Advantages of High Performance Liquid Chromatography (HPLC) for HbA1c testing

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**# 7 in the World - MEXICO  10.7 MILLION**

**Top 10: Countries/territories of number of people with diabetes (20-79 years), 2011 and 2030**

<table>
<thead>
<tr>
<th>COUNTRY/TERRITORY</th>
<th>2011 MILLIONS</th>
<th>2030 MILLIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>China</td>
<td>90.0</td>
<td>129.7</td>
</tr>
<tr>
<td>India</td>
<td>61.3</td>
<td>101.2</td>
</tr>
<tr>
<td>United States of America</td>
<td>23.7</td>
<td>29.6</td>
</tr>
<tr>
<td>Russian Federation</td>
<td>12.6</td>
<td>19.6</td>
</tr>
<tr>
<td>Brazil</td>
<td>12.4</td>
<td>16.8</td>
</tr>
<tr>
<td>Japan</td>
<td>10.7</td>
<td>16.4</td>
</tr>
<tr>
<td>Mexico</td>
<td>10.3</td>
<td>14.1</td>
</tr>
<tr>
<td>Bangladesh</td>
<td>8.4</td>
<td>12.4</td>
</tr>
<tr>
<td>Egypt</td>
<td>7.3</td>
<td>11.8</td>
</tr>
<tr>
<td>Indonesia</td>
<td>7.3</td>
<td>11.4</td>
</tr>
</tbody>
</table>
Agenda

- Benefits of using HbA1c to diagnose diabetes
- The “Gold standard” methodology for HbA1c determination
- High Performance Liquid Chromatography (HPLC) principle
- Advantages of HPLC for HbA1c testing
- What is the experience worldwide?
Benefits of using HbA1c to diagnose diabetes

1. Chronic hyperglycemia is captured by A1C but not by Fasting plasma glucose (even when repeated twice).

2. A1C is better related to cardiovascular disease than FPG.

3. Fasting is not needed for A1C assessment.

4. Standardization of A1C assay is not inferior to blood glucose assay.

5. A1C can be used for diagnosing and initiating diabetes monitoring.
Chronic hyperglycemia is captured by A1C but not by FPG (even when repeated twice).

- Fasting plasma glucose (FPG) or Oral glucose tolerance test (OGTT) gauge just a moment of a single day.

- Labeling a person with a diagnosis of diabetes has several psychological and legal implications and requires a robust and reliable approach.

- A diagnostic tool gauging chronic rather than spot hyperglycemia is certainly preferable.

- **A1C is better associated with chronic complications than FPG**
A1C is better related to cardiovascular disease than FPG.

- Cardiovascular disease (CVD) is the most frequent chronic complication of diabetes.
- CVD incidence rates 5- to 10-fold higher than with microvascular disease.
- FPG is a poor marker of future CVD events, whereas A1C is a good predictor.
Fasting is not needed for A1C assessment.

- FPG (requires overnight fasting) less reliable for diabetes diagnosis, with results sometimes falsely elevated.

- OGGT requires hours in the laboratory, with additional analytical and non-analytical costs.

- A1C can be measured anytime (provides immediate diabetes diagnosis) irrespective of fasting or feeding.
Standardization of A1C assay is not inferior to blood glucose assay.

- A1C is aligned to the Diabetes Control and Complications Trial (DCCT)/UK Prospective Diabetes Study (UKPDS) standard should the recommended target be pursued (in general <7%).

- The standardization program provides more reliable information to physicians who monitor diabetic patients

- The standardization is expected to minimize laboratory biases and is a prerequisite to **use A1C not only for monitoring but also for diagnosing diabetes**
A1C can be used for diagnosing and initiating diabetes monitoring.

- Deviation from individualized A1C targets prompts physicians to modify treatment strategies with lifestyle intervention and/or drug titration or changes.

- A1C for diagnosing diabetes has the advantage that, in subjects:
  - A1C ≥6.5% baseline A1c is already measured and deviation from target is immediately available.
  - A1C of 6.00–6.49% (high risk of diabetes), an effective prevention strategy can be immediately undertaken with a single A1C (more reliable than a single FPG to stratify the risk of the disease).
  - A1C of 5.50–5.99% plus other diabetes risk factor, counseling can be immediately offered because diabetes risk is substantial, and single A1C assessment (more reliable than single FPG to capture chronically high-normal glucose levels).
Benefits of using HbA1c to diagnose diabetes

The “Gold standard” methodology for HbA1c determination

High Performance Liquid Chromatography (HPLC) principle

Advantages of HPLC for HbA1c testing

What is the experience worldwide?
HPLC is the gold standard methodology for HbA1c determination

- The determination of **HbA1c** is an important diagnostic tool for:
  - monitoring the efficiency of dietary control
  - therapy during treatment of diabetes mellitus.

- Due to its excellent **specificity and reproducibility**, High Pressure Liquid Chromatography (HPLC) is still considered as the "**gold standard**" method for the determination of HbA1c.
HPLC is the gold standard methodology for HbA1c determination

- **Facts to also consider:**

- American Diabetes Association (ADA), recommended that HbA1c equal or more than 6.5% can now be used to diagnose diabetes.

- In 2011, World Health Organization (WHO) agreed with this recommendation.
HPLC is the gold standard methodology for HbA1c determination

- **Case study:** 205 patients with SC and diabetes was analyzed on *turbidimetric immunoassay* (non-certified method of HbA1c estimation) and *HPLC* instrument

- Some HbA1c tests give *falsely high or low readings* that can lead to the over-treatment or under-treatment of diabetes (i.e. SC trait)

- **Results:**
  - **Immunoassay** = 87.8% had HbA1c < 7%
  - **HPLC** = 16.1% had HbA1c < 7%
- **Conclusion:**

  - Immunoassay method in this study *produced lower HbA1c* (range 1-6.9% in 91.8 patients), compared to HPLC method.

  - *Falsely low readings* can lead to the under-treatment…

What methodology would you use to test HbA1c?
HbA1c can be measured by different methods.

- Ion-Exchange (HPLC)
- Cation-Exchange (HPLC)
- Boronate Affinity chromatographic
- Capillary Electrophoresis
- Enzymatic
- Immunoassay
<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Advantages</th>
<th>Challenges</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic</td>
<td>Measures Hb A\textsubscript{1c} using enzyme that specifically cleaves N-terminal valine</td>
<td>No analytical interference from Hb variants</td>
<td>Unable to detect Hb variants</td>
</tr>
<tr>
<td>Immunoassay</td>
<td>Uses antibody targeted against the glycated N-terminus of the β-chain</td>
<td>No analytical interference from the most common Hb variants using newer generation assays</td>
<td>Unable to detect Hb variants; Newer generation antibodies still susceptible to interference from rare Hb variants</td>
</tr>
<tr>
<td>Borate Affinity</td>
<td>Glycohemoglobin binds affinity resin while non-glycated hemoglobins pass through the column</td>
<td>Minimal analytical interference from Hb variants</td>
<td>Measures all glycated Hbs, not just Hb A\textsubscript{1c}</td>
</tr>
<tr>
<td>Ion-Exchange HPLC</td>
<td>Separates Hb species based on charge</td>
<td>Ability to detect the most common Hb variants</td>
<td>Prone to interference by Hb variants that co-elute with peaks of interest</td>
</tr>
<tr>
<td>Capillary Electrophoresis</td>
<td>Separates Hb species based on charge and hydrodynamic volume</td>
<td>High chromatographic resolution and resulting ability to detect many Hb variants</td>
<td>Throughput</td>
</tr>
</tbody>
</table>

Hb, Hemoglobin; HPLC, high performance liquid chromatography
HPLC is the gold standard methodology for HbA1c determination

- Regardless of methodology (whether HPLC or IA)

- Conditions that affect red cell turnover (normally 120 days) must be taken into consideration
  - hemolysis
  - significant blood loss
  - thalassemia
  - hemoglobin variants
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High Performance Liquid Chromatography (HPLC) principle
HPLC - Basic Hardware

Mobile Phase (Buffers)

Injector

Stationary Phase

Pump

Detector

BIO-RAD
Ion Exchange Chromatography

- More highly charged molecules are more tightly bound to the resin, and so travel slowly and are eluted later.
- Moderately charged molecules equilibrating between the resin and the moving buffer more readily.
- Less charged molecules bind less strongly to the resin, equilibrate with the moving buffer more readily, and so travel rapidly and are eluted sooner.
HPLC Separation

Flow

Analysis Cartridge

Fraction A: strong affinity and move slow

Fraction B: weak affinity and move fast

Retention Time
INTEGRATION PARAMETERS

Signal

Peak

Area

RT: Retention time

Time
Peak area’s

- The area bounded by an individual peak

- Expressed as a % ratio of “Total area”

- **Total area** is critical for correct integration
  - The total area indicates the degree of color intensity- or basically the hematocrit (Abs)
  - Since the HPLC detector has a specific dynamic range for absorbance if the total area is too low or too high the sample will be altered
    - Anemia, Polycythemia
    - Short sample
Chromatogram from a Bio-Rad ‘D-10’

- A0
- Labile HbA1c (Schiff base) and carbamylated Hb
- HbA1c
- HbA1a & HbA1b Minor components of HbA
- Total area 1M-4M
- A1c result
HPLC provides a **highly reliable diagnostic tool** provided the environment is **locked down** with respect to:

- Sample integrity
- Correct calibration
- Buffer concentration
- Buffer flow rate
- Column Temperature
- Resin stability

HPLC needs
To summarize

- Good quality resin
- Good integration parameters

- The separation is driven by tight control of:
  - Temperature
  - Flow rate
  - Increasing buffer strength
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Advantages of HPLC for HbA1c testing

1. Based on ‘charges’ so can **SEE different peaks**- get ‘more’ for your $

2. Not influenced by low Hb concentration - Ballion effect for IA – know not to report by Total Area Flag

3. Not influenced by heterophile Antibody

4. Provides physician the full patient picture

5. HPLC has the best CV on the CAP surveys -Superior precision (even though IA are improving)

6. See interferences influencing the HbA1c result
Hb S

Found throughout Africa - highest in Nigeria, Ghana, Gabon and Zaire
Found in Saudi Arabia and Kuwait
Found in East Central India
Thru migration/history found in the USA and Latin America

(Source: Internet)
Hb S Disease – Transfusion is usually required
Entire patient picture with HPLC –
One can ‘SEE the DIFFERENCE’
Post transfusión
- HbSC
- Este persona sin transfusión no tiene Hb A
- solo Hb S y Hb C
- Entonces no HbA1c
Physician has more information!!

Ion Exchange

Immunoassays for HbA1c – JUST a NUMBER

8.00% A1c
Advantages of HPLC for HbA1c testing

Clinical Case Study

A 14-Year-Old Boy with Chronic Cyanosis, Mild Anemia, and Limited Physical Resistance to Stress

Berndt Zur,1,2 Bernd Mayer-Hubner,2 Michael Ludwig,3 and Birgit Stoffel-Wagner1

Clinical Case Study

No abnormalities were found. Hemoglobin capillary electrophoresis (Capillary; Sebia) also revealed no abnormalities. A sample of arterial blood analyzed by co-oximetry for oxygen saturation showed a normal oxygen pressure of 94 mmHg (reference interval, 70–100 mmHg) and a decreased arterial oxygen saturation value of 84% (reference interval, >96%). Methemoglobin and carboxyhemoglobin values were normal. The partial pressure of O₂ at which hemoglobin is half-saturated (P50) in whole blood was measured with a blood gas analyzer and found to be increased [39 mmHg; normal, 26 mmHg (J)].

Clinical Case Study

Unexpected Hemoglobin A₁c Results

Alina-Gabriela Sofronescu,1 Laurie M. Williams,1 Dorinda M. Andrews,1 and Yusheng Zhu1,*
Advantages of HPLC for HbA1c testing

- Only HPLC utilizing ion exchange chromatography measures HbA1c.

- **Affinity columns measure any hemoglobin** that has glucose attached regardless of its attachment point or its structure because the column binds the glucose portion of the molecule. *Any variant hemoglobin that is present will be detected as glycated products.*

- Immunoassays also measure more than HbA1c, e.g., the Ab is reactive with HbS1c, HbC1c and HbE1c. *Glycated HbF is not detected, for most immunoassays.*

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What is the experience worldwide? - MEXICO

- Prospective study in Mexico: HPLC Retention Time as a Diagnostic Tool for Hemoglobin Variants and Hemoglobinopathies: A Study of 60,000 Samples in a Clinical Diagnostic Laboratory

- Compared RT on alkaline and acid hemoglobin electrophoresis, globin chain electro., isoelectric focusing and DNA analysis to Bio-Rad Variant II HPLC system

- Results:
  - SD and CV of RT was 1.0 (0.7%) with no statistical difference but............
Retention time on HPLC was superior to electrophoresis for the differentiation and identification of:

- 6 members of the Hb J family
- 4 members of the Hb D family
- 3 variants with electrophoretic mobilities identical or similar to that of Hb C.
- 6 variants with electrophoretic mobilities identical or similar to that of Hb S

HPLC detected 2 variants (Hb Ty Gard and Hb Twin Peaks) missed on electrophoresis.

What conclusion was made from this study?
Conclusion:

The retention time on HPLC is reliable, reproducible, and in many cases superior to conventional hemoglobin electrophoresis for the detection and identification of hemoglobin variants.

Confirmatory testing by electrophoresis can be eliminated in the majority of cases by use of retention time, proportion of total hemoglobin, and peak characteristics of HPLC.
Case report:

- 46-year-old AA man with normal CBC count
- No history of diabetes mellitus but had borderline hypertension
- 30-year smoking history of 7 cigarettes per day.
- Took no medication and had quit smoking 8 months before the current visit
- No family history of anemia and no known hemoglobinopathies
- Patient was asymptomatic with a normal hemoglobin value of 13.6 g/dL but had increased target cells on a peripheral blood smear
Results:

Boronate-affinity HPLC provided a value of 3.9% (reference range, 4.0%-6.9%), more consistent with the patient’s recent blood glucose values.

Ion-Exchange HPLC (Beta-Thal short program) – revealed an Unknown peak of 26.3% at 4.84 minutes and A2 of 5.1%.

LCMS - no mass shift in $\alpha$-globin or $\beta$-globin proteins was observed.
What is the experience worldwide?

- BA (HPLC)- does not allow recognition of Variant HgB
- Ion-Exchange (HPLC) – gives more than just a number…..
- “See the Difference” with Bio-Rad HPLC
HbA1c and MBG

- Patients could be harboring a hemoglobin variant that interferes with immunologic detection of HbA1c, one cannot know whether a patient's HbA1c levels are accurate.

- This situation might be suspected if the level of HbA1c is different than would be expected based on the results of a patient's self monitoring blood glucose (SMBG) levels.

- If possible, all patients should have at least one HPLC assay for HbA1c to rule out the presence of interfering hemoglobin variants.
High Performance Liquid Chromatography (HPLC)