Haemoglobinopathy Screening in Unknown Full Blood Samples using HPLC.

Lauryn Bailey

Date Performed: 17-11-23

Introduction

Haemoglobinopathies encompass over 1,000 genetic mutations, categorized into haemoglobin variants and thalassaemias (NHS, 2017). Sickle cell disease, a prevalent haemoglobin variant, results from a β-globin chain substitution (valine for glutamic acid), inducing abnormal polymerisation in low oxygen conditions, leading to a distinctive sickled shape. It affects approximately 1 in 2000 newborns in England, causing symptoms such as acute painful episodes and acute chest syndrome, the primary reasons for hospitalization and mortality among young patients (Howard & Davies, 2007).

Thalassaemias result from mutations causing reduced or absent globin chain production in haemoglobin. Notable types include α-thalassaemia (affecting α chains) and β-thalassaemia (affecting β chains), crucial components of adult haemoglobin. The severity varies, e.g., β0-thalassaemia entails a complete lack of β chains, while β minor thalassaemia involves a single affected β chain with minimal symptoms. Disrupted β chain production can lead to an α chain imbalance, causing bone marrow destruction and ineffective erythropoiesis (Silverberg, 2012). Severe β-thalassaemia often requires frequent blood transfusions, with regular screening to monitor treatment-related iron overload (Howard & Davies, 2007). However, the MHRA have recently authorised a new gene-therapy treatment that shows promise to cure sickle cell disease and β-thalassaemia by editing genes in the bone marrow to allow the patient to begin to create normal haemoglobin (Medicines and Healthcare products Regulatory Agency, 2023).

Symptoms of β-thalassaemia emerge around 6 months old when foetal haemoglobin levels decline and adult haemoglobin levels rise, posing risks of cardiac failure and mortality without prompt diagnosis and treatment (Howard & Davies, 2007). Given this information and the prevalence of sickle cell disease in England, emphasizing the significance of antenatal and newborn screening is vital. In high-prevalence areas, all consenting mothers undergo testing, while in low-prevalence regions like Portsmouth, screening relies on family origins and full blood count test results. Figure 1 depicts a flowchart of the decision process for testing. This is primarily undertaken by capillary gel electrophoresis (CGE) or high-performance liquid chromatography (HPLC) to determine HbF and HbA_2 levels as well as detect any haemoglobin variants. MCH (mean cell haemoglobin) and Hb (haemoglobin count) results from other haematology testing is also used. If a mother results as being positive for a clinically significant mutation, father screening is offered, followed by further pre-natal screening (NHS, 2017). This includes methods such as chorionic villus sampling and amniocentesis (Public Health England, 2018).

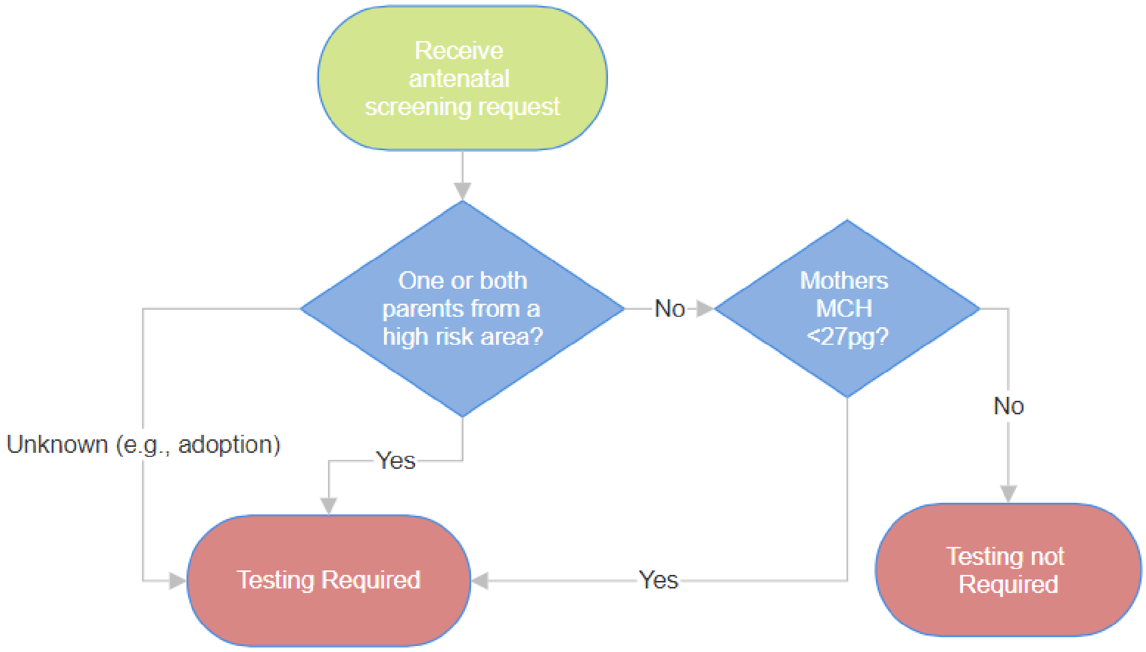

Figure 1: A flowchart depicting the decision process for testing maternal bloods for haemoglobinopathies in low-prevalence regions using information supplied by NHS (2017).

Aim

The aim of this practical is to determine the haemoglobinopathy status of three unknown samples compared to two known controls using HPLC testing and results from full blood count testing. Relevant next steps and assessment of the clinical significance of these results will be assessed along with evaluating the reliability of the results.

Method

The method followed in this practical was supplied by Greenfield (2023). Prior to the practical, two controls were prepared. The G11 Haemoglobin F&A_2 control set supplied by TOSOH, which comprises of two known levels of HbS, HbF and HbA_2, was used. 200uL of deionised water was added to each control vial using an automatic pipette and they were left to stand at room temperature for 10 minutes.

The TOSOH G11 HPLC analyser was used to automatically test the samples. It uses 3 buffers of varying salt concentration, a wash buffer, a filter, and a cation exchange column. The test count of the filter and column were checked to ensure they are within their limits for the run (300 and 750 maximum tests respectively). The remaining level of each buffer was checked also. All consumables were within their range for the number of tests necessary for this practical and no changes were needed. The analyser was then turned on using the power key on the control panel which initiated an automatic warming up procedure. Once the analyser had finished the warmup procedure, the previously reconstituted control vials could be used to prepare the control samples for testing. 10uL of each control was pipetted into separate sample cups. 500uL of deionised water was added to each cup and mixed using a pastette. Each control had a barcoded position in a rack. The control samples were added to their respective positions. The controls were then programmed into the PIANO computer software, confirming they match lot numbers to ensure the known values of HbS, HbF and HbA_2 are correct. The rack containing the control samples was then loaded onto the analyser along with 3 unknown samples. The unknown samples were mixed by gentle inversion and placed with their barcodes facing out of the rack to ensure the analyser can detect each sample. An empty 'stop rack' was added after the controls and samples to signal to the analyser that there were no more samples to be run.

The analyser performed automatic HPLC analysis on each sample and the results were reported to the PIANO software for assessing.

Results

Table 1: A table to show the results of HPLC analysis for two controls and three unknown samples alongside their given Hb and MCH results from previous testing.

Sample	HbA0	HbA_2 (%)	HbF (%)	HbS (%)	Hb (g/L)	MCH (pg)
Control Level 1	Pos	2.4	2.7	0.0	n/a	n/a
Control Level 2	Pos	6.0	6.9	26.7	n/a	n/a
Unknown 1	Pos	2.6	1.3	0.0	99	28.4
Unknown 2	Pos	4.4	1.8	0.0	68	23.4
Unknown 3	Pos	3.1	0.9	20.3	103	27.6

Table 1 shows the results of the automatic HPLC analysis performed by the TOSOH G11. It is also noted that all samples tested resulted with an appropriate total area of >1000 and <4500 and no extra unexpected peaks were detected.

Discussion

The TOSH G11 analyser is an automated analyser based on the principle of gradient elution HPLC. The sample, injected via a probe, passes through a cation exchange column where haemoglobins are retained based on ionic charge. A mobile phase of three salt solutions gradually increases salt concentration, causing haemoglobins to elute at specific times. The detector records elution times and the amount of haemoglobin at each retention time, presented as percentages in a chromatogram (Greenfield, 2023). Table 2 shows the specific retention times for each detected haemoglobin on the TOSOH G11.

Table 2: A table to show the retention times, in minutes, of haemoglobins detected by the TOSOH G11 as specified by Greenfield (2023).

Haemoglobin	Retention Time (minutes)
F	0.58-0.64
A0	1.60-2.00
A2	2.00-2.40
E	2.65-2.73
D	2.73-2.98
S	2.98-3.40
C	3.70-5.00

Table 3 shows the acceptable ranges for each control. The results shown in table 1 show that the controls worked as expected as the results are within the acceptable ranges. The total area was also within range for all samples showing that they were of strong enough concentration for analysis. The total area can be low if the patient is anaemic or if there are issues with aspiration on the analyser (Greenfield, 2023). This information helps to assure the validity of the sample results.

Table 3: A table to show the acceptable control ranges for HbF, HbA_2, and HbS at levels 1 and 2 (Greenfield, 2023).

Control	HbF (%)	HbA_2 (%)	HbS (%)
Level 1	2.3 – 2.9	2.4 – 2.8	0
Level 2	6.5 – 7.3	5.5 – 6.5	25.5 – 28.5

Wilkins' (2023) thalassaemia diagnostic guide indicates normal ranges for HbA2 (1.7-3.9%), HbF (<10%), MCH (≥27.0 pg), and Hb (≥80 g/L). Unknown sample 1 aligns with these parameters, indicating no haemoglobinopathies and obviating the need for further testing, irrespective of antenatal or non-antenatal requests.

In contrast, unknown sample 2 exhibits a HbA2 result of 4.4%, surpassing the normal reference range. Additionally, MCH is <25 pg, and Hb is <80 g/L. These findings suggest a potential β-thalassaemia carrier status. B-thalassaemia carriers, typically asymptomatic due to heterozygosity, constitute approximately 1.5% of the global population and up to 15% in regions such as Greece, Turkey, and

Cyprus (Needs et al., 2023). Non-antenatal cases often do not necessitate further testing, but the patient is made aware. However, in antenatal scenarios, paternal screening is recommended. As thalassaemias are generally recessive, both parents must be carriers for offspring to potentially manifest a symptomatic homozygous β-thalassaemia. The probabilities include a 25% chance of manifestation, a 50% chance of carrier status, and a 25% chance of having no thalassaemia. If the father is a carrier, genetic counselling is advised to elucidate risks and offer additional baby genetic testing (NHS, 2017). If the father is not a carrier, there is no risk of homozygous beta thalassaemia inheritance, with a 25% chance of carrier status for the baby, rendering further testing unnecessary. It can be beneficial to diagnose beta thalassaemia early due to its rapid onset once HbF starts to deplete meaning that treatment can be undertaken before complications arise (Howard & Davies, 2007).

Unknown sample 3 exhibits normal ranges for HbA2, HbF, Hb, and MCH. However, a detected HbS peak at 20.3% raises concerns about the reliability of HbA2 results due to potential interference from haemoglobin variants to the right of the HbA peak on the chromatogram (Greenfield, 2023). Consequently, the HbA2 result is deemed invalid. The presence of an HbA0 peak suggests the patient could be a heterozygous sickle cell carrier or a homozygous sufferer who has undergone a blood transfusion (Ganczakowski, 2023). Quantification of HbA0 is omitted due to the absence of appropriate controls (Greenfield, 2023). A sickle solubility test is essential for confirming the HbS peak. A negative result prompts referral to a lab for DNA testing. If positive, the patient is made aware. In non-antenatal cases, no further testing is needed for asymptomatic sickle cell carriers (Kanter & Kruse-Jarres, 2013). For both positive and negative solubility test outcomes in antenatal cases, a father screen is recommended to assess the likelihood of offspring inheriting homozygous sickle cell disease, necessitating early intervention to avert complications like acute chest syndrome in young patients (Howard & Davies, 2007).

There are many benefits to HPLC analysis such as the fact that it only requires a small volume of sample (5uL). As well as this, it is rapid, highly sensitive, and provides a quantitative result for the peak percentages. One of the drawbacks includes the need for manual interpretation of the chromatograms. Furthermore, some abnormal peaks can have similar retention times as normal peaks making them hard to distinguish such as HbE being similar to HbA_2. One way to eliminate these issues be to follow up with another method such as isoelectric focusing however this would be more time consuming (Gupta et al., 2009).

Conclusion

HPLC analysis efficiently detects haemoglobinopathies in both antenatal and non-antenatal cases, providing rapid results. Integration of tests like MCH and Hb values onto a single analyser could further accelerate diagnoses. Timely identification of hemoglobinopathies is crucial for initiating early treatment and optimizing patient outcomes.

References

- Ganczakowski, M. (2023) 'Procedure for authorising abnormal haemoglobinopathy results (for Haematology doctors)'. Portsmouth, UK: Portsmouth Hospital University NHS Trust.
- Greenfield, T. (2023) 'Tosoh G11 HPLC analyser Standard Operating Procedure'. Portsmouth, UK: Portsmouth Hospital University NHS Trust.
- Gupta, P. *et al.* (2009) 'Cation exchange high performance liquid chromatography for diagnosis of Haemoglobinopathies', *Medical Journal Armed Forces India*, 65(1), pp. 33–37. doi:10.1016/s0377-1237(09)80051-8.
- Howard, J. and Davies, S.C. (2007) 'Haemoglobinopathies', *Paediatrics and Child Health*, 17(8), pp. 311–316. doi:10.1016/j.paed.2007.05.001.
- Kanter, J. and Kruse-Jarres, R. (2013) 'Management of sickle cell disease from childhood through adulthood', *Blood Reviews*, 27(6), pp. 279–287. doi:10.1016/j.blre.2013.09.001.
- Medicines and Healthcare products Regulatory Agency (2023) 'MHRA authorises world-first gene therapy that aims to cure sickle-cell disease and transfusion-dependent β-thalassemia', *GOV>UK*, November. Available at: https://www.gov.uk/government/news/mhra-authorises-world-first-gene-therapy-that-aims-to-cure-sickle-cell-disease-and-transfusion-dependent-thalassemia (Accessed: 23 November 2023).
- Needs, T., Gonzalez-Mosquera, L.F. and Lynch, D.T. (2023) *Beta Thalassemia*. Available at: https://www.ncbi.nlm.nih.gov/books/NBK531481/ (Accessed: 21 November 2023).
- NHS (2017) *NHS Sickle Cell and Thalassaemia Screening Programme*, *Public Health England*. Public Health England. Available at: https://www.pnsd.scot.nhs.uk/wp-content//Antenatal_Laboratory_Handbook.pdf (Accessed: 19 November 2023).
- Public Health England (2018) *Counselling and referral for prenatal diagnosis (PND)*, *GOV.UK*. Available at: https://www.gov.uk/government/publications/handbook-for-sickle-cell-and-thalassaemia-screening/prenatal-diagnosis-guidelines#prenatal-diagnosis-procedure (Accessed: 19 November 2023).
- Silverberg, D.S. (2012) *Anemia*. London: IntechOpen. Available at: https://books.google.co.uk/books?hl=en&lr=&id=WoefDwAAQBAJ&oi=fnd&pg=PA341&dq=haemoglobin+structure+thalassemia&ots=560MGoOV5D&sig=j0vw63h0lSDB3hWJFgDoEEP9DDs&redir_esc=y#v=onepage&q=haemoglobin%20structure%20thalassemia&f=false (Accessed: 19 November 2023).
- Wilkins, J. (2023) 'Thalassaemia Diagnosis Guide'. Portsmouth, UK: Portsmouth Hospital University NHS Trust.