Binding of p-Cresylsulfate and p-Cresol to Human Serum Albumin Studied by Microcalorimetry

David Bergé-Lefranc,[†] Florence Chaspoul,[†] Raymond Calaf,[§] Philippe Charpiot,[‡,§] Philippe Brunet,[‡] and Philippe Gallice*,[†]

Laboratoire de Chimie Générale, Prévention des Risques et Nuisances Technologiques, EA 1784 Biogénotoxicité et Mutagenèse Environnementale FR3098 ECCOREV, Université de la Méditerranée, Faculté de Pharmacie 13005 Marseille, France, Laboratoire d'Hématologie-Immunologie, INSERM U608, Université de la Méditerranée, Faculté de Pharmacie, 13005 Marseille, France, and Laboratoire de Biochimie Fondamentale Moléculaire et Clinique, Université de la Méditerranée, Faculté de Pharmacie, 13005 Marseille, France

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p-Cresylsulfate, a metabolite of p-cresol, is reported as prototypic protein-bound uremic toxin, inefficiently removed by hæmodialysis. The binding between p-cresylsulfate or p-cresol and human serum albumin was studied using microcalorimetry. The results confirm that the two molecules are protein-bound. However, the affinity of p-cresylsulfate and p-cresol toward human serum albumin is moderate at 25 °C and becomes relatively weak at physiological temperature, 37 °C. The binding principally involves van der Waals type interactions, and the binding sites of the two molecules are the same or very close. The low fraction of bound toxin (13−20%) appears to be insufficient to link strong binding to poor removal of this toxin by hemodialysis.

Introduction

Compounds that accumulate in blood during kidney failure are called uremic retention solutes, and those showing adverse biological effect uremic toxins. p-Cresylsulfate, a metabolite of p-cresol was thus reported as a prototypic protein-bound uremic toxin.[1,2] p-Cresol is produced during degradation of tyrosine by intestinal bacteria.[3] This phenolic compound is conjugated principally by sulfatation in the liver, leading to the formation of p-cresylsulfate.[4,5]

p-Cresol and p-cresylsulfate have often been confused due to easy hydrolysis of the conjugated form.[6] Since p-cresol is present in the body at very low concentrations, p-cresylsulfate is today considered the uremic toxin.[7]

In healthy subjects, the concentration of p-cresylsulfate in blood is low, about 6 μM, but increases to values higher than 300 μM in patients with end-stage renal disease .[9] In these patients, the accumulation of p-cresylsulfate has been associated with impairment of the vascular lesions,[10] mortality,[11] and activation of leukocyte free radical production.[6] p-Cresylsulfate has also been implicated in the dose-dependent increase in shedding of endothelial microparticles.[12]

Elimination of this toxin through hemodialysis is poor, with only 29% removed,[13] which has been attributed to its strong binding to plasma-proteins.[14] Because human serum albumin (HSA), the main protein of human plasma, is known to bind a great variety of endogenous or exogenous compounds,[15] HSA is usually investigated in relation to the binding of ligands. Thus, HSA has been reported to be involved in the binding of the phenolic derivative uremic toxin p-cresylsulfate.[16,17] However, there is clearly a lack of information in the literature concerning this interaction and the associated mechanism. Studies simply report the bound and unbound fractions of the uremic toxin without examining the degree of affinity of the toxin for HSA and defining the binding involved.[18] Here, therefore, we investigated the binding of p-cresol and p-cresylsulfate to HSA using microcalorimetry, defining this binding thermodynamically.

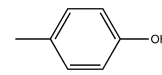
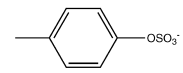
Compounds	Structure	Molar mass (g.mol^{-1})
p-cresol		108
p-cresylsulfate		187

Figure 1. Skeletal representation and molecular mass of p-cresol and p-cresylsulfate.

Materials and Methods

Reagents. p-Cresol (purity >99.7%) and HSA (A 9511, fatty acid free, minimum 97−99%, electrophoresis, molecular weight 66.5 kDa) were purchased from Sigma-Aldrich. p-Cresylsulfate was synthesized and controlled by the Laboratoire de Pharmaco-Chimie Radicalaire UMR CNRS 6264. The purity (>96%) was checked by nuclear magnetic resonance (^{1}H NMR), X-ray diffraction, and high-performance liquid chromatography (HPLC). All other reagents were of analytical grades, and solutions were made in distilled water. The skeletal formulas of the molecules are given in Figure 1. p-Cresol, p-cresylsulfate, and HSA solutions were prepared in phosphate buffer of 10 mM, pH 7.4. Ionic strength was adjusted to 0.15 or 0.30 M by addition of

* To whom correspondence should be addressed. Tel: +33 4 91 83 55 40. Fax: +33 4 91 80 94 69. E-mail: philippe.gallice@pharmacie.univ-mrs.fr.
† Laboratoire de Chimie Générale.
§ Laboratoire de Biochimie Fondamentale Moléculaire et Clinique.
‡ Laboratoire d'Hématologie-Immunologie.

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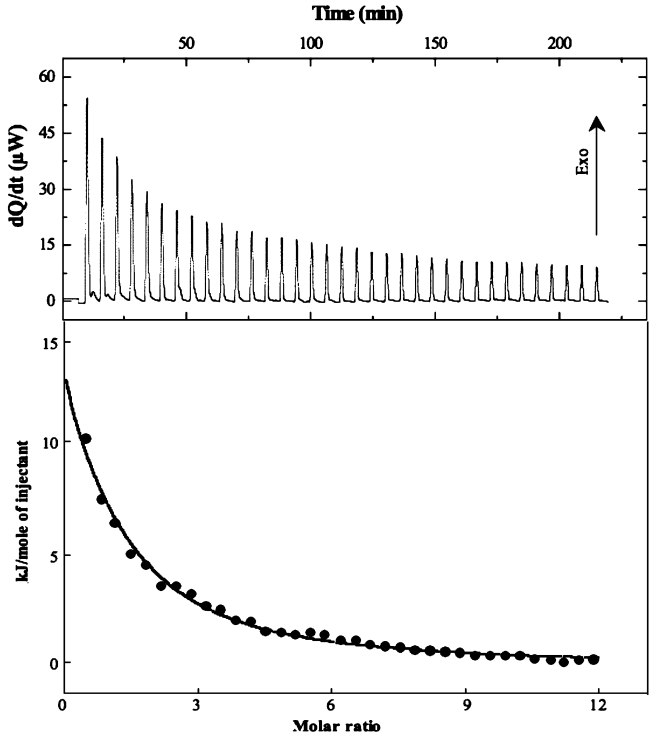

Figure 2. Typical ITC of the binding of p-cresol to HSA. Upper panel: Raw data obtained for 35 injections, each of 3 μL, of p-cresol solution (100 mM) into the sample cell containing HSA solution (1 mM) in phosphate buffer (10 mM, pH 7.4). Lower panel: Plot of processed data. The points are experimental and the solid line corresponds to the best-fit curve. The best fitting parameters are 0.98 for stoichiometry, 406 M^{-1} for K, and − 31 kJ/mol of p-cresol for $\Delta H°$.

sodium chloride (NaCl). Immediately before experiments, the pH of solutions was checked and, if necessary, adjusted to the same values by adding NaOH or HCl microvolumes. UV absorptions were measured on a Hitachi U-2001 double-beam spectrophotometer. The solution concentrations of p-cresol and p-cresylsulfate were controlled by UV absorption at $\lambda = 277$ nm, $\varepsilon_{277} = 1259$ L·mol^{-1}·cm^{-1} and $\lambda = 284$ nm, $\varepsilon_{284} = 1432$ L·mol^{-1}·cm^{-1} for p-cresol and p-cresylsulfate, respectively. The HSA concentration was determined at $\lambda = 280$ nm, $\varepsilon_{280} = 46000$ L·mol^{-1}·cm^{-1}. When pH adjustment of p-cresylsulfate was carried out, solutions were checked at 284 nm, to identify any change in absorbance that would indicate an absence of detectable p-cresylsulfate hydrolysis.

Isothermal Titration Calorimetry (ITC). Equilibrium binding experiments designed to study the interaction between uremic toxins and HSA were performed at 25 and 37 °C using a 2277 Thermal Activity Monitor calorimeter (Thermometric, Sweden) equipped with a titration unit. Data acquisition and analyses were carried out using DIGITAM 4.1 software (Thermometric, Sweden) and MS Excel.

Aliquots (3 μL) of toxin solution (p-cresol and p-cresylsulfate) were injected, at 300 s time intervals, from a 250 μL syringe into the calorimeter cell containing HSA solution (0.9 mL) to achieve a complete binding isotherm. The effective heat of binding (Q_{binding}) exchanged was obtained by subtracting the heat of dilution (measured by additional injections of p-cresol or p-cresylsulfate solution after saturation) from the heat of reaction. This heat measured is proportional to the concentration of HSA−toxin complex formed as described by eq 1:

$$Q_{\text{binding}} = V\Delta H[n\text{HSAT}] \tag{1}$$

with V being the volume of solution, ΔH being the binding enthalpy, and [nHSAT] being the complex HSA−toxin concentration where n is the number of binding sites.

Applying the law of mass action at the equilibrium, the association constant, a reflection of the affinity of toxin for HSA is expressed as

$$K = \frac{[n\text{HSAT}]}{([n\text{HSAt}] - [n\text{HSAT}]) \times ([T] - [n\text{HSAT}])} \tag{2}$$

with K being the association constant, HSAt being the total HSA concentration, T being the total toxin concentration, and n being the number of binding sites.

Combining eq 1 and 2, the association constant can be expressed as:

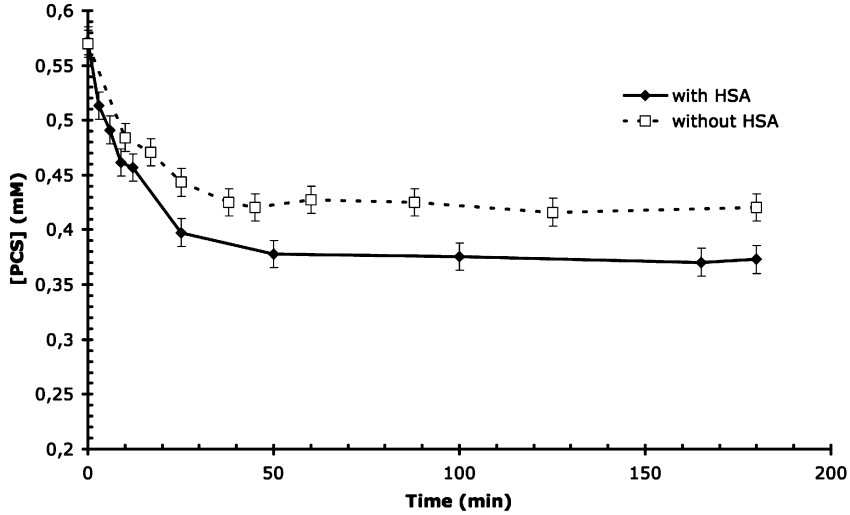

Figure 3. Equilibrium dialysis experiment results of p-cresylsulfate (PCS) with and without HSA at 25 °C. Points □ represent the mean ± standard deviation of three experiments performed without HSA, and points ◆ represent the mean ± standard deviation of three experiments with HSA (46 μM); the points are joined by an arbitrary line.

TABLE 1: Thermodynamic Parameters of Binding Determined at 25 °C[a]

compounds	K (M^{-1})	$\Delta G°$ (kJ.mol^{-1})	$\Delta H°$ (kJ.mol^{-1})	$\Delta S°$ (J.mol^{-1}.K^{-1})	n
p-cresol	495 ± 33[b]	−15.4 ± 1.2	−29.2 ± 1.2	−46.3 ± 1.0	0.98 ± 0.04
p-cresylsulfate	401 ± 38	−14.8 ± 1.5	−38.3 ± 0.3	−78.9 ± 1.5	0.98 ± 0.04

[a] K: association constant; $\Delta G°$: standard free Gibbs energy; $\Delta H°$: standard binding enthalpy; $\Delta S°$: standard entropy; n: number of sites. [b] Values are the mean ± standard error of three independent experiments.

$$K = \frac{Q_{\text{binding}}/V\Delta H}{([n\text{HSAt}] - (Q_{\text{binding}}/V\Delta H)) \times ([T] - (Q_{\text{binding}}/V\Delta H))} \quad (3)$$

From eq 3, ΔH, K, and n were accurately obtained by fitting the experimental corrected binding isotherm to a model incorporated into the DIGITAM software.

The calorimeter was dynamically calibrated before each measurement. At equilibrium, the variations of free energy ($\Delta G°$) and entropy ($\Delta S°$) were obtained from the classic thermodynamic relation:

$$\Delta G° = -RT \ln K = \Delta H° - T\Delta S°$$

with T being the temperature (Kelvin) and R being the perfect gas constant ($J \cdot K^{-1} \cdot mol^{-1}$).

In accordance with the methodology described, ITC was used in a first set of experiments to investigate the effect of ionic strength on the interaction at 25 °C.

Titrations were thus carried out in phosphate buffer at 10 mM, pH 7.4, and ionic strength adjusted to 0.15 and 0.30 M. In a second set of experiments, the temperature effect was investigated at physiological temperature 37 °C.

Finally, the competitive interaction between p-cresol and p-cresylsulfate with HSA was investigated in order to obtain information about the binding sites and the type of bond involved. For this purpose, ITC experiments were performed by injecting a p-cresylsulfate solution (0.1 M) into the calorimeter cell containing HSA (10^{-3} M) saturated at 50% with p-cresol.

Equilibrium Dialysis. Equilibrium dialysis experiments were carried out in a 3 mL "macrocell" using a rotative Dianorm apparatus, the two compartments of each cell being separated by a Diachema membrane (5000 Da) at 25 °C. At the beginning of experiments, buffer solution or HSA solution (0.46 mM) was injected into one compartment, and a p-cresylsulfate solution (0.57 mM) was injected into the other. The concentration of p-cresylsulfate over time was measured.

Statistical Analysis. Values are expressed as mean ± standard error of the mean. Statistical analysis was performed using Student's t test. $P < 0.05$ was considered significant.

Results and Discussion

Thermodynamic Analysis of the Interaction between HSA and p-Cresol or p-Cresylsulfate. A typical ITC measurement is illustrated by Figure 2. Results obtained for the interactions of p-cresol and p-cresylsulfate are reported in Table 1.

As shown in Figure 2, titration experiments show binding of p-cresol to HSA. Analogous results were obtained for p-cresylsulfate.

Results in Table 1, suggest that the affinity reflected by the K value for the two molecules is moderate, and the stoichiometry (n) is 1 mol of p-cresol or p-cresylsulfate per 1 mol of HSA. Affinity between p-cresol and HSA is, however, higher than that observed for p-cresylsulfate.

TABLE 2: Affinity Constants Determined at 25 °C in NaCl 0.3 M Solution

compounds	K (M^{-1})
p-cresol	371 ± 61[a]
p-cresylsulfate	330 ± 21

[a] Values are the mean ± Standard Deviation of two independent experiments.

The binding for both molecules is enthalpy-driven with an unfavorable entropy, suggesting a similar electrostatic interaction with HSA.[19] The difference between the $\Delta H°$ values for p-cresol and p-cresylsulfate may be due to the polarity of these molecules. Indeed, p-cresol presents a small dipole moment (μ = 1.808 D)[20] whereas p-cresylsulfate has a charged point belonging to the sulfate group.

To check whether binding becomes more profound over time, equilibrium dialysis experiments were performed. The results presented in Figure 3 show that p-cresylsulfate concentration decreases then reaches a constant value of 0.373 ± 0.003 and 0.421 ± 0.005 mM with and without HSA, respectively. From 50 to 180 min, toxin concentration is remarkably constant, indicating that equilibrium has been reached. Without HSA, the concentration value (0.421 ± 0.005 mM) is higher than with HSA (0.373 ± 000.3 mM), which reflects the binding of p-cresylsulfate to the HSA. The corresponding calculated association constant K is found to be 446 ± 54 M^{-1}. This is in perfect agreement with results obtained through ITC experiments.

Moreover with HSA, the p-cresylsulfate concentration is remarkably constant from 50 to 180 min, proving that the binding does not increase over time.

Results obtained by varying the ionic strength presented in Table 2 clearly support the electrostatic nature of the interaction. Indeed, the affinity constant values decrease with the increase of ionic strength. Affinities decrease by 25% and 18% for p-cresol and p-cresylsulfate, respectively, when ionic strength increases from 0.15 to 0.30 M. However, a contribution from hydrophobic binding cannot be excluded, in view of the interaction of indoxyl sulfate with HSA reported in previous work. Indeed, the aromatic ring of this molecule binds with a hydrophobic pocket site and the sulfate group binds with charged amino-acids.[21]

The results of the interaction performed at 37 °C are reported in Table 3.

At this temperature, the affinities of both p-cresol and p-cresylsulfate are significantly lower, whereas the $\Delta H°$ values are similar to those measured at 25 °C. These results are in good agreement with the values predicted using the fundamental Van't Hoff equation. Thus, the binding sites of HSA were energetically the same, and the protein structure was unchanged in the temperature range studied. On the basis of our results (K = 381 M^{-1} at 37 °C) and given the mean concentration of p-cresol and p-cresylsulfate (185 μM) in the uremic plasma before dialysis,[8] the calculated fraction of uremic toxin bound

TABLE 3: Thermodynamic Parameters of Binding Determined at 37 °C[a]

compounds	K (M^{-1})	$\Delta G°$ (kJ.mol^{-1})	$\Delta H°$ (kJ.mol^{-1})	$\Delta S°$ (J.mol^{-1}.K^{-1})	n
p-cresol	381 ± 16[b]	−15.3 ± 1.2	−31.3 ± 2	−51.6 ± 1.0	1.02 ± 0.04
p-cresylsulfate	233 ± 5	−14.0 ± 1.5	−37.2 ± 1	−74.8 ± 1.5	1.02 ± 0.04

[a] K: association constant; $\Delta G°$: standard free Gibbs energy; $\Delta H°$: standard binding enthalpy; $\Delta S°$: standard entropy; n: number of sites. [b] Values are the mean ± standard error of three independent experiments.

to the HSA (40 g·L^{-1}) is calculated as only 20%. If we consider an extreme case presenting hypo-albuminemia (30 g·L^{-1} of albumin) and very high concentrations of p-cresol and p-cresylsulfate (370 μM), the bound fraction is then 13%. This result contradicts the findings of previous studies reporting a strong binding for these molecules[14,17,22−25] but is in good agreement with one earlier report.[26] Such a discrepancy might be due to the methods of investigation used; ultrafiltration processes involve physical phenomena (viscosity change, equilibrium displacement, back diffusion, etc.) which have been reported to cause artifacts in measurement.[27,28] Indeed for all reactions, the complex formed and the free reagents are in apparently constant concentrations, since the equilibrium is dynamic. If free ligand is separated by using a membrane with appropriate cutoff (ultrafiltration), the ligand concentration in the reactional medium is diminished. According to the well-known Le Chatelier's principle, the equilibrium is then displaced, leading to the dissociation of the complex, which results in weaker binding. This is not in agreement with the strong binding reported for p-cresylsulfate studied by ultrafiltration,[18,29,30] which suggests that free p-cresylsulfate ultrafiltration was hindered. Indeed, during the ultrafiltration process, the increased plasma concentration in the retentate could hinder filtration of the p-cresylsulfate. In addition, this increased plasma concentration could result in a membrane potential, which would also impede p-cresylsulfate filtration. We therefore feel that relying solely on ultrafiltration to determine binding is insufficient, and questionable.

It appears to be useful to study competition between p-cresol and p-cresysulfate for HSA, to obtain more accurate information on binding sites at the molecular level. Our results show that the affinity of p-cresylsulfate decreases from $K = 401 \pm 38$ M^{-1} with HSA alone to $K = 244 \pm 23$ M^{-1} with HSA saturated 50% by p-cresol. It can thus be concluded that the molecules either share the same binding site or have binding sites very near each other. Moreover, the rather low association constant at 37 °C implies a low HSA-bound fraction of toxin. As albumin is not the sole plasma protein, other proteins may bind the uremic toxin, particularly immunoglobulin, which is known to present binding properties.[31] But it is difficult to implicate immunoglobulin because of the strong binding reported.[14,32] Indeed, taking into account the mean plasma immunoglobulin concentration (80 μM) and the mean p-cresylsulfate concentration in uremic plasma, and given a very high affinity constant ($K = 10^8$ M^{-1}), the calculated fraction of the bound toxin is only 45%.

Consequently, the binding to protein of p-cresylsulfate was not sufficient to explain the poor removal rate of this uremic toxin by hemodialysis.

Conclusions

This first thermodynamic study reports that p-cresylsulfate and p-cresol are bound to HSA involving van der Waals type interactions. The binding sites of the two molecules on HSA are the same or very close. The values of the association constants are weak, which reflects a moderate affinity of the toxins for HSA. The fraction of unbound p-cresylsulfate is thus about 80−87%. The low fraction of bound toxin (20−13%) appears to be insufficient to link strong binding to poor removal of the toxin by hemodialysis.

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References and Notes

(1) de Smet, R.; van Kaer, J.; van Vlem, B.; de Cubber, A.; Brunet, P.; Lameire, N.; Vanholder, R. *Clin. Chem.* **2003**, *49*, 470.
(2) Schepers, E.; Meert, N.; Glorieux, G.; Goeman, J.; Van der Eycken, J.; Vanholder, R. *Nephrol., Dial., Transplant.* **2007**, *22*, 592.
(3) Bone, E.; Tamm, A.; Hill, M. *Am. J. Clin. Nutr.* **1976**, *29*, 1448.
(4) Powell, G. M.; Miller, J.; Olavesen, A. H.; Curtis, C. G. *Nature* **1974**, *252*, 234.
(5) Vanholder, R. *Nephrologie* **2003**, *24*, 373.
(6) Schepers, E.; Meert, N.; Glorieux, G.; Goeman, J.; Van der Eycken, J.; Vanholder, R. *Nephrol., Dial., Transplant.* **2007**, *22*, 592.
(7) Winchester, J. F.; Hostetter, T. H.; Meyer, T. W. *Am. J. Kidney Dis.* **2009**, *54*, 792.
(8) Hida, M.; Aiba, Y.; Sawamura, S.; Suzuki, N.; Satoh, T.; Koga, Y. *Nephron* **1996**, *74*, 349.
(9) Vanholder, R.; de Smet, R.; Glorieux, G.; Argiles, A.; Baurmeister, U.; Brunet, Ph.; Clark, W.; Cohen, G.; de Deyn, P.; Deppisch, R.; Descamps-Latscha, B.; Henle, T.; Jorres, A.; Lemke, H.; Massy, Z. A.; Passlick-Deetjen, J.; Rodriguez, M.; Stegmayr, B.; Stenvikel, P.; Tetta, C.; Wanner, C.; Zidek, W. *Kindney Int.* **2003**, *63*, 1934.
(10) Meijers, B. K.; Bammens, B.; De Moor, B.; Verbeke, K.; Vanrenterghem, Y.; Evenepoel, P. *Kidney Int.* **2008**, *73*, 1174.
(11) Bammens, B.; Evenepoel, P.; Keuleers, H.; Verbeke, K.; Vanrenterghem, Y. *Kindney Int.* **2006**, *69*, 1081.
(12) Meijers, B. K. I.; Van Kerckhoven, S.; Verbeke, K.; Dehan, W.; Vanrenterghem, Y.; Hoylaerts, M. F.; Evenpoel, P. *Am. J. Kidney Dis.* **2009**, *54*, 891.
(13) Lesaffer, G.; De Smet, R.; Lameire, N.; Dhondt, A.; Duym, P.; Vanhloder, R. *Nephrol., Dial., Transplant.* **2000**, *15*, 50.
(14) Martinez, A. W.; Recht, N. S.; Hostetter, T. H.; Meyer, T. W. *J. Am. Soc. Nephrol.* **2005**, *16*, 3430.
(15) Chuang, V.; Otagari, M. *Chirality* **2006**, *18*, 159.
(16) Lesaffer, G.; De Smet, R.; D'heuvaert, T.; Belpairea, F. M.; Lameire, N.; Vanholder, R. *Life. Sci.* **2001**, *69*, 2237.
(17) Meijers, B. K. I.; Bammens, B.; Verbeke, K.; Evenepoel, P. *Am. J. Kidney Dis.* **2008**, *51*, 839.
(18) de Loor, H.; Bammens, B.; Evenepoel, P.; de Preter, V.; Verbeke, K. *Clin. Chem.* **2005**, *51*, 1535.
(19) Velazquez-Campoy, A.; Todd, M. J.; Freire, E. *Biochemistry (U.S.)* **2000**, *39*, 2201.
(20) Krishnan Namboodiri, K. P.; Viswanathan, S.; Ganesan, R.; Jyothi Bhasu, V. C. *Monatsh. Chem.* **1982**, *113*, 949.
(21) Ghuma, J.; Zunszai, P. A.; Petitpas, I.; Bhattacharya, A. A.; Otagari, M.; Curry, S. *J. Mol. Biol.* **2005**, *353*, 38.
(22) Niwa, T.; Maeda, K.; Ohki, T.; Saito, A.; Kobayashi, K. *Clin. Chim. Acta* **1981**, *110*, 51.
(23) De Smet, R.; David, F.; Sandra, P.; Van Ker, J.; Lesaffer, G.; Dhondt, A.; Lameire, N.; Vanholder, R. *Clin. Chim. Acta* **1998**, *278*, 1.
(24) Vanholder, R.; De Smet, R.; Lameire, N. *Kidney Int. Suppl.* **2001**, *78S*, 266.
(25) De Smet, R.; Van Kaer, J.; Liebich, H.; Lesaffer, G.; Verstraete, A.; Dhondt, A.; Duym, P.; Lameire, N.; Vanholder, R. *Clin. Chem.* **2001**, *47*, 5–901.
(26) Ogata, N.; Shibata, T. *Res. Commun. Mol. Pathol. Pharmacol.* **2000**, *107*, 167.
(27) Barré, J.; Chamouard, J. M.; Houin, G.; Tillement, J. P. *Clin. Chem.* **1985**, *31*, 60.

(28) Jung, D.; Mayersohn Perrier, D. *Clin. Chem.* **1981**, *27*, 166.
(29) Meijers, B. K. I.; de Loor, H.; Verbeke, K.; Evenepoel, P. *Kidney Int.* **2006**, *70*, 232.
(30) Meyer, T. W.; Peattie, J. W. T.; Miller, J. D.; Dinh, D. C.; Recht, N. S.; Walther, J. L.; Hostetter, T. H. *J. Am. Soc. Nephrol.* **2007**, *18*, 868.
(31) Routledge, P. A. *Br. J. Clin. Pharmacol.* **1986**, *22*, 499.
(32) Fagugli, R. M.; De Smet, R.; Buoncristiani, U.; Lameire, N.; Vanholder, R. *Am. J. Kidney Dis.* **2002**, *40*, 339.

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