Rapid analysis of porphyrins at low ng/l and μg/l levels in human urine by a gradient liquid chromatography method using octadecylsilica monolithic columns[☆]

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Abstract

Rapid gradient RP-HPLC method with fluorimetric detection for trace analysis of diagnostically significant porphyrins in human urine was developed for clinical and diagnostic purposes. Results show that optimized high-pressure gradient elution and monolithic column Chromolith SpeedRod RP18e enabled separation of seven urine porphyrins including baseline separation of I and III positional isomers of uro- and coproporphyrins within 3.2 min. Problems associated with high metal cation complexing ability of the analytes and common stainless steel based instrumentation were substantially reduced by use of 0.1 mol/l ammonium citrate buffer (pH 5.47) and methanol as a mobile phase components. Good reproducibilities of retention times (within ±0.36% RSD) and peak areas (from ±0.6 to ±2.5% RSD) at 5–20 μg/l level of the analytes were achieved. Determined LOQ (10 × S/N) values of diagnostically important porphyrins using fluorimetric detection (ex.405 nm/em.620 nm) were 82 pmol/l (65 ng/l, 1.30 pg/injection) for uroporphyrin I, 44 pmol/l (33 ng/l, 0.66 pg/injection) for uroporphyrin III, 50 pmol/l (40 ng/l, 0.80 pg/injection) for coproporphyrin I and 47 pmol/l (39 ng/l, 0.78 pg/injection) for coproporphyrin III. Attained LOQ concentration level is approximately 20–120 times lower than concentration of porphyrins in a urine of healthy person. Calculated LOD's (3 × S/N) were at a low ng/l levels, what enabled quantification of carry-over effect to be from 2.0% to 0.2% in each of three consecutive blank runs and from 2.5% to 7% in total after injection of mixed standard of porphyrins with 5–20 μg/l concentrations. Recovery of porphyrins at low μg/l concentration levels was from 93% to 97.5%. Devised method increases productivity of clinical laboratory from 2 to 10 times in dependence of duration of currently used method.
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Keywords: Porphyrins; Ultra-trace (ng/l; μg/l) analysis; Rapid RP-HPLC; Monolithic column; Gradient; Urine sample

1. Introduction

Porphyrins are cyclic tetrapyrole intermediates of the heme biosynthetic pathway [1]. Individual porphyrins differ by the side chain substituents and their isomers differ by the side chain substituents arrangement [2]. Urinary porphyrin excretion and pattern of distribution is the key tool for the diagnosis of the heme biosynthetic pathway disturbances [3].

Determination of individual porphyrins concentration beside the determination of porphobilinogen and 5-aminolevulinic acid in human urine is therefore cardinal and decisive information for porphyria disease diagnosis assignment [4,5].

Urinary porphyrins are analysed by numerous reliable analytical methods. This type of analysis we can define as (ultra)trace analysis of chelating constituents in a complex biological matrix, because normal level of porphyrins in human urine is up to 50 nmol/l (below 1 μg/l) for uroporphyrins and up to 250 nmol/l (below 5 μg/l) for coproporphyrins. Physico-chemical properties of urinary porphyrins and diagnostic value of uro- and coproporphyrins I and III positional isomers separation force us directly to use gradient elution involving non-productive re-equilibration

step. However, from the point-of-view of productivity of clinical laboratory rapid analysis is essential.

Methods of liquid chromatography (LC) have found widespread applicability both in research and clinical analysis of porphyrins in body fluids and tissues as is stressed in the review chapter of Lim in monography [6]. Among the chromatographic methods those based on hydrophobic effect (RP-HPLC) play dominant role. Gradient RP-HPLC on C18 columns with fluorimetric detection is the method of choice [7,10,12,14–16,19–28]. For comparison, only a few applications that use gradient RP-HPLC (C18 column) with UV–vis spectrophotometric detection [11,14,17,18,29], or electrochemical detection [41], or isocratic mode [13] were described. Phenyl substituted silica was used in works [8,9], use of silicagel in normal phase HPLC mode was published in article [50], TLC with automatic multiple development was applied in [51] and ion-exchange HPLC in works [52–54].

With respect to above mentioned productivity of RP-HPLC methods used up-to-now for determination of porphyrins (usually in a 24 h urine) take a time between 5 and 40 min. Characteristic duration of analysis as is evident from published data and chromatograms ranged from 25 to 40 min [19,22,25,27,28,30,35,38], from 15 to 25 min [7,8,15,17,20,23,29,33], from 5 to 15 min [26,32,34] and 5 min [21]. This figure-of-merit of analysis is probably influenced mainly by complexity of the porphyrin mixture, the matrix character, geometry of used columns, column permeability, sorbent particle diameter and surface chemistry.

However, at least some of these restrictions for effective increase of productivity could be solve by a commercially available monolithic columns. Inorganic monolithic sorbents are made of silica gel [30–34], they have high porosity (80–90%) and well defined bimodal pore system [35–37] of large flow-through pores (1–3 μm diameter) and small (10–20 nm diameter) pores where separation mainly takes place, resulting both in sufficient efficiency achieved even at high flow-rates of mobile phase and small pressure drop along the column. As was already shown existing HPLC methods are for clinical analysis relatively time consuming.

Availability and several years experience with monolithic silica based sorbent Chromolith (Merck) led us to development of rapid and effective RP-HPLC routine method for the analysis of urinary porphyrins. To the best knowledge of the authors no method of rapid analysis of porphyrins was published in a sources available to authors and there is no known evidence of its use.

This work is intended

- to study conditions for the application of rapid gradient HPLC (maximum 4 min per analysis cycle) using Chromolith columns for analysis of porphyrins in human urine samples;
- to propose rapid RP-HPLC method for routine analysis of porphyrins in human urine for diagnostic purposes and
- to validate the method including study of potential interferences and other adverse effects.

2. Experimental

2.1. Instrumentation

Study of the retention behaviour and evaluation of chromatographic characteristics and detectability of the porphyrins was carried out by two HPLC systems representing common instrumentation, which can be used in clinical laboratories.

Modular high-pressure gradient liquid chromatograph was composed of two high-pressure pumps type 2150, gradient controller type 2152 (both components made by Pharmacia LKB, Bromma, Sweden), auto-sampler type Gilson 232 BIO (Gilson, Villiers-le-Bel, France) provided by sampling valve Rheodyne 2010 (Rheodyne, Palo Alto, USA) with fixed volume (20 μl) sample loop. Signal of spectrofluorimetric detector type FP 920 (Jasco, Tokyo, Japan) provided by a photomultiplier sensitised for red spectral region (600 nm) was digitised by a 32-bit resolution integration sigma delta A/D converter and processed by the chromatographic data system for Windows CSW 32, v.1.4.8 (DataApex, Prague, Czech Republic). The mobile phases were degassed by a helium sparging degasser. Measured dwell volume of the chromatographic system was 100 μl. Separations were carried out on a Chromolith SpeedRod RP 18e column 55 mm × 4.6 mm. Injected sample volume was 20 μl (by a sample loop).

Low-pressure gradient HPLC system LaChrom (Merck-Hitachi, Darmstadt, Germany) consisted of pump L-7100 provided by quarternary low-pressure gradient, auto-sampler L-7200, column oven L-7300, diode-array detector L-7450A, fluorescence detector L-7480, interface D-7000, PC data station with software HSM ver.3.1 and on-line four channel solvent degasser L-7612. Measured dwell volume of the system excluding column was 1250 μl and should be considered when gradient mixing profile and chromatogram appearance is to be compared.

Digital pH meter Radiometer, type PHM 84 (Radiometer, Copenhagen, Denmark) provided by combined glass/AgCl (Hamilton Company, Reno, Nevada, USA) electrode was used for mobile phase and sample pH measurement. All used substances were weighed with the aid of digital analytical balances Mettler PE 360 (Mettler Toledo, Houston, Texas, USA). For measurement of spectral characteristics of porphyrins UV spectrophotometer Merck-Hitachi type 2001 (Merck AG, Darmstadt, Germany) was used.

2.2. Chemicals and materials

Chemicals used for buffered mobile phases preparation were of analytical grade. Citric acid, ammonia (25% m/m aqueous solution) and sodium hydroxide and methanol LiChrosolv of gradient HPLC purity were purchased from Merck AG (Darmstadt, Germany). Acetic acid was from Lachema (Brno, Czech Republic).

Water was purified to $18\,M\Omega$ using a Millipore (Bedford, MA, USA) Mili Q system. Riboflavine pro analysi was purchased from Léčiva (Prague, Czech Republic).

Porphyrin standards—uroporphyrin I octamethylester; uroporphyrin III octamethylester; methylesters of 7-, 6-, 5-carboxylated porphyrins I; tetramethylesters of coproporphyrins I and III; were form Porphyrin Products (Logan, UT, USA).

All porphyrins were alternatively separated on a Chromolith Performance RP18e column $100\,mm \times 4\,mm$, or Chromolith ROD RP18e column $50\,mm \times 4\,mm$ filled by a monolithic octadecylsilica (Merck, Darmstadt, Germany). The monolithic column is devised mainly for fast reversed-phase analysis [38]. The flow-through pore size is $2\,\mu m$ and separation takes place mainly in 13 nm mesopores. Pore volume is 1 ml/g at total porosity higher than 80%, typically 81.3%. High purity silica (metals content below $1\,\mu g/g$) has specific surface area $300\,m^2/g$ covered by octadecyl functional groups with surface coverage $3.6\,mmol/m^2$ and carbon content 18% (m/m). Residual silanol groups are endcapped and pH stability is declared to be within the range 2.0–7.5. From practical point of view we should take into the consideration also the other restrictions of the column, as is choice of mobile phase modifiers where we should avoid use of tetrahydrofuran, dimethylsulfoxide, dichloromethane, strong acids as formic acid, HCl, HNO_3, H_2SO_4 and alkalies. Due to PEEK material of the column maximum pressure is restricted to 20 MPa, however this value does not pose any restriction to use under normal working conditions, because actual pressure drop is hardly approaching this value even at flowrates as high as 10 ml/min. Column temperature is restricted to $45\,°C$ for different dilatation of the PEEK column and monolithic silica rod [40].

Results obtained on short monolithic column were compared with two other particulate short columns—column EPS C18 ($53\,mm \times 7\,mm$, $3\,\mu m$) from Alltech Associates (Deerfield, Illinois, USA) and column Purospher C18 ($55\,mm \times 4.6\,mm$, $5\,\mu m$) from Merck KGaA (Darmstadt, Germany).

2.3. Methods

Separation was carried out using a Chromolith Performance RP18e column $100\,mm \times 4\,mm$ filled by monolithic octadecylsilica, or alternatively Chromolith SpeedRod RP18e column ($50\,mm \times 4\,mm$), guarded by a Chromolith $4\,mm \times 4\,mm$ precolumn. Dead volume directly measured or calculated from the manufacturers data [38] of the Performance brand column was 1.06 ml, or 0.55 ml for SpeedRod column and is equal to the retention volume of the first eluted peak of urine sample measured by DAD. Flow-rate was adjusted to 3.00 ml/min.

Separation conditions for optimised gradient elution of porphyrins were as follows. Mobile phase A composition: aqueous ammonium acetate (2 mol/l) or ammonium citrate buffer (pH 5.47, 0.10 mol/l) containing 1% (v/v) of methanol (MeOH). Mobile phase B was 100% MeOH. Gradient program was set from 0.0 to 1.0 min isocratic 5% B in A, from 1.1 to 3.0 min linear increase from 5% B in A to 65% B in A, from 3.1 to 4.0 min linear decrease from 65% B in A to 5% B in A. Column oven temperature was maintained at $25.0 \pm 0.2\,°C$. Injection volume $20\,\mu l$ was injected by the auto-sampler.

Preparation of individual stock solutions of porphyrin standards was done by a dissolution of weighed solid standards of their methylesters in aqueous 6 mol/l HCl. After 24 h hydrolysis at laboratory temperature in the dark, UV absorbtion spectra were recorded on a Hitachi U 2001 spectrophotometer. Concentration of individual uroporphyrins and coproporphyrins in a mixed standard solutions was determined by calculatation from the known molar absorption coefficients equal to the value $5.41 \times 10^5\,l/(mol\,cm)$ for uroporphyrins and $4.89 \times 10^5\,l/(mol\,cm)$ for coproporphyrins [2,39], both valid at 405 nm. Solutions of porphyrins at low concentration levels were prepared daily fresh by a proper dilution either as standard addition to urine samples or addition to the mobile phase. Exact concentrations of individual porphyrins were for uroporphyrin I within the range 2.4–2.5 $\mu mol/l$, for uroporphyrin III 2.7–2.8 $\mu mol/l$, for coproporphyrin I 3.2–3.3 $\mu mol/l$ and for coproporphyrin III 3.6–3.7 $\mu mol/l$. Working standards were prepared daily fresh by an appropriate (at least 25-fold) dilution of the stock standard solutions in a solution of 0.1 mol/l HCl.

Structures of analysed porphyrins are shown at Fig. 1 and their selected properties are given in Table 1. Uroporphyrin I changes by decarboxylation at $180\,°C$ and by deoxidation in 1 M HCl solution to coproporphyrine I. Usually is prepared for use by hydrolysis of its methylesther. It is found together with seven-carboxylated porphyrin at increased

Table 1
Selected properties of diagnostically important porphyrins and their esters, respectively [2]

Porphyrin	Molecular formula	Molar mass (g/mol)	Melting temperature (°C)	Solubility
Uroporphyrin I (uro I)	$C_{40}H_{38}N_4O_{16}$	830.77	291–292 (OME)	OME partially soluble in DEE
Uroporphyrin III (uro III)	$C_{40}H_{38}N_4O_{16}$	830.77	267–269 (OME)	OME soluble in CHO, non-soluble in DEE
Coproporphyrin I (copro I)	$C_{36}H_{38}N_4O_8$	654.73	251–252 (OME)	OME soluble in CHO, non-soluble in DEE
Coproporphyrin III (copro III)	$C_{36}H_{38}N_4O_8$	654.73	151–154, 165–168, 176–179 (PTME), 216–219 (CuCPIII)	n.a.

Abbreviations: OME is octamethyl ester of given porphyrin, TME is tetramethyl ester, PTME is polymorphic tetramethyl ether, CuCPIII is copper complex of coproporphyrin III, CHO is cyclohexanone, DEE is diethylether, n.a. means not available.

Uroporphyrin I

Uroporphyrin III

A = CH$_2$ COOH
R = CH$_2$CH$_2$ COOH

A = CH$_2$ COOH
R = CH$_2$CH$_2$ COOH

Coproporphyrin I

Coproporphyrin III

R = CH$_2$–CH$_2$ –COOH
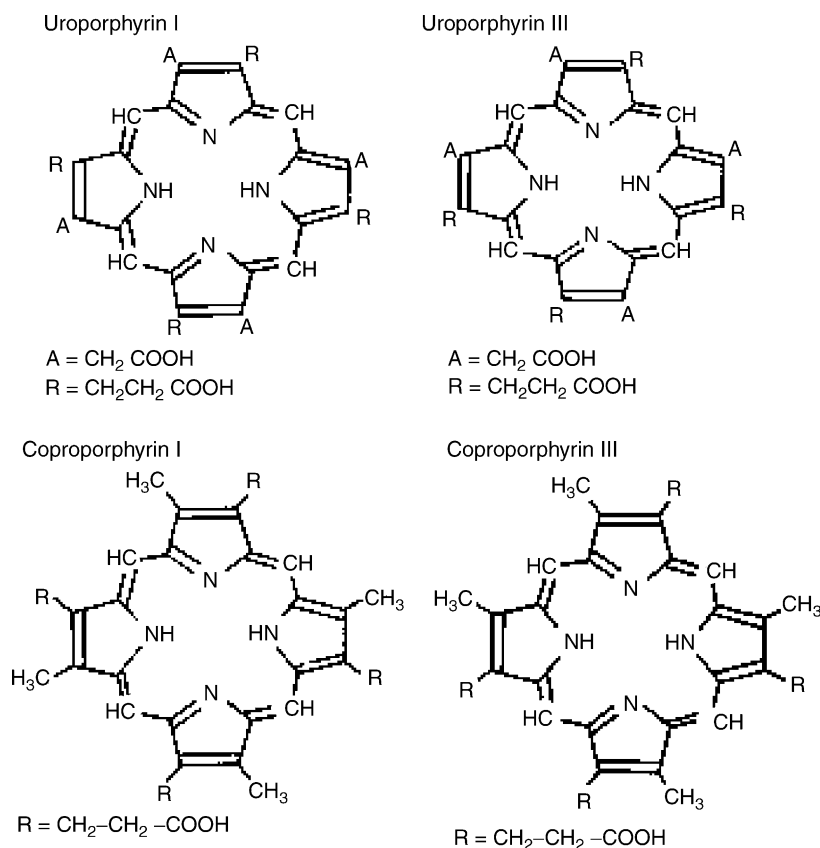
R = CH$_2$–CH$_2$ –COOH

Fig. 1. Chemical structures of diagnostically important uroporphyrins and coproporphyrins.

concentration level and with higher frequency in urine of patients with both the acute intermittent and chronic hepatal porphyria accompanied by a skin affection. Uroporphyrin III changes by decarboxylation at 180 °C and by deoxidation in 1 M HCl solution to coproporphyrine III. Uroporphyrin III is obtained enzymatically from 5-aminolevulinic acid or porphyrinobilinogen. Coproporphyrin I changes by decarboxylation at 180 °C and by deoxidation in 1 M HCl solution to coproporphyrine III. Coproporphyrin I is obtained by isolation from urine and feces, respectively, and is present in higher extent in a urine of humans having liver function faults as a consequence of inborn hyperbilirubinemia. Coproporphyrin III is obtained by isolation from urine and feces, respectively, and is present in higher extent in urine of humans having hereditary coproporphyria as-well-as at chronic poisoning by a lead. All these porphyrins are soluble in an aqueous solution of HCl, NaOH, KOH, Na$_2$CO$_3$, NH$_4$OH.

3. Results and discussion

3.1. Optimisation of separation and detection conditions

Urine porphyrins have at four pyrrole rings attached from 2 to 8 carboxylic groups altogether. Each of these porphyrins creates four positional isomers at C and D pyrrole ring in

dependence of position of acetate and propionate functional groups, respectively, for uroporphyrins and propionate and methyl, respectively, for coproporphyrins. Recently isomers I and III of uroporphyrin and coproporphyrin are the most interesting for quantitation in the human urine for the purpose of porphyrias diagnosis. Porphyrins belong to amphoteric substances with isoelectric point of at pH from 3.2 to 4.0 and its value is the characteristic of given porphyrin. Aqueous solubility of porphyrins around pI is low, at higher concentration they even precipitate. Arrangement of the functional groups is responsible also for their complex forming and chelate forming ability, respectively, with numerous metal ions e.g., Fe(II), Mg(II), Zn(II), Cu(II), Co(II) and others in a 1:1 molar ratio. Complexed forms dominate in nature (metalloporphyrin with bound Fe(II) is heme; chlorophyll is pentacoordinated complex of Mg(II) with one more valency for the other ligand).

The most sensitive detection of urine porphyrins is achieved by their fluorescence measurement, intensity of which: increases with the excitation source intensity; generally drops with decrease of pH and the lowest is at pH equal to pI; increases with lowering of temperature. Fluorescence can change according to actual matrix constituents and their concentration—e.g. presence of Mg(II), Zn(II), Cd(II), Sn(II), K(I) metal ions causes formation of fluorescing complexes, whereas their complexes with Fe(II) and Cu(II) have

lower fluorescence than free porphyrins. They are intensively coloured and due to their structure and presence of 18 delocalised π electrons out of 22 π electrons in total they absorb light in the Soret region around 400 nm [55], what makes the other rationale for their photometric detection as was already pointed-out.

Fluorimetric detection was used to obtain detailed information on chromatographic behaviour of the porphyrins in urine samples at nmol/l concentration levels. It is evident that within given set of actual conditions potential detectability and separability of porphyrins is tied each other. The retention behaviour was studied in dependence of pH value of the mobile phase in the range 3.90–5.60. As is evident from Fig. 2, within pH range from pH 4.25 to pH 5.52 change of pH has high potential to influence retention very effectively. At pH values lower than 5.00 retention of coproporphyrins under isocratic conditions is too high and content of MeOH in mobile phase must be increased above 50% (v/v) to achieve sufficiently fast separation. At the same time pH lowering causes drop of resolution of positional isomers I and III of both uro- and coproporphyrins. However, coproporphyrins are influenced in much less extent. Below pH 4.00 the isomers are not resolved and also detectability is much worth. The optimal pH 5.47 was chosen for the best separation and detection within the studied set of conditions and fixed for rapid gradient and its shape optimization. Under these conditions excitation wavelength 405 nm and emission wavelength 620 nm gave satisfactory sensitivity of detection as is discussed later. Due to a relatively high flow-rate and instant and transient character of the mobile phase change (within 1–2 min) the gradient shape is instrumentally dependent as resulted from comparison of both high-pressure and low-pressure gradient HPLC instruments.
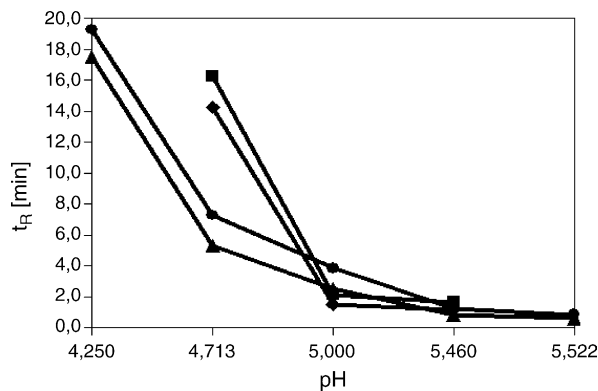

Fig. 2. Dependence of retention time of diagnostically important porphyrin isomers on pH value of mobile phases MeOH:water. pH data points represent measurement at pH values 4.250, 4.710, 5.000, 5.460 and 5.722, respectively. Uncertainty of the pH measurement was ±0.005 pH unit. Uroporphyrins were measured in MeOH:aqueous buffer (20/80) and coproporphyrins in MeOH:aqueous buffer (50/50). The buffer was citric acid and ammonium citrate (constant ionic strength 0.10 mol/l), Temperature 25 ± 0.5 °C. Assignment: (◆) uro I; (■) uro III; (▲) copro I and (●) copro III.

Rapid gradient profile used and discussed further (Fig. 3B) was selected from alternative gradients optimized by means of ChromSword. Properties of these gradients were tested by their alternative use in the two above-mentioned HPLC systems. Overall appearance of chromatogram depends on dynamic properties of device used for rapid gradient mixing, dwell volume of the system and timing of the gradient events. This is essentially critical for multi-step gradients. We can conclude that from this point-of-view robustness is attributed to a simple linear gradients and certain HPLC system. Results and discussion about influence of rapid gradient
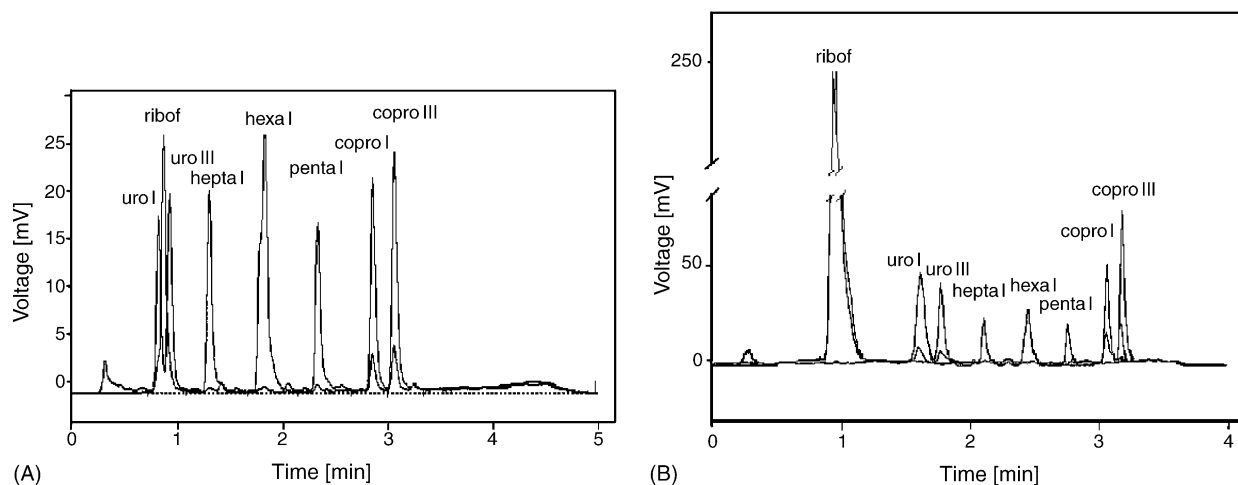

Fig. 3. (A) Comparison of chromatograms of porphyrins mixed standard (5–20 μg/l level, upper trace) and urine sample of healthy person after Vitamin B$_2$ treatment (lower trace) obtained by gradient elution optimized only with respect to the analytes resolution. For conditions except the gradient profile see part B. Note interfering peak in position of uroporphyrine I and uroporphyrine III (retention time range 0.8–1.0 min). Abbreviations—Table 2, ribof means riboflavine. (B) Comparison of chromatograms of porphyrins mixed standard (5–20 μg/l level, upper trace), standard of riboflavin (200 μg/l, lower trace) and urine sample of healthy person after Vitamin B$_2$ treatment (in the middle). Gradient elution was additionally optimized with respect to the analytes and interfering substance resolution (refer also to Section 2.3). Conditions: pH 5.47, fluorimetric detection (λ_{ex} = 405 nm and λ_{em} = 620 nm), T = 26.2 °C, p_A = 6.2–6.4 MPa, flow rate 3 ml/min. Abbreviations—Table 2, ribof means riboflavine.

shape and timing of short gradient events to chromatographic parameters of porphyrins is to be published elsewhere. Here only results from high-pressure gradients are involved.

3.2. Urine matrix related considerations

Optimization of separation parameters using the monolithic column was initiated with non-published method already established in the clinical laboratory for the analysis of porphyrins. In this gradient method aqueous buffering solution containing 2 mol/l ammonium acetate (pH 5.46) and mixed organic modifier MeOH/ACN was used. Analysis was of 7 min duration and analysis run cycle lasted 10 min. However, alternative use of the column Chromolith SpeedRod also for the other diagnostic markers analyses accompanied by a change of mobile phase (to 0.1 mol/l acetic acid and MeOH) deteriorated gradually resolution of uroporphyrins after approximately 100 runs to zero. Careful consideration have led us to a final conclusion that presence of trace metals within the HPLC system introduced both by untreated urine sample (hydrolysis and dilution are the only operations) and the construction stainless steel leaching are responsible for the problem. However, addition of 50 mmol/l EDTA into the mobile phase did not have positive impact. Consequently acetate was changed for citrate having high chelating potential. Flushing of the column with the citrate regenerated its ability to separate uroporphyrins and also the efficiency approached original values. The explanation can be derived from the information on complex formation of studied porphyrins with metal ions (mobile phase can dissolve from stainless steel mainly $Fe(II)$ but also $Cr(III)$, $Ni(II)$, $Mn(II)$, $Co(II)$ molybdenium, vanadium ions) changing their retention behaviour. Moreover these $Fe(II)$ complexes does not show fluorescence. Even low concentrations of metal ions at ng/l levels are in a molar proportion to the analysed porphyrins or can many times exceed their concentration. We suppose that high concentration even of highly pure acetate also introduced sufficient amount of trace metals into the stationary phase and maybe caused their complexation with residual silanols. This hypothesis is supported by a published articles on complex forming ability of porhyrins used as analytical reagents reviewed by Tabata and Tanaka [42], as ligands in HPLC for trace metal analysis by Shi and Fu [43], as reagents in analytical chemistry generally by Biesaga et al. [44], and porphyrins used in chromatography by Trojanowicz et al. [45]. From the set of studied urine porphyrins almost entirely coproporphyrin I complexes with $Cu(II)$ and $Co(II)$ [46], with $Cd(II)$ and $Mn(II)$ [47], with $Hg(II)$ and $Co(II)$ [48] were studied with focus to their formation kinetics and equilibria. Trace metals $Mn(II)$, $Co(II)$, $Zn(II)$, $Ni(II)$, $Cu(II)$ were analysed at approximately 0.1 mg/l levels in river water by Giovannetti and Bartocci [49] as coproporphyrin I complexes. RP-HPLC with photometric detection at 347 and 458 nm using MeOH:aqueous sodium phosphate, sodium borate and sodium acetate buffer (pH 7) was used. The complexes were eluted by at least 70% (v/v) MeOH, concentration

several times higher than is needed for elution of free coproporphyrin I at this pH.

Urine belongs to complex matrices containing up to 60 mg/l $Mg(II)$ and 150 mg/l $Ca(II)$ cations (for normal urine see, e.g. Table 3, [57]) that are also able to influence both retention and fluorescence of all carboxylated uroporphyrins due to natural selectivity of vicinal carboxylates for chelation of these metals. However, at a trace levels (mg/l) urine contains hundreds of substances and at lower concentration levels (μg/l) even thousands of substances [57] including urinary porphyrins. Citrate competes well with the porphyrins in above mentioned metal chelation equilibria.

Therefore we decided to use entirely ammonium citrate buffer (citric acid titrated in various extent by ammonia) having sufficient buffering capacity [56] within studied range of pH 3.0–6.2. Methanol was chosen as organic modifier due to lower toxicity than acetonitrile, better solubility of citrate buffer, lower tendency to precipitate proteins from biological samples and lower price. For comparison, we can point out that per 1000 analyses mobile phase expense was halved.

During analyses of urine of both healthy persons and patients we noted in some cases that an unidentified compound had been co-eluted with the uroporphyrins (Fig. 3A). This interference we noticed irregularly and randomly only in some specimens. As was traced later it was related to patients taking vitamins and we recognized the interference as riboflavin (Vitamin B_2) fluorescence. Taking this fact into consideration we successfully further optimized the conditions of rapid gradient elution and we eliminated the co-elution of riboflavin with uroporphyrins as is evident from Fig. 3B. Achieved resolution between riboflavin and uroporphyrin I is higher than 3 and all the other porphyrins are separated with resolution higher than 1.3, including separation of I and III isomers of uro- and coproporphyrins.

3.3. Quantitative aspects and method validation

For reliable and robust method applicable in clinical laboratory, reproducibility of the analyses is very important. The devised method was tested also in this respect and we can state that under given conditions uroporphyrins are the most sensitive of all analysed porphyrins to uncertainties of fast gradient timing and change of shape of the complex gradient. In the case when sudden programmed change (node) of the gradient is responsible for given analyte elution (see Fig. 4) we can observe lower repeatability of retention times. Statistical treatment of ten measurements done within two consecutive days is given in Table 2. Both retention times and peak areas repeatability is evaluated.

Our studies demonstrate that good repeatability and reproducibility is achieved with this method for urinary porphyrin concentration values of both normal and porphyric individuals. For porphyric urine sample dilution is sometime needed to shift the analytical signal into the centre of calibration curve. Calibration curves of selected porphyrins were constructed and statistically treated by least squares method of

Table 2
Statistical evaluation of reproducibility of retention times (t_R) and reproducibility of peak areas (A) in 10 consecutive runs measured within two days

Porphyrin	Average of t_R (min)	Standard deviation (t_R) (min)	t_R RSD (%)	Average of A (mV s)	Standard deviation (A) (mV s)	A RSD (%)
Uro I	1.533	0.021	1.38	73.54	1.77	2.41
Uro III	1.763	0.007	0.42	86.76	1.89	2.18
Hepta I	2.081	0.004	0.17	90.36	1.05	1.16
Hexa I	2.800	5×10^{-8}	<0.10	118.40	1.80	1.52
Penta I	2.661	0.004	0.17	78.64	0.49	0.60
Copro I	2.950	4×10^{-8}	<0.10	87.64	2.04	2.33
Copro III	3.073	0.005	0.15	111.78	1.04	0.92

Abbreviations: uro I is octacarboxylated uroporphyrin I, uro III is octacarboxylated uroporphyrin III, hepta is heptacarboxylated porphyrin I, hexa is hexacarboxylated porphyrin I, penta is pentacarboxylated porphyrin I, copro I is tetracarboxylated coproporphyrin I, copro III is tetracarboxylated coproporphyrin III.

linear regression at concentration levels ranged from 8 to 350 nmol/l. At the higher values it was nonlinear, therefore we reduced the range from 8 to 180 nmol/l (refer to Fig. 5 and Table 3)

Calculated limit of quantitation (LOQ) at ($10 \times$ S/N) for uroporphyrin I is 82 pmol/l, for uroporphyrin III is 44 pmol/l, for coproporpyrin I is 50 pmol/l and for coproporphyrin III 47 pmol/l, respectively. These values are approximately 20–120 times lower than concentration of investigated porphyrins in a human urine under physiological conditions [58]. This enables further convenient decrease of column load and decrease of influence of potential interferences (e.g. macro-constituents and/or trace metals) simply by urine dilution and contributes to column life prolongation.

The analysis of porphyrins is done with extremely low amounts from 0.2 pg/injection (LOD) to 400 pg/injection at 20 μg/l concentration level. At such low concentrations even traces of metal cations can cause unexpected and unwanted effects. Actually, we carefully verified carry-over effects by three consecutive blank runs after the standard run. Results obtained at Chromolith SpeedRod column are given at Fig. 5
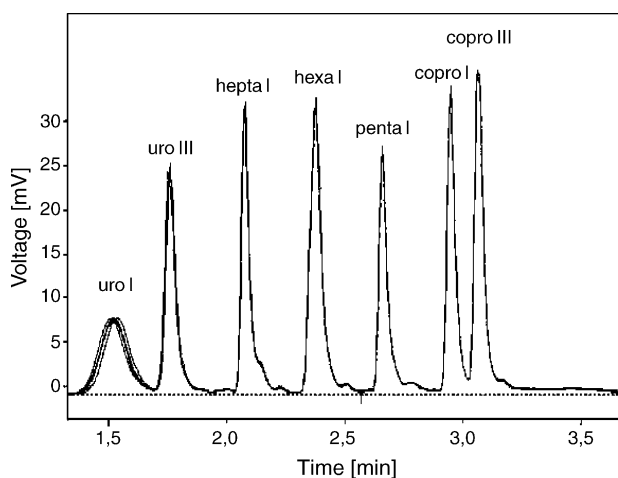
and the carry-over of individual porphyrins is quantitated in Table 4. Even at these low ng/l concentration levels analytes are separated and well resolved within 3.2 min (Fig. 6). Increase of resolution is observed for majority of porphyrins to value around 2.0 in opposite to decrease of resolution observed for uroporphyrin I and uroporphyrin III isomer separation from value 1.3 at healthy person physiological level to 0.9 at concentration approaching LOQ and LOD levels, respectively. This could be explained by the fact that for elution of uroporphyrine I gradient node is responsible.
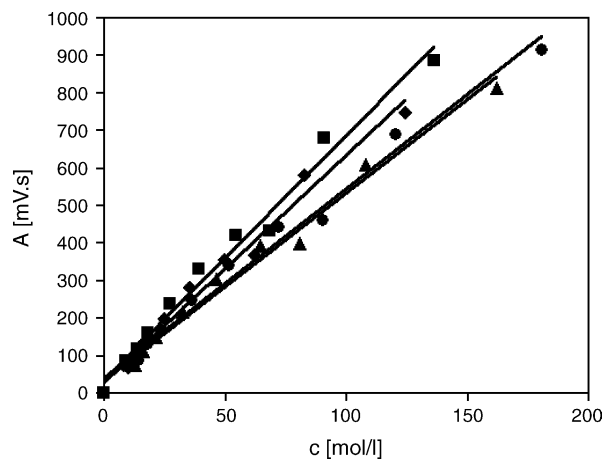

Fig. 5. Calibration curves of selected porphyrins in a concentration range 8–180 nmol/l (equal to 6.4–150 μg/l or 130 pg/injection–3 ng/injection for uroporphyrins; 5.2–118 μg/l or 104 pg/injection–2.4 ng/injection for coproporphyrins). Assignment: (♦) uro I; (■) uro III; (▲) copro I and (●) copro III.

Fig. 4. Overall reproducibility of 10 overlayed chromatographic profiles of porphyrins mixed standard (from 5 to 20 μg/l, individually) measured within two days. Gradient elution, pH 5.47, fluorometric detection ($\lambda_{ex} = 405$ nm and $\lambda_{em} = 620$ nm), $T = 25.8$–26.4 °C, $p_A = 5.3$–5.5 MPa, flow rate 3 ml/min. Abbreviations—Table 2.

Table 3
Parameters of calibration curve for linear model as obtained by linear regression (least square method)

Porphyrin	Linear regression equation	Correlation coefficient, R
Uro I	$y = 5.96x + 35.33$	0.9914
Uro III	$y = 6.42x + 42.02$	0.9925
Copro I	$y = 4.92x + 40.22$	0.9933
Copro III	$y = 4.95x + 48.02$	0.9937

Where, y is peak area in (mV s) and x is concentration in (nmol/l). *Abbreviations*: see Table 2.

Table 4
Analysis of porphyrins at 5–20 µg/l level and carry-over effect observed in three successive blank runs (blk 1, blk 2 and blk 3) expressed as relative proportion of original signal (%) for monolithic column Chromolith

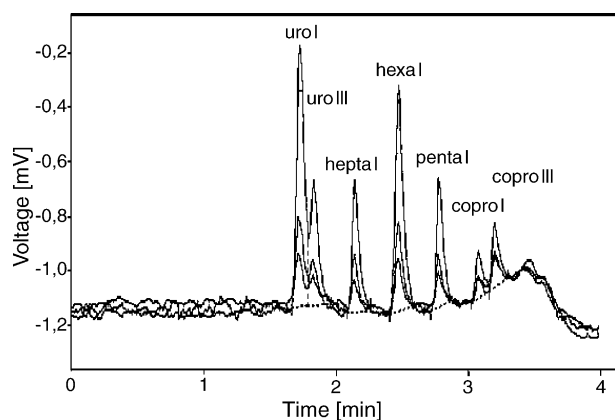| Porphyrin	t_R (min)	A (mV s)	blk 1 (%)	blk 2 (%)	blk 3 (%)
Uro I	1.72	226.706	1.6	0.6	0.3
Uro III	1.83	98.452	2.2	0.9	0.3
Hepta I	2.14	96.803	1.8	0.7	0.3
Hexa I	2.47	196.951	1.7	0.7	0.4
Penta I	2.77	84.870	1.9	0.8	0.5
Copro I	3.07	23.707	2.6	0.9	0.9
Copro III	3.20	26.379	3.3	1.8	1.2

Abbreviations: see Table 2.

Fig. 6. Chromatograms of three consecutive blank runs after injection of mixed standard of porphyrins (individuals from 5 to 20 µg/l) to Chromolith SpeedRod RP 18e, 100-times zoomed in comparison to Fig. 4. Abbreviations—Table 2.

For comparison purposes and to reveal whether the phenomena of carry-over is column dependent or not the whole experiment was run also using EPS C18 and Purospher RP18e stainless steel columns, having dimensions and overall efficiency similar to the Chromolith SpeedRod column under the otherwise identical conditions. Data evaluated for the EPS C18 particulate column given in Table 5 clearly show that at 5–20 µg/l concentration level from 3.5% to 0.2% of porphyrins is carried-over in each of three consecutive runs. Height of peaks is approaching the heights attributed to that of limit of quantitation. In a total, from 2.5% to 7% of material was carried-over regardless the column including monolithic column what could be interpreted as 93–97% recovery of porphyrins at low µg/l concentration levels. Similar results were

Table 5
Analysis of porphyrins at 5–20 µg/l level and carry-over effect observed in three successive blank runs (blk 1, blk 2 and blk 3) expressed as relative proportion of original signal (%) for Platinum EPS column

Porphyrin	t_R (min)	A (mV s)	blk 1 (%)	blk 2 (%)	blk 3 (%)
Uro I, uro III	2.31	370.565	1.3	0.5	0.2
Hepta I	2.63	100.537	0.9	0.4	0.2
Hexa I	2.94	203.609	1.1	0.4	0.3
Penta I	3.24	88.590	1.3	0.4	0.2
Copro I	3.52	19.630	2.4	0.8	–
Copro III	3.62	25.239	4.0	2.9	–

obtained for the Purospher column. The carry-over effect is not column dependent and probably not related to stainless steel frits in the metal columns. Obvious assumption about its instrumental dependence and impurities in mobile phase (constitutional and/or leached, respectively) was partly verified by the authors. Hypothesis of trace metals release due to dissolution of stainless steel sample loop by HCl (added to urine samples, see Section 2.3) was verified by its substitution for titanium sample loop. No change was observed. The other potential sources should be proved by use of non-metal wetted parts HPLC instrumentation which is at this moment not available to the authors or additional purification of basic chemicals, however this approach is not applicable to clinical laboratory work.

4. Conclusion

Given results show that proposed validated ultra-trace and rapid RP-HPLC method enables reproducible separation of seven diagnostically relevant porphyrins from human urine sample within 3.2 min. Run-to-run analysis cycle takes 4 min. Resolution of isomers I, and III for uroporphyrins and coproporphyrins, respectively, increases diagnostic value of the method. LOQ ($10 \times$ S/N) of selected porphyrins is 82 pmol/l (65 ng/l, 1.30 pg/injection) for uroporphyrin I, 44 pmol/l (33 ng/l, 0.66 pg/injection) for uroporphyrin III, 50 pmol/l (40 ng/l, 0.80 pg/injection) for coproporphyrin I, and 47 pmol/l (39 ng/l, 0.78 pg/injection) for coproporphyrin III, respectively. Limit of detection (LOD, $3 \times$ S/N) of selected porphyrins is 24.6 pmol/l (20 ng/l, 0.40 pg/injection) for uroporphyrin I, 13.2 pmol/l (11 ng/l, 0.22 pg/injection) for uroporphyrin III, 15 pmol/l (13 ng/l, 0.26 pg/injection) for coproporphyrin I, and 14.1 pmol/l (12 ng/l, 0.24 pg/injection) for coproporphyrin III, respectively. Repeatability of both retention times and peak areas is highly acceptable. Recovery of analytes is ranged from 93% to 97.5% at physiological concentration level. The proposed validated method is robust, interference free and applicable for diagnostic purposes. Introduction of this method benefiting from use of commercial monolithic column can increase productivity of clinical laboratory from 2 to 10 times in dependence of currently used method duration.

References

[1] L.R. Milgrom, The Colours of Life, Oxford University Press Inc., New York, 1997, pp. 1–22.

[2] R.M.C. Dawson, D.C. Elliott, W.H. Elliott, K.M. Jones, Data for Biochemical Research, third ed., Oxford Science Publication, Oxford, UK, 1986.

[3] C.R. Scriver, A.L. Beaudet, W.S. Sly, D.S. Vale (Eds.), The Metabolic and Molecular Basis of Inherited Disease, eighth ed., McGraw-Hill Book Co., New York, 2001 (Chapter 124).

[4] R.E. Ford, C.-N. Ou, R.D. Ellefson, Clin. Chem. 27 (3) (1981) 398.

[5] R.D. Ellefson, R.E. Ford, Regul. Toxicol. Pharmacol. 24 (1996) 119.

[6] C.K. Lim, Porphyrins: liquid chromatography, in: M. Cooke, C.F. Poole (Eds.), Encyclopaedia of Separation Science, vol. III, Academic Press, London, 2000, p. 3960.

[7] H.D. Meyer, K. Jacob, W. Vogt, M. Knedle, J. Chromatogr. 199 (1980) 339.

[8] L.L. Needham, S.L. Sirmans, J.A. Liddle, D.D. Bayse, Clin. Chem. 27 (1981) 1099.

[9] R.H. Hill Jr., S.L. Bailey, L.L. Needham, J. Chromatogr. 232 (1982) 251.

[10] R.E. Ford, C.N. Ou, R.D. Ellefson, Clin. Chem. 27 (1981) 387.

[11] B. Johansson, B. Nilsson, J. Chromatogr. 229 (1982) 439.

[12] E. Gaetani, C.F. Laureri, M. Vitto, J. Chromatogr. 231 (1982) 425.

[13] D.J. Wright, J.M. Rideout, C.K. Lim, Biochem. J. 201 (1983) 553.

[14] C.K. Lim, J.M. Rideout, D.J. Wrigt, Biochem. J. 211 (1983) 435.

[15] I.M. Johansson, F.A. Niklasson, J. Chromatogr. 275 (1983) 51.

[16] H.D. Meyer, W. Vogt, K. Jacob, J. Chromatogr. 290 (1984) 207.

[17] C.K. Lim, T.J. Peters, Clin. Chim. Acta 139 (1984) 55.

[18] L.A. Grubina, I.F. Gurinovich, E.P. Demidchik, S.V. Trofimovich, J. Chromatogr. 380 (1986) 232.

[19] M. Takayama, J. Chromatogr. 133 (1987) 313.

[20] K. Jacob, I. Kossien, E. Egeler, M. Knedel, Chromsymp. 991 (1988) 171.

[21] P.M. Johnson, S.L. Perkins, S.W. Kennedy, Clin. Chem. 34 (1988) 103.

[22] K. Jacob, E. Egeler, D. Neumeier, M. Knedel, J. Chromatogr. 468 (1989) 329.

[23] S.W. Kennedy, A.L. Maslen, J. Chromatogr. 493 (1989) 53.

[24] K. Abe, R. Konaka, Clin. Chem. 35 (1989) 1619.

[25] M.E. Hahn, T.A. Gasiewicz, J. Chromatogr. 563 (1991) 363.

[26] N. Schoenfeld, R. Mamet, Clin. Chem. 40 (1994) 417.

[27] G.G. Garcia-Vargas, A. Hernandez-Zavala, Biomed. Chromatogr. 10 (1996) 278.

[28] F.M.J. Zuijderhoudt, S.G.A. Koehorst, W.E. Kluitenberg, Clin. Chem. Lab. Med. 38 (2000) 227.

[29] C. Magi, E. Ianni, P. Rivaro, R. Frache, J. Chromatogr. A 905 (2001) 141.

[30] C. Ericson, J.L. Liao, K. Nakazato, S. Hjerten, J. Chromatogr. A 767 (1997) 33.

[31] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, J. Chromatogr. A 762 (1997) 135.

[32] M.T. Dulay, R.P. Kulkarni, R.N. Zare, Anal. Chem. 70 (1998) 5103.

[33] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, J. Chromatogr. A 797 (1998) 121.

[34] N. Ishizuka, H. Minakuchi, K. Nakanishi, N. Soga, K. Hosoya, N. Tanaka, J. High Resolut. Chromatogr. 21 (1998) 477.

[35] Q. Tang, M.L. Lee, J. High Resolut. Chromatogr. 23 (2000) 73; Q. Tang, M.L. Lee, J. High Resolut. Chromatogr. 23 (2000) 67.

[36] N. Tanaka, H. Nagayama, H. Kobayashi, T. Ikegami, K. Hosoya, N. Ishizuka, H. Minakuchi, K. Nakanishi, K. Cabrera, D. Lubda, J. High Resolut. Chromatogr. 23 (2000) 111.

[37] K. Cabrera, D. Lubda, H.M. Eggenweiler, H. Minakuchi, K. Nakanishi, J. High Resolut. Chromatogr. 23 (2000) 93.

[38] Anonymous, Chromolith RP18e Specification, Merck KGaA, Darmstadt, Germany, 2001.

[39] Ch. Sobel, C. Cano, R.E. Thiers, Clin. Chem. 20 (1974) 1397.

[40] N. Tanaka, H. Kobayashi, Anal. Bioanal. Chem. 376 (2003) 298.

[41] F. Li, C.K. Lim, T.J. Peters, Chromatographia 24 (1987) 421.

[42] M. Tabata, M. Tanaka, Trends Anal. Chem. 10 (1991) 128.

[43] Z. Shi, Ch. Fu, Talanta 44 (1997) 593.

[44] M. Biesaga, K. Pyrzyńska, M. Trojanowicz, Talanta 51 (2000) 209.

[45] M. Trojanowicz, M. Biesaga, J. Orska-Gawrys, Anal. Sci. 17 (2001) 587.

[46] R. Giovannetti, V. Bartocci, S. Ferraro, M. Gusteri, P. Passamonti, Talanta (1995) 1913.

[47] V. Bartocci, R. Giovannetti, E. Carsetti, J. Porphyrins Phthalocyanines 2 (1998) 139.

[48] R. Giovannetti, V. Bartocci, Talanta 46 (1998) 977.

[49] R. Giovannetti, V. Bartocci, J. Liq. Chromatogr. Relat. Technol. 21 (1998) 2607.

[50] N. Evans, A.H. Jackson, S.A. Matlin, R. Towill, J. Chromatogr. 125 (1976) 345.

[51] J. Blądek, A. Zdrojewski, Chem. Anal. (Warsaw) 48 (2003) 689.

[52] P. Culbreth, G. Walter, R. Carter, C. Burtis, Clin. Chem. 25 (1979) 607.

[53] K. Jacob, E. Egeler, B. Hennel, D. Neumeier, Fresenius Z. Anal. Chem. 330 (1988) 386.

[54] A. Junker-Buchheit, H. Jork, J. Planar Chromatogr. -Mo. TLC 1 (1988) 3.

[55] K.M. Smith, Porphyrins and Metalloporphyrins, Elsevier, Amsterdam, 1975.

[56] M. Ferenčík, B. Škárka, Biochemické laboratórne metódy (Biochemical Laboratory Methods, in Slovak), SNTL, Prague, 1981, p. 71.

[57] Cs. Mišľanová, M. Hutta, J.Ї Chromatogr. B 797 (2003) 91.

[58] J.T. Hindmarsh, L. Oliveras, D.C. Greenway, Clin. Biochem. 32 (1999) 609.