Clin Chem Lab Med 2010;48(11):1611–1618 © 2010 by Walter de Gruyter • Berlin • New York. DOI 10.1515/CCLM.2010.317

Research Article

Towards the development of a certified reference material for hemoglobin A_2

Renata Paleari[1], Amalia Muñoz[2] and Andrea Mosca[1,*] on behalf of the IFCC Working Group on Standardization of HbA_2 ($WG\text{-}HbA_2$)

[1] Centre for Metrological Traceability in Laboratory Medicine (CIRME), Department of Science and Biomedical Technology, Laboratorio Interdisciplinare di Tecnologie Avanzate (LITA), University of Milan, Milan, Italy

[2] Institute for Reference Materials and Measurements (IRMM), European Commission, Joint Research Centre, Geel, Belgium

Abstract

Background: In 2004, a working group on the standardization of hemoglobin A_2 (HbA_2) was created within the IFCC, with the aim of developing a reference system for this analyte. One goal was to prepare a certified reference material in collaboration with the Institute for Reference Materials and Measurements (IRMM). This paper describes the properties of a first batch of this candidate study material.

Methods: Eighty millilitre of fresh whole blood, collected from a healthy blood donor, was treated by removing plasma, white blood cells and platelets. Red cells were hemolyzed to prepare 100 vials of lyophilized material (approximately 155 mg per vial). After reconstitution, the HbA_2 content was measured with a total of seven HPLC methods, three electrophoretic techniques, and two capillary electrophoresis (CE) methods. Homogeneity was tested in a subset of five vials. Stability during storage at +4°C and –20°C was tested monthly over a period of 1 year. The commutability of this material was assessed by analysing the study material together with a set of 54 fresh blood samples, with a subgroup of the above mentioned methods, only by one routine HPLC (Bio-Rad Variant II, dual kit) and by a CE method (Beckman PA800, Analis kit), respectively.

Results: The chromatographic and electrophoretic patterns obtained by all the HPLC, electrophoretic and CE techniques did not show any difference between those obtained using the first study material and those obtained with fresh blood samples. The lot was found to be homogeneous on the basis of the content of lyophilized powder per vial. The HbA_2 concentration in the lyophilized material remained stable at +4°C and –20°C, even after 1 year of storage. After reconstitution, the HbA_2 concentration did not change for more than 2 weeks in the refrigerator at +4°C. The normalized residual of the study material, measuring the degree of its commutability was 0.9, similar to that obtained on other home prepared and some commercial controls.

Conclusions: Ideally, fresh whole blood is the best reference material in the meterological traceability chain for HbA_2 analysis. However, for a number of reasons the preparation of large batches of fresh whole blood to be used as secondary reference material for HbA_2 is not practical. In our work, we have proven that lyophilization does not appear to cause any matrix effect or inhomogeneity in the study material, which also confirmed to be commutable for the Bio-Rad Variant II (dual kit) and Beckman PA800 (Analis kit) methods. We conclude that a material similarly prepared as the current study material and value assigned with the candidate reference measurement procedure still under development will be suitable to calibrate various routine methods for HbA_2. This will result in improvement of the inter-method variability for this important biochemical marker.

Clin Chem Lab Med 2010;48:1611–8.

Keywords: commutability; HbA_2; reference materials; standardization; thalassemia.

Introduction

Thalassemia syndromes and hemoglobinopathies are among the most common genetic disorders worldwide, and constitute a major health problem affecting approximately 400,000 newborns each year (1). The largest fraction of the affected newborns carry sickle cell disease, and from 60,000 to 70,000 have major β-thalassemia. Unfortunately most affected children are born in countries with limited resources, where hereditary disorders receive little attention and prevention programmes are not well implemented.

In this regard, laboratory medicine has an important role in developing low cost tests, with high diagnostic sensitivity and specificity. The measurement of hemoglobin A_2 (HbA_2) is indeed one of the laboratory tests most useful in assessing the carrier state of β-thalassemia. An increase in this hemoglobin fraction is one of the most typical markers that allows β-thalassemia heterozygotes to be recognized. Unfortunately, in some circumstances, such as in the presence of iron deficiency, the increase in HbA_2 can not be easily detected and interpretation of the HbA_2 data is more difficult, as illustrated in greater detail in a recent review (2).

*Corresponding author: Prof. Andrea Mosca, Dip. di Scienze e Tecnologie Biomediche, Via Fratelli Cervi 93, 20090 Segrate, Milan, Italy
Phone: +39 02 5033 0422, Fax +39 02 9998 7559,
E-mail: andrea.mosca@unimi.it
Previously published online October 30, 2010

Besides the clinical interpretation of the HbA_2 data, analytical aspects have to be considered. First, there is no reference method approved for this analyte at present, apart from the minicolumn method recommended by the International Committee for Standardization in Haematology (ICSH) from 1987 (3). Moreover, no certified reference material for HbA_2 has been developed to date, besides a WHO ''reference reagent'' for HbA_2 available from the National Institute for Biological Standards & Control (89/666, NIBSC, UK). This material has an assigned a HbA_2 value of 5.3% (mass fraction) of total hemoglobin, established using the means of an international collaborative study based on liquid chromatography.

As a consequence of this lack of standardization, it is quite difficult to compare HbA_2 results obtained by different methods in different laboratories. This is because the actual methods for HbA_2 are not standardized against a common, sufficiently robust reference system. Indeed, data obtained in External Quality Assessment Schemes (EQAS) indicate that there is an urgent need to improve the reliability of HbA_2 measurements (4).

In order to accomplish this, the IFCC promoted the creation of a Working Group with the task for the development of a complete reference system for HbA_2. A traceability chain has been established to improve the accuracy of HbA_2 quantification (5). A candidate IFCC reference measurement procedure, based on a quantitative peptide mapping analysis, has been developed. However, it still needs improvements in reproducibility and robustness.

In addition, a certification strategy has been studied, and this document is the first report on a study material in order to prove its suitability for the calibration or quality control of HbA_2 diagnostic quantification kits currently available.

Materials and methods

Preparation of the lyophilized material and reconstitution for use

Whole blood was obtained from the local blood donor centre Avis (Milan, IT, Italy) following informed consent. The donor blood samples were tested and found to be negative for antibodies anti-human immunodeficiency virus (anti-HIV), hepatitis B surface antigen (HbsAg) and antibodies against hepatitis C virus (anti-HCV). After removal of plasma and leukocytes by routine procedures, erythrocytes were stored at +4°C in a standard transfusion bag in the presence of CPDA as preservative.

The procedure was essentially similar to that already described for the preparation of control hemolysate for HbA_{1c} analysis (6). Within 7 days after collection, the erythrocytes were washed three times with cold isotonic saline solution (9 g/L NaCl) by centrifugation at 1000 *g* at +4°C. Washed erythrocytes were dialysed at +4°C against isotonic saline solution. The dialysis was completed after three changes of the dialyzing solution over 48 h. Packed erythrocytes were lysed in a 5% (mass fraction) sucrose solution to obtain hemolysate with a hemoglobin concentration of 25 g/L. This hemoglobin solution was dispensed in 2 mL aliquots into 5 mL dark glass vials and lyophilized.

A total of 100 vials were prepared and aliquoted inside a sterile clean-room. The temperature was maintained at –20°C for storage of the vials immediately after filling. Stoppers were entered half way into the vials and the shelves of the lyophilizer were loaded equally. In order to avoid microbial growth, the lyophilization program was designed to achieve a residual water mass fraction $<1\%$ in the lyophilized material. After lyophilization, the vials were filled with argon and stoppers were closed. The vials were stored at –80°C until analysis.

For reconstitution of the lyophilized samples, 300 μL of distilled water was added to each vial. The reconstituted samples were left at room temperature for 30 min at room temperature before use. The reconstituted material had a total hemoglobin concentration in the range of undiluted blood samples.

Methods for hemoglobin analysis

The total hemoglobin concentration was measured using Drabkin's method (7). Spectral analysis was performed between 350 and 700 nm. The methemoglobin (MetHb) content was measured using a standard spectrophotometric method (8).

Methods for the determination of HbA_2

The HbA_2 content was measured by different HPLC, electrophoresis and capillary electrophoresis (CE) methods. HPLC methods included: Bio-Rad Variant II dual kit, Bio-Rad Variant II β-thal, Bio-Rad Variant II β-thal short program, Menarini HA-8160, Tosoh G7, Tosoh G8, open HPLC system using PolyCATA column. Electrophoresis was performed using: Sebia Hydrasys, Beckman Paragon and a manual electrophoresis procedure with elution and quantification of the HbA_2 band performed as described previously (9). CE methods were: Beckman PA800 Analis kit and Sebia Capillarys. Finally, a reverse-phase HPLC method for globin chain separation was performed as described (10). The protocol for analysis prescribed using two separate vials per method, to be reconstituted and analyzed over two different days.

All methods were performed according to the manufacturer's instructions. We wish to note that some manufacturer do not provide calibration materials to the end user, and, to this regard, the following methods were run without any calibration: PolyCATA, Sebia Hydrasis, Beckman Paragon, Beckman PA800, Sebia Capillarys, the manual electrophoresis procedure and HPLC for globin chains. In all the automated methods, the procedure for the calculation of the relative abundance of HbA_2 with respect to all hemoglobin fractions was based on the integration of the areas obtained by the chromatograms or by the electropherograms.

Testing the homogeneity of the lyophilized material

Homogeneity was tested on five out of 100 vials selected randomly from the study material batch (after argon closure). Each vial was weighed before and after reconstitution. The HbA_2 concentration was measured in each vial by the Bio-Rad Variant II (dual kit) method.

Commutability

The commutability of the study material was tested by a method comparison between the HPLC Bio-Rad Variant II (dual kit) and the CE Beckman PA800 (Analis kit). Fifty-four EDTA blood samples were analyzed using the two methods, together with a set of four control materials from various manufacturers (Bio-Rad Laboratories: Lyphocheck Hemoglobin A_2 Controls, Level 1 and 2; Menarini: Eurotrol HbA_2/Hb Var Controls; Sebia: Normal HbA_2

Control), and three home-prepared as previously described LITA 1–3 (6). The same methods were used for the study material batch and all measurements were done in duplicate. The intermethod relationship in the analysis of patient blood samples was assessed by means of linear regression and dispersion around the line was estimated as the residual standard deviation ($S_{y|x}$). The non-parametric correlation coefficient (Spearman *r*) was also calculated. For each control material, the residual was calculated and then divided by the residual standard deviation of the patient blood samples to yield the normalized residual. The normalized residual of each control material was taken as the measure of its degree of commutability (11, 12). Normalized residuals outside the ±2SD interval were considered to indicate lack of commutability.

Stability study

Stability was evaluated for both the reconstituted and lyophilized material. For the evaluation after reconstitution, different vials of lyophilized samples were reconstituted and stored at +4°C. Samples were analyzed for their HbA_2 content after 1, 2, 4, 7, 10 and 22 days of storage, together with a freshly reconstituted sample (storage time in reconstituted form at 0 days). Each sample was measured in duplicate using the Bio-Rad Variant II (dual kit) system. Possible instrument drift was checked by analysis of internal quality control (IQC) samples.

Stability studies on the lyophilized samples were performed using an isochronous design (13), consisting of the simultaneous analysis of reference and test samples. For each study, a defined set of samples was exposed for different periods of time to increased temperature, and afterwards returned to the reference temperature (–80°C). At the end of the study, all the HbA_2 measurements were performed in the same analytical run to keep analytical imprecision at a minimum. The data were analyzed by determining the regression line for the HbA_2 concentration as a function of time (14). Analysis of variance (ANOVA) was also performed on the results of stability.

Statistical analysis of the stability study results

Stability experiment data were analyzed first by a single and then by a double Grubbs tests to detect outlying individual results, as well as for averages for each vial. Two measurements were performed at each temperature. The slope of the HbA_2 concentration as a function of time was considered to be significantly different from zero if the absolute value of slope *b* divided by its uncertainty u_b ($|b/u_b|$) was larger than $t_{0.05,4} = 2.78$.

The uncertainty contribution from stability was estimated according to the following expression:

$$u_{lts} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}}$$

With x_i being the time points for the individual sample (i), x the shelf life, and RSD the relative standard deviation of the results of the stability study. The calculated relative standard uncertainty (u_{lts}) was 1%.

Linear regression analysis was performed using SigmaPlot version 11 (Systat Software Inc., Chicago, IL, USA).

Results

Characterization of the study material

Thirty vials produced in the first batch were dedicated to the quantification of HbA_2 by most of the methods currently available in order to check for the presence of abnormal fractions or degradation products formed during the lyophilization process. Figure 1 shows a typical chromatogram obtained by the Bio-Rad Variant II (dual kit) HPLC system. As can be seen, we found no difference between HPLC analysis performed on the starting material and on the final product following lyophilization. In addition, no unexpected peaks were detected in this material, compared to the chromatograms obtained on fresh blood samples, neither from healthy adults or carriers of β-thalassemia.

With regard to the total area of the chromatographic peaks, the study material did not show any significant difference from that obtained using fresh blood samples. This finding is in agreement with the total hemoglobin concentration in the study material being comparable to that of normal human blood. The chromatograms and electropherograms showed patterns similar to those on fresh human blood samples (Figure 2). Table 1 shows the average values of HbA_2 measured by all methods that were tested. All the data obtained was compatible with the HbA_2 concentration seen in healthy adults who are not carriers of β-thalassemia.

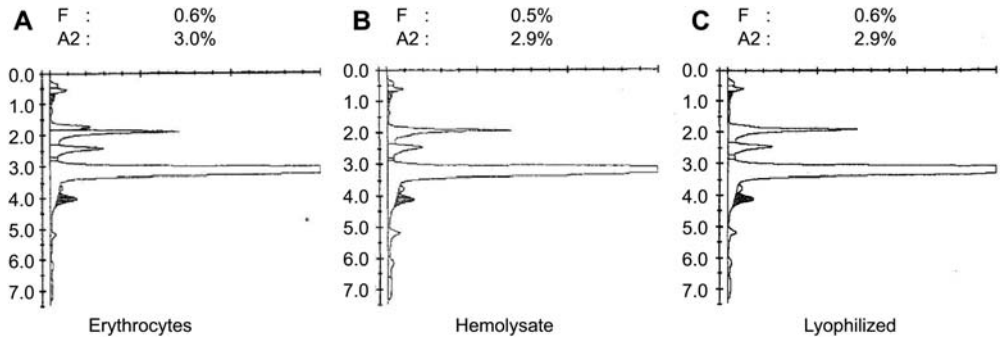

Figure 1 Anion exchange HPLC chromatograms of human erythrocytes used as starting material (A), the intermediate fraction before lyophilization (B) and the final lyophilized product (C).

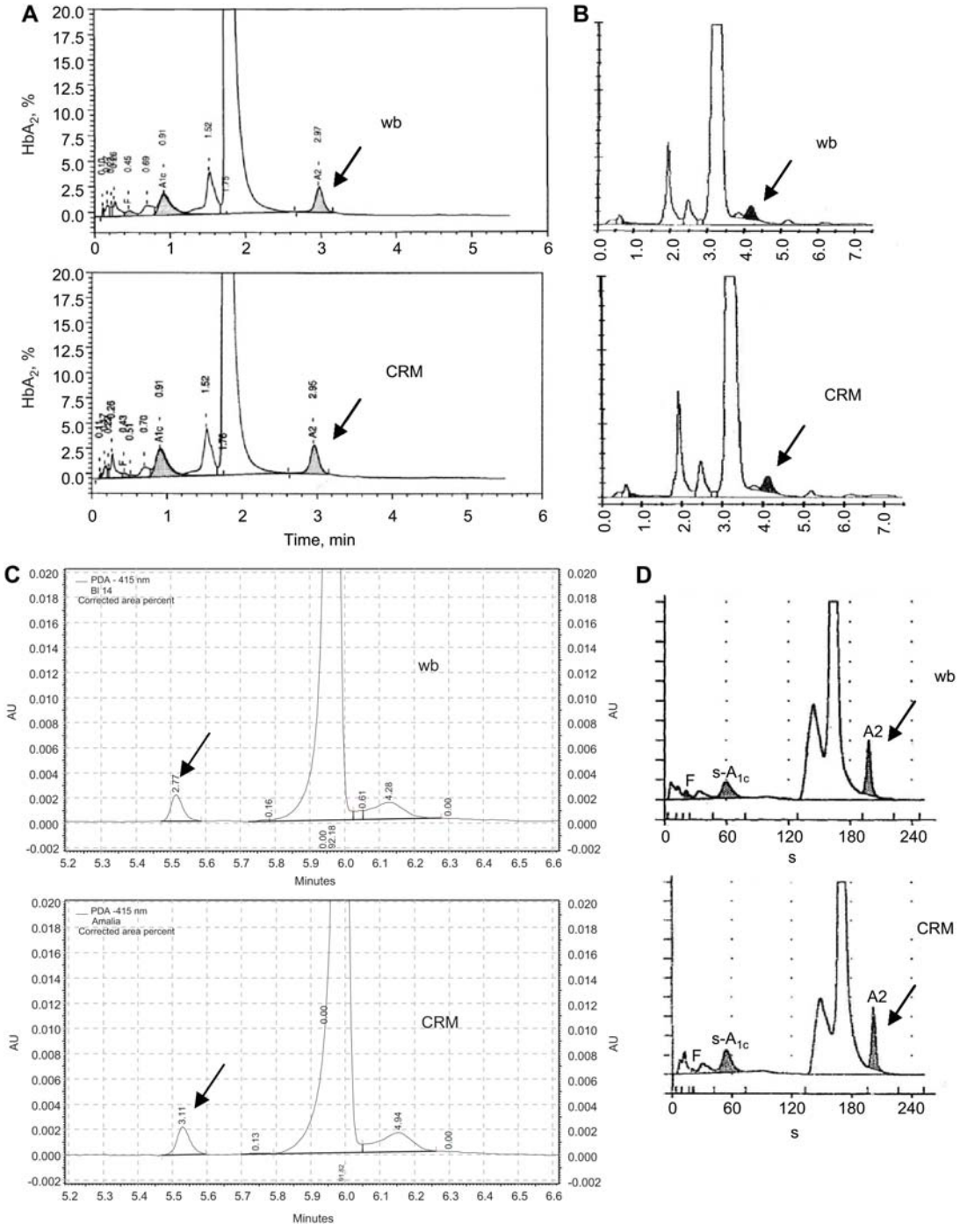

Figure 2 Analysis of the study material by various analytical techniques, compared to that of a fresh whole blood sample (wb). (A) Bio-Rad Variant II, dual kit; (B) Tosoh G7; (C) Beckman Coulter PA800; (D) A. Menarini HA 8160. The peak corresponding to HbA_2 is indicated by the arrow.

Homogeneity

The mean filling weight was 157 mg, with a coefficient of variation (CV) of 0.9%. The mean ($\pm$SD) HbA_2 concentration was 3.0%$\pm$0.1% mass fraction HbA_2 in total hemoglobin. Thus, this material can be considered as being sufficiently homogeneous after reconstitution.

Stability upon storage under various conditions

With regard to the HbA_2 content of the non-reconstituted samples, a 1-year stability study was performed in order to confirm the stability of the material upon storage at +4°C and –20°C, as shown in Figure 3A. A test for significance of the slope was performed. None of the slopes were signif-

Table 1 Characterization of the study material. All the analyses were performed in duplicate using two different vials over 2 days (total of four data points).

Method	Instrument	HbA_2, % mean ± SD
HPLC	Bio-Rad Variant II, dual kit	2.9 ± 0.1
	Bio-Rad Variant II, b-thal	2.8 ± 0.1
	Bio-Rad Variant II, b-thal, short	3.0 ± 0.1
	Menarini HA-8160	3.1 ± 0.1
	Tosoh G7	2.8 ± 0.1
	Tosoh G8	2.9 ± 0.1
	PolyCATA	2.4 ± 0.1
	RP-C4 globin chains separation	2.0 ± 0.2
Capillary	Beckman PA800, Analis kit	3.0 ± 0.1
Electrophoresis	Sebia, Capillarys	2.9 ± 0.1
Electrophoresis	Sebia Hydrasys	2.2 ± 0.1
	Beckman Paragon	2.7 ± 0.1
	Manual procedure with elution	2.7 ± 0.1

Table 2 Test for significance of slopes (95% confidence level). Slope (b), standard deviation (s_b) and b/s_b in the 1 year stability experiment.

θ, °C	b, %/month	s_b, %/month	t-Test	
			$t = b/s_b$	$t_{0.005,10}$
+4	–0.003	0.002	1.5	2.23
–20	–0.019	0.037	0.51	2.23

icantly different from zero at a 95% confidence level, as shown in Table 2.

Concerning the stability after reconstitution, the data are shown in Figure 3B. As can be seen, the reconstituted materials can be maintained for at least 2 weeks at +4°C, with no significant (95% confidence level) change in HbA_2 content. In addition, none of the typical abnormalities deriving from a degradation of the sample (i.e., presence of additional peaks, shift in the retention times, baseline drifts) were observed in the chromatograms during the entire time of storage.

Concerning the presence of hemoglobin derivatives, the MetHb concentration was also measured during storage at +4°C and at –20°C, yielding the data presented in Figure 4. As can be seen, a slight increase in MetHb concentrations can be observed after 12 months of storage at +4°C.

Finally, the materials were analyzed with regard to the stability of other hemoglobin fractions. These components are detected by the various HPLC and electrophoretic techniques used for the quantification of HbA_2. Of particular interest was the stability of HbA_{1c}, reported in Figure 5. HbA_{1c} is of particular interest since a material similarly prepared as the study material may serve as a commutable calibrator and quality control material for this analyte. Also, this fraction did not deteriorate in the pilot batch stored for up to 1 year at +4°C or at –20°C.

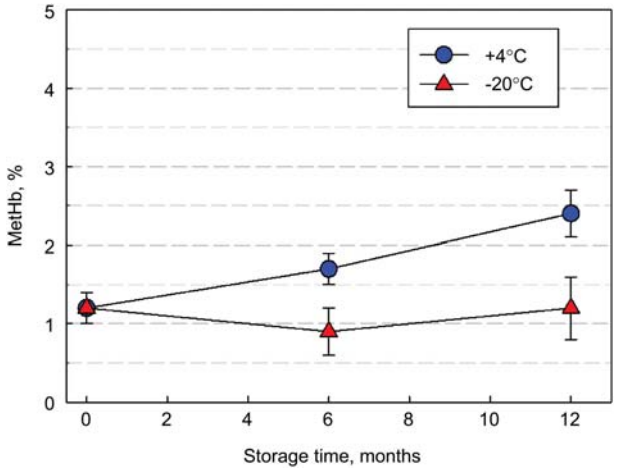

Figure 4 Methemoglobin (MetHb) content of the IRMM vials stored at +4°C and at –20°C over 1 year, and analyzed at baseline and every 6 months.
Mean and SD of three determinations are reported.

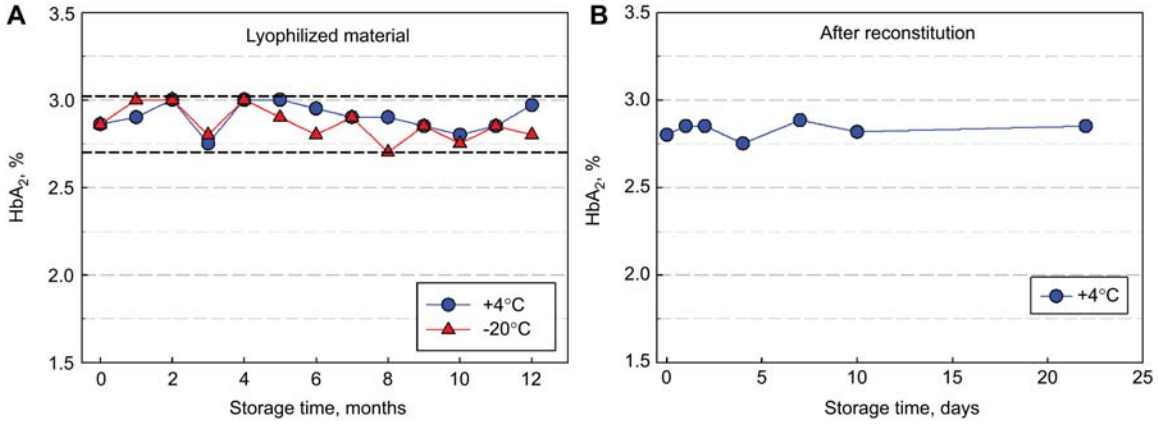

Figure 3 Stability of the candidate material.
(A) Stored in the lyophilized state at different temperatures for up to 1 year; (B) after reconstitution and storage at +4°C for up to 3 weeks. The dashed lines indicate the confidence interval of the target HbA_2 value measured before storage.

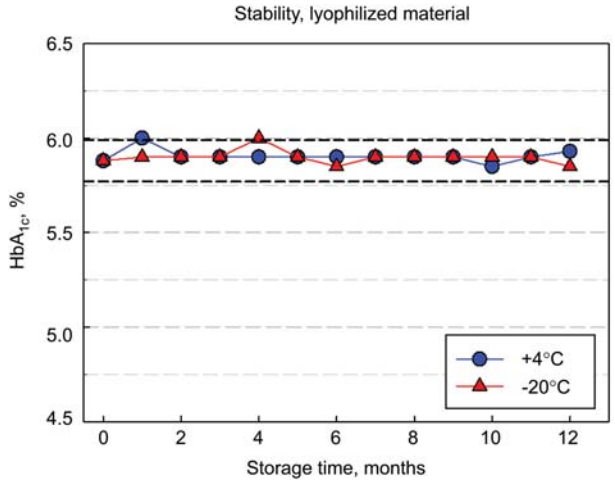

Figure 5 HbA_{1c} concentrations in aliquots of the study material after storage, as in Figure 3A.

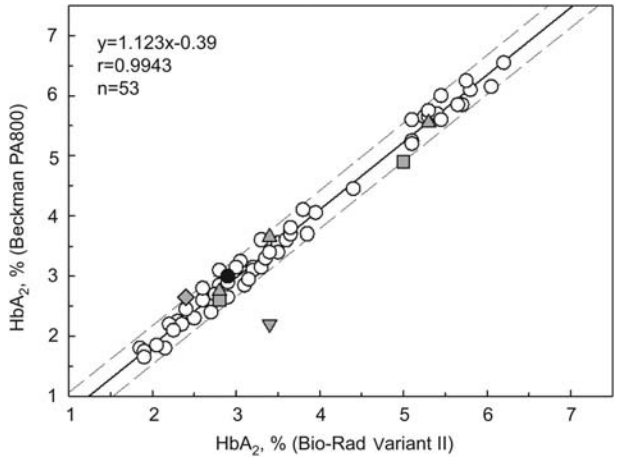

Figure 6 Commutability test on the intermethod relationship between an HPLC (*x*-axis) and capillary electrophoresis (*y*-axis) method for HbA_2.

The linear regression (full) and 95% prediction intervals (dashed) lines are reported. Empty circles, patient blood samples; gray symbols, control materials from various sources (■, Bio-Rad; ▲, LITA; ▼, Menarini; ◆, Sebia); full circle, IRMM study material.

Commutability

The intermethod differences observed in the analysis of blood samples and various control materials are shown in Figure 6, where the means of the duplicate analyses are reported. The aberrant intermethod behaviour of some non-commutable control materials are clearly shown. Table 3 reports the statistical assessment of the method comparison results, where the normalized residuals of the seven tested control materials are listed. The intermethod behaviour of the study material looks similar to that of fresh blood samples, as well as to some, but not all, tested control materials. Particularly, the Menarini control material at low HbA_2 concentration proved to not be commutable among the two tested methods.

Table 3 Main characteristics and normalized residuals of the commercially available lyophilized control materials included in this study.

No.	Product name	HbA_2, % mass fraction[a]	Normalized residual, % mass fraction
1	Bio-Rad 1	2.8	–1.0
2	Bio-Rad 2	5.0	–2.2
3	Menarini	3.4	–8.2
4	LITA 1	2.8	0.3
5	LITA 2	5.3	0.3
6	LITA 3	3.4	1.8
7	Sebia	2.4	2.6
8	IRMM study material	2.9	0.9

[a]Bio-Rad Variant II (dual-kit).

Discussion

The availability of certified reference materials to check the analytical performance of the methods is of fundamental importance for IQC and EQAS. Also, the use of a commutable calibrator is of paramount importance for the proper implementation of the in vitro diagnostics (IVD) directive. The main requirements of a certified reference material (CRM) are that the certified values be metrologically traceable and accompanied by an uncertainty statement. Moreover, adequate stability and homogeneity with respect to all the certified properties need to be verified. Ideally, the CRMs should be commutable, i.e., they must resemble the behaviour of patient samples in order to minimize calibration bias.

Ideally, pooled blood samples would be best, but these materials cannot be stored in large quantities for a long time. Therefore, some type of stabilized materials need to be provided instead. In the case of proteins, stabilization can be achieved by storing materials treated with various cryoprotectants at –20°C. Glycerol, for instance, has been successfully used for enzymes (15), and ethylene glycol was found to be very effective for glycated hemoglobin (16) and HbA_2 (17). Unfortunately, these treatments require maintaining the samples at low temperatures during storage, as well as during transport, resulting in higher transportation costs and complicated logistics.

For these reasons, it is advantageous if an appropriate lyophilization protocol could be developed which stabilizes the proteins without changing their analytical behaviour or the behaviour of the matrix. In the case of hemoglobin analysis, this treatment has been the best choice for HbA_{1c} (6, 18), and has also been applied to HbA_2. In both cases, the materials were prepared starting with whole blood, followed by removal of plasma and preparation of a hemolysate from washed red cells. In this way, all red cell proteins and other components were preserved in the final lyophilized product, thus being more like fresh blood samples than other control materials prepared from purified hemoglobins. Indeed, it is particularly important that all hemoglobin fractions initially present in the native fresh red cells be also present in the final lyophilized material, since all the presently available

methods for HbA_2 are essentially based on the separation of this hemoglobin fraction from the other hemoglobins.

Generally, lyophilized hemoglobin material can be stable for long periods when stored at +4°C or at lower temperatures, although some extra peak eluting between HbA_{1c} and HbA_0 has been reported which is probably related to the formation of an interfering compound (19). This fraction is often responsible for the apparent instability of HbA_{1c} if the analytical technique is not able to separate them properly. However, in our experience such a fraction was never observed in the study material, even after 1 year of storage.

Concerning the stability of lyophilized materials for HbA_2, similar to the study material, more experiments are planned to test it over a longer period of time (4–5 years) under conditions of accelerated degradation (at +18°C and +40°C). These tests will be performed on a new batch in the near future, probably also testing different kinds of vials or other protective gases in order to keep oxidation and denaturation of hemoglobin at a minimum. Indeed, until now, a slight increase in the formation of MetHb after 1 year of storage at +4°C was found, but this was without any consequence on the HbA_2 mass fraction. Ideally MetHb should be at the same level as in the starting material or in fresh human blood (i.e., normally <1% mass fraction out of total hemoglobin).

Finally, with regard to the commutability of the study material, our data show that this material was found to be commutable between two methods (Bio-Rad Variant II HPLC system and Beckman PA800 CE system). In the next batch, commutability will be tested with other methods, such as those listed in Table 1. However, it is very likely that the similarly-produced second batch will work properly with these other methods. Indeed, the chromatographic profile, as shown in Figure 2, is a good indication that this material behaves similarly to fresh human blood.

The development and nomination of the candidate reference measurement procedure for HbA_2, based on quantitative peptide mapping and mass spectroscopy, is required before any steps concerning the processing and certification of a reference material can be done. A reference method is needed for characterization, as already illustrated (5), in order to be able to certify a reference material that is part of a reference measurement system for procedure-defined measurands. At present, the reference method is still under development, and improvement in interlaboratory precision, arising partly from the digestion phase, is still required. A possible use of ^{13}C labelled peptides is under investigation in order to improve the precision of the peptide quantification step, in line of what has been recently developed for HbA_{1c} (20).

Acknowledgements

We wish to thank the IFCC Scientific Division for supporting the project of the Working Group for the Standardization of HbA_2. We thank particularly the following colleagues for testing the study material with various techniques, as indicated: Antonio Amato (Centro Microcitemie, Roma, I; Bio-Rad Variant II β-thal), Emmanuel Bissé (University Hospital, Freiburg, Germany; PolyCATA HPLC), Barbara Wild (University Hospital, London, UK; Bio-Rad Variant II β-thal and zone electrophoresis with manual elution), Geneviéve Hennache (Sebia, France; Sebia Capillarys and Hydrasis). Part of the work was supported by a European project (ENERCA III, AM as recipient).

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.

References

1. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bulletin of the World Health Organization 2001;79:704–12.
2. Mosca A, Paleari R, Ivaldi G, Galanello R, Giordano PC. The role of haemoglobin A2 testing in the diagnosis of thalassaemias and related haemoglobinopathies. J Clin Pathol 2009;62:13–7.
3. International Committee for Standardization in Haematology (ICSH). Recommendations for selected methods for quantitative estimation of HbA2 and for HbA2 reference preparation. Br J Haematol 1978;38:573–8.
4. Paleari R, Giambona A, Cannata M, Leto F, Maggio A, Mosca A. External quality assessment of haemoglobin A2 measurement: data from an Italian pilot study with fresh whole blood samples and commercial HPLC systems. Clin Chem Lab Med 2007;45:88–92.
5. Mosca A, Paleari R, Galanello R, Sollaino C, Perseu L, Demartis FR, et al. IFCC Working Group on Standardization of HbA2. New analytical tools and epidemiological data for the identification of HbA2 borderline subjects in the screening for beta-thalassaemia. Bioelectrochemistry 2008;73:137–40.
6. Mosca A, Paleari R, Trapolino A, Capani F, Pagano G, Plebani M. A re-evaluation of glycohemoglobin standardization: the Italian experience with 119 laboratories and 12 methods. Eur J Clin Chem Clin Biochem 1997;35:243–8.
7. Fairbanks VF, Klee GG. Biochemical aspects on hematology. In: Burtis CA, Ashwood ER, editors. Tietz Textbook of Clinical Chemistry. Philadelphia: W.B. Saunders Company, 1996:2020–1.
8. Fairbanks VF, Klee GG. Biochemical aspects on hematology. In: Burtis CA, Ashwood ER, editors. Tietz Textbook of Clinical Chemistry. Philadelphia: W.B. Saunders Company, 1996:2025–7.
9. Chanarin I, Waters DA. Haemoglobin analysis. In: Chanarin I, editor. Laboratory Haematology, an account of laboratory techniques. Churchill Livingstone Edinburgh, 1989:38–9.
10. Leone L, Monteleone M. Reversed-phase high-performance liquid chromatography of human haemoglobin chains. J Chromatogr 1985;321:407–19.
11. Van Helden WC, Visser RW, Van Den Bergh FA, Souverijn JH.

Comparison of intermethod analytical variability of patient sera and commercial quality control sera. Clin Chim Acta 1979;93: 336–47.
12. Middle JG, Libeer J-C, Malakhov V, Penttila I. Characterisation and evaluation of external quality assessment scheme serum. Clin Chem Lab Med 1998;36:119–30.
13. Linsinger TP, Pauwels J, Lamberty A, Schimmel HG, van der Veen AM, Siekmann L. Estimating the uncertainty of stability for matrix CRMsFresenius. J Anal Chem 2001;370:183–8.
14. Pauwels J, Schimmel H, Lamberty A. Criteria for the certification of internationally acceptable reference materials. Clin Biochem 1998;31:437–9.
15. Gekko K, Timasheff SN. Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. Biochemistry 1981;20:4667–76.
16. Mosca A, Carpinelli A, Paleari R, Carenini A, Bonini PA, Franzini C. Preparation and control of liquid ethylene glycole-stabilized hemolysates for glycosylated hemoglobin assay. J Clin Chem Clin Biochem 1985;23:361–4.
17. Franzini C. Ethylene glycol-stabilized haemolysates as control material in haemoglobinometry. Clin Chim Acta 1983;135:175–9.
18. Weykamp CW, Penders TJ, Muskiet FA, van der Slik W. Evaluation of a reference material for glycated haemoglobin. Eur J Clin Chem Clin Biochem 1996;34:67–72.
19. Little RR, Wiedmeyer HM, England JD, Naito HK, Goldstein DE. Interlaboratory comparison of glycohemoglobin results: College of American Pathologists survey data. Clin Chem 1991;37:1725–9.
20. Kaiser P, Akerboom T, Ohlendorf R, Reinauer H. Liquid Chromatography–Isotope Dilution–Mass spectrometry as a new basis for the reference measurement procedure for hemoglobin A1c determination. Clin Chem 2010;56:750–4.