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Determination of uremic solutes in biological fluids of chronic kidney disease patients by HPLC assay

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ABSTRACT

During chronic kidney disease (CKD), solutes called uremic solutes, accumulate in blood and tissues of patients. We developed an HPLC method for the simultaneous determination of several uremic solutes of clinical interest in biological fluids: phenol (Pol), indole-3-acetic acid (3-IAA), p-cresol (p-C), indoxyl sulfate (3-INDS) and p-cresol sulfate (p-CS). These solutes were separated by ion-pairing HPLC using an isocratic flow and quantified with a fluorescence detection. The mean serum concentrations of 3-IAA, 3-INDS and p-CS were 2.12, 1.03 and 13.03 μM respectively in healthy subjects, 3.21, 17.45 and 73.47 μM in non hemodialyzed stage 3–5 CKD patients and 5.9, 81.04 and 120.54 μM in hemodialyzed patients (stage 5D). We found no Pol and no p-C in any population. The limits of quantification for 3-IAA, 3-INDS, and p-CS were 0.83, 0.72, and 3.2 μM respectively. The within-day CVs were between 1.23 and 3.12% for 3-IAA, 0.98 and 2% for 3-INDS, and 1.25 and 3.01% for p-CS. The between-day CVs were between 1.78 and 5.48% for 3-IAA, 1.45 and 4.54% for 3-INDS, and 1.19 and 6.36% for p-CS. This HPLC method permits the simultaneous and quick quantification of several uremic solutes for daily analysis of large numbers of samples.

1. Introduction

In patients with chronic kidney disease (CKD), numerous solutes, called uremic retention solutes or uremic toxins, are retained in blood and tissues instead of being excreted by kidneys [1]. So far, about a hundred compounds have been identified in the serum of patients and classified according to their comportment during dialysis [1]. These compounds originate from different metabolic pathways, thus displaying various biochemical structures and properties [1]. The toxicity of each of them is not yet known because this requires both clinical and in vitro studies. Nonce, the toxicity is well established for homocysteine [2,3], asymmetric dimethylarginine [4,5], advanced glycation end-products [6,7], beta 2 microglobulin [8] and endothelin [9], which subsequently dispose of routine laboratory measurements. Compared to these leader toxins, others begin to emerge as cytotoxic molecules and then molecules of clinical interest [10–12]. It is the case for indoxyl sulfate (3-INDS), indole-3-acetic acid (3-IAA), and p-cresol sulfate (p-CS) for which deleterious effects have been already described [10,13].

Indole-3-acetic acid and 3-INDS increase interstitial renal fibrosis in uremic rats [14,15]. Oral administration of 3-INDS to uremic rats induces increased expression of transforming growth factor-beta 1, tissue inhibitor of metalloproteinases and pro-alpha 1 collagen which could favour the progression of the

Abbreviations: CKD, chronic kidney diseases; C_N, mean normal concentration; C_M, maximal uremic concentration; C_U, mean uremic concentration; PSN, pool of normal serums; PSHD, pool of serums of hemodialyzed patients; 3-IAA, indole-3-acetic acid; 3-INDS, indoxyl sulfate; p-C, p-cresol; p-CS, p-cresol sulfate; Pol, phenol.

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tubulointerstitial damage [16]. Indole-3-acetic acid and 3-INDS induce oxidative stress in tubular [14,15] and endothelial cells [14,17]. P-cresol sulfate induces shedding of endothelial microparticles [18], a marker of cell injury, and exhibits a pro-inflammatory effect on unstimulated leukocytes since it increases the percentage of leukocytes displaying oxidative burst activity at baseline [19]. More recently, higher levels of 3-INDS and free p-CS have been associated with cardiovascular mortality in CKD patients [20–23]. Indole-3-acetic acid overload in a rat model of chronic renal failure accelerates the loss of kidney function, glomerular sclerosis, and tubulointerstitial injury [24]. In addition, these toxins are protein-bound and consequently badly removed by conventional dialysis [13]. Only 30% of 3-INDS is removed during a dialysis session instead of 70% for water soluble molecules such as creatinine [13].

Thus, p-CS, 3-INDS and 3-IAA are expected to exhibit a clinical impact on the out-come of CKD patients. In addition, since they are produced by intestinal flora from tyrosine, phenylalanine and tryptophane and subsequently conjugated with sulfate [11,25,26], therapeutic strategies using probiotic seem to be very hopeful to decrease their concentration [27,28]. All these data make essential to be able to easily monitor their serum concentrations in CKD patients.

Several works have reported on the quantitative analysis of these solutes but none on simultaneous analysis [29–31]. The aim of our work was to develop a simple, rapid and sensitive high-performance liquid chromatographic (HPLC) method to simultaneously quantify in CKD patients the uremic solutes 3-IAA, 3-INDS, and p-CS. In addition, we quantified at the same time phenol (Pol) and p-cresol (p-C).

2. Materials and methods

2.1. Chemicals

We purchased p-cresol (p-C), phenol (Pol), p-ethylphenol internal standard (IS), acetonitrile (HPLC grade), 3-indoxyl sulfate potassium salt (3-INDS), indole-3-acetic acid sodium salt (3-IAA), sodium octanoate, the ion-pairing agent tetrabutyl ammonium iodide (TBAI), p-methylphenol, pyridine, diethylether, sodium hydroxide and chlorosulfonic acid from Sigma (Saint-Quentin Fallavier, France). Ethanol (HPLC grade), methanol (HPLC grade), sodium dihydrogen phosphate, H_2O, and sodium chloride were from Carlo Erba (Peypin, France). "HPLC grade" water was freshly prepared by passing distilled, de-ionized water through a Norganic cartridge and a 0.22 μm filter (Nalgene Labware, Brumath, France). P-cresol sulfate (p-CS) was prepared in our laboratory.

2.2. Synthesis and analysis of sodium p-cresol sulfate (p-CS)

Slight modifications were made on procedure described by Feigenbaum [32]. To an ice-cooled solution of 21 g of *p*-methylphenol (0.192 mol, 1 equiv.) in 80 mL of pyridine, 29 g of chlorosulfonic acid (0.25 mol, 1.3 equiv.) were added dropwise. The reaction mixture was then stirred for 1 h at room temperature and treated with 20 mL of a 5 N sodium hydroxide solution at 0 °C. After one night at room temperature, colorless crystals were formed and filtered off. These crystals were successively washed with diethylether and 95% ethanol, affording pure colorless p-CS crystals in 75% yield. ^{a}H NMR (200 MHz; DMSO d_6): δ 2.24 (3H, s), 7.04 (4H, s); ^{ac}C NMR (50 MHz; DMSO d_6): δ 20.5 (CH_3), 120.6 (2CH), 129.2 (2CH), 131.9 (C), 151.5 (C); MS (ESI-): m/z = 187. The X-ray diffraction data indicated that the crystal structure of the synthesized p-CS sodium salt was monohydrated.

2.3. HPLC apparatus

We separated uremic solutes by a Shimadzu Prominence HPLC system (Shimadzu, Champs sur Marne, France) with two LC20 AD pumps, DGU-A3 degasser, SIL-20A autosampler, CTO-20A column oven, CBM-20A interface, and RF-10 AXL fluorescence detector with an octyl reversed-phase column, Merck Lichrospher 60 RP Select B 5 μ, 125 mm × 4 mm (Merk Chimie, Fontenay sous Bois, France), at 35 °C.

2.4. Preparation of stock and working solutions of standards

A solution of each uremic solute was prepared. Then, a mixture containing all uremic solutes was made by pooling each solution volume to volume. Concentrations corresponding to mean normal concentration (C_N), mean uremic concentration (C_U), and maximal uremic concentration (C_M) [according the values given by the publication from the European Work Group on Uremic toxins [1]] were obtained by different dilutions (Table 1).

Table 1
Preparation of stock and working solutions (μM) for C_N–C_U and C_U–C_M calibration curves.

C_N–C_U calibration curves							
Toxin	Conc of working solution 1	Final conc in mixture	Point 1 of curve dilution 1/5	Point 2 of curve dilution 2/5	Point 3 of curve dilution 3/5	Point 4 of curve dilution 4/5	Point 5 of curve dilution 1
Pol	263	52.6	10.52	21.04	31.56	42.08	52.6
3-IAA	25.6	5.1	1.02	2.04	3.06	4.08	5.1
p-C	1017.6	203.5	40.7	81.4	122.1	162.8	203.5
3-INDS	994.8	199.0	39.8	79.6	119.4	159.2	199.0
p-CS	574.6	114.9	22.98	45.96	68.94	91.92	114.9
C_U–C_M calibration curves							
Toxin	Conc of working solution 2	Final conc in mixture	Point 1 of curve dilution 1/5	Point 2 of curve dilution 2/5	Point 3 of curve dilution 3/5	Point 4 of curve dilution 4/5	Point 5 of curve dilution 1
Pol	1052.1	210.4	42.08	84.16	126.24	168.32	210.4
3-IAA	256.1	51.2	10.24	20.48	30.72	40.96	51.2
p-C	2543.9	508.8	101.76	203.52	305.28	407.04	508.8
3-INDS	4974.1	994.8	198.96	397.92	596.88	795.84	994.8
p-CS	1436.4	287.3	57.46	114.92	172.38	229.84	287.3

3-IAA, indole-3-acetic acid; 3-INDS, indoxyl sulfate; p-C, p-cresol; p-CS, p-cresol sulfate; Pol, phenol.

2.5. Serum samples

The study included 40 healthy subjects, 16 men and 24 women, with a mean age of 65 ± 8 years, 50 hemodialyzed patients (stage 5D CKD), 33 men and 17 women, with a mean age of 65 ± 15 years and 43 non hemodialyzed CKD patients, 26 men and 17 women, with a mean age of 61 ± 15 years. This last group included patients with stage 3–5 CKD with a glomerular filtration rate (estimated with MDRD equation) ranging from 8 to 57 mL/min/1.73 m^2 (mean 26 ± 11 mL/min/1.73 m^2). All populations were recruited with informed consent from the *Centre de Nephrologie et de Transplantation Rénale* and from the *Centre d'Investigation Clinique*. All serum samples were frozen at −80 °C until use.

2.6. Sample preparation and HPLC analysis

2.6.1. Sample preparation

Serum deproteinization and bound uremic solutes displacement were made by adding 300 μl of ethanol containing 5 nmol p-ethylphenol (IS, 16 μM) to 100 μl of serum. Then, serum sample was saturated with 100 mg of NaCl and mixed vigorously. After 10 min, 700 μl of mobile phase A were added and the sample was centrifuged at 10,000 × *g* for 10 min. For spiked serum, 100 μl of standard solution and 600 μl of mobile phase A were added instead of 700 μl. We injected 20 μl serum extracts, equivalent to 1.8 μl of serum. In experiments with sodium octanoate, 100 μl of 0.24 M sodium octanoate were added to 100 μl of serum and then sample was vigorously mixed. After 10 min, serum sample was treated with ethanol as described above.

2.6.2. HPLC analysis

Mobile phase A consisted of 2.76 g/L (20 mM) NaH_2PO_4, H_2O and 1.85 g/L (5 mM) TBAI in water, and mobile phase B of acetonitrile. For elution, a 1.5 mL/min isocratic flow of 22% B in mobile phase A was used. We quantified uremic solutes and IS with fluorescence detection monitored at specific excitation (E_x) and emission (E_m) wavelengths (Pol: E_x 272, E_m 319 nm; 3-IAA and 3-INDS: E_x 278, E_m 348 nm; p-C and p-CS, IS: E_x 285, E_m 310 nm) according to the retention times. The concentrations of uremic solutes were calculated using the standard calibration curves (C_N–C_U; C_U–C_M) by Shimadzu LC solution software (version 1.21). All samples were run in duplicate, and two reference samples were included in every run.

2.7. Method validation

2.7.1. Calibration curves

For each uremic solute, two calibration curves C_N–C_U and C_U–C_M corresponding to the values ranging from C_N to C_U and from C_U to C_M, were plotted. The calibration curves C_N–C_U and C_U–C_M were obtained with a pool of normal serum (PSN) or hemodialyzed serum (PSHD), alone and spiked by point 1 to point 5. We performed Merck Lichrospher 60 RP Select B separation (n = 6) for each concentration. The data obtained were subjected to linear regression analysis to calculate the slope, intercepts, SEs of the slope and intercept, and SD.

2.7.2. Within-day and between-day reproducibility

For each uremic solute, six samples of different concentrations were made: pool of normal serum alone and spiked with point 1 and point 3 of C_N–C_U curve, and pool of hemodialyzed serum alone and spiked with point 3 and point 5 of C_U–C_M curve. For each concentration, 20 runs were performed in the same day and two runs per day during 20 separate days. The within-day and between-day differences were analyzed and compared with ANOVA test.

2.7.3. Limit of quantification

We defined the limit of quantification as signal-to-noise = 10 in the HPLC run during linear regression analysis using samples described above.

2.7.4. Recovery

Recovery tests for each toxin peak were calculated as shown below: Recovery % = [(final concentration − initial concentration) × 100)]/added concentration.

3. Results and discussion

3.1. Sample preparation and chromatographic separation

The chromatograms of the uremic solutes, obtained in 20 min, are shown in Fig. 1. The uremic solutes analyzed in this study exhibit

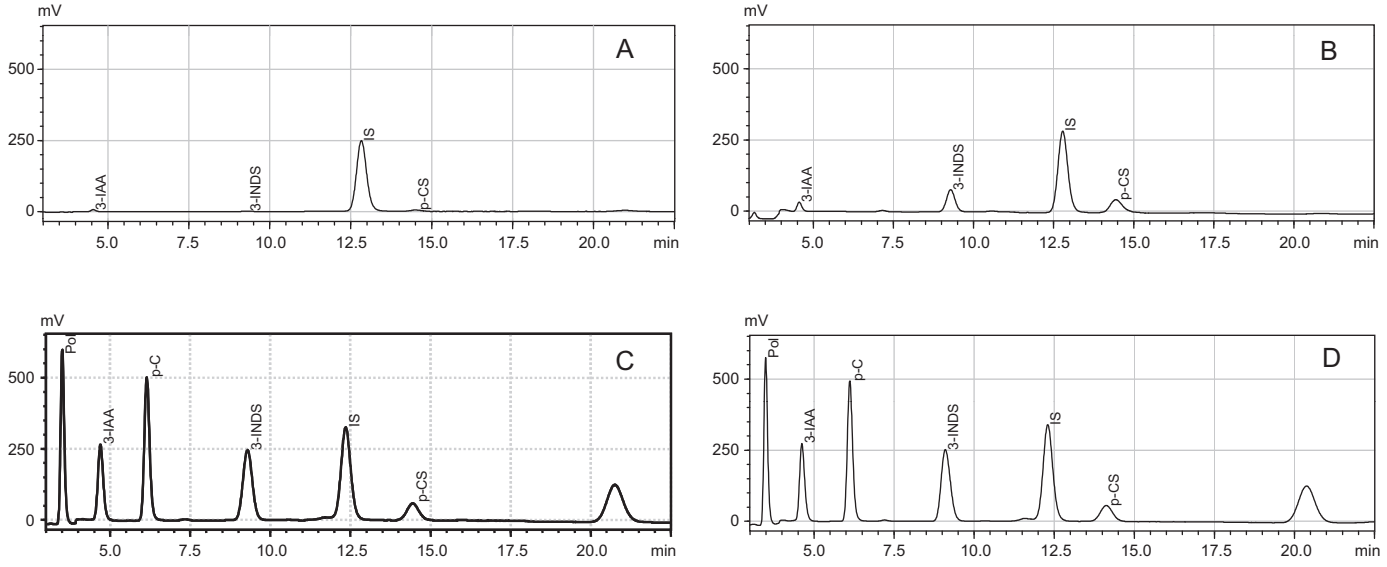

Fig. 1. Chromatograms of standard solutions and serums. Typical chromatograms obtained with serum of healthy patient (A), serum of hemodialyzed patients (PSHD) (B), point 3 of C_U–C_M calibration curve + PSHD treated without 0.24 M sodium octanoate (C) and point 3 of C_U–C_M calibration + PSHD treated with 0.24 M sodium octanoate (D) 3-IAA: indole-3-acetic acid, 3-INDS: indoxyl sulfate, p-C: p-cresol, p-CS: p-cresol sulfate, Pol: phenol.

Table 2
Effects of 0.24 M sodium octanoate on PSHD spiked with point 3 C_U–C_M calibration curve.

	Pol	3-IAA	p-C	3-INDS	p-CS
Theorical value (μM)	126.2	36.85	305.3	715.5	352.5
Without Na octanoate (n = 10)					
Mean ± SD (μM)	124.9 ± 2.1	36.25 ± 0.85	304.1 ± 3.1	711.4 ± 10.2	348.5 ± 5.8
Mean recovery (%)	98.9	98.4	99.6	99.4	98.9
Range of recovery (%)	97.2–100.6	96.1–100.7	98.6–100.6	98.0–100.9	97.2–100.5
With Na octanoate (n = 10)					
Mean ± SD (μM)	119.4 ± 2.2	35.3 ± 0.8	302.7 ± 2.9	705.4 ± 9.9	346.4 ± 5.5
Mean recovery (%)	94.6	95.8	99.2	98.6	98.3
Range of recovery (%)	92.8–96.3	93.7–97.9	98.2–100.1	97.2–100.0	96.7–99.8

3-IAA, indole-3-acetic acid; 3-INDS, indoxyl sulfate; p-C, p-cresol; p-CS, p-cresol sulfate; Pol, phenol.

different chemical properties: hydrophobic for p-C, hydrophobic and polar for Pol, ionic and hydrophobic for 3-INDS, 3-IAA, and p-CS. Given these properties, different percentages of methanol or acetonitrile in TBAI were tested (data not shown). The best separation was achieved with acetonitrile and TBAI as described in materials and methods.

We have analyzed the effect of the addition of sodium octanoate as albumin binding competitor during sample preparation as described previously [31]. The results with and without sodium octanoate showed no significant differences (Fig. 1 and Table 2). Thus, our preparation of samples with ethanol leads to the complete release of uremic solutes from proteins.

In accordance with Martinez et al., we found no p-C in serum of CKD patients [30]. In many studies, acidic treatment of serum or plasma was used to deproteinize samples before biochemical analysis [33]. This treatment is not applicable to measurement of p-CS because it leads to acidic hydrolysis of the covalent bond between the phenol ring and sulfate group and allows separation of p-cresol and sulfate [30,34]. This is why several laboratories found p-C instead of p-CS in the serum or plasma of patients.

Previous works on quantitative analysis of these solutes have been reported but none analyzed five solutes in the same assay [29,31]. A similar ion-pairing liquid chromatography assay has been developed but only 3-INDS was quantified [29]. In another study, both uremic toxins 3-INDS and p-CS were quantified but the preparation of serum samples involved three steps: first with sodium octanoate to reverse albumin binding of uremic solutes, second with acetone to deproteinize samples, and third with dichloromethane to extract [31]. This method had the disadvantage to be time consuming and required the handling of dichloromethane. Moreover, in our method to prepare serum samples, the recovery of uremic solutes was not increased by the use of 0.24 M sodium octanoate.

3.2. Method validation results

3.2.1. Linearity

A linear relationship between peak areas and concentrations was found when known amounts of uremic solutes were added to healthy and hemodialyzed serum samples as described in materials and methods. The linear relationships for p-C, Pol, p-CS, 3-IAA, and 3-INDS were between 0 and 1500 μM. Regression analysis showed $r \geq 0.996$ for all compounds (Suppl Table 1).

3.2.2. Recovery

The ranges of recovery were between 98.9 and 100.6% for Pol, 97 and 101.7% for 3-IAA, 99.3 and 100.8% for p-C, 98.1 and 101.2% for 3-INDS, and 103.6 and 109.5% for p-CS (Table 3).

3.2.3. Reproducibility

The within-day CVs were between 0.96 and 2.43% for Pol, 1.23 and 3.12% for 3-IAA, 0.95 and 1.43% for p-C, 0.98 and 2% for 3-INDS, and 1.25 and 3.01% for p-CS. The between-day CVs were between 1.43 and 5.68% for Pol, 1.78 and 5.48% for 3-IAA, 1.21 and 1.85% for p-C, 1.45 and 4.54% for 3-INDS, and 1.19 and 6.36% for p-CS (Table 4).

3.3. Sample analysis results

The mean concentrations of 3-IAA, 3-INDS, and p-CS were 2.12 (±1.24), 1.03 (±1.28), and 13.03 (±10.05) μM respectively in serum of 40 healthy subjects and 5.9 (±5.28), 81.04 (±54.44), and 120.54 (±88.8) μM in 50 hemodialyzed patients. The 43 non hemodialyzed CKD group included patients with stage 3 to stage 5 CKD and displayed the mean following concentrations for 3-IAA, 3-INDS, and p-CS respectively: 3.21 (±3.32), 17.45 (±19.13), and 73.47 (±63.82) μM. The concentrations of these uremic toxins in non

Table 3
Analytical recovery of uremic solutes values assigned to control serum in the concentration ranges C_N–C_U and C_U–C_M.

Uremic solute	Number of runs	C_N to C_U concentration range (μM)	Mean recovery %	Ranges of recoveries %
Pol	10	10.6–53.1	99.5	98.9–100.6
3-IAA	10	1.01–5.05	99.4	97–101.7
p-C	10	37–185	100	99.3–100.8
3-INDS	10	39.8–199	99.6	98.1–101.2
p-CS	10	17.5–87.7	107.1	103.6–108.9
Uremic solute	**Number of runs**	**C_U to C_M concentration range (μM)**	**Mean recovery %**	**Ranges of recoveries %**
pol	10	42.5–212.5	100	99.7–100.4
3-IAA	10	10.1–50.5	99.9	99.4–100.4
p-C	10	92.5–462.6	100.1	99.7–100.6
3-INDS	10	199–995	99.9	99.5–100.3
p-CS	10	43.9–219.3	109	108.4–109.5

Recovery % = (final concentration − initial concentration) × 100)/added concentration. C_N, concentration found in normal serum; C_U, concentration found in uremic serum; C_M, maximal concentration found in uremic serum. 3-IAA, indole-3-acetic acid; 3-INDS, indoxyl sulfate; p-C, p-cresol; p-CS, p-cresol sulfate; Pol, phenol.

Table 4
Precision of determination of uremic solutes in serum.

	PolMean ± SD (μM)CV	3-IAAMean ± SD (μM)CV	p-CMean ± SD (μM)CV	3-INDSMean ± SD (μM)CV	p-CSMean ± SD (μM)CV
Within-day (n = 20)					
PSN	0	1.89 ± 0.03 1.68	0	1.55 ± 0.03 2.00	11.27 ± 0.34 3.01
PSN + point 1	10.13 ± 0.25 2.43	2.87 ± 0.03 1.23	36.72 ± 0.53 1.43	37.47 ± 0.54 1.44	32.22 ± 0.67 2.09
PSN + point 3	30.26 ± 0.29 0.96	5.00 ± 0.09 1.82	119.84 ± 1.27 1.06	117.60 ± 1.15 0.98	81.62 ± 1.55 1.77
PSHD	0	6.24 ± 0.19 3.12	0	117.33 ± 1.84 1.57	177.81 ± 3.00 1.69
PSHD + point 3	128.72 ± 2.72 2.11	40.03 ± 0.78 1.94	306.11 ± 3.37 1.10	710.57 ± 10.34 1.31	352.90 ± 6.12 1.69
PSHD + point 5	210.62 ± 2.26 1.07	60.68 ± 0.78 1.28	500.44 ± 4.74 0.95	1120.09 ± 12.11 1.01	470.49 ± 5.89 1.25
Between-day (n = 40)					
PSN	0	1.95 ± 0.11 5.48	0	1.57 ± 0.07 4.54	11.00 ± 0.70 6.36
PSN + point 1	9.73 ± 0.26 2.71	2.97 ± 0.15 4.96	36.92 ± 0.63 1.70	37.32 ± 1.07 2.85	32.82 ± 1.28 3.90
PSN + point 3	29.49 ± 1.68 5.68	5.10 ± 0.20 3.96	120.12 ± 1.85 1.54	117.07 ± 1.69 1.45	82.40 ± 1.96 2.24
PSHD	0	6.36 ± 0.23 3.55	0	118.63 ± 2.61 2.20	180.15 ± 3.51 1.95
PSHD + point 3	127.42 ± 3.48 2.73	40.27 ± 1.09 2.71	305.04 ± 5.64 1.85	713.20 ± 12.86 1.64	351.97 ± 8.41 2.32
PSHD + point 5	209.78 ± 3.01 1.43	61.01 ± 1.09 1.78	501.16 ± 6.09 1.21	1130.25 ± 21.19 1.78	471.71 ± 5.59 1.19

For each uremic solute, six samples of different concentrations were realized: pool of normal serum alone (PSN) and spiked with point 1 and point 3 of C_N–C_U curve and pool of hemodialyzed serum alone (PSHD) and spiked with point 3 and point 5 of C_U–C_M curve. 3-IAA, indole-3-acetic acid; 3-INDS, indoxyl sulfate; p-C, p-cresol; p-CS, p-cresol sulfate; Pol, phenol.

hemodialyzed CKD patients were inversely correlated to their renal function evaluated by the MDRD equation (Fig. 2). The spearman's correlation coefficients between 3-IAA, 3-INDS, and p-CS and the MDRD were respectively −0.32 ($p < 0.05$), −0.55 ($p < 0.0001$) and −0.51 ($p < 0.001$). Highest levels of 3-IAA, 3-INDS, and p-CS were found in hemodialyzed patients. We found no Pol and no p-C in any population.

Our results show that the amount of 3-INDS, 3-IAA, and p-CS are dramatically increased in serum of CKD patients, in agreement with previous studies [20,21,29–31,35]. This could be explained by the

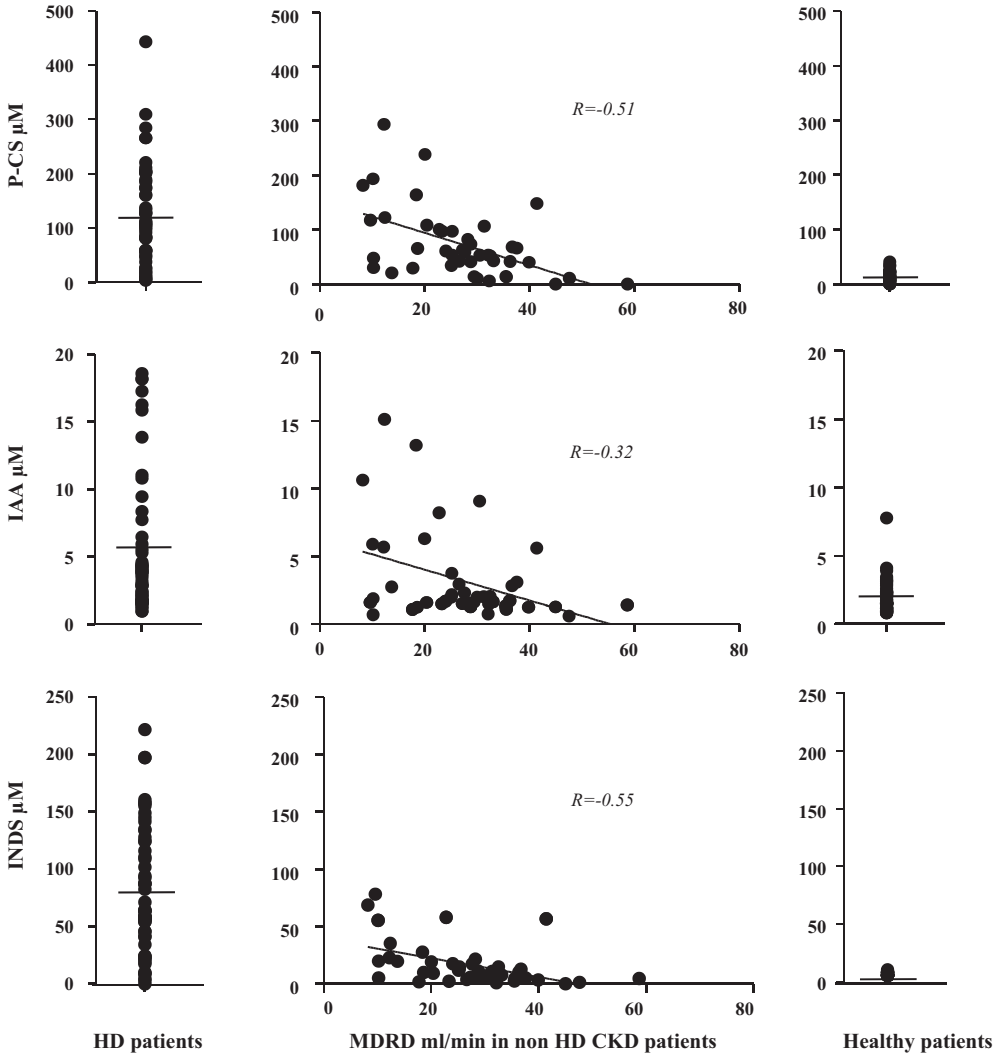

Fig. 2. Relationships between serum concentrations of uremic toxins and renal function. HD: hemodialyzed patients, 3-IAA: indole-3-acetic acid, 3-INDS: indoxyl sulfate, p-CS: p-cresol sulfate.

association between a bad renal elimination and an alteration of the intestinal flora of patients. Indeed, these patients displayed an increased aerobacteria such as *E. Coli* that mainly produced indole, the precursor of 3-INDS [25,28].

4. Conclusion

We have developed a rapid, simple and very sensitive high-performance liquid chromatographic (HPLC) method to quantify several uremic solutes in a unique sample of biological fluid: Pol, 3-IAA, p-C, 3-INDS, and p-CS. First, the preparation of samples is rapid since it involves deproteinization with ethanol and one centrifugation. Afterwards, supernatant is injected in the column, and the chromatograph corresponding to these uremic solutes is obtained in less than 20 min. Moreover, we use an isocratic flow instead of gradient flow, which avoids column re-equilibrium.

The toxic effects of these solutes have been largely demonstrated [10,13–24,35–37]. Thus, the easy and simultaneous determination of the concentration of these solutes would give the opportunity to monitor their serum levels in CKD patients. This method could be of clinical importance for the follow-up of these patients, the assessment of cardiovascular risk and the checking of blood purification adequacy during dialysis treatment.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.06.014.

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