Voxelotor Treatment Interferes With Quantitative and Qualitative Hemoglobin Variant Analysis in Multiple Sickle Cell Disease Genotypes

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Key Words: Voxelotor; Oxbryta; GBT440; HbS percentage; Sickle cell disease; Hemoglobin S; Interference

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ABSTRACT

Objectives: *Voxelotor was recently approved for use in the United States as a treatment for sickle cell disease (SCD) and has been shown to interfere with the quantitation of hemoglobin (Hb) S percentage. This study aimed to determine the effect of voxelotor on the quantitation of hemoglobin variant levels in patients with multiple SCD genotypes.*

Methods: *In vitro experiments were performed to assess the impact of voxelotor treatment on hemoglobin variant testing. Whole blood samples were incubated with voxelotor and then analyzed by routinely used quantitative and qualitative clinical laboratory methods (high-performance liquid chromatography [HPLC], capillary zone electrophoresis [CZE], and acid and alkaline electrophoresis).*

Results: *Voxelotor modified the α-globin chain of multiple hemoglobins, including HbA, HbS, HbC, HbD-Punjab, HbE, HbA_2, and HbF. These voxelotor-hemoglobin complexes prevented accurate quantitation of multiple hemoglobin species, including HbS, by HPLC and CZE.*

Conclusions: *Technical limitations in quantifying HbS percentage may preclude the use of HPLC or CZE for monitoring patients treated with voxelotor. Furthermore, it is unclear whether HbS-voxelotor complexes are clinically equivalent to HbS. Consensus guidelines for reporting hemoglobin variant percentages for patients taking voxelotor are needed, as these values are necessary for determining the number of RBC units to exchange in acute situations.*

Key Points

- Voxelotor, a new drug for sickle cell disease, interferes with the routine measurement of hemoglobin S percentage.
- Current quantitation methods may need to be altered depending on whether hemoglobin and hemoglobin-voxelotor complexes are considered clinically equivalent or distinct.
- Clinical consensus is needed to guide laboratory reporting of hemoglobin variant analyses for patients taking voxelotor.

Sickle cell disease (SCD) is a common genetic disorder, affecting approximately 100,000 Americans.[1,2] SCD is caused by mutations in the gene that encodes β-globin, which result in the production of variant hemoglobin species instead of wild-type hemoglobin A (HbA). Hemoglobin S (HbS) is a common variant, carried in the heterozygous state by 7.7% of the African American population, although symptoms typically do not occur in individuals with sickle cell trait.[3] SCD occurs when an individual is homozygous for HbS (HbSS) or coinherits HbS with another abnormal form of hemoglobin, such as hemoglobin C (HbC), hemoglobin D–Punjab (HbD-Punjab), hemoglobin E (HbE), or hemoglobin O–Arab (HbO-Arab), or with β-thalassemia. HbS has an increased propensity to polymerize in the deoxygenated state,[2] which results in damage to RBC membranes and produces the classic sickle-shaped RBCs associated with SCD. These changes to RBCs lead to vaso-occlusion and hemolysis. Consequently, patients with SCD often have chronic anemia and pain, and they may experience stroke and organ damage due to vaso-occlusive episodes, contributing to early mortality.[2]

Current treatments for the prevention of SCD symptoms include oral doses of hydroxyurea and RBC transfusions.[4] The goal of both treatment strategies is to reduce the relative percentage of HbS (HbS%) to prevent sickling events. Quantitation of specific hemoglobin species in a patient's blood can be helpful for monitoring the patient's SCD. The percentage of fetal hemoglobin (HbF%) is monitored in patients receiving hydroxyurea, and prior to an RBC exchange transfusion, the HbS% is measured to calculate the number of RBC units to transfuse. The use of these values in the clinical treatment of patients with SCD highlights the importance of the accurate measurement of hemoglobin species. Currently, the methods most commonly used in laboratories for the quantitative measurement of HbS%, as well as the quantitative assessment of other hemoglobin species, are high-performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE).[5] Acid and alkaline gel electrophoresis are also commonly used for qualitative assessment of hemoglobin variants. All techniques rely on the highly reproducible separation of different hemoglobin species to provide relative percentages of each hemoglobin present in the patient's blood.

Voxelotor (Oxbryta, previously known as GBT440) is a novel drug that was recently approved by the US Food and Drug Administration (FDA) for the treatment of SCD.[6,7] Voxelotor functions by reversibly binding to one α-globin chain within the hemoglobin tetramer, which stabilizes hemoglobin in the oxygenated state.[8,9] Given that α-globin is not specific to HbS, voxelotor also forms complexes with other hemoglobin species.[5] HbS polymerizes when deoxygenated, and thus voxelotor treatment should prevent polymerization and reduce RBC sickling and hemolysis, as well as end-organ damage experienced by patients with SCD.

It was previously reported that voxelotor interferes with hemoglobin variant analysis via HPLC and CZE analysis in a patient with HbSS who was participating in a multicenter clinical trial of voxelotor; this study confirmed via mass spectrometry that the interfering peak was an HbS-voxelotor complex.[5] The chromatogram displayed two intersecting peaks in the HbS window, which appear as a "split HbS peak." The additional peak prevented accurate quantitation of HbS% and subsequently delayed RBC exchange transfusion for this patient. The additional peaks in samples from the patient posttransfusion appeared to be HbA-voxelotor and HbF-voxelotor complexes, and they demonstrated that most (>65%) current methods used in clinical laboratories are unable to provide an accurate measurement of HbS% in a patient with HbSS SCD when HbS-voxelotor complexes are present.[5]

Here, we characterized the effect of voxelotor treatment on routine laboratory analysis and quantitation of hemoglobin variant levels in patients with the SCD genotypes who could require transfusion and were available in our laboratory during the study period: HbSC, HbSS, HbSD-Punjab (Los Angeles), HbSE, and HbSβ^0-thalassemia. We also included HbS-Kenya due to its migration pattern. We created Hb-voxelotor complexes by incubating voxelotor with residual blood samples from patients with normal or variant hemoglobins. We subsequently performed quantitative hemoglobin variant analysis on paired samples using HPLC and CZE as well as qualitative variant analysis using acid and alkaline gel electrophoresis.

Materials and Methods

To evaluate the effect that voxelotor may have on hemoglobin variant analysis for patients with SCD with varying genotypes, we collected residual whole blood samples from our routine hemoglobin variant testing. Voxelotor is known to modify hemoglobin molecules in vitro,[9] and by spiking residual blood samples with voxelotor, we were able to mimic patient treatment with voxelotor.[5] Paired voxelotor-treated (Vox+) blood samples were then analyzed by three methods used for hemoglobin variant analysis: HPLC (BioRad Variant II β-thalassemia short program), CZE (Sebia Capillarys 2 Flex-piercing Hemoglobin [E] program), and acid/alkaline gel electrophoresis (Helena SPIFE).

Sample Collection

Residual EDTA-anticoagulated whole blood samples (BD Vacutainer) from routine hemoglobin variant testing were deidentified and retained for this study. Samples were stored at 4°C in accordance with our specimen retention policy, and spiking experiments and subsequent analysis were performed within 2 weeks of sample receipt. Samples were excluded if hematocrit was less than 20%. This study was approved by the institutional review board (IRB #180370) as non–human subjects research and prior consent was not required. Blood samples were collected from patients with the following hemoglobin genotypes: HbAA, HbSC, HbSS, HbSD-Punjab, HbSE, HbSβ^0-thalassemia, and HbS-Kenya.

Treatment With Voxelotor

Blood samples were diluted to 20% hematocrit with BioRad β-thalassemia short program wash/diluent solution. To create Vox+ samples, the diluted blood was

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mixed with 67 mg/mL voxelotor (MedChemExpress) in 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to a final concentration of 300 µmol/L voxelotor. The targets for hematocrit and voxelotor concentration were selected based on previously published in vitro spiking studies.[9] An aliquot of the same sample was used as a paired control for each genotype. The paired controls were handled in the same manner as the voxelotor-spiked samples. Each paired control sample had an equal volume of voxelotor-free DMSO added to blood prior to incubation. Increasing the DMSO concentration up to 6.5-fold over the concentrations used in the study did not alter the chromatogram compared with a native sample (data not shown).

Optimal conditions for complex formation were investigated by incubating test samples for (1) 1 hour at room temperature, (2) 2 hours at room temperature, (3) 1 hour at 37°C, and (4) 2 hours at 37°C. All four conditions yielded the same chromatographic profile (see supplemental material; all supplemental materials can be found at *American Journal of Clinical Pathology* online); therefore, Vox+ samples used in this study were incubated with voxelotor for 1 hour at room temperature. The HPLC analysis shown here was performed immediately after incubation. Samples for CZE and acid and alkaline gel electrophoresis were shipped overnight at 4°C and tested within 72 hours of preparation. To determine if shipping conditions or the testing delay affected stability, another set of HbSS samples was prepared and analyzed immediately by CZE, and samples were then shipped overnight at 4°C for testing by HPLC. No differences were observed between the two sets of data (data not shown).

Qualitative and Quantitative Sample Analysis

Vox+ and control samples were analyzed by three methods: (1) cation exchange HPLC (BioRad Variant II β-thalassemia short program), (2) CZE (Sebia Capillarys 2 Flex-piercing Hemoglobin [E] program), and (3) acid and alkaline gel electrophoresis (Helena SPIFE instrument). Quantitative analysis of Vox+ and control samples was performed on paired samples using HPLC and CZE. In a quantitative analysis, vendor-provided software integrates the area under each peak present in the chromatogram. The relative percentage of each hemoglobin present is based on the peak area of that hemoglobin species divided by the total area of the chromatogram. For example, HbS% = (peak area HbS/sum of area of all peaks) × 100%. The reliability and reproducibility of quantitative measurements is best when the chromatographic peaks completely coelute or are baseline resolved. If the peaks are not well resolved, the software may make an inaccurate baseline assignment that affects the accuracy of quantitative hemoglobin results.

Results

Analysis of Vox+ blood samples containing different hemoglobin variants confirmed that voxelotor forms stable complexes with non-HbS hemoglobins in vitro **Figure 1**, **Figure 2**, **Figure 3**, and **Table 1**. Optimal test conditions were determined by incubating samples with voxelotor or DMSO for 1 hour at 25°C (supplemental material). Each chromatogram shown corresponds to a single patient sample spiked with voxelotor; however, multiple patients were tested for each genotype by HPLC and CZE with the exception of genotypes HbSD, HbSE, and HbS-Kenya. HPLC chromatograms for patients with HbSβ^0-thalassemia displayed the same change as HbSS patients upon voxelotor treatment and are shown in the supplemental material, along with HPLC chromatograms for multiple HbSS and HbSC patients. The migration pattern and retention time were consistent over all patient samples tested **Table 2**.

HPLC Analysis

The cation-exchange HPLC analysis of Vox+ samples displayed altered chromatographic profiles compared with the DMSO-treated control samples (Figure 1). The chromatogram for Vox+ HbAA had an additional distinct and resolvable peak that eluted before HbA (HbA, 64.0%; HbA-Vox, 28.5%; Figure 1A). The chromatogram for Vox+ HbSS displayed a peak that overlapped with HbS (HbS, 47.1%; HbS-Vox, 20.1%; Figure 1C). The chromatogram for Vox+ HbSC also displayed an overlapping peak with HbS (HbS, 26.6%; HbS-Vox, 6.3%; Figure 1B) and a very small distinct and resolvable peak that eluted before HbC (2.3%). The chromatogram for Vox+ HbSD displayed an extra peak before HbS and caused the HbS peak to become asymmetric (Figure 1D). In this sample, HbA was present due to a recent RBC transfusion. In addition, Vox+ HbSD was not properly quantified by the software due to the cluster of peaks (Figure 1D). For Vox+ HbS-Kenya, the chromatogram displayed a second peak that overlapped with HbS (HbS, 31.4%; HbS-Vox, 12.3%; Figure 1E), as well as additional shoulders on the Hb Kenya/A_2 and HbF peaks. The chromatogram for Vox+ HbSE displayed a peak that overlapped with HbS (HbS, 35.8%; HbS-Vox, 12.0%; Figure 1F), a shoulder on the HbE/A_2 peak, and an additional resolvable peak that eluted before the HbE/A_2 peak (6.7%).

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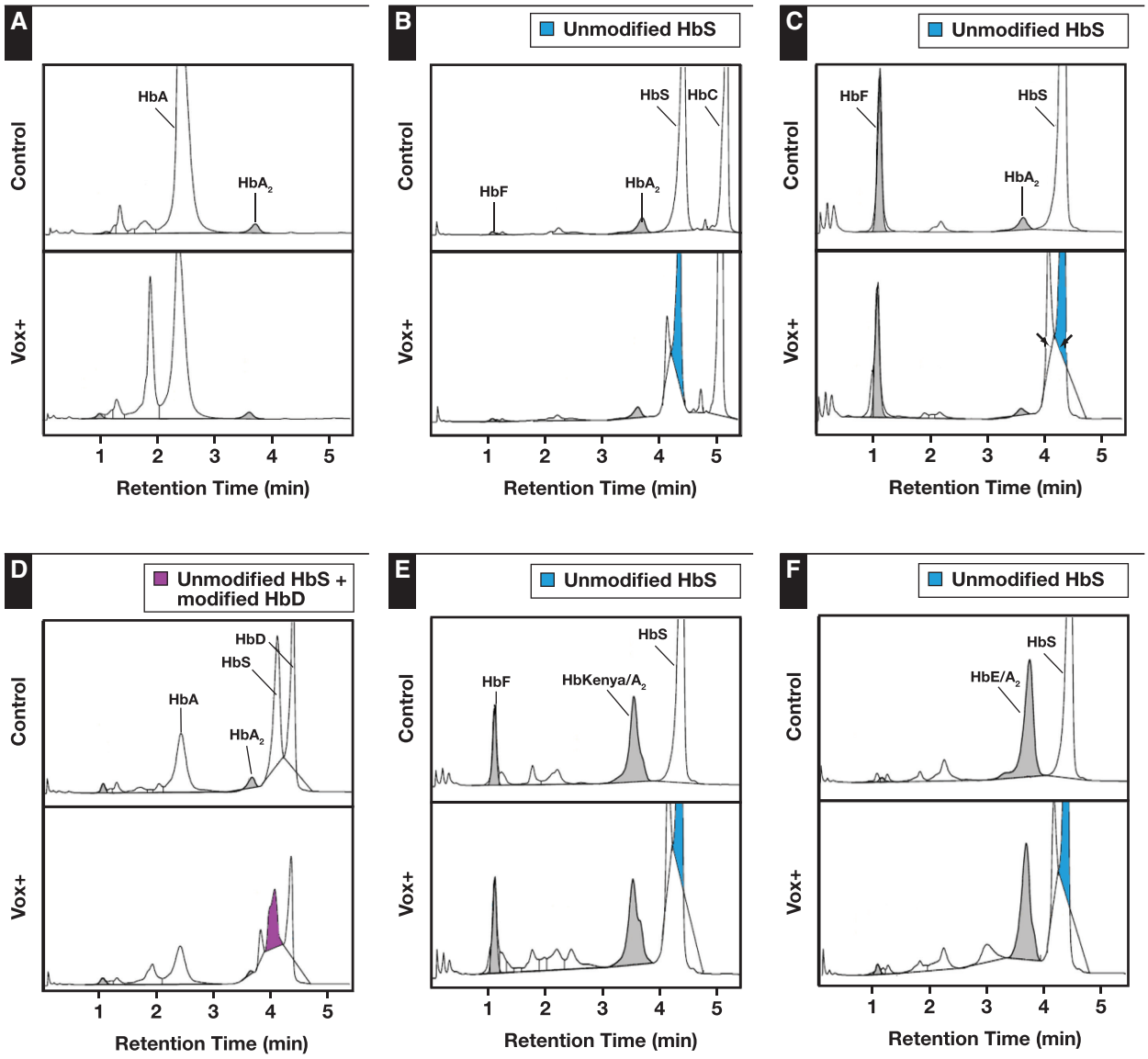

Figure 1 Cation exchange high-performance liquid chromatography analysis of whole blood samples with different hemoglobin variant profiles without voxelotor treatment (control) and with voxelotor treatment (Vox+). Hemoglobin (Hb) variants tested include HbAA (**A**), HbSC (**B**), HbSS (**C**), HbSD (**D**), HbS-Kenya (**E**), and HbSE (**F**). Arrows in **C** indicate the baseline assigned by BioRad software.

In contrast to voxelotor-modified HbS, voxelotor-modified HbA and voxelotor-modified HbC species appeared as distinct peaks on the chromatograms (Figures 1A and 1B). Interestingly, the voxelotor-modified HbC peak appeared to be much smaller relative to HbC and when compared with other unmodified/modified pairs (Figure 1B). Other voxelotor-modified hemoglobins were not resolvable from the unmodified hemoglobin with the current manufacturer settings. HbS-Kenya and HbE each displayed asymmetrical peaks following voxelotor treatment (Figures 1E and 1F). The peaks corresponding to voxelotor-modified HbS overlapped with HbS peaks (Figures 1B, 1C, 1E, and 1F).

The accuracy of quantitative HbS% measurements by the current HPLC method was assessed using the peak area for unmodified HbS in the Vox+ sample normalized to the peak area for HbS in the control sample **Table 3** and using the sum of the modified and unmodified HbS (total HbS) in the Vox+ sample normalized to the peak area of HbS in the control sample **Table 4**. When using unmodified HbS as the numerator, recovery was 63.3% in Vox+ HbSS and similar, or lower, for compound heterozygotes (57.3% for HbSC and 62.9% for HbSE) (Table 3). When using total HbS as the numerator, the recovery of HbS% in Vox+ HbSS was 90.3% and slightly lower in the

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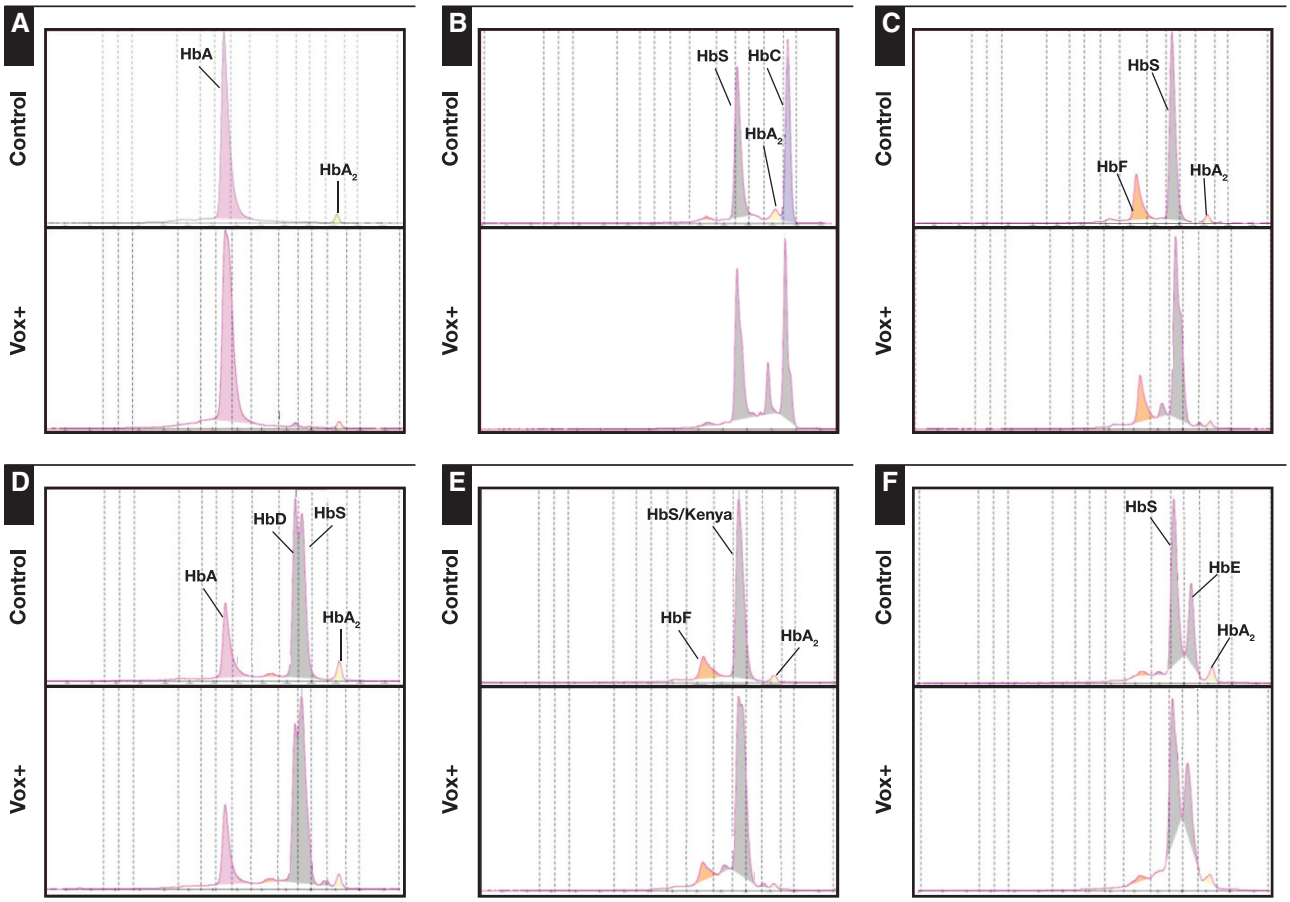

Figure 2 Capillary zone electrophoresis analysis of whole blood samples with different hemoglobin variant profiles without voxelotor treatment (control) and with voxelotor treatment (Vox+). Hemoglobin (Hb) variants tested include HbAA (**A**), HbSC (**B**), HbSS (**C**), HbSD (**D**), HbS-Kenya (**E**), and HbSE (**F**).

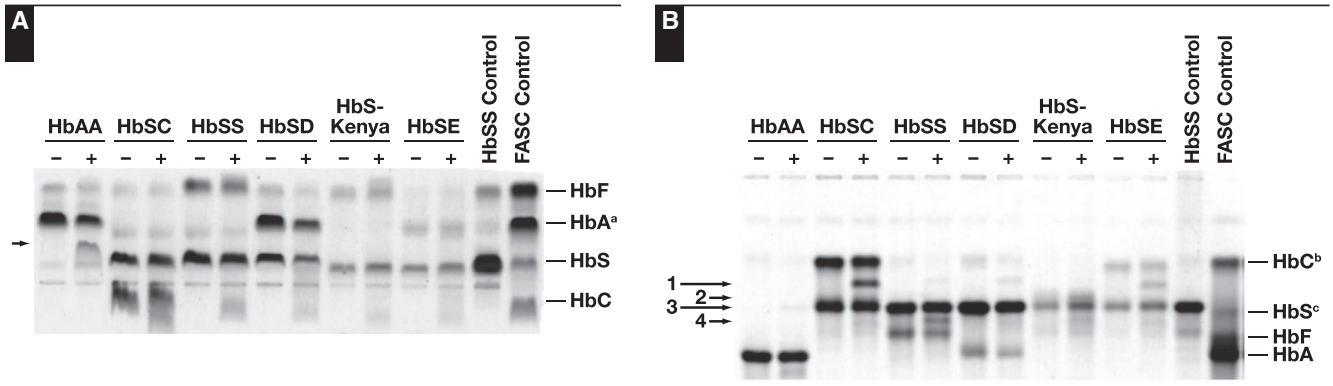

Figure 3 Acid (**A**) and alkaline (**B**) gel electrophoresis analysis of whole blood samples with different hemoglobin variant profiles without voxelotor treatment (–) and with voxelotor treatment (+). Bars on the right indicate the migration of hemoglobin (Hb) species within a control sample. Numbered arrows indicate additional bands present in voxelotor-treated samples. [a]HbD, HbE, and HbA_2 comigrate with HbA on acid gel electrophoresis. [b]HbE and HbA_2 comigrate with HbC on alkaline gel electrophoresis. [c]HbD comigrates with HbS on alkaline gel electrophoresis.

compound heterozygotes (70.9% for HbSC and 84.0% for HbSE) (Table 4). Quantitative HbS% measurements were also calculated for multiple HbSS, HbSC, and HbSβ^0-thalassemia patients and are shown in the supplemental material along with the corresponding HPLC chromatograms.

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Table 1

Summary of Differences Observed Between the Control and Voxelotor-Treated (Vox+) Samples for Different Hemoglobin Genotypes When Analyzed by Different Methods

Genotype	HPLC	CZE	Acid Gel	Alkaline Gel
HbAA	Distinct A peak (RT 1.89)	Asymmetrical peak	Additional band	Additional band
HbSS	Overlapping S peak (RT 4.11)	Asymmetrical peak Small distinct peak	No difference	Additional bands
HbSC	Overlapping S peak (RT 4.15) Small distinct C peak (RT 4.74)	Asymmetrical peak Distinct peak	No difference	Additional band
HbSD	Overlapping S peak (RT 4.08) Asymmetrical peak Additional peak (RT 3.83) Unreliable integration	Change in distribution of HbS and HbD peaks Very small distinct peak	No difference	Additional bands
HbS-Kenya	Overlapping S peak (RT 4.14) Asymmetrical peak	Asymmetrical peak	No difference	Additional bands
HbSE	Overlapping S peak (RT 4.14) Asymmetrical peak Small distinct peak (RT 2.97)	Overlapping peaks Small distinct peak	No difference	Additional bands

CZE, capillary zone electrophoresis; Hb, hemoglobin; HPLC, high-performance liquid chromatography; RT, retention time.

Table 2

Retention Times From Multiple Patient Samples Spiked With Voxelotor[a]

	Retention Time (min)			
Genotype	HbA-Vox+	HbS-Vox+	HbC-Vox+	HbA_2/E-Vox+
HbAA	1.89			
HbSS/HbSS transfused	1.95	4.09-4.14		
HbSβ^0-thalassemia		4.06-4.12		
HbSC/HbSC transfused	1.96	4.11-4.15	4.71-4.74	
HbSD transfused	1.93	Cannot resolve		
HbS-Kenya		4.14		
HbSE		4.14		2.97

Hb, hemoglobin; Vox+, voxelotor treatment.
[a]There were six HbSS patients, three HbSβ^0-thalassemia patients, four HbSC patients, and one patient for each of HbSD, HbS-Kenya, and HbSE.

CZE Analysis

Vox+ samples also displayed altered CZE profiles compared with the DMSO-treated control samples (Figure 2). The CZE profile for Vox+ HbAA displayed an asymmetric HbA peak (Figure 2A). For Vox+ HbSS, the CZE profile displayed an asymmetric HbS peak and additional peaks that migrated on either side of HbS (Figure 2C). For Vox+ HbSC, the CZE profile displayed HbS and HbC peaks that were asymmetric and a second peak that migrated close to, and overlapped with, HbA_2 (Figure 2B). The CZE profile for Vox+ HbSD displayed altered proportions of HbS and HbD peaks and produced an additional peak migrating between HbS and HbA_2 (Figure 2D). The CZE profile for Vox+ HbS-Kenya displayed a second peak that overlapped with HbS and additional small peaks migrating on either side of HbS (Figure 2E). For Vox+ HbSE, the CZE profile displayed asymmetric HbS and HbA_2 peaks (Figure 2F).

The accuracy of quantitative HbS% measurements by CZE was assessed using the sum of the modified and unmodified HbS (total HbS) in the Vox+ sample normalized to the peak area for HbS in the control sample Table 5. CZE did not resolve HbS and HbS-voxelotor complexes; therefore, the accuracy of HbS% using only unmodified HbS could not be assessed. Recovery of HbS% for each Vox+ sample tested was close to 100% (range, 95.7%-105.5%).

Table 3

Quantitation and Recovery of Unmodified HbS by High-Performance Liquid Chromatography Analysis of Whole Blood Samples With Different Hemoglobin Variant Profiles Shown in Figure 1 Without Voxelotor Treatment (Control) and With Voxelotor Treatment (Vox+)[a]

	Control	Vox+	
Genotype	HbS, %	Unmodified HbS, %	Recovery, %
HbSS	74.4	47.1	63.3
HbSC	46.4	26.6	57.3
HbSD	29.3	21.7	74.1
HbS-Kenya	58.1	31.4	54.0
HbSE	56.9	35.8	62.9

Hb, hemoglobin.
[a]Quantitation is determined by BioRad software. Accurate results rely on separation of peaks. Recovery (%) is calculated using unmodified HbS in the Vox+ sample as a percentage of HbS in the control sample (HbS (Vox+) / HbS (control) × 100).

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Table 4

Quantitation and Recovery of HbS, Using Total HbS, by HPLC Analysis of Whole Blood Samples With Different Hemoglobin Variant Profiles Shown in Figure 1 Without Voxelotor Treatment (Control) and With Voxelotor Treatment (Vox+)[a]

	Control	Vox+			
Genotype	HbS, %	Unmodified HbS, %	HbS-Voxelotor, %	Total HbS, % (Unmodified HbS + HbS-Voxelotor)	Recovery, %
HbSS	74.4	47.1	20.1	67.2	90.3
HbSC	46.4	26.6	6.3	32.9	70.9
HbSD	29.3	21.7	Cannot identify	NA	NA
HbS-Kenya	58.1	31.4	12.3	43.7	75.2
HbSE	56.9	35.8	12.0	47.8	84.0

Hb, hemoglobin; NA, not applicable.
[a]Quantitation is determined by BioRad software. Accurate results rely on separation of peaks. Recovery (%) is calculated using total HbS (HbS + HbS-voxelotor) in Vox+ sample as a percentage of HbS in the control sample (HbS + HbS-Vox (Vox+) / HbS (control) × 100).

Table 5

Quantitation of HbS in Capillary Zone Electrophoresis Analysis of Whole Blood Samples With Different Hemoglobin Variant Profiles Shown in Figure 2 Without Voxelotor Treatment (Control) and With Voxelotor Treatment (Vox+)[a]

	Control	Vox+	
Genotype	HbS, %	HbS, %	Recovery, %
HbSS	74.7	75.2	100.7
HbSC	49.5	48.0	97.0
HbSD	74.1	75.5	101.9
HbS-Kenya	84.0	88.6	105.5
HbSE	65.1	62.3	95.7

Hb, hemoglobin.
[a]Quantitation is determined by Sebia software. Accurate results rely on separation of peaks. Recovery (%) is calculated using HbS in the voxelotor-treated sample as a percentage of HbS in the control sample (HbS (Vox+) / HbS (control) × 100).

Acid and Alkaline Gel Electrophoresis

Acid gel electrophoresis displayed similar patterns for control and Vox+ samples, with one exception, Vox+ HbAA, which displayed an additional band (arrow; Figure 3A). Additional bands were observed upon alkaline gel electrophoresis for all Vox+ samples (Figure 3B). Vox+ HbAA displayed an additional band that migrated like HbS (arrow 3). Vox+ HbSS displayed additional bands that migrated on either side of HbS (arrows 1 and 4). Vox+ HbSC displayed an additional band that migrated between HbS and HbC (arrow 1). Vox+ HbSD displayed additional bands that migrated on either side of HbS (arrows 1 and 4). Vox+ HbS-Kenya displayed additional bands that migrated on either side of HbS (arrows 2 and 4). Vox+ HbSE displayed additional bands that migrated on either side of HbS (arrows 1 and 4).

Discussion

As a recent FDA-approved treatment for SCD, little is known about how voxelotor use affects laboratory testing for patients with SCD. Here we used in vitro spiking studies to mimic voxelotor treatment and assessed the impact on qualitative and quantitative hemoglobin variant analysis using three commercially available platforms commonly used in clinical laboratories. Our experiments assessed the effects of voxelotor treatment on analysis of blood from patients with some of the most common SCD genotypes, including HbSS and compound heterozygotes (HbSC, HbSD-Punjab, HbSE, HbSβ^0-thalassemia) as well as HbS-Kenya. We incubated residual blood samples with voxelotor to produce hemoglobin-voxelotor complexes and then analyzed the samples by HPLC (BioRad Variant II), CZE (Sebia Capillarys 2), and acid/alkaline gel electrophoresis (Helena SPIFE). These experiments demonstrated that voxelotor modifies the α-globin chain of many hemoglobins in vitro, including HbA, HbS, HbC, HbD-Punjab, HbE, HbF, and HbS-Kenya. This finding builds on earlier work showing that voxelotor modifies HbA and HbS in vivo[5] and HbS in vitro.[8,9]

For each hemoglobin genotype we tested, we found that voxelotor treatment altered the hemoglobin profile measured by HPLC, CZE, and alkaline gel electrophoresis, yielding additional and/or asymmetrical peaks and bands in the Vox+ samples compared with the control samples. These techniques are routinely employed in clinical laboratories for diagnosis and management of patients with SCD. Interestingly, we observed no unexpected bands when analyzing samples from patients with SCD by acid gel electrophoresis, which could be due to the dissociation of voxelotor from hemoglobin molecules under acidic conditions or to comigration of modified and unmodified hemoglobins. Our study also demonstrates that total HbS% is consistently underestimated when HPLC, but not CZE, is used to measure Vox+ samples; however, we found that CZE was unable to resolve the modified and unmodified Hb complexes, which affects the measurement of unmodified HbS%. The differences we observed in the content and reliability of quantitative results

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is particularly concerning for patients with SCD who present to the emergency department during sickle cell crisis. If these patients undergo an emergent RBC exchange transfusion, accurate quantitation of HbS% is necessary to determine the volume of RBCs required. Currently, it is not always possible to obtain an HbS quantitation prior to transfusion. In these circumstances, the patient is assumed to have 100% HbS; however, this increases the risk of alloimmunization from exposure to more donor blood products, raises the risk of iron accumulation, and uses more blood products than needed.[10,11] Assumption of 100% HbS may not be a viable long-term solution.

A key question that remains to be answered is whether Hb-voxelotor complexes should be considered clinically equivalent to the unmodified hemoglobin. For example, should HbS and HbS-voxelotor complexes be treated as equivalent for the purposes of transfusion and the total HbS% summed together (Tables 4 and 5), or should they be treated as two different species, one sickling (HbS) and one nonsickling (HbS-voxelotor) (Table 3)? For the former, our results demonstrate that CZE can provide accurate total HbS% for patients with SCD with the following genotypes: HbSS, HbSC, and HbSE (accurate quantitation for HbSD patients is not possible by CZE due to overlapping peaks).

If HbS and HbS-voxelotor are considered different species, then modification of the current FDA-approved HPLC and CZE methods will be necessary to achieve complete separation of the voxelotor-modified and unmodified hemoglobins. Alteration of the methods would also be required if there is a clinical need to assess response to voxelotor therapy by measuring HbS-voxelotor complex formation (ie, to use the complex as a marker for therapeutic drug management similar to the way HbF is used for hydroxyurea treatment).

In addition to posing a problem for quantitative measurements of hemoglobin variants, widespread use of voxelotor could also present challenges for the diagnosis of patients who transfer care to a new provider or who seek treatment from a new institution in SCD crisis. Unfortunately, there is not currently a consensus among the medical community on how to report the extra peaks or bands that are observed in samples collected from patients receiving voxelotor. When a consensus is reached about the qualitative and quantitative reporting of voxelotor complexes, it will likely have many downstream effects, potentially including changes to the laboratory's equipment that necessitate revalidation or changes to prior diagnoses in the electronic medical record. Prompt guidance from the clinical community is needed to ensure that patients taking voxelotor continue to receive accurate hemoglobin variant results and to prevent any unnecessary delays in reporting the results of these critical tests.

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