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NEW APPROCHES TO THE REMOVAL OF PROTEIN-BOUND TOXINS FROM BLOOD PLASM OF UREMIC PATIENTS

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Калит сўзлар: *оқсил билан боғланган уремик токсинлар, зардоб албумин, микрокалориметрия, активлаштирилган кўмир, фракция, гемосорбция.*

АННОТАЦИЯ

Оқсил билан боғланган уремик токсинларни ҳайдашни янги ва самарали усуллари ривожланишининг назарий жиҳатлари таъкидланди. Оқсил билан боғланган уремик токсинлар билан бирга, зардоб албуминлари-ни лиганд даражасини баҳолашда потокли ва дифференциал сканерли микрокалориметрия усули самарали эканлиги кўрсатилган. Тасдиқлаш – иш INTAS-04-082-7065 грант дастури томонидан қўллаб-қувватланди.

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АННОТАЦИЯ

Освещены теоретические аспекты разработки эффективного метода удаления белковосвязанных уремических токсинов. Показано, что методы поточной и дифференциальной сканирующей микрокалориме-трии эффективны для оценки степени лигандной загрузки сывороточного альбумина с белковосвязанными уремическими токсинами. Подтверждение – работа поддержана программой грантов INTAS-04-082-7065

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ABSTRACT

The article is elucidates the theoretical aspects of the effective method development for the removal of protein-bound uremic toxins. It is shown that the methods of flow and differential scanning microcalorimetry are effective for the evaluation of the degree of ligand loading of human serum albumin with protein-bound uremic toxins. Acknowledgment - This work supported by INTAS-04-082-7065 grant program.

The molecules of albumin isolated from blood plasma of the patients being kept on chronic dialysis are demonstrating significant alterations of conformation and complex-forming properties the correction of which by conventional methods of extracorporeal detoxification (exhaustive dialysis, treatment on synthetic Spherical Carbon Nitrogen (SCN) carbons) are practically ineffective. Deliganding of uremic albumin maybe successfully performed on conventional carbon haemosorbents upon preliminary separation of blood plasma and its dilution with acetate buffer 1:1 at pH=5.08. Treatment of the whole blood of patients onto new mass fractal deliganding carbon i.e. haemosorbents of Hemosorbents Granulated Deliganding (HSGD) trade mark These HSGD haemosorbents are quite effectively could be used for restoration of main parameters of uremic Human Serum Albumin (HSA) molecules conformation and ligand-binding activity simultaneously with hemodialysis upon the protection by locally performed citrate anticoagulation as an easier and cheaper method scheme for the removal of protein-bound uremic toxins.

There is no doubt that the problem of extracorporeal removal of protein-bound uremic toxins is the one of the most important tasks of the therapy of the terminal stage of chronic renal insufficiency [4]. Long time ago it has been stated that transport proteins of blood plasma of uremic patients, and, first of all, serum albumin possess the decreased affinity to medicinal preparations due to their overloading with protein-bound endogenous metabolites. Let's note that according to classification [24], albumin possesses 3 main centers for binding pharmaceuticals. The complex-forming properties of all these centers in albumin isolated from uremic plasma are suppressed, while the binding capacity of such plasma is decreased, respectively.

According to modem view [1, 27], the significant number of compounds being at some level protein-bound is taking part in realization of uremic toxicosis:2-methoxyresorcinol, 3–deoxyglucozone, 3-carboxy-4-methyl-5-propyl-2-furanpropionoc acid**,** Fructoselysine, Glyoxal, Hippuric acid, Homocysteine, Indoxylsulphate, Kynurenine, Kynurenic acid, p-cresol, Pentosidine, Phenol, p-

OH-hippuric acid, Retinol-binding protein, Spermidine, Spermine, Leptin, Melatonin, Ne-(carboxymethyl)lysine.

Let's select from that list 3 protein-bound uremic toxins. That are characterized by strong, medium and low affinity to human serum albumin, i.e. CMPF, indoxylsulphate and hippuric acid respectively [20].

On the opinion of Tsutsumi et al. [26], exactly these 3 metabolites, i.e. 3-carboxy-4- methyl-5-propyl-2furanpropionoc acid (CMPF), indoxylsulphate (IS) and hippuric acid (HA) are the most important inhibitors of binding of pharmaceuticals with uremic HSA. CMPF is a typical representative of urofuranoid acids possessing strongly pronounced lipophylic properties and high (near 10^8 M^{-1}) constant of association with HSA molecule. At normal state that metabolite is excreted in unaltered form in urine being involved, due to its high affinity to albumin, in active tubular secretion [9]. CMPF suppresses the activity of glutathione-S-transferase and production of iodine in rat hepatocytes [13], possesses certain nephrotoxicity and inhibits erythropoiesis [18]. That uremic toxin suppresses the binding of non-conjugated bilirubin and a number of medicinal preparations, including furosemide, with HSA [25].

In healthy donors indoxylsulphate (IS) is excreted in urine by tubular secretion. Accumulation of that metabolite possessing medium affinity to HSA, in the fluids of body of uremic patients is being thought to be linked to cerebral disfunction, disturbance of iodine metabolism and acceleration of the progression of renal insufficiency associated for example with an increased index of glomerular sclerosis [17]. Indoxylsulphate is also suppressing hepatocyte' transport of thyroxine and formation of colonies of erythroblasts and lymphoblasts in cell cultures of bone marrow [13, 12]. Among preparations and metabolites the binding of which with HSA became negatively affected in the presence of elevated concentration of indoxylsulphate, one may name diazepam, warfarin, L-tryptophane, furosemide, salicylates [5, 10, 14, 17, 19].

Hippuric acid (HA) is least of all tightly bound with HSA. As well as CMPF and IS, it suppresses the production of iodine by hepatocytes, but for manifestation

of such effect the concentration of that metabolite should be 10 fold higher than the concentration of CMPF. Among pharmaceuticals with which hippuric acid competes for transport sites of HSA, without a doubt, salicylates should be mentioned [5].

Fig. la presents the melting curve of purified (defatted) HSA received by the method of differential scanning microcalorimetry (DSMC). That method is very sensitive to the degree of HSA liganding, and therefore very suitable for evaluation of the loading of mentioned transport protein with different hydrophobic toxins and metabolites [22]. As it follows, CMPF renders the strongest influence on the results of DSMC, increasing the temperature of denaturation of albumin and transforming unimodal curve of its melting to bimodal one. One may see it especially clear, where upon the increase of CMPF/albumin relation to 2:1 the melting curve is practically identical to that for the complex "albumin - nonconjugated bilirubin" [16].

For evaluation of the degree of loading of discrete transport centers of albumin with hydrophobic metabolites, the method of flow microcalorimetry is being successfully used; it consists in the precise measurement of amount of heat excreted or absorbed during the process of complex- formation of albumin with marker ligands addressed to certain binding sites of the molecule of that protein [7]. Naturally upon the increase of the degree of occupation of each concrete binding center, an absolute value of heat (enthalpy) of complex formation is decreasing. Where the addition of IS and HA (but not CMPF) causes the apparent alterations in the heats of binding of marker ligands, exactly salicylic acid and phenol red.

In turn, the elevation of concentration (0, 60, 120, 180, 240 and 300 μmol) of CMPF which is possessing trophy toward bilirubin's binding center on albumin molecule notably elevates the content of free fraction of bromophenol blue (from 7 up to 24 %) that is considered the standard marker of bilirubin's center, thus demonstrating the increased degree of occupation exactly of that center of the molecule by this uremic toxin.

The methods of differential scanning and flow microcalorimetry in combination with the classic method of equilibrium dialysis provide complete enough information on the changes of molecular conformation and complex-forming activity of HS A molecules upon the loading by typical representatives of protein-bound uremic toxins in model studies and, consequently, they may be adequately used for evaluation of the respective alterations of HSA isolated from blood plasma of uremic patients. Especially one should note that the method of isolation of HSA from blood plasma of uremic patients should be delicate enough, i.e. it should favor the preservation of “ligand coating” of protein-carrier molecule [29].

Represents the differences in melting thermograms of albumin fractions isolated from blood plasma of healthy donors and uremic patients undergoing chronic hemodialysis treatment. That phenomenon has been studied in detail on 47 patients undergoing chronic alimentary dialysis from 6 months to 20 years [21]. As it follows from this picture the most pronounced deviations in the shape of melting curves of albumin are observed in patients undergoing dialysis from l to 5 years. The analogous results have been obtained on enthalpy of binding of marker ligands, and upon the study of spectra of fluorescence of tryptophane where the respective curve for the period of 1-5 years is the most different from that for HSA of healthy donors (data not presented).

For the first glance, the fact that upon increased duration of alimentary haemodyalysis in uremic patients the conformation and complex-forming characteristics of uremic albumin are becoming evidently better, is paradoxical. Nevertheless it could be explained that not only plasma albumin content is important prognostic factor in prediction of survival rate of dialysis patients [19] but also functional abilities of that transport protein should be taken into account.

All abovementioned points on the expediency of the active extracorporeal removal of protein bound uremic toxins that may be substantially narrowed down to the development of the methods of normalization of conformational and transport functions of the molecule of uremic albumin. More than 10 years ago Dr.

John Daugirdas [8] has described the different aspects of problem for the removal of hydrophobic uremic toxins that have been formulated as 3 questions of "Dialysis doctor":

1. For what extent the protein-bound compounds possessing toxic properties are removed upon standard dialysis, high-flax-dialysis or hemofiltration, and isn't it better to remove these compounds using peritoneal dialysis;
2. If some protein-bound components are removed not too properly, is there the proof for the fact that their accumulation in the body causes toxic effects. Also, one should distinguish toxicity caused by protein-bound toxins as they are, and toxicity, that depends on the occupation by them of special sites on serum albumin, and the elevation of concentration of free fractions of other toxic compounds displacing uremic metabolites from their normal bonds with transport protein;
3. Do the practical methods for elevating the removal of protein-bound components during hemodialysis exist? Are there some other additive methods including diet ones, that allow to decrease plasma concentration of toxic protein-bound components in patients with uremia?

The first two questions somehow have found their explanation in multiple to-day studies on the problem of protein-bound uremic toxins [28, 30, 22], but the third question of "dialysis doctor" still remains unanswered.

The reason for inefficiency of conventional dialysis is the discrepancy of the size of pores of dialysis membrane and the size of protein molecules containing tightly bound ligands. Moreover, the use of heparin activating lipoprotein lipase during dialysis is leading to enhancement of liganding of HSA molecule by non-esterified fatty acids and "deterioration" of the mode of melting curves of uremic albumin (the shift of the maximum of curve to the right) due to elevation of its loading with these hydrophobic metabolites (data not presented).

The binding capacity of transport sites of albumin, especially of the site for middle-chained fatty acids (octanoate) slightly decreases. The parameters of binding for HSA of the patients undergoing constant peritoneal dialysis are practically no different from these in patients before hemodialysis, except

unexpected increase of functional potency of the center of long- chained fatty acids tested by SDS.

At the same time, the shape of melting curves of HSA of patients undergoing hemodialysis is practically similar to that in patients undergoing peritoneal dialysis, demonstrating inefficacy of both methods for the removal of the mostly tightly bound to albumin uremic toxins [11].

Nor “depleting” (24 hours) laboratory dialysis of blood plasma of uremic patients, nor its purification on conventional synthetic carbon SCN haemosorbents doesn’t alter significantly nor the shape of melting curves of albumin, nor its complex-formation properties. At the same time it is known that one may achieve sufficiently good purification of serum albumin on standard activated carbons using pH-dependent induction of conformation of the protein [6].

It was demonstrated that albumin preparations isolated from blood plasma of uremic patients pre-treated by purification on conventional SCN-2K carbonic haemosorbent at acidic range of pH (pH=3) improved the activity of the majority of binding sites towards specific marker ligands. However, the treatment of plasma at pH=3 is too “rigorous” to preserve the native state of all proteins of uremic plasma. At the same time, at least one legalized medical technology where the adsorptive purification of blood plasma occurs at significantly lowered pH, is known [15]. That technology known as Heparin-induced Extracorporeal Low-density Lipoprotein (HELP) and directed on the removal of atherogenic lipoproteins of low and very low density, results in: 1) Separation of plasma; 2) Dilution of plasma with acetate buffer (pH=5.08) at the relation 1:1; 3) Precipitation of Lipoprotein of Low Density (LPLD) with heparin; and 4) Removal of the precipitate and heparin. Naturally, in that case we are interested only in the part concerning acidification of blood plasma.

The melting curve of uremic albumin isolated from plasma that underwent the dilution with acetate buffer (pH=5.08) and the next purification on SCN carbons, looks even somehow better than that obtained upon purification at pH=3, while the purification at pH=7 is practically ineffective. That result is in

accordance with the data of flow microcalorimetry and the data obtained by equilibrium dialysis with the dye methyl red.

So, in principle, the procedure parallel to the dialysis may be performed, when blood plasma of uremic patient is being diluted in ‘on-line’ regimen by acetate buffer, undergoes the treatment onto carbonic adsorptive columns and returns to the main contour before the entrance to the traditional system for bicarbonate dialysis, that is responsible apart from other functions for the normalization of pH and removal of the excess of acetate and water. However, such system despite its apparent perspective for the solving the tasks for the removal of protein-bound uremic toxins, still is considered too bulky and expensive than it’ll be reasonable from the practical point of view.

Recently we have developed the new type of carbonic haemosorbents specially designed for the removal of protein-bound compounds [23]. According to the results of transformation of conventional synthetic carbon obtained by carbonization and activation of phenolformaldehyde resins to the haemosorbent special toward protein-bound metabolites (nonconjugated bilirubin) that preserves the original properties toward free-soluble compounds of low molecular weight (creatinin) upon somewhat elevated capacity to the marker of compounds of middle molecular weight substance - vitamin B 12 .

Table 1. Transformation of conventional phenolformaldehyde carbon (sample 1) intodeliganding form (sample 2) [32].

Bulk weight, g/cm’	Adsorption of creatinin (M.w.=113.1), mg/g	Adsorption of vitamin B12 (M.w =71355), mg/g	Adsorption of bilirubin (M.w.=584), from 3% HSA solution, mg/g
0.33, sample 1	52.5	162.5	0.56
0.168, sample 2	52.7	191.2	4.71

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These haemosorbents produced on the base of pyrolysis of nitrogen-containing synthetic resins under the mark Haemo Somenta Granulated Deliganding (HSGD) possess so-called fractal, i.e. 'broken" structure of the innate surface where instead of standard hierarchy branching of pores (macropores-mesopores-micropores), the co-existence of all their types in each unit of the adsorbent's volume is supposed [32]. The degree of factality of carbonic adsorbents has been evaluated by an index a obtained by analysis of the curves of small-angle neuron scattering (SANS).

Table 2. Correlation between the degree of fractality of carbonic adsorbent (a) and adsorption of nonconjugated bilirubin (mg/g) from albumin-containing solution [16]

Coefficient α	Type of structure	Adsorption of bilirubin (mg/g)
-3.51	Fractal surface	0.82
-2.99	Mass-fractal	5.20
-2.80	Mass-fractal	13.40
-2.39	Mass-fractal	98.70

As it follows from the Table 10, enhancement of the values of coefficient a from-3.51 to -2.39 (the range where, possibly, the transition from superficially-fractal to mostly mass-fractal structure occurs) correlates with the sharp (more than 100-fold) increase of sorption of nonconjugated bilirubin from 3% solution of HSA. Conventional carbonic haemosorbents of SCN class (α=-3.51) possess moderate adsorptive capacity by that index (near 0.6-1.0 mg/g).High adsorptive capacity of carbonic haemosorbents of HSGD trade-mark may be explained, for example, by the short diffuse way passed by the complex "carrier-ligand" from the place of its primary position to the place of adsorptive binding of ligand and the wide range of the sizes and shapes of adsorbent' pores placed in each micro-

volume of granules which allows to find their appropriate configuration responding to actual conformational state of protein-ligand complex [2].

Application of HSGD haemosorbents allows to create the original device for effective pre- hepatocytic removal of bile components and their precursors (apparatus “Artificial liver”) [16].

Ideology of the use of deliganding mass fractal haemosorbents is, apparently, reasonable enough also for solving the tasks for the removal of non-protein-bound and weakly protein-bound uremic toxins. Comparative measurement of standard capacity of two trade-marks haemosorbents towards uric acid which is presented mostly in a free form demonstrated the high preference of HSGD over SCN haemosorbents (1750 and 490 mg/g, respectively). As it follows from the Fig. 7 a, the contact with HSGD haemosorbent completely restores the normal shape of melting curves of albumin loaded with CMPF and effectively purified the model mixtures of HSA supplemented by CMPF, indoxylsulphate and hippuric acid in 4 hours microcolumn experiments.

Experimental time of adsorption has been selected taking into account the duration of hemodialysis session.

The treatment of the total plasma of uremic patients on HSGD haemosorbents leads to the restoration of practically normal shape of melting curves of uremic albumin and complex-forming capacity of its binding sites that undoubtedly points on the high level of purification of blood plasma of uremic patients from protein-bound toxins. It’s necessary to note that all abovementioned is related only to blood plasma of dialysis patients not suffering from diabetes because in the last case the restoration of normal conformation-acceptor properties of albumin is hindered due to the presence of covalent bonds between that protein and final products of glycation and oxidation [3, 31].

So, for the removal of protein-bound toxins from the body of the majority of patients undergoing chronic supportive dialysis, the next easy scheme not requiring separation of plasma and its dilution with acetate buffer may be proposed. The problem of regulation of the levels of heparinization which are essentially different

for dialysis and haemocarboperfusion [27] is easily solved in that scheme using additional citratization of blood passing through haemosorbent [32] with the next removal of the excess of citrate and restoration of ion balance by dialysis unit.

An application of these HSGD haemosorbents in the treatment of children with multy-organ failure caused by heavy poisoning with death cap allows to reduce up to 6-fold the lethality compared with the variant of therapy based on the use of conventional carbonic haemosorbent.

All mentioned is making realistic the perspectives of effective application of carbonic mass fractal and combined adsorbents not only on the therapy of acute and chronic renal insufficiency, but al so the control of other heavy clinical states related, in particular, to the manifestation of the syndrome of systemic inflammatory response and poly-organic insufficiency.

Conclusions:

1. The methods of flow and differential scanning microcalorimetry are appropriate for evaluation of the level of loading of human serum albumin with protein-bound uremic toxins.
2. The molecule of albumin isolated from blood plasma of the patients undergoing chronic hemodialysis demonstrates significant alterations of conformation and complex-forming properties, correction of which by routinely used methods of extracorporeal etoxification (dialysis, treatment on synthetic carbons of SCN type) is practically impossible.
3. Deliganding of uremic albumin may be successfully performed on conventional carbonic haemosorbents upon preliminary separation of plasma and its dilution with acetate buffer at the relation 1:1 atpH=5.08
4. Potentially easier and economically more acceptable scheme for the removal of protein-bound uremic toxins relays in the treatment of the whole blood of patients on deliganding haemosorbents =of HSGD carbons performed simultaneously with haemodyalysis upon the protection of locally done citrate anticoagulation.

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