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Comparison of Glycated Hemoglobin Measurement with High Performance Liquid Chromatography and Immunoturbidimetry

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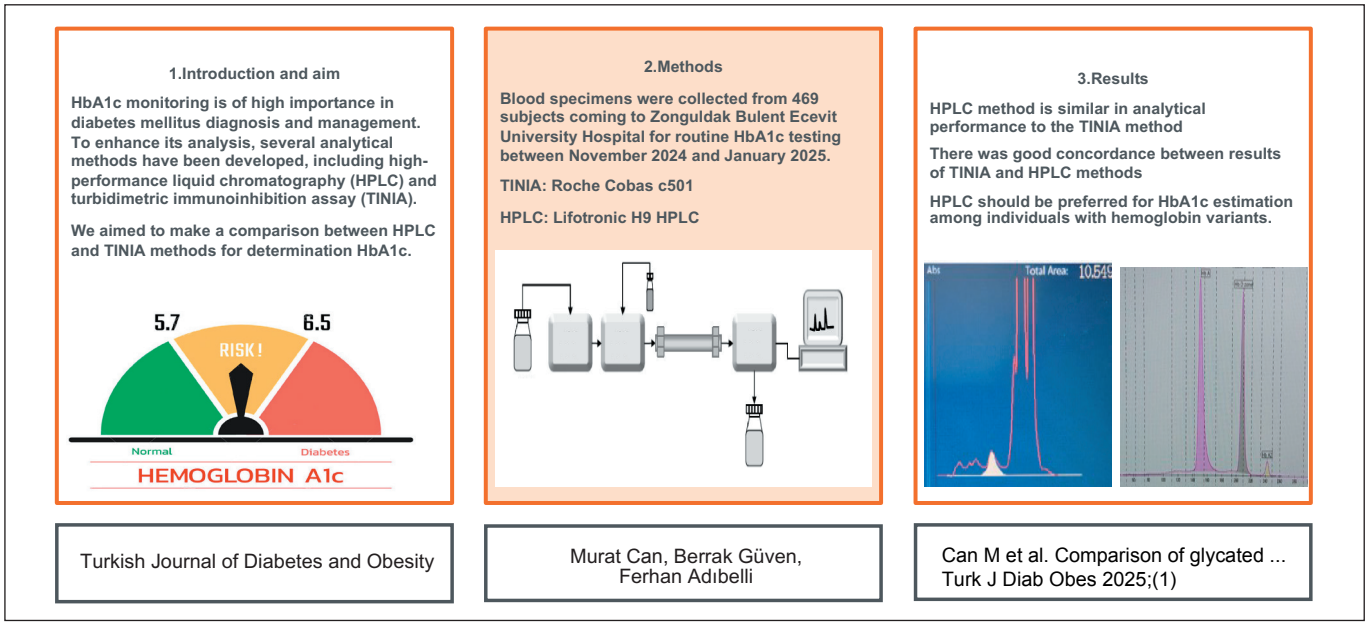
GRAPHICAL ABSTRACT



ABSTRACT

Aim: American Diabetes Association (ADA) have endorsed the use of HbA1c for diagnosing diabetes and for screening individuals at elevated risk for the disease. In our study, we aimed to evaluate the Lifotronic H9 High-Performance Liquid Chromatography (HPLC) Analyzer for the routine measurement of HbA1c in comparison to the Roche Cobas c501 method, which employs turbidimetric immuno-inhibition.

Material and Methods: Blood specimens were collected from four hundred sixty-nine subjects coming to Zonguldak Bulent Ecevit University Hospital for routine HbA1c testing between November 2024 and January 2025. Among them, two hundred ninety-seven (63.3%) were identified as female, while one hundred seventy-two (36.7%) were male. Method comparison was conducted using linear regression and Bland-Altman plots.

Results: HbA1c values obtained through the HPLC method were found to be greater than those obtained via the turbidimetric immuno-inhibition method, with measurements of 6.78±1.76 and 6.70±1.39, respectively. However, this difference was not statistically significant (p=0.461). The Passing–Bablok regression analysis indicated a slope of 0.90 (confidence interval (CI) 0.89-0.91) and an intercept of 0.59 (CI 0.50–0.67) for HbA1c, alongside a notably high correlation coefficient (r=0.974). Bland-Altman analysis conducted on repeated

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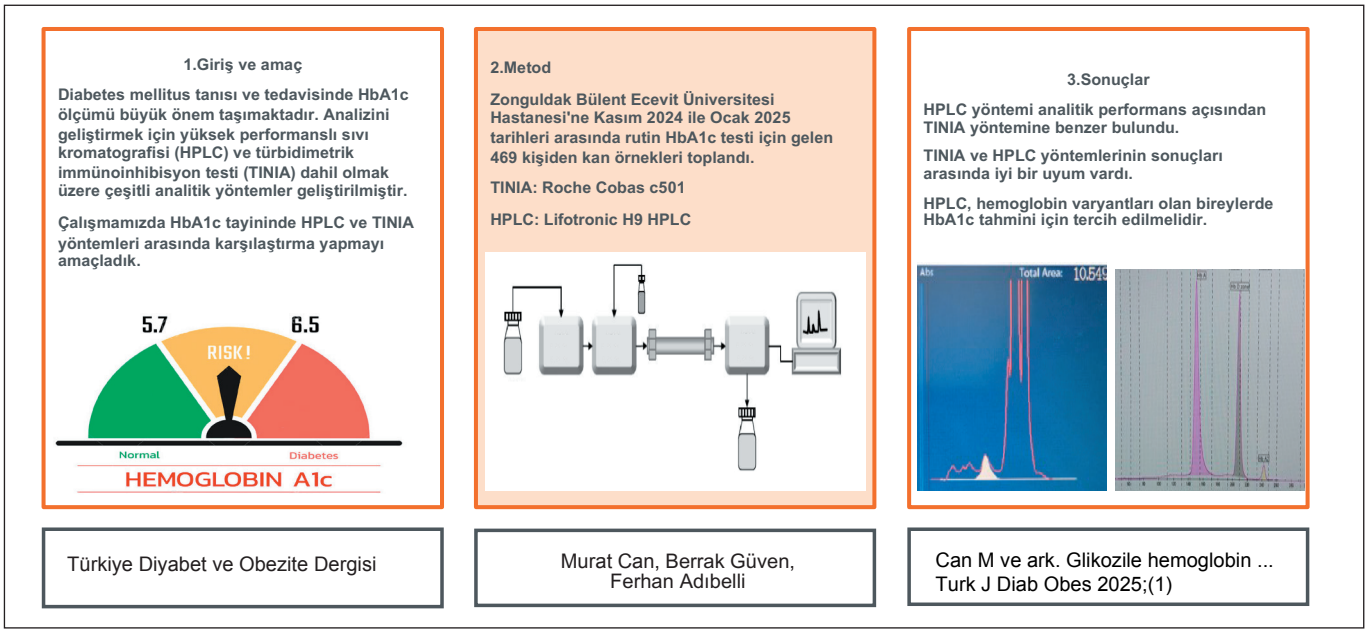
measurements across all patients indicated a strong level of agreement, characterized by a bias of +0.08% (CI 0.05–0.10). The analysis revealed that the two standard deviations (2 SD) established the lower limit at -0.47 (CI -0.52 to -0.43) and the upper limit at 0.63 (CI 0.59 to 0.68).

Conclusion: The quality and performance of HPLC and turbidimetric immuno-inhibition methods are comparable and exhibit excellent correlation. Clinicians and clinical biochemists should collaborate on the management of diabetes mellitus, focusing on diagnosis, treatment, and follow-up. This collaboration is essential for effective patient care.

Keywords: *HbA1c, Turbidimetric immuno-inhibition (TINIA), High-performance liquid chromatography (HPLC), Lifotronic H9, Roche cobas c501*

Glikozile Hemoglobin Ölçümünün Yüksek Performanslı Sıvı Kromatografisi ve İmmünturbidimetri ile Karşılaştırılması

GRAFİKSEL ÖZET



ÖZ

Amaç: Amerikan Diyabet Derneği (ADA), diyabetin teşhisinde ve hastalık açısından yüksek risk altındaki kişilerin taranmasında HbA1c'nin kullanılmasını onaylamıştır. Çalışmamızda HbA1c'nin rutin ölçümü için Lifotronic H9 Yüksek Performanslı Sıvı Kromatografi (HPLC) analizörünü türbidimetrik immün inhibisyon kullanan Roche Cobas c501 yöntemiyle karşılaştırmalı olarak değerlendirmeyi amaçladık.

Gereç ve Yöntemler: Zonguldak Bülent Ecevit Üniversitesi Hastanesi'ne Kasım 2024 ile Ocak 2025 tarihleri arasında rutin HbA1c testi için gelen dört yüz altmış dokuz kişiden kan örnekleri toplandı. Bunlardan 297'sinin (%63,3) kadın, 172'sinin (%36,7) erkek olduğu belirlendi. Yöntem karşılaştırması doğrusal regresyon ve Bland-Altman grafikleri ile yapıldı.

Bulgular: HPLC ve türbidimetrik immün inhibisyon yöntemiyle elde edilen HbA1c değerleri sırasıyla 6.78±1.76 ve 6.70±1.39 elde edildi. Ancak bu fark istatistiksel olarak anlamlı değildi (p=0.461). Passing-Bablok regresyon analizi, oldukça yüksek bir korelasyon katsayısının (r=0.974) yanı sıra, HbA1c için 0.90'lık bir eğim (güven aralığı (CI) 0.89-0.91) ve 0.59'luk bir kesişme noktası (CI 0.50-0.67) gösterdi. Tüm hastalarda tekrarlanan ölçümler üzerinde gerçekleştirilen Bland-Altman analizi, +%0.08'lik bir sapma (CI 0.05-0.10) ile karakterize edilen güçlü düzeyde bir anlaşmaya işaret etti. Analiz, iki standart sapmanın (2 SD) alt sınırı -0.47 (CI -0.52 ila -0.43) ve üst sınırı 0.63 (CI 0.59 ila 0.68) olarak belirlediğini ortaya çıkardı.

Sonuç: HPLC ve türbidimetrik immün inhibisyon yöntemlerinin kalitesi ve performansı karşılaştırılabilir ve mükemmel bir korelasyon gösterdi. Klinisyenler ve klinik biyokimyacılar, diyabetin teşhisi, tedavisi ve takibi konusunda işbirliği yapmalıdır. Bu işbirliği etkili hasta bakımı için gereklidir.

Anahtar Sözcükler: *HbA1c, Türbidimetrik immün inhibisyon (TINIA), Yüksek performanslı sıvı kromatografisi (HPLC), Lifotronic H9, Roche cobas c501*

INTRODUCTION

The International Diabetes Federation reported that in 2021, approximately 537 million people, representing 10.5% of the global population aged between 20 and 79 years, were living with diabetes. Notably, 44.7% of these individuals were unaware of their condition (1). Diabetes mellitus is defined as a metabolic disorder with diverse causes, marked by the persistent hyperglycemia and disruptions in the metabolism of carbohydrates, fats, and proteins, which arise from deficiencies in insulin secretion, insulin action, or both (2). Given the challenges associated with measuring fasting plasma glucose and the variability of glucose levels on a daily basis, the international committee and the American Diabetes Association (ADA) have endorsed the use of HbA1c for diagnosing diabetes and for screening individuals at elevated risk for the disease (3).

HbA1c constitutes the predominant form of glycohemoglobin, which is synthesized through a two-step process involving the nonenzymatic glycation of the N-terminal amino group of valine located in the β chain of hemoglobin (4). The initial step is reversible and leads to the formation of an aldimide, commonly referred to as a Schiff base. Subsequently, an Amadori rearrangement occurs, leading to the formation of a stable ketoamine, which plays a crucial role in subsequent biochemical processes. The transition to stable HbA1c is constrained by the lifespan of erythrocytes, which ranges from approximately 100 to 120 days, thereby providing an average representation of blood glucose levels over the prior 6 to 8 weeks (5). HbA1c results are universally reported in both IFCC units (mmol/mol) and derived NGSP units (percentage of total hemoglobin).

HbA1c monitoring is of high importance in diabetes diagnosis and management. To enhance the biochemical analysis, several methods have been developed, including high-performance liquid chromatography (HPLC) and turbidimetric immunoinhibition assay (TINIA). The ion exchange method of HPLC relies on the electrical charge of the globin portion of hemoglobin. In TINIA, the HbA1c antibody specifically interacts with HbA1c, allowing for quantification through a turbidimeter (6). While the turbidimetric approach is more easily integrated into biochemical instruments, is less expensive, and provides quicker results compared to HPLC, the latter is recognized for its long-term reliability, precision, and consistency (7). The Diabetes Control and Complications Trial (DCCT) and the National Glycohemoglobin Standardization Program (NGSP) have established HPLC as the standard reference method (8-9).

In this research, our objective was to evaluate the Lifotronic H9 HPLC Analyzer for the routine measurement of HbA1c in comparison to the Roche Cobas c501 method, which employs turbidimetric immuno-inhibition.

MATERIALS and METHODS

Samples

Blood specimens were collected from four hundred sixty-nine subjects coming to Zonguldak Bulent Ecevit University Hospital for routine HbA1c testing between November 2024 and January 2025. Among them, two hundred ninety-seven (63.3%) were identified as female, while one hundred seventy-two (36.7%) were male. The age range of the participants spanned from two to ninety-four years, with a mean age of 54.3 years and a standard deviation of 17.8 years. This research received approval from the Clinical Researchers Ethics Committee of Zonguldak Bulent Ecevit University (Date: November 20, 2024, Decision No: 2024/26). All procedures were conducted in compliance with ethical standards and the principles outlined in the Declaration of Helsinki.

Venous blood samples were collected from each participant by a single specialist. The collection was done following standardized protocols. The collection utilized Vacuette 21x1 1/2”-gauge sterile needles (Greiner Bio-One GmbH, Kremsmünster, Austria) along with adapters specifically designed for vacuum blood collection tubes containing K2 EDTA (Vacusera, Disera A.S., Izmir, Turkey). Following the blood collection, the tubes were inverted between eight to ten times in accordance with the manufacturer’s guidelines. Each sample was analyzed once using both systems, and the results were documented. Given that both methodologies incorporated automated dilution and hemolysis processes, no additional pretreatment was necessary.

The imprecision of the methods was tested by repeated measurements using two levels of control materials with mean HbA1c concentrations of 5.7% and 10.2%, expressed as the coefficient of variation (CV) for within-run and between-day measurements. Each control material was assayed 20 times within a single day to determine the within-run CV and 20 times on consecutive days to evaluate the between-day CV for these methods.

Tina-quant Hemoglobin A1c Gen.3

HbA1c levels were assessed using the TINIA for hemolyzed whole blood, employing Roche Diagnostics commercial kits on the Cobas c501 (Roche Diagnostics, Mannheim, Germany) analyzer. In this procedure, tetradecyltrimethylammonium bromide served as a detergent within the hemolyzing reagent, effectively mitigating interference from leukocytes, thus eliminating the need for sample pretreatment to remove labile HbA1c. The hemolysis of samples was conducted at a dilution ratio of 1/90. Follow-

ing dilution and mixing with the reagent on an automated analyzer, the HbA1c present in the sample interacts with anti-HbA1c antibodies, resulting in the formation of soluble antigen-antibody complexes during the preincubation phase. Subsequently, polyhaptens are introduced to react with excess anti-HbA1c antibodies, leading to the creation of an insoluble antibody-polyhapten complex, which can be quantified turbidimetrically at wavelengths of 340 nm and 660 nm. Concurrently, hemoglobin levels in the hemolyzed sample are measured bichromatically at 376 and 660 nm during the preincubation phase. After determining their respective concentrations, the percentage of HbA1c is calculated using the formula (HbA1c/Hemoglobin) x 100. The measurable range for % HbA1c is reported to be between 4.2% and 20.1% at a typical hemoglobin concentration of 13.2 g/dL.

Lifotronic Glycated Hemoglobin

The Lifotronic H9 Hemoglobin Analyzer (Lifotronic Technology, Shenzhen, China) is an advanced fully automated high-performance liquid chromatography (HPLC) device designed for the determination of HbA1c levels. This analyzer operates in accordance with the manufacturer's guidelines, utilizing original commercial kits to analyze fresh human whole blood samples. The underlying assay principle employs ion-exchange chromatography to effectively separate various hemoglobin molecules, which are subsequently detected using a visible light detector at wavelengths of 415 and 500 nm. The system automatically samples five microliters of whole blood, which is then hemolyzed and injected into the HPLC column. The analyzer boasts a throughput of 27 samples per hour, with each sample processed in approximately 130 seconds. To facilitate the elution of hemoglobin species from the HPLC column, three elution buffers with varying ionic strengths are employed. The standardization of the results is aligned with the DCCT and the IFCC HbA1c reference methods, as recognized by the NGSP.

Statistical Methods

Statistical analyses were conducted utilizing SPSS Statistics Version 19.0 and MedCalc software packages. The Kolmogorov-Smirnov test was employed to confirm the normality of distribution for continuous variables. To compare means between groups, two-sample t-tests were applied, while the Mann-Whitney U test was used to evaluate differences in medians. Pearson correlation coefficients and linear regression equations were computed to assess the linear relationships among the parameters. The Bland-Altman test was implemented to determine the agreement between HbA1c results, with 95% confidence interval limits of agreement. Additionally, Cohen's kappa coefficient (κ) was calculated to measure the concordance between the methods, with the kappa values categorized as follows: poor (0-0.21), fair (0.21-0.40), moderate (0.40-0.60), good (0.61-0.80), and very good (0.81-1.00) agreement (10). The sample size was calculated using G Power 3.1.9.4 software (Franz Foul, Kiel College of Applied Sciences, Kiel, Germany). The effect size was 0.80 (large), with a p-value of 0.05, a power of 95%, and a total sample size of at least 74 subjects.

RESULTS

HbA1c values obtained through the HPLC method were found to be greater than those obtained via the TINIA method, with measurements of 6.78±1.76 and 6.70±1.39, respectively. However, this difference was not statistically significant (p=0.461). The overall and average HbA1c values from the study are illustrated in Figure 1.

A comparative analysis of the Lifotronic H9 Hemoglobin HPLC and the Cobas c501 TINIA revealed a strong agreement in the results derived from 469 whole blood samples. The Passing-Bablok regression analysis indicated a slope of 0.90 (confidence interval (CI) 0.89-0.91) and an intercept of 0.59 (CI 0.50-0.67) for HbA1c, alongside a notably high correlation coefficient (r=0.974) (Figure 2).

Bland-Altman analysis conducted on repeated measurements across all patients indicated a strong level of agreement, characterized by a bias of +0.08% (CI 0.05-0.10). The analysis revealed that the two standard deviations (2 SD) established the lower limit at -0.47 (CI -0.52 to -0.43) and the upper limit at 0.63 (CI 0.59 to 0.68) (Figure 3). Furthermore, the kappa coefficient assessing the correlation between the Lifotronic H9 Hemoglobin HPLC and the Cobas c501 TINIA for HbA1c was found to be excellent, with a value of κ = 0.88 (CI 0.87 to 0.89).

Within-run and total the coefficients of variation (CV) are summarized in Table 1. When the data obtained from the

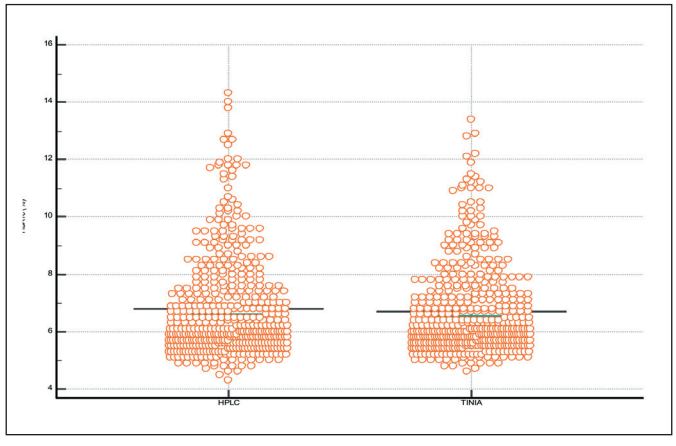


Figure 1: HbA1c values of the patients

Table 1. Precision study of TINIA and HPLC

%HbA1c, n=20		Within-Run			Between-Day		
	Method	Mean (%)	SD (%)	CV (%)	Mean (%)	SD (%)	CV (%)
Control 1	TINIA	5.82	0.08	1.37	5.95	0.14	2.35
	HPLC	5.79	0.06	1.03	5.73	0.13	2.26
Control 2	TINIA	10.26	0.09	0.87	10.40	0.25	2.40
	HPLC	10.19	0.05	0.49	10.37	0.24	2.33

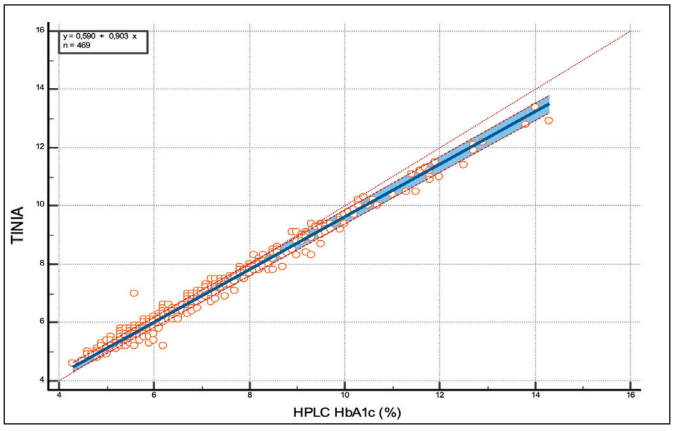


Figure 2: A comparative analysis of HbA1c levels

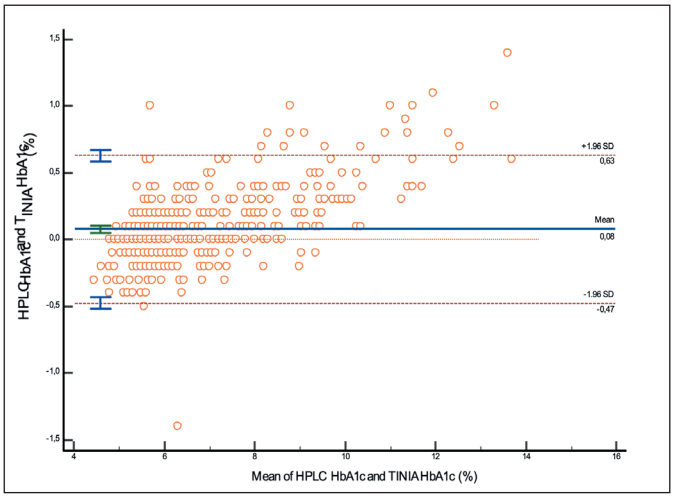


Figure 3: The means, mean differences, and agreement limits (bias ± 2 SD) of HbA1c results according to Bland-Altman analyses

control level 1 and 2 were examined, the CV of within-run and between-day for HPLC method was lower than TINIA method, respectively.

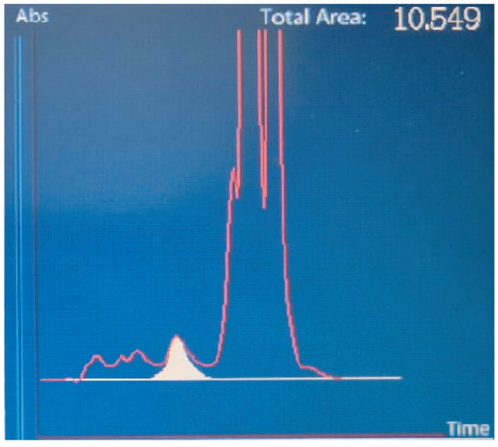
DISCUSSION

HbA1c plays a pivotal role in the management of diabetes mellitus, which necessitates a reliable and efficient method for its analysis. The performance characteristics of two different methods have been analyzed and compared.

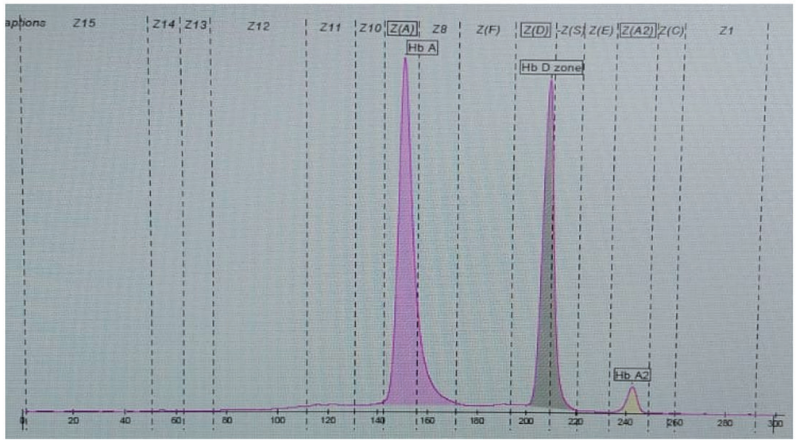
Dhingra et al. compared HbA1c values obtained using the Roche C6000 third-generation TINIA and Bio-Rad D10 HPLC methods among non-dialysis CKD patients, revealing a very strong positive correlation between TINIA and HPLC (r = 0.928). The agreement between the methods was substantial (Cohen's kappa 0.657; $p < 0.0001$) (11). Gilani et al. measured 100 patients on an ADVIA 1800 with third generation TINIA and the Bio-Rad Variant II Turbo system by HPLC, finding that TINIA correlated well with HPLC (r = 0.996) (12). Altawallbeh et al. compared the Roche c501 third generation TINIA method with the ADAMS HA-8180V and Tosoh HPLC instruments for 100 whole blood samples; they found a very high correlation coefficient between TINIA and ADAMS HA-8180V HPLC (r = 0.997) and between TINIA and TOSOH HPLC (r = 0.998) (13). Genc et al. compared the cation-exchange method used by the Arkray Adams HA-8160 HPLC analyzer with TINIA second (2917 patients) and third generation (103 patients) assays. They found that these methods correlated well with each other: the correlation between HPLC and second generation TINIA was represented by the equation ($y = 1.091x - 0.363$; $r^2 = 0.96$), while the correlation between HPLC and third generation TINIA was represented by ($y = 0.96x + 0.02$; $r^2 = 0.98$) (14). The TINIA third-generation HbA1c assay is the same as the second-generation assay, except that a detergent is added to the reagents to improve accuracy. Our results are similar to previous studies, which found that both the HPLC and third-generation TINIA methods yielded comparable results.

The estimated mean difference between the methods was +0.08% (CI 0.05-0.10). Our comparison data showed that 20 out of 469 HbA1c results were within ±5% between HPLC and TINIA, thereby meeting the 2019 NGSP certification criterion. Furthermore, the kappa coefficient was found to be excellent, with a value of $\varkappa = 0.88$ (CI 0.87 to 0.89). These results are consistent with published studies, which demonstrate that the HPLC method is similar in analytical performance to the TINIA method (11-13).

The anti-HbA1c antibodies utilized in the TINIA method, approved by the International Federation of Clinical Chemistry (IFCC), show no cross-reactivity with HbA0, HbA1a,



4.8% HbA1c in HPLC analysis with Hb Variant Alarm



Further Capillary Electrophoresis analysis of this patient with HbD

Figure 4: Representative chromatogram of HbD variant patient

HbA1b, acetylated Hb, carbamylated Hb, glycated albumin, or labile HbA1c (15). Importantly, the immuno-inhibition method is unaffected by HbF, HbS, HbD, HbA2, and carbamylated hemoglobin (16). The Lifotronic H9 Hemoglobin HPLC analyzer provides a graph of each run, which should be reviewed for the presence of a hemoglobin variant window before releasing the reports. In the comparison study, one patient triggered a variant alarm; when we performed capillary electrophoresis on this patient, we found 39.4% Hb D (Figure 4). This patient had 4.8% in HPLC, and when we measured it with TINIA, this value was 5.8%. As a matter of principle, care must be taken when interpreting HbA1c results from patients with hemoglobin variants, as these variants can affect the accuracy of the results. Whenever it is suspected that the presence of an Hb variant affects the correlation between the HbA1c value and glycemic control, HbA1c must not be used for the diagnosis of diabetes mellitus.

The data obtained from our samples show no clinically significant difference between the HPLC and TINIA methods. Both methods satisfied the recommended precision (CV%) for HbA1c assays, which is <2.5%, as specified by the IFCC; however, the HPLC method was more precise than the TINIA method, with the lowest within-run and total CV values (17). These results are consistent with published studies, which demonstrate that the HPLC method is similar in analytical performance to other HbA1c methods, including the TINIA method CV (12,18).

In conclusion the quality and performance of HPLC and TINIA are comparable and exhibit excellent correlation. Depending on the method and technique used, hemoglobin variants could interfere with HbA1c analysis in various ways, a significant portion of which may be asymptomatic. HPLC is superior to TINIA because it directly detects the HbA1c peak when chromatograms are abnormal. Thus, HPLC should be preferred for HbA1c estimation among individuals with hemoglobin variants. As a result, we reported the first measurement of the compatibility of the Lifotronic H9 HPLC system in terms of HbA1c levels using TINIA. Clinicians and clinical biochemists should collaborate on the management of diabetes mellitus, focusing on diagnosis, treatment, and follow-up. This collaboration is essential for effective patient care.

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The Authors Contributions
Conceptualization: **Murat Can, Berrak Güven,** Methodology: **Murat Can, Berrak Güven,** Formal analysis and investigation: **Murat Can, Berrak Güven, Ferhan Adıbelli,** Writing - original draft preparation: **Murat Can, Berrak Güven,** Writing - review and editing: **Murat Can, Berrak Güven**

Conflict of Interest
The authors state that they have no conflicts of interest to disclose.

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Ethics Committee Approval
Ethics committee approval (Date: November 20, 2024, Decision No: 2024/26) was obtained from Clinical Researchers Ethics Committee of Zonguldak Bulent Ecevit University.

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