Automated Quantification of Free Hemoglobin in Hematology Samples

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Detection of cell-free, (extra-cellular) hemoglobin in plasma of samples submitted to core laboratories for routine cell counting has potential clinical advantages. The presence of cell-free hemoglobin can indicate significant hemolysis, which could occur either as a consequence of the blood collection process (in vitro hemolysis), or could represent the presence of intravascular free hemoglobin.
Intravascular free hemoglobin could be secondary to intravascular hemolysis, or could possibly be due to the administration of hemoglobin-based blood substitute.
Detection of hemoglobin-based blood substitute could soon become important, as these products are currently being used in some countries for enhancing perfusion in hypoxic tissues secondary to severe trauma. Stability and storage requirements make these products an extremely attractive alternative to blood in certain remote clinical settings, and combat zones.
As these products become available, the anti-doping agencies will need to incorporate measurement tools into their testing procedures, in order to screen for the presence of hemoglobin-based blood substitutes.
Detecting significant hemolysis in the sample sent for hematology cell counting, could further alert clinicians to exercise caution when interpreting other results from the same patient.

(Sysmex J Int. 17: 30-34, 2007)

Key Words Extra-cellular Hemoglobin, Cell-free Hemoglobin, Hemoglobin-based Blood Substitutes, Intravascular Hemolysis

Received 3 November, 2006; Accepted 6 November, 2006

INTRODUCTION

Since May 2005, the RET-H_e (Reticulocyte Hemoglobin Equivalent) has been available for use on the Sysmex XE-2100 (K#050589), broadening the availability of a tool for assessing reticulocyte hemoglobin content (Substantially equivalent to CHr parameter[1)]on Advia-120). Measurement of reticulocyte hemoglobin content in picograms is now an alternative to the use of transferrin saturation (T sat) testing in renal dialysis patients according to the latest revisions in the KDOQI (Kidney Disease Outcomes Quality Initiative) clinical practice guidelines for anemia in chronic kidney disease, from the National Kidney Foundation[2)].
By directly measuring the mean hemoglobin content (in picograms) of Red Blood Cell precursors (Reticulocytes), early stages of iron deficiency may be identified[3-5)], at a time that other traditional biochemical parameters are non-informative. The measurement of reticulocyte hemoglobin content is a direct assessment of the incorporation of iron into erythrocyte hemoglobin and thus a direct estimate of the functional availability of iron to the erythron. The mean intracellular hemoglobin content is an all inclusive measure of both the availability of iron, and the introduction of iron into intracellular hemoglobin. The absence of significant interference with analyte concentrations by commonly occurring inflammatory conditions, is another potential advantage over indirect biochemical testing.

Table 1 *Origins and formulae for the calculation of the Total HGB (Spectrophotometric), Cellular HGB and Hemoglobin in solution (cell-free HGB)*

Total HGB	Spectrophotometric HGB
Cellular HGB	((RBC-H_e*(RBC-O - Ret#)) + (RET-H_e*Ret#)
Free HGB	Spectrophotometric HGB - Cellular HGB

Cellular parameters available on the Ret Master software package include RET-H_e, RBC-H_e, and RBC-O (Fluorescent Optical Red Blood Cell Count).
The traditional Hemoglobin concentration is determined spectrophotometrically, and as such represents both cellular and free Hemoglobin in the sample.
With the availability of the new Ret Master parameters, cellular Hemoglobin concentrations can be measured. Cellular Hemoglobin is the sum of the red blood cell and reticulocyte hemoglobin concentrations, as determined by fluorescent optical methods, in combination with the Ret Master software application.
Cell-free Hemoglobin can be calculated by subtracting the cellular Hemoglobin concentration from the Total spectrophotometric Hemoglobin concentration (***Table 1***).

We have previously shown that there is an excellent level of agreement[1)] between Advia CHr and Sysmex RET-H_e. RET-H_e and Advia CHr ; Y=1.04X-1.06; r^2 = 0.88 and between the RBC-H_e and Advia CH parameters; Y=0.93X+1; r^2 = 0.84.

The RBC-H_e and CH parameters determine the mean red blood cell hemoglobin content (in picograms). Examining both the precursors, and mature red cells provides an opportunity to detect and monitor acute changes in cellular hemoglobin status.
In order to assess the performance of the Sysmex XE 2100 as regards detection and quantification of cell-free Hemoglobin, dilutions were performed on commercially manufactured Hemoglobin-based blood substitute (Hemopure, Bovine Hemoglobin Glutamer from Biopure Corporation), and on whole blood samples spiked with known concentrations of free Hemoglobin, created from red cell lysis of normal human blood by repeated freeze-thaw cycles.

MATERIAL AND METHODS

Hemopure® blood substitute at a concentration of 12.6 g/dL were obtained from Biopure® Corporation (Cambridge, MA, USA).
Varying proportions of blood substitute and normal blood were mixed to produce samples with a range of cell-free hemoglobin concentrations from zero to 10 g/dL.
In addition, blood was collected from a healthy donor (Approximately 40mL). Centrifugation and separation of the red cells was followed by multiple washes and freeze -thaw cycles. The resulting hemoglobin solution was diluted in order to create a workable concentration of 15.3 g/dL. The hemoglobin product was mixed with whole blood from the same donor, (Hemoglobin concentration of 15.7 g/dL) in order to create a range of cell-free hemoglobin samples to duplicate those of the Hemopure dilutions, with a range of cell-free hemoglobin concentrations from zero to 8 g/dL.
Reference Range data was collected from 126 normal volunteers.

RESULTS

Hemopure Dilutions:

The results of Hemopure dilutions are shown in ***Fig. 1*** and ***Table 2*** and ***3***.

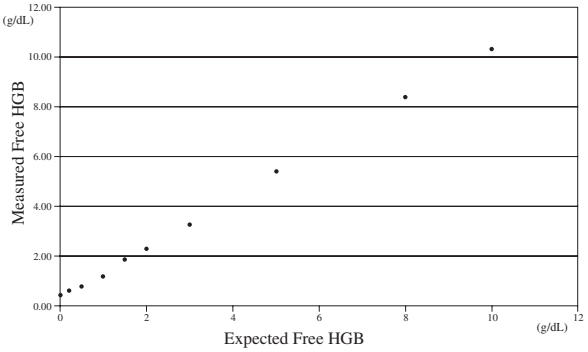

Fig. 1 *Graphic representation of the data in* ***Table 2****.*

Table 2 *Results of dilutional experiment involving varying concentrations of Hemopure hemoglobin based blood substitute, with 4 replicates at each concentration tested.*

XE 2100 Cell Free HGB (Hemopure)					
Expected (g/dL)	Means	Result 1	Result 2	Result 3	Result 4
0	0.40	0.68	0.04	0.23	0.65
0.2	0.56	0.74	0.43	0.36	0.70
0.5	0.72	0.73	0.33	1.05	0.76
1	1.15	1.07	1.18	0.91	1.42
1.5	1.82	1.63	1.56	2.07	2.02
2	2.26	1.83	2.31	2.02	2.87
3	3.23	3.15	3.53	3.19	3.04
5	5.37	5.41	5.44	5.36	5.26
8	8.39	8.27	8.41	8.56	8.33
10	10.33	10.43	10.20	10.28	10.39

Table 3 *The experimental design for Hemopure with a specified hemoglobin concentration of 12.6g/dL, diluted in whole blood with a concentration of 15.7g/dL.*

Hemopure Dilutions (12.6g/dL) in Whole Blood (15.7g/dL)

	Sample composition		Total Hemoglobin			Cellular HGB			Cell-Free HGB		
	Whole Blood volume	Hemopure volume	Expected	Assay Result	2SD	Expected	Assay Result	2SD	Expected	Assay Result	2SD
Level 1	5.00	0.00	16.10	16.08	0.19	16.10	15.67	0.67	0	0.40	0.63
Level 2	4.92	0.08	16.04	16.03	0.10	15.84	15.47	0.33	0.2	0.56	0.38
Level 3	4.80	0.20	15.96	16.00	0.00	15.46	15.28	0.59	0.5	0.72	0.59
Level 4	4.60	0.40	15.82	15.90	0.00	14.82	14.75	0.43	1	1.15	0.43
Level 5	4.40	0.60	15.68	15.88	0.10	14.18	14.06	0.48	1.5	1.82	0.52
Level 6	4.21	0.79	15.54	15.70	0.16	13.54	13.44	0.75	2	2.26	0.90
Level 7	3.81	1.19	15.27	15.50	0.00	12.27	12.27	0.42	3	3.23	0.42
Level 8	3.02	1.98	14.71	15.03	0.10	9.71	9.66	0.20	5	5.37	0.16
Level 9	1.83	3.17	13.88	14.15	0.12	5.88	5.76	0.17	8	8.39	0.26
Level 10	1.03	3.97	13.32	13.65	0.12	3.32	3.32	0.11	10	10.33	0.21

Whole Blood Dilutions:

The results of whole blood dilutions are shown in ***Fig. 2*** and ***Table 4*** and ***5***.

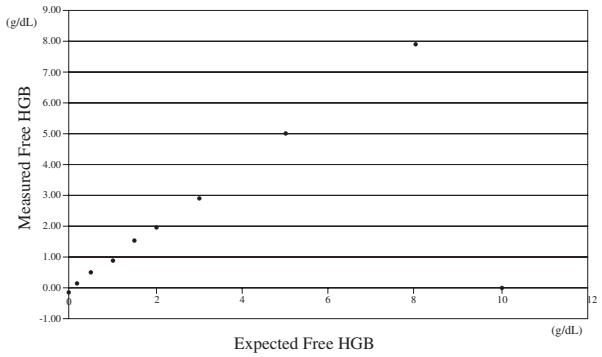

Fig. 2 *Graphical representation of the data in* ***table 4****.*

Table 4 *Results of dilutional experiment involving varying concentrations of whole blood sample lysis, with 4 replicates at each concentration tested.*

XE 2100 Cell Free HGB (Whole Blood Hemolysate)					
Expected (g/dL)	Means	Result 1	Result 2	Result 3	Result 4
0	-0.09	-0.20	0.08	-0.07	-0.17
0.2	0.21	0.13	0.30	0.46	-0.04
0.5	0.55	0.77	0.35	0.65	0.44
1	0.93	0.92	0.68	1.20	0.90
1.5	1.58	1.55	1.16	1.65	1.94
2	1.95	2.26	1.88	2.03	1.63
3	2.93	2.82	2.91	3.05	2.95
5	5.03	5.18	4.93	4.99	5.03
8	7.89	7.88	7.84	7.79	8.07

Table 5 *The experimental design using lysed red blood cells with a specified hemoglobin concentration of 15.3g/dL, diluted in whole blood from the same donor and having a concentration of 15.7g/dL.*

Hemopure Dilutions (12.6g/dL) in Whole Blood (15.7g/dL)

	Sample composition		Total Hemoglobin			Cellular HGB			Cell-Free HGB		
	Whole Blood volume	Hemopure volume	Expected	Assay Result	2SD	Expected	Assay Result	2SD	Expected	Assay Result	2SD
Level 1	5.00	0.00	15.70	15.55	0.20	15.70	15.64	0.21	0	-0.09	0.25
Level 2	4.93	0.07	15.69	15.60	0.16	15.49	15.39	0.28	0.2	0.21	0.43
Level 3	4.84	0.16	15.69	15.58	0.19	15.19	15.03	0.21	0.5	0.55	0.38
Level 4	4.67	0.33	15.67	15.58	0.10	14.67	14.65	0.35	1	0.93	0.42
Level 5	4.51	0.49	15.66	15.60	0.00	14.16	14.02	0.64	1.5	1.58	0.64
Level 6	4.35	0.65	15.65	15.53	0.10	13.65	13.57	0.56	2	1.95	0.53
Level 7	4.02	0.98	15.62	15.50	0.16	12.62	12.57	0.04	3	2.93	0.19
Level 8	3.37	1.63	15.57	15.40	0.00	10.57	10.37	0.22	5	5.03	0.22
Level 9	2.39	2.61	15.49	15.18	0.10	7.49	7.28	0.23	8	7.89	0.24

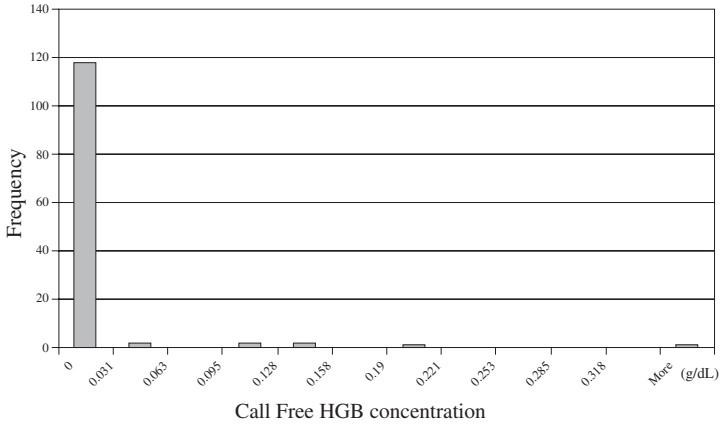

Fig. 3 *Graphical representation of reference range data collected from 126 normal individual.*

Table 6 *Reference Range Data from 126 normal subjects.*

Reference Range Data (n=126)	
Mean	0.01
SD	0.04
Max	0.35
Min	0
Range	0 – 0.09

Reference Range:

The results of reference range are shown in ***Fig. 3*** and ***Table 6.***

DISCUSSION

Traditional methods for quantifying Hemoglobin rely on spectrophotometry at characteristic wavelengths. Recent advances in cell counting allow quantification of reticulocyte and mature red blood cell hemoglobin content. This in turn allows separate quantification of intra-cellular and extra-cellular Hemoglobin. The product of the mean red blood cell hemoglobin content, and the fluorescent optical red blood cell count, allows accurate estimation of the cellular hemoglobin concentration.

Hemopure consists of chemically stabilized bovine hemoglobin formulated in a balanced salt solution. On a gram-for-gram basis, this cross-linked hemoglobin carries the same amount of oxygen as the hemoglobin in red blood cells. However, these linked hemoglobin molecules circulate in plasma, and are smaller, have lower viscosity (resistance to flow) and more readily release oxygen to tissues than red blood cells[6-7]. Consequently, they can potentially carry oxygen at low blood pressure and can carry oxygen through constricted or partially blocked blood vessels to areas of the body that red blood cells cannot reach due to their larger size.

The concentration ranges tested during this experiment (0-10.28 g/dL) cover the desired reportable ranges achievable during clinical use of hemoglobin-based blood substitutes.

An additional clinical application relates to the detection of red blood cell destruction in samples sent for laboratory analysis. Physiological intracellular potassium levels are much higher than serum potassium levels, due to the actions of the Na+/ K+ ion exchange pump in cell membranes. Only about 2% of total body potassium remains in the extracellular compartment. The extracellular potassium concentration plays a critical role in maintaining cell membrane resting potential. Extracellular potassium concentration therefore must be kept within a very narrow range, in order to avoid arrythmias or even cardiac arrest.

Detecting significant hemolysis in the sample sent for hematology cell counting, could further alert clinicians to exercise caution when interpreting potassium results from the same patient.

The reference range for the measurement of cell-free Hemoglobin is between zero and 0.09 g/dL as determined by measuring results on 126 normal volunteers.

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