Original Article

Evaluation of Glycated Hemoglobin Results in Different Anticoagulant Materials and Methods

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Abstract

Background: Samples for glycated hemoglobin (HbA1c) measurements should be collected in ethylenediaminetetraacetic acid (EDTA) tubes, and only a few studies were available for the assessment of its measurements by another anticoagulant tube. There are different methods with different principles for the measurement of HbA1c in addition to high-performance liquid chromatography (HPLC) standard method, and one of these methods is ion exchange chromatography (IEC) which depends on the charge differences principle. **Aim of the study:** The aim is to compare the effect of different anticoagulant additives (EDTA, lithium heparin, and sodium citrate) on HbA1c value and to compare the results of HbA1c obtained by the IEC method with those by the HPLC method. **Materials and Methods:** This case methodology study investigated the effect of different anticoagulant additives on HbA1c values on 40 diabetic patients and compared the results of HbA1c values between HPLC method and IEC method on another 40 diabetic patients. **Results:** The study showed that there were non-significant differences in HbA1c mean values among different types of these anticoagulant materials and non-significant differences in glycated hemoglobin mean values between the methods of HPLC and IEC, and sensitivity and specificity for these techniques were 97.3% and 100% and 97.1% and 100%, respectively. **Conclusion:** This study showed that HbA1c can be tested with lithium heparin or sodium citrate as an alternative to EDTA tube, and the IEC method can be used as one of the main methods for the assessment of HbA1c.

Keywords: Anticoagulants additives, diabetes mellitus, glycated hemoglobin, high-performance liquid chromatography, ion exchange chromatography

Introduction

Diabetes mellitus (DM) is a metabolic syndrome characterized by elevated blood glucose resulting from defective insulin secretion, action, or both. The chronic elevation in blood glucose in diabetes is linked to long-term disruption, malfunction, and loss of various organs, including the kidneys, eyes, nerves, heart, and vessels.[1] The vast majority of patients with DM belongs to one of the two main types: DM type 1, which is linked to insulin deficiency, or DM type 2, which is linked to peripheral tissues resistance for insulin action. Additionally, women who develop diabetes during pregnancy are known as having gestational diabetes, and there are also other specific types of diabetes.[2]

Diabetes could be diagnosed using one of the following four methods[1]: HbA1c equal to or more than 6.5% using a National Glycohemoglobin Standardization Program-Certified Method (NGSP),[2] a fasting blood glucose equal to or more than 126 mg/dL (7.0 mmol/L)[3] or an oral glucose tolerance testing at 2 h after load (75 g of glucose) with blood glucose level equal to or more than 200 mg/dL (11.1 mmol/L),[4] and diabetes clinical symptoms plus a random blood sugar value equal to or more than 200 mg/dL (11.1 mmol/L), each of them should be confirmed by using one of the first three methods on a subsequent day.[3]

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The term glycated hemoglobin refers to a hemoglobin compound formed when glucose (a reducing sugar) interacts with the amino group of hemoglobin (the globin part). Glycation is the process of addition of a sugar residue to amino part of proteins by non-enzymatic way. Human hemoglobin of adult (Hb) made up normally from HbA (97%), HbA2 (2.5%), and HbF (0.5%).[4]

Glycated hemoglobin (HbA1c) is mainly used in the following clinical applications: (A) *Diabetes monitoring*: HbA1c is a more effective form of tracking long-term diabetes control than random blood glucose. It provides a glycemic index along the whole lifespan of the red blood cells which is 120 days.[5] It has been shown that lowering HbA1c to less than 7% reduces retinopathy, neuropathy, and microvascular complications related to diabetes. Therefore, the target level of HbA1c for adults should generally be lower than 7%.[3] (B) *Diabetes diagnosis*: The American Diabetes Association (ADA) in 2010 recommended glycated hemoglobin (HbA1c) level equal to or more than 6.5% (48 mmol/mol) for diagnosis of diabetes and 5.7–6.4% (39–46 mmol/mol) for identifying people with high risk for development of DM in the future (known as prediabetes) in 2010.[6]

The majority of commercial HbA1c testing kits require samples to be obtained in an anticoagulant tube called ethylenediaminetetraacetic acid (EDTA). The main anticoagulant additives used routinely are heparin, EDTA, and citrates. EDTA tubes mainly used for hemocytometry, heparin is preferred anticoagulant additive in clinical chemistry, whereas sodium citrate is used for testing coagulation. These anticoagulants are available in different forms as powder, solids, and crystallized or may be as lyophilized liquid.[7]

EDTA is a good chelating agent as it binds divalent cations (calcium), so this causes inhibition of enzymatic reactions encountered in the clotting process. This additive is used in hematologic testing,[1] including applications in transfusion medicine,[2] drug measurements inside the cells, for example, cyclosporine or tacrolimus,[3] glycated hemoglobin testing,[4] DNA isolation study,[5] and in molecular quantitative or qualitative techniques used in virus determinations as it acts to preserve the blood cellular components. EDTA is not applicable for the measurement of calcium, magnesium, and iron because it chelates them leading to incorrect results in most photometric analysis.[8]

The heparin anticoagulant additive, which is a mucoitinic polysulfuric acid, is usually used in small amounts as effective anticoagulant additive, with no significant influence on many determinants.[9] This one is the preferred anticoagulant additive for many laboratory tests using plasma or whole blood samples because of its relatively low concentration of cation, lower chelating properties, and little effects on water shifting. It is the only type of anticoagulant tubes that can be used for blood collecting systems for pH, electrolytes, blood gases, and ionized calcium determinations.[10] Because of the presence of fibrinogen, which migrates together with two monoclonal proteins, the heparin additive cannot be used for coagulation studies and is not approved for protein electrophoresis or cryoglobulin detection.[11]

Sodium citrate is another type of anticoagulant additives which can be used for coagulation study. The College of American Pathologists (CAP) recommended the use of sodium citrate in concentration of 3.2% for coagulation testing. To reverse the calcium binding effect, this is done by recalcifying the blood or plasma to its natural state. The reversible action of this tube makes it highly recommended for factor assays and clotting studies. Citrate also showed to have little effects on blood cells or platelets, so that it is used to assess platelets aggregation.[11] Citrated tubes are also used for erythrocyte sedimentation rate (ESR), prothrombin time (PT), and activated partial thromboplastin time (APTT) testing.[9]

Many methods have been identified for the analysis of GHbs. The majority of these methods separates glycated from non-glycated hemoglobin using techniques that depend on the differences in charges [high-performance liquid chromatography (HPLC), ion exchange chromatography (IEC), electrophoresis, and iso-electric focussing] or differences in hemoglobin structure (immunoassay or affinity chromatography).[12] There are also chemically based techniques (enzymatic, photometry, and spectrophotometry) used for this purpose. Nowadays, capillary electrophoresis or an enzymatic assay becomes commercially available methods for specific measurements of HbA1c.[4]

One of the main methods for measuring HbA1c that relies on charge difference is IEC, which allows for the separation of Hb from its components depending on the iso-electric point difference between HbA1c and HbA0. Separation is done depending on variations in ionic interactions between the cationic exchange group on the resin surface of the chromatography column and the Hb parts in the specimen.[13] Some Hb family members can cause interference with this method (e.g., the Schiff base, carbamylated Hb, and hemoglobin variants).[14] The presence of HbF causes increased levels of glycated hemoglobin, whereas the presence of Hb S and C causes decreased levels, so that these hemoglobin variants may lead to false results of HbA1c.[3]

Another technique using charge differences in separation and analysis of HbA1c is HPLC method. In 1996, the National Glycohemoglobin Standardization Program (NGSP) was developed to standardize glycated hemoglobin results compared to those obtained from the Diabetes Control and Complications Trial using the HPLC method, depending on it as the gold standard method. Its required short testing time ranges from 3 to 5 min. All

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HPLC-dependent methods are known to have coefficient of variation (CV) lower than 3.0% as seen with the survey done in 2014 by the College of American Pathologists (CAP).[4] In contrast, the device used for this technique is costly, difficult to work with, and time-consuming; therefore, it always requires professional personnel to work with, making it impossible and not cost-effective to be used in most clinical laboratories.[15]

Aims of the study

1- To compare the effect of different anticoagulant additives (EDTA, lithium heparin, and sodium citrate) on HbA1c value;
2- To compare the results of HbA1c using charge separation-based methods: IEC method with the gold standard method, i.e., the HPLC.

Materials and Methods

Eighty patients with DM who are diagnosed by consultant physicians according to the Americans Diabetes Association were involved in this study.

The study included two parts: the first one investigated the different anticoagulant additives (EDTA, lithium heparin, and sodium citrate) effect on HbA1c levels and included 40 patients (group A). The second part (group B) included the remaining 40 patients and intended to compare the results of HbA1c values between the two methods: HPLC standard method and IEC method. Patients were also subdivided according to their gender and ages.

An aliquot of 5–7.5 mL of venous blood sample collected from antecubital fossa veins was aspirated from each one of the included patients. Samples from patients of group A were divided and collected into three different disposable anticoagulant tubes: EDTA, lithium heparin, and sodium citrate tubes and each one of these tubes was used for the measurement of HbA1c using the same IEC method.

Sample from each patient of group B was transferred into the same type of anticoagulant additive EDTA tube and used for the measurement of HbA1c by two different methods (HPLC method by Bio-Rad D10 device and IEC method by Ram 500 device).

Instruments used in this study included two main devices for HbA1c analysis, which were as follows:

(A) *Ram* 500 *full automated HbA1C analyzer*: Ram 500 is a HbA1C autoanalyzer using low pressure IEC method for the measurement of HbA1c, using whole blood or diluted blood sample with 5–20 µL volume. The test takes 3 min time to report the result and 4.5 min with cleaning the column and recovery time. At first, the samples were hemolyzed by using melting hemolytic agent or the cleaner. Secondly, there are three gradients elution between HbA1c and unglycated hemoglobin: buffer 1, buffer 2, and buffer 3, using gradient elution to separate the glycation parts (HbA1ab, HbA1c) and unglycation parts (HbA0). Thirdly, the absorbance of elutes is tested consecutively online by using a colorimetric method to get the chromatogram. The recommended visible wavelength used in this method should be 415 nm. Then, the HbA1c and HbA0 absorbance area is calculated using the integral principle.[16]

(B) *D*-10 *System for Hemoglobin Testing*: The D-10 device is a fully automated system that is used for both glycated Hb measurement and testing for hemoglobinopathies. This system uses whole blood sample with 5 µL volume and limited to 10 samples. The results of glycated Hb were reported within 3 min. The D-10 system depends on chromatographic principle for the separation of analytes using the ion exchange HPLC. The samples dilution is done automatically on this device. Then the sample is injected into the analytical cartridge. To increase the ionic strength buffer gradient delivered by this system, the Hb ionic interactions with the cartridge material are needed for separation. After hemoglobin separation, it passes through the filter photometer flow cell which leads to absorbance changes that is measured at 415 nm.[17]

Statistical analysis

The data of this study were analyzed through SPSS program version 23. Frequencies, mean, standard deviation, 95% confidence interval, analysis of variance, complete randomized design, *t*-test, least significant difference, receiver operating characteristics (ROC), and area under the curve (AUC) were used to determine the significant differences of HbA1c levels according to the parameters in this study at the 0.05 level of significance ($P \leq 0.05$).

Results

The study included 80 patients divided into two groups with the age range 38–70 years, and the mean age was 53.78 ± 7.52 years. The mean value and the range of blood HbA1c were 8.78 ± 2.61 and 4.9–15.4%, respectively.

Results of group A study population

The mean (±SD) values of HbA1c % of patients of group A with different types of anticoagulant tubes—EDTA, lithium heparin, and sodium citrate—were 8.90 ± 2.72%, 8.70 ± 2.58%, and 8.73 ± 2.58%, respectively, and demonstrated a non-significant difference among them, $P > 0.05$ [Figure 1].

There are three different HbA1c glycemic control subgroups: <7% (well glycemic control), 7–8% (fair glycemic control), and >8% (poor glycemic control). The mean comparison values of HbA1c (%) between and within type of tubes (EDTA, lithium heparin, and sodium citrate) of the HbA1c glycemic control group (<7%

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group) were 6.16 ± 0.625, 6.27 ± 0.563, and 6.30 ± 0.625, respectively. Similarly, the mean of the 0.7–0.8 group was 7.41 ± 0.26, 7.52 ± 0.32, and 7.57 ± 0.28, and the mean of the >0.8 group was 11.15 ± 2.30, 11.19 ± 2.01, and 10.99 ± 2.17, respectively. All these results showed non-significant difference within each group ($P > 0.05$) [Figure 2].

Results of group B study population

The mean comparison of HbA1c (%) levels between HPLC and IEC methods was 8.41 ± 2.01 and 7.80 ± 2.11, respectively; these results demonstrated a non-significant difference between them, $P > 0.05$ [Table 1].

The mean comparison of HbA1c (%) values between and within HPLC and IEC methods among HbA1c glycemic control groups was as follows: for the <7% group it was 6.01 ± 0.39 and 5.91 ± 0.56; for the 7–8% group it was 7.35 ± 0.30 and 7.4 ± 0.33; for the >8% group it was 10.07 ± 1.62 and 9.88 ± 1.66, respectively. All these results showed statistically non-significant difference within each glycemic control group of HbA1c ($P>0.05$) [Figure 3].

The ROC curves for HbA1c (%) using HPLC and IEC methods are shown. The AUCs were 0.98 (95% CI 0.94–1.00) and 0.97 (95% CI 0.94–1.00), respectively [Figure 4(a) and (b) and Table 2]. The sensitivity and specificity for the HPLC and IEC methods were 97.3% and 100% and 97.1% and 100%, respectively.

Discussion

Effect of different anticoagulant tubes on HbA1c measurements

In the first part of this study, the HbA1c values for group A patients, who had a wide range of HbA1c values ranging from good control to poor control levels, were

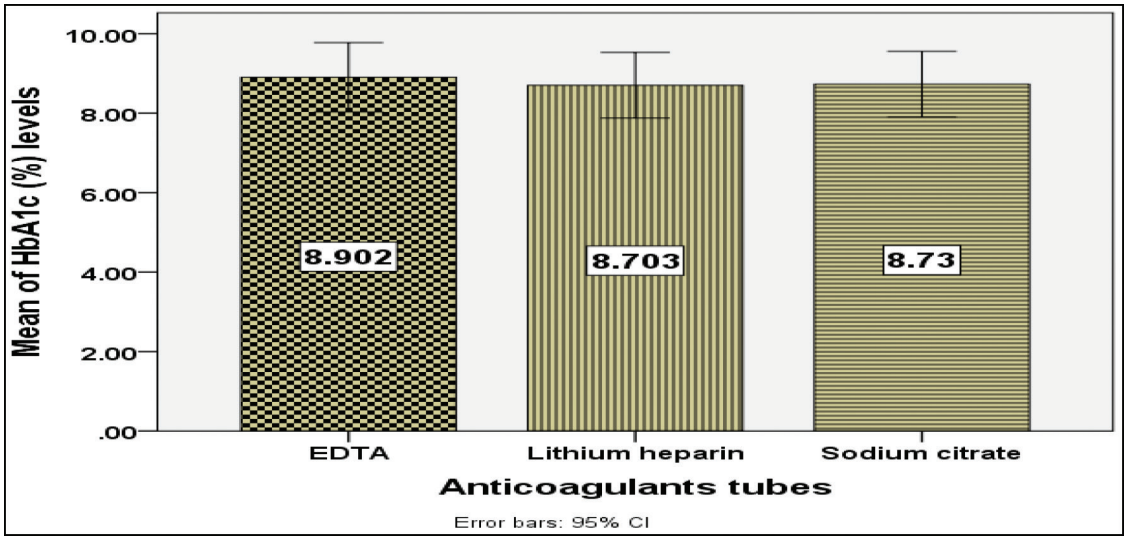

Figure 1: Mean and standard deviation of HbA1c (%) levels related to the type of anticoagulant tubes. *NS =*P*-value >0.05. Non-significant

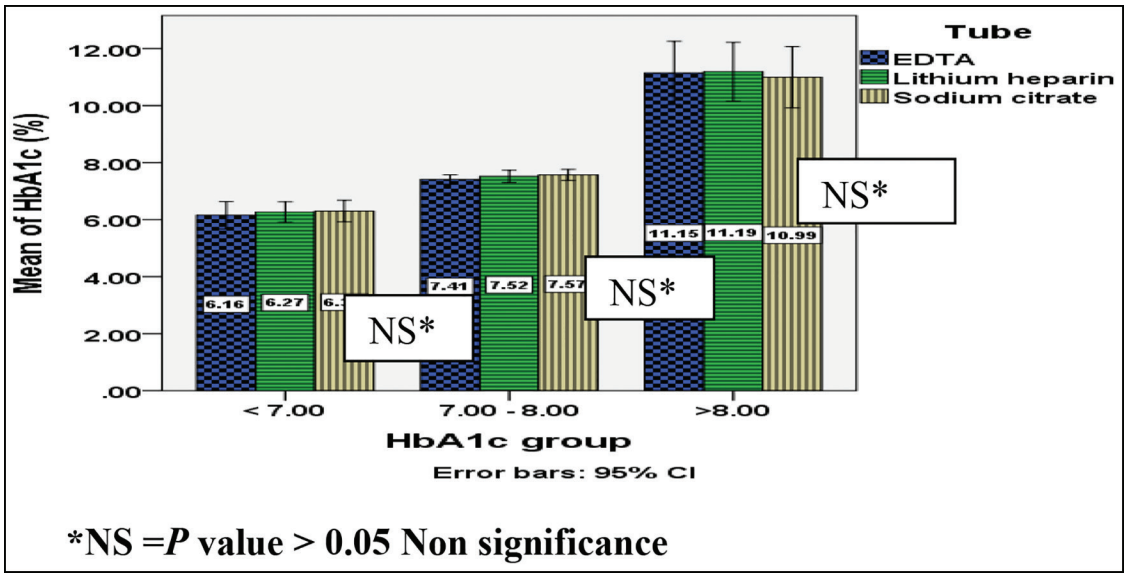

Figure 2: Mean comparison of HbA1c (%) levels between anticoagulant tubes with respect to the HbA1c group. *NS=*P*-value >0.05. Non-significant

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measured using three different types of anticoagulant tubes (EDTA, lithium heparin, and sodium citrate) and found that there was no significant difference in glycated hemoglobin results among these anticoagulant tubes. Similar results were obtained by the studies by Park and Park in 2016[18] and Boonlert *et al.* in 2010,[19] who observed non-significant difference in HbA1 values when lithium heparin tube is used in comparison with that with EDTA.

The Indian study done by Sarmah and Sharma[20] also showed non-significant differences in HbA1c level results using the same three anticoagulant tubes used in this study. Mailankot *et al.*[21] and Biswas *et al.*[22] also reported that there was no effect of anticoagulant tubes on HbA1c value, but their studies were limited by six and three subject samples, respectively.

The explanation for these results may be due to the fact that erythrocytes were lysed before glycated Hb value estimation and the isolation and resolution processes of glycated hemoglobin were not affected by anticoagulant materials.[22]

HbA1c measurements using IEC in comparison to HPLC

HPLC is the most reliable method used in measurements of HbA1c but it is a costly method and not available in every clinical laboratory; there are many methods for measurements of glycated hemoglobin with different principles, and one of the lowest cost methods is IEC which depends on charge difference for separation of glycated hemoglobin as in the HPLC method.

In the second part of this study, 40 samples of HbA1c collected in EDTA tubes from group B diabetic patients were measured by the HPLC method (D10 SYSTEM) and IEC method (Ram 500 HbA1c autoanalyzer), and the study showed no statistically significant difference between these two methods (*P*-value >0.05) even with slightly higher results obtained by the HPLC method. The IEC method showed excellent sensitivity and specificity close to that seen in the HPLC method.

In contrast, Rukmini *et al.*[23] showed that there was a significant difference between IEC and HPLC methods (*P*-value <0.05) and the sensitivity of IEC was also excellent (94%) but with lower specificity (62.4%) when compared with the ICE method results of the present study (sensitivity = 97.1% and specificity = 100%), whereas Razi *et al.*[24] used three different methods to measure HbA1c in comparison to the HPLC method. One of them is IEC (by DS5 device) and lower sensitivity is obtained than that seen by the IEC method in the current study but specificity is excellent.

Table 1: Mean ± SD and range values of HbA1c (%) levels for HPLC and IEC methods

Type of method	No.	Mean ±	SD	Range		Statistical significance
				Minimum	Maximum	
HPLC	40	8.41 ±	2.01	5.6%	13.5%	*NS
IEC	40	7.80 ±	2.11	5.1%	13.6%	

*NS = *P*-value > 0.05, non-significant

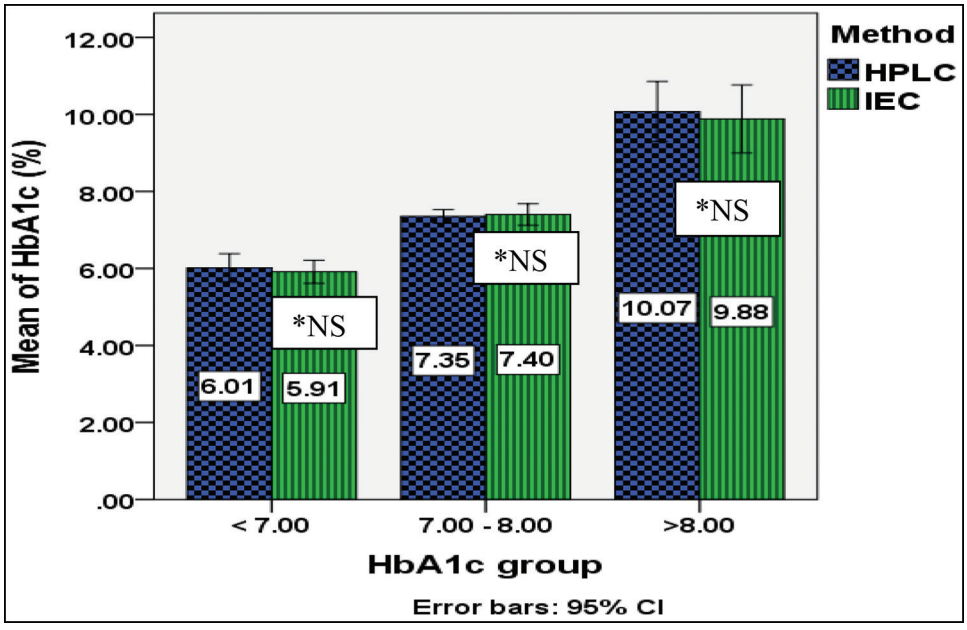

Figure 3: Mean comparison of HbA1c (%) levels between and within HPLC and IEC methods

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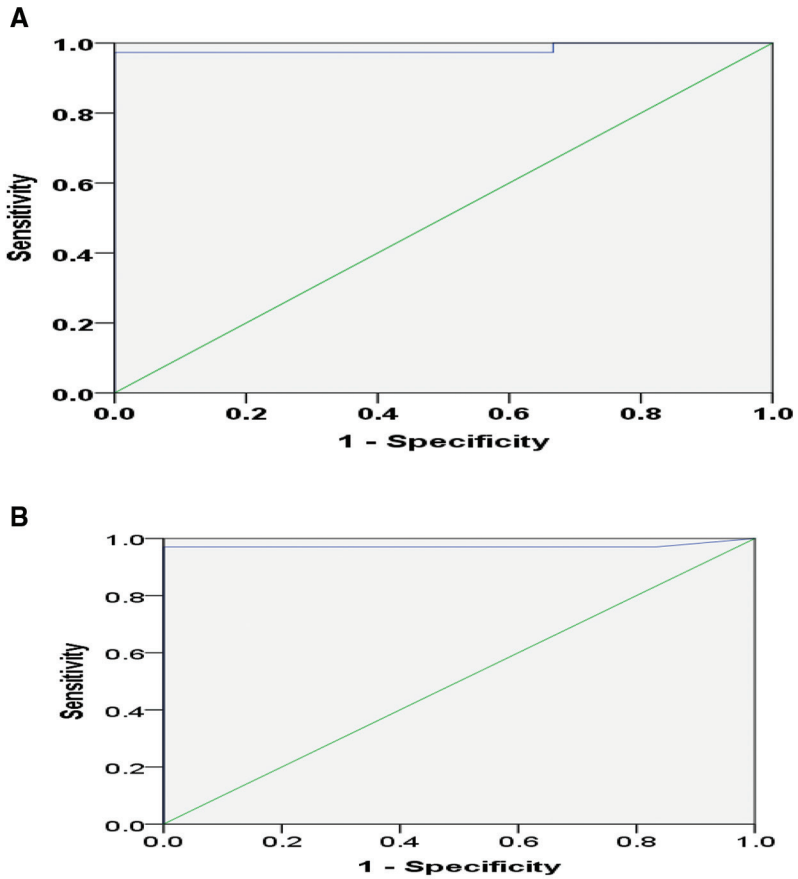

Figure 4: (a) ROC curve of HbA1c estimation by the HPLC method. (b) ROC curve of HbA1c estimation by the IEC method

Table 2: AUC obtained for HPLC and IEC methods

Method	Area	*P**-value	95% confidence interval		Sensitivity	Specificity
			Lower bound	Upper bound		
HPLC	0.98	0.006	0.94	1.00	97.3%	100%
IEC	0.97	0.000	0.92	1.00	97.1%	100%

The explanation to these variations between the current study and the previous two studies even when all of them used the same method principle is not so clear, but may be related to differences in calibrations or specifications of these devices and other different clinical laboratory work conditions which can affect the results.

The other two methods studied by Razi *et al.* were immunoassay and the boronate affinity chromatography; the immunoassay showed slightly lower sensitivity and specificity than the IEC method in this study, whereas boronate affinity chromatography showed lower sensitivity but comparable specificity so that the IEC method in this study had the more comparable results to standard HPLC method than other different methods used in glycated hemoglobin measurements.

Conclusion

1. This study showed that there was no effect for different anticoagulant additive tubes on glycated Hb values, so that it can be measured by lithium heparin or sodium citrate as an alternative to EDTA when needed.
2. The IEC method showed comparable results for HbA1c measurements compared with those of HPLC, which shows statistically non-significant differences, with slightly lower result of HbA1c obtained by the IEC method but with no significant effect on clinical evaluation of the patients; therefore, when it comes to cost and ease of preparations and use, the IEC method can be used as one of the main methods to assess HbA1c in clinical laboratories with acceptable and comparable results to the HPLC method.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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