Automated quantitative mass spectrometry-based approaches for the diagnosis of beta thalassemia in a clinical trial

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Overview

Aims. To evaluate the most appropriate mass spectrometry-based method for automated, high throughput screening of haemoglobin (Hb) disorders, which could replace the existing HPLC and IEF methods in clinic in the UK.

Methods. Two different methods have been evaluated on a large set of clinical samples in a clinical trial using a triple quadrupole mass spectrometer:
1.) Direct infusion intact globin chain analysis
2.) Multiple reaction monitoring (MRM) targeted peptide analysis

Results and Conclusions. A total number of 2017 patient blood samples have been analysed in a 3 month mirrored test with current screening methods. The results show excellent correlations with existing methods for HbA_2 quantitation, and additional variants have been identified which were not picked up by the current screening methods.

Introduction

Disorders of the haemoglobin protein are the most common type of inherited disorders, and pose a significant healthcare problem. Screening programs for haemoglobinopathies currently employ chromatography and electrophoretic techniques, which provide presumptive identification of clinically important haemoglobin disorders, and quantitation of HbA_2 values as possible indication for Beta-thalassemia with the cut-off value of 3.5%. A definitive characterisation of the disorders requires protein sequence elucidation or DNA analysis. The development of a rapid population screening method for haemoglobinopathies, which could also provide definitive diagnosis, is of significant interest to the healthcare profession. Mass spectrometry is currently used to characterise a range of variants but has not been used as a population screen. In this work, mass spectrometry-based approaches to haemoglobinopathy detection were compared and contrasted to determine the most appropriate method for a rapid diagnostic screen.

The structure of haemoglobin is shown in **Figure 1.** The disorders of Hb fall into 2 main groups.

1. Structural variants (potentially around 1000):
Alteration in the primary globin protein structure. Common clinically significant Hb variants are: S, C, D-Punjab, O-Arab, E and Lepore.

> What can MS offer? Identification based on mass differences observed due to amino acid changes.

2. Thalassemias (potentially around 300):
A gene mutation that causes production of an insufficient amount of normal structure globin chains.

> What can MS offer? Quantification of δ-chain:β-chain ratios, from intact chain or peptide intensities (**Figure 2**) *can be used as a marker for β-thalassemia disease.*

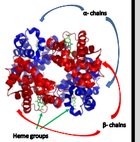

Figure 1. Structure of Haemoglobin

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β  1  VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPKVKAHGKKVLGAFSDG  75
δ  1  VHLTPEEKTAVNALWGKVNVDAVGGEALGRLLVVYPWTQRFFESFGDLSSPDAVMGNPKVKAHGKKVLGAFSDG  75

β  76 LAHLDNLKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFGKEFTPPVQAAYQKVVAGVANALAHKYH  146
δ  76 LAHLDNLKGTFSQLSELHCDKLHVDPENFRLLGNVLVCVLAVNFGKEFTPQMQAAYQKVVAGVANALAHKYH  146
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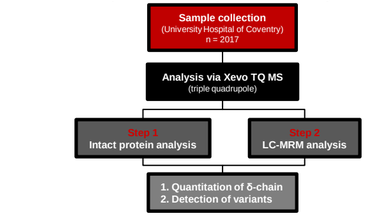
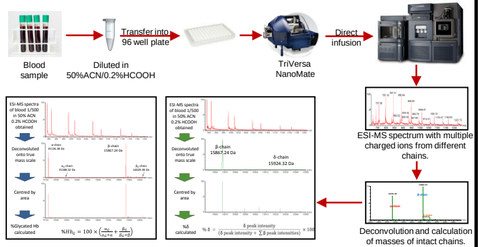
Figure 2. Differences between the amino acid sequence of β- and δ-globin chains

Material and Methods

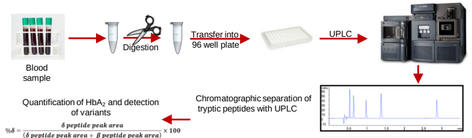
An overview of the 2 optimised methodologies used in this study are shown below:



Step 1. Direct infusion analysis of intact haemoglobin



Step 2. LC-MRM analysis of tryptic digest of blood



Peptide	Precursor ion (Da)	Product ion 1 (Da)	Product ion 2 (Da)
Hb6-T2	468.8	390.2	876.1
Hb6-T2	480.4	390.2	688.3
Hb6-T3	657.5	758.4	887.5
Hb6-T3	628.8	829.5	944.5
Hb6-T5	1030	1015.5	1102.5
Hb6-T5	1023	928.5	1116.5

The applied MRM transitions for T2, T3 and T5 peptides.

Variant type	Position of mutation	Amino acid change	Mass change (Da)	Precursor ion (Da)
Hb S	β6 (βT1)	Gluβ→Val	-30	461.8
Hb C	β6 (βT1 new peptide)	Gluβ→Lys	-1	694.4
Hb E	β26 (βT3 new peptide)	Gluβ→Lys	-1	916.8
Hb O-Arab	β121 (βT13 new peptide)	Gluβ→Lys	-1	625.3

Clinically significant variants, related mutation position and precursor ion used for the MRM analysis.

Results: Intact Protein Analysis

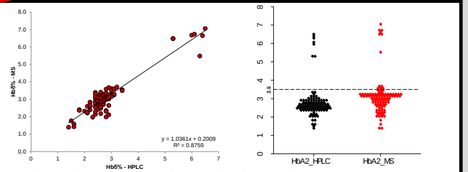


Figure 3. Correlation and distribution analysis of HbA_2 values measured with HPLC and intact δ/ß-chain analysis by direct infusion. (A) The chart presents the degree of correlation between the determined HbA_2 levels of samples from batch C (n = 151) based on intact δ/ß-chain with the HPLC values measured by the hospital (R^2 = 0.87). **(B)** The chart represents a comparison of the distribution of the determined HbA_2 levels of samples from batch C (n = 151) based on intact δ/ß-chain and HPLC values measured by the hospital. The general pattern of the distribution for both measurements are similar but the calculated HbA_2 levels based on the intact protein are slightly higher when compared to the HPLC values. A total number of 7 samples (including 2 standards) show elevated levels of HbA_2 in both measurement methods.

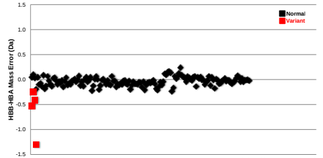


Figure 4. Mass error of the intact α/ß-chain measurements with direct infusion. The graph represents ±0.2 Da window for normal samples (black dots) and the samples with higher mass error indicate the possibility of abnormalities

Batch ID	Sample No.	Intact Protein Average R^2	Batch ID	Sample No.	Intact Protein Average R^2
C	155	0.68	I	114	0.82
D	108	0.90	J	114	0.74
E	280	0.70	K	144	0.72
F	251	0.62	L	166	0.74
G	93	0.61	M	180	0.81
H	228	0.65			

Table 1. The R^2 values of the correlation analysis of the HbA_2 level measurements from HPLC and intact protein analysis of the different sample batches in the clinical trial. The R^2 values obtained by plotting the HbA_2 levels measured by HPLC versus the calculated levels from intact δ/ß-chain analysis. The average R^2 across all batches was 0.73.

Results: LC-MRM Analysis

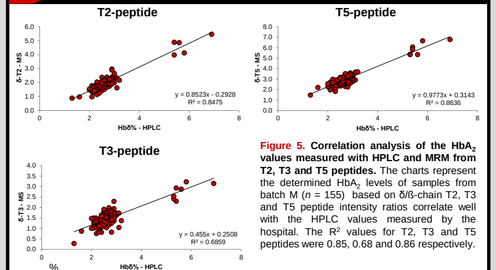


Figure 5. Correlation analysis of the HbA_2 values measured with HPLC and MRM from T2, T3 and T5 peptides. The charts represent the determined HbA_2 levels of samples from batch M (n = 155) based on δ/ß-chain T2, T3 and T5 peptide intensity ratios correlate well with the HPLC values measured by the hospital. The R^2 values for T2, T3 and T5 peptides were 0.85, 0.68 and 0.86 respectively.

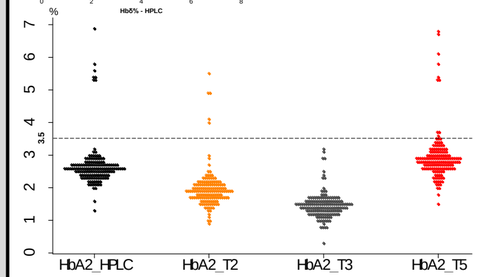


Figure 6. Distribution of HbA_2 values measured with HPLC and MRM from T2, T3 and T5 peptides. The chart represents a comparison of the distribution of the determined HbA_2 levels of samples from batch M (n = 155) based on δ/ß-chain T2, T3 and T5 peptide intensity ratios as well as HPLC values measured by the hospital. The general pattern of the distribution for each of the 4 different measurements are similar but the calculated HbA_2 levels based on the T2 and T3 peptides are lower when compared to the HPLC values.

Batch ID	Sample No.	MRM Average R^2 (T2)	MRM Average R^2 (T3)	MRM Average R^2 (T5)	Batch ID	Sample No.	MRM Average R^2 (T2)	MRM Average R^2 (T3)	MRM Average R^2 (T5)
B	136	0.65	0.43	0.90	H	228	0.65	0.83	0.89
C	155	0.26	0.77	0.88	I	114	0.30	0.86	0.71
D	108	0.05	0.44	0.91	J	114	0.66	0.59	0.90
E	280	0.47	0.79	0.81	K	144	0.46	0.90	0.80
F	251	0.30	0.69	0.77	L	166	0.34	0.25	0.88
G	93	0.11	0.47	0.79	M	180	0.72	0.72	0.76

Table 2. The R^2 values of the correlation analysis of the HbA_2 level measurements from HPLC and MRM analysis of the different sample batches in the clinical trial. The R^2 values obtained by plotting the HbA_2 levels measured by HPLC versus the calculated levels from MRM analysis of the T2, T3 and T5 peptides. The average R^2 values across all batches for T2, T3 and T5 peptides are 0.44, 0.65 and 0.84 respectively.

Clinical condition	Patient No.	Proportion	Analysis method	
			Intact protein	MRM
SS	18	0.9%	Indication for all.	All detected.
AS (+transfused SS)	52	2.6%	Indication for all.	All detected.
SC	3	0.15%	Indication for all.	All detected.
AC	6	0.3%	Indication for all.	All detected.
AD	10	0.5%	9	-
CC	1	0.05%	Indication for all.	All detected.
AE	1	0.05%	Indication for all.	All detected.
AO-Arab	1	0.05%	Indication for all.	All detected.
Beta thalassemia	1	0.05%	Indication for all.	All detected.
High F	4	0.2%	Indication for all.	-
Other abnormalities picked up by HPLC*	16	0.8%	Not investigated.	-
Variants only picked up by MS	6	0.3%	Indication for all.	-
Misdiagnosed by HPLC**	3	0.15%	Indication for all.	All detected.
Not confirmed by MS	1	0.05%	No indication.	-
Normal	1892	93.8%	Indication for all.	All detected.

* HPHF, Alpha-zero thalassemia, A2var, Hb Lepore carrier, unknown bands
** Elevated HbF was a β-chain variant, and HbD was HbG-Philadelphia

Table 3. List of clinical trial samples with haemoglobin abnormalities. A total number of 2017 samples were analysed in this study and the table shows the number of patients with haemoglobin related abnormalities and the efficacy of the two MS-based strategies in detecting these. The intact protein analysis gave indications for the presence of variants which required to be confirmed by MRM analysis. A total number of 6 samples classed as normal by the hospital, show the presence of haemoglobin abnormalities when analysed by MS approaches and the nature of these were later identified in 5 samples. Two of the samples classed as AD by the hospital were misdiagnosed and were actually carriers of an α-chain variant which was detected by mass spectrometry.

Conclusions

- An intact globin chain analysis was carried out for HbA_2 level determination in diluted whole blood on 2017 clinical samples, with good average correlation to HPLC values obtained.
- Mass error plots from intact protein analysis gave indication for the presence of α- and ß-chain variants which were then further investigated and characterised.
- Haemoglobin variants not identified using current screening methods were able to be characterised (*i.e.* HbJ-Baltimore and HbJ-Broussais).
- The MRM-based method gives an acceptable correlation using T3 peptides, and an excellent correlation when using T5 peptide ratios for HbA_2 level determination.
- The MRM-based method confirmed the presence of HbS, HbC, HbE, and HbO-Arab. The method can potentially be modified to monitor for the presence of other α- and ß-chain variants such as HbG Philadelphia which was later added to our method.
- Further optimisation for screening of the HbD-Punjab mutation via MRM analysis is necessary.
- The methods all show promise for diagnostic application providing definitive diagnosis and confirmation of the presence of clinically significant variants.
- The MS-based screening method has been semi-automated and can potentially be fully automated in future to be introduced for clinical use in a screening hospital.

Acknowledgment

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