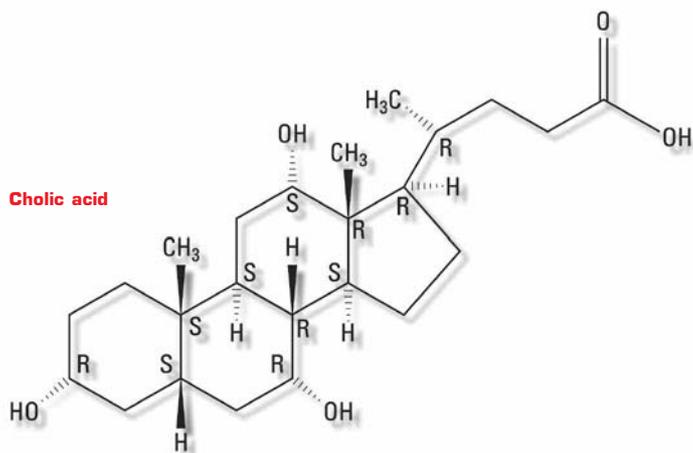


Simultaneous determination of tryptophan in pretreated human blood



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In the treatment of patients with kidney and liver problems, extracorporeal blood cleaning processes are very important forms of therapy in modern medicine. The currently applied practice of dialysis with three 4 – 6 hour treatment sessions per week is a pragmatic compromise between the ability to cope with this form of treatment and the well-being of the patient and is based on empirical data that can be transferred to nearly all dialysis patients.

The duration and frequency of a dialysis treatment must be applied in such a way that a maximum of enriched pathogenic metabolic products or toxins are removed from the patient's circulatory system while, at the same time, taking care that important blood constituents are not unnecessarily enriched. Special emphasis, in this

case, is on protein-bound metabolic products and toxins, the so-called uremic toxins. The analytes presently under investigation – tryptophan, phenol, p-cresol and cholic acid – are used as representative markers for other important medical indicators.

When important metabolic organs such as the liver and/or kidneys are not functioning properly, a multitude of metabolic waste

products will accumulate in the body. As long as these waste products are low molecular mass compounds, soluble in water and do not exhibit high degrees of protein binding, they can be removed via dialysis treatment. Some of these metabolic products are, however, lipophilic or are bound to proteins and therefore cannot be eliminated via the usual dialysis procedures. At the same time, these types of compounds are not usually co-detected in the clinical-chemical routine laboratory, so that clinicians will have no experimental process parameters at their disposal.

Phenol and p-cresol are formed as metabolic end products of the degradation of the amino acids tyrosine and phenylalanine by intestinal bacteria. When liver or kidney failure occurs, phenol and p-cresol accumulate in the blood. Although the patho-physiological significance is still largely unclear, p-cresol seems to negatively impact the intestinal endothelial

barrier function, reduce endothelial cell proliferation as well as impair the response of the endothelium to pro-inflammatory stimuli.

Interestingly enough, a prospective clinical study recently showed a significant correlation between the serum level of free p-cresol and mortality probability of patients due to chronic haemodialysis therapy. This has further increased the interest in the possibility of p-cresol monitoring.

Liver failure will, among others, lead to a strong imbalance in the amino acid metabolism. In particular, the essential amino acid tryptophan has already been correlated for over 40 years to the pathogenesis of liver coma, wherein the free tryptophan level is noticeably elevated in the serum of these patients. Changes in the tryptophan concentration in human blood or different endogenous sera have been observed

Individual steps in the development of an HPLC/DAD/MS method for the identification, separation and quantification of cholic acid, phenol, p-cresol and tryptophan in human blood

Step I: Development of an isocratic HPLC/DAD/MS method for the identification and separation of the analytes tryptophan, phenol and p-cresol.	Step II: Development of an isocratic HPLC/DAD/MS method for the identification of the analyte cholic acid
Step III: Combination and conversion of both isocratic HPLC/DAD/MS methods into a gradient HPLC/DAD/MS method for the identification and separation of the analytes tryptophan, phenol, p-cresol and cholic acid	
Step IV: Optimization of sample pre-treatment. Complete removal of cellular and further solid sample constituents, precipitation and removal of proteins, precipitation and removal of cholesterol from the blood sample and quantitative extraction of the analytes tryptophan, phenol, p-cresol and cholic acid in the diluted blood plasma phase.	
Step V: Calibration of the HPLC/DAD/MS analysis method for the quantification of the analytes tryptophan, phenol, p-cresol and cholic acid	
Standard-calibration based on a statistically sufficient number of PBS buffer solutions containing various amounts of an analyte-sample standard	Standard-addition calibration based on a statistically sufficient number of diluted human blood samples containing various amounts of an analyte-sample standard
Step VI: Statistical comparison and testing of the calibration procedure via an F-test and T-test. Evaluation of both methods for the quantification of the analytes.	

tryptophan, phenol, p-cresol and cholic

HPLC/DAD/MS method



Left: Dipl.-Ing. Jürgen Müller M.Sc., right: Prof. Dr. Hermann Büttner

in correlation with metabolic diseases such as Parkinson or Alzheimer.

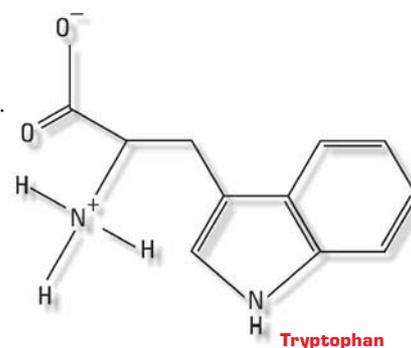
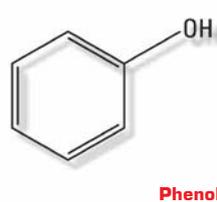
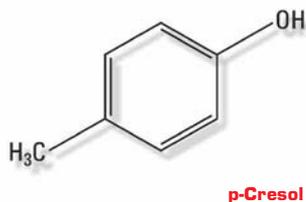
Bile acids, such as cholic acids whose serum concentrations are strongly elevated due to failing excretory functions, are other possible markers for liver insufficiency. The blood level of bile acids, as indicated by cholic acid as being the most frequent representative of these steroid derivatives, provides information on possible interferences in the cholesterol metabolism.

A fast, simultaneous and simple analytical determination of these lipophilic and protein-bound markers will therefore provide a strong indication of the efficiency of haemodialysis, in turn enabling an optimum treatment process that is customized to each patient.

Method

An HPLC/DAD/MS method has been developed for the simultaneous determination of tryptophan, phenol, p-cresol (4-methylphenol) and cholic acid in human

blood. These four analytes represent a selection of hydrophobic albumin-bonded uremic metabo-



lites in human blood. Blood constitutes approximately 8 % of the body weight; for an adult weighing 80 kg, this is approximately 6.4 liters.

Fresh blood appears to be quite fluid although it consists of more than 40 % of solid components such as blood cells. The fluid part is called plasma, which contains numerous proteins necessary for blood clotting and functioning of the immune system. The most abundant plasma protein is albumin, which has a number of different transport functions and can bind to a multitude of compounds, among others the four analytes tryptophan, phenol,

p-cresol and cholic acid, under investigation in the present study.

Due to the complexity of the blood matrix, a clear strategy was required in order to develop a robust HPLC method with high analytical reproducibility and high recovery. Method development was carried out in a series of individual steps. Breaking down the method development process was aimed at achieving a clear and thereby straightforward procedure. The method was, in the first place, aimed at the simultaneous determination of tryptophan, phenol, p-cresol and cholic acid in a human blood matrix.

The analysis of a variety of blood sample types was, at this point, not yet considered. The available control blood sample (500 mL) originated from a healthy person.

In the first sample pretreatment step, the blood solids were removed. Simultaneously, during this step, all soluble proteins should, if possible, be denatured and precipitated. The most abundant soluble protein is albumin, which in the blood acts as a carrier for a large part of the respective analytes. The procedure must therefore ensure that all analytes that are adsorbed to particulate matter and to all soluble proteins are completely desorbed while quantitatively remaining in the plasma solution. In the subsequent step the cholesterol that has remained in solution is removed. A stock solution containing all four compounds is

being used for the preparation of the measuring solutions for the standard-addition calibration series.

In the suggested analytical method, the analytes are separated using a semi-micro gradient HPLC (Shimadzu Liquid Chromatograph LC-10AD; HPLC column: Multispher 120 RP 18 HP-3 μm , mobile phase A: methanol, mobile phase B: 1 N formic acid). Detection is carried out in series, first via UV-VIS spectrometry (Shimadzu DAD SPD-M10A diode array detector) and subsequently via mass spectrometry (Shimadzu LCMS-2010

single-quadrupole mass spectrometer). For ionization APCI (atmospheric pressure chemical ionization) as well as ESI (electrospray ionization) are well suited. The combination of both independent detection modes – diode array and mass spectrometry – results in a high degree of analytical control and reliability especially with respect to the complex sample matrix. The duration of a single measurement takes almost thirty minutes. ▶

Results

For the quantitative determination of the analytes tryptophan, phenol, p-cresol and cholic acid in blood, two methods were used – the standard-calibration and the standard-addition method. Calibration data must, in principle, be free from outliers. The resulting values of the respective corresponding sample series were compared with each other and statistically evaluated using the F- and T-tests. In this way, a distinct agreement between each of the independent sample series for the analytes tryptophan, phenol p-cresol and cholic acid could be observed. According to the F- and T-tests, the four measuring series of the standard calibration could be pooled. When comparing the standard-calibration method in PBS buffer with the standard-addition method in the pretreated blood, a slight matrix-dependent deviation was detected.

The detection limits for tryptophan, using the standard-calibration method were 0.049 mg/L in the matrix-adapted calibration standard PBS-buffer and 0.145 mg/L in human blood.

The detection limits for phenol in the PBS-buffer were 0.097 mg/L and 0.068 mg/L in human blood. The detection limits for p-cresol

HPLC/DAD/MS method for simultaneous analysis of tryptophan, phenol, p-cresol and cholic acid in pretreated human blood				
Analyte	Reference (PBS-buffer) Detection limit analyte (experimental) in mg/L	Pretreated blood sample Detection limit analyte (experimental) in mg/L	Normal concentration Analyte in blood (empirical) in mg/L	Pretreated blood sample Concentration analyte (experimental) in mg/L
Tryptophan	0.049	0.145	13.7 ± 4.5	9.25
Phenol	0.097	0.068	0.6 ± 0.2	< 0.069
p-Cresol	0.027	0.068	0.6 ± 0.2	1.23
Cholic acid	0.063	0.044	< 0.068	0.679

were 0.027 mg/L in the reference standard and 0.068 mg/L in human blood.

The detection limits for cholic acid were 0.063 mg/L in the reference and 0.044 mg/L in human blood.

The detection limits are approximately one order of magnitude lower than the measured concentrations of the analytes in blood samples of a healthy person, and the literature values for average normal concentrations of these compounds in blood.

Discussion

The developed HPLC/DAD/MS method enables the reliable simultaneous determination of tryptophan, phenol, p-cresol and cholic acid in pretreated human blood.

The method is convincing through its simplicity, as derivatization of the analytes is not necessary. All four analytes can be

unequivocally determined in their native form, which saves time and money. In combination with an efficient automation of the sample pretreatment, the proposed method is one step towards the development of a "semi-online" HPLC determination of selected toxins and metabolic products in blood during a patient dialysis treatment.

In this application, tryptophan, phenol, p-cresol and cholic acid are examples for other important medical markers whose fast and simultaneous determination in blood can enable optimal patient-customized checking and control of dialysis treatment in order to reduce the physical stress to a minimum. This can restore a certain amount of quality of life to dialysis patients.

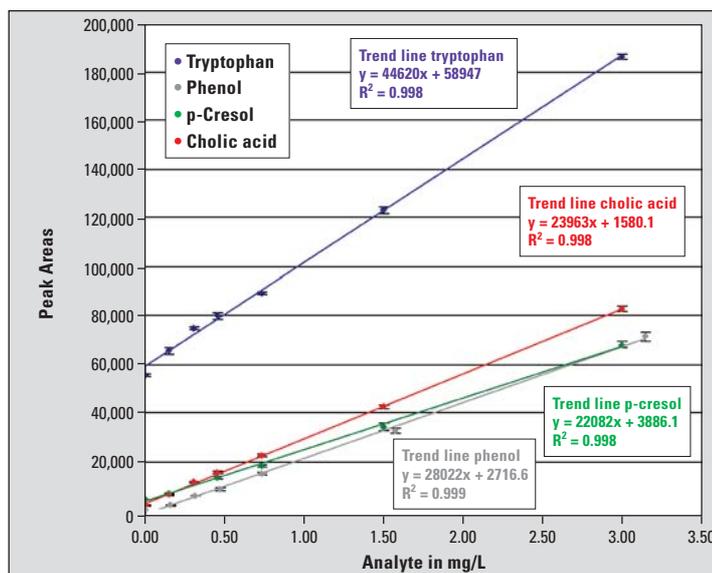
In conclusion, it should be noted that the development of a technical implementation of the analysis during patient-dialysis should be

coupled to clearly defined and reproducible conditions: an automated, efficient and hygienic sample pretreatment technique combined with a high-performance HPLC/DAD/MS system that can handle the entire analysis sequence – from blood sampling, subsequent removal of cellular and other solid blood constituents, precipitation and removal of most proteins, precipitation and removal of cholesterol, the quantitative extraction of the analytes in the diluted blood plasma phase up to the dilution and injection into the HPLC/DAD/MS system. Such a technical implementation is proposed using Shimadzu's Bio-Sample Analysis System Co-Sense® BA.

We will gladly send you further information. Please note the appropriate number on your reply card. Info 312



Co-Sense BA



Tryptophan, phenol, p-cresol and cholic acid in pretreated human blood (standard-addition method)