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Drug protein binding in chronic renal failure: Evaluation of nine drugs

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Drug protein binding in chronic renal failure: Evaluation of nine drugs. In this study, changes of protein binding of nine drugs were evaluated. In addition, theophylline and phenytoin, the two drugs with the most substantial and progressive decrease in protein binding, were further studied by high performance liquid chromatography (HPLC)-fractions of ultrafiltrate of normal and uremic serum, in an attempt to identify substances causing drug protein binding inhibition. There was a marked decline of the protein binding of theophylline, phenytoin and methotrexate (dialyzed patients vs. normals: −20.1, −16.0 and −15.1%, respectively). There was a rise in the protein binding of propranolol, cimetidine and clonidine. The changes observed for diazepam, prazosin and imipramine were less marked. For phenytoin, theophylline, methotrexate and diazepam, protein binding was inversely correlated to the serum creatinine ($r = 0.87$, 0.80, 0.79 and 0.67, $P < 0.001$), and a less pronounced but still significant positive correlation was found for clonidine ($r = 0.46$, $P < 0.01$). Ultrafiltrate, obtained during a hemofiltration session, inhibited protein binding of theophylline and phenytoin in a dose dependent way. After separation of this ultrafiltrate by HPLC, it appeared that for both theophylline and phenytoin at least a part of this inhibitory activity corresponded to the elution zone of hippuric acid. For theophylline two other inhibitory zones were further recognized: one corresponding to the elution zone of NaCl and one in which the responsible substance remained unidentified. Hippuric acid in solution inhibited protein binding of theophylline and phenytoin in a dose dependent way. In conclusion, protein binding of several drugs currently used in renal failure is affected in parallel with renal function, which might affect the therapeutic effectiveness of the drugs. Furthermore, hippuric acid appears to play an important role in the defect of the protein binding of theophylline and phenytoin.

The binding of drugs to plasma proteins in uremia remains a source of major concern. The knowledge of binding percentages and possible displacement effects is clinically important in the adjustment of the administered dose to avoid intoxication and/or undertreatment, in particular since the pharmological action of drugs is dependent on their free, unbound fraction. In contrast, the clinical monitoring of blood concentrations of most drugs is based on determinations of total (bound plus unbound) concentrations; thus changes in the fraction of bound/unbound drug should be taken into account to avoid possible side effects and to maximize drug effectiveness.

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Renal failure is thought to be associated with a change in protein binding of drugs. The available literature applies to several drugs [1–9], but a number of substances that are frequently used in renal failure have not been evaluated. Furthermore, studies investigating the factors that may be responsible for changes in drug protein binding have been scarce, and some of them based on indirect approaches, such as the analysis of normal urine [10], or the use of non-biological solutions [11].

Therefore, the present study was undertaken to evaluate dynamic changes of protein binding of nine drugs that are commonly used in renal failure patients. In addition, a further study was undertaken in which theophylline and phenytoin, the two drugs with the most substantial and progressive decrease in protein binding, were submitted to further evaluation by high performance liquid chromatography (HPLC)-fractions of ultrafiltrate of normal and uremic serum, in an attempt to identify substance(s) responsible for the drug protein binding inhibition.

Methods

Protein binding of normal and uremic serum

Sample collection and analysis. Serum was obtained from 11 healthy volunteers, 18 non-dialyzed patients with renal failure and 11 dialyzed patients, prior to the start of dialysis. Of the dialyzed group, five patients were treated by conventional hemodialysis with Cuprophan membranes (Bravo 500 or 512, surface area 1.06 m^2, Bellco, Mirandola, Italy), whereas the remaining six patients were receiving hemodiafiltration therapy with AN 69S membranes (Biospal S 3000, surface area 1.20 m^2, Hospal, Lyon, France), and a substitution volume of 10 liters. Heparinization was performed with a heparin brand containing no benzyl alcohol as preservative. Patients involved in the study were selected for no intake of salicylates or related drugs. Blood samples in the dialyzed patients were collected at the start of dialysis. The results of the Cuprophan and AN69S groups were pooled, since there were no major differences. In addition to drug binding, the serum creatinine, total protein and albumin concentration were also determined in the same samples by the Jaffé-reaction, the biuret and the brome chresol acid methods, respectively.

Protein binding studies. Studies were performed with radiolabeled theophylline, clonidine, imipramine, diazepam, cimetidine, propranolol, prazosin and methotrexate (Amersham In-

ternational, U.K.) and diphenylhydantoin (New England Nuclear, Boston, Massachusetts, USA).

The choice of the drugs was inspired by: 1) their current use in renal failure and 2) their easy availability as radiolabeled substance.

Protein binding was evaluated in vitro by mixing 500 μl of serum with 200 μl of one of the drug solutions, followed by incubation at 37°C for 30 minutes. Two aliquots of this mixture (100 μl each) were transferred into a small plastic vial containing 5 ml of scintillation cocktail (Opti-fluor, Packard, Brussels, Belgium), for analysis of total drug concentration in a liquid scintillation counter (Packard Tri-carb B 2450). Free drug was separated from the bound fraction by centrifugation (1800 × g for 15 min) and ultrafiltration through an anisotropic hydrophyllic ultrafiltration membrane (Centrifree microportion system, Amicon, Danvers, Massachusetts, USA). Aliquots (100 μl) of the filtrate were processed for scintillation counting as described above, for the estimation of free drug concentration.

DPM (disintegrations per minute) were calculated from the measured CPM values in the radioactive solutions by a ESR (external standard ratio) method. The mean of duplicate determinations was established. Protein binding was calculated as a percentage (bound fraction versus total).

None of the drugs under study was bound to the centrifree ultrafiltration membrane by more than 10%, except for imipramine (quantity retained on the membrane = 24.3%). Retention for phenytoin and theophylline was 4.1 and 0.1%, respectively.

Influence of ultrafiltrate on protein binding

Ultrafiltrate was collected during hemofiltration of uremic patients with the AN 69S membrane. The influence of ultrafiltrate on protein binding of theophylline and phenytoin was studied by lyophilizing 0.5, 1 and 2 ml of ultrafiltrate, and adding it to 500 μl of pooled normal serum. Protein binding was evaluated and the percentage difference versus pooled normal serum was determined. The sodium content of each of the samples studied was measured by standard methods before lyophilization.

Influence of NaCl on protein binding

Five hundred μl of NaCl containing solutions (50, 100, 200, 300, 400, 500 and 600 mEq/liter) were lyophilized and the dry substance was then added to 500 μl of normal serum; protein binding of theophylline and phenytoin was assessed.

When theophylline and phenytoin were diluted in a solution containing 600 mEq/liter of sodium, no major change in the binding of the two drugs to the centrifree ultrafiltration membrane was observed, compared to a dilution in isotonic saline.

Influence of HPLC eluate on protein binding

Normal sera (N = 3), and ultrafiltrates (N = 4) obtained during hemofiltration were further evaluated. These were submitted to a preliminary ultrafiltration over a Centrifree filter, as a preparative procedure before HPLC. Two ml samples were submitted to each HPLC-run. HPLC determinations were undertaken with two LKB 2150 high pressure pumps and an LKB 2152 gradient controller (LKB, Bromma, Sweden). The analyses were performed on a RSil reverse-phase C18 preparative column with a particle size of 10 μ, a length of 25 cm and an inner diameter of 10 mm (Alltech Europe, Eke, Belgium). A guard column (5 cm × 4.6 mm) packed with Pellicular C18 (10 μm) was used to protect the main column from contaminants. Detailed operating conditions during this procedure have been described elsewhere [12]. The Valco injector (Valco Instruments Co., Houston, Texas, USA) was provided with a loop of 2 ml. The solvent gradient was linear from 100% ammonium formate (0.05 μmol/liter, pH 4.0) to 54% highly purified methanol (Alltech Associate Inc., Deerfield, Illinois, USA) within 45 minutes. Flow rate was 3 ml/min. The solvent and the column were kept at room temperature. Ultraviolet detection was performed by a UV photometer (LKB 2238 Uvicord SII, Bromma, Sweden) at 254 nm. Peaks were registered by an LKB 2210 two pen recorder (LKB, Bromma, Sweden). Three ml eluate fractions collected during the first 30 minutes of the HPLC-procedure, were sampled, lyophilized and rediluted either in 500 μl water for the determination of the concentration of Na^+, K^+ and Cl^-, and osmolality, or in 500 μl of normal serum for the determination of protein binding of theophylline and phenytoin as described above.

Identification of substances responsible for protein binding inhibition

To further identify substances potentially responsible for the binding inhibition, the HPLC pattern of several known uremic solutes was studied separately before and after addition to uremic ultrafiltrate. These included: creatinine (20 mg/100 ml), pseudo-uridine (0.90 mg/100 ml), uric acid (0.95 mg/100 ml), indoxyl sulphate (1.60 mg/100 ml) and hippuric acid (3.0 mg/100 ml). HPLC was performed after 3 ml of this solution was added to 1 ml of H_2O, and was processed as described above. The same solution was also added to 1 ml of uremic ultrafiltrate, and analyzed. To assess the influence of ultrafiltrate, 1 ml of ultrafiltrate and 3 ml of H_2O were mixed, and processed and analyzed in the same way.

Finally, five known uremic solutes were diluted in water to different concentrations, equal to or above those currently observed in renal failure: urea (200, 300, 400 and 500 mg/100 ml), creatinine (5, 10, 15, and 20 mg/100 ml), indoxyl sulphate (10, 20, 50, 100 and 200 mg/100 ml), hippuric acid (10, 20, 50, 100 and 200 mg/100 ml) and pseudo-uridine (0.25, 0.50, 1, 2, and 5 mg/100 ml). Five hundred μl of these solutions were lyophilized, added to 500 μl of normal serum and protein binding of both phenytoin and theophylline was determined.

Statistical evaluations

Values are given as means ± SD. Statistical comparison between different groups was performed by Wilcoxon's test for unpaired values. Regression-correlation analysis was obtained by Spearman's test. Statistical significance was accepted for $P < 0.05$.

Results

Protein binding of normal and uremic serum

The percentage protein binding of the nine drugs under study for different degrees of renal failure is shown in Table 1. There was a marked and gradual decline of the protein binding of theophylline, phenytoin and methotrexate which for hemodialyzed patients reached values that were decreased by 20.1%, 16.0% and 15.1%, respectively, when compared to normals (P

Table 1. Percent protein binding according to renal function

	S_{Cr} *<1.3 mg/100 ml* $N = 9$	S_{Cr} *1.3 to 3.0 mg/100 ml* $N = 6$	S_{Cr} *3.0 to 6.0 mg/100 ml* $N = 6$	S_{Cr} >6.0 mg/100 ml $N = 7$	Hemodialyzed patients $N = 12$
Theophylline	63.6 ± 1.9	62.5 ± 4.9	52.3 ± 4.6[c]	48.4 ± 8.7[c]	43.5 ± 9.5[c]
Phenytoin	91.6 ± 0.7	87.9 ± 1.4[c]	82.4 ± 1.8[c]	81.7 ± 2.7[c]	75.6 ± 6.2[c]
Methotrexate	42.8 ± 1.6	44.5 ± 5.7	40.4 ± 6.0	36.2 ± 9.1[a]	27.7 ± 7.7[c]
Diazepam	98.3 ± 1.0	98.1 ± 0.3	97.8 ± 0.4[a]	97.1 ± 1.1[b]	96.6 ± 1.1[c]
Prazosin	94.0 ± 0.5	92.5 ± 0.6[c]	91.1 ± 0.3[c]	89.9 ± 0.6[c]	92.4 ± 3.1
Imipramine	96.2 ± 0.8	97.4 ± 0.7[b]	97.4 ± 0.2[c]	96.7 ± 0.6	96.2 ± 1.3
Propranolol	90.2 ± 2.0	95.1 ± 1.1[c]	93.2 ± 1.5[b]	94.1 ± 1.8	93.6 ± 1.2[c]
Cimetidine	8.9 ± 2.2	14.7 ± 4.8[c]	17.1 ± 2.7[c]	15.5 ± 3.7[c]	14.4 ± 3.0[c]
Clonidine	44.4 ± 7.1	54.1 ± 5.4[a]	50.0 ± 3.4[a]	52.4 ± 6.7	56.2 ± 6.8[c]

Values are expressed as means ± SD; S_{Cr} is serum creatinine.
[a] $P < 0.05$
[b] $P < 0.02$
[c] $P < 0.01$, vs. $S_{Cr} < 1.3$ mg/100 ml (Wilcoxon's test for unpaired values).

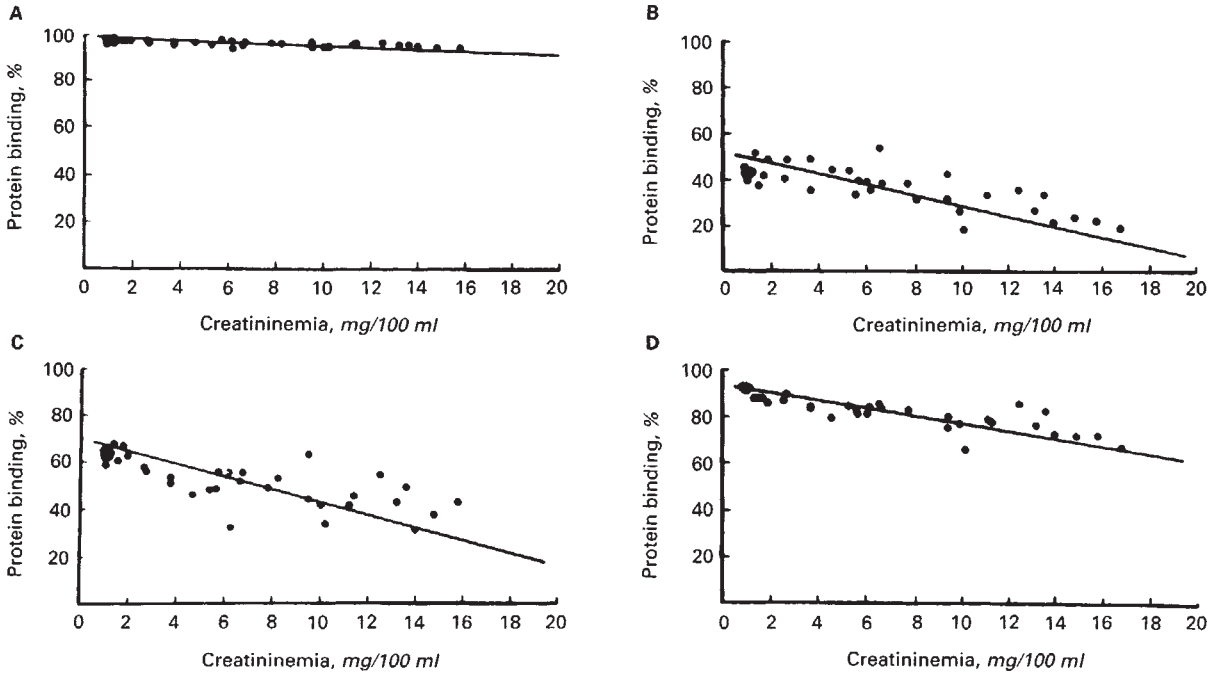

Fig. 1. *Correlation-regression analysis (N = 40) between creatinemia and the protein binding of four drugs: diazepam (A), methotrexate (B), theophylline (C), and phenytoin (D).* The characteristics of the different regression lines were as follows:
- Diazepam: creatininemia = 30.7 − (3.03 × protein binding); $r = 0.67$, $P < 0.001$
- Methotrexate: creatininemia = 22.3 − (0.43 × protein binding); $r = 0.79$, $P < 0.001$
- Theophylline: creatininemia = 26.0 − (0.37 × protein binding); $r = 0.80$, $P < 0.001$
- Phenytoin: creatininemia = 56.6 − (0.60 × protein binding); $r = 0.87$, $P < 0.001$.

< 0.01 for all). The decrease in protein binding for diazepam was less marked, although statistically significant (98.3 compared with 96.6%). This alteration corresponded to a 100% rise in the free fraction (from 1.7 to 3.4%). The protein binding of prazosin and imipramine in dialyzed patients was not significantly different from the control group, although minor but significant differences were observed in non-dialyzed patients with renal failure. Prazosin was characterized by a slight decline of protein binding, whereas imipramine showed a minor increase. There was a rise in the degree of protein binding of propranolol, cimetidine and clonidine, when renal failure progressed.

For diazepam, methotrexate, theophylline and phenytoin, there was a highly significant negative correlation between serum creatinine levels and protein binding (Fig. 1, $r = 0.67$, 0.79, 0.80 and 0.87, respectively, $P < 0.001$). For clonidine, a positive correlation was found that was also significant, albeit to a lesser extent ($r = 0.46$, $P < 0.01$). The correlation was not significant for cimetidine, imipramine, prazosin and propranolol ($r = 0.22$, 0.26, 0.26 and 0.28, respectively, $P > 0.05$). The

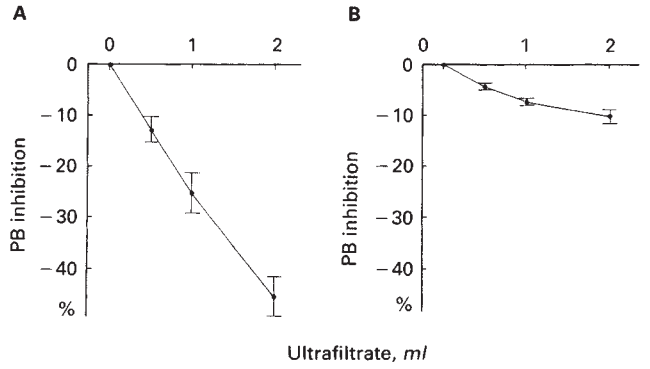

Fig. 2. *Percentage inhibition of protein binding (PB) versus normal when lyophilisate of 0.5, 1 and 2 ml of ultrafiltrate is added to normal serum, for theophylline (A) and phenytoin (B).*

respective mean values of serum protein for the different groups were 7.4 ± 0.6 (S_{Cr} < 1.3 mg/100 ml), 7.3 ± 0.6 (S_{Cr}: 1.3 to 3.0 mg/100 ml), 7.3 ± 0.5 (S_{Cr}: 3.0 to 6.0 mg/100 ml), 7.4 ± 0.5 (S_{Cr} > 6.0 mg/100 ml) and 6.6 ± 0.7 g/100 ml (hemodialyzed patients). Only the latter value was significantly different from the result obtained in healthy controls (S_{Cr} < 1.3 mg/100 ml, P < 0.05). The respective serum albumin concentrations were 4.4 ± 0.3, 4.3 ± 0.5, 4.3 ± 0.5, 4.5 ± 0.3 and 4.1 ± 0.8 mg/100 ml. None of these values were significantly different. No correlation could be found between serum protein and albumin concentration on one hand and the protein binding of any of the drugs on the other.

Influence of ultrafiltrate on protein binding

The influence of ultrafiltrate of uremic serum obtained during hemofiltration on the protein binding of phenytoin and theophylline was evaluated. Lyophilized ultrafiltrate (N = 5) was added to normal serum and the degree of protein binding inhibition was evaluated (Fig. 2). There was a gradual dose-dependent decrease of the protein binding for both theophylline and phenytoin with increasing quantities of added lyophilisate (0.5, 1 and 2 ml). After the addition of 2 ml lyophilisate, we observed a fall in protein binding towards control of 43.0 ± 3.3% for theophylline and of 12.1 ± 1.2% for phenytoin. The sodium concentration in the different samples studied was 125 ± 6, 248 ± 11 and 500 ± 23 mEq/liter for the lyophilisates of 0.5, 1 and 2 ml, respectively. The inhibitory effect of ultrafiltrate was thus more pronounced for theophylline than for phenytoin, but this may in part be attributed to the progressively increasing sodium content in the mixtures under study.

Influence of NaCl on protein binding

When NaCl was added to normal serum in varying concentrations, theophylline protein binding was inhibited progressively in a dose dependent way (Table 2), but for a similar NaCl concentration the protein binding inhibition was less pronounced here than in the dose response studies with ultrafiltrate (Table 3). This suggests that the presence of sodium alone in these ultrafiltrates was not sufficient to explain the entire effect of theophylline protein binding inhibition observed in the lyophilisates of different volumes of ultrafiltrate, and that there are still one or more other substances that are present in ultrafiltrate that may inhibit theophylline protein binding.

Phenytoin protein binding was not influenced.

Table 2. Effect of different NaCl concentrations on protein binding

NaCl concentration *mEq/liter*	Relative changes in PB vs. normal (Δ%)	
	Theophylline	Phenytoin
50	−1.2 ± 0.1	−1.3 ± 0.1
100	−4.3 ± 0.5	−1.3 ± 0.1
200	−11.2 ± 1.1	−1.0 ± 0.1
300	−17.6 ± 2.0	−0.6 ± 0.1
400	−23.9 ± 2.5	−1.4 ± 0.1
500	−27.1 ± 3.6	−1.6 ± 0.3
600	−31.1 ± 4.3	−1.4 ± 0.1

Table 3. Relative importance of NaCl in theophylline protein binding inhibition in ultrafiltrate dose response studies

Lyophlized volume *ml*	NaCl concentration *mEq/L*	Expected inhibition of protein binding	Registered inhibition of protein binding
0.5	125	−6.0[b]	−13.5
1	248	−14.4[b]	−26.3
2	500	−27.1	−43.0

[a] According to the measured NaCl concentration.
[b] Extrapolated from the data in Table 2.

Influence of HPLC eluate on protein binding

Ultrafiltrate of normal and uremic serum was submitted to HPLC elution, and the influence of each of the eluted fractions on theophylline and phenytoin protein binding was evaluated. Two characteristic chromatograms (one of normal and one of uremic serum), and the respective degree of inhibition of the different fractions on phenytoin and theophylline protein binding are illustrated in Figure 3.

For normal serum, there is an inhibition of theophylline protein binding in fraction 5-8, whereas phenytoin protein binding is not influenced. Ultrafiltrate of uremic serum inhibits theophylline protein binding in fractions 5 to 7, 9 to 10 and 27 to 28, and phenytoin protein binding in fractions 27 to 28. Fractions 27 to 28 correspond to the elution zone of hippuric acid.

The mean values of protein binding inhibition of the 30 fractions under study for normal serum samples and uremic ultrafiltrates are summarized in Table 4. This table also illustrates the concentration of Na^+ in the samples after lyophilization and redilution in water. Overall, these results are similar to those displayed in Figure 3. HPLC-fractions of ultrafiltrate of normal serum do not influence the protein binding of phenytoin. There is, however, a marked inhibition of theophylline protein binding in fractions 5 to 7, with a maximum in fraction 5. The evaluation of ultrafiltrate of uremic serum revealed that phenytoin protein binding is inhibited in fractions 27 and 28. Theophylline protein binding is inhibited in fractions 5 to 10 with a maximum in fraction 5 and another maximum in fractions 9 to 10. There is also an important inhibitory activity up to a value of 18.6 ± 16.3% in fractions 27 and 28. Fraction 5 contains the highest concentration of Na^+. Similarly, the concentration

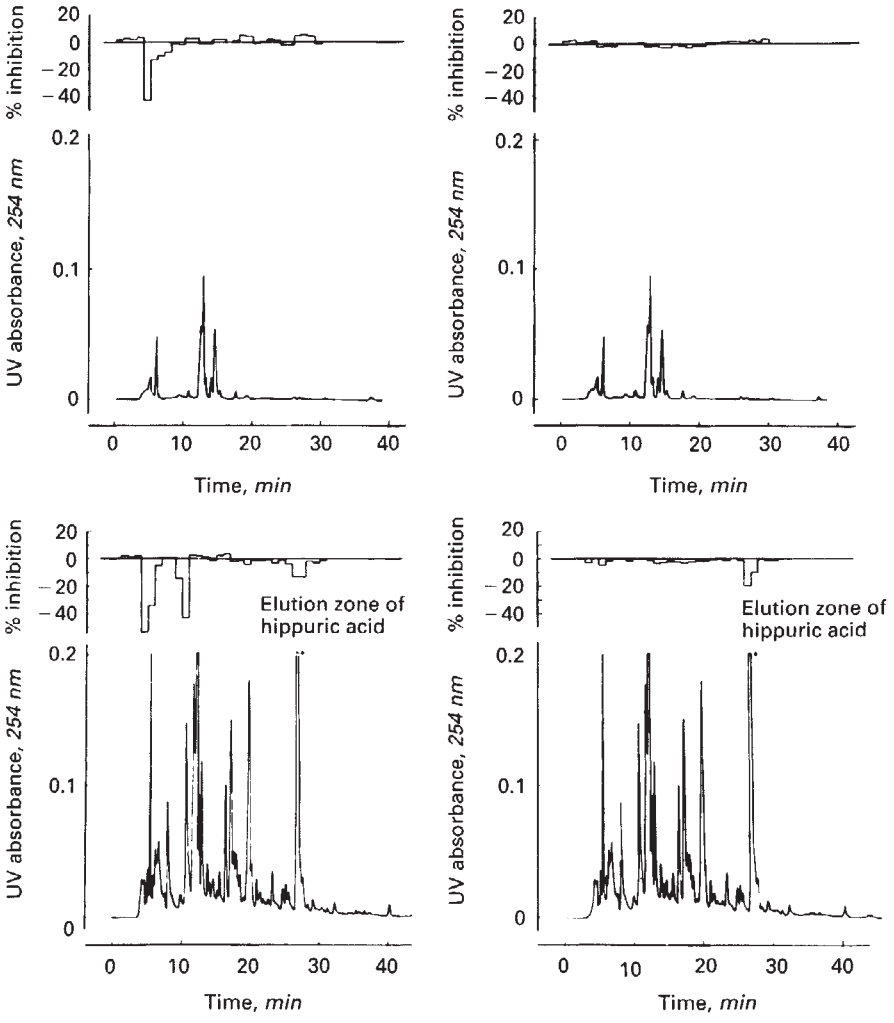

Fig. 3. *High performance liquid chromatogram of ultrafiltrate of normal (upper panel) and uremic serum (lower panel) with corresponding UV-absorbance (below) and percent protein binding inhibition of the different fractions (above) for theophylline (left) and phenytoin (right).* For normal serum ultrafiltrate, theophylline protein binding is inhibited in one elution zone, whereas phenytoin binding is not influenced. For ultrafiltrate of uremic serum, theophylline protein binding is inhibited in 3 different elution zones. Phenytoin binding is inhibited in only one elution zone. The asterisk indicates the elution zone of hippuric acid.

of the other electrolytes (K^+, Cl^-) and the osmolality were high in this fraction both in the chromatograms of normal and uremic serum ultrafiltrate.

Identification of substances responsible for protein binding inhibition

When chromatographing creatinine, pseudo-uridine, uric acid, indoxyl sulphate and hippuric acid, it appeared that three of these known uremic solutes were eluted on HPLC near the zones with inhibitory activity on protein binding of theophylline and/or phenytoin (Fig. 4A). Creatinine and pseudo-uridine were eluted near to fraction 9-10, and hippuric acid eluted exactly in fraction 27 and 28. The elution zone of these substances corresponded to the elution zone of a major HPLC-peak when uremic ultrafiltrate was chromatographed (Fig. 4B). When adding a standard solution of these substances to uremic ultrafiltrate, the height of the existing peaks was increased, without the creation of new peaks (Fig. 4C), suggesting that the observed peaks corresponded exactly to the above uremic solutes.

The respective effects of varying concentrations of urea, creatinine, indoxyl sulphate and pseudo-uridine on protein binding of theophylline and phenytoin is illustrated in Table 5. Urea, creatinine and pseudo-uridine exerted no influence on the protein binding of both theophylline and phenytoin. Indoxyl sulphate influenced protein binding, but only in a concentration in excess of 50 mg/100 ml. The influence of hippuric acid on theophylline and phenytoin protein binding is illustrated in Figure 5. Hippurate inhibited protein binding of phenytoin and theophylline in a dose dependent manner. The inhibitory effect was most pronounced for theophylline.

Discussion

In a first part of this study, the protein binding of several drugs, that are currently used during chronic renal failure, is evaluated. For some drugs, such as phenytoin [1–7] and diazepam [8], our findings are a confirmation of earlier reports, showing a decrease in protein binding. For most other substances, no data on protein binding with progressive renal dysfunction is available.

Five drugs (theophylline, phenytoin, methotrexate, diazepam and prazosin) are characterized by a decrease of their protein binding by up to 20% when renal function is progressively lost. This means a relative increase of the free, active fraction of the

Table 4. Characteristics of different HPLC elution fractions of ultrafiltrate of normal ($N = 3$) and uremic serum ($N = 4$)

	Normal serum			Uremic serum		
Elution fraction	PB[a] theophylline ($g\Delta\%$ vs. control[b])	PB phenytoin ($\Delta\%$ vs. control)	Na^+ mEq/liter	PB theophylline ($\Delta\%$ vs. control)	PB phenytoin ($\Delta\%$ vs. control)	Na^+ mEq/liter
1	4.3 ± 3.8	0.5 ± 1.2	0	−1.1 ± 5.5	−0.6 ± 1.1	0
2	5.1 ± 3.5	0.7 ± 1.4	0	−3.9 ± 8.8	−0.6 ± 0.9	0
3	3.4 ± 2.3	0.5 ± 0.3	0	−9.8 ± 17.6	−1.5 ± 1.2	0
4	4.7 ± 1.0	1.4 ± 0.3	0	−9.5 ± 16.6	−0.4 ± 1.0	0
5	−43.4 ± 0.2	−3.2 ± 4.7	408.0 ± 33.9	−39.5 ± 14.2	−4.6 ± 0.9	441.3 ± 5.9
6	−12.2 ± 1.9	−2.0 ± 0.1	106.5 ± 10.6	−19.4 ± 15.7	−2.1 ± 2.3	120.3 ± 21.3
7	−9.2 ± 1.7	−0.5 ± 0.1	9.0 ± 0.1	−10.7 ± 14.3	−0.9 ± 0.9	8.7 ± 11.1
8	−3.9 ± 1.4	−0.6 ± 0.8	3.5 ± 0.7	−6.8 ± 14.3	−0.9 ± 0.3	3.3 ± 0.4
9	−0.5 ± 0.6	−0.6 ± 0.8	2.0 ± 0.1	−12.6 ± 7.9	−0.7 ± 0.6	2.3 ± 0.4
10	−0.6 ± 0.8	−0.3 ± 0.3	1.5 ± 0.7	−14.8 ± 18.6	−0.4 ± 0.3	0.7 ± 0.4
11	4.8 ± 2.6	0.3 ± 0.6	1.0 ± 0.1	−1.5 ± 3.0	−0.8 ± 1.0	0
12	3.5 ± 0.7	0.9 ± 0.2	1.0 ± 0.1	−1.3 ± 2.4	−0.5 ± 0.6	0
13	1.8 ± 5.5	−0.9 ± 0.8	0	−1.2 ± 2.2	−1.6 ± 1.4	0
14	1.1 ± 4.3	−1.4 ± 0.1	0	−0.9 ± 0.9	−1.7 ± 1.2	0
15	4.2 ± 4.3	−1.6 ± 0.6	0	−1.7 ± .7	−1.3 ± 1.0	0
16	3.7 ± 3.9	−1.1 ± 0.9	0	−0.4 ± 3.0	−1.4 ± 1.0	0
17	3.7 ± 5.8	−0.2 ± 0.2	0	−1.3 ± 1.2	−1.5 ± 1.1	0
18	1.4 ± 1.5	0.3 ± 1.1	0	−1.9 ± 3.0	−1.8 ± 1.2	0
19	4.0 ± 1.6	−0.4 ± 1.6	0	−2.5 ± 2.5	−1.9 ± 0.8	0
20	3.0 ± 2.4	0.4 ± 0.3	0	−1.2 ± 2.1	−1.4 ± 0.4	0
21	−0.1 ± 0.2	0.0 ± 0.5	0	−1.1 ± 1.8	−1.1 ± 0.4	0
22	2.9 ± 5.2	1.9 ± 0.3	0	−1.4 ± 2.6	−0.8 ± 0.2	0
23	3.3 ± 2.7	1.3 ± 0.2	0	−2.1 ± 2.7	−1.3 ± 1.0	0
24	3.7 ± 4.0	1.8 ± 0.2	0	−1.6 ± 2.8	−0.4 ± 0.6	0
25	0.5 ± 5.0	−1.1 ± 2.2	0	−2.1 ± 2.5	−0.8 ± 0.6	0
26	0.9 ± 5.7	0.5 ± 0.9	0	−2.0 ± 1.5	−0.9 ± 0.5	0
27	5.8 ± 0.8	1.1 ± 0.1	0	−15.9 ± 7.8	−10.0 ± 7.6	0
28	2.8 ± 4.2	1.0 ± 0.9	0	−18.6 ± 16.3	−3.9 ± 3.8	0
29	2.4 ± 2.9	0.6 ± 1.1	0	−2.9 ± 2.5	−1.9 ± 2.7	0
30	0.0 ± 2.6	1.1 ± 1.8	0	−3.8 ± 2.9	−1.0 ± 0.5	0

[a] PB, protein binding
[b] Control, ultrafiltrate of serum of healthy persons

drug; since for most drugs total (bound plus unbound) but not free concentrations are monitored, these data suggest that for these drugs, in renal failure, lower total drug serum concentrations should be pursued than under normal conditions, and that at "normal" total drug concentrations toxic side effects may be expected.

The fall in drug binding capacity for diazepam and prazosin by only ± 2% for dialyzed patients compared to healthy controls, appears at the outset less important than the decrease for theophylline, phenytoin and methotrexate. It should however be stressed that the protein binding in healthy controls of both diazepam and prazosin is extremely high, so that apparently minor changes in the bound fractions may induce important disturbances in the free, unbound and active fractions. For diazepam for example, the free fraction increases from 1.7% in healthy controls to 3.4% in dialyzed patients. This corresponds to a twofold rise of the available active drug and may have important therapeutic consequences.

For drugs in which a decrease in protein binding with progressive renal failure was noted, there was a highly significant negative correlation between protein binding and creatinine level (Fig. 1), suggesting that the mechanisms involved may progressively become more important with the loss of renal function.

Several pathophysiological systems have been proposed to explain this decrease in protein binding during the course of renal failure, such as a decrease in proteinemia or albuminemia [1], changes in protein structure [1, 13] and/or competitive inhibition of binding by uremic accumulation products [4–7, 10, 11, 14, 15].

Several protein structures are known to bind drugs and other substances, such as albumin, lipoproteins, gammaglobulin and alpha$_1$-acid glycoprotein. The plasma protein that probably is most prominent with this role is albumin. Our study demonstrated no significant change of plasma albumin concentration with progression of renal failure and there was no significant correlation between protein binding on one hand, and protein and albumin concentration on the other. Similarly, Kinniburgh and Boyd found no major changes in the structure of serum proteins, with progressive uremia [16]. It is likely therefore that protein concentration and/or structure play a minor role in drug protein binding in renal failure, and that other solutes which accumulate in renal failure may rather interfere in the competitive binding of drugs to protein.

This hypothesis was confirmed by the finding of a dose dependent decrease of theophylline and phenytoin protein binding by ultrafiltrate of uremic serum.

In a second part of the study, we therefore tried to discern

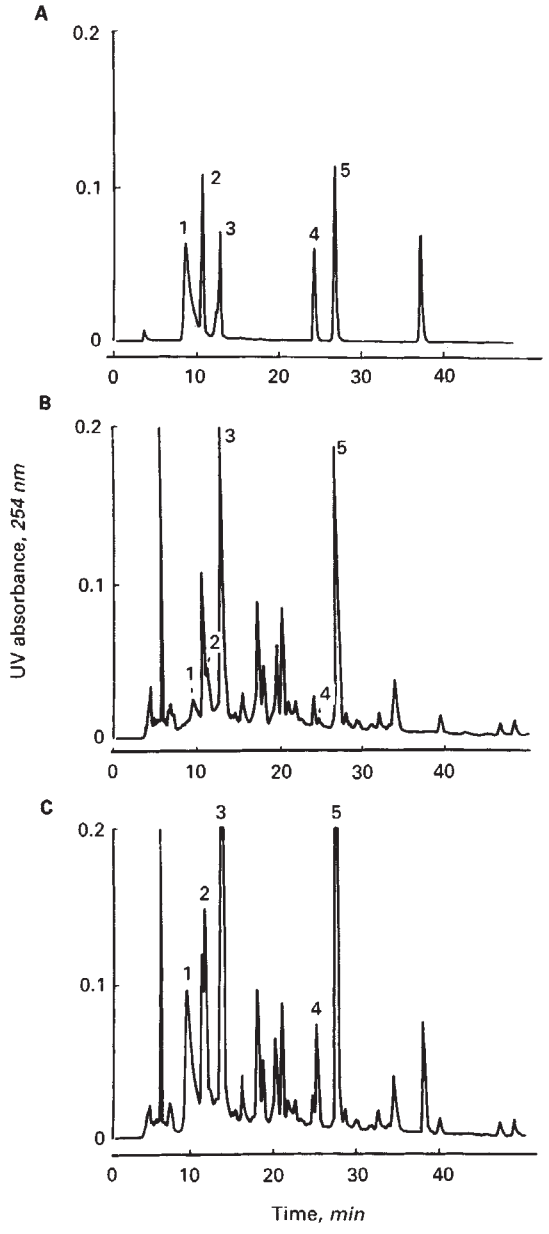

Fig. 4. *High performance liquid chromatogram of a standard solution (A) containing creatinine (1), pseudo-uridine (2), uric acid (3), indoxyl sulphate (4) and hippuric acid (5), of uremic ultrafiltrate (B), and of uremic ultrafiltrate after the addition of the standard solution (C).* Each of these substances gives rise to a distinct peak. Their elution zone corresponds to the elution zone of existing peaks on HPLC of uremic ultrafiltrate. After addition of the standard solution to uremic ultrafiltrate, the height of existing peaks is increased without the creation of new peaks. Creatinine and pseudo-uridine are eluted in the proximity of elution zones 9 and 10, and hippuric acid is eluted exactly in fractions 27 and 28.

Table 5. Influence of different concentrations of urea, creatinine, indoxyl sulphate and pseudo-uridine on protein binding

Substance	Concentration	Relative changes in PB vs. normal Δ%	
		Theophylline	Phenytoin
Urea	200 mg/100 ml	−2.2 ± 4.3	−1.0 ± 0.3
$N = 5$	300 mg/100 ml	−3.0 ± 3.6	−0.4 ± 0.2
	400 mg/100 ml	−2.1 ± 3.1	−0.4 ± 0.2
	500 mg/100 ml	−2.8 ± 3.3	−0.6 ± 0.2
Creatinine	5 mg/100 ml	−3.8 ± 4.7	0.1 ± 0.5
$N = 5$	10 mg/100 ml	−3.7 ± 4.1	0.6 ± 0.1
	15 mg/100 ml	−4.1 ± 4.4	0.1 ± 0.1
	20 mg/100 ml	−3.9 ± 3.4	0.2 ± 0.1
Indoxyl sulphate	10 mg/100 ml	−0.6 ± 1.3	−0.3 ± 0.3
$N = 5$	20 mg/100 ml	−1.9 ± 0.6	−1.3 ± 0.2
	50 mg/100 ml	−4.7 ± 1.5	−5.1 ± 0.4
	100 mg/100 ml	−11.8 ± 1.5	−14.7 ± 0.4
	200 mg/100 ml	−24.9 ± 1.6	−21.2 ± 0.5
Pseudo-uridine	0.25 mg/100 ml	−1.6 ± 1.2	−1.4 ± 0.2
$N = 5$	0.50 mg/100 ml	−5.7 ± 1.1	−1.1 ± 0.1
	1.00 mg/100 ml	−4.8 ± 2.1	−1.2 ± 0.1
	2.00 mg/100 ml	−6.3 ± 1.6	−1.4 ± 0.1
	5.00 mg/100 ml	−3.6 ± 1.7	−1.4 ± 0.7

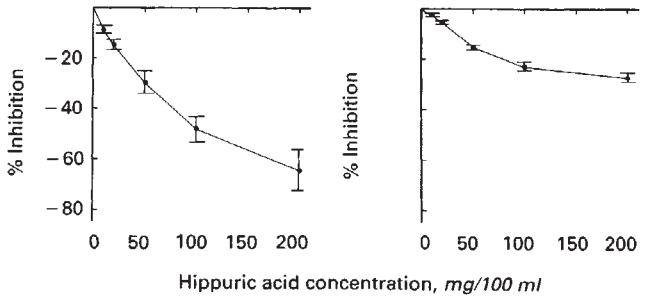

Fig. 5. *Effect of different concentrations of hippuric acid, added to normal serum after lyophilization, on protein binding of theophylline (left) and phenytoin (right).*

which factors were responsible for this inhibition, by evaluating the influence of 30 different HPLC fractions of ultrafiltrate on protein binding. This approach allowed the direct evaluation of protein binding inhibitors in uremic biological fluids. The most important finding of this part of the study was a substantial decrease of both phenytoin and theophylline protein binding in the HPLC fractions 27 to 28, the elution zone of hippuric acid, suggesting that the major dialyzable and/or ultrafiltrable factor inhibiting the protein binding of these two drugs is hippurate. Earlier indirect results obtained after the study of urine of healthy persons [10, 17] and of solutions partially mimicking the composition of uremic serum [11], had suggested the inhibitory influence of hippurate on phenytoin protein binding. Our data confirm these earlier results, by a direct evaluation and indicate that the negative influence of hippurate is not limited to phenytoin alone, but that at least one other drug is similarly affected.

Although the metabolic origins of hippuric acid are multiple, and precursors of the substance may be found in coffee, tea, fruits and may be produced by intestinal flora and endogenously [18], one source specific to dialysis patients is benzyl alcohol used as a preservative in some heparin brands which is entirely metabolized to hippurate [19].

In consequence, brands of heparin containing benzyl alcohol should be used with care in patients on theophylline and phenytoin. Monitoring of plasma hippurate levels might also be advisory in such patients.

It should be stressed that our dialysis patients do not use heparin that contains benzyl alcohol, so that it may be assumed that the protein binding inhibition observed in the present study is even less pronounced than is likely in patients using brands of heparin containing benzyl alcohol.

To allow a correct estimation of the magnitude of the hippurate related protein binding inhibition effect, it is important to stress that the hippuric acid concentrations of the ultrafiltrates in the present study averaged 7.53 ± 2.94 mg/100 ml (means ± SD, unpublished results). In a recent study from our lab, serum hippuric acid concentration was found to be between 2.5 to 37.2 mg/100 ml in hemodialyzed uremics (mean value: 9.7 ± 8.5 mg/100 ml) [20].

Extrapolation of these data to Figure 5, reveals that similar concentrations of hippuric acid would result in a protein binding inhibition in dialyzed patients by maximum 10 and 5% for theophylline and phenytoin, respectively, suggesting that other ligands may play an additional role as well.

Apart from the elution zone of hippuric acid, two other zones of binding inhibition were observed in the theophylline studies. One zone of binding inhibition corresponded to a number of fractions containing mainly NaCl. In both normal and uremic ultrafiltrate (Table 4) a marked parallelism between protein binding inhibition of theophylline and the electrolyte content in the fifth and nearby fractions was present; this suggests that the protein binding inhibition of theophylline in these fractions may be related to the electrolyte content. Sodium chloride in different concentrations also caused a dose dependent inhibition of theophylline protein binding (Table 2).

These data emphasize the importance of taking into account sodium concentrations in protein binding studies and point to the effectiveness of HPLC for the fractionation of serum samples since with this technique, electrolytes are clearly separated from the elution fractions containing the major organic substances.

A last zone of inhibitory activity was seen in fractions 9 and 10 of HPLC eluate; we were able to recognize only two UV-absorbing uremic solutes in or near to these elution fractions: creatinine and pseudo-uridine. Neither of these compounds inhibited protein binding, when dissolved in concentrations comparable to those observed in renal failure and in normal serum (Table 5). It is conceivable that another as yet unidentified compound plays an inhibitory role in this fraction.

In view of the fact that no new substances responsible for drug protein binding inhibition were found, except perhaps for an unidentified fraction eluting in HPLC fractions 9-10, our data suggest that at least some of the binding inhibitors are highly protein-bound as such, so that they do not appear in measurable amounts in the ultrafiltrate. Alternatively, loss of the inhibitor, due to binding to the filtration membrane or failure to elute from the HPLC-column, should be considered.

Indoxyl sulphate has been suggested as being responsible for a part of the protein binding inhibition of diazepam, warfarin and phenytoin [11, 21–23]. The role of indoxyl sulfate in the protein binding defect of phenytoin could not be confirmed in the present study. We found no inhibitory activity in the HPLC elution zone of indoxyl sulphate (fractions 24-25, Fig. 3), and although this substance was shown to decrease theophylline and phenytoin protein binding, at concentrations from 50 to 100 mg/100 ml (Table 5), these concentrations are, however, much higher than those found in renal failure patients [11], or even uremic coma [24].

Of the drugs studied, four drugs (propranolol, cimetidine, clonidine and to a certain extent also imipramine) showed increased protein binding with progressive renal failure. An increase in drug protein binding decreases the available free concentration and modifies the therapeutic effect of the drugs in a way that their activity is diminished. Thus, for these drugs higher total concentrations should be pursued in renal failure, if no free concentrations can be measured. The possibility exists that, by some mechanism, the number of binding sites for certain drugs increases with the progressive loss of renal function. The fact that all drugs in this study which show a rise in protein binding with renal failure are basic might be a possible explanation, since basic drugs might bind preferentially to alpha$_1$-acid glycoprotein (AAG) [25, 26], and it is recognized that AAG concentrations rise during renal failure [8]. A similar binding increase associated with a rise in serum AAG concentration has also been observed for imipramine after severe burn injury [27], in cardiac patients [28], and for propranolol in rats with severe inflammation [29].

It is notable that results of drug protein binding were similar in the patients treated with hemodialysis and hemodiafiltration, so that the data were pooled. This observation might lead one to suppose that middle molecules or poorly dialyzed but easily filtered solutes are not responsible for the binding inhibition of renal failure. However, the results considered are pre-dialysis values, so that it is difficult to make any definite conclusion on the direct influence of factors related to the dialysis technique as such on drug protein binding.

The present data of altering relations between bound and unbound fractions of drugs during the progression of renal failure emphasize the importance of monitoring free rather than total drug fractions in uremic patients. When monitoring total drug concentrations, and if protein binding is decreased, a high normal total drug level corresponds to an increased level of free and active drug, and vice versa.

Finally, it could be argued that for four substances (theophylline, methotrexate, clonidine and cimetidine) data concerning renal failure are not available in the literature because these drugs are poorly bound in normal subjects, so that they were not submitted to further evaluation. It should be stressed however, that in renal failure for a drug such as theophylline, a decrease in protein binding from 63.6 to 43.5% (Table 1), will result in a change in maximum allowable total drug serum concentration from 20 to ± 13 μg/ml, which may have important clinical implications, in the search for optimal non-toxic treatment schedules.

In conclusion, our study indicates that:

1. The protein binding of propranolol, clonidine, cimetidine and to a lesser extent imipramine is increased with progressive renal failure, while the protein binding of diazepam, methotrexate, phenytoin and theophylline is decreased.
2. There is a highly significant negative correlation between the binding inhibition of the latter four drugs and creatinine concentration, suggesting a relationship between the progressive accumulation of uremic solutes and the protein binding defect in renal failure. This binding defect is most important for theophylline and phenytoin.

3. Lyophilization of different volumes of ultrafiltrate inhibits the binding of theophylline and phenytoin in a dose dependent way.
4. Sodium chloride inhibits the protein binding of theophylline, and the salt content of fractions should always be taken into account when undertaking protein binding studies.
5. HPLC is a simple and useful method to separate NaCl from the other organic compounds, especially uremic retention products.
6. Hippuric acid appears to be the major dialyzable and/or ultrafiltrable substance affecting protein binding of theophylline and phenytoin.

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