Course notes: Urinary Proteins

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Abstract

Urinary proteins measurements have basically been used for the diagnosis and monitoring of nephrourological diseases. The pattern of urinary protein excretion can be used to identify the cause of the disease and to classify proteinuria. Urine proteins testing usually involves a screening test to detect excess protein (dipstick, colorimetric or turbidimetric methods) at the beginning, a qualitative determination (electrophoresis with all its variants including immunofixation) and finally a quantitative evaluation (immunoassay). An especially important issue is the study of Bence Jones proteins - free monoclonal light chains – which are a significant marker of the malignant monoclonal gammopathy.

Rezumat

Determinarea proteinelor urinare se foloseşte în principal pentru diagnosticul şi monitorizarea bolilor nefro-urologice. Tabloul excreției proteinelor urinare poate fi utilizat pentru identificarea cauzei bolii şi pentru a clasifica proteinuria. Testarea proteinelor urinare implică în mod obişnuit un test de screening pentru a detecta excesul de proteine (dipstick, metode colorimetrice sau turbidimetrice), o determinare calitativă (electroforeză cu toate variantele sale, incluzând imunofixarea) și, în final, o evaluare cantitativă (immunoassay). O importanță specială o are studiul proteinelor Bence Jones - lanțuri uşoare libere monoclonale - care constituie un marker semnificativ al gammapatiei monoclonale maligne.

Introduction

Kidney regularizes the chemical composition of biological fluids through selective filtering of blood, which takes place through glomerular basement membranes[11]. The movement of molecules through the membrane is dependent on the size, plasmatic concentration and their electrical charge. In the healthy kidney, most of the protein molecules are too large (molecular weight over 20 000 Da) for crossing the basement membrane. Proteins with small molecular masses (under 20 000 Da) easily cross the glomerular capillary wall, are resorbed by the proximal tube and only small amounts are excreted[5].

Violating the basement membrane can alter its permeability, which causes large protein molecules (such as albumin with molecular mass 65 000 Da and negative charge) to reach the urine and to determine the proteinuria.

The term "proteinuria" is characterized by high glomerular permeability to plasma macromolecules which are normally unfiltered. Proteinuria is defined as urinary excretion of protein greater than 150 mg/ day (10-20 mg /

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dL) in adult and 140 mg/m2 body surface area-to-child[4].

After González-Buitrago and col.[8], urine was defined as the "fluid biopsy" of the kidney and the urogenital tract.

High protein concentration in the urine is due normally to[14]:

- Glomerule`s loss of ability to retain large protein molecules;
- Proximal tubes` inability to resorb and catabolize proteins, when the glomerule`s function is normal;
- The injury of the glomerular and tubular function;
- Abnormal excretion of urine through the urinary tract;
- The combination of the conditions listed.

Types of proteinuria

I. Physiological

The total proteins of a physiological urine are about 50-150 mg/24h, in which a value of less than 30 mg/24h consists of albumin, the so-called normoalbuminuria[4]. Separating the proteins of the physiological urine in proportions, we get 1/3 albumin and 2/3 globulin.

Orthostatic proteinuria is a benign form of proteinuria that can occur in the prolonged standing, and disappears after the clinostatism[18].

II. Pathological

A. Transient proteinuria is the most common form. This excess of protein is caused by a functional disorder that can be a temporary change of the glomerular hemodynamics, after which follows a benign, self-limited course[18].

B. Persistent proteinuria reflects kidney diseases or systematic disorders. This protein may be accompanied by other abnormalities as hematuria or bacteriuria[17].

B1. Renal type

***Glomerular* type**[2,8] involves the alteration of the glomerular basement membrane permeability with the passage of macromolecules and plasma proteins (albumin, immunoglobulins). Glomerular proteinuria may be selective, semi-selective or non-selective, whether or not containing IgG and other proteins with higher molecular weight. Microalbuminuria is the slightly increased and persistent excretion of albumin in the urine. Abnormal presence of albumin in the urine is a marker of preventive risk for chronic kidney disease or a kidney injury.

Tubular type[4] is quantitively moderate and with relatively little albuminuria. Tubular proteinuria is characterized by excretion of proteins with low molecular weight (Tamm-Horsfal) the glomerular filtration remaining normal. Among the proteins with low molecular weight that are eliminated there are the β2 microglobulin as a major component and light chains of immunoglobulins.

The Bence Jones proteins are a classic example of tubular proteinuria, in which a protein of low molecular weight is filtered through the glomeruli and is eliminated due to the reduced tubular resorbtion.

The Bence Jones proteins occur in some pathological conditions like multiple myeloma, as a result of the proliferation of a kind of immunoglobulin produced by plasmocytes, which leads to the formation of an excess of monoclonal free light chains of immunoglobulins that can be found in urine and plasma. Structurally, these proteins are fragments of immunoglobulins, which result from the removal of the heavy chains of an immunoglobulin.

Mixed type (tubulo-glomerular) appears as a secondary phenomenon due to the overload of the tubular system in cases of severe glomerular proteinuria, or in diseases that in addition to glomeruli involve simultaneously kidney interstitial tissue[11]. It is a significant proteinuria from quantitave point of view and contains albumin and microproteins as well[4].

B2. Prerenal

Prerenal proteinuria is due to the cell

disintegration of proteins with low molecular weight, which is formed in blood circulation and eliminated physiologically via kidneys[4] .

Overflow proteinuria is due to an excess of proteins with low molecular weight (free light chains, lysozyme, myoglobin, hemoglobin) which are filtered through normal glomeruli at a speed exceeding the resorbtion capacity of the proximal tubes [2,5,8,18].

B3. Postrenal

Postrenal proteinuria involves the appearance of proteins in the urine, after their existence in the kidney, proteins that come in plasma or lymph[4]. All plasma proteins occur including those with molecular weight of more than 500 000 (which can hardly pass through the basement membrane) with red blood cell[11].

Investigation methodology

A. Collection and preservation (4)

The quantitative determination of urinary proteins is made from fresh urine, harvested in a clean collector or on urine preserved at 4°C, up to 7 days (except IgG). For the determination of 24h urine an antimicrobial agent is added to (azide solution or tymol). Repeated frosting and defrosting is not recommended, for some proteins are distorted. For qualitative determinations urine is centrifuged 10 min at 3000 r.p.m.

B. The screening test and the determination of total proteins

The screening test

Dipstick is a semi quantitative test commonly used to detect proteinuria. It consists of a tape impregnated with a colouring solution (usually blue tetra bromine phenol) and a buffer that brings the pH to 3. The protein present in urine binds to the dyestuff that changes color depending on the protein pH. This method can not detect the microalbuminuria.

The quantitative determination of the total proteins (proteinuria)

1. The thermocoagulation test, which involves the urine precipitation at 56°C and its dissolution at 100°C, has only historical value[4,13], giving false positive results in the presence of polyclonal light chains in excess and even the Bence Jones proteins do not always precipitate at high temperatures[9].

2. The turbidimetric methods are used less and less, involving the urine precipitation with an acid at high temperatures (sulfosalicilic, trichloracetic, acetic acid with acetate buffer, acid, tannin, senzentonium chloride) and the measuring of the turbidimetric precipitate[5,2,13].

3. Colorimetric methods are used in greater extent, their principle being based on the formation of a coloured derivate (biuretic like), or on the binding a dyestuff (as in the case of Brilliant Blue Coomasie, tannins)[4]. Colorimetric methods have sensitivity and good repeatability, acceptable reproducibility, being superior to the turbidimetric ones, although in both cases, the detection of the Bence Jones proteins is inconsistent.

C. Qualitative determination methods

1. **Monodimensional electrophoresis**

Urinary protein separation based on the electrical load is carried out in agar gel or cellulose acetate. In this case it is very difficult to characterize the type of protein, because the proteins of tubular origin have similar mobility to those of glomerular origin. By this separation there are proteins that have the same mobility. The Bence Jones proteins appear as a single, discreet band, or sometimes as multiple bands (corresponding to oligoelements or rarely to a biclonal gammopathy), which can be confused with β2 or α2 microglobulin, when the kidney injury can produce an additional proteinuria[15] with transferrin or intact immunoglobulins . Therefore these separations must be checked with specific methods. In conclusion, this type

of electrophoretic separation has reduced sensitivity to detect small concentrations of Bence Jones proteins.

Urinary protein separation based on molecular weight are carried out in polyacrylamide or agar gel containing *sodium dodecyl* sulfate. In the case of polyacrylamide electrophoresis sometimes the urine concentration is necessary. This option allows the separation of proteins with low molecular weight, generally speaking those of tubular origin, and of the proteins with high molecular weight, generally speaking those of glomerular origin. So, using this electrophoretic model we can characterize the tubular, glomerular and mixed proteinuria. Based on the molecular weight, this variation of electrophoresis views monomers of free light chains as well (monoclonal, polyclonal), easy to be distinguished from intact immunoglobulins. The electrophoregrams are coloured with Coomasie Brilliant Blue or Ag salts. The separation on agar- *sodium dodecyl* sulfate does not allow the differentiation of monoclonal free light chains from the polyclonal ones. Also, the polymer forms of the Bence Jones proteins comigrate and affect their quantitative determination. To remove this impediment 2-mercaptoethanol may be used as a reducing agent before electrophoresis and a depolimerizing marker that migrates along. Despite these impediments, electrophoresis on agar- *sodium dodecyl* sulfate presents an increased resolution, sensitivity and good reproducibility.

2. Isoelectric focusing electrophoresis

This method allows the separation of proteins in relation to their isoelectric point. Isoelectric focusing electrophoresis is not ideal for complex mixtures of proteins, because the individual proteins can provide with multiple bands and the interpretation is difficult.

3. Immunoblotting

This method is quantitatively similar to the immunofixation[10] or even more sensitive, superior to the isoelectric focusing electrophoresis. After the electrophoretic separation of the proteins, they are transferred to Nitrocellulose bands and are identified by their reaction with marked antibodies. In other words, this method gives information about the presence, molecular weight and / or the quantity of the antigen, combining protein separation - via the gel electrophoresis – with the specific identification of antigens by antibodies.

4. Capillary electrophoresis

The capillary electrophoresis method is superior to the zone electrophoresis one, it is fast, automatically separates the molecules of the liquid medium on the basis of molecular weight, electric load, hydropho6bicity and does not require urine concentration. The method uses the silica capillary, and the direct reading of the fractions is done with an UV detector at 214 nm[14]. If a quantitative assessment is necessary, this is done by immunosubstraction. The capillary electrophoresis has an enormous clinical potential, but is less sensitive and less flexible than the traditional electrophoretic methods, including the isolelectric focusing one.

5. Bidimensional electrophoresis

According to its name, bidimensional electrophoresis involves the electric focusing electrophoresis in the first place, when the separation takes place based on the isolelectric points and electrophoresis on polyacrylamide with *sodium dodecyl* sulfate in the second place (when the electrophoregram is located at 90° to the first stage) when the separation takes place on the basis of protein molecular masses. This method is superior to zone electrophoresis, because it enables the reporting to two parameters, the isoelectric point and the molecular weight. The Bence Jones proteins are identified by immunoblotting.

The human normal urine, having a low concentration of protein, and a high amount of salts and metabolic scrap, which interferes in particular in the phase of isolelectric focusing electrophoresis, requires the urine concentration which is crucial for the bidimensional electrophoresis on polyacrylamide[19].

The methods of urine concentration involve the following:

- The precipitation (ethanol, methanol, acetone, acetonitrile acetic acid, trichloracetic acid) [7, 19] or enrichment with beds or columns of affinity for chemical separation[6];
- Lyophilization[16], ultracentrifugation, centrifugal filter[19], for physical separation.

Bidimensional electrophoresis is optimal for the physical-chemical characterization of the Bence Jones proteins, for it separates the free light chains and the fragments corresponding to discrete spots, in terms of proteins distortion before they are submitted for analysis.

6.Immunofixation

Immunofixation identifies the light, free or connected κ and λ chains. After the electrophoretic separation takes place, insoluble immune complexes are formed between proteins and monospecific antibodies anti IgG,anti IgA,anti Ig M,anti κ or anti λ. This method is more sensitive than the electrophoresis, quick and relatively easy interpreted.

D. Methods of quantitative determination

The Bence Jones proteins are determined quantitatively using monoclonal antibodies, or antisera enriched with free light chains specific antibodies. If one wishes only a relative quantitative assessment the chemical determination can be used[3]. This can be done by identifying the total proteins with the Bence-Jones proteins, when they prevail, or it can be calculated as a percentage from the total proteins according to the density of the electrophoresis band.

The immunochemical methods are: immunonephelometry, immunoturbidimetrics, radio immunoanalysis, enzymatic immunoanalysis.

The accuracy of the quantitative methods at the limits of the dynamic field, clinically very relevant, is poor (remission is determined at low level, progression at high level). There is no substance of reference for monoclonale light chains, which makes accuracy to be affected. The quantification methods of urinary free light chains are not standardized and the results may vary significantly among different methods. Despite these limitations, the immunochemical methods monitor the clone during treatment, provided to take account of those shown above.

The κ/λ immunochemical report reveals a semi quantitative correlation referring to the quantity of monoclonal free light chains.

More recently it appeared that in Bence Jones myeloma serum free light chains are correlated with changes in the excretion of urinary free light chains. Moreover, the nephelometric determination of serum free light chains is more sensitive than the urinary determination[1].

Turbidimetric or nephelometric determination of serum free light chains is 500 times more sensitive than the protein electrophoresis, is fully automatic and quantitative. As a result of these arguments, this method of determining is recommended in the recent years[12].

Conclusions

- The amount of proteins in the urine is partly related to the extension of the kidney disorder.
- The nature of specific proteins makes proteinuria reflect the location of the nephrotic disorder (glomerular and / or tubular) and the pathogenesis of renal dysfunction.
- The investigation of the proteinuria with reliable techniques and modern equipment does not exclude however the fact that the results interpretation shall always be integrated into the clinico-pathological context.

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