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(54) **Method for determining the presence of free light chains in urine samples, complex of preparations for performance of the method and related reagent.**

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EP 0 336 472 B1

Description

It is known that an immunoglobulin is schematically made up of two heavy chains and two light chains. Determinations of the presence of the free light chains, also called proteins by Bence Jones, in the urine is of great interest from the diagnostic viewpoint.

The free light chains are present in traces in the serum of normal subjects but are practically absent in the urine. The presence of said light chains in the urine is an indication of the existence of a pathological condition, particularly of immunological nature.

Actually the presence of free light chains in urine presupposes their anomalous increase in the serum of the subject but, given their low molecular weight, free light chains pass through the glomerular filter and do not persist in the blood. It is therefore necessary to perform an indirect investigation, ascertaining their presence in the urine.

The presence of free light chains in the urine, which is the consequence of the increase thereof in the blood, is associated with immunological pathologies which can be summarized as (a) the presence of monoclonal free light chains, i.e. immunoproliferative illnesses such as multiple myeloma, micromolecular myeloma, Waldenström's macroglobulinemia, chronic lymphatic leukemia and primitive amyloidosis; and (b) the presence of polyclonal free light chains, i.e. hyperimmune illnesses such as systemic lupus erythematosus, acute rheumatoid arthritis and secondary amyloidosis.

Diagnostic methods based on ascertainment of free light chains in the urine are of great interest but at present are blocked by the difficulties of performance of such an investigation. At present the most widely used method calls for electrophoretic analysis of the concentrated urine.

This technique necessarily requires concentration of the sample because of the relatively small percentage of free light chains in the organic liquid even with serious pathological conditions of the subject. Electrophoretic examination of the unconcentrated sample results in unacceptably low sensitivity and the resulting unreliability. The time necessary for concentration is added however to the time required for electrophoretic analysis with the obvious drawbacks. The analysis performed on the concentrated samples undoubtedly raises the reliability of the results without however achieving reasonable certainty. On the samples which prove suspect under electrophoresis it is therefore very advisable to perform immunofixation or immunoelectrophoresis tests, the laboriousness and cost of which are known, to achieve truly satisfactory levels of sensitivity and hence reliability of the

analysis results.

A different method of detecting the presence of free light chains in urine sample is described in Scand. J. Clin. Lab. Invest. 1984, 44(2), 173-6 which discloses the turbidimetric measurement of Bence-Jones proteins with anti-light chain antisera. Said document teaches to concentrate the Bence-Jon proteins from the urine and no indication of the relative amount of anti-light chain antisera is given. However this piece of prior art evaluates that this kind of measurement results in an artifact caused by different polymeric forms of light chains.

The Journal of Immunological Methods 39 (4), 355-362 (1980) also describes the turbidimetric measurement, but only of kappa- and lambda-IgG in serum and with two different monoclonal antibodies.

EP-A-0 044 219 discloses the analysis of kappa and lambda-IgG via precipitation with two monoclonal antibodies specific to two specific antigenic sites on the IgG.

The analysis of Bence-Jones proteins carried out by standard methods other than turbidimetric analysis is described, for example, in US-A-3 907 502.

The object of the invention is to propose a qualitative and quantitative ascertainment method for the presence of free light chains in urine which would be easy to perform in extremely short times with a high degree of sensitivity, especially in comparison with the times required in the analysis processes presently used.

In addition it is an object of the invention to make available to the user a complex of substances in kit form suitable for putting into practice the proposed diagnostic method.

The invention is based on the observation that an antigen-antibody reaction is usable for determination of the presence of free light chains in the urine without prior concentration, leading to turbidity values which allow appraisal of the presence of such chains with significant qualitative and quantitative reliability. In particular the invention proposes a diagnostic method based on ascertainment of the concentration of light chains in the urine comprising the phases (a) centrifugation of the urine sample and separation of the overflowing, (b) addition to the sample of an anti free light chain antiserum reagent operating with an excess of antibodies, and (c) appraisal of turbidity of the reacted sample.

In particular if a quantitative appraisal is required the method calls for addition of the antiserum reagent to a calibration sample containing free light chains in a predetermined quantity to obtain calibration curves for the quantitative analysis procedure by turbidity comparison.

The anti light chain antiserum is obtainable by any of the known procedures for the purpose, generally from an animal immunized with free light chains.

Two types of light chain are known, conventionally denominated kappa and lambda, which can be used for immunization of animals to obtain corresponding antisera.

Such an antiserum reacts with all of the antigen sites of the light chain including those which can be defined as 'hidden', when the light chain is linked to the heavy chain. This antiserum then shows both the linked light chains and the free light chains.

It is possible to obtain an anti free light chain antiserum by separating therefrom the antibodies turned toward the antigenic sites not 'hidden' by the light chain; this can be achieved by reacting the anti free light chain antiserum with whole immunoglobulins and recovering the antibodies which have not reacted. Such an antiserum reacts only with kappa or lambda free light chains respectively and does not react with linked light chains.

To better clarify the characteristics of the method in accordance with the invention a practical embodiment thereof is described below.

A kit of products necessary for performing the analysis in accordance with the invention typically includes the following components.

(a) Anti free light chain antiserum reagent consisting of dilute antiserum, e.g. in concentrations of 20% anti free kappa light chain antiserum, 20% anti free lambda light chain antiserum, and 60% of a 4% solution of PEG 6000 in a physiological buffer solution (PBS) (phosphate buffer at pH 7.4) It is advantageous to add a preservative such as 0.1% Sodium Azide.

(b) Calibrators.

These can be used as positive controls of the qualitative procedure and as calibrators for the quantitative procedure.

The samples used come from patients with secreting micromolecular myeloma. In the absence of any reference method in the literature electrophoresis was performed on the urinary proteins, noting the presence of a large band subsequently typified with immunofixation as free kappa and lambda light chains accompanied by a barely visible band of albumin. The quantity of total proteins is therefore considered to practically coincide with the quantity of free light chains. The dosage of the total proteins was performed by the Bradford method. The samples are freeze-dried with addition of preservative (1% Sodium Azide) to be diluted with distilled water for use, then with PBS to plot the standard curve for the quantitative procedure.

(c) Reagent without antiserum.

The composition of this reagent is the same as that of the antiserum reagent but without the latter and to be used in the control and calibration procedure as standard white.

The operating procedure for performance of the analysis in accordance with the invention can be summarized as follows.

The reagents with and without antiserum are brought to surrounding temperature, filtering the volume of the former necessary for the analysis if it should not be limpid.

The reagent without antiserum is mixed in a test tube with the calibrator and the resulting reacted substance must be limpid.

Then the antiserum reagent is mixed with the calibrator and the reacted substance must be turbid.

If the readings are not those expected it is due to a procedural error or an anomaly of the reagents and these must therefore be discarded.

After centrifugation of the sample it is treated with antiserum reagent. Turbidity is a sign of the presence of kappa or lambda light chains or both.

The details of the procedures for performance of the antiserum-sample reaction are not given here as they are the usual ones followed in carrying out antigen-antibody reactions. It is clear that operation will be with an excess of the latter and, for doubtful or negative samples, an extra quantity of urine can be added to the reaction test tube, then appraising whether said addition modifies the turbidity appreciably.

For instrumental quantity-reading the calibration curve must be constructed repeating the analysis procedure with antiserum reagent on samples obtained from solutions in various percentages of calibrator in saline buffer solution.

For example turbidity values can be determined on 20, 40, 60, 80 and 100% calibrator solutions. It is thus possible to show on a Cartesian chart the concentrations of free light chains of the calibrator solutions on the basis of the optical density in comparison with the standard white, measuring with a suitable instrument such as the Mod. 336 Biotron Photometer or the Cobas Bio Analyser (Roche).

As mentioned above the concentration of free light chains in the calibrator presented as freeze-dried to be reconstituted by dilution in a predetermined quantity of distilled water is known.

An alternative to the abovementioned procedure is possible using not a single kappa and lambda anti free light chain antiserum reagent but two separate kappa and lambda anti free light chain reagents respectively. In this manner there are obtained separate results of concentration of the two types of chains when desired for particular diagnostic or experimental requirements. Nothing

else changes in the abovementioned procedure. In this case it is advisable to prepare in advance kappa and lambda calibrators respectively for the checks preceding the sample analysis procedure and construction of the calibration curves for the quantitative analysis.

The results of the analysis were verified by performance of a conventional electrophoretic examination of the samples analysed in accordance with the invention, obtaining confirmation of the correctness of the results obtained by the latter. Samples which proved negative under conventional electrophoretic examination proved positive when concentrated 10 to 50 times depending on the practices of the different laboratories.

The singular and astonishing effectiveness of the method is demonstrated by the fact that a concentration of approximately 4 mg/dl of light chains was evaluated with it, demonstrating that the use in this specific field of the antigen-antibody reaction technique has lead to satisfactory results for the problem of identification of free light chains in urine.

Practically the only limit to the method is its inability to distinguish between monoclonal and polyclonal light chains as is possible using electrophoretic analysis with immunofixation.

However, the positive nature of the analysis for only one of the two light chains, kappa or lamda, can be considered a reasonably certain sign of monoclonality of the chains present in the sample.

But this limitation is largely compensated for by the speed, reliability and simplicity of performance of the method in accordance with the invention compared with the one previously used for this purpose. Only on the samples which proved positive under analysis in accordance with the invention will it be required to perform further immunoelectrophoretic analyses or with immunofixation to determine the nature of the free light chains if desired.

On this point it should be noted that in this type of diagnostic analysis the positivity is to be considered, if not rare, certainly very infrequent statistically. It is thus very useful to have available the method in accordance with the invention which permits a first screening of the samples with very high sensitivity and reliability of the result because the examinations can be pursued further on the limited number of samples which proved positive on analysis.

Claims

1. Method of determination of the presence of free light chains in undiluted and unconcentrated samples of urine comprising the phases (a) centrifugation of the urine sample and sep-

aration of the overflowing for performance of the determination, (b) addition to the sample of an anti light chain antiserum reagent operating with excess antibodies, and (c) appraisal of the turbidity of the reacted sample.

2. Method in accordance with claim 1 wherein the sample is reacted successively with kappa and lambda anti free light chain antisera respectively.
3. Method in accordance with claim 1 characterized in that the turbidity of the reacted sample is appraised by comparison with the product of reaction of the antiserum reagent with a calibrator having a predetermined content of free light chains.
4. Method in accordance with claim 3 characterized in that a calibration curve is constructed for the comparison, instrumentally appraising the turbidity value of the reaction product of the antiserum reagent with samples made up of calibrator solutions in various concentrations.
5. Method in accordance with claim 1 characterized in that the reagent is obtained by animal immunization with free light chains.
6. Method in accordance with claim 1 characterized in that the calibrator is obtained from the urine of patients with micromolecular myeloma.
7. Complex of products in kit form for determining the presence of free light chains in unconcentrated urine samples consisting of (a) an anti free light chain antiserum reagent, (b) a reagent without antiserum with a composition essentially the same as that of the antiserum reagent, and (c) a calibrator consisting of a substance containing free light chains in predetermined concentration.
8. Complex of products in accordance with claim 7 characterized in that said antiserum reagent contains anti kappa and lambda free light chain antibodies.
9. Complex of products in accordance with claim 7 characterized in that said antiserum reagent is contained in the complex of products as separate quantities of kappa anti light chain antiserum and lambda anti light chain antiserum.
10. Complex of products in accordance with claim 7 characterized in that said calibrator consists

of a freeze-dried substance from the urine of patients affected with micromolecular myeloma and is to be reconstituted with distilled water before use.

Patentansprüche

1. Verfahren zur Bestimmung der Gegenwart von freien leichten Ketten in unverdünnten und unkonzentrierten Urinproben, umfassend die Phasen (a) Zentrifugieren der Urinprobe und Abtrennen des Überstands, um die Bestimmung durchzuführen, (b) Zugabe eines Antileichte-Kette-Antiserum-Reagenzes zu der Probe, wobei mit einem Überschuß von Antikörpern gearbeitet wird, und (c) Bewertung der Trübung der umgesetzten Probe. 5
2. Verfahren nach Anspruch 1, worin die Probe sukzessive mit jeweils kappa- und lambda-Anti-freieleichte-Kette-Antiserum umgesetzt wird. 10
3. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die Trübung der umgesetzten Probe durch Vergleich mit dem Produkt der Reaktion des Antiserum-Reagenzes mit einem Eichmittel bewertet wird, das einen vorbestimmten Gehalt an freien leichten Ketten aufweist. 15
4. Verfahren nach Anspruch 3, dadurch gekennzeichnet, daß für den Vergleich eine Eichkurve erstellt wird, wobei der Trübungswert des Reaktionsprodukts von Antiserum-Reagenz mit Proben, die aus Eichlösungen verschiedener Konzentrationen hergestellt wurden, instrumentell bewertet wird. 20
5. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Reagenz durch Immunisierung von Tieren mit freien leichten Ketten erhalten wird. 25
6. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Eichmittel aus dem Urin von Patienten mit mikromolekularem Myelom erhalten wird. 30
7. Komplex von Produkten in Form eines Kits zum Bestimmen der Gegenwart von freien leichten Ketten in unkonzentrierten Urinproben, bestehend aus (a) einem Anti-freie-leichte-Kette-Antiserum-Reagenz, (b) einem Reagenz ohne Antiserum mit einer Zusammensetzung, die im wesentlichen dieselbe ist, wie diejenige des Antiserum-Reagenzes und (c) einem Eichmittel, das aus einer Substanz besteht, die 35

freie leichte Ketten in vorher bestimmten Konzentrationen enthält.

8. Komplex von Produkten nach Anspruch 7, dadurch gekennzeichnet, daß das Antiserum-Reagens Anti-kappa- und Anti-Lambda-freieleichte-Kette-Antikörper enthält. 40
9. Komplex von Produkten nach Anspruch 7, dadurch gekennzeichnet, daß das Antiserum-Reagenz in dem Komplex von Produkten als separate Portionen von kappa-Anti-leichte-Kette-Antiserum und lambda-Anti-Leichte-Kette-Antiserum enthalten ist. 45
10. Komplex von Produkten nach Anspruch 7, dadurch gekennzeichnet, daß das Eichmittel aus einer gefriergetrockneten Substanz aus dem Urin von Patienten besteht, die an mikromolekularem Myelom leiden, und daß er vor der Verwendung mit destilliertem Wasser rekonstituiert wird. 50

Revendications

1. Méthode pour déterminer la présence de chaînes légères libres dans des échantillons d'urine non dilués et non concentrés comprenant les phases (a) de centrifugation de l'échantillon d'urine et de séparation du surnageant afin de pouvoir accomplir l'analyse, (b) d'addition à l'échantillon d'un réactif composé d'un antiserum anti-chaîne légère opérant avec un excès d'anticorps, et (c) d'appréciation de la turbidité de l'échantillon après réaction. 55
2. Méthode selon la revendication 1, dans laquelle on fait réagir successivement l'échantillon avec des antisérums anti-chaîne légère libre, respectivement kappa et lambda. 60
3. Méthode selon la revendication 1, caractérisée en ce que la turbidité de l'échantillon après réaction est appréciée par comparaison du produit de réaction du réactif composé d'un antiserum avec un étalon ayant une teneur déterminée en chaînes légères libres. 65
4. Méthode selon la revendication 3, caractérisée en ce que l'on construit une courbe d'étalonnage pour la comparaison, en appréciant de façon instrumentale la valeur de turbidité du produit de réaction du réactif composé d'un antiserum avec des échantillons composés de solutions étalon à des concentrations diverses. 70
5. Méthode selon la revendication 1, caractérisée en ce que le réactif est obtenu par immunisa- 75

- tion d'un animal avec des chaînes légères libres.
6. Méthode selon la revendication 1, caractérisée en ce que l'étalon est obtenu à partir d'urine de patients présentant un myélome micromoléculaire. 5
7. Ensemble de produits sous forme d'un kit pour la détermination de la présence de chaînes légères libres dans des échantillons d'urine non concentrés constitués (a) d'un réactif composé d'un antisérum anti-chaîne légère libre, (b) d'un réactif sans antisérum de composition essentiellement identique à celle du réactif composé de l'antisérum, et (c) d'un étalon constitué d'une substance comprenant des chaînes libres légères selon une concentration déterminée. 10
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8. Ensemble de produits selon la revendication 7, caractérisé en ce que le réactif composé d'un antisérum contient des anticorps anti-chaîne légère libre kappa et lambda. 25
9. Ensemble de produits selon la revendication 7, caractérisé en ce que le réactif composé d'un antisérum est contenu dans l'ensemble de produits sous forme de quantités distinctes d'antisérum anti-chaîne légère kappa et d'antisérum anti-chaîne légère lambda. 30
10. Ensemble de produits selon la revendication 7, caractérisé en ce que ledit étalon consiste en une substance lyophilisée provenant de l'urine de patients affectés d'un myélome micromoléculaire et devant être reconstituée avec de l'eau distillée avant utilisation. 35
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