ORIGINAL ARTICLE

Performances Evaluation of Four Systems for Homocysteine Determination by LC-MS/MS Reference Method

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SUMMARY

Background: The aim of this study is to verify the analytical performance of four homocysteine detection systems made in China and to explore the comparability of homocysteine detection systems by isotope dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) reference method.
Methods: The intra-batch precision, inter-batch precision, accuracy, and linear range of four homocysteine detection systems were evaluated. The ID-LC-MS/MS reference method was used to evaluate the comparability and accuracy of fresh frozen serum samples in four different detection systems of homocysteine. The ID-LC-MS/MS reference method is used to assign samples as calibrators to calibrate each system. The variation and deviation of fresh serum samples between different systems before and after calibration were compared.
Results: The intra-batch imprecision of the four detection systems was less than 5%, and the coefficient of variation of inter-batch imprecision was less than 6.7%. The precision met the clinical requirements. Before calibration, the results measured by detection system 2 are consistent with the ID-LC-MS/MS reference method, which meets the requirements of accuracy verification. The regression equation of $R^2 \geq 0.975$ in the regression equation of linear analysis of the four systems, the linearity of the four detection systems is good in the range of evaluation concentration, and all of them can meet the declared linear range. The absolute average bias of fresh serum measured by the four detection systems after calibration decreased from 3.76 µmol/L, 0.96 µmol/L, 1.30 µmol/L, -1.56 µmol/L to 0.31 µmol/L, 0.28 µmol/L, 0.4 µmol/L, 0.40 µmol/L, respectively. The relative average bias decreased from 22.6%, 7.50%, 11.0% and -8.50% to 1.98%, 1.78%, 2.59%, 2.34%, respectively. After calibration, the slope and intercept of the regression curve of the fresh serum measured by the four detection systems and the reference method are closer to 1 and 0 than before calibration.
Conclusions: The precision, reference interval, and linear evaluation of the four detection systems are good. The ID-LC-MS/MS reference method assigning fresh frozen serum samples as calibrators can improve the accuracy and comparability of the results of different detection systems.
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KEY WORDS

INTRODUCTION

Homocysteine (Hcy) is an antioxidant and a non-protein amino acid containing mercaptan, which is involved in maintaining the biological function of metabolism and formed in muscle and liver [1,2]. It is an important intermediate in the metabolism of methionine and cysteine. In the 1960s, it was first assumed that increased homocysteine levels could lead to leukemia, Alzheimer's disease, and cardiovascular diseases, such as atherosclerotic arterial disease and atherosclerotic thrombovascular disease [3-5]. Hyperhomocysteine (Hyper-Homocysteinemia, HHcy) is an independent risk factor for cardiovascular disease, which is closely related to H-type hypertension, coronary heart disease, stroke, and other diseases [6-9]. Hcy mainly comes from the metabolism of methionine, that is the demethylation methionine to form Hcy. There are two main metabolic pathways of Hcy which are the methylation to form methionine and serine to form amine sulfide. The important cofactors are folic acid, vitamin B_{12}, and vitamin B6 [10]. The content of Hcy in blood is an important indicator of human health [11]. There are two forms of Hcy in plasma, including binding Hcy and free Hcy [12]. The Hcy detected in serum is called total Hcy. The increase of Hcy concentration will make human DNA that cannot be well repaired, leading to a higher risk of disease [13,14]. In addition, it can also cause heavy metals and free radicals and other metabolic wastes to accumulate in the body, leading to inflammation and cell damage [4]. Therefore, it is very important to detect the content of Hcy in the human body for the prevention, diagnosis, and treatment of the above diseases.

The detection methods and principles of Hcy mainly include radioimmunoassay, high performance liquid chromatography (HPLC), enzyme cycle, gas chromatography-mass spectrometry (GC-MS), enzyme-linked immunosorbent assay (ELISA), automatic fluorescence polarization immunoassay, and liquid chromatography tandem mass spectrometry (LC-MS/MS) [15-17]. However, these methods have their own advantages and disadvantages. The operation of radioimmunoassay is tedious, and the detection reagent is contaminated by radiation, which is harmful to the human body, it limits the popularization and use of this method [10]. Enzymatic cycling method is by far the most widely used method in clinic, which has the advantages of low cost and good repeatability in the detection of Hcy. The principle of enzymatic cycling method for the determination of Hcy is as follows: oxidized homocysteine is transformed into free Hcy under the action of TCEP. Free Hcy reacts with S-adenosylmethionine (SMA) to form

SAH and methionine under the action of Hcy methyltransferase, and SAH is hydrolyzed into adenosine and Hcy by S-adenosylhomocysteine hydrolase. The generated Hcy can produce a cyclic reaction. Adenosine is hydrolyzed to ammonia and hypoxanthine, which converts NADH to NAD^+ under the action of glutamate dehydrogenase. The concentration of Hcy in the sample is proportional to the conversion rate of NADH.

GC-MS and HPLC methods require advanced equipment and are expensive, complex, and time-consuming operations [18]. Different Hcy detection methods have different characteristics and performance. Among them, GC-MS and ID-LC-MS/MS were recognized as reference methods by the Joint Committee on Traceability of Laboratory Medicine (JCTLM) [19,20]. The ID-LC-MS/MS method has the advantages of high sensitivity, accurate method, no strict separation for sample preparation, and the measured value can be directly traced to the basic unit-molar of the substance in the international unit system [16]. At present, the field of inspection standardization uses it as a recognized first-level reference measurement principle for the establishment of reference methods, which is a method with absolute measurement properties.

In order to explore the quality of routine Hcy clinical detection systems, this study used ID-LC-MS/MS as a referenced method to evaluate the accuracy of enzymatic cycling method in detecting serum Hcy level. Four domestic mainstream manufacturers' kits were purchased from four companies. The precision, accuracy, linear analysis, and reference interval of four detection systems were evaluated. Among them, the accuracy of four Hcy detection systems was evaluated by using the ID-LC-MS/MS reference method established in the calibration laboratory of our medical school as the comparison method. At the same time, it discusses how to use the reference method to assign fresh serum samples as calibrators to calibrate different detection systems, in order to achieve the comparability of the measurement results of different detection systems.

MATERIALS AND METHODS

Analyzers and reagents

Four detection kits were used to obtain Hcy measurements: detection kit-1 (Zhongyuan biotechnology Co., LTD, Chongqing, China), detection kit-2 (Maccura biology Co., LTD, Sichuan, China), detection kit-3 (Mindray medical technology Co., LTD, Shenzhen, China), detection kit-4 (SHANGHAI KEHUA BIO-ENGINEERING Co., LTD, Shanghai, China), Roche Cobas 8000 C702 biochemical analyzer (Roche Diabetes Care GmbH, Mannheim, Germany), Sedorius CP225D analytical balance (Germany), Waters TQ-S tandem ACQUITY UPLC liquid chromatography-mass spectrometry (Milford, MA, USA), DL-homocysteine, DL-[2d8]-homocysteine, DL-dithiothreitol (DDT), ammonium bicarbonate (ABC) were purchased from Sigma-

Aldrich (St. Louis, MO, USA). Water was obtained from a Milli-Q system (Millipore Co., Bedford, MA, USA).

The analyzer and related reagents used for this evaluation are used in accordance with the manufacturer's instructions and are calibrated only once according to their standard operating procedures before any sample analysis is done.

Samples

The serum samples were collected from Guangdong Hospital of traditional Chinese Medicine, and approved by the hospital ethics committee, the infectious indicators (hepatitis B virus surface antigen, hepatitis C virus antibody, human immunodeficiency virus antibody, and syphilis antibody) were negative. Different concentrations of serum samples were collected and stored in the refrigerator at -80°C. Twenty-five individual sera of different concentrations and 5 mixed sera (used to calibrate 4 clinical detection systems). Three concentration levels of human serum matrix Hcy candidate reference materials are provided by the National Institute of Metrology (NIM) of China.

Precision

Four kits were loaded on the Roche Cobas 8000 C702 biochemical analyzer and calibrated with their original calibrators, and the precision was tested with Hcy samples of high and low levels.

Intra-batch precision: Hcy samples at high and low levels were repeatedly tested 20 times at the same time. Inter-batch precision: Hcy samples at high and low levels were repeated 4 times a day for 5 consecutive days.

Accuracy

Twenty-five serum samples were detected by the four detection systems 3 times successively. The accuracy of the four detection systems was evaluated by taking the mean value and taking the results of the ID-LC-MS/MS reference method as the target value. The results measured by ID-LC-MS/MS reference method were taken as the X axis, and the four conventional detection systems were used as the Y axis for regression analysis. The measurement result of the reference method is taken as the X axis, the offset rate of the conventional detection method and the reference method is taken as the Y axis, and the average bias and absolute average bias of the conventional method are less than 1/2 TEa as the judgment standard.

Linearity

Normal saline was selected as the low value sample of 0 μmol/L (L), to collect fresh serum with normal appearance, no hemolysis, lipids and jaundice, and the concentration was close to the upper limit of the linear range, and the high value sample (H) was prepared according to the following proportion: 5L magnum, 1L magic, 1L magic, 2H camera, 2L magic, 3H, 3H, 3H, 1L, 1L, 4H, 5H. Repeat the measurement on the computer 2 times and take the average value. Compared with the expected concentration, the curve fitting was carried out.

Establishment of LC-MS/MS method as a reference method

According to the suggestion of JCTLM, the ID-LC-MS/MS method is used as a reference method for Hcy detection. In this study, the ID-LC-MS/MS method of Hcy is established according to the study reported by Liu et al. [21]: the positive ion mode of ESI was selected, m/z $136 \rightarrow 90$ was used as the detection ion pair of Hcy, and m/z $140 \rightarrow 94$ was used as the internal standard of detection ion pair. Fresh frozen serum samples were used to verify the precision of the reference method. The samples were tested 3 times a day for 2 consecutive days. The coefficient of variation (CV) of 10 times was required to be less than 1%. The accuracy of the test results of the reference method is verified with the reference material SRM 1955, and the three test results are required to be in the range of "identified value + uncertainty".

Method comparison

ID-LC-MS/MS reference method was used to verify the accuracy of 25 serum samples and assign values to 5 serum calibrators. Each sample serum was measured 3 times a day for 2 days. Five serum samples assigned by the reference method were used as calibrators, and four clinical detection reagents were calibrated by a Roche biochemical analyzer. At the same time, the accuracy of 25 serum samples were determined by four kits on a Roche biochemical analyzer, and the average value was taken after determinations were repeated 3 times. The ID-LC-MS/MS reference method of homocysteine was used as the comparison method to evaluate the accuracy of four detection kits.

Statistical analysis

Excel 2016 and SPSS 19.0 software were used for statistical analysis. The outliers of the precision evaluation test were tested by Grubbs method, and the offset between the conventional detection system and the reference method was evaluated by an improved Bland-Altman graphic analysis method.

RESULTS

Results of performance evaluation of ID-LC-MS/MS reference method

The coefficient of variation (CV%) of the ID-LC-MS/MS reference method is less than 1%, and meets the requirement of less than 1/4 allowable total error (TEa, 5.0%), indicating that the reference method has excellent precision. The ID-LC-MS/MS reference method detects the standard working liquid to draw the standard curve. The linear regression equation $Y = 1.1589Y - 0.0204$, $R = 1.000$, indicates that the linearity of the

Table 1. Results of LC-MS/MS detection of Hcy reference materials.

Reference materials	ID-LC-MS/MS (µmol/L, s, n = 3)	Target value (µmol/L)	Bias, %
Hcy-1	28.67 ± 0.10	28.80 ± 1.10	-0.45
Hcy-2	17.77 ± 0.12	17.93 ± 0.57	-0.89
Hcy-3	14.62 ± 0.13	14.38 ± 0.46	1.67

Table 2. The result of the assignment to the sample by the reference method (µmol/L).

Samples	Results	Uncertainty degree (k = 2)	Samples	Results	Uncertainty degree (k = 2)
1	7.62	0.33	14	20.45	0.69
2	8.31	0.19	15	21.60	1.23
3	9.26	0.28	16	24.60	0.56
4	10.00	0.51	17	25.51	0.63
5	10.43	0.40	18	25.91	0.13
6	11.00	0.22	19	28.04	0.71
7	11.42	0.25	20	23.77	0.74
8	13.47	0.36	21	11.62	0.23
9	14.35	0.67	22	15.51	0.57
10	16.45	0.73	23	19.42	0.60
11	16.81	0.32	24	22.2	0.72
12	17.33	0.79	25	26.51	0.45
13	19.14	0.53			

Table 3. Precision of four Hcy clinical detection systems (µmol/L).

	Level 1				Level 2			
	Intra-batch		Inter-batch		Intra-batch		Inter-batch	
	$x \pm s$, CV (%)		$x \pm s$, CV (%)		$x \pm s$, CV (%)		$x \pm s$, CV (%)	
1	9.74 ± 0.18	1.81	9.71 ± 0.45	4.61	19.3 ± 0.33	1.71	19.99 ± 0.51	2.55
2	10.2 ± 0.18	1.85	9.99 ± 0.16	1.56	15.9 ± 0.34	2.15	14.94 ± 0.46	3.05
3	11.3 ± 0.15	1.28	10.7 ± 0.25	2.35	16.64 ± 0.18	1.06	15.73 ± 0.5	3.20
4	8.52 ± 0.09	1.12	8.25 ± 0.42	5.13	18.8 ± 0.49	2.61	18.5 ± 7.08	5.82

reference method is good. As shown in Table 1, the results of the ID-LC-MS/MS reference method for candidate targets are all within the allowable range of their recognized values, indicating that this reference method can accurately detect the concentration of serum Hcy in samples. Then the reference method was used to determine 25 serum comparison samples 3 times on two consecutive days. The result of the assignment to the sample by the reference method was shown in Table 2.

Results of performance evaluation of four detection systems for Hcy detection
Precision
As shown in Table 3, all results are within the acceptable range. The intra-batch imprecision (CV%) and inter-batch imprecision (CV%) of the two levels of quality control products detected by the four detection systems were 1.06 - 2.61% < 1/4 TEa (5.00%) and 1.56 - 5.82% < 1/3TEa (6.67%), respectively.

Table 4. The results of measurement comparison between four detection systems and reference methods.

Analyzer	Fitted equation	Bias (%, µmol/L)	Medicine decide level µmol/L	Calculated value µmol/L	Deviation	Criteria	Accept-ability	Results
1	Y = 1.1581X + 1.0411	22.8%, 3.69 µmol/L	15	18.4	22.67%,3.4 µmol/L	10.0% or 1.25 µmol/L	no	no pass
	R² = 0.9976		45	53.2	18.22%, 8.2 µmol/L		no	
2	Y = 0.935X - 2.1029	8.2%, 1.01 µmol/L	15	16.1	7.33%, 1.1 µmol/L		yes	pass
	R² = 0.9974		45	44.2	-1.78%, -0.8 µmol/L		yes	
3	Y = 0.8818X + 3.3426	12.0, 1.35 µmol/L	15	15.6	4.13%, 0.6 µmol/L		yes	no pass
	R² = 0.9982		45	40.2	-10.69%, -4.8 µmol/L		no	
4	Y = 0.8827X + 0.4819	-8.2%, -1.49 µmol/L	15	13.7	-8.53%, -1.3 µmol/L		yes	no pass
	R² = 0.9788		45	40.2	-10.67%, -4.8 µmol/L		no	

Table 5. Results of comparison between four detection systems and reference methods after calibration.

Analyzer	Fitted equation	Bias (%, µmol/L)	Medicine decision level µmol/L	Calculated value µmol/L	Deviation	Criteria	Accept-ability	Results
1	Y = 0.962X + 0.6479	1.98%, 0.31 µmol/L	15	15.1	0.52%, 0.1 µmol/L	10.0%, 1.25 µmol/L	yes	pass
	R² = 0.9968		45	43.9	-2.36%, -1.1 µmol/L		yes	
2	Y = 0.9956X - 0.0022	1.78%, 0.28 µmol/L	15	14.9	-0.45%, 0.1 µmol/L		yes	pass
	R² = 0.9973		45	44.8	-0.44%, 0.2 µmol/L		yes	
3	Y = 1.0168X - 0.5647	2.59%, 0.40 µmol/L	15	14.7	-2.08%, -0.3 µmol/L		yes	pass
	R² = 0.9964		45	45.2	0.43%, 0.2 µmol/L		yes	
4	Y = 0.9879X - 0.0504	2.34%, 0.40 µmol/L	15	14.8	-1.56%, -0.2 µmol/L		yes	pass
	R² = 0.9955		45	44.4	-1.33%, -0.6 µmol/L		yes	

Accuracy

Table 4 and Figure 1 shown the comparison of the measurement results of the four Hcy clinical detection systems with the ID-LC-MS/MS reference method. The fitting equation was calculated, the system bias at the medical decision level was calculated, and the 1/2 TEa was used as the judgment standard. The results showed that the measurement results of detection systems 1, 3, and 4 were inconsistent with those of the reference method, and the average biases were 22.6%, 11.0%, and -8.50%, respectively. and the absolute average biases were 3.7 µmol/L, 1.30 µmol/L, and -1.56 µmol/L, respectively (Table 4 and Figure 1). At the medical decision levels of 15 µmol/L and 45 µmol/L, the deviation
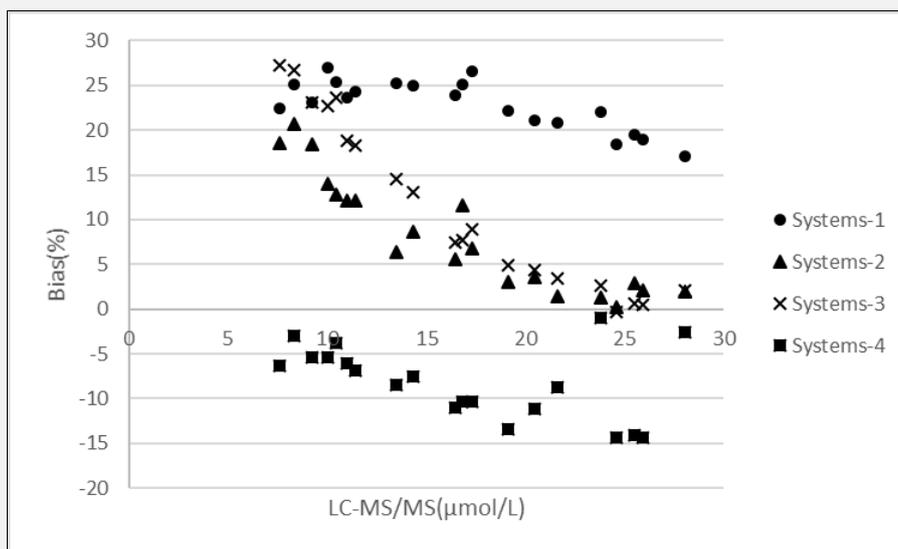
Figure 1. Bias diagrams of measurement results of four detection systems and ID-LC-MS/MS reference methods.
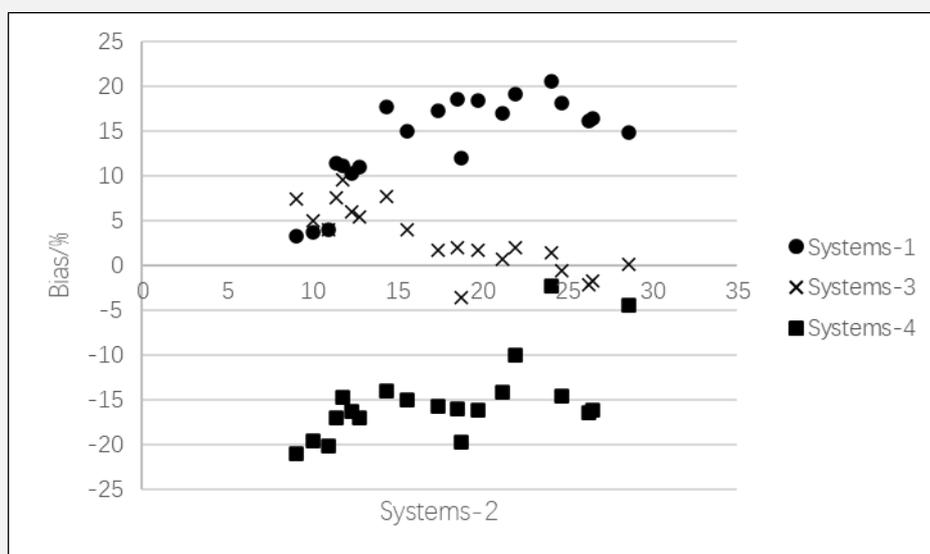

Figure 2. Bland-Altman diagram of detection system 2 and the other three detection systems.

was more than 1 µmol/L, which could not meet the accuracy requirements. Compared with the reference method, the average bias of the measurement result of detection system 2 is 7.50%, and the absolute average bias is 0.96. At the medical decision level, the calculated results are all less than 1/2 TEa, which meets the requirements of accuracy verification.
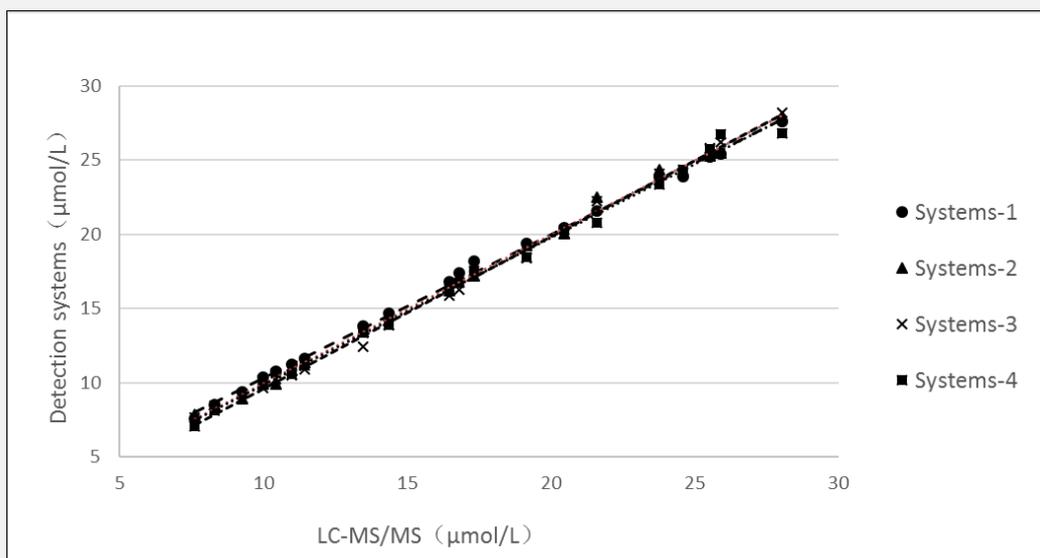
Figure 3. The measurement results of the four detection systems are compared with the reference methods after calibration.
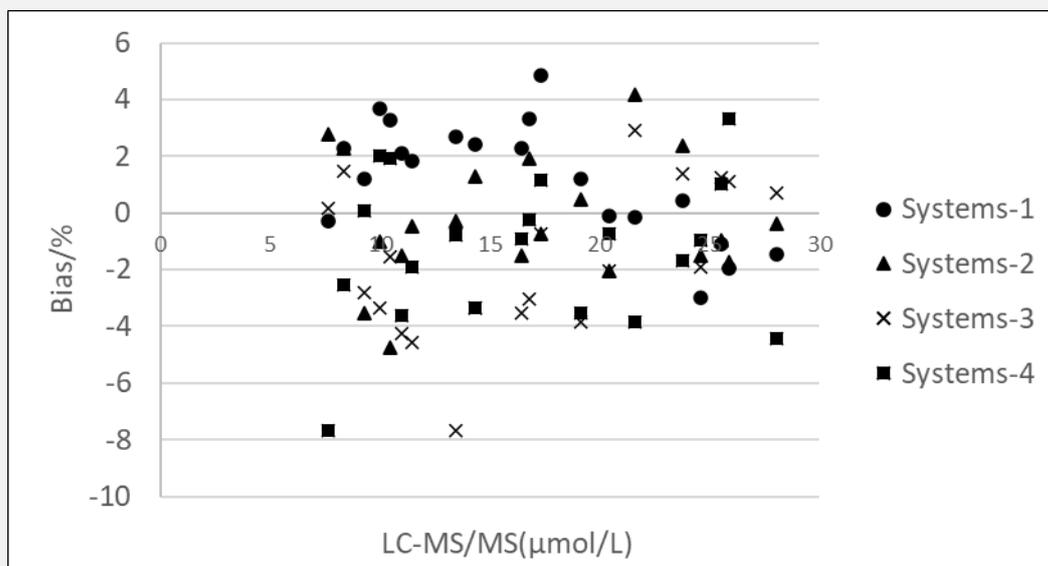

Figure 4. Bland-Altman diagrams of four detection systems and reference methods after calibration.

Linearity

The four detection systems take the theoretical value as the X axis and the measured value as the Y axis. The polynomial regression analysis is carried out by using SPSS software. There is no significant difference between the nonlinear coefficients (b2, b3) and 0. The binary first order equation is drawn. The linear regression equations of the four detection systems are as follows:

$Y = 1.002X - 0.6833$ (systems-1, $R^2 = 0.9971$), $Y = 0.9692X + 0.5524$ (systems-2, $R^2 = 0.9995$), $Y = 0.9947X + 0.0262$ (systems-3, $R^2 = 0.9990$), $Y = 0.9948X + 0.3095$ (systems-4, $R^2 = 0.9997$). The slope (a) of the four systems is between 0.9392 and 1.002. The intercept (b) is between -0.6833 and 0.5524, $R^2 \geq 0.975$. The linearity of the four detection systems is good in the range of evaluation concentration, and they all reach or are close to the linear range claimed by their respective manufacturers.

Method comparison

Twenty samples of serum were determined simultaneously by four kinds of conventional detection systems, and through the previous accuracy evaluation, the performance evaluation of detection system 2 met the requirements. Therefore, taking the measurement results of detection system 2 as the X axis, the improved Bland-Altman graphic analysis method was used for the analysis. As shown in Figure 2, the results of the four methods were inconsistent, and there were obvious differences among the four detection systems. The measurement result of detection system 1 is significantly higher than that of the other three detection systems, and the measurement result of detection system 4 is significantly lower than that of the other three detection systems.

The serum matrix mother calibrator assigned by the ID-LC-MS/MS reference method was used as the calibrator. After calibrating the four detection systems, 25 serum samples assigned by the reference method were detected. Table 5 and Figure 3 show that after calibration, the average absolute bias of the four detection systems were 0.31 μmol/L, 0.28 μmol/L, 0.40 μmol/L, and 0.40 μmol/L, respectively, and the average relative bias are 1.98%, 1.78%, 2.59%, and 2.34%, respectively. At the same time, the deviation of the four Hcy detection systems at the medically determined levels of 15 μmol/L and 45 μmol/L were all less than that of 1/2 TEa. Even after the clinical detection system was calibrated with the serum matrix mother calibrator assigned by the reference method, the results measured by the four detection systems were verified by the accuracy (Figure 4).

DISCUSSION

The ID-LC-MS/MS method has the advantages of high sensitivity, accurate method, and the measured value can trace to the basic unit-molar of the substance in the international unit system [17]. At present, the field of inspection standardization uses it as a recognized first-level reference measurement principle for the establishment of reference methods [21]. JCTLM reported that the ID-LC-MS/MS is a reference method for Hcy detection [19,20]. Through the performance evaluation of the reference method for Hcy detection, the precision of the reference method can be controlled within 1%, and the

accuracy verification is also within the allowable range of the recognized value (Table 1). Therefore, ID-LC-MS/MS meets the requirements of the comparison method for the accuracy evaluation of serum Hcy. In order to understand the quality of routine Hcy clinical detection systems, four domestic mainstream manufacturers' kits were purchased. The precision, accuracy, linear analysis, and reference interval of four detection systems were evaluated respectively. The results show that the precision, linear range, and reference interval evaluation results of the four detection systems can meet the requirements (Table 3, Table 4, and Figure 1), and only the detection system 2 meets the requirements (Table 4, Table 5, and Figure 2). The accuracy of the other three detection systems is not consistent with the reference method, and there is an overall phenomenon of high or low (Table 4 and Figure 1). It is necessary to explore the reasons for the failure of the accuracy of the three detection systems. According to the precision, linear evaluation, and reference interval verification results, it is considered that the incomparability of the accuracy results may be related to the calibration bias. For the instructions and calibration statements of the four detection systems, the calibrations of the four detection systems are traced to the NIST SRM 1955 reference material [22]. At present, the calibration value scheme with the reference method project is as follows: after the upper-level calibrator calibrates the instrument, the commercial calibrator is measured many times to get a pre-value, and then the clinical samples determined by the reference method are used for comparison. The setting value of the detection systems calibrator is adjusted according to the comparison results to ensure that the clinical results are accurate and comparable. The uncertainty of the substance or measurement procedure increases with the transmission of the traceability chain [23-25]. On the other hand, manufacturers who do not have the ability of reference measurement can only carry out quantity traceability by using the reference laboratory that provides services, but it is still difficult to transfer or calibrate each batch of reagents or calibrations. As a result, the calibration of the detection systems may be inaccurate, and it is speculated that the incomparable accuracy of the three detection systems may be related to the inaccuracy of the calibration [26,27]. In order to verify the reason of inference and realize the comparability of measurement results between different detection systems, and explore the comparability of measurement results, this experiment uses the ID-LC-MS/MS reference method to assign fresh serum samples as calibrators [28-30] and recalibrate the four detection systems. The results show that the fresh serum calibrator assigned by the ID-LC-MS/MS reference method meets the requirements of clinical detection performance (Table 2). After calibration, the comparability of measurement results is analyzed. The linear regression equation calculation results of the four detection systems and reference methods show that the coefficient of variation at the medical decision level of 15 μmol/L and

45 μmol/L is less than 2.0%, and the average offset is less than 5.0%, which is far less than the prescribed allowable range, and the comparison results are acceptable (Table 5, Figure 3, and Figure 4). Therefore, there is a deviation in the target value of the calibrator of detection system 1, 3, and 4, which needs to be re-adjusted.

In summary, the precision, linearity, and reference interval performance of the four conventional detection systems are good. After calibrating the conventional calibration system with the reference method assigned serum as calibrator, the comparability of the measurement results can be achieved. At the same time, in order to trace the clinical test results to international units, kit manufacturers have reference measurement ability or can work with units with reference measurement ability to verify the traceability of products by evaluating the target value of product calibrations. It can not only improve the quality and competitiveness of products, but also achieve the reliability and comparability of measurement results between different testing systems.

Declaration of Interest:
The authors declare that there are no conflicts of interest.

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