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Study on the Hook Effect in the Detection of Beta2-Microglobulin with Different Reagents in Fully Automated Biochemical Analyzers

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Abstract: *Objective:* To investigate the hook effect in the detection of beta2-microglobulin (β2-MG) with different reagents as well as on two fully automated biochemical analyzers and their solutions. *Methods:* Using immunoturbidimetric assay for β2-MG as the research object, β2-MG levels were detected by different concentration gradients of β2-microglobulin samples in Liedemann, Mack, and Myriad reagents of three manufacturers on two automatic biochemical analyzers, and the difference of the hook effect was comparatively analyzed. *Results:* Leadman reagent showed the hook effect on the Beckman AU5800 automated biochemistry analyzer, while both Maccura and Mindray reagents did not show the hook effect. After the experiments, we found the limit value of the pre-zone check of Leadman reagent, and changed the parameters of the instrument, when the limit value of the pre-zone check was reached, the instrument automatically diluted the specimen five times and then detected it again. After changing the parameters of the instrument, the correlation between the three methods of detecting samples of different concentrations was $r > 0.99$. *Conclusion:* Before selecting the application of immunoturbidimetric reagents, we have to carry out the risk assessment of the hook effect and selectively set the parameters of the pre-zone check based on the highest concentration that may occur in the clinic; for the items that may have the hook effect, we have to selectively set the parameters of the pre-zone check when the ratio of average reaction rate and the immediate reaction rate is at the limit value, and the phenomenon of antigen excess may occur, the instrument will carry out automatic dilution before detection, so as to avoid the issuance of erroneous results of high-value samples due to the hook effect.

Keywords: β2-microglobulin; Automatic biochemical analyzer; Immunoturbidimetric assay; Hook effect

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1. Introduction

Beta2-microglobulin (β2-MG) is a polypeptide with low relative molecular mass (118 000); it is a non-glycosylated protein, a small molecule globulin produced by platelets, lymphocytes, and polymorphonuclear cells, which is widely found in human blood, saliva, urine, colostrum, and cerebrospinal fluid [1,2]. β2-MG can be freely filtered by glomeruli and is almost exclusively reabsorbed by the proximal tubule and degraded into smaller peptides and amino acids. Its level in the blood is constant and very low in normal people. When kidney

injury occurs, the glomerular filtration rate is decreased and the level of β2-MG in the blood is increased, so the increase of β2-MG in the blood is a sensitive indicator for the clinical detection of glomerulopathy and early renal injury [3,4]. When inflammation occurs, a large number of T-lymphocytes are secreted, which increases the production and release of β2-MG [5]. In recent years, several studies have found that β2-MG has a dramatic effect on immune and neuromodulatory functions, e.g., it can alter brain development and cognitive function by regulating neurogenesis and synaptic plasticity [6]. Elevated levels of β2-MG can be found in renal failure, autoimmune diseases, multiple myeloma, lymphoma, and solid tumors. β2-MG is important in the diagnosis and treatment of a wide range of diseases, so it is important to measure β2-MG levels for research purposes.

Most of the contemporary medical tests for β2-MG levels are immunoassays using the antigen-antibody reaction mechanism; because latex-enhanced immunoturbidimetric assays have obvious advantages in terms of measurement range, test cost, convenience, etc., they are more commonly used than chemiluminescent assays, which are superior to enzyme-linked immunosorbent assays, magnetic particulate enzyme-linked immunosorbent assays, time-resolved fluorescent immunoassays, and radioimmunoassays.

Therefore, the immunoturbidimetric assay is most commonly used in fully automated biochemical analyzers. When the immunoturbidimetric assay is performed on an automatic biochemical analyzer, the absorbance value measured under the condition of constant antibody concentration in the reaction system and moderate antibody excess is proportional to the concentration of the substance to be measured. In immunoturbidimetric assays, the antigen-antibody complex is formed by the specific binding of antigen and antibody, and the complex increases with the concentration of the sample within a certain range. If the sample to be tested is high, the reaction signal will be weakened due to the excess of the antigen, and a false low value will appear, which is also known as the hook effect [7-9]. Extremely high results are likely to occur in advanced renal disease and multiple myeloma, where the high dose hook effect is likely to occur in the test, leading to the issuance of erroneous results, which will affect the clinical diagnosis, treatment, and prognosis [10]. Since the results of the hook effect due to antigenic overdose may vary for different manufacturers and reagents, this paper compares the results of three different manufacturers' reagents for β2-MG on two fully automated biochemistry analyzers, analyses the risk of incorrect results due to the hook effect, and seeks to reduce this risk and issue correct results.

2. Materials and methods

2.1. Experimental materials and instruments

The three β2-MG assay kits were from Beijing Leadman Biochemistry Co., Ltd. (linear 0–18 mg/L), Maccura Biotechnology Co., Ltd. (linear 0.4–80 mg/L), Shenzhen Mindray Biomedical Electronics Co. (linear 0.2–18 mg/L). The two analyzers used in this study were the Beckman Coulter AU5800 automated biochemical analyzer and the Mindray BS2000 automated biochemical analyzer.

2.2. Serum samples

Clinical serum samples with β2-MG levels ranging from 1.3 to 25.0 mg/L were obtained, serum was required to be clear in appearance, free of hemolysis, jaundice, and lipohemorrhagic phenomena, frozen at -20°C, and removed from the refrigerator to rethaw at room temperature at the time of use; as well as some of the linear substances purchased from Beijing Leadman Biochemistry Co., Ltd.

2.3. Experimental methodology

2.3.1. Measurement procedures

(1) Beckman Coulter AU5800 automated biochemistry analyzer: Measurement wavelength 546 nm, pho-

tometric point 18–34, measurement mode was 2-point endpoint method, calibration mode was 6-point calibration (Logit-log (4P) fitting).

(2) Mindray BS2000 automated biochemical analyzer: Measurement wavelength 540 nm, photometric point 14–27, measurement mode was endpoint method, calibration mode was 6-point calibration (Spline fitting).

2.3.2. Linear range analysis of three β2-MG assay kits

The samples of Leadman linear substances were diluted into five different gradient concentrations by means of multiplicative dilution, and the samples of each dilution concentration were measured twice, and the mean values of the test results were obtained respectively. The linear regression equation was calculated using the dilution concentration as the independent variable and the mean value of the test results as the dependent variable, and the absolute or relative deviation of linearity was calculated at the same time.

2.3.3. Three β2-MG assay kits for linear substances

Three β2-MG kits were used to detect β2-MG in 13 linear substance samples, and the data were analyzed by the Beckman Coulter AU5800 automated biochemistry analyzer and Mindray BS2000 automated biochemistry analyzer according to the steps of reagent instructions. Based on the measurement results, the correlation analysis between the measurement data and the linear substance data was performed using the linear substance data as a reference.

2.3.4. Precision analysis of three β2-MG assay kits

Two different levels of high and low-quality control were selected for testing, and the measurement was repeated 15 times for each concentration of the samples, and the size of the deviation between the measured value and the fixed value of the samples was calculated to assess the precision of the reagents.

2.3.5. Hook effect analysis and dose-effect curve plotting

49 linear substances with different concentrations were taken and tested on a Beckman Coulter AU5800 automated biochemical analyzer with Leadman β2-MG reagent. The test was repeated three times for each sample, and the dose-effect curves were plotted with the mean value of the response absorbance results of the three assays as the vertical coordinate, and the theoretical concentration of the Leadman β2-MG reagent solution as the horizontal coordinate. The threshold for the appearance of the hook effect was calculated and the parameters were reset.

2.3.6. Solution validation

13 linear substances with different gradient concentrations and 25 clinical samples were taken and detected by Leadman β2-MG detection kit and Maccura β2-MG detection kit in Beckman Coulter AU5800 automated biochemical analyzer and Mindray BS2000 automatic biochemical analyzer and supporting reagents. The correlation and deviation analyses were performed to verify the correlation between the assay data and the fixed serum data, using the theoretical data of the fixed serum as a reference.

3. Results and analyses

3.1. Linear range analysis of three β2-MG assay kits

As shown in **Figure 1**, in the concentration range of 1–80 mg/L, after the gradient dilution of the samples, the correlation between the measured mean values and the theoretical values was good, with the correlation coefficients r greater than 0.975, and the theoretical values were similar to the measured mean values, with the deviation of < 10%. The linear range of the Maccura β2-MG test kit in the Beckman Coulter AU5800 automated

biochemical analyzer was 0.4–80 mg/L, with a wide linear range. The correlation coefficient of the measured mean value and the theoretical value was good, with the correlation coefficient of r = 0.99, and there was no hook effect. The measured mean value of the reagents for the BS2000 automated biochemical analyzer has a good correlation with the theoretical value. The application of Mindray directional coupling latex technology fundamentally solves the clinical problems of low sensitivity and hook effect, although the linear 0.2–18 mg/L is more than the linear instrument alarm, the instrument is set to exceed the linear concentration, and it can be automatically diluted five times, so it can be reported to the range of 90 mg/L, and it has already been done in the highest clinical concentration without the hook effect. Leadman reagent concentration ranges beyond the 20 mg/L measured value and the fixed value of the difference is very far, and the overall curve has a clear downward trend, there is a clear hook effect. The results are presented in **Table 1**.

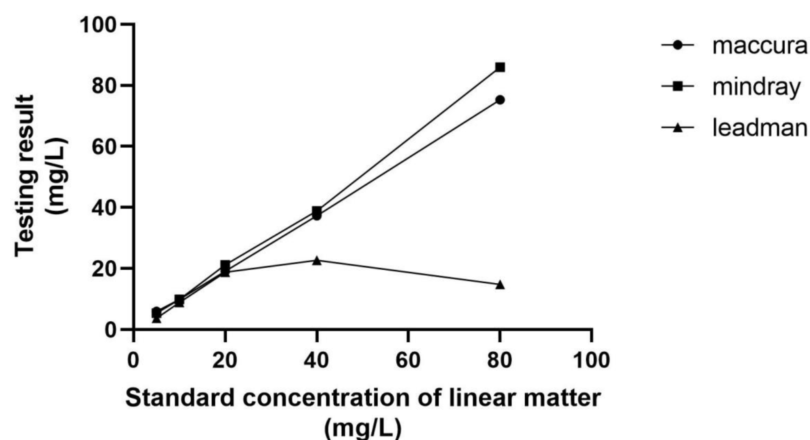

Figure 1. Results of linear range analysis of three β2-MG assay kits

Table 1. The results compared with the theoretical concentration

Theoretical values (mg/L)	Leadman (mg/L)	Relative deviation (%)	Mindray (mg/L)	Relative deviation (%)	Maccura (mg/L)	Relative deviation (%)
80	14.79	-81.51	75.26	-5.92	85.93	7.41
70	16.76	-76.06	67.53	-3.53	74.76	6.8
60	14. 36	-76.07	59.12	-1.47	61.64	2.73
50	21.36	-57.28	48.47	-3.06	49.17	-1.66
40	22.7 74	-43.15	37.31	-6.73	38.88	-2.8
30	21.22	-29.27	27.62	-7.93	26.8	-10.67
25	19.97	-20.12	23.59	-5.64	23.72	-5.12
22	18.79	-14.59	20.13	-8.5	21.24	-3.45
20	17.37	-13.15	18.74	-6.3	18.7	-6.5
15	15.07	0.47	14.71	-1.93	16.56	10.40
10	8.84	-11.6	9.89	-1.1	9.85	-1.5
5	3.71	-25.8	5.32	6.4	5.34	6.8
2.5	2.3	-8	2.67	6.8	2.64	5.6
	r = 0.46		r = 0.99		r = 0.99	

3.2. Precision analysis of three β2-MG assay kits

The reproducibility of the samples at two different levels, high and low, showed that the within-run and between-run precisions were < 6%, which is of high precision, as shown in **Table 2**.

Table 2. Degree of precision

Precision (%)	Leadman	Maccura	Mindray
Within-run precision (level l)	4.3	4.7	4.6
Within-run precision (level 2)	3.9	4.4	4.2
Between-run precision (level l)	4.8	5	4.5
Between-run precision (level 2)	5.2	5.3	5.7

3.3. Hook effect analysis

49 specimens with different concentrations of β2-MG were assayed with the Leadmann β2-MG test kit at a linear range of 0–18 mg/L. The mean values of the response absorbance of each sample were plotted as the vertical coordinates, and the theoretical concentration of β2-MG was plotted as the horizontal coordinate to form a dose-response curve (**Figure 2**). The correlation coefficient between the measured mean values of 0–20 mg/L and the theoretical values was good, $r = 0.987$. The response absorbance increased with the increase of antigen concentration within a certain concentration, and after reaching the equilibrium concentration, the response absorbance decreased with the increase of antigen concentration when β2-MG was more than 20 mg/L and decreased with the increase of antigen concentration when the concentration of the specimen was about 80 mg/L. When the concentration of the specimen was about 12 mg/L, the response absorbance decreased with that of β2-MG and increased with that of β2-MG. When the specimen concentration is about 80 mg/L, the reaction absorbance is similar to that of the concentration of 12 mg/L, which leads to a concentration of 80 mg/L when the machine gives an incorrect result; at this time, it is necessary to carry out the pre-zone parameter setting, the alarm prompts the appearance of the pre-zone reaction, or else there may be a leakage of the detection of the sample of high concentration.

As shown in **Figure 2**, the measured data were significantly lower than Leadman's fixed value, and for the detection of ultra-high values of β2-MG, there was a phenomenon of smaller detection results. It can be seen that the data obviously deviate from the regression equation, when excluding the measured value and the fixed value deviation (concentration > 20 mg/L) is too large samples, in the further correlation analysis, the correlation coefficient $r > 0.975$, the data correlation is very good, indicating that the concentration > 20 mg/L measurement value has anomalies, may be due to the hook effect.

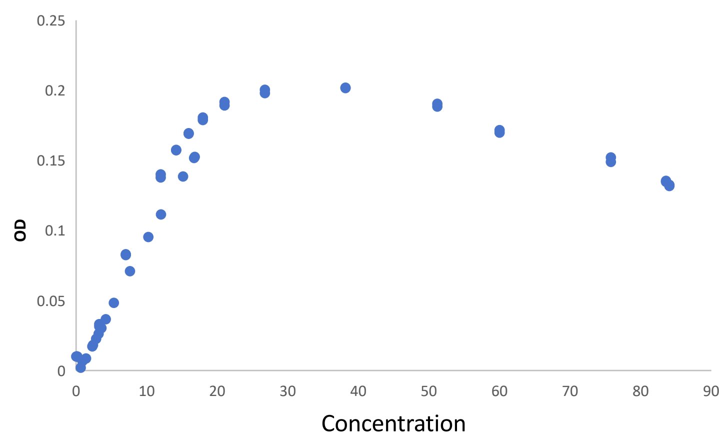

Figure 2. Dose-effect curve of Leadman β2-MG reagent

3.4. Hook effect solution

In Leadman reagents, the reaction rate ratio method is used based on the dose-response curve. For each concentration, a pre-zone detection curve is plotted according to the reaction curve and the photometric points set by the pre-zone parameters. At the boundary value of the equivalent band, the boundary value of the pre-zone test value is identified and the instrument parameters are set. When the ratio of the average reaction rate to the instantaneous reaction rate is at the boundary value, indicating an antigen-excess phenomenon, the instrument automatically performs a 5-fold dilution. Different concentrations of β2-MG were prepared using Leadman linear substances, respectively, and detected with high linear Maccura β2-MG reagent and Mindray BS2000 automated biochemistry analyzer supporting reagents for comparison and verification of the detection rate of pre-zone reaction, and the three methods of determination were basically the same as the theoretical fixed value, with a correlation $r > 0.99$, and a deviation of 10% or less, which had high accuracy (**Table 3**). Meanwhile, 25 clinical samples were tested, the correlation between the results of the Leadman reagent and Mindray reagent was $r = 0.998$, and the correlation between the results of the Leadman reagent and Maccura reagent was $r = 0.999$, which indicated that the Leadman reagent was able to successfully solve the effect of hook effect by using the dilution method.

Table 3. The results compared with the theoretical concentration of linear matter

Theoretical values (mg/L)	Leadman (mg/L)	Relative deviation (%)	Mindray (mg/L)	Relative deviation (%)	Maccura (mg/L)	Relative deviation (%)
80	82.29	2.86	75.26	-5.92	85.93	7.41
70	71.12	1.6	67.53	-3.53	74. 76	6.8
60	60.81	1.35	59.12	-1.47	61.64	2.73
50	47.87	-4.26	48.47	-3.06	49. 17	-1.66
40	37.11	-7.23	37.31	-6.73	38. 88	-2.8
30	27.16	-9.47	27.62	-7.93	26. 8	-10.67
25	23.33	-6.68	23. 59	-5.64	23. 72	-5.12
22	20.94	-4.82	20. 13	-8.5	21. 24	-3.45
20	18.48	-7.6	18.74	-6.3	18. 7	-6.5
15	16.13	7.53	14.7	-1.93	16. 56	10.4
10	9.83	-1.7	9.89	-1.1	9.85	-1.5
5	5.27	5.4	5.32	6.4	5.34	6.8
2.5	2.69	7.6	2.67	6.8	2.64	5.6
	$r = 0.99$		$r = 0.99$		$r = 0.99$	

4. Discussion and conclusion

The hook effect is an unavoidable problem of immunoturbidimetric reagents, in the process of this study, in addition to considering the differences in reagent kits, we also comprehensively considered the measurement differences in the instrument, according to the results to determine the differences in the hook effect and its solution. At the same time, through this study, we have more accurate hook effect data, which can be used to guide the dilution of antigen-limited specimens in the clinical testing process, to avoid the inaccurate detection results of ultra-high-value specimens due to differences in the detection instrument during testing.

When there is an excess of antibodies, the absorbance increases as the antigen increases, but when the antigen of the substance to be measured is extremely elevated, instead the absorbance decreases. For abnormal results, if the concentration of the antigen is too high and is on the post-zone of the antigen-antibody reaction, the instrument's absorbance is a low value, resulting in erroneous results [11]. In the immunoturbidimetric determination of some indicators, the hook effect is unavoidable, the machine often cannot recognize and send out false results.

Generally, the solutions for the hook effect of the immunoturbidimetric method are the following: (1) Antigen re-addition method: the sample plus R1 reagent is used to start the reaction, through the setting of the detection of absorbance at the point m; add R2 reagent (components for a small amount of serum containing antigen dilution), at the point n to calculate the difference in absorbance before and after the addition of R2 reagent. If R2 is added, the absorbance increases, indicating that there is no antigen overdose. If R2 is added and the absorbance decreases, an excess of antigen has occurred. This method is relatively accurate but costly. The reagents, antibody concentrations, and formulations from different manufacturers vary, resulting in different reaction performances. Reagent replacement requires resetting, and there are significant differences between batches. Additionally, the numerous steps involved reduce testing speed. (2) Reaction rate ratio: Sample plus R1 reagent is used, and the R2 reagent is added after a few minutes, at this time, there is the specific binding of antigens and antibodies to the reagent. The ratio of the average reaction rate and the instantaneous reaction rate is compared with the set limit value, and the ratio is used to judge whether the reaction is in the zone of pre-zone reaction. This method is low cost, has fewer reaction steps, and more applications, but the detection principle varies for different instruments, and there may be differences between different models of instruments from the same manufacturer; the setup is more complicated, and a lot of experiments and experience are needed to find the limit value of the pre-zone detection for the reagents from different manufacturers, this method is also the solution for the pre-zone phenomenon of Leadman β2-MG Reagent in the present experiment. For the Leadman β2-MG test reagent, in the detection of pre-zone reaction, the instrument will automatically dilute the specimen five times after the report can be done to 90 mg/L range, which has met the clinical needs. We suggest that if the concentration is lower than the upper limit of the linear range when the hook effect occurs, we should set the parameters of the pre-zone and set the alarm, and the instrument will dilute the specimen and then test again. We should try to use the dilution method to solve the hook effect problem, that is, the results of the measurement of samples beyond the linear range of the sample as a super high-value sample, its gradient times the ratio of dilution, each dilution is re-measurement, until the dilution of the sample measured value in the linear range, the final value multiplied by the number of times the dilution of the sample, that is, the true value, the results show that the dilution method has been successful in solving the impact brought about by the hook effect.

In latex-enhanced immunoturbidimetric technology, latex microspheres increase the particle size of the antigen-antibody complex, greatly improving detection sensitivity. The direction in which the antibody is coupled to the microspheres determines whether the antibody is effective or not. The Fab segment of the antibody facing outward is the most ideal, but there is a problem—the direction in which the antibody is attached to the microspheres cannot be controlled. When the antibody is randomly attached to the microspheres, the activity is not completely lost, but it is not fully utilized, and there is a greater risk to the production batch and reagent stability. The traditional latex coupling process is cumbersome, time-consuming, and prone to errors; the coupled antibodies are random, and fewer antibodies are effectively coupled; and a large number of free antibodies are not coupled to the spheres, which is a waste of antibody materials. Mindray reagents add directional agents, additives with stronger affinity for Fc segment are encapsulated on the surface of microspheres, thus making the antibody connection to the microspheres directional, changing the interaction relationship between the mi-

crospheres and the antibody, optimizing the contact relationship between the interface of the antibody and the sphere, and increasing the efficiency of the antibody coupling, so that most of the antibody's Fab segments are facing outwards, and the acceptable amount of antigen will be greater than the actual amount of antigen, making the occurrence of antigen excess less likely. The application of Mindray directional coupling latex technology fundamentally solves the clinical problems of low sensitivity and hook effect, although the linearity (0.3–18 mg/L) can be exceeded by 5-fold automatic dilution. The reportable range reaches 90mg/L, which has been achieved in the highest clinical concentration without the hook effect.

Comparison of the results of β2-MG detection by the three reagents revealed that the difference between the results after setting the pre-zone reaction parameters and the results of Maccura detection and the theoretical value of Leadman calibrated products was small, and the difference between the detection value of Mindray detection and the theoretical value of Leadman calibrated products was slightly larger, probably due to the fact that Maccura and Leadman calibrated products have the same traceability, and Mindray calibrated products have the inconsistency of the traceability with the other two manufacturers. Through the optimization of analytical parameters, the automatic identification of antigen overdose and automatic dilution and retesting of immunoturbidimetric test items can be realized, which can give full play to the advantages of intelligent and high-throughput detection of the automatic biochemical analyzer, effectively overcome the limitations of the detection system, avoid the "leakage and misdetection" of abnormal high-value results, and ensure the quality of the test [12].

In immunoturbidimetric analysis, we should choose reagents with a wider linear range or directional coupling latex-enhanced immunoturbidimetric reagents when possible. For the risk assessment of the hook effect before applying the reagents, we base it on the highest concentration of the clinical hook effect that might occur for the project. We combine this with the concentration at the hook point to selectively set pre-zone parameters. The set parameters should have a relatively high linear range compared to others. Once the parameters are set, we should verify the detection rate and false detection rate by collaborating with manufacturers with higher linear ranges. We should also adjust the parameters promptly according to clinical needs. When a pre-zone alarm occurs, the reaction curve should be re-validated to avoid fluctuations caused by specimen conditions or other reasons, thereby preventing the issuance of incorrect results.

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Disclosure statement

The authors declare no conflict of interest.

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