Hemoglobin analysis by tandem mass spectrometry

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National Conversation: Tandem Mass Spectrometry in Newborn Screening
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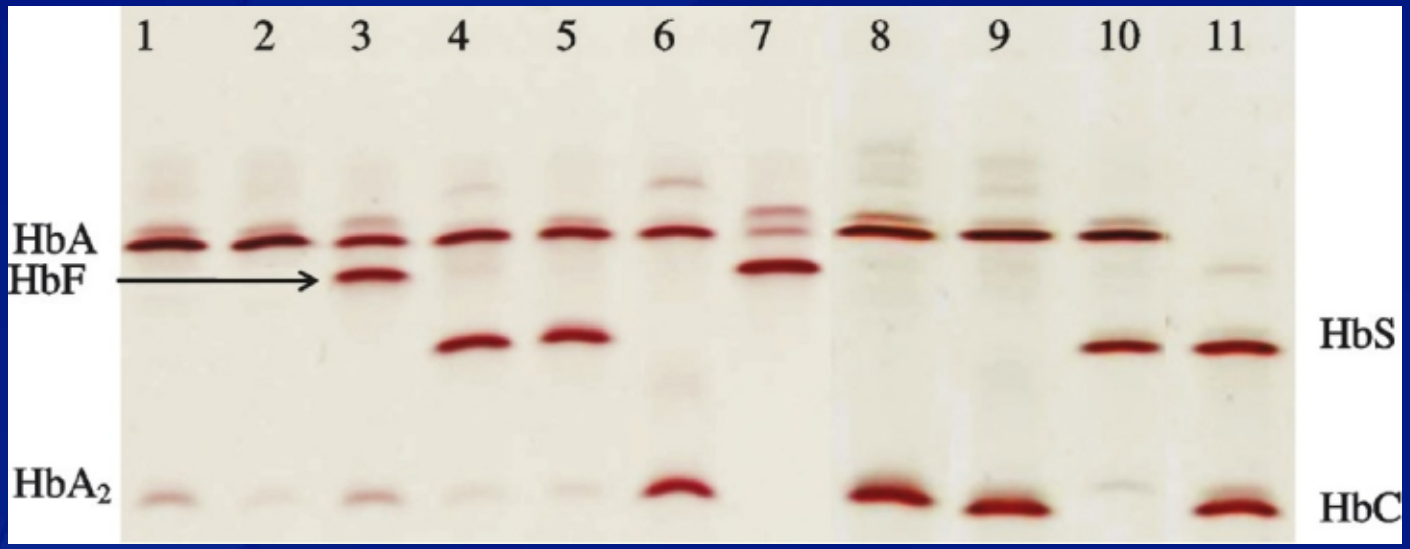
National Center for Environmental Health
Division of Laboratory Sciences

OVERVIEW

- Traditional hemoglobin analyses: IEF, HPLC-UV, DNA
- FIA-ESI-MS/MS hemoglobin analysis
- HPLC-ESI-MS/MS hemoglobin analysis
- Future directions

Hemoglobin analysis by isoelectric focusing (IEF)

Identification basis: co-migration

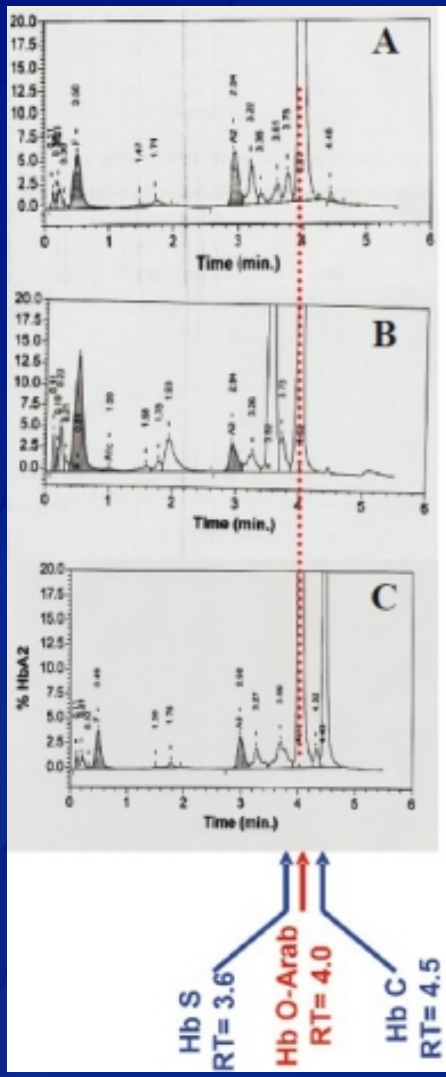


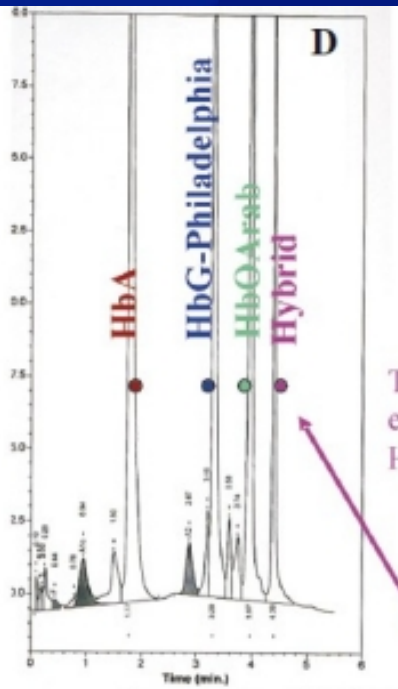
1: β thalassemia trait
2: Normal adult
3: Homozygous β^+ thalassemia
4: Hb A / Hb Korle Bu (D73N)
5: Hb A / Hb D-Punjab (E121Q)
6: Hb A / Hb O-Arab (E121K)
7: Normal newborn
8: Hb A / Hb E (E26K)
9: Hb A / Hb C (E6K)
10: Hb A / Hb S (E6V)
11: Hb S / Hb C

Wajcman and Moradkhani, Abnormal haemoglobins: detection and characterization, Indian J. Med. Res. 134, 538-546, 2010.

Hemoglobin analysis by HPLC-UV

Identification basis: retention time





The hybrid Hb ($\alpha_2^{G\text{-}Phil}\beta_2^{O\text{-}Arab}$) elutes at a RT similar to that of HbC.

	LA1c	---	0.4	0.803	9081
	A1c	7.4*	---	0.861	49003
	P3	---	2.7	1.524	55745
●	Ap	---	36.1	1.786	756921
	A2	2.2	---	2.924	48069
●	Unknown	---	21.8	3.325	455953
	Unknown	---	1.4	3.628	28380
	Unknown	---	1.6	3.785	33438
●	Unknown	---	18.9	3.992	396504
●	C	---	10.8	4.423	225498

Wajcman and Moradkhani, Abnormal haemoglobins: detection and characterization, Indian J. Med. Res. 134, 538-546, 2010.

Hemoglobin analysis by DNA methodologies

Identification basis: PCR / microarray

PCR-based methods:

Sanger sequencing of HBA1, HBA2, and HBB

("gold standard"-potentially identifies any mutation)

ASO (Allele-specific oligonucleotide hybridization)

ARMS (Amplification refractory mutation system)

PCR-RFLP (Restriction fragment length polymorphism)

Gap-PCR (PCR amplification across breakpoints)

Microarray-based methods:

Nanochip®(Nanogen, San Diego, CA, USA)

APEX (Arrayed primer extension)

SBE (Single-base extension)

MLPA (Multiple ligation probe amplification)

Hemoglobin analysis by different methodologies

Analysis	Advantages	Disadvantages
IEF	High throughput Inexpensive	Presumptive diagnosis (co-migration)
HPLC-UV	High throughput Inexpensive	Presumptive diagnosis (elution time)
DNA (Microarray, exon seq., Whole-gene seq., etc.)	Definitive diagnosis (amplicon / sequence)	Moderate throughput High equipment cost High reagent cost Expert analyst
ESI-MS/MS (Including trypsin digestion)	Definitive diagnosis (elution time w/ HPLC, precursor *m/z*, product ion scan) Low reagent cost	Moderate throughput High equipment cost Expert analyst

Hemoglobin: Monomer amino acid sequence β-globin and α-globin mutants

β MVHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDA
VMGNPKVKAHGKKVLGAFSDGLAHLDNLKGTFATLSELHCDKLHVDPENFRLL
GNVLVCVLAHHFGKEFTPPVQAAYQKVVAGVANALAHKYH

Globin	Name	HGVS* traditional	HGVS* recommended
Beta	HbS	(c.20A>T, p.E6V)	(c.20A>T, p.E7V)
Beta	HbC	(c.19G>A, p.E6K)	(c.19G>A, p.E7K)
Beta	HbE	(c.79G>A, p.E26K)	(c.79G>A, p.E27K)
Beta	HbD-Los Angeles	(c.364G>C, p.E121Q)	(c.364G>C, p.E122Q)
Beta	HbO-Arab	(c.364G>A, p.E121K)	(c.364G>A, p.E122K)
Alpha	HbG-Philadelphia	(c.[207C>G or 207C>A], p.N68K)	(c.[207C>G or 207C>A], p.N69K)

α MVLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLS
HGSAQVKGHGKKVADALTNAVAHVDDMPNALSALSDLHAHKLRVDPVNF
KLLSHCLLVTLAAHLPAEFTPAVHASLDKFLASVSTVLTSKYR

HGVS: Human Genome Variation Society, http://www.hgvs.org/

Tryptic peptides of β-globin

Trypsin cleaves proteins C-terminal to Arg (R) and Lys (K)

VHLTPEEK	βT1-Met	*m/z* 952.5*
SAVTALWGK	β T2	*m/z* 932.5
VNVDEVGGEALGR	β T3	*m/z* 1314.7
FFESFGDLSTPDAVMGNPK	β T5	*m/z* 2058.9

* *m/z* values of peptides predicted with Protein Prospector: http://prospector.ucsf.edu/prospector/mshome.htm

Tryptic peptides of β-globin mutants

Trypsin cleaves proteins C-terminal to Arg (R) and Lys (K)

VHLTPVEK	ST1-Met	*m/z* 922.5
SAVTALWGK	β T2	*m/z* 932.5
VNVDEVGGK	ET3	*m/z* 916.5
FFESFGDLSTPDAVMGNPK	β T5	*m/z* 2058.9

* *m/z* values of peptides predicted with Protein Prospector: http://prospector.ucsf.edu/prospector/mshome.htm

Newborn Blood Spot Screening for Sickle Cell Disease by Using Tandem Mass Spectrometry: Implementation of a Protocol to Identify Only the Disease States of Sickle Cell Disease

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Clin Chem. 2014 Feb;60(2):373-80

Goal: Identify only sickle-cell disease (Hb SS), not carriers (Hb AS) or variants

Tryptic digest of DBS punches: SpOtOn kit (Clinical Diagnostics) with labeled peptide

Flow injection analysis: CH_3CN / H_2O (50:50) with 0.025% HCOOH, 2 minutes

MS/MS: Single-reaction monitoring of selected peptides (most are 2^+ precursors)

Data analysis: ratios of peptide signals

e.g. S:A = (461.7→472.2) / (476.7→502.2)

Hemoglobin analysis by FIA-ESI-MS/MS (Wales)

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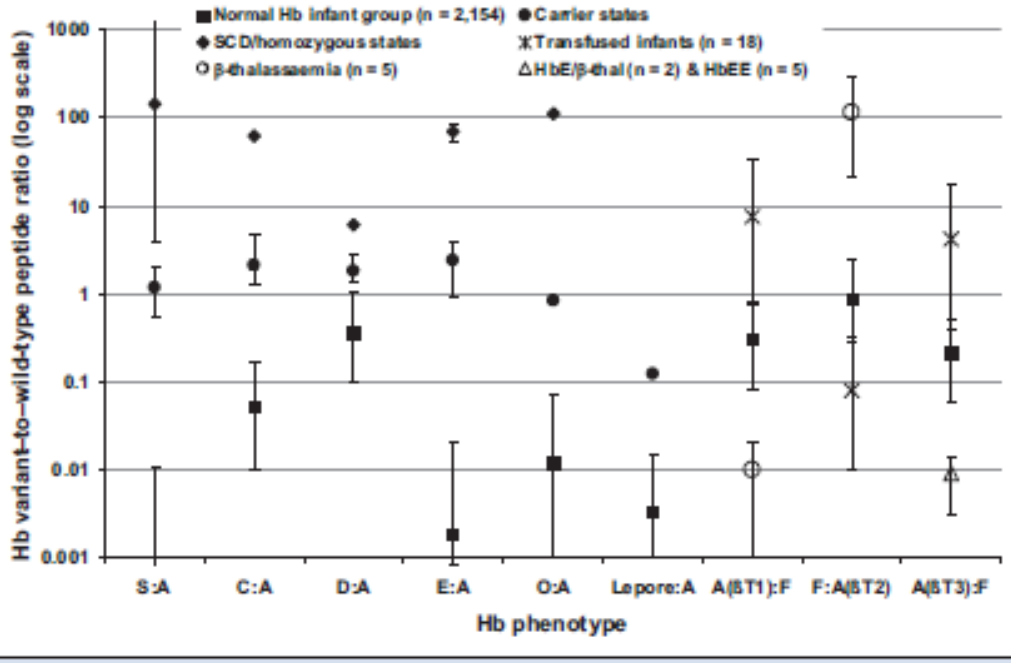


Fig. 1. Cutoffs for the target Hb variant–to–wild-type peptide ratios.

Data show the mean (1st and 99th centiles) for the ratios (logarithmic scale) in the normal hemoglobin infant group and the HbS infant carrier group and the mean (lowest and highest values observed) for the various Hb variant phenotypes. Samples from cases of β-thalassemia and HbE/β-thalassemia were included from both the Great Ormond Street and Birmingham Laboratories.

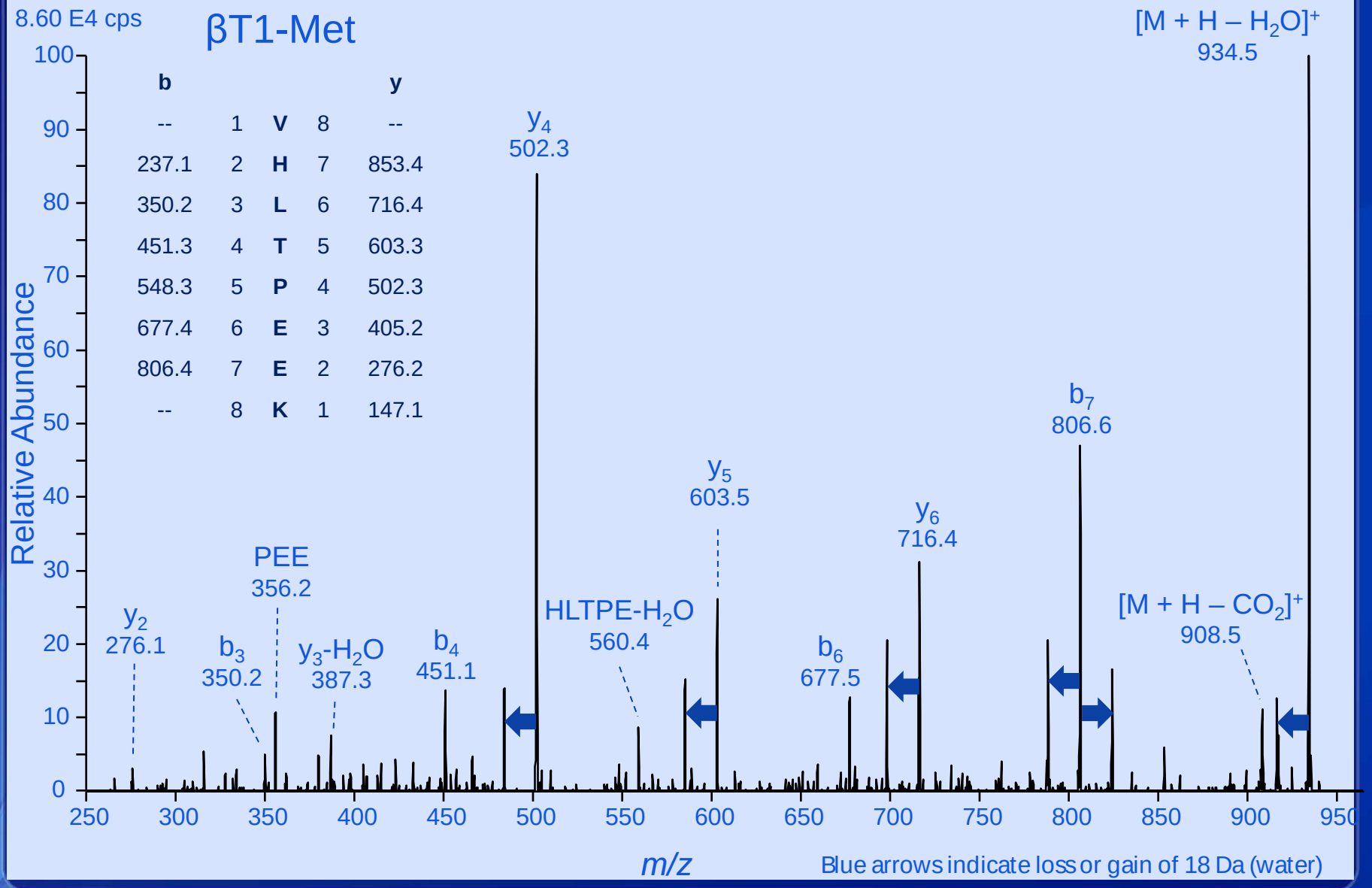
Hemoglobin analysis by HPLC-ESI-MS/MS at CDC

- Punch (3.2 mm) DBS; controls are HbAS adult and HbFA umbilical cord blood
- Trypsin digestion of punches in 96-well plate (50ºC for 30 min)
- Digestion solution contains stable isotope labeled peptide
- Stop digestion with 3 µL of HCOOH per well
- HPLC: Jupiter Protea C12 column (2 x 50 mm), A = 1:99 CH_3CN / H_2O 0.1% HCOOH, B = CH_3CN 0.1% HCOOH, 10 min linear gradient from 0 to 35% B
- MS/MS: Thermo LTQ-XL ion trap, positive ion mode, single-reaction monitoring

α-globin: T1, T10
β-globin: T1, T3, T12
γ-globin: T3, T13
Variants: ST1, CT1, ET3, DT12, OT12, GT10
Internal standard: cut and un-cut (VHLTP[+6]EEKSAVTAL)

Cost: $0.30 per sample

Product ion scan: *m/z*952.5 @6.2 minutes

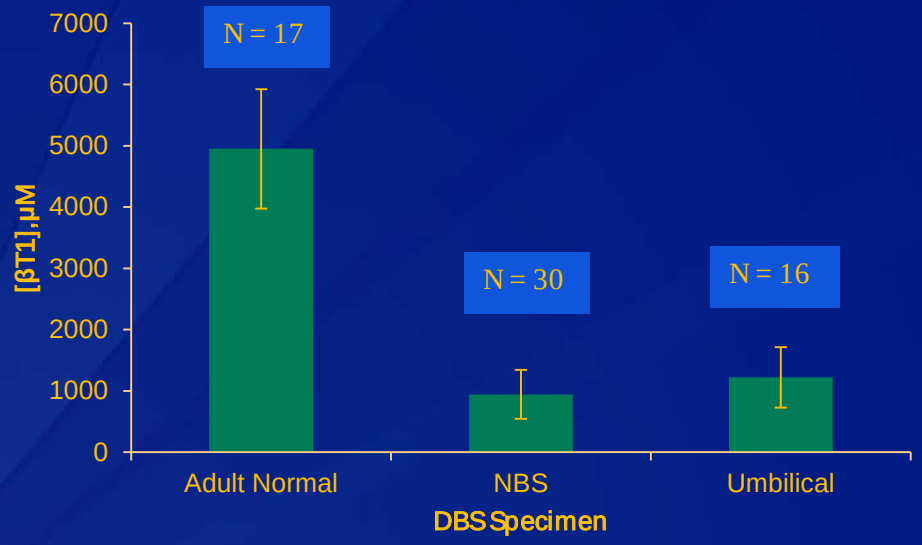


Hemoglobin analysis by HPLC-ESI-MS/MS at CDC

Variant identification: SRM signal at appropriate elution time
(HbS, HbC, HbE, HbD-Los Angeles, HbO-Arab, HbG-Philly)

Beta-thalassemia identification: quantitation of βT1 peptide

(952.5→502.3) / (958.5→508.3) x [IS, μM] x Dilution Factor



NBS DBS: 944 ± 399 μM

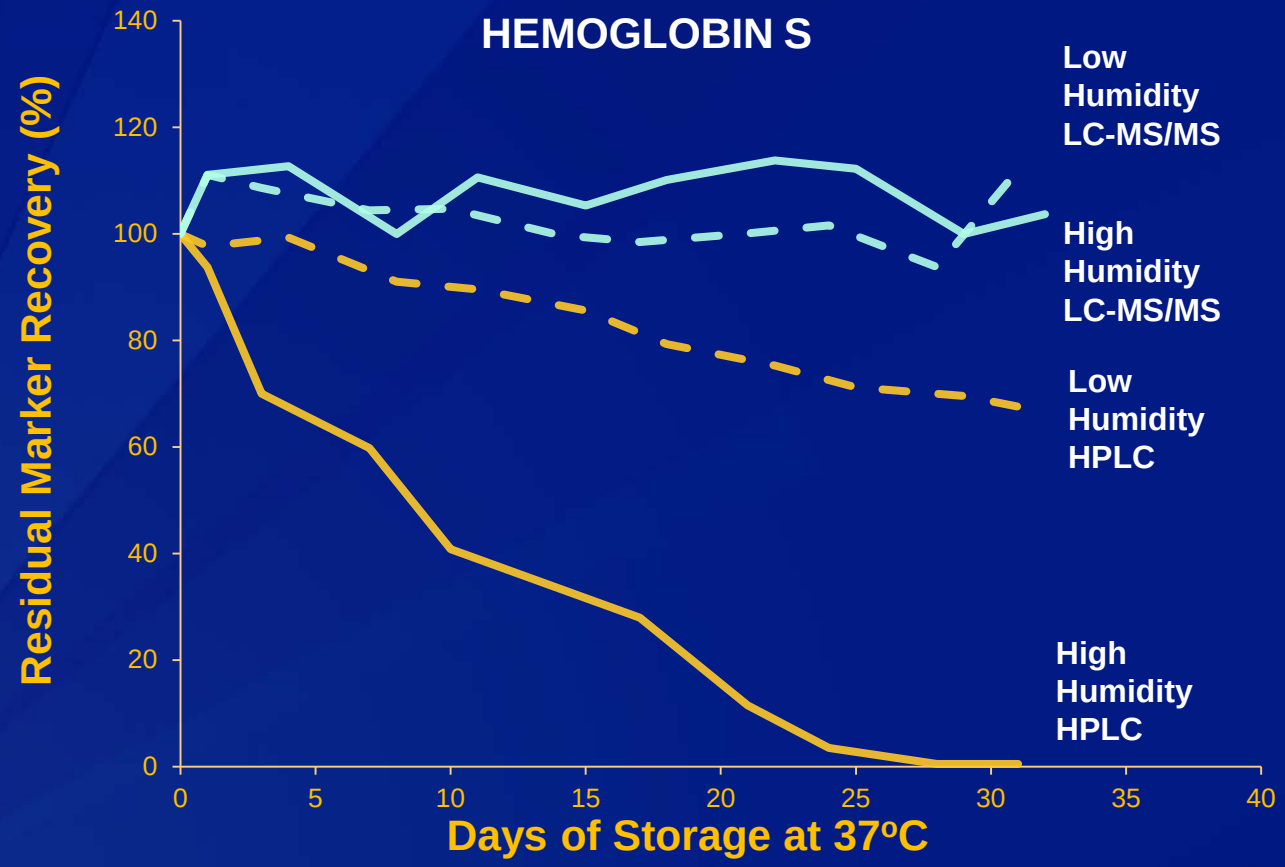
HbE / β^o: 511 μM
HbS / β^+: 85 μM
HbS / β^+: 134 μM

Analysis of DBS stored under high humidity

B.W. Adam, C.A. Haynes, D.L. Chafin, V.R. De Jesus, Clin Chim Acta, 429 (2014) 59-60.

Challenge: Storage of DBS without desiccant under high humidity conditions

Solution: Analysis of hemoglobin by HPLC-ESI-MS/MS



Hemoglobin analysis by ESI-MS/MS

- Higher specificity than IEF or HPLC-UV analyses
- Faster and less expensive than DNA sequencing
- Simple extraction of DBS punches with trypsin digestion
- Can use FIA-ESI-MS/MS (Wales), ion ratios to identify HbSS
- Can use HPLC-ESI-MS/MS (CDC), quantitation to identify thalassemias
- Suitable for specimens compromised by high humidity

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