Original Article

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Seminal Plasma Examination Test Result Agreements Using the WHO Manual Recommendation: With Semi-Automatic and Automatic Methods

William William, M.D.[1], Agustinus Agustinus, M.D.[2,3], Hartono Kahar, M.D.[4], Pety Narulita, M.D.[2], Ninik Darsini, M.D.[3], Sri Musta'ina, M.Sc.[3], Atika Atika, M.H.[5], Supardi Supardi, M.D.[2]

[1]Department of Medical Biology, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta 14440, Indonesia.

[2]Andrology Study Program, Faculty of Medicine Universitas Airlangga, Surabaya 60132, Indonesia.

[3]Department of Medical Biology, Faculty of Medicine Universitas Airlangga, Surabaya 60132, Indonesia.

[4]Department of Clinical Pathology, Faculty of Medicine Universitas Airlangga, Surabaya 60132, Indonesia.

[5]Department of Public Health and Preventive Medicine, Faculty of Medicine Universitas Airlangga, Surabaya 60132, Indonesia.

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Abstract:

Objective: To analyze the suitability of the measurement results of neutral alpha-glucosidase and fructose in seminal plasma, between the manual method recommended by the World Health Organization (WHO) Sixth Edition and the semi-automatic method, using Biosystems BTS 350 and the automatic method using Biosystems A15.

Material and Methods: This study involved 47 subjects at the Andrology Outpatient Department of Dr. Soetomo General Academic Hospital; from October 2022 and February 2023. The measurement of neutral alpha-glucosidase and fructose levels in seminal plasma was conducted, via three different approaches: the manual method, as recommended by the WHO Sixth Edition, the semi-automatic Biosystems BTS 350, and the automatic Biosystems A15. The manual method recommended by the WHO Sixth Edition was employed as the reference system.

Contact: William William, M.D.
Department of Medical Biology, School of Medicine and Health Sciences,
Atma Jaya Catholic University of Indonesia, Jakarta 14440, Indonesia.
E-mail: william@atmajaya.ac.id

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Results: The average disparity in neutral alpha-glucosidase levels among the manual method, as recommended by the WHO Sixth Edition, compared to the semi-automatic and automatic methods were recorded as: -1.0215 IU/l and -2.3428 IU/l, respectively. Similarly, the fructose levels exhibited differences of -2.1234 mmol/l and 2.2834 mmol/l. Notably, within the method comparisons, 44 values of neutral alpha-glucosidase levels fell within the limit of agreement, while fructose levels showed 44 and 45 values within the agreement limit.

Conclusion: The results obtained from the manual method, as recommended by the WHO Sixth Edition, demonstrated agreement with the measurements of neutral alpha-glucosidase and fructose obtained when using both the semi-automatic and automatic methods.

Keywords: fructose, neutral alpha-glucosidase, plasma seminal biochemistry

Introduction

Infertility refers to the inability of a couple to conceive, even after one year of regular unprotected sexual intercourse[1]. Male factors contribute to approximately half of infertility cases, and semen analysis plays a crucial role in diagnosing these conditions[2]. To gain further insights, additional tests can be conducted to assess biochemical levels in seminal plasma. Specifically, neutral alpha-glucosidase (NAG) and fructose measurements can provide information as to the function of the epididymis and seminal vesicles, respectively[3,4]. Furthermore, analyzing biochemical levels in seminal plasma can help establish specific diagnoses, such as the presence of infection or obstructions within the male reproductive tract[5,6]. Specifically for NAG, the examination of this enzyme can even serve as an early predictor of patency and natural pregnancy after microsurgical vasoepididymostomy[7].

Currently, the widely used method for seminal plasma biochemical examination follows the guidelines outlined in the World Health Organization (WHO) Laboratory Manual for the Examination and Processing of Human Semen, Sixth Edition. This method involves centrifugation and incubation processes, which are time-consuming and require precision[5]. The complexity of this procedure often leads to longer waiting times for patients and potentially impacts the efficiency of doctors.

To address these challenges, there is a need for a fast, practical and cost-effective examination method that provides reliable results. In recent years, semi-automatic and automatic tools have been developed to measure biochemical levels in seminal plasma[8,9]. To assess the suitability of these approaches, this study aimed to compare the results obtained from the manual method, as recommended by the WHO Sixth Edition, with those obtained using semi-automatic and automatic methods. This evaluation will provide valuable insights into the accuracy and efficiency of each method.

Material and Methods

Study design

This research was conducted as an analytical observational study in a laboratory setting. The study population consisted of 47 patients who sought treatment at the Andrology Clinic of Dr. Soetomo General Academic Hospital in Surabaya; from October 2022 to February 2023. This quantity fulfills the minimum requirement of 40 samples, according to the EP09-A3 protocol. Prior to commencing the study, ethical approval was obtained from the Ethics and Research Committee of the hospital: approval code 0480/KEPK/IX/2022.

Patient recruitment

During the research period, individuals seeking fertility treatment at the Andrology Clinic of Dr. Soetomo General Academic Hospital in Surabaya were provided with detailed explanations and given the opportunity to participate voluntarily in the study until the desired sample size was achieved. Prior to enrollment, patients received comprehensive counseling and a thorough explanation of the research procedures, after which they were requested to provide written informed consent.

Seminal plasma collection

Ejaculate samples were obtained from male participants via masturbation, having met the predetermined inclusion and exclusion criteria. To be included in the study, participants were required to abstain from sexual activity for a period ranging from 48 hours to 7 days[5], and their semen volume needed to exceed 0.5 ml. Samples exhibiting hematospermia and leucospermia were excluded. The collected ejaculate samples were placed in a provided clean container and subsequently incubated at 37°C until liquefaction occurred.

Following liquefaction, the samples underwent centrifugation at 3,000 g for 20 minutes to separate the seminal plasma from their cellular components[5]. The resulting seminal plasma was then carefully preserved in a freezer at -20°C until analysis. The content of neutral alpha-glucosidase and fructose in seminal plasma remains stable at this temperature[5].

The manual method recommended by the WHO Sixth Edition

The Manual method, as recommended by the WHO Sixth Edition, was employed as the reference system; due to its standardization and endorsement by the WHO as the method for measuring biochemical levels in seminal plasma.

Principal of neutral alfa-glucosidase activity[5]

Under specified conditions (pH=6.8, temperature=37°C), alpha-glucosidase catalyzes the conversion of the substrate 4 (para)-nitrophenyl-α-D-glucopyranoside (PNPG) to α-D-glucopyranoside and 4-nitro phenol (PNP). The yellow color of the latter product is measured spectrophotometrically at a 405 nm wavelength. As the reaction buffer contains sodium dodecyl sulfate (SDS), the acid form of α-glucosidase (originating from the prostate) is selectively inhibited. This allows specific determination of neutral enzyme activity.

$$\text{PNPG} + \text{alpha-glucosidase} \longrightarrow \text{alfa-D-glucopyranoside} + \text{PNP (yellow)}$$

Principal of fructose measurement[5]

Under the influence of heat and low pH, fructose forms a colored complex with indole, which absorbs light at a wavelength of 470 nm.

$$\text{Fructose} + \text{indole} \xrightarrow{T\uparrow,\ pH\downarrow} \text{colored complex}$$

Semiautomatic method

Prior to quantifying the concentrations of neutral alpha-glucosidase and fructose, the seminal plasma and corresponding reagents were manually mixed. The mixing process was conducted before utilizing the Biosystems BTS 350 instrument (Barcelona, Spain) for the measurement.

Principal of neutral alpha-glucosidase measurement[10]

The neutral alpha-glucosidase enzyme plays a role in the following reactions to produce a colored complex that can be measured using a spectrophotometer.

$$\text{Maltose} \xrightarrow{\alpha\text{-glucosidase}} 2\alpha\text{-D-Glucose}$$

$$\alpha\text{-D-Glucose} + \tfrac{1}{2}O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{Gluconate} + H_2O_2$$

$$2\ H_2O_2 + 4\text{-Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4\ H_2O$$

Principal of fructose measurement[11]

After fructose has gone through the following reaction steps, the NADPH level, which is equivalent to fructose, can be measured using a spectrophotometer.

$$\text{D-Fructose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Fructose-6-phosphate} + \text{ADP}$$

$$\text{Fructose-6-phosphate} \xrightarrow[\text{isomerase}]{\text{Phosphoglucose}} \text{Glucose-6-phosphate}$$

$$\text{Glucose-6-phosphate} + NADP^+ \xrightarrow[\text{dehydrogenase}]{\text{Glucose-6-phosphate}} \text{Gluconate-6-phosphate} + NADPH + H^+$$

Automatic method

In general, the underlying reaction principle employed in the automatic method is comparable to that of the semi-automatic method. However, when utilizing the Biosystems A15 automated instrument (Barcelona, Spain) for measuring neutral alpha-glucosidase and fructose levels, special preparatory steps; such as sample dilution and mixing with reagents, are not necessary. This streamlined process allows for more efficient and convenient measurements via the automated tool.

Statistical analysis

Quantitative data obtained in this study were subjected to analysis using the Statistical Package for the Social Sciences (SPSS) software, version 25.0. The findings of the analysis are depicted in Bland-Altman plots, which provide a visual representation of the results[12].

Results

The patient's characteristics

Patient's characteristics are presented in Table 1.

Table 1 Patient characteristics

characteristics	Value (n=47)
Mean of Age±S.D. (years)	31,64±5,42
Mean of Ejaculatory Abstinence ±S.D.(days)	4,49±1,28
Mean of Ejaculate Volume±S.D. (ml)	2,966±0,7334

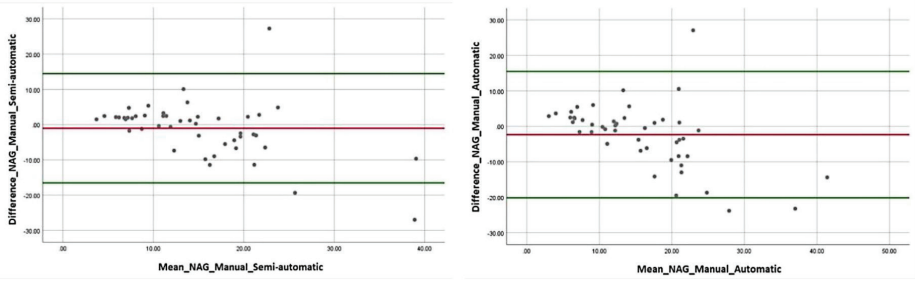
S.D.=standard deviation, ml=mililiter

Comparison of the results of the neutralal pha-glucosidase measurement

The average disparity between the manual method suggested by the WHO Sixth Edition and the semi-automatic method was found to be -1.02 IU/l, with an upper limit of 14.46 IU/l and a lower limit of -16.51 IU/l (Figure 1a). Among the values obtained, 44 fell within the limit of agreement. Similarly, the mean difference between the manual method recommended by the WHO Sixth Edition and the automatic method was determined to be -2.34 IU/l, with an upper limit of 15.47 IU/l and a lower limit of -20.16 IU/l (Figure 1b). Additionally, 44 values aligned within the limit of agreement.

Comparison of the results of fructose measurement

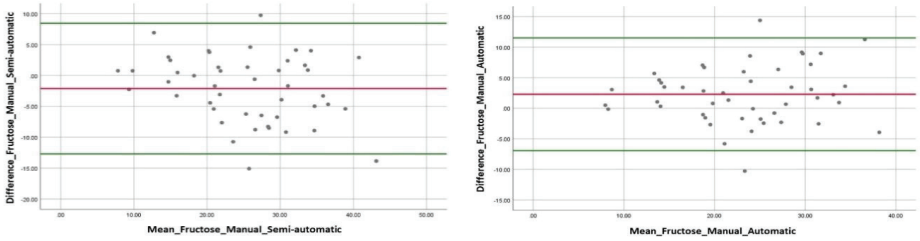
The average discrepancy between the manual method, as recommended by the WHO Sixth Edition, and the semi-automatic method was observed to be -2.12 mmol/l, with an upper limit of 8.45 mmol/l and a lower limit of -12.69 mmol/l (Figure 2a). Among the measured values, 44 fell within the limit of agreement. Similarly, the mean difference between the manual method, as recommended by the WHO Sixth Edition, and the automatic method was found to be 2.28 mmol/l, with an upper limit of 11.49 mmol/l and a lower limit of -6.93 mmol/l (Figure 2b). Furthermore, 45 values were within the limit of agreement.



(a) (b)

NAG=neutral alpha-glucosidase

Figure 1 Bland-Altman plot between the neutral alpha-glucosidase measurement of (a) the manual method, as recommended by the WHO Sixth Edition, and the semi-automatic and (b) the manual method, as recommended by the WHO Sixth Edition, and the automatic method



(a) (b)

Figure 2 Bland-Altman plot between the fructose measurement of (a) the manual method, as recommended by the WHO Sixth Edition, and the semi-automatic and (b) the manual method, as recommended by the WHO Sixth Edition, and the automatic method

Discussion

Patient's characteristics

The subjects in this study had a mean age of 31.64 years. This indicates the average age at which male infertility patients begin seeking treatment and semen analysis is conducted. The mean ejaculatory abstinence period for the subjects in this study was 4.49 days. This aligns with the requirements stated in the WHO Sixth Edition guidelines, which mandate a recommended ejaculatory abstinence period of two to seven days[5]. Meanwhile, the mean ejaculate volume in this study was 2.966 ml. This is consistent with the findings of Levitas et al., who reported a mean ejaculate volume in men aged 30-35 years to be 3.51±1.76 ml, and Kumar et al., who reported a mean ejaculate volume of 2.74±1.48 ml in men within the age group of 29-35 years[13,14].

Comparison of the results of neutral alpha-glucosidase measurement

Evaluation of neutral alpha-glucosidase levels using both semi-automatic and automatic methods yielded satisfactory outcomes compared with the manual method recommended by the WHO Sixth Edition. The mean difference in concentration measurements between the

manual and semi-automatic method was determined to be -1.0215 IU/l (-1.0215 mIU/ml), while between the manual and automatic method, it was 2.3428 IU/l (-2.3428 mIU/ml). These values remain within acceptable clinical ranges when compared to the reference value for neutral alpha-glucosidase in seminal plasma; which is >20 mIU/ejaculate according to WHO guidelines[5]. The Bland-Altman plot revealed that 44 difference sample values fell within the limit of agreement for both comparison methods. This indicated that both semi-automatic and automatic methods accurately measure neutral alpha-glucosidase levels.

Both semi-automatic and automatic methods utilize the kinetic reaction principle to measure neutral alpha-glucosidase levels in seminal plasma, and use glucose as the seminal plasma background[15]. Glucose has been evaluated as an alternative for castanospermine[13]. The reaction between quinoneimine and other compounds can be specific to the analytical method employed. In the presence of hydrogen peroxide, quinoneimine can react with 4-aminoantipyrine and phenol to form a colored complex[16]. The quinoneimine color complex is typically stable and exhibits good light absorption properties, allowing for accurate and sensitive detection[16,17].

A study in stallions demonstrated that during storage at -20°C, the activity of α-glucosidase was approximately 90% of the initial activity during the first week, followed by subsequent decreases of around 10%, 30%, and 40% after 10, 20, and 30 days. After a storage period of 3 months, the α-glucosidase activity became approximately 30-40% of the initial activity[18]. However, due to the focus of this study on instrument suitability, these changes in activity can be disregarded.

In both semi-automatic and automatic methods, the machine assesses the rate of color change in the sample when reacting with the reagent[10]. This differs from the manual method, as recommended by the WHO Sixth Edition, which measures the absorbance of the final color change (endpoint) in the sample after incubation with reagents for 120 minutes[5]. Despite these differences, both reaction principles yielded comparable measurement values. This finding aligns with the study conducted by Lu et al., wherein neutral alpha-glucosidase levels were measured using the semi-automatic method[8]. The researchers reported a positive correlation between the results of the neutral alpha-glucosidase test using manual (glucose oxidation) and semi-automatic methods. However, it is important to note that this present study also included measurements using the automatic method, which was not explored in the aforementioned study.

Comparison of the results of fructose measurement

The assessment of fructose levels, using both semi-automatic and automatic methods, demonstrated favorable outcomes compared to the manual method; as recommended by the WHO Sixth Edition. The mean difference in concentration measurements between the manual and semi-automatic methods was found to be -2.1234 mmol/l, while between the manual and automatic methods, it was 2.2834 mmol/l. Overall, these values do not significantly impact the clinical interpretation when compared to the reference value of fructose in seminal plasma (>8.33 mmol/l)[19]. The Bland-Altman plot further confirms that 44 and 45 difference sample values fell within the limit of agreement for the respective comparison methods.

Unlike the manual method, as recommended by the WHO Sixth Edition, which relies on high temperature and low pH to produce a color change, the measurement of fructose concentration in the semi-automatic and automatic methods utilizes the enzymatic reaction principle and measures the equivalent amount of NADPH molecules formed at the end of the reaction[11,20,21]. Some of these reactions are part of the pentose phosphate pathway[22].

These findings align with the study conducted by De Jong et al., wherein fructose levels in seminal plasma were measured using an automatic method on the Beckman

Coulter AU400[23]. This instrument utilizes the principle of an indirect reaction through glucose as an intermediary, which is similar to the reaction principle employed in both the semi-automatic and automatic methods utilized in this study[10,11,23]. However, it is important to note that the aforementioned study did not include measurements of fructose levels using a semi-automated method; as was performed in this present study.

To the best of the authors' knowledge, this study represents the first attempt to compare the levels of neutral alpha-glucosidase and fructose in seminal plasma using manual, semi-automatic, and automatic methods. However, it is important to acknowledge the limitations of this study. The sample size in this study is relatively small; therefore, a larger sample size is required for future studies. Additionally, several other biochemical parameters in seminal plasma that were not covered in this study could also be further investigated.

Conclusion

The results obtained from the manual method, as recommended by the WHO Sixth Edition, demonstrate agreement with the measurements of neutral alpha-glucosidase and fructose obtained using both the semi-automatic and automatic methods.

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Conflict of interest

The are no potential conflicts of interest to declare.

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