialyzed patients, suggests that HCB-contaminated dialysate water may not be the only, or even the main source of accumulation.
According to Bommer and Ritz [1987] the hazards of pesticide contamination are difficult to define, and widely discrepant recommendations of legal limits have been proposed by the Association for the Advancement of Medical Instrumentation, and the World Health Organization. The same author stated that "this difficulty is compounded by the inadequacy of most analytical techniques to monitor water for these compounds".

The study described here is detailed by Rutten [1988]. The method we used, proved to be suitable for quantitation of trace concentrations of HCB and *p,p'*-DDE in serum samples.

6.2 QUANTITATION OF POSSIBLY TOXIC POLYOLS IN UREMIC SERA BY GAS CHROMATOGRAPHY

6.2.1 Introduction

Different authors have observed elevated levels of polyols in uremic serum, using gas chromatography or enzymatic methods [Pitkänen,1972; Clements et al.,1973; Blumberg et al.,1978]. Clements et al. [1973] reported that raised plasma levels of myo-inositol, a cyclic hexitol, were related to progression of polyneuropathy in uremic patients, as measured by electroencephalography (EEG) and motor-nerve conduction velocity (MNCV). Myo-inositol is a common serum and tissue compound and is a constituent of phospholipids present in nerve tissue, the phosphatidyl-inositols. Myo-inositol is catabolized in great part in the normal kidney. The compound accumulates in the serum of patients with impaired kidney function, not only as a result of "failure to excrete", but also because of impaired degradation. Hitherto, the etiology of uremic polyneuropathy is unknown. An inverse correlation of indices of neuropathy with residual renal function has been reported by Nielsen [1973].

6.2.2 Experimental

Gas chromatography mass spectrometry. A profiling technique based on capillary gas chromatography, has been developed in the past [Schoots et al.,1979a]. The procedure proved to be suitable especially for the analysis of aldoses, aldonic acids, and polyols. Sample pretreatment consisted of ultrafiltration, evaporation, and silylation. Pre- and postdialysis sera of uremic patients, and sera of normal persons were analyzed. Seventy peaks were detected within 45 min in the profiles (Fig.#6.3). Twenty-four components were identified by means of electron-impact and chemical ionization mass spectrometry [Schoots,1979b]. The compounds erythritol, arabinitol, glucitol, and myo-inositol were quantitated.

Patients and sera. We analyzed the sera of 14 uremic patients, who were dialyzed with cuprophan and polyacrilonitril membranes in the Ghent University Hospital. Three of them showed a severe neuropathy (fibular MNCV < 27 m/s).

Electromyography. These measurements were done on the left fibular nerve, using a Neurodial VCN 760 (ABG-SEMCA) EMG-apparatus.

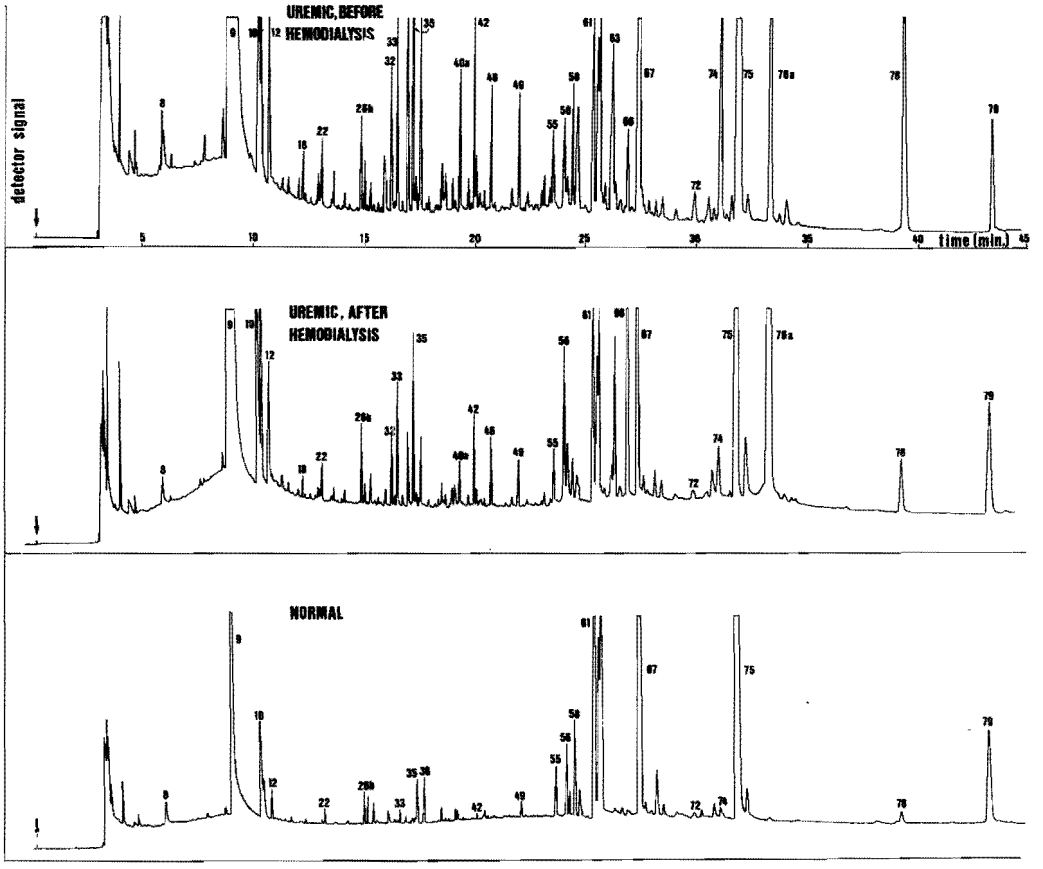


Fig.#6.3. Gas chromatographic profiles of ultrafiltered uremic serum before and after hemodialysis treatment, compared to a similar profile of normal serum.

6.2.3 Results and Discussion

A gas chromatogram of the serum of one of the neuropathic patients is shown below (Fig.#6.4). Very high serum levels of glucitol and myo-inositol were observed in this patient (549 μmol/L and 1983 μmol/L respectively), as compared to normal values.

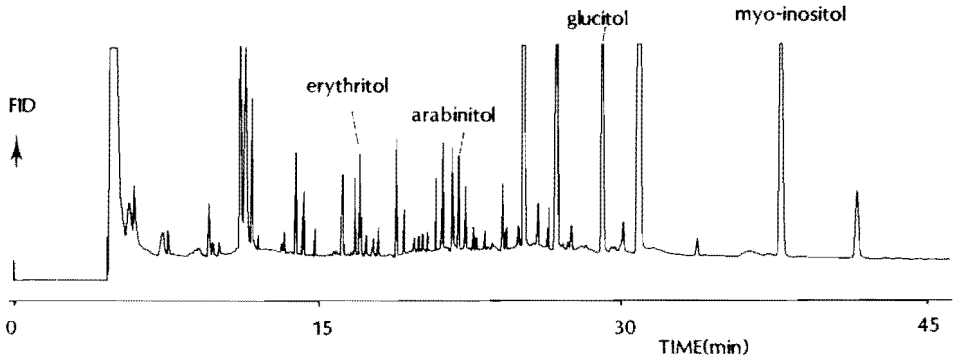


Fig.#6.4. Gas chromatographic analysis of ultrafiltered serum from a patient with polyneuropathy.

Reportedly, normal serum concentrations are between 24 and 63 μmol/L for myo-inositol, and between 13 and 18 μmol/L for glucitol, as reported by different authors [Pitkänen,1972; Clements et al.,1973; Blumberg et al.,1978; Aloia,1973]. While serum concentrations of glucitol were inversely correlated to MNCV ($r=-0.58$, $P<0.04$), those of myo-inositol were not (Spearman correlation). The concentrations of the two solutes were significantly correlated ($r=0.76$, $P<0.002$).

Table#6.2 summarizes the pre- and postdialysis concentrations of erythritol, arabinitol, glucitol, myo-inositol, and creatinine (the latter compound measured conventionally).

Table#6.2. Polyol Concentrations in Sera of Eight Uremic Patients Before and After Hemodialysis Treatment. Dialysis Ratios[a].

COMPOUND (No of C-atoms)	SERUM LEVEL[b]		DIALYSIS RATIO
	BEFORE HD Mean (SD)	AFTER HD Mean (SD)	
Erythritol (4)	36.8 (15.5)	18.0 (11.5)	2.04
Arabinitol (5)	25.6 (6.6)	10.5 (8.6)	2.43
Glucitol (6)	32.4 (17.5)	11.5 (6.6)	2.82
Myo-inositol (6)[c]	1511 (756)	367 (556)	4.12
Creatinine (n.a.)[d]	1238 (319)	726 (195)	1.70

[a]Pre- to postdialysis concentration ratio. [b]All concentrations in µmol/L. [c]cyclic structure. [d]n.a. not applicable in the present context.

It was interesting to note that, with these patients, the value of the pre- to postdialysis concentration ratio (Dialysis Ratio) of the polyols was positively related to the carbon chain length. This was also observed in another study [Schoots et al., 1979a]. Dialyzer clearances of compounds in a homologous series such as these alditols are expected to be inversely proportional to the molecular mass and thus to carbon chain length [Farrell and Babb,1973]. The more pronounced decrease of serum concentrations of the higher molecular mass polyols may be explained by a higher mass transfer resistance across biological membranes between compartments. This may implicate a multipool kinetic behavior. Further indication for this was a considerable "rebound" of serum concentrations postdialysis.

6.2.4 Conclusions

The GC-method that was developed, proved to be suitable for the analysis of polyols and organic (sugar) acids. The polyols could be quantitated. The serum concentration of glucitol was negatively correlated to motor-nerve conduction velocity. The pre- to postdialysis concentration ratios of the polyols suggest a molecular mass-dependent multipool kinetic behavior.

6.3 IDENTIFICATION OF GC-ANALYZED COMPOUNDS BY EI/CI MASS SPECTROMETRY

6.3.1 Introduction

Gas chromatographic profiling of uremic serum was discussed in chapter#6.2. Mass spectrometric identification of peaks can be done in the electron-impact(EI), or chemical ionization(CI) mode. While EI generally leads to extreme fragmentation, especially with trimethylsilylated(TMS) compounds, CI could be a complementary technique for identification as it is expected to result in less fragmentation and more molecular mass information.

To our knowledge the literature on CI mass spectrometry of trimethylsilylated carbohydrates and organic acids is rather limited. Krutzsch and Kindt [1979] identified TMS-dipeptides with isobutane CI-MS, and Budzikiewicz and Meissner [1978] published isobutane CI-spectra of a series of TMS-amino acids. Ariga et al.[1978] reported on CI-MS of methoxime-TMS derivatives of some D-hexoses by means of ammonia CI-MS, while Horton and Wander [1974] analyzed various types of derivatives of sugars by ammonia CI-MS.

Johnson et al.[1978] utilized pyridine as a reagent gas for the characterization of glucuronides. Hogg and Nagabhushan [1972] studied the CI-spectra of *O*-acetyl derivatives of monosaccharides and disaccharides with different reagent gases.

In the following, CI mass spectrometry of aldoses, alditols, aldonolactones, aldonic acids and Krebs cycle acids occurring in uremic sera, will be described.

6.3.2 Experimental

Sample preparation and gas chromatographic profiling was carried out as described previously [Schoots et al.,1979]. Carrier gas (He) velocity was between 20 and 25 cm/s. Glass capillary columns, coated with SE-30 stationary phase, were used. Separations were carried out using temperature programming: 110°C for 2 min, programming rate was 5°C/min, end temperature was 210°C, maintained during 30 min. Samples were injected through a "moving needle" solids injector.

GC-MS analysis was performed on a Model 4000 dual EI/CI quadrupole mass spectrometer (Finnigan/MAT, Sunnyvale, CA, USA). Isobutane, purity grade CH35 (l'Air Liquide, Paris, France) was used as the reagent gas in the CI-mode. It was introduced into the ion source via the make-up line of the instrument. Isobutane ionizer pressure in CI was 10 Pa gauge reading, unless indicated otherwise. A Pt/Ir capillary served as the GC/MS interface, which was maintained at a temperature of 245°C.

Mass spectrometer operational conditions were: electron energy 70 eV in both EI and CI, source temperature was 250°C and 220°C in EI and CI respectively, quadrupole offset voltage was 5V, multiplier voltage was 1.7 kV.

6.3.3 Results and Discussion.

Fig.#6.5 shows a total ion current(TIC) chromatogram of a trimethylsilylated uremic serum sample. The identified peaks, their molecular masses (as TMS-derivatives), and highest mass ions in EI and CI are tabulated below. A number of them have been identified earlier by Bultitude and Newham [1975], using GC-MS with packed columns.

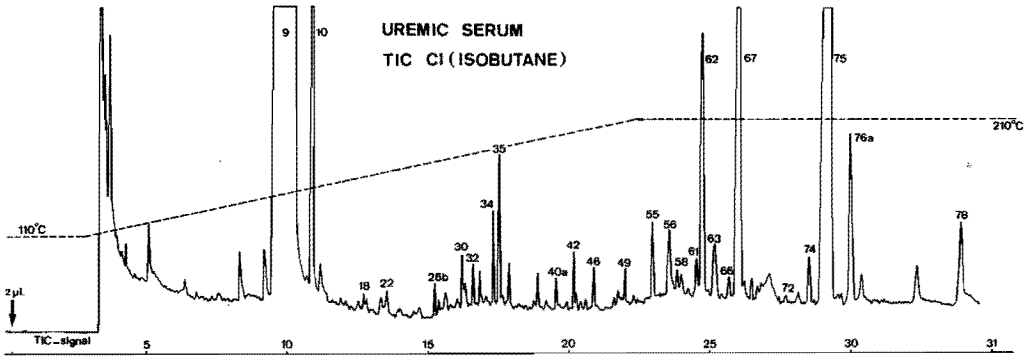


Fig.#6.5. Total ion current chromatogram of an ultrafiltered uremic serum sample. Chemical ionization, with isobutane as the reagent gas (10 Pa), was applied.

Table#6.3. Compounds Identified in Uremic Serum by GC-MS.

PEAK NO. (Fig.6.3)	COMPOUND	TMS-GROUPS	MOL. MASS[a]	HIGHEST MASS ION (m/z) in:	
				CI(i-C_4H_{10})	EI
9	Urea	2	204	205	204
10	Phosphoric acid	3	314	315	314
11	Glycerol	3	308	309	293
18	Tartronic acid[b]	3	336	337	292
22	Threonine	3	335	336	320
26b	Homoserine[b]	3	335	336	320
30	Δ-Pyrrolidone-5-carboxylic acid	2	273	274	258
32	Threitol[b]	4	410	411	320
33	Erythritol	4	410	411	320
35	Erythronic acid	4	424	425	379
40a	Tartaric acid	4	438	439	423
42	2-Deoxy-erythro-pentonic acid	4	438	439	423
49	Arabinitol	5	512	513	320
55	Arabinonic acid	5	526	527	333
56	Citric acid	4	480	481	465
58	Fructose	5	540	451	437
61	Galactose	5	540	451	435
63	3-Deoxy-arabino-hexonic acid[b]	5	540	-	345
66	Glucono-1,4-lactone	4	466	467	466
67	α-D-glucose	5	540	451	435
72/73	Mannitol/Glucitol	6	614	615	421
75	β-D-glucose	5	540	451	435
76a	Mannonic/Gluconic acid	6	628	613	435
78	Myo-inositol	6	612	613	507

[a]Molecular mass of the trimethylsilylderivative. [b]Tentatively identified.

Influence of reagent gas pressure

To study the reagent gas pressure dependence of CI spectra, the following pure compounds were silylated and analyzed by GC-MS: fructose, arabinitol, arabinonic acid, tartaric acid, and malic acid (the last compound was not detected in uremic sera; it was included in this study as were arabinose and 1,5-gluconolactone). The influence of pressure on various parameters is plotted in Fig.#6.6. The ion t-$C_4H_9^+$ (m/z 57) is predominant at higher pressures, while $C_3H_3^+$ (m/z 39) and $C_3H_7^+$ (m/z 43) become less important. Isobutane TIC decreases markedly at pressures over 20 Pa, which explains why TICs of the solutes decrease in a similar manner.

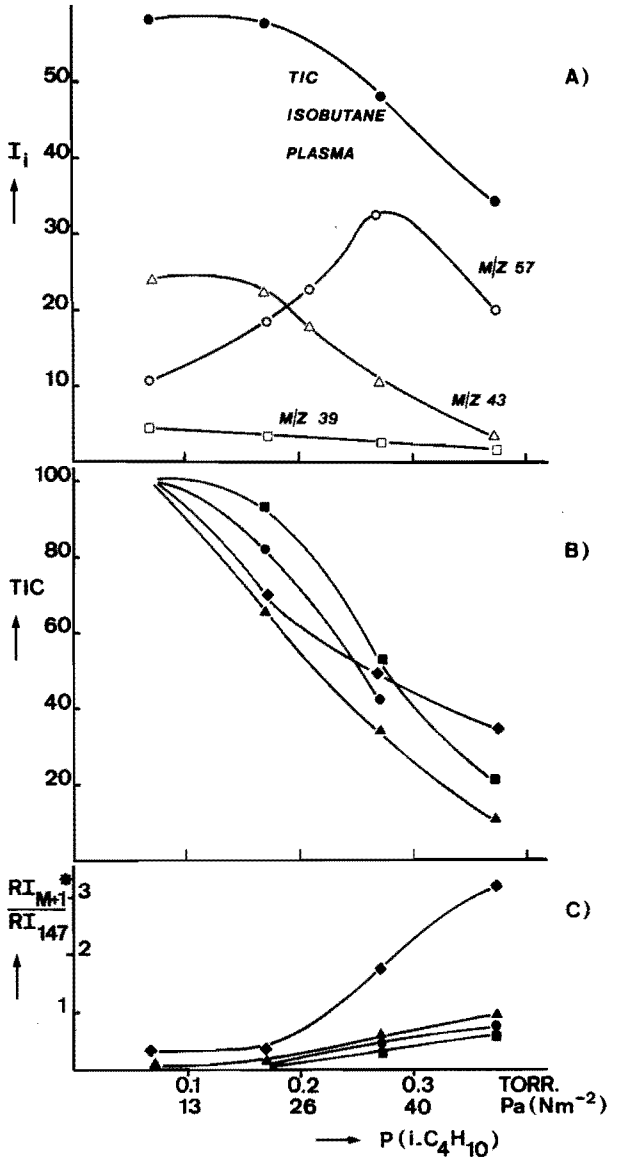


Fig.#6.6. (a) isobutane plasma composition as a function of pressure. (b) total ion currents of arabinitol, fructose, tartaric acid, and arabinonic acid (top>down), versus reagent gas pressure. (c) Variation of CI-character of the mass spectra of the same solutes with pressure, when expressed in Rel.Abundance (M+1)+/Rel.Abundance(m/z 147) ratio. The upmost trace is for tartaric acid.

The CI character of the spectra as a function of ionizer pressure was expressed as the abundance ratio of the protonated molecular ion (if applicable; with fructose $[M-90]^+$ was used), and the characteristic EI-ion at m/z 147 ($[Me_3SiO{=}SiMe_2]^+$). The carbohydrate-related polyhydroxy compounds showed a slight increase with pressure. This effect was more pronounced with tartaric acid and the other di- and tricarboxylic acids, fumaric, malic, and citric acid (not shown). In all experiments care was taken that the pressure of the sample was kept below 0.1% of isobutane pressure.

Comparison of EI and CI spectra

The identified compounds belong to different solute classes: aldoses, alditols, aldonic acids, aldonolactones, Krebs cycle acids, and amino acids. EI and CI(10 Pa) spectra of these classes are compared separately.

Aldoses.

Parallel to the EI spectra [DeJongh et al.,1969], the isobutane CI spectra of the aldoses show abundant ions at m/z 73 (Si^+Me_3), 147 ($Me_3Si^+O{=}SiMe_2$), 191 ($(Me_3SiO)_2C^+H$), 204 ($(Me_3SiO\text{-}CH)_2^+$) and 217 ($Me_3SiO\text{-}CH{=}CH\text{-}C^+H\text{-}OSiMe_3$).

In the arabinose EI-spectrum m/z 133 is the highest mass ion, representing $[M\text{-}CH_3\text{-}TMSOH]^+$. The observed abundance ratio of m/z 204 and 217 is characteristic for a furanose ring structure, both in EI and CI modes. No protonated molecular ion was found in CI. Ions corresponding to $[M+1\text{-}n$x$TMSOH]^+$ appear at m/z 349, 259, and 169 respectively, m/z 349 being the highest mass ion. The ions m/z 333 $[M+1\text{-}CH_4\text{-}TMSOH]^+$ and m/z 243 $[M+1\text{-}CH_4\text{-}2TMSOH]^+$ also appear in the CI-spectrum, in a higher abundance.

For the hexoses galactose and fructose (pyranose and furanose structures, respectively) highest mass ions were observed at m/z 435 and 437 respectively under EI. The CI spectra show enhanced abundances in the high mass region. The highest mass observed is at m/z 451 ($[M+1\text{-}TMSOH]^+$ or $[M-90]^+$). In the CI-spectra of these aldoses the $[M+1\text{-}n$x$90]^+$ ions were also abundant (m/z 451, 361, 271). The m/z 437 ion, in both EI and CI, is assumed to correspond to $[M\text{-}CH_2OTMS]^+$ or $[M+1\text{-}CH_3OTMS]^+$. The ions at m/z 103 ($[CH_2{=}OSiMe_3]^+$) and 117 ($[CHOCH_2OSiMe_2]^+$), resulting from ring cleavage under EI conditions, were not seen in the CI spectra of galactose and fructose. In general it can be concluded that at higher reagent gas pressures the loss of protonated functional groups (TMSOH) is favoured over rearrangements or ring cleavage processes.

Alditols

The alditol CI spectra also have a number of ions in common with the corresponding EI spectra [Petersson,1969]. Rearrangements result in the usual ions at m/z 73 and 147. Chain cleavage leads to the ion series m/z 103, 205, 307, 409, and 511, depending on chain length. These ions are accompanied by satellites at 90 amu lower, corresponding to loss of TMSOH [m/z 217, 319, and 421]. These ions are also found under EI conditions.

Erythritol (a straight chain C-4 polyol), arabinitol (C-5), and glucitol (C-6) exhibited $[M+1]^+$ ions at m/z 411, 513 and 615 respectively, and $[M+1-nx90]^+$ ions were observed too. In Fig.#6.7 the EI and CI spectra (at 10 Pa and 36 Pa reagent gas pressure) for arabinitol are shown.

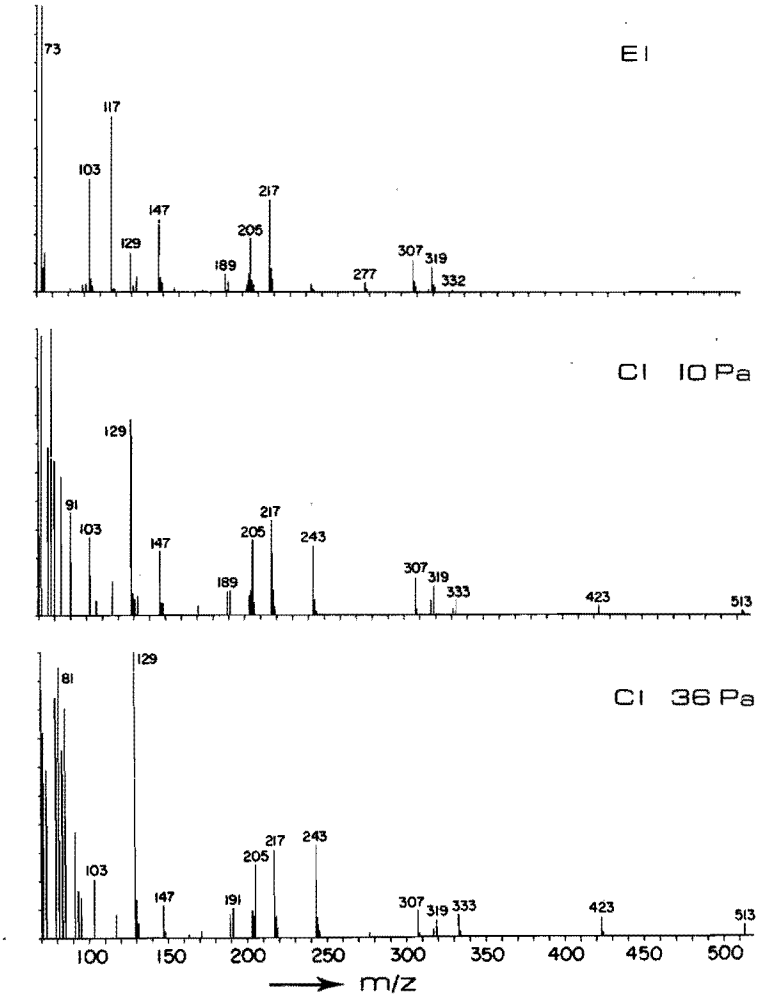


Fig.#6.7. Electron impact and chemical ionization mass spectra of trimethylsilylated arabinitol.

Aldonic acids

Arabinonic acid yields a $[M+1]^+$ ion. However, gluconic acid showed a highest mass ion at m/z 613 ($[M+1-CH_4]^+$). Apart from the known rearrangement ions, the CI spectra of the aldonic acids demonstrated the presence of m/z 292, similar to the corresponding EI-spectra [Petersson,1970]. This ion is the result of a McLafferty-type rearrangement of a trimethylsilyl group, occurring with some hydroxy carboxylic or dicarboxylic acids. Ions at m/z 319, 217 and 205 can be rationalized similarly as in the alditol spectra.

Aldonolactones

The EI spectra reveal molecular ions at m/z 466 for both glucono-1,4- and -1,5-lactones. In CI $[M+1]^+$ ions occur in higher abundance.

Krebs cycle acids

Abundant $[M+1]^+$ ions are observed in CI, whereas EI mainly exhibit $[M-CH3]^+$.

Amino acids

The spectra of the identified amino acids were in accordance with those published by Leimer et al.[1977], and Budzikiewicz and Meissner [1978]. Abundances of various ions were different probably as a result of different operational conditions.

In conclusion, chemical ionization mass spectra of all compounds under study show (protonated) molecular ions, except the aldoses and gluconic acid, while the EI spectra generally contain very few ions in the higher mass region.

At higher pressures (e.g. 36 Pa), the loss of one or more TMSOH groups becomes the prevailing fragmentation pathway.

The total ion current appeared to be inversely proportional to reagent gas pressure. This can be explained by assuming a limitation of the penetration of electrons in the ion source.

Chemical ionization with isobutane as the reagent gas proved to be a good complementary technique to EI in the identification of trimethylsilylated carbohydrates and organic acids in sera of uremic patients.

Intermediate ionizer pressures (10-25 Pa) must be chosen to maintain a reasonable TIC of the sample molecules, and to obtain relatively abundant high mass ions.

SUMMARY
(General conclusions)

This study shows, in combination with earlier findings [Mikkers,1980; Schoots,1982], that gel filtration cannot be used as an *analytical* technique for the determination of so-called middle molecules. Application of this technique for the *isolation* of middle molecules is not the most rational approach. Dialysis and ultrafiltration techniques will be more appropriate. Substances, behaving as if they have middle molecular mass in gel filtration analysis, not necessarily do so in cuprophan dialysis and *vice versa* (chapter 2).

It was shown by gas chromatography and mass spectrometry, that serum concentrations of the environmental contaminant hexachlorobenzene were significantly higher in blood sera of dialyzed and nondialyzed uremic patients, than in those of normal persons (chapter 6).

The HPLC profiling technique that was developed, proved to be reliable in more than 3000 analyses and meets the requirements of repeatability, relevance of analyzed compounds, mildness to thermolabile compounds, and possibility of automation and appropriate quantitation. Characteristic "uremic" profiles were obtained with this procedure. Twenty peaks were consistently present, nine of which were (tentatively) identified by enzymatic, mass spectrometric, and other methods. These compounds were: creatinine, pseudouridine, uric acid, *p*-hydroxyhippuric acid, hippuric acid, tyrosine, indoxylsulfate, tryptophan and 3-indoleacetic acid. For stable uremic patients, the profiles were rather constant longitudinally during several months (chapter 4).

The serum concentrations of the abovementioned solutes, and the unknowns, were determined, and compared, in uremic patients on HD and on CAPD treatment. Both total and "free" concentrations were determined. The concentrations of the tRNA catabolite pseudouridine were shown to be significantly higher in sera of patients treated with continuous ambulatory peritoneal dialysis (CAPD), than in sera of uremic patients on hemodialysis (HD). Three possible explanations have been proposed: 1) a low relative permeability of the peritoneum for pseudouridine; 2) increased specific turnover of tRNA, e.g. as a result of differences of protein synthesis in HD and CAPD treated patients; 3) the occurrence of non-clinical peritonitis, accompanied by cell death.

Protein binding levels of the HPLC-analyzed compounds in sera of CAPD and HD treated patients were determined. They were not significantly different between groups. Thus, protein binding is not expected to be a cause of different weekly clearances of uremic solutes in CAPD and HD treatment (chapter 5.2).

The accumulation of the analyzed uremic solutes was studied in relation to residual renal function in a group of nondialyzed and a group of dialyzed uremic patients. Generally two types of accumulation curves were observed. One showing a "sudden" concentration increase at about 20 mL/min residual creatinine clearance (type A), and another type where this occurred at 60 mL/min or less (type B). While the second type is representative for solutes that are excreted mainly by glomerular filtration (urea, creatinine, pseudouridine), the first in most cases probably reflects a tubular secretory mechanism (e.g. hippuric acid, *p*-hydroxyhippuric acid). The division of solutes with respect to type of accumulation curve, coincides mainly with a grouping found in principal component analysis of characteristic uremic compounds in sera of dialyzed patients. It was shown that most compounds showing a concentration increase at only 20 mL/min were significantly correlated to residual renal function in dialyzed patients with residual creatinine clearances below 5 mL/min. The results suggest that

these solutes are still excreted mainly by tubular secretion in nephrons of the remaining renal mass. Therefore residual renal function may prove to be important, not for the remaining glomerular filtration of so-called middle molecules, but for the effective clearance of "organic acid-like" substances. In literature there is convincing evidence of the toxicity of these compounds at concentrations found in sera of dialyzed uremic patients (chapter 5.3).

Interdependencies between twenty HPLC-analyzed uremic compounds were studied by principal component analysis (chapter 5.6). With this procedure a reduction of the dimensionality of the variable space can be obtained. Three groups of solutes could be distinguished, the relative concentrations of which varied between patients. This may be of importance to dialysis practice, in the perspective of adequate dialysis for individual patients. The groups of uremic solutes distinguished in principal component analysis could be interpreted in terms of chemical structure and in terms of the mechanism by which they are excreted by remaining nephrons. These solutes also tended to have different *in vitro* dialyzabilities, any protein binding taken into account.

The dialyzer clearances of a number of HPLC-analyzed uremic compounds with dialyzer clearances below 90 mL/min (on 0.8 m^2 membranes), any protein binding taken into account, were shown to benefit from dialysis on larger surface area membranes; more than do solutes such as urea, creatinine, or uric acid. The concerning compounds have (or are expected to have) aromatic or indolic structure. Dialyzer behavior of unknown and proteinbound solutes was expressed in terms of an apparent molecular mass ($M_{r,app}$). A number of solutes had apparent molecular masses higher than 300 in their native form, while others did so as a result of protein binding. From the definition these solutes are to be called "middle molecules". With a single exception all native solutes had (apparent) molecular masses well below M_r 1000. Really low clearances are to be expected only for the proteinbound solutes indoxylsulfate, 3-indoleacetic acid, UKF6 and UKF7. Protein binding is expected to be the most important factor determining low dialyzer clearances for the analyzed compounds (chapter 5.4).

Solutes could be grouped by their concentration decrease during *in vivo* hemodialysis treatment. Compounds from one group had pre-to-post dialysis concentration ratios (dialysis ratios) comparable to that measured for creatinine. Another group including the "organic acid-like substances", had higher dialysis ratios than creatinine, probably as a result of multicompartmental behavior. A third group of (protein bound) compounds had low dialysis ratios as a result of protein binding (chapter 5.5).

In a study of the relation between uremic solute concentrations and neurophysiological indices of uremia (chapter 5.7) it was found that there is a statistically significant correlation between serum lactate dehydrogenase (LDH), serum GOT, and serum total calcium on one side and Hoffmann reflex latencies on the other. This was also illustrated in principal component analysis of the neurophysiological and routine biochemical parameters together. Weak correlations were found between peroneal and ulnar nerve conduction velocities and concentrations of *p*-hydroxyhippuric acid and the unknown fluorescent UKF7A. In principal component analysis of the HPLC and neurophysiological variables together, the latter were associated to a principal component including the same "organic acid-like" HPLC variables, as were found as a group in principal component analysis mentioned above for a different patient group. The clinical significance of these findings remains subject to further study.

In conclusion, from the multidirectional, multicomponent, and multivariate analyses presented here a possible importance of a group of "organic acid-like" substances emerges. These substances were shown to have the following features:

- their concentrations (as a group) vary independently between patients, compared to those of classical markers, such as urea, creatinine, and uric acid. This concerns the *structure* of the dialyzed patients group with respect to the serum concentrations of accumulated solutes.
- they (probably) have aromatic or indolic structure.
- they show intermediate or low *in vitro* dialyzer clearances, mainly due to protein binding.
- their blood serum concentrations in both dialyzed and non-dialyzed uremic patients depend significantly on residual renal function.
- probably they are excreted by tubular secretion both in dialyzed and non-dialyzed patients.
- there are indications that they follow multicompartmental behavior in the patient-artificial kidney system.
- they are statistically related to the neurophysiological parameters MNCV and Hoffmann-reflex latencies.
- the hippurates tended to have lower blood concentrations in CAPD patients than in HD treated patients (although not statistically significant).

It has been reported in literature [Grantham et al.,1978] that these "organic acid-like" substances are toxic at concentrations found in sera of uremic patients, mainly by inhibiting ion transport across cell membranes in renal tubular cells, liver tissue, choroid plexus, other tissues, and possibly the blood-brain barrier. Therefore they may be of more general significance in the etiology of the multi-organ disturbances underlying uremia.

It seems worthwhile to introduce a marker of uremic accumulation of, residual renal function-related, "organic acid-like" compounds in addition to urea or creatinine. This could contribute to a better definition of uremic "toxicity" and adequate dialysis. *p*-Hydroxyhippuric acid or hippuric acid might prove to be suitable. A choice should be based on considerations of protein binding, measurability, variation with diet, and (mode of) endogenous generation.

Furthermore, the results indicate that it may be useful to find selective methods of blood purification to remove the accumulated "organic acids". Especially in those patients, with very small (e.g. below 2 mL/min) residual creatinine clearances, such a selective method might be a useful addition to straightforward dialysis for substances such as urea and creatinine.

SAMENVATTING
(Algemene konklusies)

Op grond van deze studie, en eerdere bevindingen [Mikkers,1980; Schoots et al.,1982], kan worden gekonkludeerd dat gelfiltratie niet geschikt is als analytische techniek voor de bepaling van zogeheten "middle molecules". Het toepassen van deze techniek als primaire methode voor de isolatie van "middle molecules" is niet de meest rationele benadering. Dialyse- en ultrafiltratietechnieken zullen daarvoor meer geschikt zijn. Stoffen die zich bij elutie in gelfiltratiekolommen gedragen alsof ze een middelgrote molekuulmassa hebben, zullen dat niet noodzakelijkerwijs ook doen bij dialyse op cuprofaan membranen, en *vice versa* (hoofdstuk 2).

Met behulp van gaschromatografie en massaspektrometrie kon worden aangetoond dat de koncentraties van de in het miljeu aanwezige stof hexachlorobenzene (HCB) signifikant hoger zijn in sera van gedialyseerde en niet-gedialyseerde nierpatienten, dan in die van normale personen (hoofdstuk 6).

De ontwikkelde HPLC-profileringstechniek bleek betrouwbaar in meer dan 3000 analyses. Ze voldoet aan eisen van reproduceerbaarheid, relevantie van geanalyseerde stoffen, geschiktheid voor de analyse van thermolabiele stoffen, en de mogelijkheid to automatiseren. De gedetekteerde komponenten konden op adekwate wijze worden gekwantificeerd. Er werden karakteristieke "uremische profielen" verkregen met deze techniek. Twintig pieken waren steeds aanwezig in de chromatogrammen van uremische sera. Hiervan konden er negen (voorlopig) worden geïdentificeerd met behulp van massaspektrometrische, enzymatische, en andere methoden. De geïdentificeerde stoffen zijn: kreatinine, pseudouridine, urinezuur, *p*-hydroxyhippuurzuur, hippuurzuur, tyrosine, indoxylsulfaat, tryptophan en 3-indolazijnzuur. De profielen van stabiele patienten waren tamelijk konstant over perioden van enkele maanden (hoofdstuk 4).

De serumkoncentraties van de bovengenoemde stoffen werden bepaald, en vergeleken, in patienten behandeld met hemodialyse en met CAPD. Zowel de totale koncentraties als de niet-eiwitgebonden koncentratie werden gemeten. De koncentraties van de tRNA-kataboliet pseudouridine in sera van CAPD-patienten, waren signifikant hoger dan die in sera van hemodialyse(HD)-patienten. Hiervoor werden drie mogelijke verklaringen voorgesteld: 1) een relatief lage permeabiliteit van het peritoneum voor pseudouridine; 2) een toename van de specifieke omzetting van tRNA, bijvoorbeeld tengevolge van een grotere eiwitsynthese; 3) het optreden van celdood bij optredende vormen van niet-klinische peritonitis.

De eiwitbinding van de HPLC-geanalyseerde komponenten in sera van CAPD en hemodialysepatienten werden bepaald. Er bleken geen signifikante verschillen te zijn tussen de twee groepen patienten. Daarom zullen eventuele verschillen in wekelijkse klaringen van eiwitgebonden uremische stoffen in sera van CAPD en HD patienten niet verklaard kunnen worden uit verschillen in eiwitbinding.

De serum koncentratie-opbouw van de geanalyseerde komponenten werd bestudeerd in haar afhankelijkheid van de residuele nierfunktie, bij zowel gedialyseerde als niet-gedialyseerde nierpatienten. Hierbij werden twee typen koncentratie-kreatinineklaring kurven waargenomen. De eerste, waarbij de serumkoncentraties niet noemenswaardig verhoogd waren tot aan een residuele kreatinine-klaring van ongeveer 20 mL/min (type A), en een tweede waar dit punt lag bij 60 mL/min (type B). Type B werd waargenomen bij stoffen die hoofdzakelijk worden uitgescheiden door middel van glomerulaire filtratie (ureum, kreatinine, pseudouridine), terwijl type A waarschijnlijk een mechanisme van tubulaire sekretie weerspiegelt (hippuurzuur, *p*-hydroxyhippuurzuur,

3-indolazijnzuur, indoxylsulfaat). De verdeling van de geanalyseerde stoffen naar type koncentratie-kreatinineklaring kurve, kwam in grote lijnen overeen met een verdeling die was gevonden met behulp van principale komponenten analyse op serumkoncentraties van stoffen in sera van dialysepatienten. De koncentraties van de meeste stoffen met type A kurven, bleken signifikant te korreleren met de residuele kreatinine-klaring in gedialyseerde patienten (met klaring<5 mL/min). Hieruit zou kunnen worden gekonkludeerd dat deze stoffen ook in gedialyseerde patienten nog steeds worden uitgescheiden door middel van sekretie, en dat dit mechanisme, dat Tm-gelimiteerd is, bij de heersende serumkoncentraties van de "organische zuur-achtige" stoffen, nog steeds niet verzadigd is. Dit is in overeenstemming met gegevens uit de literatuur betreffende *p*-aminohippuraat. Daarom zou de residuele nierfunktie bij gedialyseerde nierpatienten wel eens belangrijk kunnen blijken te zijn, niet zozeer vanwege de residuele glomerulaire filtratie van "middle molecules", dan wel voor de effektieve verwijdering van de "organische zuren"; juist vanwege de aangetoonde toxiciteit van laatstgenoemde komponenten (hoofdstuk 5.3).

De samenhang van de koncentraties van twintig HPLC-geanalyseerde uremische stoffen, werd bestudeerd met behulp van principale komponenten analyse (hoofdstuk 5.6). Met behulp van deze methode is het mogelijk om de dimensie van de variabelenruimte te reduceren. Er werden globaal drie groepen stoffen (principale komponenten) onderscheiden, waarvan de relatieve "koncentraties" verschilden tussen de patienten. Dit zou van belang kunnen zijn voor de dialysepraktijk, in het perspektief van adekwate dialyse bij de individuele patient. De onderscheiden groepen konden worden geïnterpreteerd in termen van chemische struktuur, en in termen van hun uitscheidingsmechanisme in resterende nefronen. Ook waren de *in vitro* dialysatorklaringen, mede berekend aan de hand van gemeten eiwitbinding, verschillend.

De dialysator-klaringen, gekorrigeerd voor eventuele eiwitbinding, van een aantal uremische stoffen waren lager dan 90 mL/min (op 0.9 m2 cuprofaan). Dit waren voornamelijk stoffen met een (verwachte) aromatische of indolische struktuur. De dialysatorklaringen van deze stoffen bleken sterker te verbeteren bij toepassing van membranen met een groter oppervlak, dan die van ureum, kreatinine en urinezuur. Het dialysegedrag van onbekende en eiwitgebonden stoffen werd uitgedrukt in een schijnbare molekuulmassa ($M_{r,app}$). Enkele komponenten vertoonden een schijnbare molekuulmassa groter dan 300 in hun ongemodificeerde vorm, terwijl andere dit deden tengevolge van eiwitbinding. Naar de definitie, zouden deze stoffen dus "middle molecules" moeten worden genoemd. Een enkele uitzondering daargelaten, hadden alle ongemodificeerde stoffen een schijnbare molekuulmassa ruim beneden 1000. Zeer lage klaringen (dus hoge schijnbare molekuulmassa's) zijn alleen te verwachten voor de sterk eiwitgebonden stoffen zoals indoxylsulfaat en 3-indolazijnzuur. Eiwitbinding is naar verwachting dus de belangrijkste faktor die leidt tot lage klaringen van de geanalyseerde stoffen (hoofdstuk 5.4).

De stoffen konden ook worden geklassificeerd naar de mate waarin hun serumkoncentraties dalen gedurende hemodialysebehandeling. Eén groep vertoonde voordialyse/na-dialyse koncentratieverhoudingen (dialyseverhouding), vergelijkbaar met die gemeten voor kreatinine. Een andere groep , waarin de "organische zuren" voorkwamen, vertoonde hogere dialyseverhoudingen, waarschijnlijk tengevolge van een multikompartiment-gedrag. Een derde groep bestond uit stoffen met een lage dialyse verhouding als gevolg van eiwitbinding (hoofdstuk 5.5).

De (kor)relaties tussen serumkoncentraties van uremische stoffen en neurofysiologische indices van uremie, werden bestudeerd (hoofdstuk 5.7). Er werd gevonden dat serum LDH, SGOT en serum totaal calcium niveau's signifikant, en positief, korreleren met H-M intervallen (Hoffmann reflex-"latencies"). Dit werd ook gevonden met behulp van principale komponenten analyse. Zwakke, maar signifikante korrelaties werden waargenomen tussen motorische zenuwgeleidingssnelheden en serumkoncentraties van *p*-hydroxyhippuurzuur en de onbekende fluorescerende stof UKF7A. In principale komponenten analyse van de koncentraties van

HPLC-geanalyseerde stoffen en neurofysiologische parameters, bleken deze laatste parameters te associëren met de koncentraties van de "organische zuren". De klinische relevantie van deze waarnemingen kan onderwerp zijn van verdere studie.

Op grond van de multidirectionele, multikomponent, en multivariate analyses beschreven in dit rapport, kan worden gesteld dat het belang van een groep aromatische (en indolische) zuren naar voren komt. Een aantal eigenschappen van deze stoffen worden hieronder nog eens samengevat:

- hun relatieve "groeps"-koncentraties ten opzichte van die van de klassieke markers ureum, kreatinine, en urinezuur, zijn verschillend in verschillende patienten. Dit betreft dus de *struktuur* van de gedialyseerde patientengroep met betrekking tot de koncentraties van akkumulerende stoffen.
- ze hebben (waarschijnlijk) een aromatische of indolische struktuur.
- ze vertonen lage of middelgrote *in vitro* dialysatorklaringen, hoofdzakelijk tengevolge van eiwitbinding.
- de serumkoncentraties blijken signifikant samen te hangen met de mate van residuele nierfunktie.
- ze worden waarschijnlijk uitgescheiden door middel van tubulaire secretie in zowel gedialyseerde als niet-gedialyseerde nierpatienten.
- er zijn aanwijzingen dat ze verdeeld zijn over twee of meerdere kompartimenten.
- de serumkoncentraties van deze stoffen hangen statistisch gezien samen met de neurofysiologische parameters: maximale motorische zenuwgeleidingssnelheid en H-M interval. Hogere koncentraties vallen samen met slechtere neurofysiologische uitslagen.
- de hippuraten vertoonden enigszins lagere serum koncentraties in patienten behandeld met CAPD, in vergelijking met hemodialysepatienten (alhoewel niet statistisch signifikant).

In de literatuur [Grantham et al.,1978] is beschreven dat de "organische zuren" toxisch zijn bij koncentraties die worden aangetroffen bij gedialyseerde nierpatienten. Deze toxiciteit wordt hoofdzakelijk toegeschreven aan een inhibitie van ionen-transport door celmembranen. Deze inhibitie is aangetoond voor tubulus-cellen van de nier, in lever weefsel, in de choroid plexus, in andere weefsels, en mogelijk door de "blood-brain" barriere. Daarom kunnen deze stoffen van meer algemeen belang zijn bij het ontstaan van multi-orgaan ontregelingen die ten grondslag liggen aan *uremie*.

Het lijkt nuttig om een "marker" te introduceren, naast ureum en/of kreatinine, voor de beschrijving van uremische akkumulatie van "organische zuren". Dit zou kunnen bijdragen tot een betere definitie van uremische "toxiciteit" en adekwate dialyse. De keuze van een marker moet worden gebaseerd op kennis van een aantal eigenschappen zoals eiwitbinding, analyseerbaarheid, invloed van dieet op serumkoncentraties, en de wijze van endogene produktie.

Verder indiceren de huidige resultaten dat het nuttig kan zijn om selektieve methoden van bloedzuivering te ontwikkelen, voor de verwijdering van de "organische zuren". Zulke methoden zouden met name kunnen worden toegepast bij patienten met een zeer lage residuele creatinine klaring (b.v. lager dan 2 mL/min), als een aanvulling op de konventionele dialyse van ureum, kreatinine en dergelijke.

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LIST OF SYMBOLS

A	membrane surface area, cm^2
b	gradient steepness parameter
C	concentration, µmol/L
C_0	concentration at time t=0, µmol/L
C_{BO}	dialyzer blood outlet concentration, µmol/L
C_{Bi}	dialyzer blood inlet concentration, µmol/L
C_{DO}	dialyzer dialysate outlet concentration, µmol/L
C_f	"nonbound" concentration of solute, µmol/L
C_{pl}	plasma concentration mL
C_{st}	standardized concentration
C_t	total concentration (bound + free), µmol/L
C_{ur}	creatinine concentration in urine, µmol/L
CV	coefficient of variation, %
$\overline{\Delta C}$	log-mean concentration difference
D	dialysis ratio
D_B, D_M, D_D	diffusion coefficient (in blood, membrane, and dialysate, cm^2/s
d_M	membrane thickness, µm
δ_B,δ_D	apparent blood and dialysate boundary layer thicknesses
ΔS	solvent strength difference
F	flow rate mL/min
G	band compression factor in gradient elution
G	solute generation rate, g/min
g	acceleration of gravity, m/s^2
H_0	overall mass transfer coefficient, cm/min
K	equilibrium constant
K_0	equilibrium constant of unsubstituted compound
K_B	dialyzer clearance, based on blood side concentrations, mL/min
K_D	dialyzer clearance, mL/min
K_d	distribution coefficient in gel chromatography

K_R	residual solute clearance, mL/min
k	solute distribution coefficient between membrane and blood
k	urea dialyzer clearance, mL/min
k'	capacity factor
k_A, k_B	capacity factor in solvent A, B.
λ_i	i^{th} eigenvalue
$\dot{M}$	amount removed per unit time, g/min, μmol/min
M_r	molecular mass
$M_{r,app}$	apparent molecular mass
m/z	ion mass to charge ratio in mass spectrometry
μ	equilibrium distribution coefficient of free solute
N	theoretical plate number
P	significance level
PBL	protein binding level, %
PC	peak capacity
PC	principal component
Q_B	blood flow, mL/min
Q_D	dialysate flow, mL/min
R_0	overall resistance to mass transfer, min/cm
RCC	residual creatinine clearance, mL/min
R_s	resolution in chromatography
r	correlation coefficient
ρ	protein binding coefficient
SD	standard deviation
t	time
t_{coll}	urine collection time, min
t_G	gradient time in gradient elution HPLC
V	body distribution volume, mL (L)
V_0	extraparticle fluid volume (gel chromatography), void volume, mL
V_1	retention volume of first peak, mL
V_e	elution volume in gel chromatography, mL
V_i	total internal fluid volume of gel, mL
V_m	column mobile phase volume, mL
V_{ur}	urine volume, mL
V_z	retention volume of last peak, mL

w_{ij}	weighting coefficient of j^{th} variable on i^{th} principal component
x_j	score on j^{th} variable

LIST OF ABBREVIATIONS

3-IAA	3-indoleacetic acid
BSTFA	bis-trimethylsilyltrifluoroacetamide
CANCORR	SAS canonical correlation procedure
CAPD	continuous ambulatory peritoneal dialysis
CI	chemical ionization
CI-MS	chemical ionization mass spectrometry
CLIN-set	set of routine clinical biochemistries
CNS	central nervous system
CREA	creatinine
cAMP	cyclic adenosine monophosphate
D_2O	deuteriumoxide
DIF	data interchange format
EI	electron impact ionization
EP	electrophoresis
EPA/NIH	Environmental Protection Agency / National Institutes of Health
FAB	fast atom bombardment
FACTOR	SAS procedure factor and principal component analysis
FD	field desorption
FFA	free fatty acids
GC	gas chromatography
GC/MS	gas chromatography / mass spectrometry
GE	gradient elution
H-M interval	Hoffmann reflex latency

HCB	hexachlorobenzene
HD	hemodialysis
Hipp	hippuric acid
HPLC	high performance liquid chromatography
HPLC-set	set of concentrations of HPLC-analyzed compounds
HPLC1	first canonical variate in HPLC-set
hx	hypoxanthine
Ind	indoxylsulfate
ITP	isotachophoresis
LDH	lactate dehydrogenase
MM	middle molecules
MNCV	maximal motor nerve conduction velocity
MS	mass spectrometry
Na-K-ATPase	Na-K-adenosinetriphosphatase
NERVE-set	set of neurophysiological parameters
NERVE1	first canonical variate in NERVE-set
NSA	naphtalene sulfonic acid
organic acid-like	excreted by the "organic acid tubular secretory mechanism"
P	inorganic phosphate
P	probability level, significance
PAH	*p*-aminohippurate
PC#	principal component #
PCA	principal component analysis
PCB	polychlorinated biphenyls
PSI	pseudouridine
PTH	parathyroid hormone (parathormone)
p,p'-DDE	1,1-di(4-chlorophenyl)-2,2-dichloroethene

*p*OHH	*p*-hydroxyhippuric acid
RCC	residual creatinine clearance
REG	SAS regression procedure
RIA	radioimmunoassay
RIVM	Rijks Instituut voor de Volksgezondheid en Milieuhygiene
RO	reverse osmosis
SGOT	serum glutamate oxaloacetate transaminase
SGPT	serum glutamate pyruvate transaminase
SLDA	stepwise linear discriminant analysis
STEPDISC	SAS stepwise discriminant analysis procedure
TCA	trichloroacetic acid
TIC	total ion current
TMS-group	trimethylchlorosilane-group
TRIS	tris(hydroxymethyl)aminomethane
Trp	tryptophan
TSK	Toyo Soda Company
t-,m-,r-RNA	transfer-, messenger-, ribosomal- Ribonucleic acid
UF	ultrafiltration
UK#	unknown UV-absorbing compound
UKF#	unknown fluorescent compound
Uric	uric acid
UV	ultraviolet
WHO	World Health Organization

Authors' Publications

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Vanholder R, Van Landschoot N, De Smet R, Schoots A, Ringoir S: Drug protein binding in chronic renal failure: evaluation of nine drugs, and direct identification of inhibitory ligand. *Kidney Int submitted.*

Schoots AC, De Vries PMJM, Thiemann R, Visser SL, Oe PL. Correlations of biochemical and neurophysiological indices of uremia. *Submitted for publication.*

ACKNOWLEDGEMENTS

This is an integral study, parts of which have been carried out in cooperation with the medical institutions mentioned below. Results from these studies have been, or will be, published together with the participators. Many persons have contributed to the realization of parts of the study described here. They are dialysis patients, nurses, technical staff, and medical staff from the clinics involved, and people from the Laboratory of Instrumental Analysis at the Eindhoven University of Technology.

The following medical institutions were involved:

- Department of Nephrology, University Hospital of Ghent, Belgium. (Prof.Dr. S.M.G. Ringoir, Dr. R. Vanholder).
- Department of Internal Medicine, Hemodialysis Unit, Free University Hospital, Amsterdam, The Netherlands. (Dr. P.L. Oe, and Drs. P.M.J.M. De Vries).
- Department of Clinical Neurophysiology, Free University Hospital, Amsterdam, The Netherlands. (Prof. Dr. S.L. Visser).
- Department of Internal Medicine and Dialysis Unit, St.Joseph-Hospital, Eindhoven, The Netherlands. (Dr. P.G.G. Gerlag).
- Department of Internal Medicine and Dialysis Unit, Catharina-Hospital, Eindhoven, The Netherlands. (Dr. A.W. Mulder).

The indicated persons, attached to these institutions, are gratefully acknowledged for their contributions; especially Prof. Dr. S.M.G. Ringoir, for his enduring enthusiasm. Mrs. Rita De Smet (Ghent University, Nephrological Laboratory) and Mrs. Chris Vonk (St.Josephziekenhuis, Dialysis Unit, Eindhoven), thank you for your wonderful participation.

At the Eindhoven University of Technology many persons were involved in some way, and I want to thank all those who contributed positively to the realization of the studies described in this dissertation. I am indebted to Prof. Dr. Ir. C.A. Cramers and Prof. Dr. Ir. F.M. Everaerts for the opportunity to work on this subject. Thanks to Dr. Frans E.P. Mikkers. We did a great part of the "middle molecule" studies together, and we had fruitful discussions in the early years. Mr. Hans Vlems is gratefully acknowledged for his enthusiastic and skillful efforts to create computing facilities (both hardware and software) for the purpose of this study. I want to thank Mr. Geert-Jaap Scherpenzeel for his

assistance in recording some of the mass spectra. Ir. Gerard A.F.M. Rutten is gratefully acknowledged for his advice and contributions concerning gas chromatographic analyses. Thanks also to Dr. Jan B. Dijkstra from the Computing Centre of this institute, who advised on the application of statistical procedures. Mr. Kees P. Schoots expertly read through a draft version of this manuscript, to check for linguistic errors. He also did the metaphrasing of the Huygens-poem to the English language. Last, but certainly not least, I want to mention the enthusiastic participation of the following graduate students and trainees: Nicoline Beyer, Nicolé Gerrits, Astrid Giesbers, Marcel Gladdines, Monique Greymans, Wilbram Hazejager, Hittjo Homan, Marco Karman, Premradjen Krishnasing, Jack Peeters, Jacqueline Peeters, Paul Pieters, and Robert Thiemann.

To my relatives and friends,

If (it were) Not For You.

CURRICULUM VITAE

The author was born in 1951 in Terneuzen, The Netherlands. After having lived for six years in the city of The Hague, he moved to Tilburg where he followed his secondary education, HBS-B, at the "Willem II" Lyceum. In 1978, Ad Schoots graduated at the Eindhoven University of Technology to get his "ir" degree, at the Faculty of Chemical Engineering. Since then, he is working as a researcher in the Laboratory of Instrumental Analysis of the same Institute. His main field of interest is analytical (bio)chemistry, and he has been engaged for several years in the so-called Nierprojekt ("Kidney project"). In 1986 he was a member of the Organizing Committee of the International Symposium on "Uremic Toxins", held in Ghent.

Even the pain counts.

Erroneous figures are given in the fourthcolumn of Table#5.10 in this dissertation. The correct table is given below.

Table#5.10. K_B, H_0A and R_0 Values of UV-absorbing and Fluorescent Compounds.

COMPOUND	KB (mL/min) Mean (SD) n			HOA (mL/min) Mean (SD) n			RO (min/cm) Mean
	0.9 m^2	1.8 m^2	Δ %	0.9 m^2	1.8 m^2	Δ %	0.9 m^2
Urea	135(8) 16	148(9) 16	10	273(35) 16	338(54) 16	24	33
Creatinine	118(13) 14	141(9) 16	20	213(46) 14	304(45) 16	43	42
Pseudouridine	90(9) 11	109(15) 8	21	133(21) 11	185(43) 8	39	68
Uric acid	116(10) 16	147(12) 16	27	204(34) 16	336(64) 16	65	44
UK4	92(7) 15	108(14) 16	17	138(16) 15	180(35) 16	30	65
UK5	104(11) 14	118(21) 13	14	169(30) 14	219(67) 13	30	53
p-OH-hipp.acid	83(7) 17	111(6) 16	34	119(15) 17	189(17) 16	59	76
Hippuric acid	90(7) 16	118(6) 14	31	134(15) 16	207(18) 14	55	67
UKF1	70(9) 9	n.a.	-	93(16) 9	n.a.	-	97
UKF3	64(8) 16	88(5) 16	38	83(13) 16	129(10) 16	55	108
UKF4	56(9) 10	73(8) 13	30	70(14) 10	99(15) 13	41	129
UKF5[a]	34(2) 6	n.a.	-		n.a.	-	
Indoxylsulfate	100(5) 16	128(6) 14	28	157(13) 16	243(24) 14	55	57
Tryptophan	n.a.	127(14) 6	-	n.a.	243(50) 6	-	74
UKF6	100(5) 10	n.a.	-	158(13) 10	n.a.	-	57
UKF7A	90(8) 15	117(13) 10	30	134(18) 15	208(41) 10	55	67
UKF7[a]	80(22) 6	n.a.	-		n.a.	-	
UKF8	73(4) 13	108(11) 13	48	98(8) 13	182(37) 13	86	92
3-Ind.ac.acid	89(12) 14	108(14) 15	21	133(27) 14	180(39) 15	35	68

[a] with QB=200 mL/min and QD=600 mL/min

n.a. = not available

STELLINGEN

1. De literatuur geeft geen eenduidig antwoord op de vraag of de serum eiwitbinding van tryptophan toe- dan wel afneemt tijdens hemodialysebehandeling.

   Farrell PC, Grib NL, Fry DL, Popovich RP, Broviac JW, Babb AL.
   Trans Am Soc Artif Int Organs 1972;18:268-276.

   Saito A, Niwa T, Maeda K, Kobayashi K, Yamamoto Y, Ohta K.
   Am J Clin Nutr 1980;33:1402-6.

2. De vergelijkingen voor de piekkapaciteit in isokratische en gradient elutie vloeistofchromatografie, gegeven door Giddings en Snyder, zijn onjuist.

   (*1*) Snyder LR. Gradient elution. In: Horvath Cs (Ed), *High Performance Liquid Chromatography, Advances and Perspectives*, vol.1. New York, Academic Press;1980.

   (*2*) Giddings JC. *Anal Chem* 1967;39:1027-1028.

3. De inhoud van het kranteartikel met als kop: "TH Twente dicht bij nier-dialyse van een uurtje" is onverantwoord en duidt op wetenschapsvoorlichting van de slechte soort.

   Trouw 11 jan 1985.

4. In de door Horváth et al. gegeven historische beschouwing van het displacement principe zou een verwijzing naar de ontwikkeling van kapillaire displacement electroforese niet hebben misstaan.

   (*1*) Horváth Cs, Nahum A, Frenz J. *J Chromatogr* 1981;218:365-393.

   (*2*) Martin AJP, Everaerts FM. *Proc Roy Soc Lond* A 1970;316:493-514.

5. Het beschikbaar komen van toegankelijke personal computer software voor geavanceerde statistiek zal de werkgelegenheid van statistici bevorderen.

6. De term "digital chromatography" is misleidend.

7. " ..just letting the GCs work .." (*1*) is nodig om " .. modelling done on the Yeti" (*2*), te voorkomen.

(*1*) Gotch FA.
Int Symp "Uremic Toxins". Ghent;1986.

(2) Farrell PC. Discussion. In: Brunner H, Mann H. What remains of the "middle molecule" hypothesis today?
Contr Nephrol Basel, Karger, 1985;44:14-39.

8. Simulatie met behulp van de personal computer moet niet zó ver gaan dat met het zitten voor het beeldscherm zinvolle aktiviteit wordt gesimuleerd.

9. Het toepassen van ethanol als "solutizer" ter bevordering van de dialyse efficiëntie van eiwitgebonden stoffen, zoals gesuggereerd door Dorson, zal leiden tot een nieuwe vorm van dialysekater.

Dorson WJ, Pizziconi VB, Sizto CN, Radnoti RP, Zarembinski CJ, Aniuk LM. *Trans Am Soc Artif Int Organs* 1980;26:116-119.

A.C. Schoots, Eindhoven, 26 april 1988.