روش های اندازه گیری هموگلوبین گلیکوزیله

Hb A1c measurements methods

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NGSP
National Glycohemoglobin Standardization Program

main goal NGSP
Harmonising Hb A1c testing

- A Better A1c test means better diabetes care

- **The purpose of the NGSP is to standardize Hemoglobin A1c test results** to those of the Diabetes Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS) which established the direct relationships between HbA1c levels and outcome risks in patients with diabetes

2010 Consensus Statement on the Worldwide Standardization of HbA1c

- 1- HbA1c test results should be standardised worldwide, including the reference system and results reporting.
- 2- The IFCC reference system for HbA1c represents the **only valid anchor to implement standardisation of the measurement**.
- 3- HbA1c results are to be reported by clinical laboratories worldwide in SI (Système International) units (mmol/mol – no decimals) and derived NGSP units (% - one decimal), using the IFCC-NGSP master equation (DCCT units).
- 4- HbA1c conversion tables including both SI (IFCC) and NGSP units should be easily accessible to the diabetes community.
- 5- Editors of journals and other printed material are strongly recommended to require that submitted manuscripts report HbA1c in both SI (IFCC) and NGSP/DCCT units.
- 6- The reportable term for glycated hemoglobin is HbA1c, although other abbreviations may be used in guidelines and educational material (A1C).
- 7- The above consensus recommendations apply through 2011, when they will be discussed again at the next consensus meeting at the IDF meeting in Dubai December 2011

Role of the Laboratory in diabet

- diagnosis & Management of diabet

Glucose

Glycated proteins

HbA1c

Fructosamine

Urinary proteins/microalbuminuria

Uses of HbA_{1c} Analysis

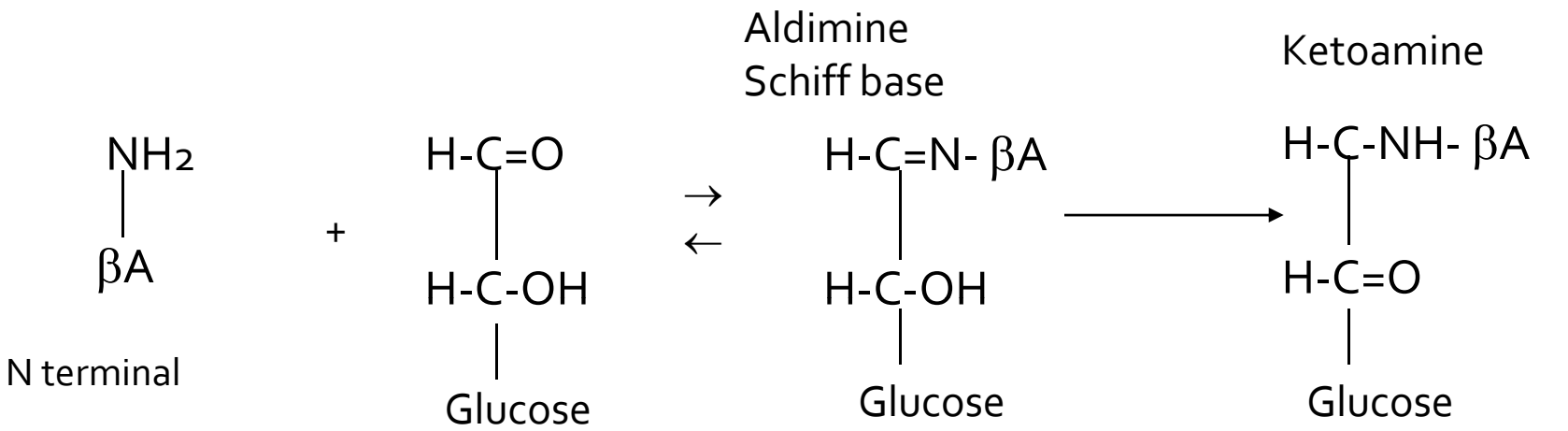
- 1- Monitor glycemic control
  - patient management
  - clinical guidance and audit
  - clinical trial design
- 2- Independent risk factor for MI in non-diabetic patients
- 3- Estimate of recovery from infection in ICU

What is Hemoglobin A1c?

- The higher the blood sugar, the faster HbA1c will be formed, resulting in higher HbA1c levels.
- Red blood cells circulate 60-120 days, and the HbA1c level is in part affected by blood sugar levels over a **three-month period.**
- **However, it is heavily weighted to levels over the past 45-60 days.**

Glycated Haemoglobin Analysis

- Haemoglobin HbA 97%, HbA_2 2.5 % and HbF 0.5%
- Several minor haemoglobins migrate more rapidly than HbA in an electric field, called **HbA1, made up of HbA1a + HbA1b + HbA1c**.
- **Condensation of glucose and the N-terminal valine of each beta chain of haemoglobin is HbA1c**



HbA + glucose $\rightleftarrows$ Pre-HbA1c $\longrightarrow$ HbA1c

rapid

slow

مراحل شكل گيری هموگلوبين گليكوزيله پايدار

هموگلوبين گليكوزيله به طور غير آنزيمي طي يك واكنش دو مرحله اي ايجاد مي گردد
مرحله اول سريع و قابل برگشت بوده و بستگي به غلظت گلوكز محيط دارد و حاصلش ماده آلديمين ناپايدار (Labile Aldimine)است كه طي زمان تدريجا به كتوآمين پايدار كه همان هموگلوبين گليكوزيله است تبديل مي گردد.

اكثر روشهاي اندازه گيري اين كتو آمين پايدار را مي سنجند و نه محصول ناپايدار اوليه را كه بيشتر تحت تاثير رژيم غذايي اخير بيمار است.

2002 National Academy of Clinical Biochemistry Guidelines

- Interassay CV < 5% (ideally < 3%)
- 2 control materials with different mean values
- Verify values below lower limit of reference interval by repeating testing
- Verify values above 15% by repeat testing
- Schiff base (labile HbA_{1c}) should be removed before assay
- Reference range 4 - 6%

Precision Requirements for HbA_{1c} Methods

2002 NACB Guidelines

Clin Chem 2002;

Interassay CV 5% (ideally 3%)

Precision Requirements for HbA_{1c} Methods

Kolatkar et al

Clin Chem 1994; 40: 1608-9

- Intensive diabetes management requires very precise testing of glycohemoglobin .

- **CV should be in the range of 2 – 3%**

Precision Requirements for HbA_{1c} Methods

Skeie et al. Clin Chem 2001; 47: 1212-7

Patients were asked to indicate change in HbA_{1c} from 9.4% that they considered a true (real) change; from their responses we derived patient-derived quality specifications for $HbA_{1c.}$

Conclusion:

HbA_{1c}
increasing CV 3.1%
decreasing CV 3.2%

Precision Requirements for HbA_{1c} Measurements

Physician Expectations:

- Diabetic endocrinologists were asked to state their expectations of reproducibility and significant differences between HbA_{1c} results.
- **The conclusion was that an analytical variation > 3% could give clinically significant HbA_{1c} results**.

Precision Requirements of HbA_{1c} Methods

Phillipou's approach. Clin Chem 1993;

- **Based on biological variation of 1.7%**
- **Practical working CV of 3%**

Summary of Precision Requirements for HbA_{1c} Measurements

1. NACB: ideally < 3% (<5% tolerated)

2. Kolatkar: CV should be < 2 - 3%.

3. Skeie: Patient-derived specifications
   3.1% (HbA_{1c} increasing)
   3.2% (HbA_{1c} decreasing)

4. Skeie: Physician response 3-5%.

5. Physician: preferably CV of < 3%.

6. Phillipou: practical CV of 3%.

Critical Differences for HbA_{1c} at Different Analytical CVs at a HbA_{1c} of 7%

Analytical CV %	Critical Difference %	Difference in HbA_{1c}
2	7.2	0.5
3	9.5	0.7
4	11.9	0.8
5	14.5	1.0

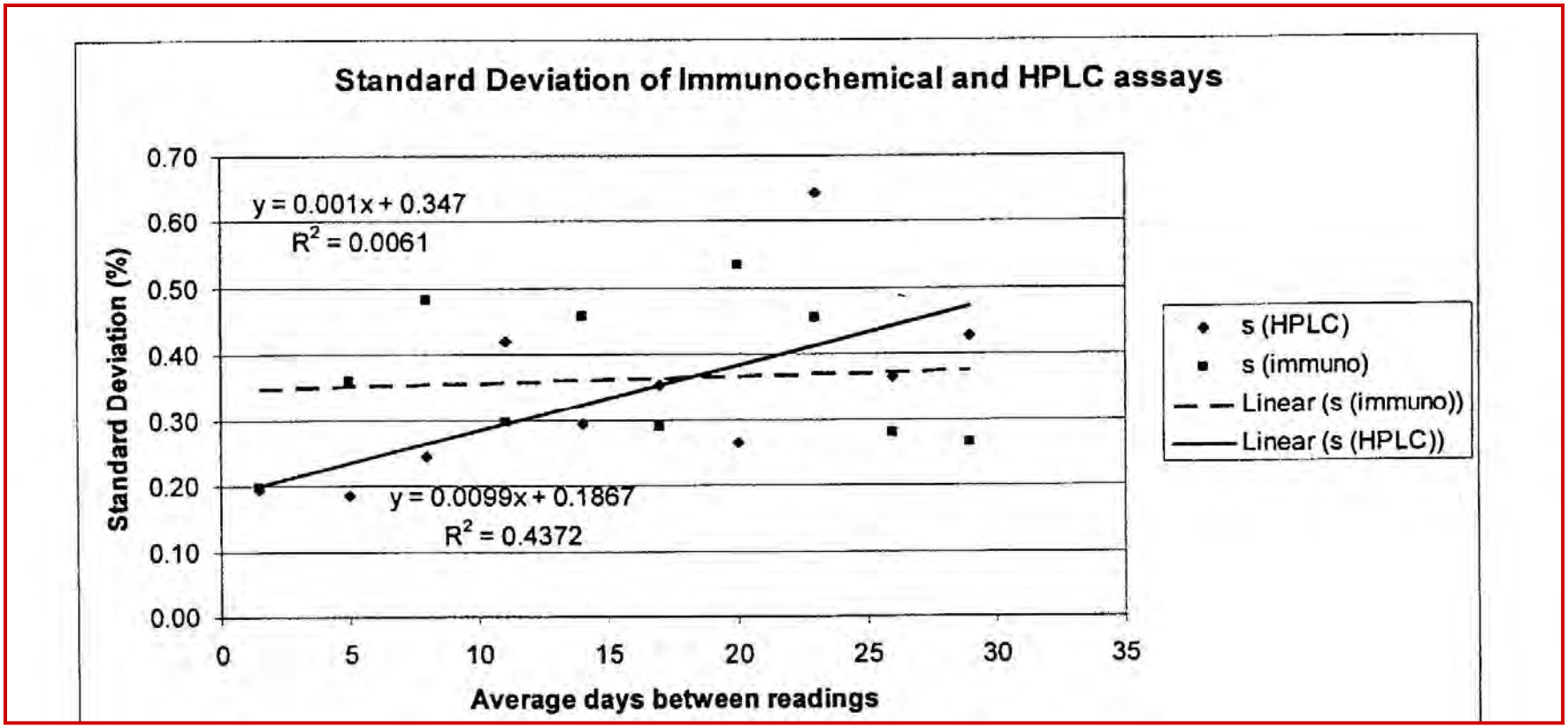
Performance of HbA_{1c} Methods on CAP Program

Glycohemoglobin HbA_{1c} – Percent

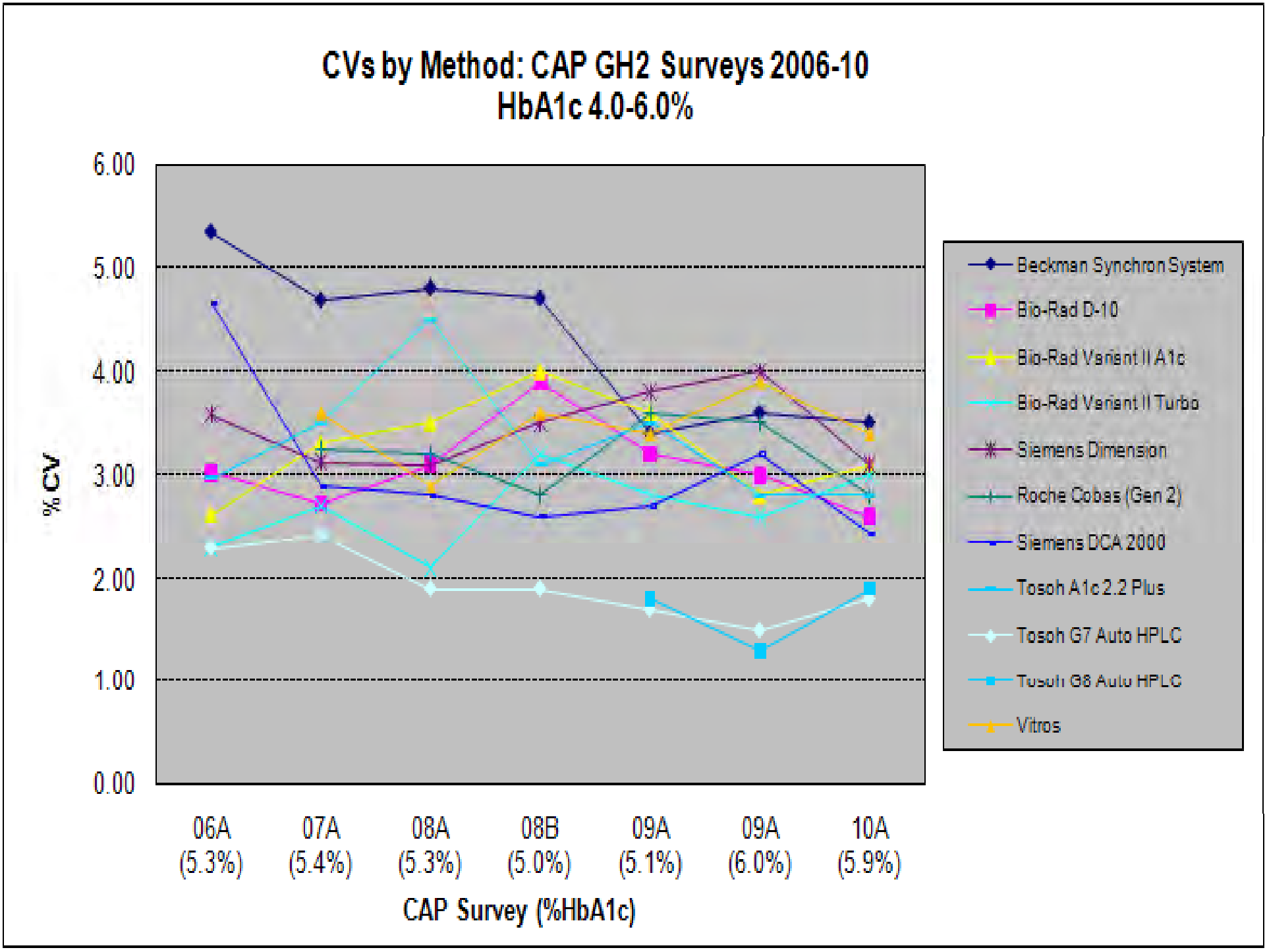
GH2-03

METHOD	NO. LABS	MEAN	S.D.	C.V.	MEDIAN	LOW VALUE	HIGH VALUE
ABBOTT AEROSET	5	-	-	-	7.4	7.2	8.3
ABBOTT ARCHITECT	8	-	-	-	7.1	6.8	7.2
BAYER ADVIA 1650/2400	17	7.15	0.47	6.6	7.0	6.5	8.0
BAYER DCA 2000/2000+	174	7.22	0.21	2.9	7.2	6.7	7.7
BECKMAN SYNCHRON SYST	277	6.99	0.31	4.5	7.0	6.1	7.9
BIO-RAD D-10	74	7.71	0.17	2.2	7.7	7.4	8.1
BIO-RAD DIASTAT	29	7.31	0.36	5.0	7.3	6.6	8.1
BIO-RAD VARIANT	32	7.37	0.23	3.1	7.4	6.9	7.8
BIO-RAD VARIANT II	299	7.65	0.24	3.2	7.6	7.0	8.4
BIO-RAD VARIANT II TURBO	17	7.62	0.19	2.6	7.6	7.3	8.0
DADE BEHRG DIMENSION	420	7.52	0.25	3.3	7.5	6.8	8.3
METRIKA A1cNOW	13	7.22	0.56	7.7	7.2	6.2	8.2
OLYMPUS AU SYSTEMS	15	7.21	0.47	6.6	7.3	6.5	8.0
PRIMUS HPLC (AFFINITY)	22	7.34	0.21	2.8	7.3	6.8	7.8
PRIMUS NYCOCARD	5	-	-	-	7.6	7.5	7.8
ROCHE COBAS INTEGRA	267	7.66	0.29	3.7	7.7	6.9	8.6
ROCHE/HITACHI	84	7.02	0.26	3.7	7.0	6.4	7.7
TOSOH A1C 2.2 PLUS	215	7.78	0.21	2.8	7.8	6.8	8.8
TOSOH G7 AUTO HPLC	168	7.56	0.14	1.8	7.6	7.2	8.0
REFERENCE METHOD *		7.40					

Precision Requirements for HbA_{1c} Measurements



Average SD of HPLC (diamonds) and immunoassay (squares) vs. time between testing. The y-intercept represents the average analytic SD, while graphs represent both analytic and physiologic variation.


CVs by Method: CAP GH2 Surveys 2006-10
HbA1c 4.0-6.0%
% CV
6.00
5.00
4.00
3.00
2.00
1.00
0.00
06A (5.3%)
07A (5.4%)
08A (5.3%)
08B (5.0%)
09A (5.1%)
09A (6.0%)
10A (5.9%)
CAP Survey (%HbA1c)
Beckman Synchron System
Bio-Rad D-10
Bio-Rad Variant II A1c
Bio-Rad Variant II Turbo
Siemens Dimension
Roche Cobas (Gen 2)
Siemens DCA 2000
Tosoh A1c 2.2 Plus
Tosoh G7 Auto HPLC
Tosoh G8 Auto HPLC
Vitros

شرایط آماده سازی تست هموگلوبین گلیکوزیله

نکته اول :

آزمایش هموگلوبین گلیکوزیله در هر ساعت روز و بدون نیاز به ناشتا قابل اجام است و بر خلاف قند خون به تغذیه و فعالیت بدنی و حالات روحی بستگی ندارد

نکته دوم :

ابتلا به انواع بیماری التهابی یا غیر التهابی می تواند منجر به افزایش کاذب هموگلوبین گلیکوزیله گردد لذا اندازه گیری هموگلوبین گلیکوزیله در شزایط بیماری بهتر است صورت نپذیرد

Specimen Collection and Storage

Patients need not be fasting. Venous blood should be collected in tubes containing **EDTA,oxalate, or fluoride**.

Sample stability depends on the assay method.

Whole blood may be stored at **4 °C for up to l week.**

Above 4°C, Hb Ala+b increases in a time- and temperature-dependent manner, but Hb Alc is only slightly affected.

Storage of samples at -20°C is not recommended. For most methods, whole blood samples **stored at -70 °C are stable for at least 18 months.**

Heparinized samples should be assayed within 2 days and may not be suitable for some methods of analysis (e.g., electrophoresis).

توصیه انجمن رسمی دیابت ایران

از آنجایی که روشهای اندازه گیری هموگلوبین گلیکوزیله در آزمایشگاههای مختلف متفاوت است، نتایج این آزمایش نیز متفاوت خواهد بود.

به عنوان مثال در یک آزمایشگاه ممکن است نتیجه ۶درصد نرمال تلقی گردد و در آزمایشگاه دیگر ابنرمال لذا طبق نظر انجمن رسمی دیابت ایران **بهتر است افراد دیابتیک آزمایشات هموگلوبین گلیکوزیله را به طور ثابت در یک مرکز آزمایشگاهی معتبر انجام دهند** تا نتایج مقایسه ای دارای ارزش تفسیر بالینی باشد

انجمن دیابت امریکا توصیه مي نماید که هموگلوبین گلیکوزیله حداقل دو نوبت در سال در بیماران دیابتیکي که قند خونشان تحت کنترل است آزمایش شود و براي بیماراني که روش درماني آنها عوض شده یا قند خونشان تنظیم نشده چهار بار طي يك سال صورت گیرد

Reference Intervals
Hb- A1c & Hb-A1

	Mean (%)	Interval (%)
Hb AI (A1a+b+c)	6.5	5.0-8.0
Hb AIc only (DCCT – equivalent)	4.5	4.0-6.0

مقادیر ایده آل هموگلوبین A1C در سنین مختلف	
بالای ۱۸ سال	کمتر از ۷ درصد
بین ۱۸-۱۲ سال	کمتر از ۸ درصد
بین ۱۲-۶ سال	کمتر از ۸/۵ درصد
زیر ۶ سال	کمتر از ۹ درصد

reference intervals A1c

The effects of age on reference intervals are controversial
.Some studies show age-related increases
(0.1 % per decade after age 30), and other reports show no increase.
Results are not affected by acute illness.
Intraindividual variability is minimal.

In patients with poorly controlled diabetes mellitus, values may extend to twice the upper limit of the reference interval or more but rarely exceed 15%.

Values greater than 15% should prompt further studies to determine the possible presence of variant hemoglobin.

There is no specific value of **Hb Alc below which the risk of diabetic complications is eliminated completely**.

The ADA states that the **goal of treatment should be to maintain Hb Alc less than 7%. (Some organizations recommend an Hb Alc target of less than 6.5%.)**

These values are applicable only if the assay method is certified as traceable to the DCCT reference.

Each laboratory should establish its own nondiabetic reference interval

ADA Guidelines

Treatment goal normal or near normal with HbA_{1c} < 7%.
Correlation between HbA_{1c} and mean plasma glucose levels.

	Mean plasma glucose	
A1C (%)	mg/dL	mmol/L
6	135	7.5
7	170	9.5
8	205	11.5
9	240	13.5
10	275	15.5
11	310	17.5
12	345	19.5

رابطه افزایش هموگلوبین گلیکوزیله
با افزایش عوارض دیابت

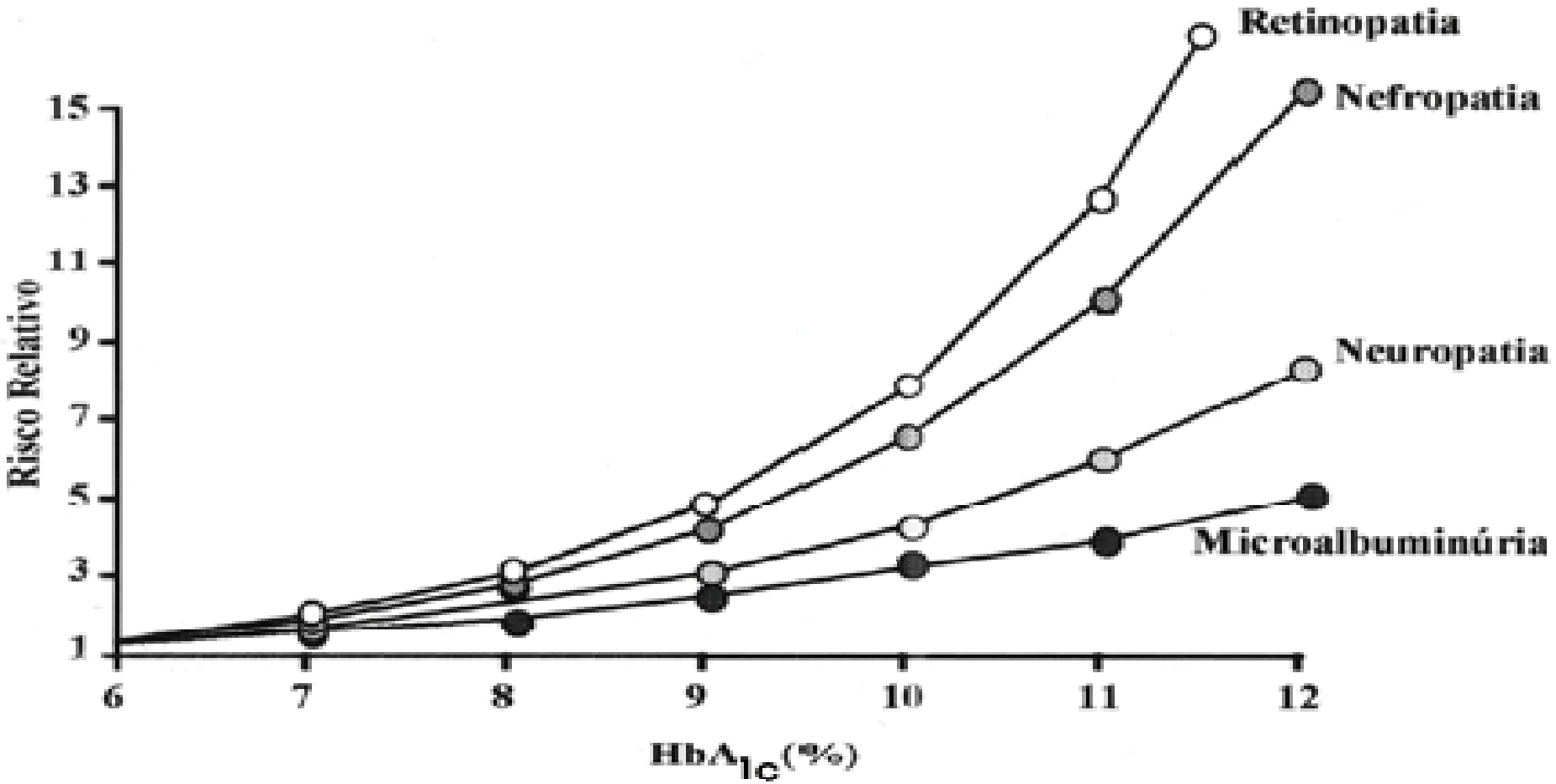


Figura 1. Relação da glico-hemoglobina/HbA_{1c} e risco de complicações microvasculares. Adaptado de Skyler JS. **Endocrinol Metab Clin 1996**;25:243-54.

High

- Newly diagnosed diabetes mellitus.
- Uncontrolled diabetes mellitus.
- Non-diabetic hyperglycaemia: acromegaly, phaechromocytoma, thyrotoxicosis, Cushing's syndrome.
- Splenectomy.
- Alcoholism.

Low

- Haemolytic anaemia: congenital (for example, spherocytosis and elliptocytosis), haemoglobinopathies, acquired haemolytic anaemias—for example, drug induced (dapsone, methyldopa).
- Chronic blood loss.
- Chronic renal failure (variable).

False positive & Negative (A1c)

موارد زمینه ساز مثبت کاذب A1c

سه ماه سوم حاملگی / تداخل با هموگلوبین های استیله یا کاربامیله / مصرف کورتون

موارد زمینه ساز منفی کاذب A1C

۱-هر عامل کاهش دهنده طول عمر گلبول قرمز یا ایجاد کننده همولیز (انمی های همولیتیک) / هموراژی / اورمی / سالیسیلات با دوز بالا / هموگلوبینوپاتی ها / الکلیسم مزمن / فقر آهن / مسمومیت سرب / اسپلنکتومی / نگاهداری نادرست نمونه

۲-سطح هموگلوبین گلیکوزیله در تابستان پایین تر از زمستان است چون میانگین قند بیمار در تابستان پایین تراز زمستان است .

۳-اورمی به دلیل افزایش سطح هموگلوبین کاربامیله منجر به کاهش کاذب هموگلوبین گلیکوزیله می گردد و مصرف سالیسیلات ها به دلیل تشکیل هموگلوبین اتیله شده سطح توتال هموگلوبین گلیکوزیله را کاهش می دهد

نکته : احتمال سقط خودبخودی در زنان باردار دیابتیک با درجه کنترل ضعیف سه برابر بیشتر از زنان باردار دیابتیک با کنترل خوب می باشد .

با توجه به میل ترکیبی بالا هموگلوبین گلیکوزیله به اکسیژن که حدود ۱۰ برابر بیشتر از هموگلوبین طبیعی است در نتیج بیمار با افزایش هموگلوبین گلیکوزیله دچار یک هیپوکسی مزمن می گردد که در نهایت با ثبات آن منجر به پلی سیتمی اکتسابی و جبرانی در فرد می گردد و نهایتا ممکن است به یک هیپر تانسیون سیستولیک نیز منجر گردد

نکته : طی مطالعات تائید شده کاهش سطح همگوبین گلیکوزیله باعث کاهش ریسک رتینوپاتی دیابتیک ها به طور واضح می گردد به عنوان مثال ۱۰% کاهش هموگلوبین گلیکوزیله (از ۸% به ۷% درصد) منجر به کاهش ۴۰% درصدی ریسک پیشرفت رتینوپاتی می گردد .

.

The Physiological Problem

- Normal red cell survival: 120 days
- β thalassemia reported at 90 – 96 days
- Hemoglobin variants have decreased life cycle (HbS reported at 90 days)
- Preferential expression of normal HbA compared to variant

Hemoglobin Variant Interference - Analytical

Interferences we can see:

hemolysis	-	K^+, glucose, LD
lipemia	-	many chemistries
icterus	-	acetaminophen

Interferences we cannot see:

ascorbic acid - glucose, cholesterol, triglyceride, uric acid methods

heterophile antibody - all immunoassays

***In general, we do not have a clue if these are present in a sample*.**

Conclusions

- The G7 (when the HbF peak is properly recognized) and Variant II methods show minimal interference in HbA_{1c} measurement up to HbF of ~30%.
- The DCA 2000, 2.2+ and CLC330/385 methods underestimate HbA_{1c} in the presence of elevated HbF, especially at HbF levels > 20%.
- **Physicians need to be aware of potential interferences when interpreting HbA_{1c} results.**

Effect of Elevated Fetal Hemoglobin on HbA1c Measurements: Five Common Assay Methods compared to the IFCC Reference Method. C. Rohlfing, S. Connolly, J. England, R. Little. Dept. of Pathology & Anatomical Sciences, U of Missouri School of Medicine, Columbia MO.

Interference from HbF – DKML initial results

Patient	% HbF	HbA_{1c} HPLC	HbA_{1c} Immoassay	HbA_{1c} Immoassay
A	22.4	6.9	4.7	4.9
B	15.0	5.5	4.3	4.7
C	3.4	5.7	5.9	5.7

Limitations of HbA_{1c} Analysis

"Hemoglobinopathies may interfere with GHb analysis independent of their effects on erythrocyte survival."

"Moreover, hemoglobin variants cannot be identified by immunoassay."

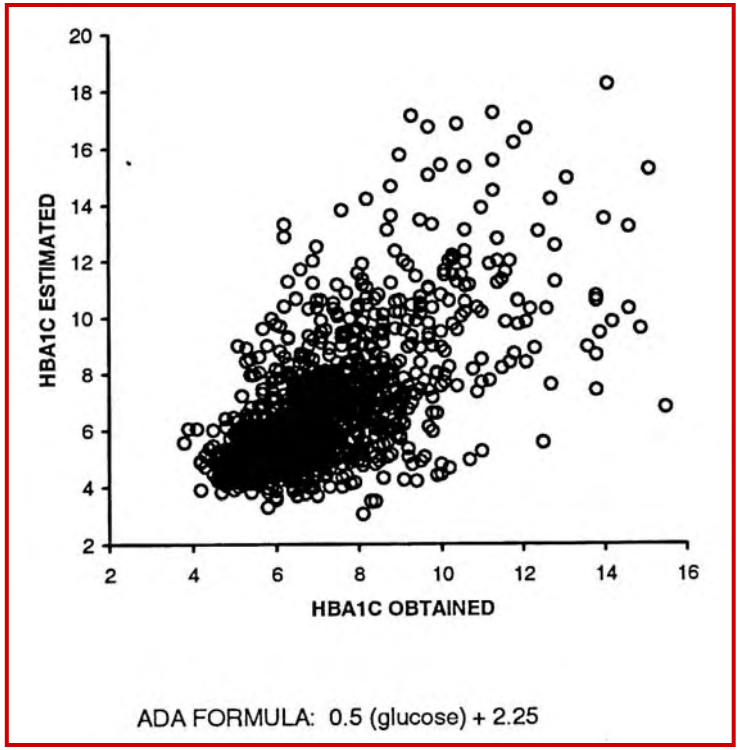
Sacks DB. Clin Chem 2003; 49: 1245-7.

Use of Calculated HbA_{1c}

ADA equation rearranged:

HbA_{1c} (calculated) = 0.5 (glucose) + 2.25 (SI units)

HbA_{1c} (calculated) = 0.0285 (glucose) + 2.15 (traditional units)



ADA/CDA Recomendations for Measurement of HbA_{1c}

Frequency at least:

- **2 times/year** in patients meeting treatment goals or with stable glycemic control

- **4 times/year in patients whose therapy has changed or who are not meeting glycemic controls**

Interference

- Icterus :
- Lipemia
- **Hemoglobin variants S and C** have no effect on the assay when they exist in the heterozygous forms HbAS and HbAC.
- In homozygous **Hb SS or Hb CC** patients do not have HbA present or HbA1c thus criteria other than monitoring of HbA1c must be used to assess long term diabetic control in these patients.

- HbF levels upto 30 % do not interfere

What do the test results mean?

- The HbA1c is linearly related to the average blood sugar over the past 1-3 months (but is somewhat weighted to the past 2-4 weeks).
  Mean Plasma Glucose (mg/dl) = (HbA1c X 35.6) - 77.3
  (Rohlfing CL and coworkers. Diabetes Care 25:275-278, 2002)
- **The HbA1c is strongly associated with the risk of development and progression of microvascular and nerve complications.**
- **High HbA1c (>9.0-9.5%) is associated with very rapid progression of microvascular complications.**

Methods for determining glycated haemoglobins

- 1-based on **charge differences:**
- ion-exchange chromatography
- HPLC
- electrophoresis
- isoelectric focusing
- 2-based on **structural differences**
- affinity chromatography
- immunoassay.
- 3- **Chemical methods** a third option rarely used.

Methods for determining glycated haemoglobins

- **Ion-exchange chromatography**
- Measures HBA1 – total glycated haemoglobins (A1a + 1b + 1c)
- **HPLC** Both HbA1c and HbA1 can be reported,
- **Electrophoresis** can measure HbA1c but less specific .
- **Isoelectrophoresis** HbA1c adequately resolved from HbA1a1, HbA1b and S and F.

روش سنتی اندازه گيري هموگلوبين گليكوزيله رنگ سنجی كالريمتري

اين روش براساس آزادسازي گلوكز اتصال يافته به هموگلوبين درحرارت جوش به شكل هيدروكسي متيل فورفورال واندازه گيري آن دريك واكنش رنگي با اسيد تيوباربيتوريك مي باشد **اين روش بسيار سخت و زمان بر بوده و تحت تأثير متغيرهاي مختلفي قراردارد**

دو متغير مهم در روش كالريمتری هموگلوبين گليكوزيله :

۱-نوسان زمان جوش به طور جدی در نوسان جواب ها موثر است

۲- ناپايداری رنگ نهائی در حرارت اطاق

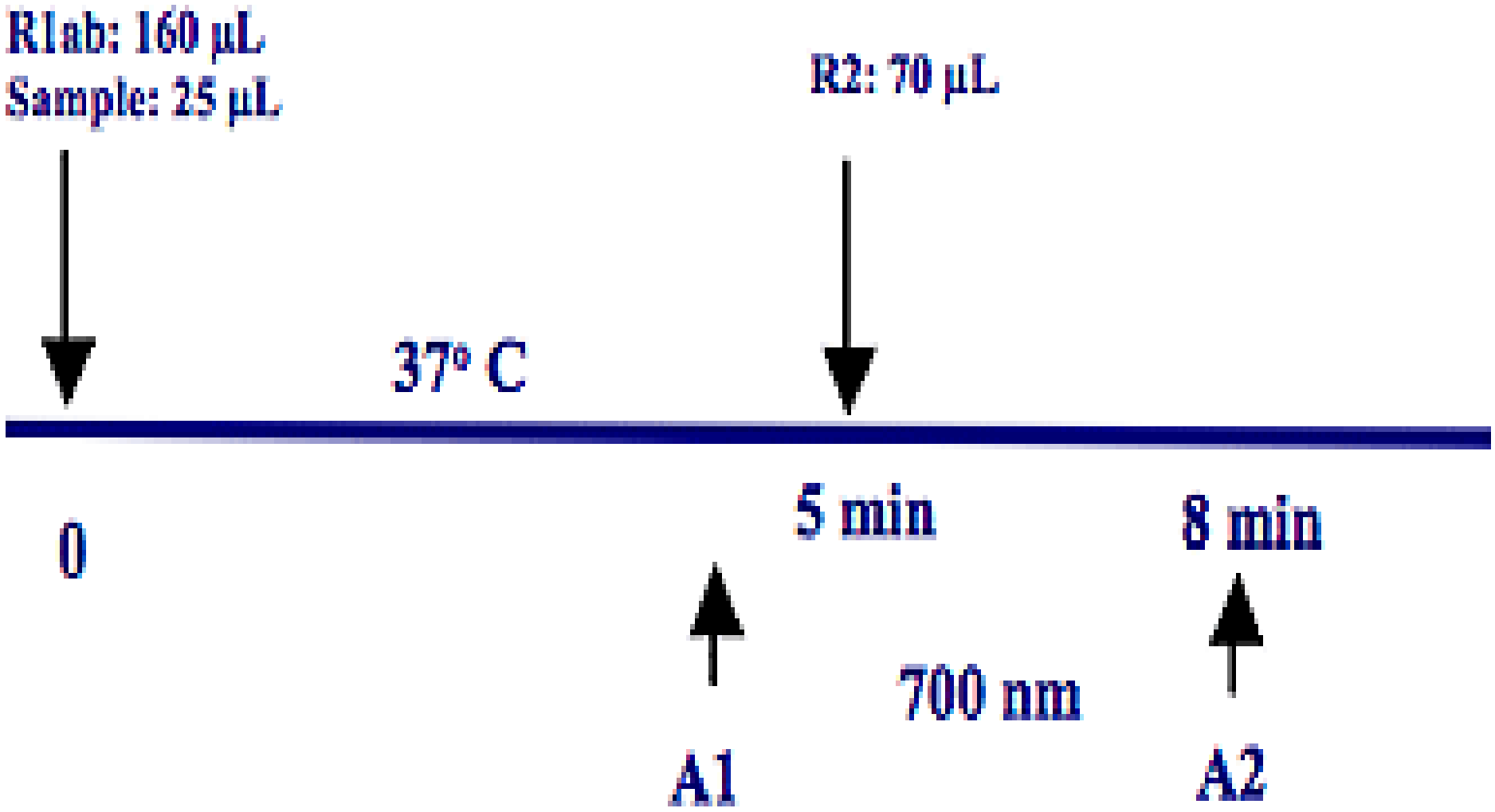
Direct Enzymatic HbA1c

- Diazyme's NSGP and IFCC Certified **Direct Enzymatic HbA1c assay has no interference from hemoglobin variants including HbC, HbS, HbE, and caramylated acetylated Hb or labile HbA1c.**
- This unique freedom from interference combined with unparalleled **precision (intra and inter % CV values less than 2.6%) means that Diazyme's Direct Enzymatic HbA1c assay provides unique performance advantages over conventional immunoassay and chromatography methods**.
- Diazyme's patented enzymatic method measures HbA1c directly using only a single instrument channel and **completely eliminates the latex particles cuvette contamination often seen in HbA1c immunoassay methods**.
- This reduces instrument maintenance requirements.

Direct Enzymatic HbA1c

- Diazyme's Direct HbA1c can be used on most automated chemistry analyzers and reagent transfer can be eliminated for most chemistry systems with instrument specific packaging options including Roche Hitachi 917 series, Olympus AU (400/600/640/680), Beckman Synchron CX, LX and DXC analyzers. The assay correlates well with conventional methods such as the Tosoh™ HPLC and Roche™ Tina-Quant methods and has linear range of 4%-12%. The assay is stable for 15 months from date of manufacture when stored @ 2°-8°C.

Direct Enzymatic HbA1c



Direct Enzymatic HbA1c

- **Assay Principle**
  Whole blood samples are lysed with a lysing buffer and the released hemoglobin molecules are digested with a protease mix. **This process releases amino acids including glycated valines from the hemoglobin beta chains. Glycated valines then serve as substrates for specific recombinant fructosyl valine oxidase (FVO) enzyme which specifically cleaves N-terminal valines and produces hydrogen peroxide in the presence of selective agents.**
- This, in turn, is measured using **a horseradish peroxidase (POD) catalyzed reaction and a suitable chromagen**. The HbA1c concentration is expressed directly as %HbA1c by use of a suitable calibration curve in which the calibrators have values for each level in %HbA1c.

There are more than 30 different methods for the determination of GHbs.

These methods separate hemoglobin from GHb using techniques based on *charge differences* (ion-exchange chromatography, HPLC, electrophoresis,and isoelectric focusing), *structural differences (affinity* chromatography and immunoassay), or *chemicalanalysis* (photometry and spectrophotometry).

Regardless of the method, the result is expressed as a percentage of total hemoglobin.

Analysis by chemical techniques is rarely used and is not addressed further here. (Interested readers are referred to earlier editions of this book.)

Method selection A1c

The selection of method by a laboratory is influenced by several factors, including:

1-sample volume,
2-patient population,
3-cost.
4-It is advisable to consult clinicians in this process.

- The ADA recommends that laboratories should use only GHb assays that are certified by the National Glycohemoglobin Standardization Program (now known as the NGSP) as traceable to the DCCT reference.

The results demonstrate that in 2003 virtually all laboratories used immunoassay or ion-exchange chromatography. (in usa)

Hb AlC was measured by more than 99% of laboratories (Table 25-7).

Total GHb and Hb Al measurements had virtually disappeared.

These results reflect significant changes from the methods used in 1995 when affinity chromatography was the most common analytical method (Table 25-7). Also, only 60% of laboratories reported Hb Alc in 1995.

In addition, the variation among mean values-both between and within methodsand imprecision were substantially lower in 2003.

Ion-Exchange Minicolumns

Ion-exchange chromatography separates hemoglobin variants on the basis of charge. The cation exchange resin (negatively charged), packed in a disposable minicolumn, has an affinity for hemoglobin, which is positively charged.

The patient's sample is hemolyzed and an aliquot of the hemolysate is applied to the column.

A buffer is applied and the eluent collected. The ionic strength and pH of the eluent buffer are selected so that **GHbs are less positively charged than Hb A, do not bind as well to the negatively charged resin,** and are **therefore eluted first**.

The GHbs-Ala + Alb+ Alc expressed collectively as Hb AI- are measured in a spectrophotometer.

- A second buffer of different ionic strength can be added to the column to elute the more positively charged main hemoglobin fraction. This is read in the spectrophotometer and GHb is expressed as a percentage of total hemoglobin.

- Methods that **separate Hb A1a+b from Hb Alc by using two different buffers** have also been described

Most of the current commercial ion-exchange methods use HPLC .

In all ion-exchange column methods, it is important to ***control the temperature of the reagents and columns to obtain*** **accurate and reproducible results.**

This is best done by thermostatting the columns. Alternatively, a temperature correction factor can be applied if the room temperature differs from the specified optimum. In addition,

rigid control of pH and ionic strength must be maintained.

Sample storage conditions are also important.

High-Performance Liquid Chromatography

Hb Alc and other hemoglobin fractions can be separated by HPLC, which employs cation-exchange chromatography.

- **Several fully automated systems are commercially available**.

Assays require only 5 microL of whole blood, and fingerstick samples can be collected in a capillary tube for analysis.

Anticoagulated blood is diluted with a hemolysis reagent containing borate.

Samples are incubated at 37°C for 30 minutes to remove Schiff base and inserted in the autosampler.

A step gradient using three phosphate buffers of increasing ionic strength is passed through the column.

Detection is performed at both 4 I5 and 690 nm, and results are quantified by integrating the area under the peaks.

Analysis time is as short as 3 to 5 minutes.

All HPLC methods had CVs less than 3.5% in a 2003 CAP survey.

Hb Alc by HPLC was used for analysis of all patient samples in the DCCT71and UKPDS

Electrophoresis (A1c)

- Agar gel electrophoresis on whole-blood hemolysates at pH 6.3 provides good resolution of Hb A and Hb AI.
- The gel contains negatively charged moieties that interact with the hemoglobin. After 25 to 35 minutes, the GHb separates on the cathodic side of Hb A.
- Quantification is performed by scanning densitometry at 415 nm.
- **Results generally agree well with those obtained by HPLC or column chromatography, but are less precise.**
- Minor variations in pH, ionic strength, or temperature have little effect on results. Hb F migrates to the same region as Hb AI and causes a falsely increased Hb AI value, but Hb Sand Hb C do not

Isoelectric Focusing (A1c)

The hemoglobin variants separate on isoelectric focusing on the basis of their migration in gel containing a pH gradient.

Ampholines in the pH range of 6 to 8 establish the gradient in I-mm-thick acrylamide gel slabs .

Hb Alc is adequately resolved from Hb A1a +A1b & S, and F.

Results showed close agreement with other methods. The equipment is expensive and is not widely used in the United States, but is more popular in Europe

Immunoassay (A1c)

Assays for Hb Alc have been developed using antibodies raised against the Amadori product of glucose (ketoamine linkage) plus the first few (four to eight) amino acids at the N-terminal end of the chain of hemoglobin.

widely used assay measures Hb Alc in whole blood by inhibition of latex agglutination.

Immunoassay (A1c)
reverse latex agg (immuno turbidometry)

The agglutinator, a synthetic polymer containing multiple copies of the immunoreactive portion of Hb Alc' binds the anti-Hb Alc monoclonal antibody that is attached to latex beads.

This agglutination produces light scattering, measured as an increase in absorbance.

Hb Alc in the patient's sample competes for the antibody on the latex,inhibiting agglutination, thereby decreasing light scattering

Immunoassay (A1c)

Enzyme immunoassays using monoclonal antibodies are commercially available and most exhibit low imprecision

These assays have been shown to correlate well with HPLC. The antibodies do not recognize labile intermediates or other GHbs (such as Hb Ala or Hb Alb) because both the ketoamine with glucose and the specific amino acid sequences are required for binding. Similarly, other hemoglobin variants, such as Hb F, Hb A2, Hb S, and carbamylated hemoglobin are not detected.

The procedure has been adapted for capillary blood samples using a bench-top analyzer with reagent cartridges designed for use in physicians' office laboratories.

Error in HbA_{1c} by Immunoassay

Patient 28 y f with known C trait

	Roche	Variant II	HPLC
HbA_{1c}%	8.8	7.0	6.3

At HbA_{1c} of 6%, Variant II bias is +0.82% (+0.85 at 7%).

Little (Clin Chem 2004; 50S6; D-12)

Bias from true HbA_{1c} value of 6.15%.

	Roche	Variant II	HPLC
Bias %	43	13.3	2.4

What are the differences, advantages and disadvantages of the HPLC versus immuno-turbidimetric HbA1c assays?

- Diabetes Control and Complications Trial (DCCT)
- The assays should achieve very similar values and are both calibrated to the DCCT standard. Upper limits of normal for all HbA1c assays is about 6.2 but can vary slightly between specific machines.

HPLC assay

- Assay technique = High Performance Liquid Chromotography (HPLC)
  Storage limit (at 4°C) = One week
- *Advantages:*
- Assay type used in DCCT
- Traditional Standard (more "clinically" pure)

Immuno-turbidimetric assay

- Assay technique = Immuno-turbidimetric
  Storage limit (at 4°C) = One month
- *Advantages:*
- More "analytically" pure approach to measurement
- Longer storage time possible

روش فروکتوز آمین

جهت کنترل قند خون در دوره زمانی کوتاه(۳ تا ۶ هفته)

مزیت روش فروکتوز آمین به روش هموگلوبین گلیکوزیله:

۱- سادگی تست و استفاده از سرم به جای نمونه خون کامل
۲- هزینه پایین تر
۳ - انجام روتین تست با تجهیزات خودکار و تسریع در جوابدهی
٤ - تست فروکتوز آمین بهترین جایگزین تست هموگلوبین گلیکوزیله در بیماران هموگلوبینوپاتی ها و آنمی همولیتیک می باشد .

عیوب روش فروکتوز آمین:

۱- زمان کوتاهتر کنترل نسبت به روش هموگلوبین گلیکوزیله
۲- تغییرات سطح فروکتوز آمین به نسبت تغییرات سطح پروتین سرم خصوصا در بیماری های حاد و کلیوی
۳- اگر سطح پروتئین سرم کمتر از ۳ گرم باشد نباید از این روش ها استفاده شود

In addition,fructosamine may be useful in patients with hemoglobin variants, such as Hb S or Hb C, that are associated with decreased erythrocyte lifespan where GHb is of little value.

Gross changes in protein concentration and half-life may have large effects on the proportion of protein that is glycated. Thus fructosamine results may be invalid in patients with nephrotic syndrome, cirrhosis of the liver, or dysproteinemias,

It is generally accepted that the test should not be performed when serum albumin is less than 30 gIL.

Reference Intervals fructoseamine

- **Values in a nondiabetic population range from 205 to 285 micmol/L.**

- **The reference interval corrected for albumin is 191 to 265 micmol/L**

Standardization of Methods for determining glycated haemoglobins

- **Diabetes Control and Complications Trial (DCCT)**
- HbA1c measurement systems have been standardized through a process of alignment with the original DCCT method. This has been undertaken by the **US National Glycohemoglobin Standardisation Program (NGSP) .**

- **UK Consensus Statement**
- Glycemic control is best measured by HbA1c
- The method should be a DCCT –aligned HBA1c method
- **The assay should have acceptable**
- **within assay precision <3%**
- **between assay imprecision <5%**

- Sample EDTA whole blood stable 1 week at 4°C
- HbA1c half life 35 days
- **1% increase in %HbA1c** is equivalent to a rise in **average blood glucose of 35 mg/dL.**
- International Expert Committee says HbA1c should be the diagnostic test for diabetes.
- **The value of ≥ 6.5% decision point**
- 6.0-6.4% indicate individuals at high risk of developing diabetes

What are total glycosylated hemoglobin and HbA1c?

- **HbA1c** = (0.705 * TGHb) + 1.117

- **TGHb** = (1.325 * HbA1c) - 0.803.

Why is HbA1c important?

- 1- It is a simple way to evaluate **average glucose levels over the past two to four weeks.**
- 2-It is the best single test for evaluating the risk for glycemic damage to tissues (e.g., nerves, and small blood vessels in the eyes and kidneys) and, thus, risk of complications of diabetes.
- 3- Clinical trials, such as the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) have shown that **improving HbA1c measures will decrease the development and progression of eye, kidney and nerve complications in both type 1 and type 2 diabetes**.