High Performance Liquid Chromatography vs Immunoassay for HbA1c testing

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Objectives

By the end of this session, you will be able to:

1. Describe different needs of IA (immunoassay)

2. Understand IA and HPLC differences

3. Discuss why HPLC is a superior methodology for HbA1c
Agenda

- Theory behind the needs of a good immunoassay
  - Types of assays and why so many

- Theory of HPLC

- HbA1c testing in the lab - methodologies
# 6 in the World - MEXICO  16.4 MILLION

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What is an immunoassay (IA) ?

- A test that uses Antigen (Ag) and Antibody (Ab) complexes that can generate a measurable result thru some signal. The unknown analyte in an IA that is being measured can be an Ag or Ab.

- This is different than a basic colorimetric test which just uses the analyte being measured and some chemical to generate a color change (ie. Creatinine – Jaffe reaction uses Picric Acid)
Optimal Strategy of Immunoassay Design

- Allosteric effects - minimize
- Sample volume - optimize
- Incubation time - optimize
- One epitope
- No non-specific binding
Bond strength / Binding energy

Hydrogen Bonding

Electrostatic

Van der Waals

Hydrophobic

H₂O
Epitope (multiple) Antigen determinant
Types of Assays

Another example of confounding nomenclature

- Homogeneous vs. Heterogeneous
- Immunometric vs. ‘competitive’
- Reagent excess vs. reagent limited
- Rate or endpoint
- **Homogeneous** simply means that bound(B) and free(F) Ab are discriminated without separation. There is a change in signal due to the insolubility of the Ag-Ab complex detection.

- **Immunometric** is typically when Ab is in excess and Abs are against different epitopes of Ag. (reagent excess)

- **Competitive** is typically when the Ab is limited so as to have a certain # of Ab sites for the unknown Ag and the conjugated Ag to ‘fight’ or ‘compete’ for. The dose response curve is inversely proportional to concentration.

- **Equivalence** This is when a visual precipitate of Ag - Ab complexes forms. Examples of this are RID, Immunoelectrophoresis, Nephelometry and Turbidimetry.
Why the diversity?

- Permutations of:
  - Ab
  - Molecular Size
  - Calibration Methods
  - Separation Systems
  - Signal Generation
Homogeneous Assay

- Simplicity
- NO separation of B/F - typically not as sensitive as a heterogeneous assay
- Good for drug monitoring - Why? Because therapeutic drugs circulate at high enough levels as not to be overly concerned with sensitivity
- Suitable for uncomplicated automation
- Examples
  - Agglutination
  - Nephelometry
  - Turbidimetry
  - Fluorescent Polarization
Heterogeneous Assay

Who says size isn’t important?

- Hapten + Peptide - MacroMolecule (Alice in Wonderland or pantalla)
- Immunometric typically refers to reagent excess or Ab excess
- Separation between B/F

- Basically 3 Types of heterogeneous immunoassays
  - A. Competitive Assay with Solid Phase Separation (small molecular weight SMW)
  - B. Immunometric Assay (for detecting Antigen) (LMW)
  - C. Immunometric Assay (for detecting Antibody)
Sandwich Assay (non competitive)
Competitive Assay
Turbidimetric Assay (HbA1c)

**Competitive binding principle**

1. Hemolysate + Particles in Reagent 1 → Reaction 1

   - Total Hb and HbA1c in hemolyzed EDTA blood bind with the same affinity to particles in R1. The amount of binding is proportional to the relative concentration of both substances in the blood.

2. Antibodies in Reagent 2/3 + HAMA (poly Ab) + GAMA (poly Ab) → Reaction 2
Considerations

- What is the assay used for?
  - Detection
  - Quantification
  - Monitoring

- Stability (lot-to-lot variation)

- Are epitopes altered?
Do immunoassays measure the analyte?

They estimate (quantitatively) by a direct comparison with standard material
What, Where, How, of Standard Material

Definitive Method (REAL ‘Reference Method’)

- ’Well established’ method can be used as reference
- ‘Well established’ does NOT mean better
- Need a commonality across the board
  - ISO
  - NCCLS
  - WHO
  - NIBSC
  - For HbA1c – NGSP / IFCC
To be MONOCLONAL or POLYCLONAL?

- **Monoclonal Ab** - unending supply of Ab with a **SINGLE SPECIFICITY = MONO**

- **HYBRID + OMA**

- **IDENTICAL CLONE** that has same Ig class, allotype, the same variable region, structure, idioype, affinity and specificity for a given epitope. (WOW - no wonder it revolutionized immunodiagnistics! (as well as therapeutics)
Monoclonal Ab production scheme
POLYCLONAL Antibodies

Multivalent Antigen Complex + Polyclonal Antibodies → Antibody-Antigen Complex
- **Mixture** of Abs with different affinities can be a "double edged sword"

- Some can have low affinity due to nature of polyclonal makeup - can have High Dose Tolerance” (depends on frequency of immunization to the animal) ie. – cause of Lot-to-lot variation
I hope they bleed me at the right time for proper **polyclonal antibody** production

Yo tambien
Major Labeling Schemes or Signal Generation

RIA
Radioimmunoassay $^{125}\text{I}, ^{14}\text{C}$

EIA
Enzyme Immunoassay
Alkaline phosphatase
Horseradish peroxidase
They have a free amino left open for conjugation
FIA Fluorescent

FPIA Fluorescent Polarization

- Analyte referred to as Ligand
- When tracer is bound to Ab the tumbling is slowed
- Emitted light is more polarized
Assay imprecision caused by intrinsic and extrinsic factors

- B/F Separation - *incomplete separation* has been the main cause of imprecise results.

- Detection - How good *(stable)* is the signal being used? Detector?

- What *flavor* is the Antibody? (MAb or PolyAb)

- Manipulation errors in assay design
- Cross reactivity
  - Steroid Hormones

- Interference (The Matrix)
  - EDTA

- High Dose Hook Effect
  - Tumor Markers

- HAMAs ‘raton’ (SAMAs, ‘oveja’ GAMAS ‘cabra’)
  - False Pos and/or False Neg – HbA1c
HPLC principle

**High Performance Liquid Chromatography**
“and Moses stretched out his hand over the sea…..and the waters were divided.”

separated
COLUMN CHROMATOGRAPHY

Proteins are often fractionated by column chromatography. A mixture of proteins in solution is applied to the top of a cylindrical column filled with a permeable solid matrix immersed in solvent. A large amount of solvent is then pumped through the column. Because different proteins are retarded to different extents by their interaction with the matrix, they can be collected separately as they flow out from the bottom. According to the choice of matrix, proteins can be separated according to their charge, hydrophobicity, size, or ability to bind to particular chemical groups (see below).
Basic Hardware

Mobile Phase (Buffers)

Injector

Stationary Phase

Detector

Pump

Pump
HPLC principle in plain language

- Separating components of a mixture based on chemical or physical properties:
  
- In the case of hemoglobin
  - Charge (ion) differences
  - Hence we use “Ion exchange” chromatography
    CE – HPLC
  - Intercambio de cargas
Cation Exchange Chromatography

- Separates Hb based on charge differences

- Positively charged Hb are separated by their absorption on a negatively charged stationary phase in a column

- The cations (positive charge) in the mobile phase (buffers with increasing ionic strength) compete with the absorbed Hb eluting them off

- The fractions are detected optically by a spectrophotometer that measures the concentration of Hgb in each fraction which is quantified by calculating the area under the peaks
The 2 system buffers have different charge
- Buffer A is low Ionic strength
- Buffer B is higher Ionic strength
- Controlled blending generates a “Continuous” gradient of increasing charge – or “Step” gradient

The analytical resin has a – charge (carboxyl group)
- Why it is called cation exchange
- Which creates a competitive binding environment

Different Hb types have different charges
- Varying from very weak to very strong
- Hb Barts very weak, Hb C very strong
HPLC Separation

Flow

Analysis Cartridge

Fraction A: strong affinity and move slow

Fraction B: weak affinity and move fast
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
Chromatogram from a Bio-Rad ‘D-10’
The separation process

Buffer A flowing

Analytical cartridge
Carboxyl group (•) on resin

Detector

C’Gram
Any Hb with ionic strength less than buffer A passes straight through (Barts)

Other Hb’s and buffer A bind to Carboxyl group on resin
The separation process

- Gradual increase of buffer B
  Increases ionic strength

- Further Hb displacement caused by increased ionic strength of buffer mix
The separation process

Then 100% buffer B

- Complete Hb displacement along with any residual lower ionic strength buffer
The separation process

Final end of separation
Flush with buffer A

Analytical cartridge

Detector

C’Gram
INTEGRATION PARAMETERS

- Signal
- Peak
- Area
- RT: Retention time
- Time
To summarize

- Good quality resin
- Good integration parameters

- The separation is driven by tight control of:
  - Temperature
  - Flow rate
  - Increasing buffer strength
Now.....LETS LOOK At HbA1c
Red Blood Cells

- RBCs play a vital role in oxygen transportation to all the organs of the body and also the removal of carbon dioxide.

- Normal RBCs have a lifespan of 80 - 120 days (A1c)
Transition of the hemoglobin chains

EMBRYO

α₂ε₂
ζ₂γ₂
ζ₂ε₂

FETUS

α₂γ₂(HbF)
ε
γ
β
δ

ADULT

α₂γ₂ (HbF)
α₂δ₂ (HbA₂)
α₂β₂ (HbA)

Ratio (%)

Antenatal

Birth

Post-natal

0 3 6

0 10 20 30 40 50
Normal Hemoglobin Structure

- Composed of 4 subunits:
  - 2 α and 2 β chains = Hb A
  - 2 α and 2 δ chains = Hb A2
  - 2 α and 2 γ chains = Hb F = Fetal hemoglobin

- Normal individual:
  - 95% HbA
  - < 2% HbF
  - 1.5 - 3.5% HbA2

- A number of chemically modified hemoglobin structures can be present in the blood HbA1c
  - Carbamylated hemoglobin
  - Acetylated hemoglobin

**TODOS TIENEN CARGAS DIFFERENTES**
Percentage in whole blood depends upon:

1) Duration of glucose exposure to hemoglobin
2) Turnover rate of the RBCs
3) Concentration of glucose
   - **weighted** mean
   - prior 1 - 4 weeks determine 50%
   - prior 5 - 8 weeks determine 25%
   - prior 9 - 18 weeks determine final 25%
PATHWAY FOR THE MALLIARD REACTION

Protein (Hgb)  \( \text{NH}_2 \)

Glucose  \( \text{CH}_2\text{OH} \)

Protein  \( \text{CH}_2 \)

Amadori Products

\( \text{Oxidation} \)

Advanced Glycation Endproducts

Protein  \( \text{NH} \)

\( \text{CH}_2 \)

\( \text{C} = \text{O} \)

\( \text{OH} \)
GLYCOHEMOGLOBIN

- Potential glycation sites:
  - N-terminal amino acids of the four polypeptide chains
  - Free epsilon-amino groups of lysine within chains
- Most reactive site = N-valine terminal of beta chains (60% of bound glucose)
- Called HbA1c
 analyticaL methods

- **Ion exchange chromatography** - charge differences due to binding with glucose which changes isoelectric point of hemoglobin

- **Immunoassay** - mono or polyclonal antibodies directed against glycated N-terminal group of beta-chain of hemoglobin

- **Affinity chromatography** - reaction between bound glucose and immobilized boronic acid
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 operator will be altered
    - Anemia, Polycythemia
    - Short sample
Example from Bio-Rad’s HPLC instruments

- VII beta-Thal 1.0 – 3.0 Million
- VII Dual 1.5 – 3.5 Million
- D-10 1.0 – 4.0 Million
- D-10 1.0 – 5.0 Million
- Vnbs 1.0 – 3.5 Million

Mas o menos como hematocrit
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 LA

(Source: Internet)
Hb S
Single nucleotide polymorphisms is expected 1:1000 to 1:100 bases
Entire patient picture with HPLC – 
One can ‘SEE the DIFFERENCE’

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Concentration:

| % A1c | 4.1 |

HbA1c% Degree of Glucose Control

> 8  Action Suggested
< 7  Goal
< 6  Non-diabetic level
VII Bio-Rad

- Post transfusión
  - HbSC
- Este persona sin transfusión no tiene Hb A
  - solo Hb S y Hb C
- Entonces no HbA1c
Patient with abnormal Hb

HPLC - Ion Exchange

Immunoassay

7.3% A1c
Una pintura vale mil palabras

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A1c Sleep 04/24/2012 01:49:52PM

Graph showing the A1c levels.
Clinical Case Study

A 14-Year-Old Boy with Chronic Cyanosis, Mild Anemia, and Limited Physical Resistance to Stress
Berndt Zur,¹ Bernd Mayer-Hubner,² Michael Ludwig,¹ and Birgit Stoffel-Wagner¹

Clinical Case Study

3) The abnormal hemoglobin is clearly visible

No abnormalities were found. Hemoglobin capillary electrophoresis (Capillary; Sebia) also revealed no abnormalities. A sample of arterial blood analyzed by cooximetry 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)].

Clinical Case Study

Unexpected Hemoglobin A₁c Results
Alina-Gabriela Sofronescu,¹ Laurie M. Williams,¹ Dorinda M. Andrews,¹ and Yusheng Zhu¹
SHORT COMMUNICATION
Advantage of HbA1c Assay by HPLC D-10 Versus Cobas Integra 400 in a Population Carrier for HbS and HbC

KAHENA BOUZID 1, AFEF BAHLOUS 1, WAFA FERJANI 1, EYA KALAI 1
Benefits 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.

HbA1c and MBG

• Because the patient could be harboring a hemoglobin variant that interferes with immunologic detection of HbA1c, one cannot know a priori 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.
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