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INFLUENCE OF VARIOUS FACTORS ON DETERMINATION OF CERULOPLASMIN LEVEL IN HUMAN BLOOD PLASMA IN CLINICAL DIAGNOSTICS

Polyarush S.V.[1], Masaidova I.B.[2], Ryskulov F.T.[2]
[1] LBM Medical Centre, Tashkent, Uzbekistan,
[2] Institute of Immunology and human genomics

XULOSA

Ushbu ishda ceruloplazminning miqdoriy aniqlanishini ikkita usulning qiyosiy tahlili o'tkazildi: immunoferment tahlili (ELISA) va biokimyoviy spektrofotometriya. Imunologikusullarceruloplazminning apoformalari bilan antitanalarning o'zaro ta'siri sababli natijalarni yuqori ko'rsatishi ehtimoli aniqlangan. Olingan natijalar ceruloplazminning ifodasi bilan bog'liq genetik buzilishlarning laborator diagnostikasini optimallashtirishda muhim ahamiyatga ega.

Usullar. *Tadqiqotda bemorlar qon namunalarida ceruloplazmin darajalarini miqdoriy aniqlash uchun ELISA va biokimyoviy spektrofotometriya usullari qo'llanildi. Har bir usulda aniqlik va ishonchlilikni ta'minlash maqsadida standart eritilgan eritma va so'rilish o'lchovlari qo'llanildi.*

Natijalar. *Olingan ma'lumotlar shuni ko'rsatdiki, ELISA ko'pincha spektrofotometriya bilan solishtirganda ceruloplazmin yuqoriroq darajalarini beradi. Bu farq klinik amaliyotda diagnostic usullarni ehtiyotkorlik bilan tanlash zaruriyatini ta'kidlaydi, natijalarni noto'g'ri talqin qilish ehtimolini bartaraf etish maqsadida.*

Kalit so'zlar: *ceruloplazmin (CP), apoceruloplazmin, biokimyoviy usullar, proteoliz ingibitorlari, yallig'lanish, biomarkerlar, apoCP.*

РЕЗЮМЕ

В данной работе проведен сравнительный анализ двух методов количественного определения церулоплазмина: иммуноферментного анализа (ELISA) и биохимической спектрофотометрии. Установлено, что иммунологические методы могут давать завышенные результаты из-за взаимодействия антител с апоформами церулоплазмина. Полученные результаты имеют важное значение для оптимизации лабораторной диагностики генетических нарушений, связанных с экспрессией церулоплазмина.

Материалы и методы. *В исследовании использовались ELISA и биохимическая спектрофотометрия для количественного определения уровней церулоплазмина в образцах пациентов. Для каждой методики были применены стандартные разведенные растворы и измерения поглощения, чтобы обеспечить точность и надежность результатов.*

Результаты. *Полученные данные показали, что ELISA часто давал более высокие уровни церулоплазмина по сравнению со спектрофотометрией. Это различие подчеркивает необходимость тщательного выбора диагностических методов в клинической практике, чтобы избежать потенциальной интерпретации результатов.*

Ключевые слова: *церулоплазмин (CP), апоцерулоплазмин, биохимические методы, ингибиторы протеолиза, воспаление, биомаркеры, apoCP.*

Ceruloplasmin (CP) is a blue alpha-2 serum glycoprotein with a molecular mass of 150,000-160,000 g/mol that binds 90-95% of plasma copper. It contains 6-7 copper ions per molecule and exhibits ferroxidase, amine oxidase and superoxidase activities, participating in copper and iron metabolism [1; P.1–23, 7; P. 1100-1112]. The CP gene is located on chromosome 3 and encodes a protein of 1046 amino acids consisting of eight subunits, each of which attaches one copper ion. Ceruloplasmin is produced by hepatocytes and contains a carbohydrate component (2-8% by weight) including several oligosaccharide chains [4; P. 77-84].

Determination of CP concentration is important for the diagnosis of genetic diseases such as Wilson-Conovalov disease and Menkes disease, and as a marker for Parkinson's disease and some cancers [1; P.1–23, 2; P.1860–1866]. Normal serum CP levels are 20-50 mg/dl. Current methods of determination include immunoturbidimetry, enzyme immunoassay, radial immunodiffusion test, and bichromatic assay, each of which has its own drawbacks. For example, the bichromatic method requires caution due to benzidine toxicity and does not detect apoceruloplasmin [5; P. 499–506, 6; P. 6082–6092]. Further research is needed to improve methods for determining the concentration of ceruloplasmin and its forms [3; P. 590–596].

Methods of determination

Immunological methods for the determination of ceruloplasmin (CP) concentrations are widely used in diagnostics, but have drawbacks. The main problem is the overestimation of CP concentrations due to antibody reaction with subunit forms of the apoenzyme resulting from dissociation of the 'mature' CP octamer. This leads to elevated CP concentrations in patients with Wilson's disease and other liver diseases. Normally, CP levels are reduced in this disease, and low values (< 5-7 mg/dl) may

indicate the presence of disease.

Importantly, CP is also an inflammatory response protein and is elevated in tumour and inflammatory conditions. The main methods for determining CP in clinical practice include ELISA and immunotubidimetry. The ELISA method may lead to partial dissociation of CP subunits due to the lengthy steps involved, whereas immunoturbidimetry is a faster method of analysis.

THE AIM OF THIS STUDY is to compare methods for the quantification of ceruloplasmin (CP), such as enzyme-linked immunosorbent assay (ELISA) and biochemical spectrophotometry.

STUDY OBJECTIVES

The study involved 250 children aged 1 year and older who were examined by geneticists. The control group consisted of 250 children without family history and clinical manifestations of diseases associated with ceruloplasmin metabolism. The parents of the patients gave informed consent to participate in the study. The final diagnosis of the children was made by qualified geneticists.

Determination of CP concentration by ELISA method

The concentration of ceruloplasmin was determined using a commercially available HUMAN CERULOPLASMIN ELISA Assay Kit. Five ml of venous blood was collected from each subject from both groups on an empty stomach. The ELISA procedure included the following steps: addition of 100 µl of samples and calibrators to wells, incubation at room temperature, addition of biotin-labelled antibodies and incubation at 37°C. Washing was then performed, substrate solution was added and incubated to show dye. The optical density was measured at 450 nm and the concentration was determined from the calibration graph. The sensitivity of the kit is 75 pg/ml with the required dilution of samples in million fold.

Immunoturbidimetric assay

The CP content was determined using a Ceruloplasmin Turbidimetry spectrophotometric kit. The method is based on the ability of ceruloplasmin to form a complex with p-phenylphenylenediamine (PPD) in Tris buffer solution, resulting in a colour change. Absorbance measurement using a spectrophotometer allows the concentration of CP to be determined from a calibration curve. The method is versatile, easy to use and widely used in clinical diagnosis. The serum sample was used without prior dilution immediately after collection and centrifugation.

Analysis of ceruloplasmin concentration

The assay involved adding 7 µl of sample or calibrator to reagent R1, mixing and recording the first optical density value (A1) using an automated programme. Then 200 µl of reagent R2 was added and the second optical density value (A2) was read after 2 minutes. The concentration of ceruloplasmin was calculated using the formula:

$$\frac{(\mathrm{A2}-\mathrm{A1})\mathrm{sample}}{(\mathrm{A2}-\mathrm{A1})\mathrm{calibrator}} \times \text{Calibrator concentration } = \frac{\mathrm{mg}}{\mathrm{dL}}\text{Ceruloplasmin}$$

According to the kit specifications, the sensitivity limit was 3.27 mg/dl and the linearity range was up to 120 mg/dl.

RESULTS AND DISCUSSION

Our study tested two types of inhibitors designed to prevent proteolytic cleavage and dissociation of the ceruloplasmin globule. Ten patient samples divided into three groups were used to analyse the efficacy of the inhibitors. In the first group no inhibitors were used, while in the second and third groups inhibitors were added.

The results of the analysis showed that samples in the first group (no inhibitor) showed the highest values, ranging from 23.3 to 29.7. Specifically, samples with numbers 27 and 124 showed the best results with ceruloplasmin concentrations of 23.4 and 29.7, respectively.

In the second group where protease inhibitors were used, the mean values decreased significantly, ranging from 17.4 to 24.1, with sample number 106 giving the highest value at 22.3. This indicates the effect of the inhibitors in reducing the ceruloplasmin concentration.

The third group containing the complete inhibitors showed intermediate results which were between the first two groups, ranging from 20.9 to 27.2. Samples numbered 124 and 106 gave the highest results of 27.2 and 25.7 respectively.

Thus, the action of protease inhibitors markedly decreased the concentration of ceruloplasmin compared to samples without inhibitors, but their use gave intermediate results that confirm the importance and effectiveness of using inhibitors for research purposes.

In the initial stages of the study, we used an ELISA kit for the determination of CP, but found significant variation in results for both clinical samples and standards. This may be due to the low sensitivity of the kit. The ELISA method is time-consuming and requires a multistep process, which may lead to disintegration of the CP octamer and increased errors due to the need for strong dilution of samples.

The preservation of the globular structure of CP is crucial, and the use of ELISA can lead to dissociation of the protein into subunits, which is likely responsible for the variability in results. By conducting further studies, we have switched to a biochemical method for the determination of CP.

CONCLUSION

In this work, a comparative evaluation of methods for quantitative determination of ceruloplasmin: enzyme-linked immunosorbent assay (ELISA) and biochemical spectrophotometry was carried out. It was found that the ELISA method is inefficient and unreliable due to dissociation of high molecular weight proteins, which leads to inaccurate results. The findings are in agreement with other studies such as Aisen P. and William C. Cho, concerning the analysis of transferrin.

REFERENCES

1. An, Y. The role of copper homeostasis in brain disease / Y. An, S. Li, X. Huang, X. Chen, H. Shan, M. Zhang // International Journal of Molecular Sciences. – 2022. – Vol. 23. – P. 1–23.
2. Członkowska, A. Accuracy of the radioactive copper incorporation test in the diagnosis of Wilson disease / A. Członkowska, M. Rodo, A. Wierzchowska-Ciok, L. Smolinski, T. Litwin // Liver Int. – 2018. – Vol. 38. – P. 1860–1866.
3. Dong, Y. Role for Biochemical Assays and Kayser-Fleischer Rings in Diagnosis of Wilson's Disease / Y. Dong, R.M. Wang, G.M. Yang, H. Yu, W.Q. Xu, J.J. Xie, Y. Zhang, Y.C. Chen, W. Ni, Z.Y. Wu // Clin. Gastroenterol. Hepatol. – 2021. – Vol. 19. – P. 590–596.
4. Harris, Z. L. Chapter 9 − Ceruloplasmin / Z. L. Harris // Clinical and Translational Perspectives on Wilson Disease. – Academic Press, 2019. – P. 77-84.
5. Martinez, X. Visualizing protein structures─tools and trends / X. Martinez, M. Chavent, M. Baaden // Biochem. Soc. Trans. – 2020. – Vol. 48, no. 2. – P. 499–506.
6. Patel, C. K. Biomolecular Condensation of Trypsin Prevents Autolysis and Promotes Ca2+-Mediated Activation of Esterase Activity / C. K. Patel, T. K. Mukherjee // Biomacromolecules. – 2024. – Vol. 25, no. 9. – P. 6082–6092.
7. Sánchez-Monteagudo, A. Wilson's Disease: Facing the Challenge of Diagnosing a Rare Disease / A. Sánchez-Monteagudo, E. Ripollés, M. Berenguer, C. Espinós // Biomedicines. – 2021. – Vol. 9. – P. 1100-1112.