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Study by means of high-performance liquid chromatography of solutes that decrease theophylline/protein binding in the serum of uremic patients

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Abstract

Substantial changes in protein binding of drugs occur during the progression of renal insufficiency. Protein-bound uremic solutes play a role in the inhibition of drug protein binding. We previously demonstrated that hippuric acid in uremic ultrafiltrate was an inhibitor of the theophylline protein binding. The present study was undertaken to extend the yield of protein-bound uremic solutes by displacing ligands in uremic serum from their binding sites by five deproteinization methods. The inhibitory effect on theophylline protein binding of the deproteinized uremic serum was higher than with ultrafiltrate ($p<0.05$). The influence of 30 semi-preparative HPLC fractions from deproteinized uremic serum on the theophylline protein binding was evaluated to identify the responsible compounds and to compare their relative individual impact. The theophylline protein binding was calculated as a percentage (bound versus total). The most important decrease of the protein binding was observed in HPLC fractions 6, 10 to 13, 15 and 28 with protein binding of: 61.5±10.8, 64.5±7.6, 60.9±10.1, 47.5±3.3, 60.0±6.7, 60.7±6.3 and 61.3±6.9%, respectively versus 69.1±2.4% for control serum ($p<0.05$). The responsible compounds were characterized in the fractions by co-elution: 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), indole-3-acetic acid, indoxyl sulfate, hippuric acid, *p*-hydroxyhippuric acid and tryptophan. Their concentration was determined by analytical HPLC and a solution containing these compounds at the same concentration as in deproteinized uremic serum was composed. This solution was added to control serum and decreased the theophylline protein binding from 69.0±4.4% to 61.3±1.3%, which was less important than in genuine uremic serum (44.4±3.8%, $p<0.05$). Dose–response curves with the characterized compounds revealed that the most important role in binding inhibition could be attributed to hippuric acid and CMPF. Our data suggests that the yield of protein binding inhibiting compounds is more important with deproteinized uremic serum than with uremic ultrafiltrate. The identified uremic compounds are not entirely representative for the decreased protein binding of theophylline, indicating that additional factors than those identified in this study affect the protein binding as well.

Keywords: Drug protein binding; Uremic retention solutes; Hippuric acid; CMPF; Theophylline

1. Introduction

Substantial changes in protein binding of drugs occur during the progression of renal insufficiency. Although some basic drugs show an increased binding, the most frequent alteration is a decrease of binding of mainly acidic drugs [1]. Various low-molecular-mass compounds in serum of uremic patients are protein bound [2,3]. Although some of them have been proposed to play a role in the

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inhibition of drug protein binding [4,5], few studies directly evaluate their presence in uremic biological fluids and compare their importance in relation to each other. In one of the few studies evaluating this problem, we demonstrated a role for hippuric acid [6]. The conclusion in this study is however limited by the fact that uremic ultrafiltrate and only a restricted number of semi-preparative HPLC fractions were studied. Whereas uremic ultrafiltrate undoubtedly contains uremic retention solutes, these are by definition not protein-bound; the bulk of protein-bound solutes were thus not available for evaluation. Furthermore, high-performance liquid chromatography (HPLC) was limited to the first eluates obtained during application of a linear gradient from 100% ammonium formate buffer to buffer–methanol (40:60). Such an approach does not yield the compounds with the strongest protein binding because these are mostly eluting at higher methanol concentration [7].

The present study was thus undertaken to evaluate the effect of deproteinized uremic serum on the protein binding of theophylline. A maximum displacement of the uremic ligands from their binding sites was obtained by five deproteinization methods and the yield was compared to conventional ultrafiltration. The influence of all HPLC fractions (up to a methanol concentration of 100%) of deproteinized uremic serum and of uremic ultrafiltrate on protein binding of theophylline was further evaluated. The responsible compounds in the inhibiting fractions were co-eluted at the same retention time with known compounds and their concentration was determined by analytical HPLC.

2. Experimental

2.1. Patients

A 20-ml blood sample was obtained from five healthy volunteers and from twelve hemodialyzed patients; the latter samples were obtained prior to the start of a dialysis session. Seven patients were treated with polysulfone (Rapido BLS 634, Sorin/Bellco, Mirandola, Italy) and five with hemophan membranes (GFS 1.6, Gambro, Lund, Sweden). Patients were anticoagulated with regular heparin (Heparin Leo, Leo Pharmaceutical, Ballerup, Denmark), containing no benzyl alcohol as preservative. Patients who took digoxin, theophylline, phenytoin, allopurinol and salicylates or related drugs were excluded from the study. Six hours before the collection of the blood, the intake of coffee, tea or chocolate as well as smoking were prohibited. The local ethics committee approved the study.

2.2. Reagents

All reagents were obtained from Sigma (St. Louis, MO, USA), except 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) which was a kind gift from G. Spiteller (Bayreuth, Germany). *p*-Hydroxyhippuric acid was obtained by the courtesy of A. Schoots (TU, Eindhoven, Netherlands); the latter compound was only available during the initial phase of the study. Water and methanol (HPLC grade) were purchased from Acros Organics (New Jersey, USA), ammonia solution was obtained from BDH (Poole, UK). [8-^{14}C]-Theophylline (250 μCi) was prepared by Amersham (Little Chalfont, UK). The reagents for the determination of urea, serum total protein and albumin were purchased from Sigma Diagnostics (St. Louis, MO, USA), the creatinine reagent was from Analis (Namur, Belgium).

2.3. Protein binding studies

Studies were performed with radiolabeled theophylline. The drug was dissolved with 5 ml methanol (2%) and further diluted (1:30) in an isotonic NaCl solution. The choice of theophylline was inspired by previous studies on a panel of drugs where the most substantial changes in protein binding had been observed for this compound (protein binding ±32% lower in hemodialysis patients compared to healthy controls) [6].

Protein binding was evaluated in vitro by mixing 0.25 ml serum with 50 μl theophylline, followed by incubation at 37°C for 30 min. The protein binding of theophylline was studied by ultrafiltration as described previously [6].

2.4. Dose–response studies

2.4.1. Influence of uremic ultrafiltrate

Ultrafiltrate from uremic serum pools was prepared by ultracentrifugation through Centrifree ultrafiltration membranes with a molecular mass (M_r) cut-off of 30 000.

The influence of uremic ultrafiltrate on protein binding of theophylline (evaluating the effect of free unbound fraction of solutes in uremic serum) was studied by adding control serum and theophylline to lyophilized uremic ultrafiltrate (Lyophilisator, GT2, Amsco/Finn-Aqua, Brussels, Belgium). To obtain a dose–response curve, different volumes (0.10, 0.25, 0.50, 1.00 and 1.50 ml) were lyophilized. The dry residues were redissolved in 0.25 ml control serum, 50 μl theophylline was added to each vial and the protein binding was assessed.

2.4.2. Influence of NaCl

By lyophilizing increasing volumes of uremic ultrafiltrate will contain increasing amounts of NaCl. For uremic ultrafiltrates ($n=3$), the respective sodium concentrations were 59±1 (0.10 ml), 137±2 (0.25 ml), 270±6 (0.50 ml), 541±6 (1.00 ml), 838±9 mmol/l (1.50 ml) (Klina System E2A, Beckman, CA, USA).

The effect of an increasing NaCl concentration on the protein binding of theophylline was therefore evaluated separately. Identical volumes of a 137 mmol/l NaCl solution as those used for the study of uremic ultrafiltrate (0.10, 0.25, 0.50, 1.00 and 1.50 ml), were lyophilized resulting in an added sodium concentration in each sample of respectively 55, 137, 274, 548 and 822 mmol/l. Control serum and theophylline were added to the dry substance and the protein binding was assessed.

2.4.3. Influence of deproteinized uremic serum

Uremic solutes were displaced from their protein binding sites using five different deproteinization methods, as previously described in detail [3]: (a) heat denaturation, (b) acetonitrile extraction, (c) trichloroacetic acid (TCA) deproteinization, (d) acid precipitation/acetonitrile extraction, (e) bilirubin displacement. In this earlier study these five methods appeared to be the most efficient for releasing bound solutes.

After these deproteinization procedures, samples corresponding to a volume of 0.10, 0.25, 0.50, 1.00 and 1.50 ml uremic serum were lyophilized, 0.25 ml control serum and 50 μl theophylline were added and the protein binding was evaluated.

This procedure was followed to compare the influence of the total (bound plus unbound) to the free (unbound) uremic solutes, obtained by ultracentrifugation.

2.5. Semi-preparative HPLC procedure – isolation and characterization of uremic compounds that decrease the theophylline protein binding

Uremic ultrafiltrates and deproteinized uremic sera were fractionated by semi-preparative HPLC, with a chromatograph consisting of two high-pressure pumps (2150), a gradient controller (2151), a solvent degasser (Degasys DG-1310) from Pharmacia (Bromma, Sweden) and two detectors in series: a UV detector at 254 nm (2238 Uvicord SII, Pharmacia) and a fluorescence detector adjusted on an excitation at 280 nm and an emission at 340 nm wavelength (RF530, Shimadzu, Tokyo, Japan). The analyses were performed on a Rsil RP C_{18} semi-preparative column (25 cm×10 mm) with a particle size of 10 μm (Biorad, Eke, Belgium). A guard column (5 cm×4.6 mm) with the same material was used to protect the main column. The injector (Valco, Houston, TX, USA) was provided with a loop of 2 ml. The solvent gradient was linear from 100% ammonium formate buffer (50 mmol/l, pH 4.0) to 60% methanol at 45 min and to 100% methanol at 60 min. The flow was 3 ml/min. The solvent and the column were kept at room temperature. The elution fractions were collected every 2 min during the 60 min of the HPLC run (Multirac 2111, Pharmacia).

2.5.1. Influence of HPLC fractions of uremic ultrafiltrate and deproteinized uremic serum

The eluents methanol and ammonium formate buffer were removed from the 30 fractions by evaporation in a vacuum centrifuge and lyophilization. The freeze–dried samples were then redissolved in 0.25 ml control serum for the determination of theophylline protein binding.

2.5.2. Influence of the HPLC eluents

To exclude the possible effects from the solvents, a blank HPLC procedure was run. The fractions were lyophilized and applied to the theophylline protein binding analysis.

2.5.3. Elution of trichloroacetic acid

As TCA-treated samples showed a more important inhibition of protein binding than other deproteinized samples, the same concentration TCA as used in the deproteinization of uremic serum (50 μl TCA of 612 mmol/l was added to 2 ml water), was submitted to HPLC as described above and the effect of the obtained fractions on the protein binding was evaluated.

2.5.4. Elution of bilirubin

After the addition of large amounts of bilirubin to serum for deproteinization, it may be possible that an excess of non-protein bound bilirubin remains present in the ultrafiltrate. The possibility should be considered that this bilirubin per se interferes with theophylline protein binding. To exclude this possibility, bilirubin was added to normal serum [3], and submitted to HPLC with UV detection. Furthermore, the bilirubin elution fractions were dried, after which control serum and theophylline were added to the dry substance and the protein binding was assessed.

2.5.5. Dose–response effect of HPLC fractions of heat-deproteinized uremic serum

To increase the concentration of uremic solutes in the HPLC fractions, 4 ml heat-deproteinized uremic serum was fractionated, lyophilized and processed as described above; the results were compared with the effect of similar lyophilized fractions obtained from 2 ml heat-deproteinized uremic serum, dissolved in 0.25 ml normal serum.

2.5.6. Influence of normal ultrafiltrate

To demonstrate that particularly ''uremic compounds'' were responsible for the effect on the protein binding of theophylline; the semi-preparative HPLC method was applied to normal ultrafiltrate. A 2-ml sample of normal ultrafiltrate from control serum was prepared by ultracentrifugation with a Centrifree filter, fractionated by semi-preparative HPLC and evaluated for its effect on protein binding.

2.5.7. Characterization of substances responsible for protein binding inhibition

To characterize substances potentially responsible for the binding inhibition, the co-elution of several known uremic compounds with peaks in the chromatogram of deproteinized uremic serum was performed. The standard solution included: 42 μmol/l CMPF, 2210 μmol/l creatinine, 409 μmol/l hippuric acid, 102 μmol/l *p*-hydroxyhippuric acid, 29 μmol/l hypoxanthine, 17 μmol/l indole-3-acetic acid, 40 μmol/l indoxyl sulfate, 41 μmol/l pseudouridine, 25 μmol/l tryptophan, 133 μmol/l tyrosine and 71 μmol/l uric acid. All compounds were measured with UV detection, except indole-3-acetic acid, indoxyl sulfate and tryptophan, which were measured with fluorescence detection. To characterize the uremic compounds in the fractions, HPLC was performed on samples that had been submitted to one of the following procedures: (1) addition of 1 ml of the standard solution to 3 ml HPLC-grade water; (2) addition of 1 ml HPLC-grade water to 3 ml heat-deproteinized uremic serum; (3) addition of 1 ml of the standard solution to 3 ml heat-deproteinized uremic serum. Peaks in the heat-deproteinized uremic serum were identified as a given compound when co-elution with a known substance of the standard solution was observed.

2.6. Analytical HPLC procedure – concentration of uremic compounds in semi-preparative HPLC fractions

To measure the concentration of the compounds that had been characterized in the fractions with a decreased protein binding, the semi-preparative HPLC fractions were submitted to analytical HPLC; the latter was applied with the same procedure as mentioned above, except for the columns which were filled with 5 μm particles (C_{18}, Ultrasphere ODS, Beckman, San Ramon, USA) and for the flow-rate which was 1 ml/min. An integrator (2221, Pharmacia) registered peak heights.

Uremic serum was treated as described above. The lyophilized fractions containing known uremic solutes which decreased protein binding obtained from 2 ml uremic ultrafiltrate or deproteinized uremic serum were redissolved in 1 ml water. A stock standard solution containing 417 μmol/l CMPF,

1637 μmol/l hippuric acid, 1020 μmol/l *p*-hydroxy-hippuric acid, 571 μmol/l indole-3-acetic acid, 318 μmol/l indoxyl sulfate and 196 μmol/l tryptophan was diluted 1/16, 1/8, 1/4 and 1/2. One ml of internal standard of 48 μmol/l naphthalene sulfonic acid was added to 1 ml sample or standard solution and 50 μl was injected on the column. A calibration line was made for each substance and the concentrations in the fractions were determined.

2.7. Uremic solutes

2.7.1. Dose–response effects of solutes on theophylline protein binding

The following five known uremic solutes were dissolved and diluted to different concentrations, in the range of those currently observed in renal failure: CMPF (104, 208, 417, 833 and 1667 μmol/l), hippuric acid (409, 1637, 2456, 3274 and 4093 μmol/l), indole-3-acetic (29, 57, 285, 571 and 1142 μmol/l), indoxyl sulfate (40, 199, 398, 796 and 1592 μmol/l) and tryptophan (49, 122, 245, 490 and 979 μmol/l). The solutions were lyophilized and added to control serum.

2.7.2. Prepared solution mimicking the composition of deproteinized uremic serum

To know whether the observed inhibition of protein binding by the deproteinized uremic sera was entirely attributable to the compounds identified by the characterization procedure, the inhibitory effect of a solution containing these compounds at the total concentrations found in uremic serum were compared to drug protein binding in the original uremic serum pool and in control serum and at concentrations 2-, 4-, 6- and 8-times higher than in uremic serum to evaluate a dose–response effect.

2.8. Statistical evaluations

Values are given as means±standard deviation (SD). Statistical comparison between individual means of variables were performed with the Wilcoxon's test for paired values and Mann–Whitney *U* test for unpaired values. Analysis of variance (ANOVA) was performed for groups of variables.

3. Results

3.1. General characteristics of the uremic and control sera

In the control sera the mean concentrations were: urea 5±2 mmol/l, creatinine 75±27 μmol/l, total protein 7.2±0.3 g/100 ml and albumin 4.1±0.4 g/100 ml.

The mean concentrations in the pre-dialysis sera of the 12 patients were: urea 28±6 mmol/l, creatinine 1070±265 μmol/l, total protein 6.6±0.4 g/100 ml and albumin 3.9±0.3 g/100 ml.

3.2. Dose–response studies

3.2.1. Influence of uremic ultrafiltrate

A progressive decline of theophylline protein binding was found when increasing amounts of lyophilized uremic ultrafiltrate ($n=7$) were added to control serum; statistical significance was found from 0.25 ml added lyophilized volume on ($p<0.05$) (Table 1).

3.2.2. Influence of NaCl

Saline by itself also had an inhibitory effect on the theophylline binding, which however was less important than for uremic ultrafiltrate. When 55, 137, 274, 548 and 822 mmol/l lyophilized NaCl ($n=6$) were added to control serum, the protein binding decreased from 67.1±1.3 to 62.7±2.2, 60.7±1.8, 57.7±2.7, 54.8±3.3 and 53.8±1.9% (ANOVA, $p<0.001$). A significant difference with uremic ultrafiltrate was found for the two highest concentrations of NaCl ($p<0.05$).

3.2.3. Influence of deproteinized uremic serum

A progressive decline in theophylline protein binding versus control was found when increasing quantities of lyophilized uremic samples, obtained with the five deproteinization methods, were added to control serum (Table 1) (ANOVA, $p<0.05$ versus ultrafiltrate for all methods). The most pronounced decrease of protein binding was obtained with trichloroacetic acid deproteinization ($n=5$). Furthermore, a significant decrease versus the ultrafiltration method was also found for all lyophilized volumes with acetonitrile extraction ($n=7$) and bilirubin

Table 1
Dose–response effect of lyophilizate of uremic ultrafiltrate and deproteinized uremic serum on theophylline protein binding[a]

Methods to obtain uremic ultrafiltrate or deproteinized uremic serum	Percentage protein binding					
	Lyophilized volumes (ml)					
	0.0	0.10	0.25	0.50	1.00	1.50
Ultrafiltration	69.2±3.8	64.7±1.6	61.9±4.4*	55.8±5.6*	47.2±6.0*	41.1±5.1*
Deproteinization						
Heat denaturation	68.7±4.1	63.2±2.8	59.6±6.4 *	46.4±8.4 * °	36.9±5.5 * °	33.2±3.3 * °
Trichloroacetic acid deproteinization	67.0±3.0	29.6±3.8 * °	18.2±1.6 * °	13.1±2.4 * °	15.2±2.9 * °	18.0±2.5 * °
Acetonitrile extraction	67.4±4.6	56.2±0.9 °	56.6±4.2 * °	47.6±3.9 * °	36.5±3.7 * °	29.5±2.1* °
Acid precipitation/acetonitrile extraction	68.9±1.9	67.2±0.8	60.8±1.8 *	54.9±0.6 *	29.5±2.9 * °	23.6±1.3 * °
Bilirubin displacement	66.3±2.1	60.5±1.9 °	52.9±1.8 * °	43.3±2.9 * °	33.1±4.4 * °	31.7±5.6 * °

[a] * $p<0.05$ versus control serum (0.0 ml), ° $p<0.05$ versus ultrafiltration.

displacement ($n=5$); for 0.25, 0.50, 1.00 and 1.50 ml with heat denaturation ($n=7$); and for 1.00 and 1.50 ml with acid precipitation/acetonitrile extraction ($n=6$) ($p<0.05$). Overall, these data suggests that the yield of protein binding inhibiting compounds was more important with deproteinization.

3.3. Semi-preparative HPLC

3.3.1. Influence of HPLC fractions of uremic ultrafiltrate and deproteinized uremic serum

Table 2 illustrates the protein binding values for each of the 30 HPLC fractions eluted from the uremic ultrafiltrate or the deproteinized uremic serum samples. A significant inhibition versus control serum was observed in fractions 3, 6, 12 and 13 from ultrafiltration ($n=9$); in fractions 3, 4, 6, 10 to 13 and 28 from heat denaturation ($n=8$); in fractions 3, 5 to 9, 12 and 13 from trichloroacetic acid ($n=7$); in fractions 3, 10, 12, 15 and 28 from acetonitrile extraction ($n=7$); in fractions 3, 10, 12, 13 and 28 from acid precipitation/acetonitrile extraction ($n=7$); in fractions 3, 4, 6 and 12 from bilirubin displacement ($n=7$). Representative chromatograms of the semi-preparative HPLC separation of the heat-deproteinized uremic serum and of uremic ultrafiltrate are illustrated in Fig. 1.

When considering these results fraction by fraction, a number of changes could be attributed to interfering factors. Overall, the most important changes were registered in fraction 3; this fraction however contains sodium. NaCl as such affects theophylline protein binding; also in fraction 4 a minor quantity of sodium was found. A substantial inhibition of protein binding was found in HPLC fractions 5 to 9 obtained from the trichloroacetic acid method. HPLC analysis of similar quantities of TCA alone, revealed a theophylline protein binding of respectively 35.4, 7.9, 12.1, 30.0 and 50.0% for fractions 5 to 9. Subsequently, inhibition found in those fractions can largely be explained by the presence of TCA. Deproteinization of serum by the bilirubin displacement method resulted in non-protein bound bilirubin in the serum filtrate. This non-protein bound bilirubin eluted in fractions 6 and 7 and decreased the theophylline protein binding from 70.9±1.5 to 65.0±2.0% ($n=3$).

The genuine inhibitory effect on the theophylline protein binding was most pronounced in fraction 12 in all methods. When comparing the individual fractions of the five deproteinization methods versus ultrafiltration, after exclusion of the abovementioned fractions with potential interference, additional protein binding inhibition by deproteinization was obtained in fractions 11 and 28 for heat denaturation, in fraction 15 for acid precipitation/acetonitrile extraction and in fraction 13 for bilirubin displacement ($p<0.05$) (Table 2).

No inhibitory effect was observed when the lyophilized fractions of a blank HPLC run were submitted to the protein binding experiments.

3.3.2. Dose–response effect of HPLC fractions of heat-deproteinized uremic serum

The semi-preparative HPLC separation of extra concentrated heat-deproteinized uremic serum ($n=2$)

Table 2
Percentage protein binding of theophylline after the addition of lyophylized HPLC fractions of uremic ultrafiltrate or deproteinized uremic serum[a]

	Method					
	Ultrafiltration	Heat denaturation	Trichloroacetic acid deproteinization	Acetonitrile extraction	Acid precipitation/ acetonitrile extraction	Bilirubin displacement
Control	69.2±1.3	69.1±2.4	68.6±0.9	68.9±1.1	70.4±1.0	70.2±1.9
Fractions						
1	69.9±1.8	70.3±2.9	70.1±2.5	69.5±1.0	69.7±2.1	70.4±1.8
2	70.7±2.0	70.9±3.0	66.6±9.3	69.7±1.6	71.4±1.5	68.8±6.8
3	37.4±2.7*	38.6±4.4*	39.5±2.3*	31.9±5.9*	44.1±7.6*	38.3±4.0*
4	66.3±5.2	63.6±4.7*	66.1±8.9	67.3±2.3	68.6±2.0	65.5±7.5*
5	69.9±2.4	67.8±4.2	16.2±4.3*	69.1±2.0	70.1±1.8	65.9±8.2
6	66.8±1.8*	64.6±6.8*	18.6±8.1*	68.3±2.5	69.6±1.6	61.5±10.8*
7	69.8±1.9	69.5±3.5	24.1±10.7*	68.3±2.7	71.5±1.8	67.0±9.7
8	68.9±2.1	63.9±6.9	32.5±8.2*	67.2±1.9	70.6±0.9	67.7±3.4
9	67.6±2.7	65.9±4.9	55.6±13.6*	67.3±2.8	67.8±3.3	68.0±1.3
10	68.2±2.3	66.0±2.8*	66.0±3.0	66.5±2.0*	64.5±7.6*	68.6±1.6
11	69.2±2.0	60.9±10.1*°	68.5±1.2	65.7±4.3	70.5±1.3	66.9±5.0
12	61.0±5.1*	58.0±7.7*	47.5±3.3*	52.4±10.2*	55.1±10.1*	57.5±9.1
13	62.1±4.9*	60.0±6.7*	60.3±6.3*	66.0±5.0	68.0±2.7*	61.6±11.5* °
14	70.1±1.4	69.4±5.8	69.5±1.3	67.6±3.4	70.6±1.4	68.6±5.2
15	70.6±1.4	67.0±4.8	66.8±2.5	60.7±6.3* °	71.9±2.3	68.2±7.8
16	69.3±2.9	68.3±5.2	69.0±1.7	68.8±3.5	69.9±1.9	69.2±3.1
17	69.4±3.0	67.4±5.4	69.7±1.3	67.2±6.7	69.9±2.0	70.3±2.1
18	69.1±3.4	71.2±4.7	70.0±1.6	68.6±6.8	70.7±1.6	72.8±4.1
19	70.0±2.0	70.0±3.9	69.6±1.5	69.2±2.1	70.7±1.7	73.0±7.7
20	69.8±3.1	68.7±3.4	70.6±1.8	69.0±3.2	70.9±1.4	70.3±2.5
21	69.2±4.0	68.3±6.6	69.7±1.3	70.6±2.6	70.8±1.1	71.1±2.8
22	71.1±2.7	69.6±3.0	70.6±1.7	71.7±4.6	70.7±1.4	70.3±2.4
23	71.2±1.7	69.9±2.1	69.8±1.2	70.7±2.8	71.2±1.3	70.6±1.9
24	70.7±2.1	70.2±2.3	70.2±1.1	69.7±2.8	71.1±2.5	70.6±1.9
25	70.9±1.9	70.2±2.5	69.9±1.6	70.3±2.4	71.2±1.5	70.6±2.1
26	70.9±2.2	70.7±2.8	69.9±2.6	70.4±4.0	71.9±2.1	70.8±1.5
27	69.0±2.1	70.0±3.6	68.6±2.3	70.4±3.6	71.3±2.1	70.9±2.1
28	69.5±3.2	61.3±6.9* °	69.0±1.9	65.7±4.6*	69.1±1.9*	70.3±2.8
29	70.7±1.7	67.6±4.8	69.6±2.0	70.1±2.8	71.0±3.5	70.6±2.4
30	69.9±1.8	70.0±3.5	69.4±1.4	68.7±5.9	71.7±1.9	69.7±2.3

[a] * $p<0.05$ versus control, ° $p<0.05$ versus ultrafiltration.

resulted in a more pronounced decrease of protein binding in several fractions, compared to the less concentrated sample (ANOVA, $p<0.01$) (Table 3).

3.3.3. Influence of normal ultrafiltrate

The lyophilized fractions of normal serum ultrafiltrate ($n=3$) did not decrease the protein binding of theophylline, except in fraction 3 where NaCl eluted. A representative chromatogram of normal ultrafiltrate is given in Ref. [6].

3.3.4. Characterization of substances responsible for protein binding inhibition – co-elution of deproteinized uremic serum with known retention compounds

When chromatographing the standard composed for the spiking procedure, 10 of the 11 compounds co-eluted with peaks of deproteinized uremic serum. The fractions in which they eluted were fraction 4 for creatinine, 5 for pseudouridine, 6 for uric acid, 7 for hypoxanthine, 9 for *p*-hydroxyhippuric acid, 11 for indoxyl sulfate, 12 for hippuric acid and

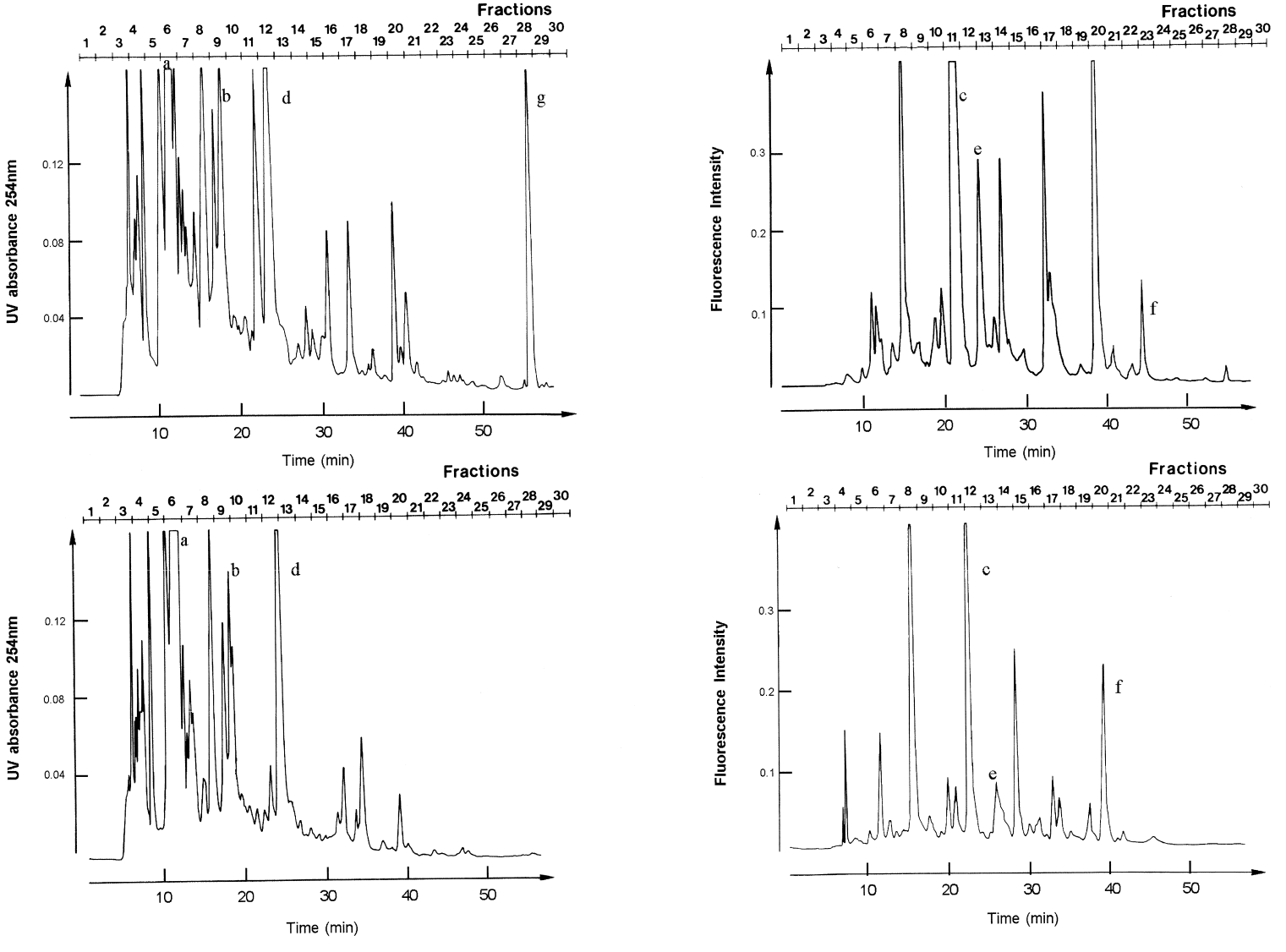

Fig. 1. Representative chromatograms of heat-deproteinized uremic serum (above) and uremic ultrafiltrate (below); the left panel represents the UV detection and the right panel gives the fluorescence detection. The yield of peaks and peak heights are markedly more important for the deproteinized uremic serum. (a to f) Peaks corresponding to the identified compounds; a=uric acid, b=*p*-hydroxyhippuric acid, *c*=indoxyl sulfate, d=hippuric acid, e=tryptophan, f=indole-3-acetic acid and g=CMPF.

Table 3
Effect on theophylline protein binding of HPLC fractions of heat-deproteinized uremic serum and of 2× concentrated heat-deproteinized uremic serum added to control serum

Fraction	Protein binding (%)	
	Heat denaturation method	Heat denaturation method[a] (sample 2× concentrated)
9	65.9±4.9	56.9±4.0
12	58.0±7.7	32.9±8.9
14	69.4±6.7	53.7±4.7
15	67.1±4.8	55.7±4.0
20	69.6±3.0	63.7±5.0
28	61.3±6.8	44.3±9.2

[a] * $p<0.01$ by ANOVA.

tryptophan, 20 for indole-3-acetic acid, and 28 for CMPF (Table 4).

A decreased theophylline protein binding was not observed in fractions 4, 5 and 7, excluding a role for creatinine, pseudouridine and hypoxanthine. The protein binding was significantly different versus control in fraction 6 obtained from ultrafiltration and heat denaturation (Table 2); however uremic ultrafiltrate and deproteinized serum showed a similar peak height for uric acid; therefore the role of uric acid could also be excluded (Fig. 1).

Uremic retention compounds in the fractions 9, 11, 12, 20 and 28 inhibited theophylline protein binding either with the ultrafiltrated and deproteinized samples or with the concentrated heat denatured samples (Table 4); they were shown to contain *p*-hydroxyhippuric acid, indoxyl sulfate, hippuric acid, tryptophan, indole-3-acetic acid and CMPF.

3.4. Analytical HPLC – concentration of uremic solutes in semi-preparative HPLC fractions

Only six substances (*p*-hydroxyhippuric acid, indoxyl sulfate, tryptophan, hippuric acid, indole-3-acetic acid and CMPF) were retained as potential inhibitors. Their concentrations were determined by the same chromatographic procedure as described in a previous study [3], with a minor modification to detect CMPF ($n=3$). The compounds, except CPMF, have been identified with diode array detection (DAD) (photodiode array detection) in an earlier work [3].

In deproteinized uremic serum, in general higher concentrations for the six solutes were found than in uremic ultrafiltrate, pointing out their protein bound nature (Table 5). The highest concentrations were: *p*-hydroxyhippuric acid (fraction 9) 155±9 μmol/l, indoxyl sulfate (fraction 11) 132±2 μmol/l, hippuric acid (fraction 12) 532±38 μmol/l, tryptophan (fraction 12) 26±3 μmol/l, indole-3-acetic acid (fraction 20) 29±24 μmol/l and CMPF (fraction 28) 233±32 μmol/l. These concentrations were used in the prepared uremic solution, mimicking the com-

Table 4
Characterization of substances in semi-preparative HPLC fractions of deproteinized uremic serum

Elution fraction	Substances in spiking solution	Co-elution with peaks in deproteinized uremic serum	Fractions with a decreased protein binding	Peaks increasing in height in deproteinized uremic serum vs. uremic ultrafiltrate	Detection method
4	Creatinine	+	−	−	UV[a]
5	Pseudouridine	+	−	−	UV
6	Uric acid	+	+	−	UV
7	Hypoxanthine	+	−	−	UV
7	Tyrosine	−	−	−	FL[b]
9	*p*-Hydroxyhippuric acid	+	+	+	UV
11	Indoxyl sulfate	+	+	+	FL
12	Hippuric acid	+	+	+	UV
12	Tryptophan	+	+	+	FL
20	Indole-3-acetic acid	+	+	+	FL
28	CMPF	+	+	+	UV

[a] UV=UV detection at 254 nm.
[b] FL=Fluoresence detection at 280 nm/340 nm as excitation/emission wavelengths.

Table 5
Concentrations (μmol/l) of six identified substances in uremic ultrafiltrate and deproteinized uremic serum

Substance	Method					
	Ultrafiltration (n=3)	Heat denaturation (n=5)	Trichloroacetic acid deproteinization (n=5)	Acetonitrile extraction (n=3)	Acid precipitation/ acetonitrile extraction (n=4)	Bilirubin displacement (n=4)
p-Hydroyhippuric acid	135±14	155±9	153±6	113±27	165±16	144±6
Indoxyl sulfate	10±4	105±8	88±6	132±2	26±3	43±16
Hippuric acid	418±25	524±16	446±29	532±38	491±12	442±11
Tryptophan	7±4	25±1	26±3	24±3	10±3	8±4
Indole-3-acetic acid	4±1	22±2	6±1	29±24	4±1	9±3
CMPF	0.00	233±32	5±2	95±2	99±16	4±1

position of deproteinized uremic serum (see Section 2.7.2).

3.5. Uremic solutes

3.5.1. Dose–response effects of solutes on theophylline protein binding

The dose–response curves for hippuric acid, CMPF, indoxyl sulfate, indole-3-acetic acid and tryptophan are illustrated in Fig. 2 (for each concentration, n=3). There was not enough *p*-hydroxyhippuric acid available to perform dose–response studies.

An intermutually significant difference in protein binding was observed with increasing concentrations of CMPF, hippuric acid (ANOVA, $p<0.01$) and of indoxyl sulfate (ANOVA, $p<0.05$). No significantly different protein binding was observed for tryptophan and indole-3-acetic acid.

3.5.2. Prepared solution mimicking the composition of deproteinized uremic serum

A solution mimicking the composition of deproteinized uremic serum, containing the compounds characterized in the present study, showed a depression for the unconcentrated sample (61.30±1.30%) (n=3), which was lower than the protein binding observed in control serum (69.0±4.4%) (n=6) ($p<$ 0.05). An inhibition comparable to that of genuine uremic serum (44.4±3.8%) (n=6) was only observed after a four-fold concentration of the sample (each n=3) (Fig. 3).

4. Discussion

This study evaluates the effect of protein-bound solutes, retained in the serum of hemodialyzed end-stage renal failure patients, on the protein binding of theophylline. As demonstrated before [6], the addition of lyophilized uremic ultrafiltrate to control serum inhibited protein binding of theophylline in a dose dependent way. In addition, inhibition was more pronounced if uremic serum was first deproteinized, but the yield was slightly different and depended on the deproteinization method used. Separation of the deproteinized uremic serum by semi-preparative reversed-phase HPLC in 30 fractions, revealed that some specific fractions were playing a more important role in this binding inhibition. The protein binding was most decreased in fraction 3; this was attributed to NaCl. We were able to identify by co-elution experiments some of the compounds eluting in these fractions and six compounds were considered to be potential candidates to affect protein binding of theophylline: CMPF, hippuric acid, *p*-hydroxyhippuric acid, indole-3-acetic acid, indoxyl sulfate and tryptophan. After determination by analytical HPLC of the concentration of the identified compounds in the fractions, an uremic solution, mimicking the total concentration of these compounds in uremic serum, was prepared to evaluate their cumulative effect. This procedure indicated that protein binding of theophylline is dependent on more factors than those identified in this study alone. Dose–response curves with the identified compounds, revealed that the main patho–physiological role for the identified ligands can be attributed to

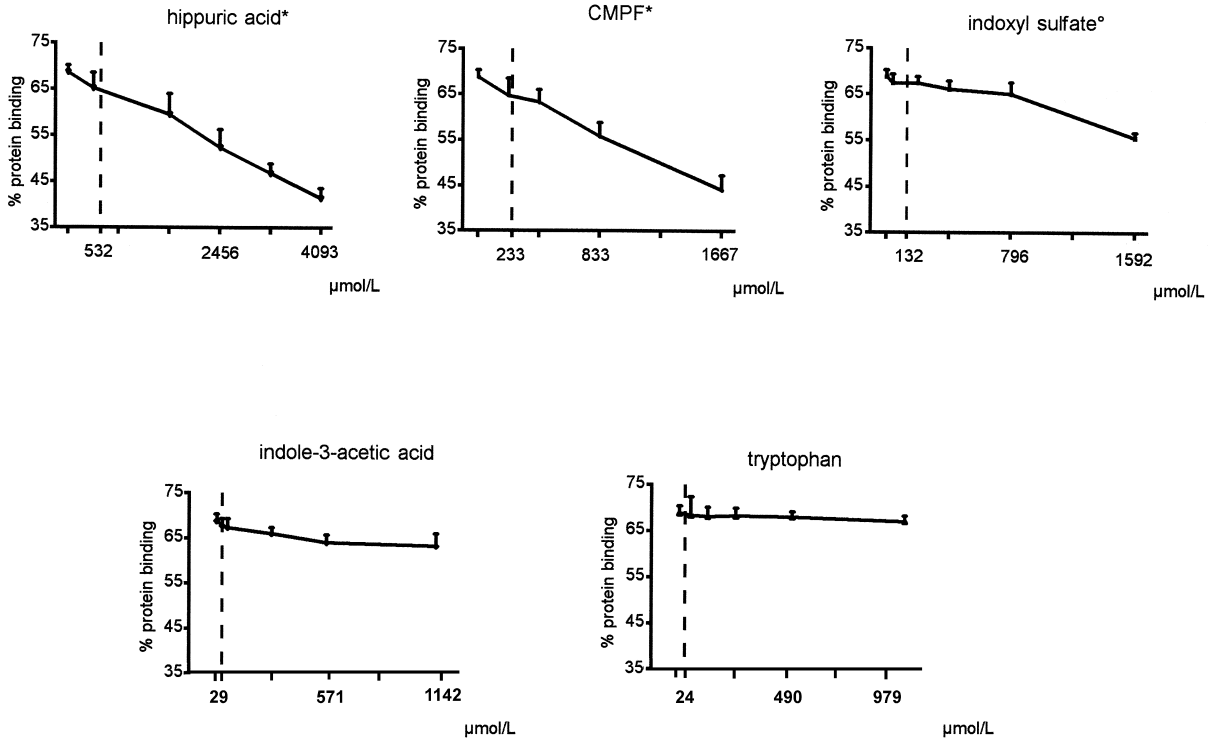

Fig. 2. Effect on theophylline protein binding of increasing concentrations of uremic retention solutes added to control serum intermutual compared, * $p<0.01$ and ° $p<0.05$ by ANOVA. The dotted lines indicate the concentration of each compound in the prepared uremic solution.

hippuric acid and CMPF, whereas indoxyl sulfate might also play an inhibitory role. Indole-3-acetic acid and tryptophan were less important, at least at the concentrations found in the uremic serum of uremic patients.

In a previous study from our group, directly evaluating the uremic factors responsible in the inhibition of drug protein binding, only hippuric acid could be demonstrated to play an active role [6]. The latter study was however undertaken only on uremic ultrafiltrate, which can be supposed to contain mainly free non-protein bound solutes. Hence, the more important the protein binding of a given substance, the lower its yield with ultrafiltration. Later studies revealed that active deproteinization of uremic serum increased the yield of protein-bound compounds [3]. Therefore, in the present study, uremic sera were deproteinized before their evaluation.

Furthermore, in the initial study from our group, HPLC was limited to the eluates of an ammonium formate buffer–methanol (40:60) gradient [6]. The HPLC elution of a given compound depends on its hydrophobicity and on the procentual concentration of the nonpolar solvent in the gradient. Hydrophobic compounds are often at the same time strongly protein-bound. Therefore, in the present study, the semi-preparative HPLC elution was continued until a 100% methanol concentration was obtained. These two modifications of the protocol enabled us to characterize several additional compounds: *p*-hydroxyhippuric acid, indoxyl sulfate, tryptophan, indole-3-acetic acid and CMPF.

When mimicking the composition of deproteinized uremic serum, based on the serum concentration of the compounds identified in this study, an inhibition not as in genuine uremic serum important could be observed. The higher protein binding with the prepared uremic solution compared to the genuine uremic serum suggests that additional ligands than those identified at present compete with drug protein

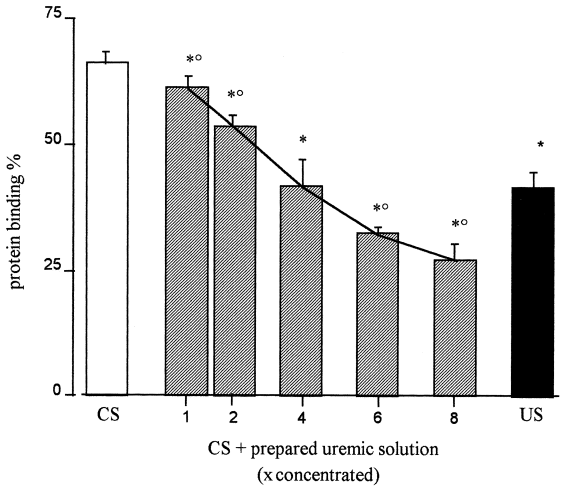

Fig. 3. Protein binding of theophylline in control serum, in control serum after the addition of 1×, 2×, 4×, 6× and 8× concentrated prepared uremic solution, (for composition, see Table 4) and in uremic serum; * $p<0.01$ (ANOVA), ** $p<0.01$ versus control serum and ° $p<0.05$ versus uremic serum (Mann–Whitney U test).

binding. One can speculate about the nature of these substances; it is clear that some of the fractions inhibiting theophylline protein binding contain more peaks than those identified at present.

Furthermore, protein-bound compounds or groups of substances, which are not detected by UV absorbance or fluorescence, could also play a role, such as free fatty acids [8], or polyamines [9]. Free fatty acids increase the unbound theophylline [10,11]; however, no increased concentration of fatty acids is observed in pre-dialysis uremic samples [12]. No data are as yet available on the effect of polyamines on theophylline protein binding. More sophisticated detection and identification methods, such as photodiode-array detection, light scattering detection, liquid chromatography–mass spectrometry or gas chromatography–mass spectrometry and nuclear magnetic resonance might be helpful to reveal the true nature of additional compounds that affect theophylline protein binding.

An alternative explanation for the lower protein binding in uremic serum compared to the prepared solution of known uremic compounds could be an altered configuration of albumin or of other binding proteins in the plasma of patients with end-stage renal disease. From older studies, it appears that alterations of albumin structure, if any, are insufficient to change rug protein binding in end-stage renal disease [13,14]. Recently, structure modification of proteins by glycosylation (advanced glycosylation end products – AGEs) has been emphasized [15]. Albumin is one of the potential (major) targets for AGE binding [16]. AGE-modification might alter the capacity of albumin to bind drugs [17]. Oxidation and carbamylation may also induce structural modifications of albumin [18–20].

Several peaks in fractions not inhibiting theophylline protein binding show a rise in concentration after deproteinization (Fig. 1), indicating that many retention solutes bind to other binding sites than theophylline, and hence do not compete with theophylline protein binding.

Although enhanced free concentration in end-stage renal disease (ESRD) is possibly compensated by alternative metabolic pathways such as increased hepatic metabolism and/or decreased intestinal absorption [21], changes in protein binding of drugs in ESRD might increase drug toxicity, as the free, unbound compound is biologically active [22]; in addition many metabolic pathways are suppressed in uremia as well. The risk of toxicity is further enhanced by the current trend to monitor total drug concentration, without taking into account eventual shifts in free fraction. The cumulative result of this toxicity is difficult to estimate, as uremic patients are often treated by multimedication, with drugs that are at least in part cleared by the kidneys, so that a complex but potentially morbid condition ensues. Further studies are needed to define ore clearly the impact of these changes on patient outcome.

It is concluded that among uremic compounds mainly hippuric acid and CMPF displace theophylline from its protein binding sites. A prepared uremic solution decreased less the theophylline protein binding than genuine uremic serum, suggesting that additional, not yet identified factors play also a role in the inhibition of uremic protein binding.

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