Some Observations on Haemoglobin A_2

Barbara J Bain

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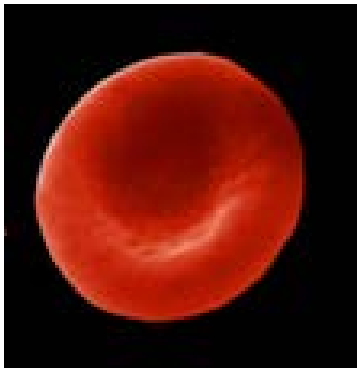
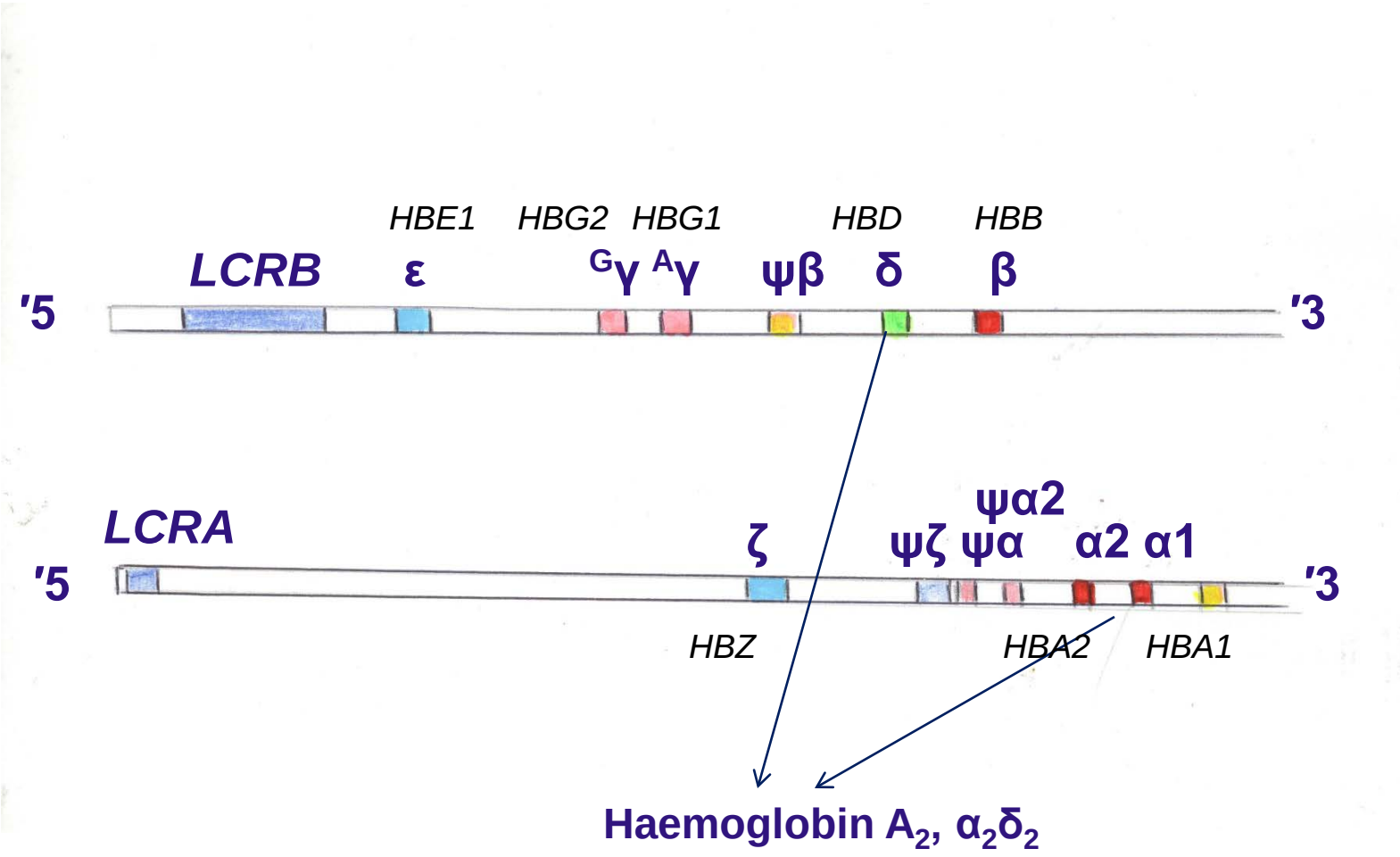
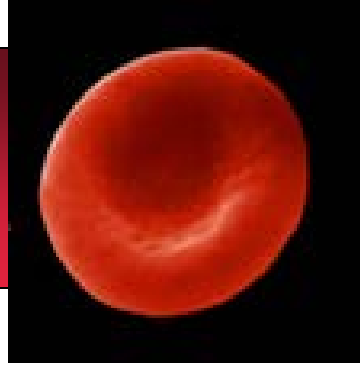


Image from www.dsc.discovery.com

Haemoglobin A_2



Haemoglobin A_2 function



- Higher oxygen affinity than haemoglobin A
- Otherwise similar function
- Inhibits the polymerisation of haemoglobin S

Haemoglobin A_2 synthesis

- Not synthesised in reticulocytes
- Half life of mRNA is less than a third of that of β chain mRNA
- Transcription of *HBD* is 50 times less efficient than transcription of *HBB*
- Adult rate of synthesis reached between one and two years of age
- Pancellular distribution

Genetic control

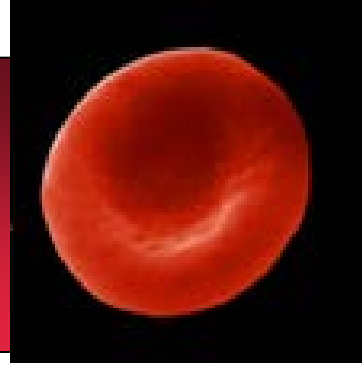
- Correlates weakly with the percentage of F cells ($r = 0.14$, $p < 0.01$), both relating to alleles of a SNP at 6q23.3
- Production of both γ and δ globin decreases as proerythroblasts mature
- Also correlates with SNPs in the region of the beta *HBB* at 11p15.4

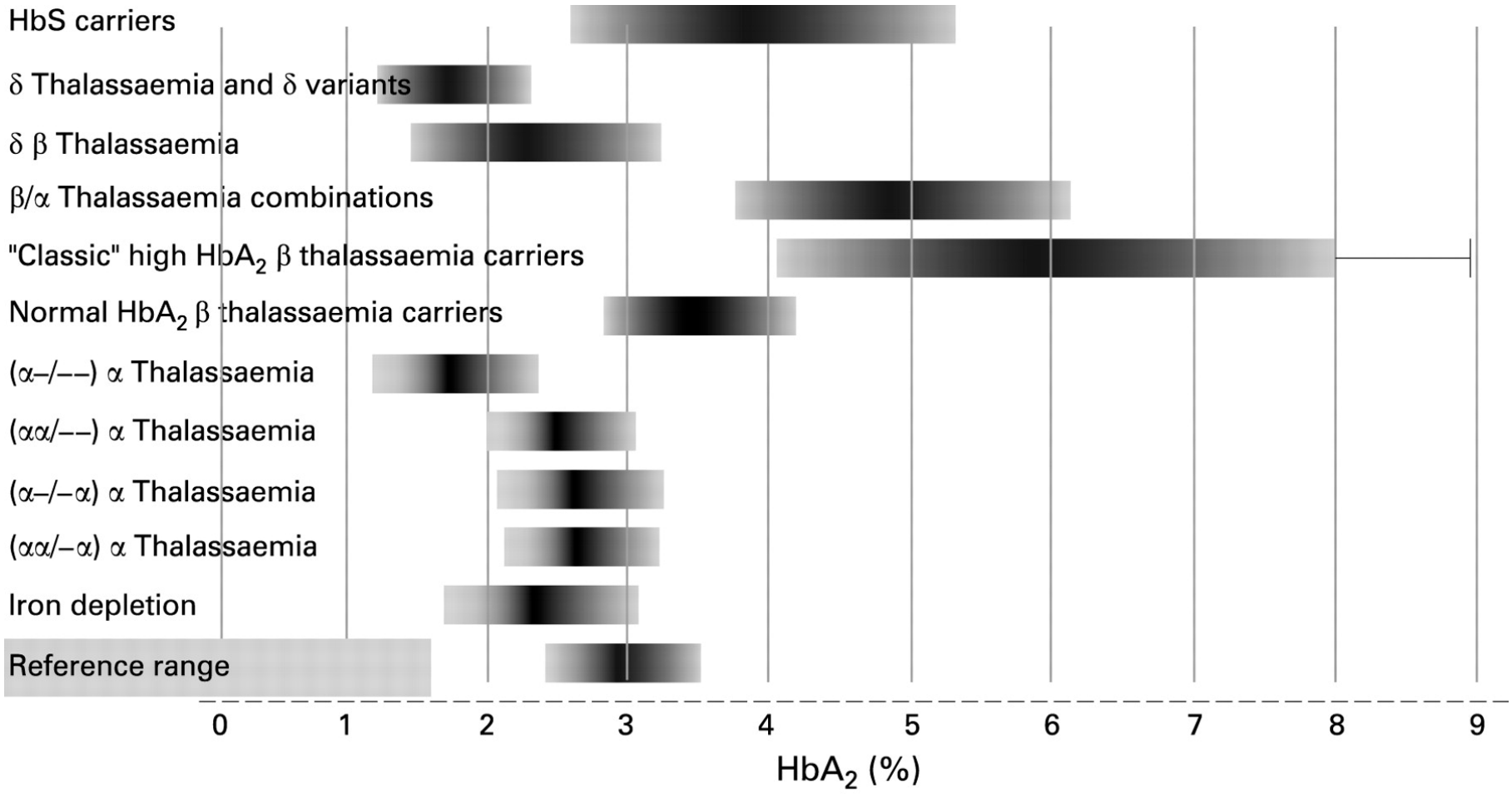
Thein SL, Garner C, Rooks H, Spector T and Menzel S (2012) HbA2 levels in adults are influenced by two distinct genetic mechanisms. *Haematologica*, 97, Suppl. 1, 249.

Haemoglobin A_2

- Important in the diagnosis of β thalassaemia heterozygosity
- Results may be inaccurate, imprecise or both
- Many inherited and acquired characteristics influence the A_2 percentage

Haemoglobin A_2





Mosca A et al. J Clin Pathol 2009;62:13-17 (BioRad HPLC, based on 100 normal and 670 others)

Haemoglobin A_2

- Although precise methods are available, accuracy is problematical
- Not only do normal ranges and mean normal values differ between methods and manufacturers, they may also differ between two instruments made by the same manufacturer
- With some methods it may be impossible to measure A_2 in some patients

Haemoglobin A_2

Analytical objectives

- SD of about 0.05%
- Duplicates should be within 0.2% of each other
- CV should be about 2% in the normal range and about 1% in the β thalassaemic range

Haemoglobin A_2

Method with unsatisfactory precision and accuracy

- Alkaline electrophoresis followed by scanning densitometry (imprecise and also percentage overestimated)

Haemoglobin A_2

Methods with satisfactory precision

- Alkaline electrophoresis followed by elution
- Microcolumn chromatography
- High performance liquid chromatography (HPLC)
- Capillary electrophoresis

Haemoglobin A_2

Variation in precision between methods

Precision of duplicate estimates of haemoglobin A_2 percentage by HPLC, microcolumn chromatography (MC) and scanning densitometry

Method	**Number of samples**	**CV**
HPLC	5	1.0%
MC	5	2.4%
Scanning densitometry	10	10.5%

From Head CE, Conroy M, Jarvis M, Phelan L and Bain BJ (2004) Some observations on the measurement of haemoglobin A_2 and S percentages by high performance liquid chromatography *i*n the presence and absence of thalassaem*i*a. *J Clin Pathol,* **57**, 276-280.

Haemoglobin A_2

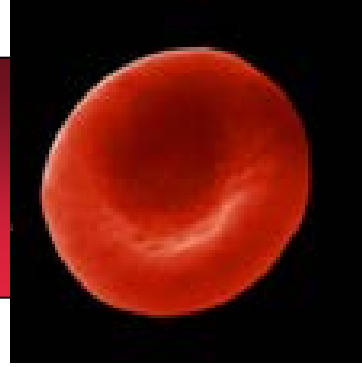
Comparison of precision between methods

CV of replicate measurements (within run) and duplicate measurements (between run) showing good precision with both methods

	HPLC	CE	n
Within-run CV	0.278	0.219	10
Between-run CV	0.128	0.194	8

From Borbely, Phelan, Szydlo and Bain (2012) Capillary zone electrophoresis for haemoglobinopathy diagnosis, *in press*, *J Clin Pathol*

Haemoglobin A_2



- There is a significant difference between methods for normal samples, capillary electrophoresis (CE) giving a somewhat higher result than HPLC in this study

A_2 estimate on 78 normal antenatal samples (normal indices, no variant haemoglobin)

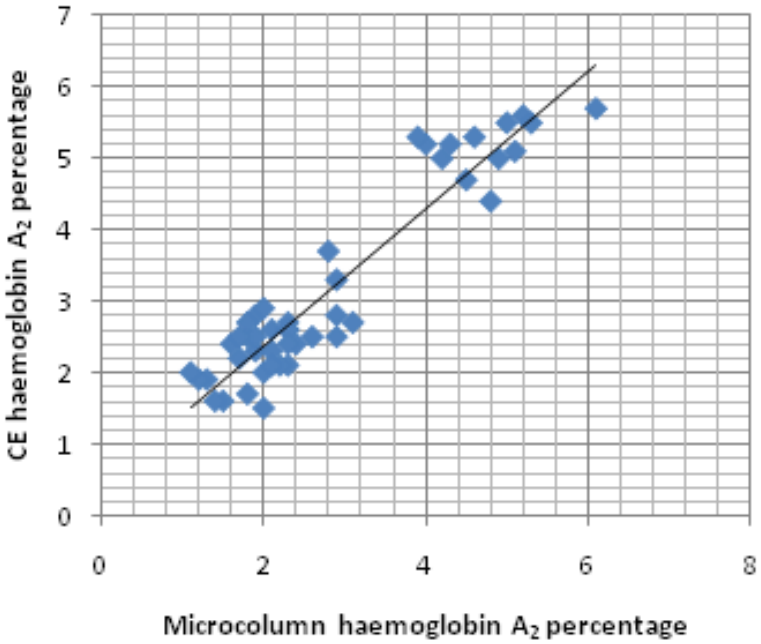
Method	Mean	95% range	Significance of difference
HPLC	2.65	2.25-3.05	p< .0001
CE	2.77	2.34-3.20	

From Borbely, Phelan, Szydlo and Bain (2012) Capillary zone electrophoresis for haemoglobinopathy diagnosis, *in press*, *J Clin Pathol*

Haemoglobin A_2

- When three methods are used (HPLC, CE and microcolumn chromatography) there is good correlation but a significant bias between methods
- The graphs that follow are for 46 specimens selected to have a A_2 percentages below, within and above the normal range

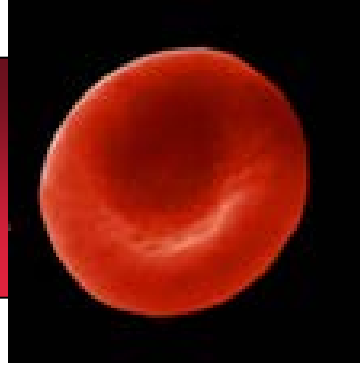
Haemoglobin A_2

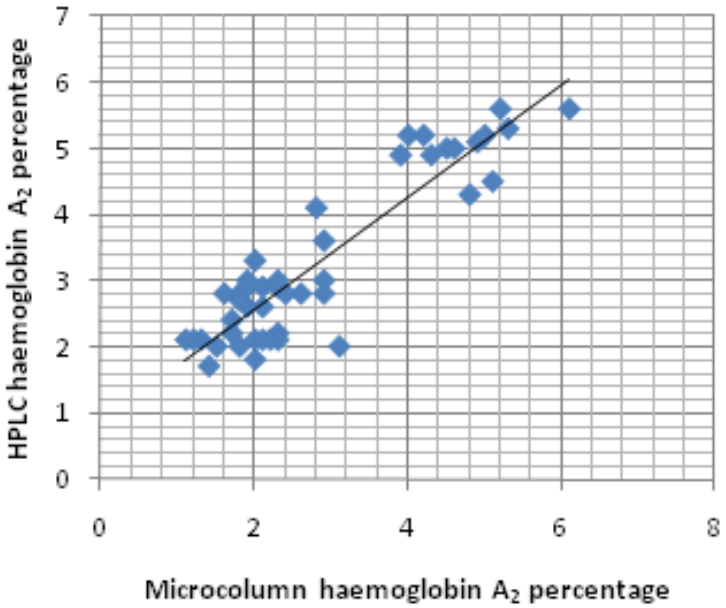


	Mean A_2	
MC	2.83%	r = 0.941, p <0.001
CE	3.17%	

From Borbely, Phelan, Szydlo and Bain (2012) Capillary zone electrophoresis for haemoglobinopathy diagnosis, *in press*, *J Clin Pathol*

Haemoglobin A_2

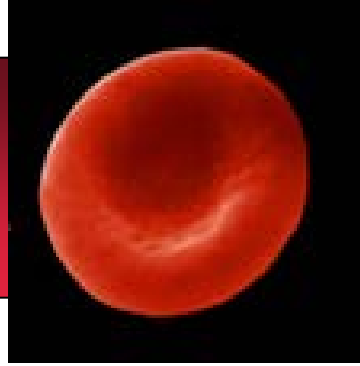


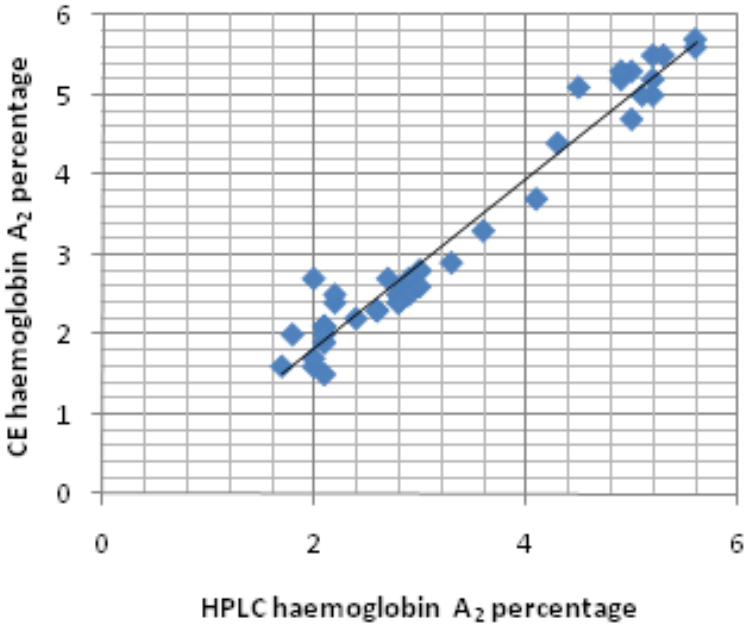


	Mean A_2	
MC	2.83%	r = 0.912, p <0.0001
HPLC	3.27%	

From Borbely, Phelan, Szydlo and Bain (2012) Capillary zone electrophoresis for haemoglobinopathy diagnosis, *in press*, *J Clin Pathol*

Haemoglobin A_2

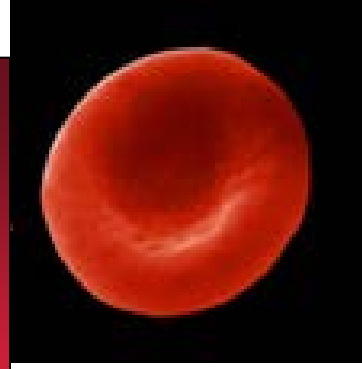




	Mean A_2	
CE	3.17%	r = 0.978, p <0.03
HPLC	3.27%	

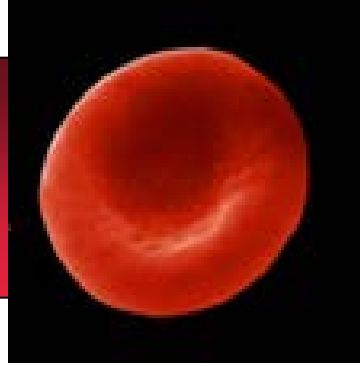
From Borbely, Phelan, Szydlo and Bain (2012) Capillary zone electrophoresis for haemoglobinopathy diagnosis, *in press*, *J Clin Pathol*

An inherited characteristic influencing haemoglobin A_2 measurement



- Sickle cell haemoglobin leads to an overestimation of A_2 by HPLC
- Post-translationally modified haemoglobin S has the same retention time as A_2
- This does not occur with capillary electrophoresis

Haemoglobin A_2



HPLC in sickle cell trait

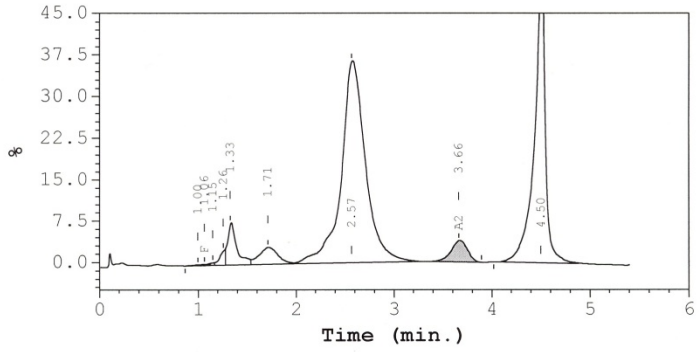
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	1.00	1048
F	0.1*	---	1.06	2061
Unknown	---	0.2	1.15	3080
Unknown	---	0.9	1.26	16287
P2	---	4.1	1.33	73824
P3	---	3.4	1.71	62032
Ao	---	52.7	2.57	952857
A2	3.8*	---	3.66	67933
S-window	---	34.8	4.50	630014

Total Area: 1809136

F Concentration = 0.1* %
A2 Concentration = 3.8* %

*Values outside of expected ranges

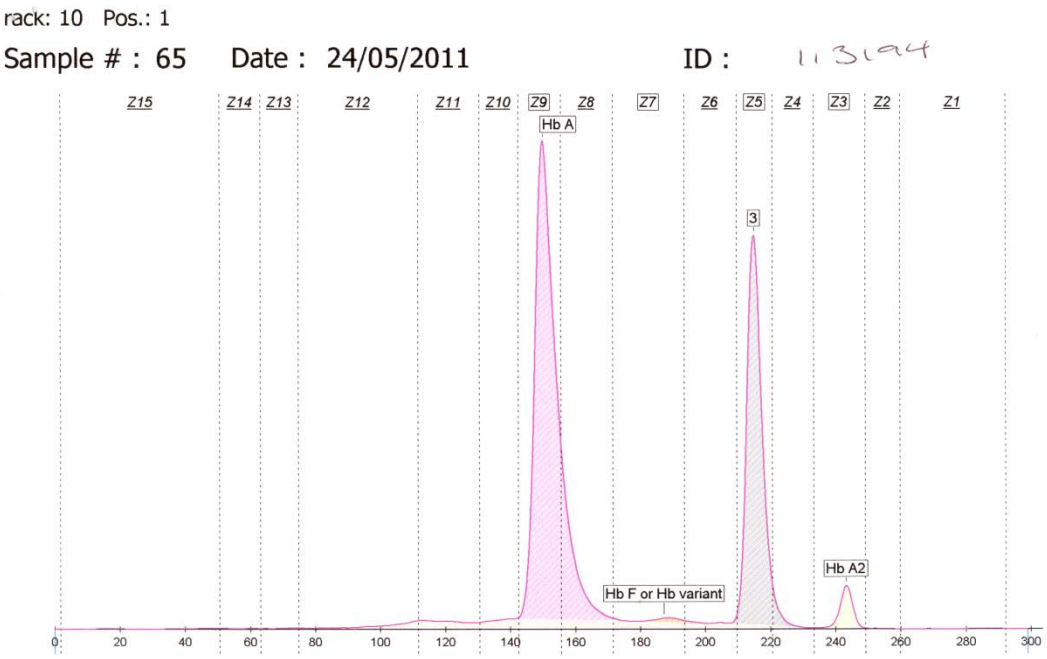
Analysis comments:



From Bain, Wild, Stephens and Phelan, Variant Haemoglobins, Wiley-Blackwell, 2010

Haemoglobin A_2

Capillary electrophoresis in sickle cell trait (not paired samples)



Name	%	Normal Values %
Hb A	**61.8**	
Hb F or Hb variant	**0.6**	
3	**34.5**	
Hb A2	**3.1**	

From Borbely, Phelan, Szydlo and Bain (2012) Capillary zone electrophoresis for haemoglobinopathy diagnosis, *in press*, *J Clin Pathol*

Haemoglobin A_2

Inaccuracy of haemoglobin A_2 estimated in the presence of haemoglobin S

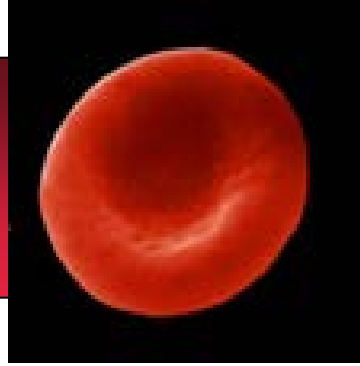
Mean, standard deviation, and 95% range of measurements of haemoglobin A_2 percentage in 73 patients with sickle cell trait when measured by HPLC, microcolumn chromatography (MC) and scanning densitometry

Method	**Mean (SD)**	**95% range**
HPLC	4.4% (0.34)	3.7–5.1%
MC	3.3% (0.36)	2.6–4.0%
Scanning densitometry	3.6% (0.70)	2.2–5.0%

HPLC,MC difference $p<0.001$; HPLC, densitometry difference $p < 0.01$); MC, densitometry difference $p > 0.05$

From Head CE, Conroy M, Jarvis M, Phelan L and Bain BJ (2004) *J Clin Pathol,* **57**, 276-280.

Haemoglobin A_2



- This inaccuracy in the presence of haemoglobin S does not occur with capillary electrophoresis (CE), which gives significantly lower results than HPLC
- Mean of A_2 in AS samples by CE 3.01%
- Mean of A_2 in AS samples by HPLC 3.50%
- Significance of difference $p < 0.0001$

From Borbely, Phelan, Szydlo and Bain (2012) Capillary zone electrophoresis for haemoglobinopathy diagnosis, *in press*, *J Clin Pathol*

Haemoglobin A_2

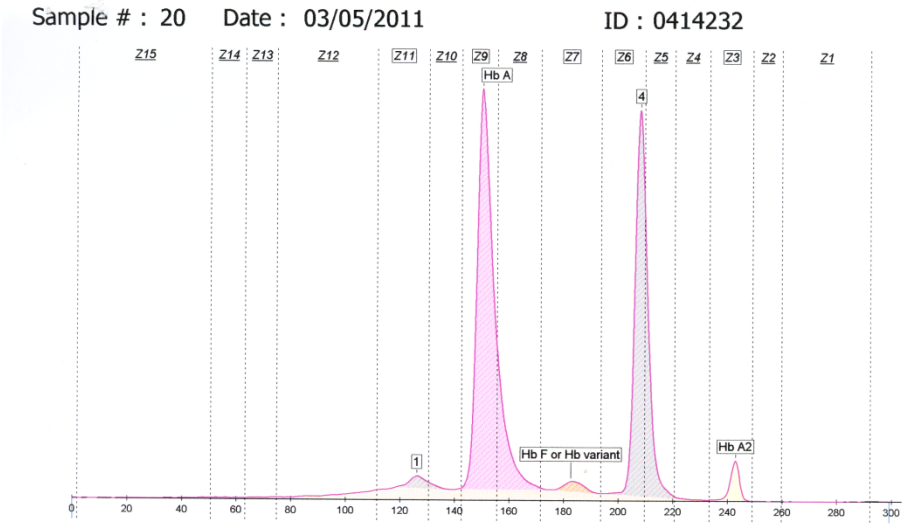
- Haemoglobin A_2 is also underestimated by HPLC in comparison with CE in patients with haemoglobin D Punjab heterozygosity
- Mean values were 2.07% by HPLC and 2.77% by CE on three samples

From Borbely, Phelan, Szydlo and Bain (2012) Capillary zone electrophoresis for haemoglobinopathy diagnosis, *in press*, *J Clin Pathol*

Haemoglobin A_2

Capillary electrophoresis

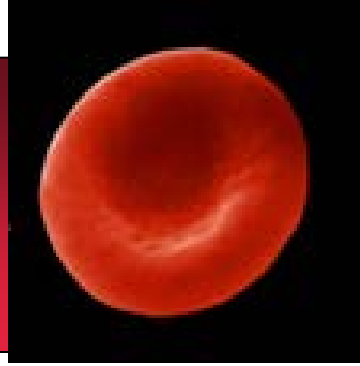
- Haemoglobin D Punjab heterozygote – good separation of A_2 and D peaks



Name	%	Normal Values %
1	**1.8**	
Hb A	**54.9**	
Hb F or Hb variant	**1.6**	
4	**38.9**	
Hb A2	**2.8**	

From Borbely, Phelan, Szydlo and Bain (2012) Capillary zone electrophoresis for haemoglobinopathy diagnosis, *in press*, *J Clin Pathol*

Haemoglobin A_2



HPLC

- Haemoglobin D Punjab heterozygote – poor separation of A_2 and D peaks
- A_2 underestimated

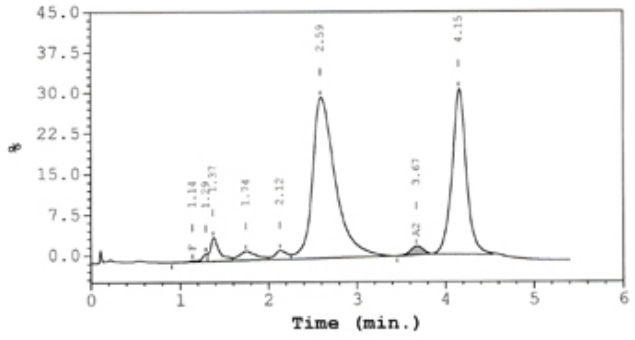
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.1*	---	1.14	1735
Unknown	---	0.6	1.29	7602
P2	---	3.2	1.37	40510
P3	---	2.4	1.74	30771
Unknown	---	1.7	2.12	20844
Ao	---	55.2	2.59	695575
A2	1.6*	---	3.67	19397
D-window	---	35.2	4.15	444023

Total Area: 1260457

F Concentration = 0.1* %
A2 Concentration = 1.6* %

*Values outside of expected ranges

Analysis comments:

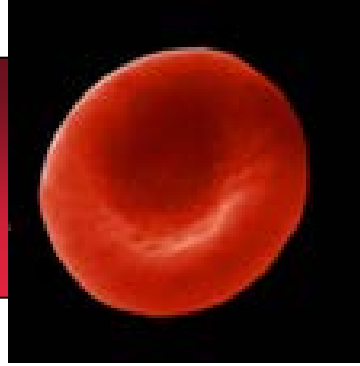


From Bain, Wild, Stephens and Phelan, Variant Haemoglobins, Wiley-Blackwell, 2010

Haemoglobin A_2

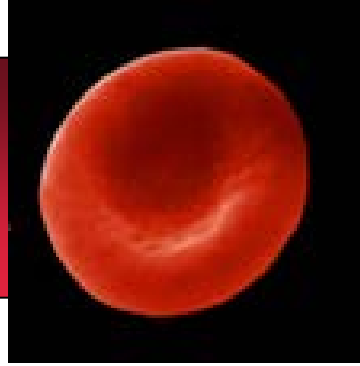
- The haemoglobin A_2 results by HPLC in the presence of haemoglobin S or D-Punjab are given as examples of inaccuracy
- They are not of importance in diagnosis

Haemoglobin A_2 and HIV

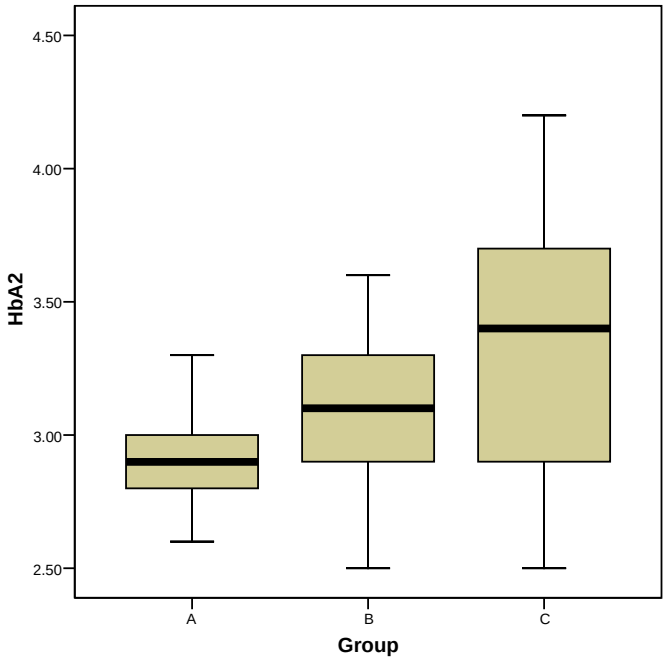


- Results may be accurate but misleading
- Haemoglobin A_2 is raised by HIV infection and raised further by zidovudine treatment

Haemoglobin A_2 and HIV

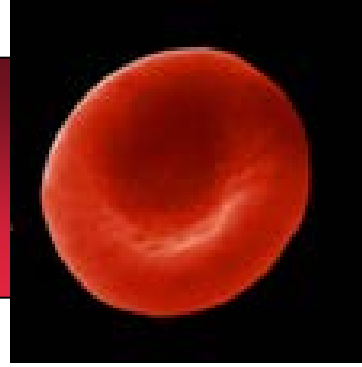


- Normal volunteers, mean Hb A_2 2.897%
- HIV-infected patients, not on antiretroviral treatment, mean Hb A_2 3.094%
- Significance of difference $p < 0.001$
- HIV-infected patients, on antiretroviral treatment (HAART), mean Hb A_2 3.4%



Wilkinson, Bain, Phelan and Benzie (2007) *AIDS*, **14**, 37-42.

Haemoglobin A_2 and HIV

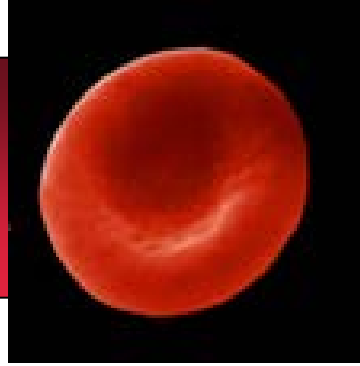


Percentage of individuals with Hb A_2 more than 3.3%

- Normal volunteers, 0%
- HIV-infected patients, not on antiretroviral treatment, 13.3% (p 0.017 cf normals, p <0.001 cf. on treatment)
- HIV-infected patients, on antiretroviral treatment (HAART), 53.3% (p < 0.0001 cf normals)

Wilkinson, Bain, Phelan and Benzie (2007) *AIDS*, **14**, 37-42.

Haemoglobin A_2 and HIV



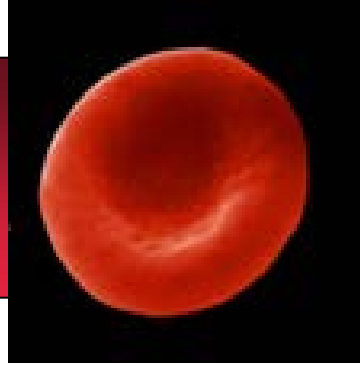
Similar observations had previously been made by others

- Routy JP, Monte M, Beaulieu R, Toma E, St-Pierre L and Dumont M (1993) Increase of hemoglobin A2 in human immunodeficiency virus-I-infected patients treated with zidovudine. *Am J Hematol*, **43**, 86-90.
- Galactéros F, Amaudric F, Préhu C, Feingold N, Doucet-Populaire F, Sobel A and Rosa J (1993) Acquired unbalanced hemoglobin chain synthesis during HIV infection. *C R Acad Sci III*, **316**, 437-440.
- Howard J, Henthorn JS, Murphy S and Davies SC (2005) Implications of increased haemoglobin A2 levels in HIV-positive women in the antenatal clinic. *J Clin Pathol*, **58**, 556-558.

Haemoglobin A_2 – some more problems

- Delta chain variants
- Delta thalassaemia
- Alpha chain variants

Haemoglobin A_2'



Haemoglobin A_2 prime heterozygosity

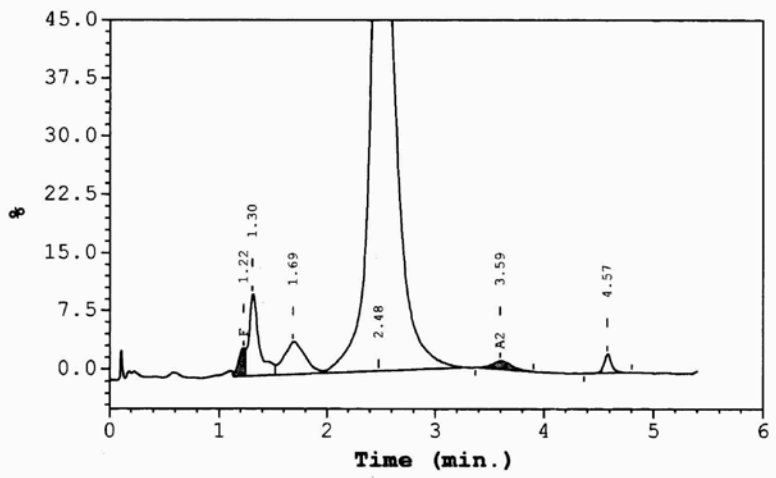
F	1.1	---	1.22	16414
P2	---	5.1	1.30	71537
P3	---	4.1	1.69	58485
Ao	---	87.8	2.48	1241603
A2	1.0*	---	3.59	13383
S-window	---	0.9	4.57	12223

Total Area: 1413646

F Concentration = 1.1 %
A2 Concentration = 1.0* %

*Values outside of expected ranges

Analysis comments:



Haemoglobin A_2'

- Co-elutes with haemoglobin A_2 on microcolumn chromatography
- Separates on cellulose acetate electrophoresis, capillary electrophoresis and HPLC
- Need to distinguish form haemoglobin S carryover

Haemoglobin A_2'

- In a CAP proficiency survey only a third of laboratories using HPLC identified A_2 [Joutovsky *et al*. 2004]

Haemoglobin A_2'

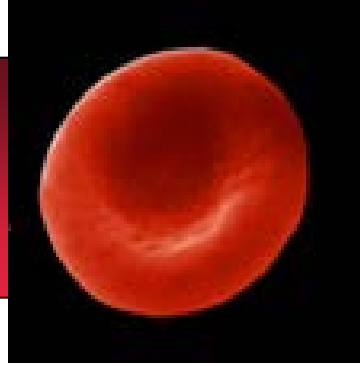
- Variant delta chain is synthesised at somewhat reduced rate

A2′	A2	Significance	Reference
1.3 (0.94-1.66)	1.7 (1.56-1.84)		Van Kirk 2005
1.24 (0.89-1.59)	1.58 (1.25-1.9)	p<0.0001	Abdel-Gadir 2008

Van Kirk R, Sandhaus LM, Hoyer JD (2005) The detection and diagnosis of hemoglobin A2' by high-performance liquid chromatography. *Am J Clin Pathol*, **123**, 657-61.

Abdel-Gadir D, Phelan L and Bain BJ (2009) Haemoglobin A_2' and its significance in β thalassaemia diagnosis. *Int J Lab Hematol*, **31**, 315-319.

Haemoglobin A_2'



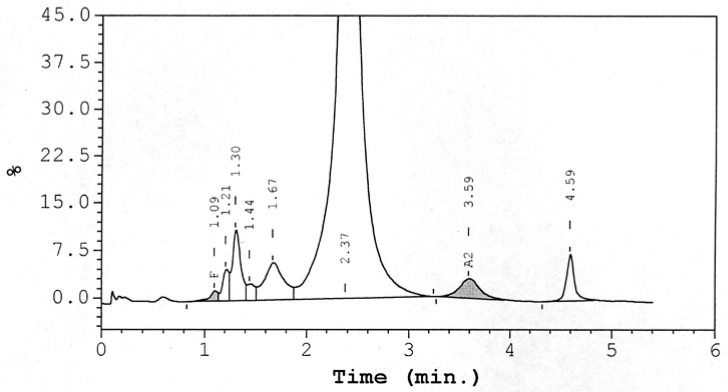
Haemoglobin A_2 prime plus β thalassaemia trait

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.5	---	1.09	9567
Unknown	---	1.3	1.21	22251
P2	---	3.7	1.30	64598
Unknown	---	0.8	1.44	14465
P3	---	4.5	1.67	78283
Ao	---	84.2	2.37	1465577
A2	3.1	---	3.59	44847
S-window	---	2.4	4.59	41401

Total Area: 1740990

F Concentration = 0.5 %
A2 Concentration = 3.1 %

Analysis comments:



With thanks to Ms Joan Henthorn

From Bain, Wild, Stephens and Phelan, Variant Haemoglobins, Wiley-Blackwell, 2010

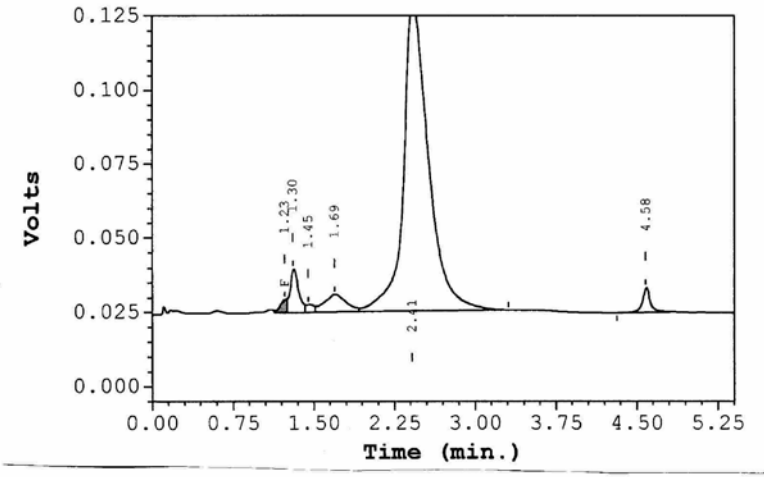
Haemoglobin A_2'

Haemoglobin A_2 prime homozygosity

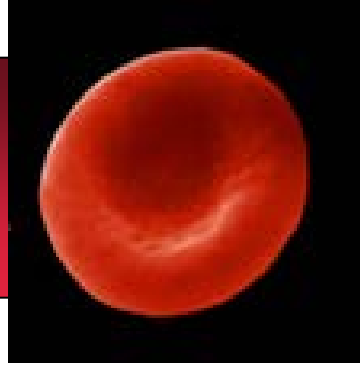
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	1.0	---	1.23	20061
P2	---	4.1	1.30	79150
Unknown	---	0.7	1.45	14091
P3	---	4.3	1.69	83699
Ao	---	87.6	2.41	1695288
S-window	---	2.2	4.58	42688

Total Area: 1934978

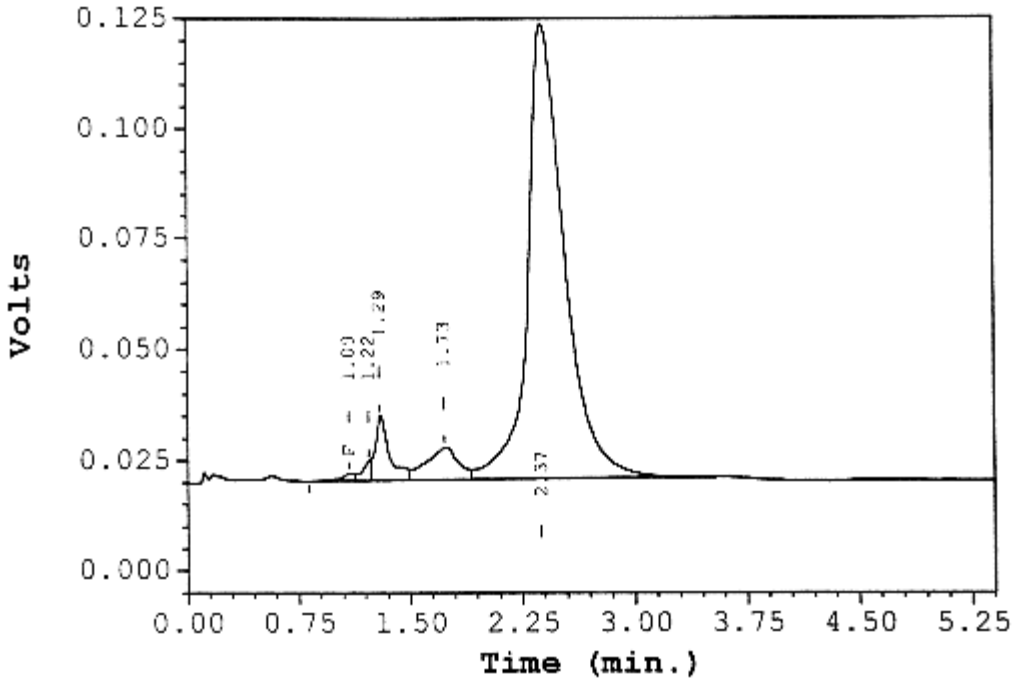
F Concentration = 1.0 %
A2 Concentration = %



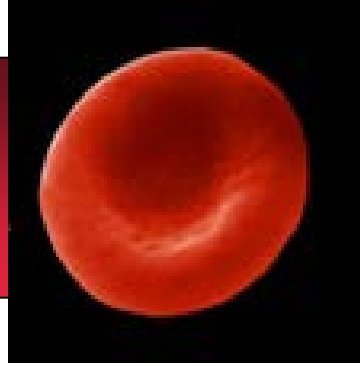
Haemoglobin A_2



Delta^0 thalassaemia homozygosity



Haemoglobin A_2



- Alpha chain variants – some of your A_2 is missing
- Haemoglobin G Philadelphia

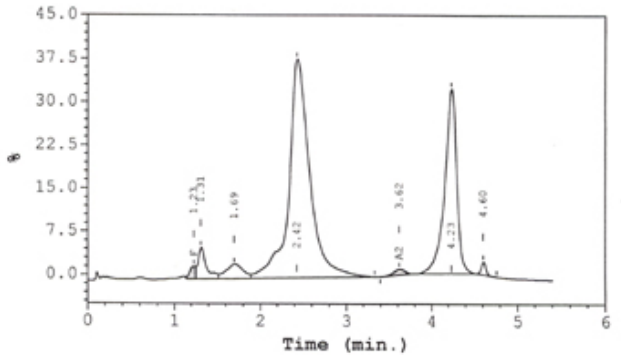
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	1.0	---	1.23	25854
P2	---	3.4	1.31	88450
P3	---	3.2	1.69	83353
Ao	---	60.6	2.42	1560836
A2	1.0*	---	3.62	23802
D-window	---	30.0	4.23	773315
S-window	---	0.8	4.60	20111

Total Area: 2575721

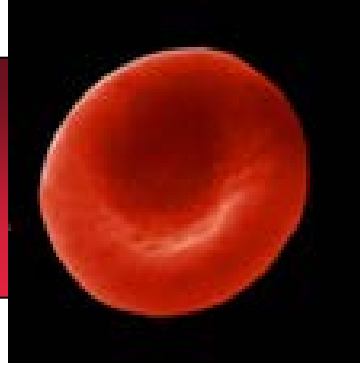
F Concentration = 1.0 %
A2 Concentration = 1.0* %

*Values outside of expected ranges

Analysis comments:



Haemoglobin A_2



- Alpha chain variants – some of your A_2 is missing
- Haemoglobin Buffalo

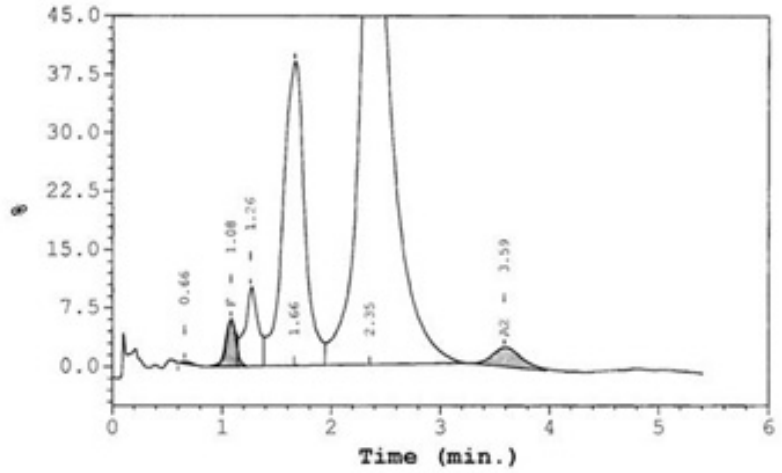
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.1	0.66	1724
F	1.9*	---	1.08	43877
P2	---	4.0	1.26	97958
P3	---	25.3	1.66	613626
Ao	---	66.7	2.35	1620511
A2	2.4	---	3.59	50506

Total Area: 2428202

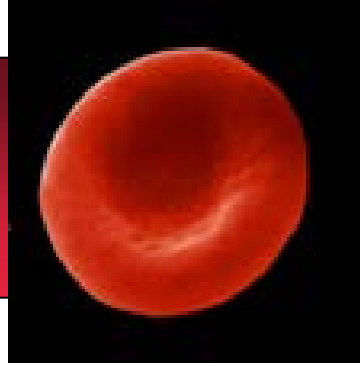
F Concentration = 1.9* %
A2 Concentration = 2.4 %

*Values outside of expected ranges

Analysis comments:



Haemoglobin A_2



- Alpha chain variants – some of your A_2 is missing
- Haemoglobin Buffalo
- The A_2 variant is lost in the A peak

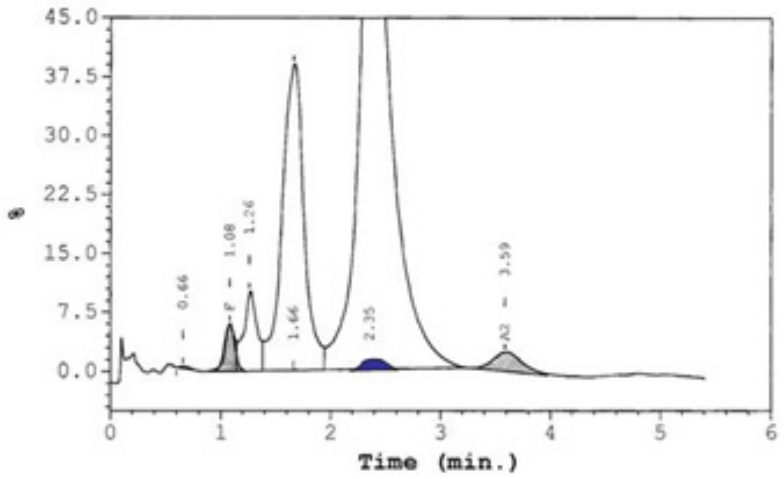
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.1	0.66	1724
F	1.9*	---	1.08	43877
P2	---	4.0	1.26	97958
P3	---	25.3	1.66	613626
Ao	---	66.7	2.35	1620511
A2	2.4	---	3.59	50506

Total Area: 2428202

F Concentration = 1.9* %
A2 Concentration = 2.4 %

*Values outside of expected ranges

Analysis comments:



Factors affecting haemoglobin A_2 percentage

- Having given some examples, I shall now summarise
  - Factors lowering A_2
  - Factors elevating A_2

Factors lowering haemoglobin A_2 percentage

Inherited conditions

- Delta and delta-beta thalassaemia
- Alpha thalassaemia (including haemoglobin H disease)*
- Haemoglobin Lepore
- Alpha chain variant
- Delta chain variant

* When α chain production is reduced αβ dimers form preferentially compared with αδ

Factors lowering haemoglobin A_2 percentage

Acquired conditions

- Iron deficiency anaemia*
- Anaemia of chronic disease*
- Sideroblastic anaemia*
- Lead poisoning*
- Juvenile myelomonocytic leukaemia
- Acquired haemoglobin H disease

…….. and ….

*Actual or functional iron deficiency reduced α chain synthesis

Factors lowering haemoglobin A_2 percentage

- Some AML, particularly erythroleukaemia
- Some aplastic anaemia
- Hypothyroidism

Factors elevating haemoglobin A_2 percentage

Inherited conditions

- Some unstable haemoglobins
- Thalassaemic haemoglobinopathies, e.g. haemoglobin E
- Haemoglobin S heterozygosity (small genuine increase) and homozygosity
- Vietnamese/South-East Asian type of deletional hereditary persistence of fetal haemoglobin (which spares the δ gene)

Factors elevating haemoglobin A_2 percentage

Inherited conditions

- Inherited high A_2
  - Mutation in δ promoter
  - *KLF1* mutation (e.g. 3.3-4.1%)
- Triple α reported (Perseu *et al*. 2011) but not confirmed (Stephens *et al*. 2012)
- Pseudoxanthoma elasticum
- Some CDA (Israeli Bedouins)
- Down's syndrome (up to 80 days of age)

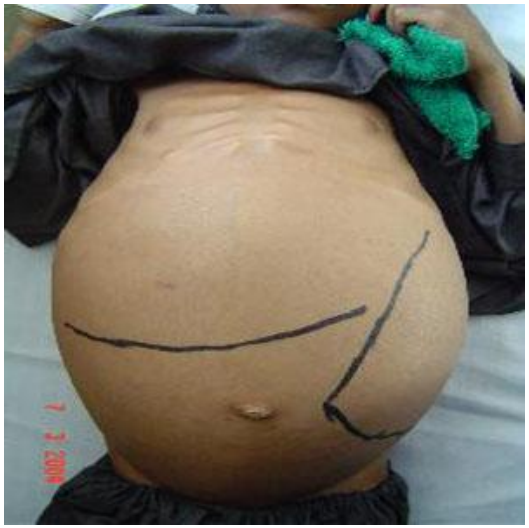
Factors elevating haemoglobin A_2 percentage

Acquired conditions

- HIV infection
- Treatment of HIV infection
- Some cases of megaloblastic anaemia
- Hyperthyroidism

Conclusions

- Not so simple
- We need accurate and precise laboratory methods
- We need careful interpretation



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