

Examination of the accuracy of the quantitative in-house kit for determining zinc concentration in seminal fluid

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Abstract:

Zinc in seminal fluid originates primarily from the prostate gland. It is pivotal for male sexual function because it affects the quantity, quality and mobility of sperm. **Materials and methods:** the semen samples were obtained from 300 male partners of infertile couples who attended the Department of Biomedicine and Genetics at the Hanoi Medical University between the ages of 18 and 50 years; they were then analysed for routine seminal parameters. They were collected and analysed according to WHO 2010 guidelines. Seminal fluid was centrifuged at 1,500 rpm for 10 minutes, and floating fluid collected for zinc quantification using spectroscopy with 5-Br-PAPS was used as a color indicator. In a pH 8.6-buffer solution, in a buffered media, zinc reacts with specific complexing 5-Br-PAPS form a stable color compound. The optical density is directly proportional to the concentration of zinc in the semen. The results yielded a linear regression model of $y = 0.0666x + 1.2026$ with a correlation coefficient of $r=0.9956$. The calibration function was $y = 0.9977x$ with $R^2=0.9995$. The repeatability was $SD=0.004$, and the coefficient of variation was $CV%=0.27%<5%$. In terms of intermediate precision, the standard deviation was $(SD)=0.01$, and the coefficient of variation was $CV%=0.64%<5%$. Trueness was $t_{ex}=2.076 < t_t=2.262$. Specificity and sensitivity were 100% at 64x dilution. Specificity and sensitivity were 100% and 99.05% respectively. A significant correlation was discovered between the two methods, with $r=0.975$ and $p<0.001$; the average difference between the two methods was 0.0002. **Conclusion:** successfully completed the kit for determining zinc concentration in semen by the colorimetric method.

Keywords: male infertility, seminal zinc, seminogram, spectrophotometric method.

Classification number: 3.2

Introduction

Infertility is “a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse” (by WHO) [1, 2]. The incidence of infertility in the world is about 13 to 18%; the causes for men and women are similar [3]. Recently, aside from tests involving semen analysis, genetic testing and hormonal methods, biochemical tests involving fructose and zinc have become more common the diagnosis and treatment of infertility [4]. They have therefore been established as reliable indicators of human male fertility. Understanding the role of these indicators can help to enable a deeper understanding of the mechanisms that cause male infertility [5, 6].

Zinc is an essential factor for the male reproductive system. Zinc deficiency in the reproductive system causes hypogonadism and gonadal hypofunction [7, 8]. Zinc is crucial for maintaining normal functioning of the testes and prostate, affecting the quantity, quality and mobility of sperm [9, 10]. In Vietnam, tests for concentrations of zinc in seminal plasma were used in 2013 at the Hanoi Medical University Hospital, and these tests are increasingly popular in clinics. The necessity of using test kits to quantify zinc in semen is increasing. However, in Vietnam, no units nor establishments currently produce this test. Therefore, all tests must be imported from abroad with many intermediate costs, often increasing the cost of the test.

Materials and method

Objects

The study subjects were semen samples of male patients who visited for examination, counseling and testing semen at the Genetic Counseling Center and the Urology and Urology Clinic at Hanoi Medical University Hospital from June,

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2017 to March, 2018. The procedure for collecting semen samples was conducted in accordance with the guidance of the World Health Organization in 2010. Participants were required to abstain from sex for 2 to 5 days. The semen was deposited into a sterile vial with no spermicide and analysed within 2 hours of sampling.

The formula used for a sample size for a descriptive study by S.K. Luanga and Lemeshow [11] is as follows:

$$n = Z_{1-\alpha/2}^2 \frac{p(1-p)}{\epsilon^2 p^2}$$

In this equation $Z_{1-\alpha/2}$: confidence factor (with 95% confidence, $Z_{1-\alpha/2}=1.96$); $\alpha=0.1$ (reliability); $\epsilon=0.10$; $p=95\%$ (reference process precision), n =number of experiments required, calculated by 21; a round of 30 was executed.

To calculate the sample size to determine sensitivity, specificity, and equivalence the following were used:

$Z_{1-\alpha/2}$: confidence factor (with 95% confidence, $Z_{1-\alpha/2}=1.96$).

According to Zahoor Ahmed and colleagues, in 2010, the percentage of men with low zinc concentrations in the azoospermia and oligospermia groups was $p=25\%$ [12]; ϵ is 0.2, and $n=1.96^2 \times 0.25 \times (1-0.25)$: $(0.2 \times 0.25)^2=288.12$, rounded to 300.

The sample size of 300 was employed to increase accuracy.

On the same sample of semen, zinc concentrations were measured using two methods: one involved the IVD kit (Zinc 5-Br-PAPS, Spinreact company, Spain), and one involved the improvement kit. The difference between the two kits was based on a Pearson correlation, T-test, and Bland-Altman plot.

The selection criteria required semen samples from male patients of reproductive ages between 18 and 50 years without acute illness who consented to participate in the study.

For the exclusion criteria, men with genital cancers, men with HIV, syphilis, and gonorrhoea, men suffering from acute illness or mental illness, and men who did not consent to enroll in the study were excluded.

Method

The principle of the method involved direct colorimetric testing without deproteinisation of the sample. In a pH 8.6 buffer solution, zinc reacts with 5-Br-PAPS complexes and produces stable color. The optical density is directly

proportional to the concentration of zinc in the semen.

Measuring the concentration of zinc:

Materials:

Buffer A: Sodium bicarbonate (200 mmol/l), Sodium citrate (170 mmol/l), Dimethylglyoxime (4 mmol/l), Triton-X100 (1%), 5-Br-PAPS (0.08 mmol/l).

Buffer B: Salicyldoxime (2,9 mmol/l).

Working buffer, pH-8.6 (C):4A:1B.

Zinc standard (Merk).

Zinc color 5-Br-PAPS (Spinreac, Spain).

The procedure for zinc quantitative testing in semen:

The steps for the procedure of zinc quantitative testing in semen are as follows:

+ Step 1: the semen sample is centrifuged at 1,500 rpm for 10 minutes. This step is used to settle the sperm cells down to the bottom; only the top of the semen containing the zinc for testing is then gathered, as the sperm is not used in the test.

+ Step 2: 200 μ l of supernatant is added into 200 μ l TCA 370 μ mol/l, mixed thoroughly, and centrifuged at 10,000 rpm for 10 minutes. This step is used to remove the protein.

+ Step 3: 100 μ l of supernatant is into 2 ml of working buffer. This is then incubated at room temperature for 5 minutes.

+ Step 4: optical density (OD) is measured at 530 nm wavelength and 1 cm curvature. For control, 100 μ l of distilled water is substituted for semen. The color of the solution remained unchanged within 1 hour.

$[Zn] \mu\text{mol/l} = (\text{OD sample}/\text{OD blank}) \times C \text{ zinc standard } (\mu\text{mol/l})$.

The sensitivity and specificity are calculated according to the following formula:

$$\text{The sensitivity (\%)} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \times 100\%$$

$$\text{The specificity (\%)} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}} \times 100\%$$

Statistical analysis involved data processing using SPSS software version 20.0. The mean values were compared using student-t-tests. The analysis is to be meaningful when the coefficient was $p<0.05$.

Regarding ethical considerations, the research was approved by the ethical council of Hanoi Medical

University. The patient completely voluntarily participated in the study. All of the information from the database was kept under strict confidentiality. No names were recorded.

Results

Construction of linear regression equations and calibration function

Linear regression equations were used to assess whether the color intensity of the mixture is proportional to the concentration of zinc in the seminal plasma. The linear regression equation was used to calculate zinc concentrations in seminal plasma based on measured photometer densities.

The regression equation of $y = 0.0666x + 1.2026$ was used, the correlation coefficient of $r=0.9956$ was employed (Fig. 1).

The calibration function is constructed with the standard zinc concentration threshold of 0; 0.5; 1; 1.5; 2 g/l the OD density was measured corresponding to each standard zinc concentration threshold based on the completed test procedure and the calibration curve was established as in Fig. 2.

The calculation function equation is $y = 0.9977x$; $R^2=0.9995$.

Determining the accuracy of the kit (Table 1)

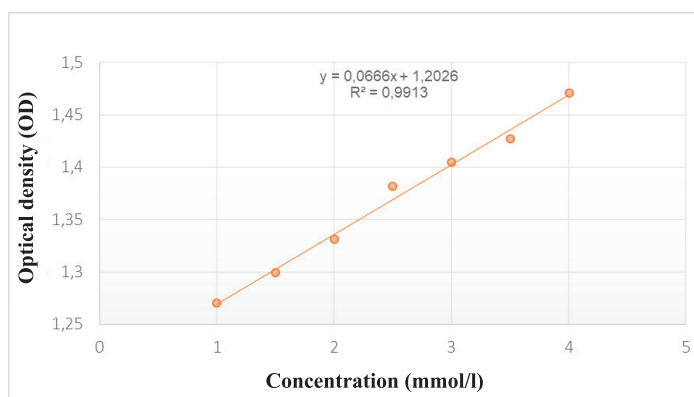


Fig. 1. Linear regression equations.

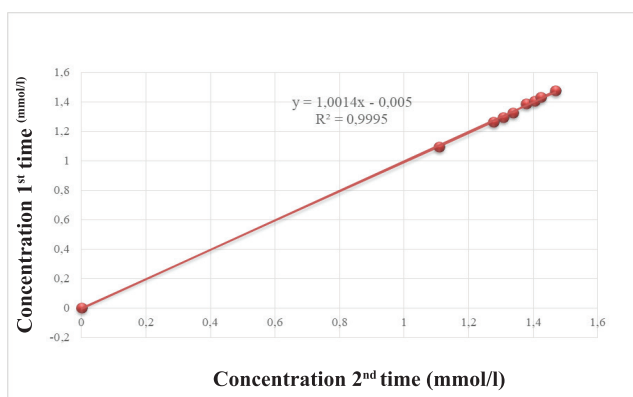


Fig. 2. Calibration curve.

Table 1. Repeatability, intermediate precision and trueness of the in-house kit.

Measurement	Zinc standard concentration (mmol/l)	Repeatability		Intermediate precision			Trueness	
		OD	The zinc concentration measured (mmol/l)	Zinc concentrations measured by different technicians (mmol/l)			OD	The zinc concentration measured (mmol/l)
				Technicians 1	Technicians 2	Technicians 3		
1	1.53	1.352	1.527	1.559	1.523	1.530	1.332	1.521
2	1.53	1.354	1.529	1.524	1.527	1.524	1.335	1.524
3	1.53	1.358	1.533	1.504	1.524	1.534	1.333	1.522
4	1.53	1.352	1.527	1.532	1.525	1.526	1.334	1.523
5	1.53	1.36	1.536	1.513	1.532	1.534	1.340	1.530
6	1.53	1.362	1.538	1.537	1.533	1.524	1.341	1.531
7	1.53	1.356	1.531	1.527	1.536	1.534	1.343	1.533
8	1.53	1.351	1.525	1.514	1.530	1.529	1.338	1.528
9	1.53	1.353	1.528	1.520	1.536	1.532	1.343	1.533
10	1.53	1.356	1.531	1.544	1.528	1.536	1.336	1.525
SD		0.004		0.01				
CV%		0.27		0.64			$T_{ex}=2.076$	

Repeatability: based on the results in the Table 1, a standard deviation (SD) of =0.004 and a coefficient of variation of CV%=0.27 were calculated. The variation coefficient of the in-house kit is within the allowable limits (CV%<5%).

Regarding the intermediate precision, based on the results obtained from the table above, standard deviation of (SD)=0.01 was obtained, so that the coefficient of variation was CV%=0.64%. The coefficient of variation lies within the CV%<5% limit.

Based on the above results, $t_{ex}=2.076$ was calculated. In addition, since $t_t=2.262$, $t_{ex} < t_t$ the testing standards were achieved.

Sensitivity and specificity (Tables 2, 3)

Table 2. Statistics of zinc quantitative results in three groups of patients.

Zinc concentration	The in - house kit	Commercial kit
Group 1 (Control group)		
Below normal concentration	43	43
Normal	57	57
Group 2 (Group with some abnormal seminal indexes)		
Below normal concentration	40	38
Normal	60	62
Group 3 (Azoospermia group)		
Below normal concentration	73	73
Normal	27	27

Table 3. Index of indicators to calculate sensitivity and specificity.

	True positive	False positive	True negative	False negative
Group 1	57	0	43	0
Group 2	60	0	38	2
Group 3	27	0	73	0
Total	208	0	90	2
Sensitivity	99.05%			
Specificity	100%			

Comment:

- True negative: below normal zinc concentrations when tested by both kits.
- False negative: below normal zinc concentrations when tested by the in-house kit above normal zinc concentrations when tested by the commercial kit.
- True positive: normal zinc concentrations when tested with both kits.
- False positive: normal zinc concentrations when tested by the in-house kit; below normal zinc concentrations when tested by the commercial kit.

Sensitivity and specificity were calculated according to the following formula:

$$\text{The sensitivity (\%)} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \times 100\%$$

$$\text{The specificity (\%)} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}} \times 100\%$$

The sensitivity of the in-house kit is 99.05%, and the specificity of the self-mixing kit is 100%.

Assessing the capability of detecting the concentration of zinc between the in-house kit and the commercial standard kit (Fig. 3)

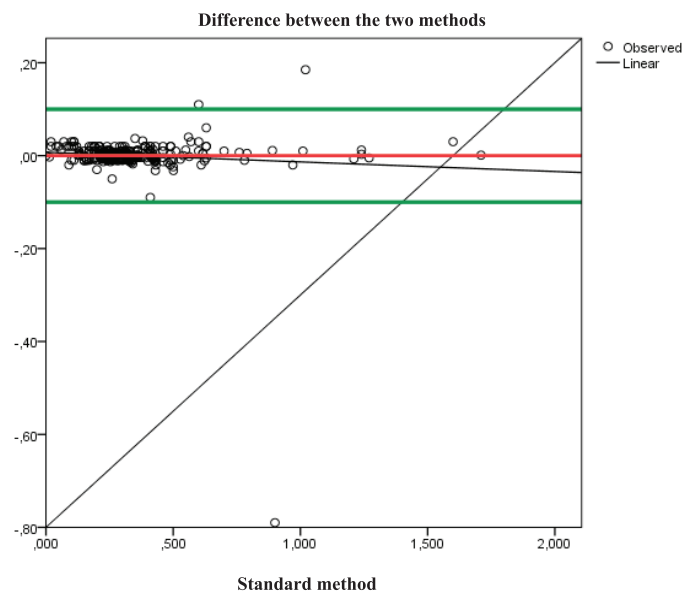


Fig. 3. Bland-Altman diagram assessing the capability of detecting zinc concentrations between the in-house kit and the commercial standard kit.

- Use semen samples of 300 patients. Each sample quantifies 2 times: using a commercial standard kit and in-house kit (the list of 300 patients tested is included in the appendix).

- The Pearson test results demonstrate a strong correlation between the concentrations of zinc measured by the two kits, with $r=0.975$ (0.983-0.995), $p<0.001$.

The in-house kit tends to yield higher results than the commercial standard kit. The difference between the two methods of detection is random (the value points are dispersed and follow no pattern), and the error deviation is not related to the zinc quantification results.

The mean difference between the two methods was minimal, or (0.0002) close to 0, with a standard deviation of 4.94%. Most cases were within ± 1.96 standard deviations.

Discussion

Concentrations of zinc in semen can be detected using the method involving 5-Br-PAPS. At suitable pH levels, zinc reacts with 5-Br-PAPS, creating a chelate with stable colors. The darkness of the mixture's color is proportional to the quantity of zinc in semen. Concentrations of zinc can be measured by the optical density of this mixture.

The researchers proceeded to measure the optical density two times in the sequence 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0 mmol/l, obtaining the average. Ms-Excel was used to draw the linear line.

The linear regression equation is $y = 0.0666x + 1.2026$, zinc concentration is indicated by the independent variables, x , and optical density is the dependent variable, y .

The correlation coefficient of $r=0.9956$, and $0.995 < r < 1$ indicate a strong positive relationship between zinc concentrations and optical density.

• About construction of calibration function

When mixing chemicals, due to subjective or objective reasons, error factors may yield different results between the two tests. In this kit, to minimise these factors and to ensure result stability between the tests, each time chemicals were mixed, the researchers proceeded to build the calibration function.

The calibration function was $y = 0.9977x$, and the correlation relation coefficient was $R^2=0.9995$. Such as computational, quantitative results when using chemical batch after need with coefficient 1, meaning that no additional coefficients. No significant difference in the test results is apparent between the different batches of chemical tests.

The calibration function equation was $y = 0.9977x$, and the correlation coefficient was $R^2=0.9995$.

• Accuracy

In trials, particularly in quantitative testing, numerous error factors affect the test, producing inaccurate results. Therefore, to control these confounding factors, it is necessary to apply precision. Precision results only depend upon the random error factor that is not related to the actual results of the sample. When the precision of the standard deviation is lower, the variance is greater.

Precision is based on three parameters, including repeatability, intermediate precision, and reproducibility. This study only involved experiments that calculated repeatability and intermediate precision; since there is no reference laboratory, it is not possible to calculate the reproducibility.

In this study, the in-house kit has a repeatability with a CV% coefficient of 0.27%, so that the coefficient of variation does not exceed 5%, which indicates that it satisfies the requirements of the analysis.

The calculation of the intermediate yielded, the coefficient of variation of $CV%=0.64\%$. This coefficient of variation is also valid for not more than 5%. Therefore, the process also meets the requirements of the analysis.

Therefore, when the effect of random error elements is the same, the concentration measured under different conditions has a tolerable range.

• About the trueness

The trueness of the method demonstrates that the degree of proximity between the result obtained and the actual value or accepted value is true (μ).

By experimentally testing the trueness, the result obtained is $t_{ex}=2.076$. Additionally, the table indicates that, the t value obtained is 2.262. This means that $t_{ex} < t_t$, and the concentration of zinc measured from this method exert the same effect as

the actual concentration of the sample. The process achieves the required accuracy of an analysis.

A good test kit should have high sensitivity and specificity, which means that the kit has low false positives and false negatives. These are two important criteria for evaluating the quality of an analysis kit.

The kit achieves a specificity of 100% and a sensitivity of 100% as well. It is therefore possible to use this kit to quantify zinc in semen with high reliability.

To reassert the accuracy of the in-house kit, this study continued to compare the results obtained by the kit with those of the standard kit.

Results demonstrated significant correlations between the two kits ($r=0.975$; $p<0.001$) and that, the mean difference between the two methods was 0.0002, equivalent to 4.94%. This difference was not statistically significant.

The chart illustrates that the difference is completely random and independent from the standard scale.

In the kit, proteins were transformed using TCA 370 mmol/l. TCA is a non-poisonous, common, and easy to purchase acid. Transforming proteins before mixing them with color indicators limits the possibility of proteins reacting to the color indicator and produces a mixture of inaccurate color. In this manner, the accuracy of obtained results is considerably improved.

Moreover, the in house test kit uses only simple, low-cost, common, and easy-to-buy chemicals and fewer, chemicals than commercial kits. The IVD kit used in this study is the Zinc 5-Br-PAPS Test (Spain), which is being used widely to measure zinc in semen in laboratories today. This kit also follows the principle of the colorimetric method, but indol is used as an expensive chemical that is difficult to purchase in Vietnam and requires the use of a color-rendering stopper. In addition, the kit must be imported from abroad, through many intermediate stages, resulting in a high cost of testing. This means that this kit is more suitable for use in Vietnam, where determination of zinc concentration in seminal fluid is in high demand; however, the average annual income remains only average.

With the achieved advancements, the researchers hope

their kit can soon be the subject of quantity production, replacing currently imported kits in health institutions.

However, this study has, only tested the kit on a laboratory scale. Assessment on an industrial scale is fundamental for the kit to become subject to quantity production.

Conclusions

Successfully complete the procedure to create the zinc quantification kit using the colorimetric method.

Calibration function: $y = 0.9977x$; correlation coefficients $R^2=0.9995$.

- Accuracy

- Repeatability: $SD=0.004$, coefficient of variation $CV%=0.27%<5%$.

- Intermediate precision: $SD=0.01$, coefficient of variation $CV%=0.64%<5%$.

- Trueness: $t_m=2.076 < t_c=2.262$.

- The sensitivity of the self-mixing kit is 99.05%, and its specificity is 100%.

- The results indicate a strong correlation between the two methods ($r=0.975$; $p<0.001$); the average difference between the two kits is 0.0002, equivalent to 4.94%. The difference has no statistical significance. Most cases yielded zinc concentration data within ± 1.96 standard deviations.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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