

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

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November 2013

HPLC



BIO-RAD



7 in the World - MEXICO 10.7 MILLION

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

COUNTRY /TERRITORY	2011 MILLIONS	COUNTRY /TERRITORY	2030 MILLIONS
1 China	90.0	1 China	129.7
2 India	61.3	2 India	101.2
3 United States of America	23.7	3 United States of America	29.6
4 Russian Federation	12.6	4 Brazil	19.6
5 Brazil	12.4	5 Bangladesh	16.8
6 Japan	10.7	6 Mexico	16.4
7 Mexico	10.3	7 Russian Federation	14.1
8 Bangladesh	8.4	8 Egypt	12.4
9 Egypt	7.3	9 Indonesia	11.8
10 Indonesia	7.3	10 Pakistan	11.4



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 $\geq 6.5\%$** 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)



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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%



HPLC is the gold standard methodology for HbA1c determination

- **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?



HPLC is the gold standard methodology for HbA1c determination

HbA1c testing in the lab – methodologies...

- HbA1c can be measured by different methods.
 - Ion-Exchange (HPLC)
 - Cation-Exchange (HPLC)
 - Boronate Affinity chromatographic
 - Capillary Electrophoresis
 - Enzymatic
 - Immunoassay



Hb A_{1c} method characteristics

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

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



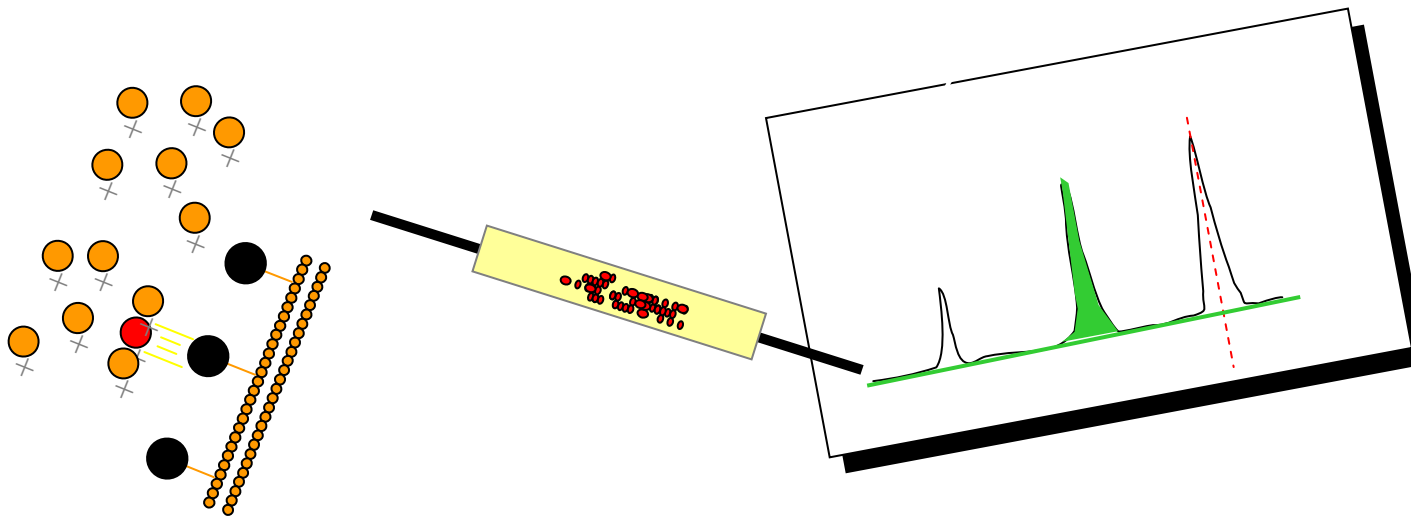
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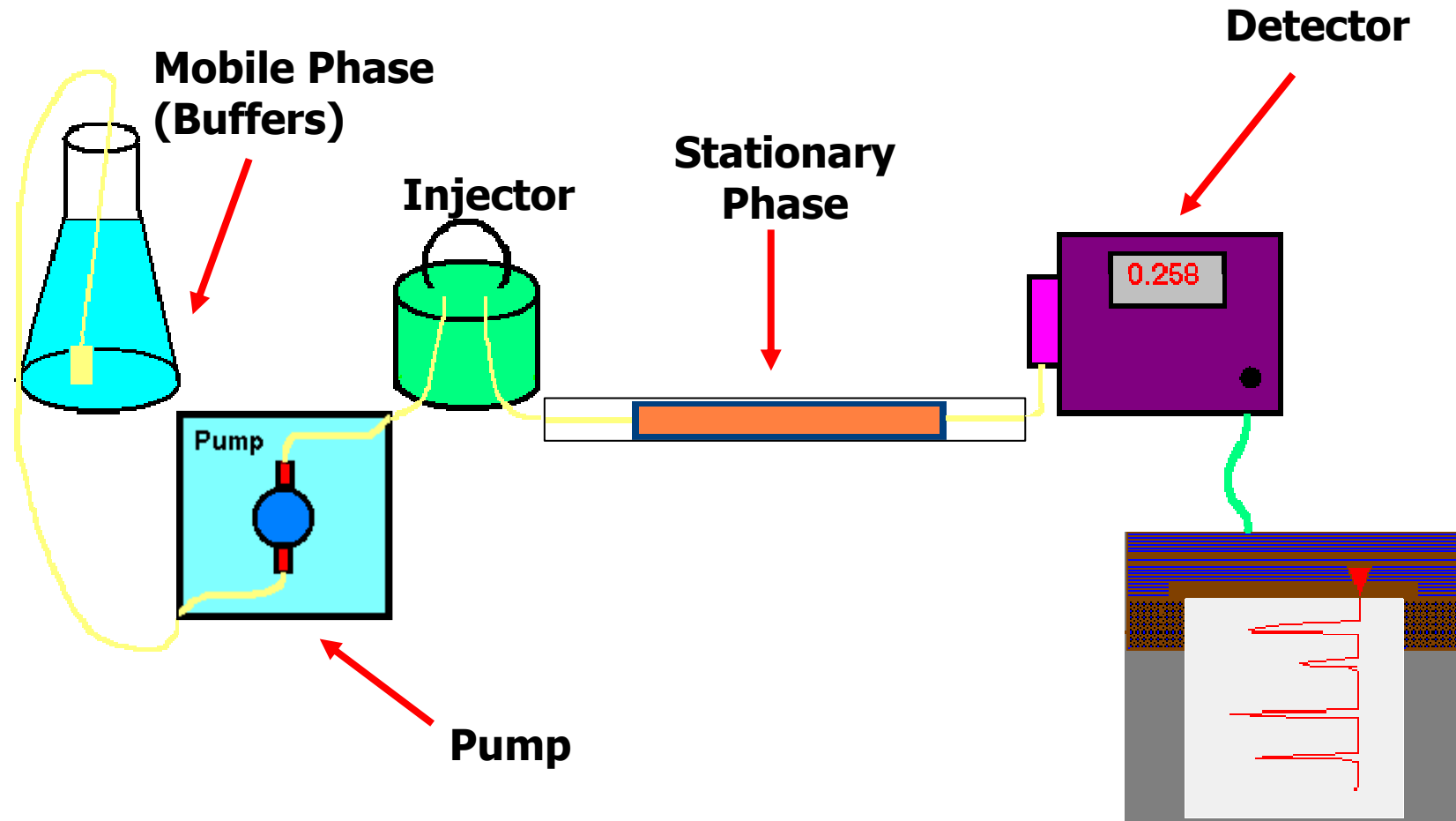
HPLC principle

High Performance Liquid Chromatography



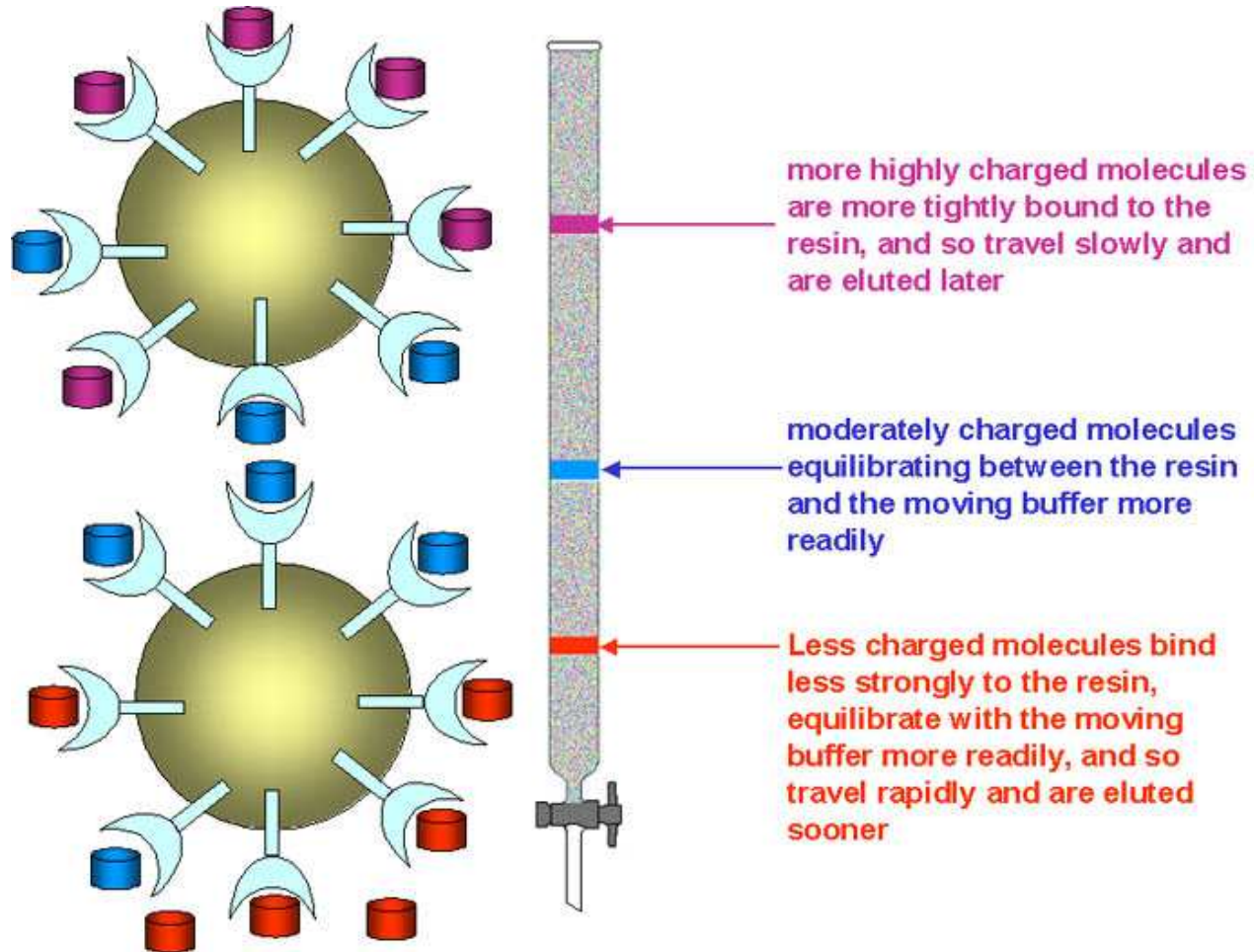


HPLC - Basic Hardware



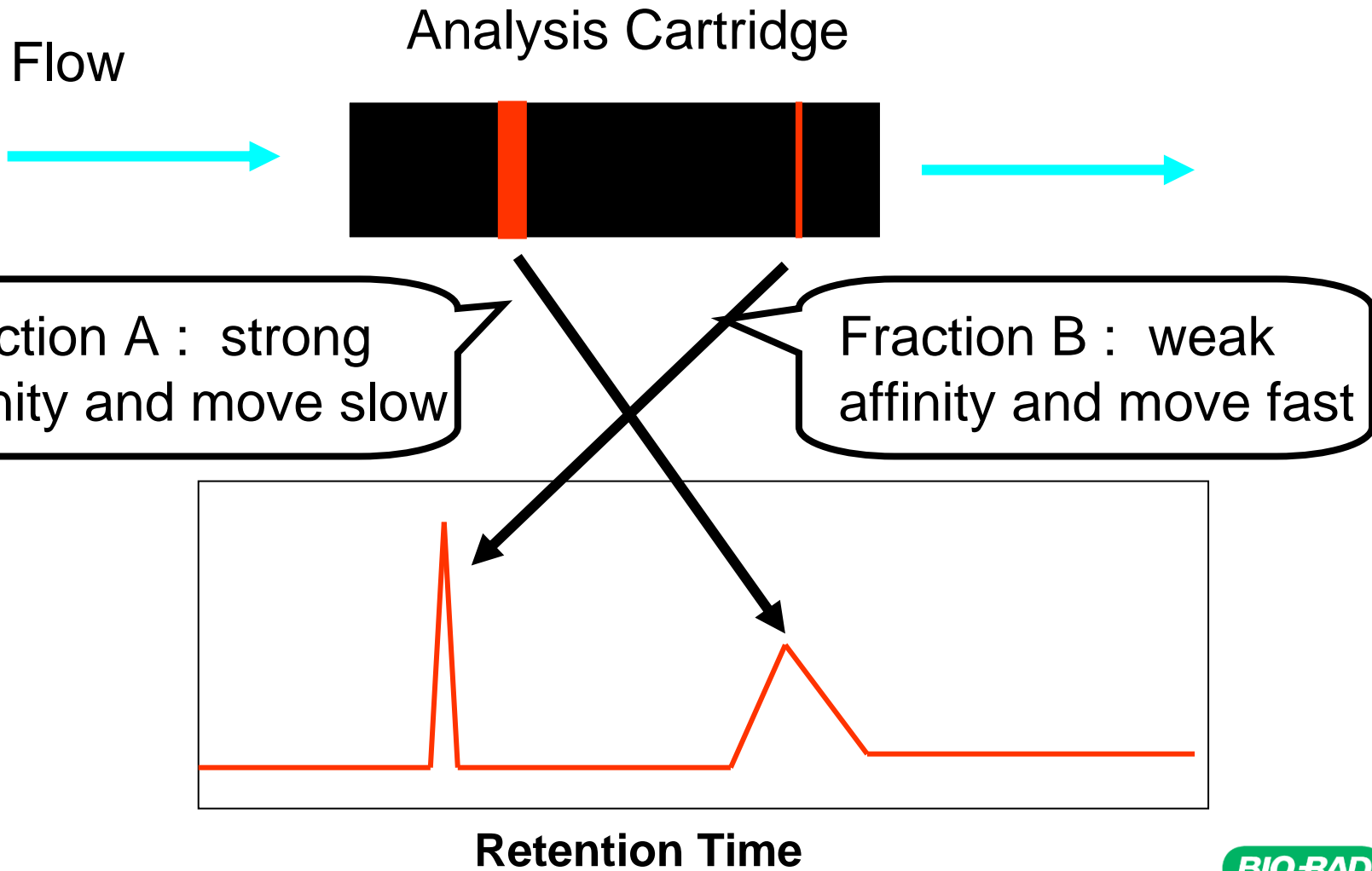


Ion Exchange Chromatography



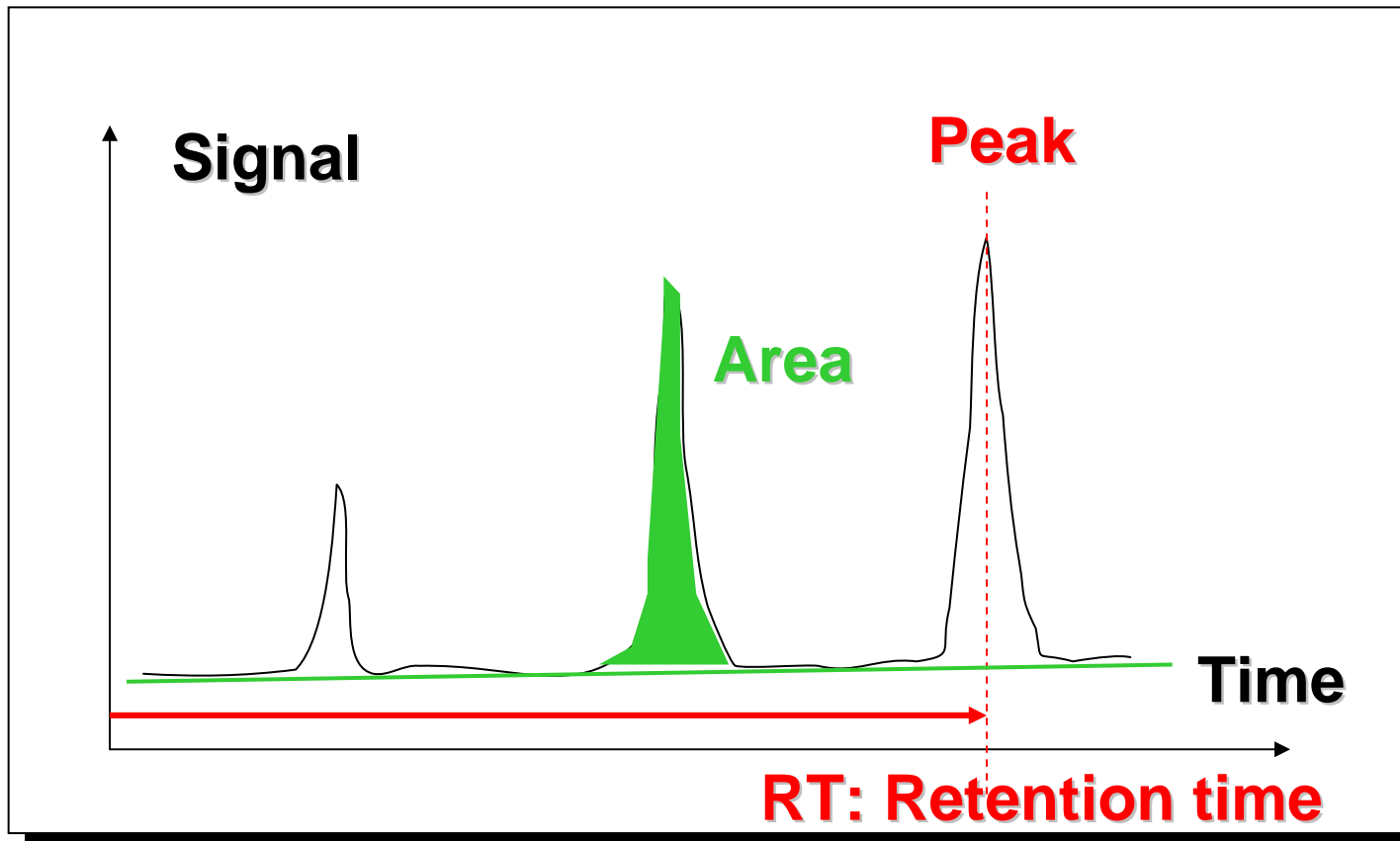


HPLC Separation





INTEGRATION PARAMETERS



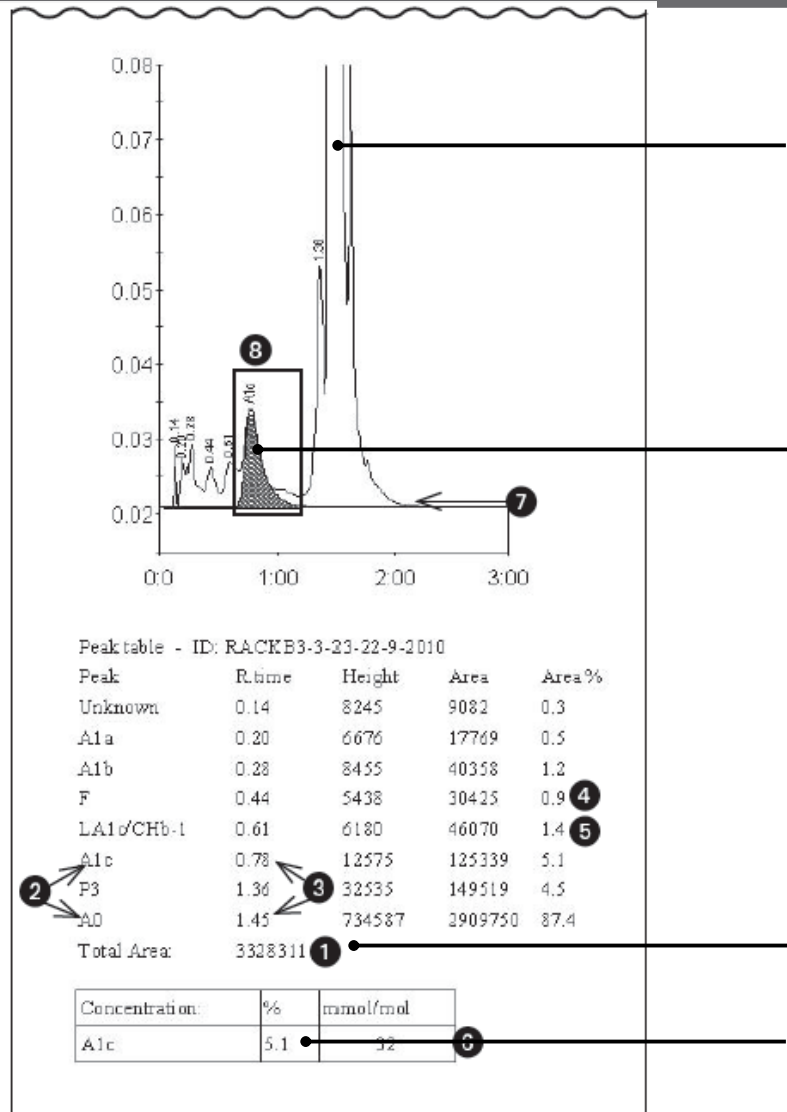


HPLC - Peak ID & quantification parameters

- 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

- 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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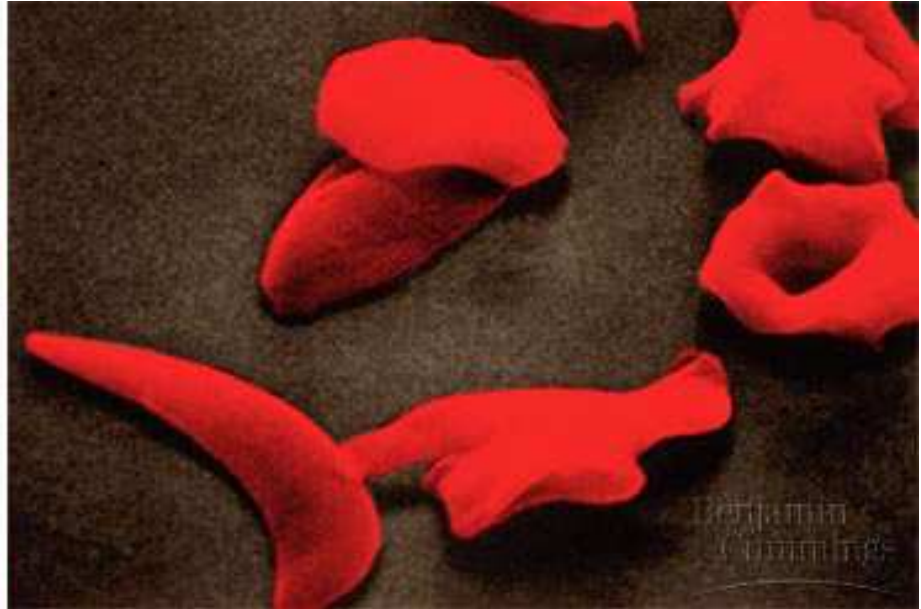
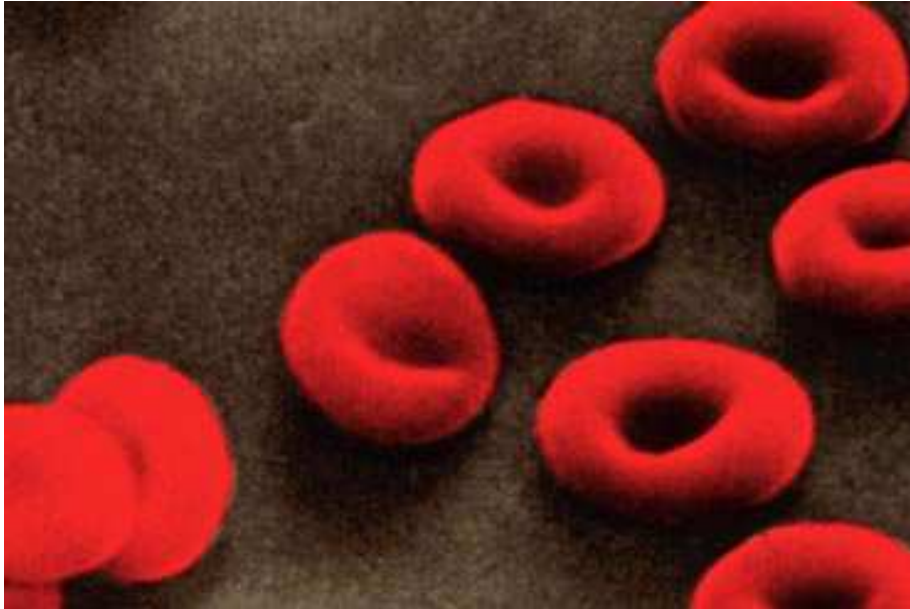


Advantages of HPLC for HbA1c testing

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



(Source: Internet)

Found throughout Africa- highest in Nigeria, Ghana, Gabon and Zaire

Found in Saudi Arabia and Kuwait

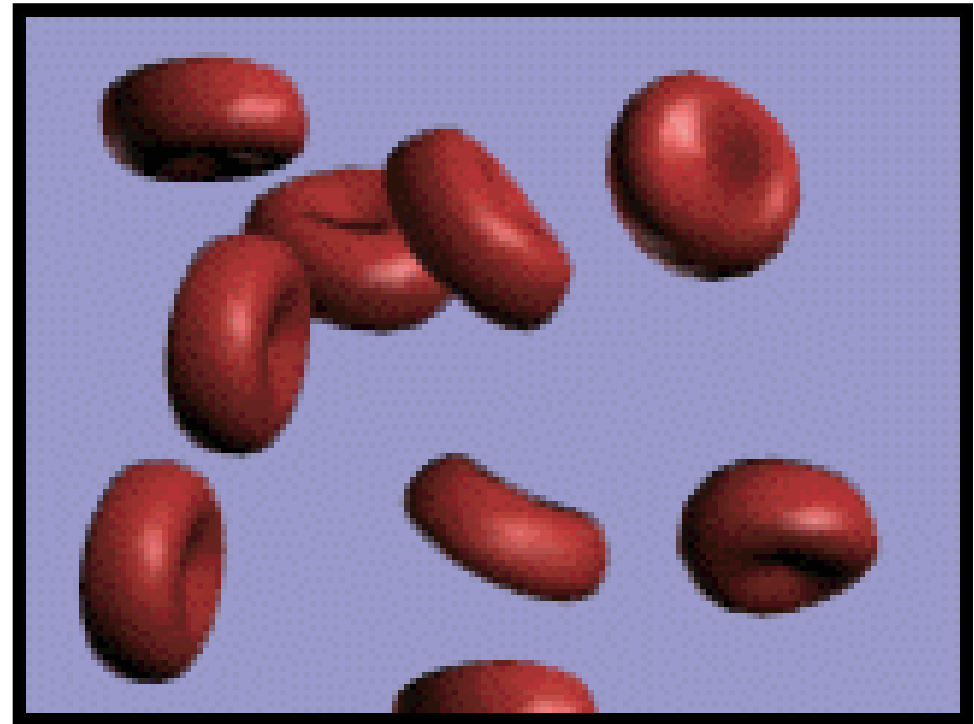
Found in East Central India

³⁰ Thru migration/history found in the USA and **Latin America**



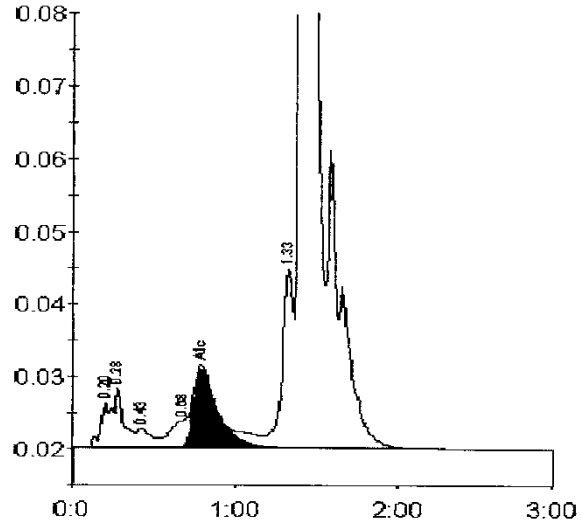


Hb S Disease – Transfusion is usually required



Patient report

Bio-Rad DATE: 08/17/2005
 D-10 TIME: 01:17 PM
 S/N: #DA3J255004 Software version: 3.07-1
 Sample ID: NP
 Injection date 08/17/2005 01:17 PM
 Injection N°: 1 Method: HbA1c
 Rack N°: 02 Rack position: 1



Peak table - ID: NP

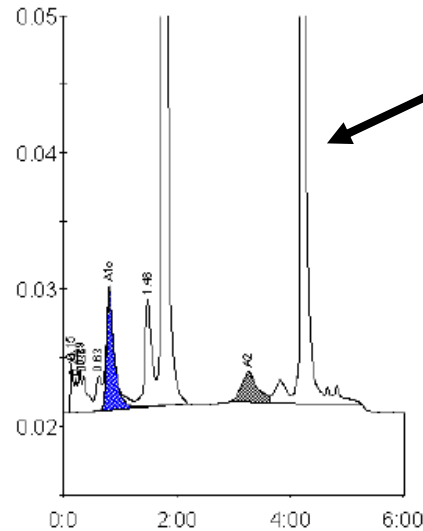
Peak	R.time	Height	Area	Area %
A1a	0.20	6342	20628	0.7
A1b	0.28	8345	41758	1.4
F	0.43	2722	18291	< 0.8
LA1c/CHb-1	0.68	3874	27304	0.9
A1c	0.80	10879	114524	4.1
P3	1.33	24821	122408	4.0
A0	1.41	637907	2716085	88.7
Total Area:		3060997		

Concentration:	
% A1c	4.1

HbA1c% Degree of Glucose Control
 > 8 Action Suggested
 < 7 Goal
 < 6 Non-diabetic level

Patient report

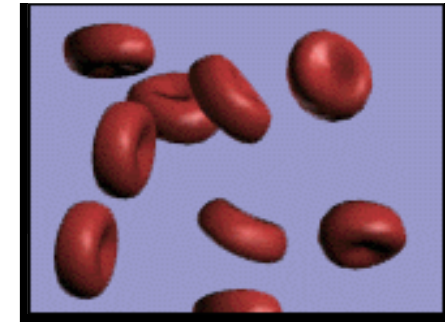
Bio-Rad DATE: 11/20/2006
 D-10 TIME: 11:10 AM
 S/N: #DA2G078002 Software version: 3.50-A1
 Sample ID: AS_SAMPLE
 Injection date 10/25/2006 05:31 PM
 Injection #: 117 Method: HbA2/F
 Rack #: E2 Rack position: 7



Peak table - ID: AS_SAMPLE

Peak	R.time	Height	Area	Area %
Unknown	0.15	3750	10754	0.7
A1a	0.22	2557	9867	0.7
A1b	0.29	3152	8824	0.6
Unknown	0.36	2646	17199	1.2
LA1c/CHb-1	0.63	2567	18201	1.3
A1c	0.81	8776	84553	11.1
P3	1.48	7783	68800	4.8
A0	1.75	140605	729917	50.8
A2	3.26	2192	43128	3.1
S-Window	4.20	75253	444531	31.0
Total Area:		1435775		

Concentration:	
% A1c	11.1
% A2	3.1



Entire patient picture with HPLC –
 One can ‘SEE the DIFFERENCE’





SEE the DIFFERENCE

VII Bio-Rad

- Post transfusion
 - HbSC
 - Este persona sin transfusion no tiene Hb A
 - solo Hb S y Hb C
 - Entonces no HbA1c

Bio-Rad CDM System 10995

Bio-Rad Variant II Instrument #1

PATIENT REPORT

V2_BThal

Patient Data

Sample ID: Unknown
 Patient ID:
 Name:
 Physician:
 Sex:
 DOB:

Analysis Data

Analysis Performed: 18/04/2001 16:39:38
 Injection Number: 196
 Run Number: 23
 Rack ID: 0006
 Tube Number: 6
 Report Generated: 18/04/2001 16:46:07
 Operator ID:

Comments:

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.8	---	1.08	31822
Unknown	---	0.6	1.20	23746
P2	---	3.9	1.27	151428
P3	---	4.7	1.61	183209
Ao	---	52.3	2.37	2046844
A2	3.8*	---	3.61	132100
S-window	---	17.4	4.52	680827
Unknown	---	0.3	4.84	10371
C-window	---	16.6	5.17	651240

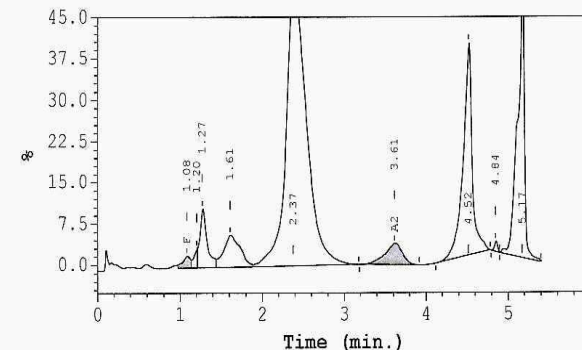
Total Area: 3911587

F Concentration = 0.8 %

A2 Concentration = 3.8* %

*Values outside of expected ranges

Analysis comments:

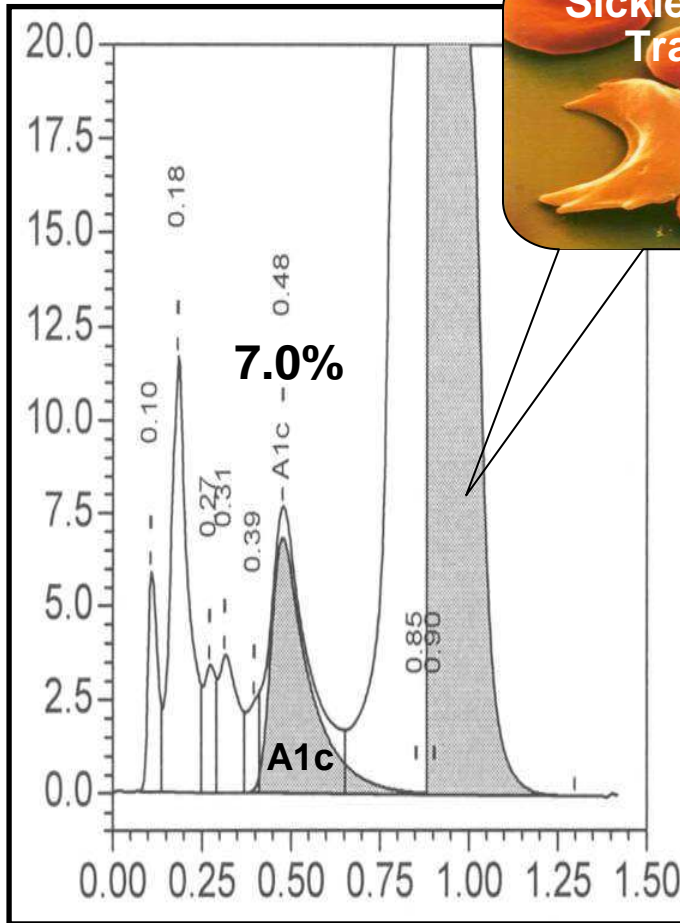




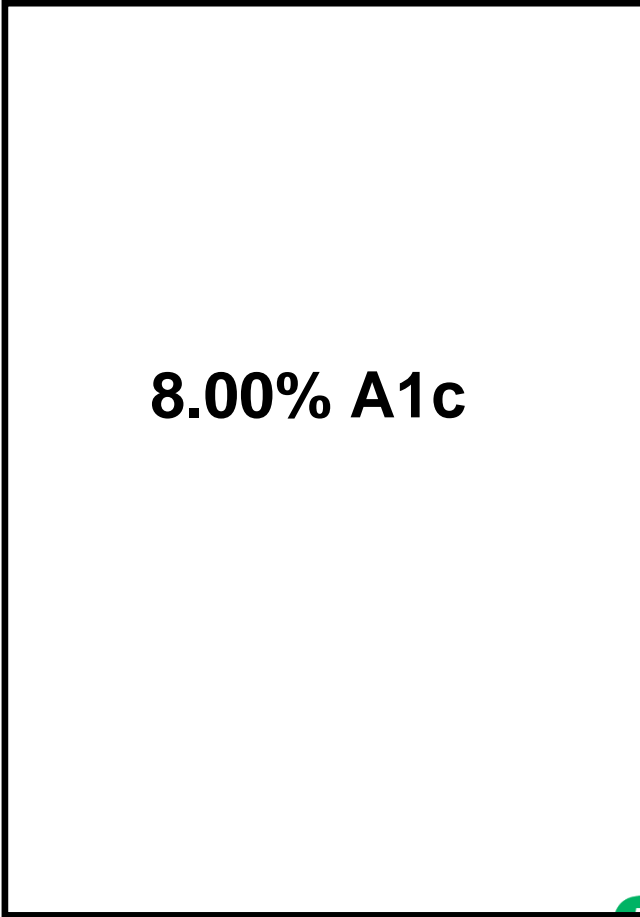
Immunoassays for HbA1c – JUST a NUMBER

Physician has more information!!

Ion Exchange



Immunoassay





Advantages of HPLC for HbA1c testing

Clinical Chemistry 58:2
332–336 (2012)

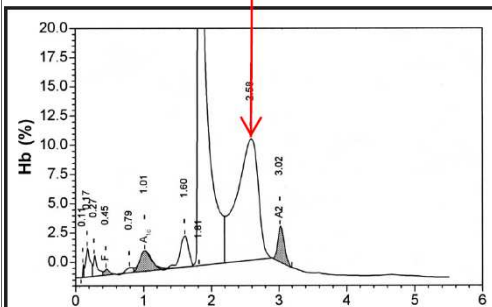
Clinical Case Study

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

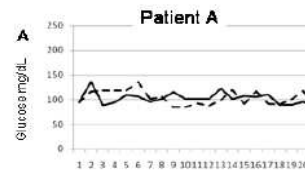


No abnormalities were found. Hemoglobin capillary electrophoresis (Capillarys; Sebia) also revealed no abnormalities. A sample of arterial blood analyzed by co-oximetry for oxygen saturation showed a normal oxygen pressure of 94 mmHg (reference interval, 70–100 mmHg) and a decreased arterial oxygen saturation value of 84% (reference interval, >96%). Methemoglobin and carboxyhemoglobin values were normal. The partial pressure of O₂ at which hemoglobin is half-saturated (P_{50}) in whole blood was measured with a blood gas analyzer and found to be increased [39 mmHg; normal, 26 mmHg (1)].

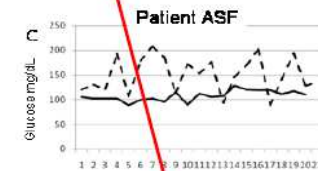
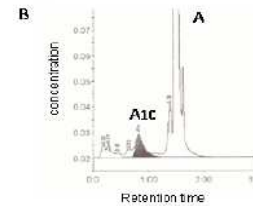
CLINICAL PRACTICE

HbA1c Does Not Always Estimate Average Glucose

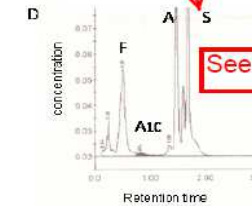
KRISTINA J BEHAN, JANE MERSCHEN



Average glucose = 5.7 mmol/L
(103 mg/dL)
Immunoassay HbA1c = 5.7%
HPLC HbA1c = 6.2%



Average glucose = 7.2 mmol/L
(130 mg/dL)
Immunoassay HbA1c = 4.7%
HPLC HbA1c = 7.0%



See the difference!

Clinical Chemistry 57:2
153–157 (2011)

Clinical Case Study

Unexpected Hemoglobin A_{1c} Results

Alina-Gabriela Sofronescu,¹ Laurie M. Williams,¹ Dorinda M. Andrews,¹ and Yusheng Zhu^{1*}



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.**

Clin Chem 2009; 55(No. 6 Supplement): A92.



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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.....



What is the experience worldwide?

- **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?



What is the experience worldwide?

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.



What is the experience worldwide? - CA

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



What is the experience worldwide?

- **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 α -globin or β -globin proteins was observed



What is the experience worldwide?

Scuderi et al / HbA_{1c} INTERFERENCE BY HEMOGLOBIN SHELBY

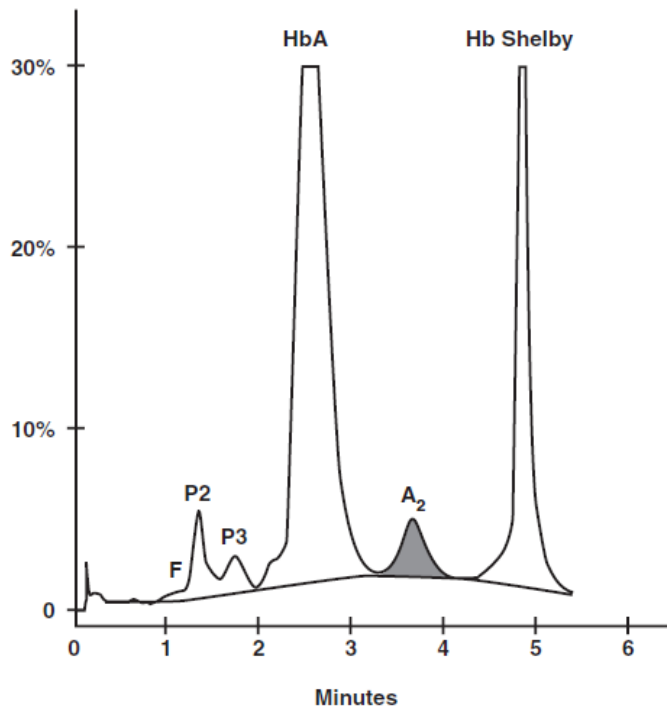


Figure 2 Hemoglobin (Hb) Shelby trait. High-performance liquid chromatography (VARIANT Beta Thal Short, Bio-Rad Laboratories, Hercules, CA) showing the standard peaks from left to right: HbF (fetal), P2, P3, HbA (61.7%) at 2.51 minutes, HbA₂ at 3.63 minutes, and the Hb Shelby peak (26.3%) at 4.84 minutes. Note also that HbA₂ is elevated at 5.1% (0.05).

- 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)

