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HbA1c Levels On High Performance Liquid Chromatography (HPLC) And Fluorescence Immunoassay (FIA) Methods

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ABSTRACT
Hemoglobin A1c (HbA1c) is an ideal parameter for the diagnosis and control of diabetes mellitus, reflecting the average blood glucose level through the non-enzymatic reaction between glucose and hemoglobin. Although HPLC is the gold standard of HbA1c measurement, this method is expensive and complex. This study aims to evaluate HbA1c measurement using Fluorescence Immunoassay (FIA), which is faster, simpler, and cost-effective. This study is a comparative study with a cross-sectional design to compare HbA1c levels using HPLC and FIA methods. The sample consisted of 30 patients of Klinik Utama Pramita Surabaya, with venous blood taken using EDTA tubes. Data analysis was performed using paired t-test or Wilcoxon test based on data distribution. Method validation included correlative analysis, sensitivity, specificity, and accuracy using IBM SPSS Statistics 25. The results showed that HPLC and FIA methods produced similar HbA1c distributions. Wilcoxon test showed no significant difference between the two methods (p-value =0.094). The sensitivity of the FIA method was 94.4%, specificity 100%, PPV 100%, NPV 92.9%, and accuracy 96.7. This study shows that the HPLC and FIA methods provide similar results in the measurement of HbA1c levels, with high levels of sensitivity, specificity, PPV, NPV and accuracy. The overall results support the use of the FIA method in the measurement of HbA1c for diabetes diagnosis and management, given its good analytical performance and compatibility with the HPLC method.

Keywords: HbA1c, HPLC, FIA

INTRODUCTION
HbA1c is an optimal parameter for the diagnosis and management of Diabetes mellitus as the concentration of HbA1c is directly proportional to the typical blood glucose level (N. Saqib., 2022). HbA1c is a product of a minor proportion of hemoglobin formed through a non-enzymatic interaction between the aldehyde group of the glucose molecule's open structure and the free amino group at the valine terminus of the hemoglobin beta chain. (Zi-min., 2023). This reaction results in the creation of unstable intermediates and Schiff bases, referred to as labile hemoglobin. Post-Amadori rearrangement, hemoglobin is stabilized in its ketoamine configuration, leading to the formation of HbA1c. (Maesa et al., 2016). Since 2010, HbA1c has been used as a diagnosis of diabetes. Reference methods for HbA1c have been authorized by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). The methods that have been approved include electrospray ionization mass spectrometry and High Performance Liquid Chromatography (HPLC). Additionally, a two-dimensional approach with ultraviolet (UV) detection is employed in conjunction with HPLC and capillary electrophoresis (CE). (Kuna et al., 2018).

HbA1c screening has many analytical approaches. The National Glycohemoglobin Standardization

Program (NGSP) improved the results of HbA1c testing. As a quality improvement measure, the American Diabetes Association (ADA) recommends that laboratories use NGSP-certified methods for HbA1c analysis (Hyeokjun., 2023).

NGSP certified method for HbA1c analysis. In addition, there are also measurements of HbA1c in blood by various laboratory methods. Significant bias was found among the analysis methods. For HbA1c, the total allowable error was 3.0% according to biological variation, and 6.0% according to NGSP (Arne., 2015), (Dildar et al., 2021).

High Performance Liquid Chromatography (HPLC) chemical separation and analysis methods are highly sophisticated and widely used in various fields of science, such as biochemistry, analytical chemistry, pharmacy, and life sciences. (Raad., 2023) More efficient and rapid separation is made possible by the use of high pressure on the liquid mobile phase, which is the basic principle of HPLC (James., 2022). The sample is introduced into a liquid column filled with a suitable stationary phase. The sample components interact with the stationary and mobile phases, resulting in a separation based on differences in affinity to the two phases (Urvi., 2022). After the analyte exits the column, it is detected with an ultraviolet detector. Software known as a data management system converts and records the signal, which is then displayed in the form of a chromatogram (Pulok., 2019), (Böttcher et al., 2019).

Fluorescence Immunoassay (FIA), also referred to as the immunochromatographic test (ICT), rapid diagnostic test (RDT), or lateral flow immunoassay technique (LFIA), has emerged as one of the most efficient analytical platforms for testing strategies due to its minimal infrastructure requirements. (Ge., 2023). FIA is an ICT-based bioanalytical method used to find the target substance. Samples are loaded onto a standalone device, read with a fluorescence Immunoassay analyzer and results are obtained within minutes (Di Nardo et al., 2021).

In the study (Chaila et al., 2022) a comparison of HbA1c data acquired from three analytical procedures (enzymatic, turbidimetric, and capillary electrophoresis) against HPLC indicated that the average values from the four methods were statistically significant, however not clinically meaningful. It may be stated that these three technologies exhibit minimal variability and high correlation relative to HPLC.

The HPLC method is the gold standard method for HbA1c measurement (Priya., 2016). However, this method is expensive, time-consuming, and requires technical skills that are difficult for every laboratory to perform (Robin., 2022). So the author is interested in knowing the measurement of HbA1c using another method, namely the Fluorescence Immunoassay (FIA) method which is more concise, fast and lower cost.

RESEARCH METHOD

This study employs a comparative research design with a cross-sectional approach to evaluate HbA1c levels using High-Performance Liquid Chromatography (HPLC) and Fluorescent Immunoassay (FIA) methods. The cross-sectional design was chosen to compare the two methods simultaneously.

The study population consists of patients from Klinik Utama Pramita Surabaya, with a sample of 30 patients selected based on Roscoe's hypothesis, suggesting a sample size range of 30 to 500 for research. Venous blood samples were collected using EDTA tubes. Participants were patients at Klinik Utama Pramita Surabaya, and consent forms along with plain language statements were provided to ensure informed consent.

Venous blood samples were collected and processed using HPLC and FIA methods for HbA1c measurement. The data were analyzed using comparative and

correlative statistical methods, with HPLC as the gold standard. Descriptive statistics (mean, standard deviation) were calculated. Normality tests determined the appropriate statistical tests: paired t-tests for normally distributed data and Wilcoxon signed-rank tests for non-normally distributed data. Validation included correlative analysis between FIA and HPLC results, sensitivity, and specificity tests using IBM SPSS Statistics 25.

The hypothesis tested were Ho: There is no difference in HbA1c levels between HPLC and FIA methods, and Hi: There is a difference in HbA1c levels between HPLC and FIA methods. Ethical clearance was obtained on April 01, 2024, meeting the seven WHO 2011 standards: Social Value, Scientific Value, Fair Distribution of Burden and Benefits, Risk, Inducement/Exploitation, Confidentiality and Privacy, and Informed Consent, as per the CIOMS 2016 Guidelines.

RESULT AND DISCUSSION

The study analyzed HbA1c levels using HPLC and FIA methods from 30 samples (13 males and 17 females). For the HPLC method, the HbA1c values ranged from 4.0% to 14.2% with a mean of 7.2% and a standard deviation of 2.2%. For the FIA method, the values ranged from 4.3% to 13.8% with a mean of 7.1% and a standard deviation of 2.0%.

Table 1. Comparison table of HPLC and FIA method results

Method	∑ Sample	∑ Male	∑ Female	Lowest Value	Highest Value	Average	Standard Deviation
HPLC	30	13	17	4.0%	14.2%	7.2%	2.2%
FIA	30	13	17	4.3%	13.8%	7.1%	2.0%

In terms of classification, 20.0% of the HPLC samples were normal, 20.0% were prediabetes, and 60.0% were diabetes. For FIA, 26.7% were normal, 16.7% were prediabetes, and 56.7% were diabetes.

Table 2. Comparison of HbA1c Classification of HPLC and FIA Methods

Classification*	HPLC method		FIA method	
	∑ Sample	Percentage	∑ Sample	Percentage
Normal (≤ 5.6)	6	20,0%	8	26,7%
Prediabetes (5.7 - 6.4)	6	20,0%	5	16,7%
Diabetes (≥ 6.5)	18	60,0%	17	56,7%
TOTAL	30	100,0%	30	100,0%

Data Analysis

Normality tests using the Shapiro-Wilk method indicated that both HPLC and FIA data did not follow a normal distribution ($p < 0.05$). Therefore, a non-parametric Wilcoxon test was used for comparative analysis.

Wilcoxon Test

The Wilcoxon test showed no significant difference in HbA1c levels between HPLC and FIA methods ($p = 0.094$), indicating that both methods provide comparable results.

Sensitivity, Specificity, PPV, NPV, and Accuracy

These evaluations are essential for assessing the performance and reli-ability of diagnostic tests. According to recommendations (Younes, Al Ghwairi, Da'As, et al., 2023), patients with HbA1c

levels ≥6.5% re-ceive intensive treatment. To evaluate clinical application, we compared HbA1c results from the FIA method with those from the reference method (HPLC) using a cutoff of 6.5% for HbA1c

Table 3. Cross-tabulation between HPLC and FIA methods

***Cut off*: ≥6.5**		**HPLC**	
		Positive	Negative
FIA	Positive	17	0
	Negative	1	12

After calculating from the table above, the values of true positive, false negative, true negative, and false positive are then entered into the calculation so that the sensitivity value is 94.4%, specificity 100%, PPV 100%, NPV 92.9%, and accuracy 96.7%.

DISCUSSION

The aim of this study was to confirm the differences in HbA1c measurements between the High-Performance Liquid Chromatography (HPLC) method using the Bio-Rad D10 instrument and the Fluores-cence Immunoassay (FIA) method using the SD Biosensor F200 in-strument. The analytical performance was tested according to prede-termined criteria, including comparison tests, sensitivity, specificity, accuracy, and linearity.

We compared the analytical performance of two different methods for measuring HbA1c. Given the role of HbA1c as a recommended bi-omarker for diabetes diagnosis (diabetes, ≥6.5%) and diabetes compli-cation management (treatment target, <7%), tests with high analytical precision are needed to differentiate between the upper limit of the non-diabetic range (<5.7%), pre-diabetes (5.7-6.4%), and the aforemen-tioned targets (Perkeni, 2021).

Based on the initial study data, it appears that the HPLC and FIA methods for measuring Hemoglobin A1c (HbA1c) yield similar results. The means and standard deviations of both methods are nearly identi-cal, indicating that both methods have comparable accuracy in HbA1c measurement. In the comparative test using the Wilcoxon test, a signifi-cance value of 0.094 was obtained, indicating no significant difference in HbA1c levels between the HPLC and FIA methods. This suggests that the FIA method can be reliably used alongside HPLC for HbA1c measurement in clinical settings.

Our findings align with those of Dildar et al. (2021), who found min-imal differences between HPLC and Particle Enhanced Immunoturbi-dimetry (PEIT) methods in HbA1c analysis, with both methods show-ing strong correlation. Similarly, Khan et al. (2012) found no significant differences between HPLC and immunological methods for HbA1c measurements up to a level of 10.0, further supporting the reliability of immunoassay methods.

The calculated sensitivity, specificity, PPV, NPV, and accuracy for the FIA method were 94.4%, 100%, 100%, 92.9%, and 96.7%, respec-tively. This high performance indicates that the FIA method is highly reliable for diagnosing diabetes, correctly identifying diabetic and non-diabetic patients with high accuracy. These results are consistent with those of Younes et al. (2023), who reported high sensitivity and speci-ficity for the Finecare™ HbA1c Rapid Test using the LFIA method compared to the Cobas Pro c503 reference method.

Novel Insights and Study Implications

This study introduces a new approach to HbA1c measurement by val-idating the FIA method against the established HPLC method. Our findings suggest that FIA could be a viable alternative to HPLC, offer-ing similar accuracy with potentially faster and more cost-effective re-sults. This

could have significant implications for clinical practice, par-ticularly in resource-limited settings where access to HPLC instruments is limited.

Study Limitations

Despite the promising results, our study has several limitations. The sample size of 30 patients may not be representative of the broader population, and further research with larger sample sizes is needed to confirm these findings. Additionally, our study was conducted at a single clinic, and multicenter studies would help validate the generali-zability of the results.

Future research should also explore the long-term reliability of FIA in various clinical scenarios and investigate potential discrepancies in HbA1c measurements at higher levels, as suggested by previous studies. Addressing these limitations will help establish FIA as a standard method for HbA1c measurement in clinical chemistry.

CONCLUSION AND RECOMMENDATION

Conclusion: In this study, we investigated the levels of HbA1c using two different methods: High Performance Liquid Chromatography (HPLC) and Fluorescence Immunoassay (FIA). Our findings reveal that both methods yield comparable results, with no significant difference between the measured HbA1c levels. Specifically, the FIA method demonstrated excellent performance, exhibiting high sensitivity, speci-ficity, PPV, NPV, and overall accuracy. Therefore, we can confidently conclude that the research question regarding the comparability of HbA1c measurements between HPLC and FIA methods has been an-swered.

Recommendations for Future Research:

1. **Implications**: Our study has important implications for clinical practice. Given the comparable performance of the FIA method and its cost-effectiveness, practitioners should consider adopting it as an alternative to HPLC for HbA1c measurement in diabetes management.
2. **Further Investigation**: While our findings are promising, further research is warranted. Future studies could validate these results in different patient populations, including those with extreme Hb levels or hemoglobin variants. Additionally, exploring the FIA method's performance in various clinical contexts would enhance its applicability.

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