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Binding of bromocresol green and bromocresol purple to albumin in hemodialysis patients

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Abstract

Background: Colorimetric albumin assays based on binding to bromocresol purple (BCP) and bromocresol green (BCG) yield different results in chronic kidney disease. Altered dye binding of carbamylated albumin has been suggested as a cause. In the present study, a detailed analysis was carried out in which uremic toxins, acute phase proteins and Kt/V, a parameter describing hemodialysis efficiency, were compared with colorimetrically assayed (BCP and BCG) serum albumin.

Methods: Albumin was assayed using immunonephelometry on a BN II nephelometer and colorimetrically based on, respectively, BCP and BCG on a Modular P analyzer. Uremic toxins were assessed using high-performance liquid chromatography. Acute phase proteins (C-reactive protein and α_1-acid glycoprotein) and plasma protein α_2-macroglobulin were assayed nephelometrically. In parallel, Kt/V was calculated.

Results: Sixty-two serum specimens originating from hemodialysis patients were analyzed. Among the uremic toxins investigated, total para-cresyl sulfate (PCS) showed a significant positive correlation with the BCP/BCG ratio. The serum α_1-acid glycoprotein concentration correlated negatively with the BCP/BCG ratio. The BCP/BCG ratio showed also a negative correlation with Kt/V.

Conclusions: In renal insufficiency, the BCP/BCG ratio of serum albumin is affected by multiple factors: next to carbamylation, uremic toxins (total PCS) and α_1-acid glycoprotein also play a role.

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Keywords: albumin; bromocresol green; bromocresol purple; carbamylation; para-cresyl sulfate; uremic toxins.

Introduction

Albumin is an important marker for predicting nutritional status, morbidity and mortality in hemodialysis patients [1]. Colorimetric serum albumin assays based on binding to bromocresol purple (BCP) and bromocresol green (BCG) are known to yield discrepant results in end-stage renal disease (ESRD) [2]. Dyes may bind to albumin due to weak van der Waals forces. In the majority of the ESRD population, the serum albumin concentrations obtained with BCG appear to be more reliable in comparison with BCP-based measurements, which yield falsely low serum albumin concentrations [3–6]. However, in a recent paper, evaluating 24 serum albumin measurement procedures (3 immunochemical, 9 BCG and 12 BCP methods) in patients without renal disease and with kidney failure before hemodialysis, larger biases were observed with BCG than with BCP, when compared with the reference measurement procedure (Roche Tina-quant immunochemical procedure). Thus, BCP was proposed as the preferred agent for standardization of serum albumin results using dye-binding methods [7]. In patients with a nephrotic syndrome, the increased amount of α_2-macroglobulin is a major factor for positive bias of BCG-based serum albumin assays [8]. As BCG shows an affinity for α- and β-globulins [9, 10], BCP should be used to determine the serum albumin concentrations in nephrotic syndrome [8].

At this moment, the underlying reasons for the discrepancy between BCG- and BCP-based serum albumin assays are not yet completely resolved. As progressive renal insufficiency induces many changes and causes accumulation of several compounds in plasma, the binding of serum albumin to dyes in ESRD patients may be simultaneously affected by many factors. Carbamylation has been identified as one of the confounders of BCP-based serum albumin assays as blood urea concentrations rise in advanced renal insufficiency. Carbamylation is a non-enzymatic, posttranslational modification of proteins by isocyanate, a urea dissociation product [11]. Cyanate binds irreversibly to proteins and neutralizes positively charged lysines, leading to changes in protein structures [12]. The two binding sites of albumin for BCP possess a lysine residue, which can be carbamylated by isocyanate, resulting in lower measured serum albumin concentrations [2, 13]. Carbamylation of albumin reduces its ability to bind ligands, especially drugs [14]. Assessing carbamylation can be considered as a good marker for evaluating hemodialysis efficiency. Being a baseline parameter of dialysis adequacy, Kt/V represents the cleared blood volume related to the distribution volume of urea [15].

In renal insufficiency, middle-sized and protein-bound molecules accumulate in plasma [16–18]. A myriad of compounds, called uremic toxins, which are excreted by the healthy kidneys under normal condition [19], are protein bound. Para-cresol originates from the phenylalanine and tyrosine metabolism by intestinal bacteria and is partly converted to para-cresyl sulfate (PCS) and para-cresyl glucuronide (PCG) [20, 21]. It binds to albumin and shows structural resemblance to BCG and BCP [21]. This compound is a marker for cardiovascular disease in ESRD [22, 23]. Indoxyl sulfate (IxS) is a tryptophan metabolite, which is converted into indole by intestinal bacteria. In the liver, indole is metabolized into IxS [24]. Also indole-3-acetic acid is produced by intestinal bacteria [21]. 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF) inhibits binding of drugs to albumin [6]. Thus, besides carbamylation of the binding sites for BCP [2], the presence of uremic toxins may be a confounder of the falsely low serum albumin concentrations [4].

Finally, the plasma protein spectrum undergoes changes in renal failure. Serum concentrations of α_1-acid glycoprotein (and in particular the strongly concanavalin A-reactive α_1-acid glycoprotein fractions) are higher in hemodialyzed and uremic patients than in control subjects [25]. In patients with renal insufficiency, α_1-acid glycoprotein is qualitatively different from normal α_1-acid glycoprotein [26]. The influence of α_1-acid glycoprotein on BCG or BCP assays has not yet been investigated in patients with ESRD.

In the present study, the dye binding of albumin in renal insufficiency will be investigated in detail. The effects of the various potential compounds (dialysis efficiency, uremic toxins and plasma proteins) will be compared.

Materials and methods

Sixty-two ESRD patients (36 men, 26 women; median age, 71 years; IQR, 62–80 years), treated with chronic hemodialysis, were enrolled in the study. Blood, sampled before the start of a hemodialysis session, was centrifuged (10 min, 3000×*g*, room temperature), and serum was obtained. Routine blood parameters such as urea and creatinine were determined. Albumin was assayed using immunonephelometry on a BN II nephelometer (Siemens Medical Solutions, Erlangen, Germany) as gold standard. In parallel, serum albumin was assayed colorimetrically, using BCG- and BCP-based dye binding assays on a Modular analyzer (Roche, Mannheim, Germany). In order to compare the relative binding of both dyes with albumin, the ratio between BCP and BCG results was calculated. Acute phase proteins [C-reactive protein (CRP) and α_1-acid glycoprotein] and plasma protein α_2-macroglobulin were assayed nephelometrically on a BN II nephelometer (Siemens Medical Solutions, Erlangen, Germany), using commercial antisera (Siemens Medical Solutions).

Single-pool Kt/V (spKt/V) was calculated according to Daugirdas [27]:

$$\mathrm{spKt/V} = -\mathrm{LN}\left(\frac{\mathrm{BUN}_{\mathrm{post}}}{\mathrm{BUN}_{\mathrm{pre}}} - 0.008 \cdot t\right) + \left(4 - 3.5 \cdot \frac{\mathrm{BUN}_{\mathrm{post}}}{\mathrm{BUN}_{\mathrm{pre}}}\right) \cdot \frac{\mathrm{UF}}{\mathrm{BW}},$$

where BUN_{pre} and BUN_{post} are the pre- and posthemodialysis blood urea nitrogen concentrations, UF is the ultrafiltration volume and BW is the postdialysis body weight. Measured Kt/V values were extrapolated to a weekly based Kt/V_{week} to account for different dialysis strategies.

In parallel, seven uremic toxins were determined with high-performance liquid chromatography (HPLC): IxS, PCS, PCG, indol-3-acetic acid (IAA), CMPF, hippuric acid (HA) and uric acid (UA). Prior to analysis, serum samples were denaturated at 95 °C, followed by filtration using a 30-kDa cutoff molecular filter (Centri-free Micropartition Devices, Amicon Inc., Beverly, MA, USA). For the determination of the free fractions, denaturation was preceded by filtration (Millipore, Billerica, MA, USA). The HPLC analyzers consisted of a Waters Alliance 2695 device (Waters, Zellik, Belgium) and two detectors in series: a Waters 996 photodiode array detector and a Waters 2475 fluorescence detector. The separation was performed at room temperature on a reversed-phase XBridge C8 column (3.5 µm, 150 mm × 4.6 mm, Waters) with an Ultrasphere ODS guard column (5 µm, 45 mm × 4.6 mm, Beckman Instruments, Miami, FL, USA). The mobile phase consisted of a 50-mM ammonium formate buffer (mobile phase A, pH 3.0) and methanol (mobile phase B). HA and CMPF were analyzed by UV detection at 245 and 254 nm, respectively, whereas PCS and PCG ($\lambda ex = 264$ nm, $\lambda em = 290$ nm), and IAA and IxS ($\lambda ex = 272$ nm, $\lambda em = 340$ nm and 374 nm, respectively) and the internal standard fluorescein (λex: 443 nm, λem: 512 nm) were determined by fluorescence detection [28, 29]. *In vitro* addition of PCS and IxS to serum of healthy subjects ($n = 5$) was achieved by adding PCS (Sigma, St Louis, MO, USA) and IxS in phosphate-buffered saline (0.1 mol/L, pH 7.4) to serum. The study was approved by the Local Ethics Committee (2015/0932, Belgian registration number B670201525559).

Statistical analysis was carried out using the program MedCalc version 15.5 (MedCalc Software, Mariakerke, Belgium). Normality of distributions was tested using the D'Agostino Pearson test. To investigate the correlation between two non-normal continuous variables, a rank correlation test was carried, whereby Spearman's rho was calculated. A p-value <0.05 was considered *a priori* to be statistically significant.

Results

The predialysis serum specimens of the hemodialysis patients were assayed for albumin using immunonephelometry (range, 27.1–48.5 g/L), BCP and BCG. Both Roche dye binding methods showed lower concentrations as compared with the immunonephelometric method [mean ± SD, 37.7 ± 4.6 g/L (immunonephelometry), 31.9 ± 5.7 g/L (BCP) and 36.7 ± 6.9 g/L (BCG)]. The following equations illustrate the relationship between the immunonephelometric method, BCP and BCG: y (albumin, BCP, g/L) = 0.5728 (albumin, immunonephelometric method, g/L) + 10.3216 (r = 0.4640, p = 0.0001) and y (albumin, BCG, g/L) = 0.5865 (albumin, immunonephelometric method, g/L) + 14.5969 (r = 0.3929, p = 0.0016). The BCP/BCG ratio was compared with the concentrations of α_1-acid glycoprotein, α_2-macroglobulin and CRP. The observed α_1-acid glycoprotein concentration range in serum (median, 1.32 g/L; IQR, 0.94 g/L–1.52 g/L) largely exceeded the reference range of 0.39–1.15 g/L. The serum concentrations of α_2-macroglobulin and CRP (reference value) ranged from 0.6 to 2.6 g/L (1.3–3.0 g/L) and from 0.5 to 65 mg/L (<5.0 mg/L), respectively. Regression analysis revealed a significant correlation between the BCP/BCG ratio and the α_1-acid glycoprotein protein concentration. The regression equation was y (BCP/BCG ratio) = 0.8920 – 0.227log(α_1-acid glycoprotein, g/L), r = –0.6279, p < 0.0001 (Figure 1). By contrast, the serum CRP and α_2-macroglobulin concentrations did not show a correlation with the BCP/BCG ratio.

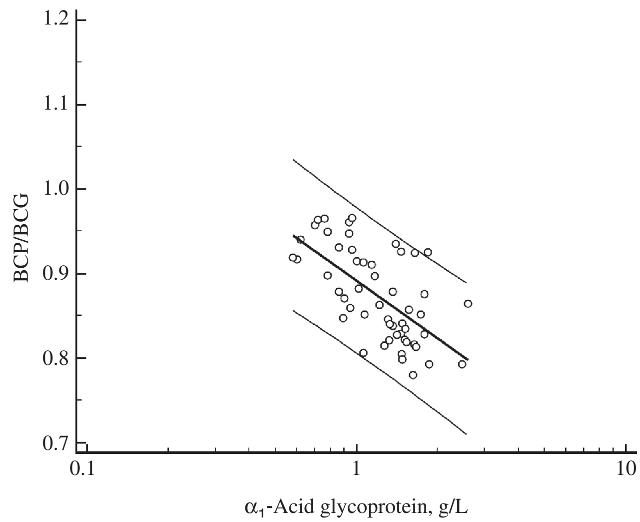

Figure 1: Regression analysis of α_1-acid glycoprotein and the BCP/BCG ratio in serum of hemodialysis patients.
The regression equation is y (BCP/BCG ratio) = 0.8920 – 0.227 log(α_1-acid glycoprotein, g/L), r = –0.6279, p < 0.0001.

In order to assess the relative importance of the various uremic toxins for the BCP/BCG ratio, multiple regression was applied in which the BCP/BCG ratio was compared with the concentrations of CMPF, free and total HA, free and total IAA, free and total IxS, free and total PCS, free and total PCG and UA. Among the investigated uremic toxins, only total PCS showed a significant correlation with the BCP/BCG ratio: y (BCP/BCG ratio) = 0.8427 + 0.01019 (total PCS, μmol/L), r = 0.3362, p = 0.0076 (Figure 2). *In vitro* addition of PCS (final concentration ranging from 0 to 0.2 mmol/L) to serum of healthy subjects resulted in a small increase of the BCP/BCG ratio. In particular, BCP-based albumin concentrations were more affected by adding PCS, in comparison with the results obtained with the BCG-based method. As expected, addition of IxS did not result in a significant change in albumin results.

The BCP/BCG ratio showed a good correlation with weekly Kt/V: y (BCP/BCG ratio) = 1013–0.064 (Kt/V_{week}), r = 0.6996, p < 0.001. Similarly, *in vitro* carbamylated

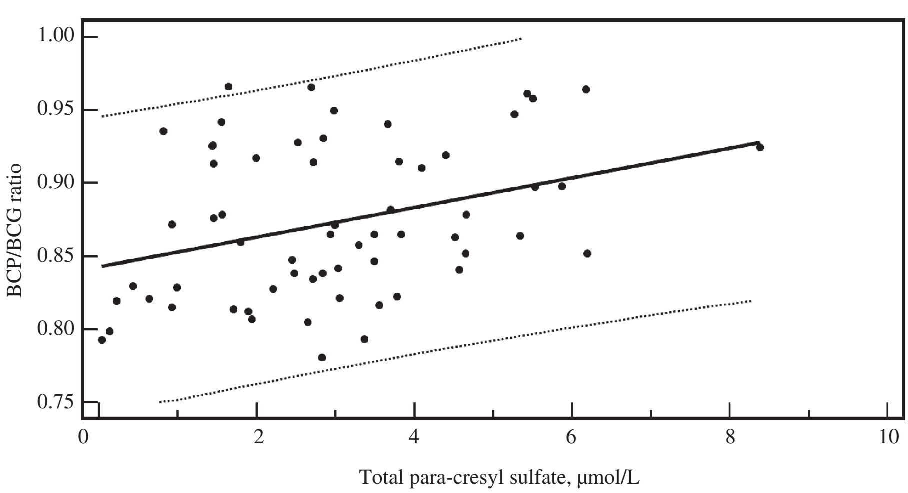

Figure 2: Regression analysis of total para-cresyl sulfate and the BCP/BCG ratio in serum of hemodialysis patients.
The regression equation is y (BCP/BCG ratio) = 0.8427 + 0.01019 (total para-cresyl sulfate, μmol/L), r = 0.3362, p = 0.0076.

Table 1: Multiple regression analysis with the BCP/BCG ratio as a dependent variable.

		β (SE)	p-Value
BCP/BCG ratio	Kt/V	−0.0618 (0.0102)	<0.0001
$r^2=0.57$, $p<0.001$	Total para-cresyl sulfate	0.0083 (0.0032)	0.013

serum showed an effect on the BCP/BCG ratio. Multiple regression analysis confirmed that Kt/V and PCS were independent predictors of the BCP/BCG ratio. Table 1 summarizes the parameters, which significantly affect the BCP/BCG ratio.

Discussion

As illustrated in the present study, the BCP/BCG ratio in patients with terminal renal insufficiency may be determined by multiple factors. The influence of urea on the discrepant results between immunonephelometry, and BCP has already been demonstrated, which is attributable to carbamylation [2]. A good correlation between the percentage of carbamylated albumin and the global carbamylation of blood proteins in uremic subjects has been observed [30]. Due to the carbamylation-induced structural changes, incorrect protein function may lead to a variety of health problems such as cardiovascular disease, the most common cause of death in patients with renal failure [12, 30]. Carbamylation of proteins by elevated blood urea concentrations can activate mesangial cells to a profibrogenic phenotype, accelerating the progression to kidney failure [31]. In addition, carbamylated LDL plays a pivotal role in atherosclerosis [32].

In a multiple regression model, only Kt/V_{week} and total PCS remained significant independent confounders of the BCP/BCG ratio. Both parameters highlight the association between the BCP/BCG ratio and the presence of circulating toxins in renal insufficiency. A fairly negative correlation between Kt/V and the BCP/BCG ratio was demonstrated. This is in agreement with the observations of Kok et al. [2], who linked the BCP/BCG ratio with carbamylation. It is of note, however, that although Kt/V is reflecting urea kinetics, it is not representative for the kinetics of other uremic toxins, of which concentrations were found more depending on protein equivalent of nitrogen appearance and/or residual renal function [33]. In addition, it has already been demonstrated that no interfering substance is present or introduced during hemodialysis as comparable BCP-based albumin concentrations were measured in pre- and posthemodialysis plasma samples [2].

Independent from carbamylation, the BCP/BCG ratio partly depended on the serum concentration of uremic toxins. Among the spectrum of investigated toxins (free and total IAA, PCS, PCG, HA, IxS and total CMPF), important differences were observed. Only total PCS correlated significantly with the BCP/BCG ratio. This might imply that BCP has affinity for the same albumin binding site as PCS has (Sudlow's site II), in contrast to CMPF (Sudlow's site I). From previous binding competition experiments, clearly showing superior binding capacity of PCS versus IxS, IAA and HA, it is however hard to explain that the correlation between BCP and PCS is due to mutual competition [34]. The in vivo results were confirmed by *in vitro* spiking of PCS to human serum. In contrast to BCP, BCG-based assays were less affected by PCS. The affinity constant of PCS towards albumin is 6 μM [35].

Also the altered protein spectrum in renal insufficiency and in particular the increased serum α_1-acid glycoprotein concentrations may play a role in explaining the BCP/BCG ratio in hemodialysis patients. The correlation between the BCP/BCG ratio, CRP and α_1-acid glycoprotein points towards a link between inflammatory reactions [36] and the BCP/BCG ratio. In agreement with the findings of Xu et al. [10], α_1-acid glycoprotein was identified as a dye binding protein. However, in the multiple regression analysis, α_1-acid glycoprotein was not withheld as an independent confounder of the BCP/BCG ratio. In hemodialysis, the values exceed the reference range. In contrast to the findings of Ueno et al. [8], the α_2-macroglobulin concentration did not affect the BCP/BCG ratio. This can be explained by the fact that in patients with a nephrotic syndrome, the α_2-macroglobulin/albumin ratio is higher than in the hemodialysis population.

In the present study, we have demonstrated that the ratio of serum albumin concentrations obtained with BCP and BCG is affected by multiple factors. Next to carbamylation, also uremic toxins (in particular PCS) and α_1-acid glycoprotein affect the binding.

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