ELSEVIER

Journal of Chromatography B, 700 (1997) 269–274

JOURNAL OF CHROMATOGRAPHY B

Short communication

# Microchromatographic quantitation of hemoglobin A levels in phenotypes of sickle cell-beta$^+$ thalassemia

I. Sweeting[a], B.E. Serjeant[b], P.W. Thomas[b], G.R. Serjeant[b,*]

[a]*Subdepartment of Haematology, Department of Pathology, University of the West Indies, Kingston, Jamaica*
[b]*Medical Research Council Laboratories, University of the West Indies, Kingston, Jamaica*

Received 29 May 1996; received in revised form 16 April 1997; accepted 24 April 1997

**Abstract**

The inheritance of the sickle cell gene in combination with a gene for $\beta^+$ thalassemia results in a spectrum of sickle cell-$\beta^+$ thalassemia syndromes with varying levels of hemoglobin A (HbA). Some severe sickle cell-$\beta^+$ thalassemia syndromes have small amounts of HbA, which may be difficult to quantitate in the presence of fetal hemoglobin. A microcolumn chromatographic method, using 0.5 *M* Tris–acetic acid developers with varying pH values from 9.0 to 6.0, appears to adequately quantitate small amounts of HbA. This method is relatively simple and cheaper than high-performance liquid chromatography, a major consideration in developing countries. © 1997 Elsevier Science B.V.

*Keywords:* Hemoglobin A

## 1. Introduction

Hemoglobin bands may be quantitated after separation by electrophoresis or chromatography [1]. Both methods accurately quantitate the minor hemoglobin component hemoglobin $A_2$ ($HbA_2$), but electrophoresis may not satisfactorily separate small amounts of HbA in the presence of fetal hemoglobin (HbF). Quantitation of HbA may be useful in investigating the different phenotypes of sickle cell-$\beta^+$ thalassemia, which give rise to four hemoglobin bands, $HbA_2$, HbS, HbF and HbA. In Jamaica, the diversity of ethnic groups results in a broad spectrum of sickle cell-$\beta^+$ thalassemia syndromes. The predominant type of sickle cell-$\beta^+$ thalassemia among people of West African origin results from substitutions at the −29 or −88 sites [2,3] and manifests 20–30% HbA. Jamaicans originating from India, China and the Mediterranean carry more severe $\beta^+$ thalassemia genes, resulting in lower levels of HbA. The amount of HbA in sickle cell-$\beta^+$ thalassemia is believed to reflect the severity of the underlying $\beta^+$ thalassemia mutation and influences the clinical course. A simple method for quantitating HbA levels would therefore be clinically useful.

Early methods for separating HbA from HbF relied on complex chromatographic procedures that were slow and required multiple developing buffers [4]. This separation was accelerated with the introduction of glycine–KCN–NaCl developing buffers [5] and was further improved by 0.05 *M* Tris–HCl buffers [6], although this method was only used in the presence of low HbF concentrations. High-performance liquid chromatography (HPLC) giving fast, precise separation superseded these methods [7] but the cost of equipment and reagents are high. We

*Corresponding author.


*PII* S0378-4347(97)00270-3

describe a cheap simple method for the quantitation of HbA, which achieves satisfactory separation of HbA and HbF within 12 h, using 0.05 *M* Tris–acetic acid buffer as the developer, and microcolumns that were made in the laboratory. This method may provide a reliable and cheaper alternative to HPLC in countries with limited resources.

## 2. Experimental

### *2.1. Patients*

The patients attended the Sickle Cell Clinic of the University Hospital of the West Indies (Kingston, Jamaica), which treats approximately 250 patients with sickle cell-$\beta^-$ thalassemia syndromes. The diagnosis of sickle cell-$\beta^-$ thalassemia was based on the presence of $HbA_2$, HbS, HbF and HbA on cellulose acetate electrophoresis, $HbA_2$ levels greater than 3.5% and family study, where possible [8]. None of the patients had been transfused within four months of blood collection. Venepuncture samples from 78 patients with sickle cell-$\beta^-$ thalassemia were used in the study.

### *2.2. Equipment*

Column chromatography equipment included a peristaltic pump, a fraction collector, buffer gradient tanks and a spectrophotometer.

### *2.3. Validation procedure*

This method was validated by comparing assays with values obtained by cation-exchange HPLC at the Medical College of Augusta in Georgia, USA (courtesy of Dr. Titus Huisman). Approximately 80 ml of pooled umbilical cord blood (providing a high HbF, low HbA sample) and 80 ml of pooled blood from individuals with a normal hemoglobin (AA) genotype (providing a high HbA, low HbF sample) were mixed in varying proportions to provide twenty samples with an estimated HbA range from 25–100%. These samples were split into two aliquots, one assayed locally with the described method and the other sent, on ice, to Augusta, GA, USA.

### *2.4. Method*

Microcolumns were prepared from Pasteur pipettes by plugging them with non-absorbent cotton and

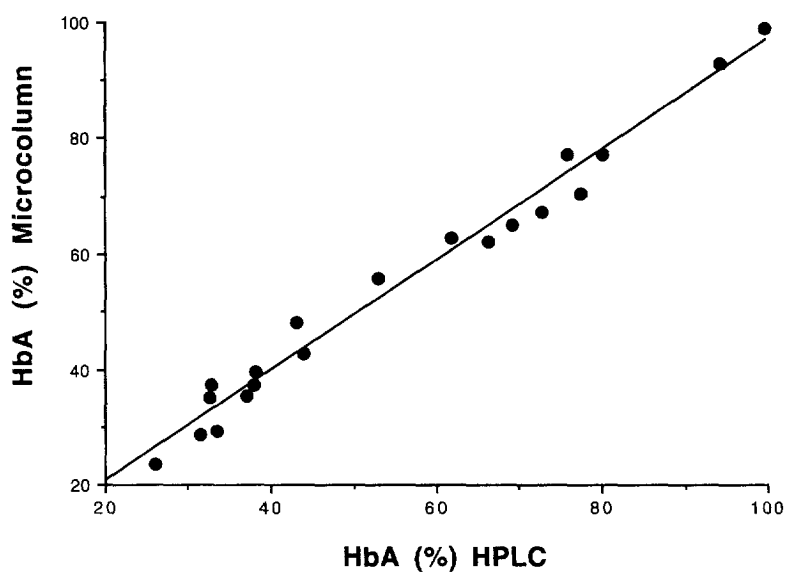


Fig. 1. Comparison of HbA levels estimated by the microcolumn method and by HPLC.

filling them with a slurry containing 6–7 g of DE-52 cellulose ion exchanger, washed in 30 ml of 0.05 *M* Tris–acetic acid buffer, titrated to pH 9.0 to remove the fines. This produced a column of approximately 6.5 cm in length, to which 100 μl of hemolysate (titrated to a concentration of 0.18–0.24 g/dl) was applied to the top of the column. The column was perfused with a developer gradient containing 30 ml of 0.5 *M* Tris–acetic acid developer, pH 9.0 (9 ml of 5% acetic acid/l) in tank 1 and 50 ml of 0.05 *M* Tris–acetic acid developer, pH 8.0 (35 ml of 5% acetic acid/l) in tank 2. After elution of the $HbA_2$ and HbS bands (approximately 2.5 h after the start of the gradient), 80 ml of the pH 8.0 developer were added to tank 2. After elution of the HbA (approximately 6–7 h later), 40 ml of the developer, titrated to pH 6.0 (50 ml of 5% acetic acid/l), were added to tank 2 to elute HbF. KCN was not added to the developers. The flow-rate was maintained at 0.16 ml/min and the effluent was collected in 3 ml fractions and the absorbance was determined in a spectrophotometer at 415 nm.

## 3. Results and discussion

Comparison of HbA levels by this method and by HPLC (Fig. 1) shows a close correlation throughout the range 25–100% HbA (correlation coefficient, $r$=0.98). The separation of HbA from high levels of

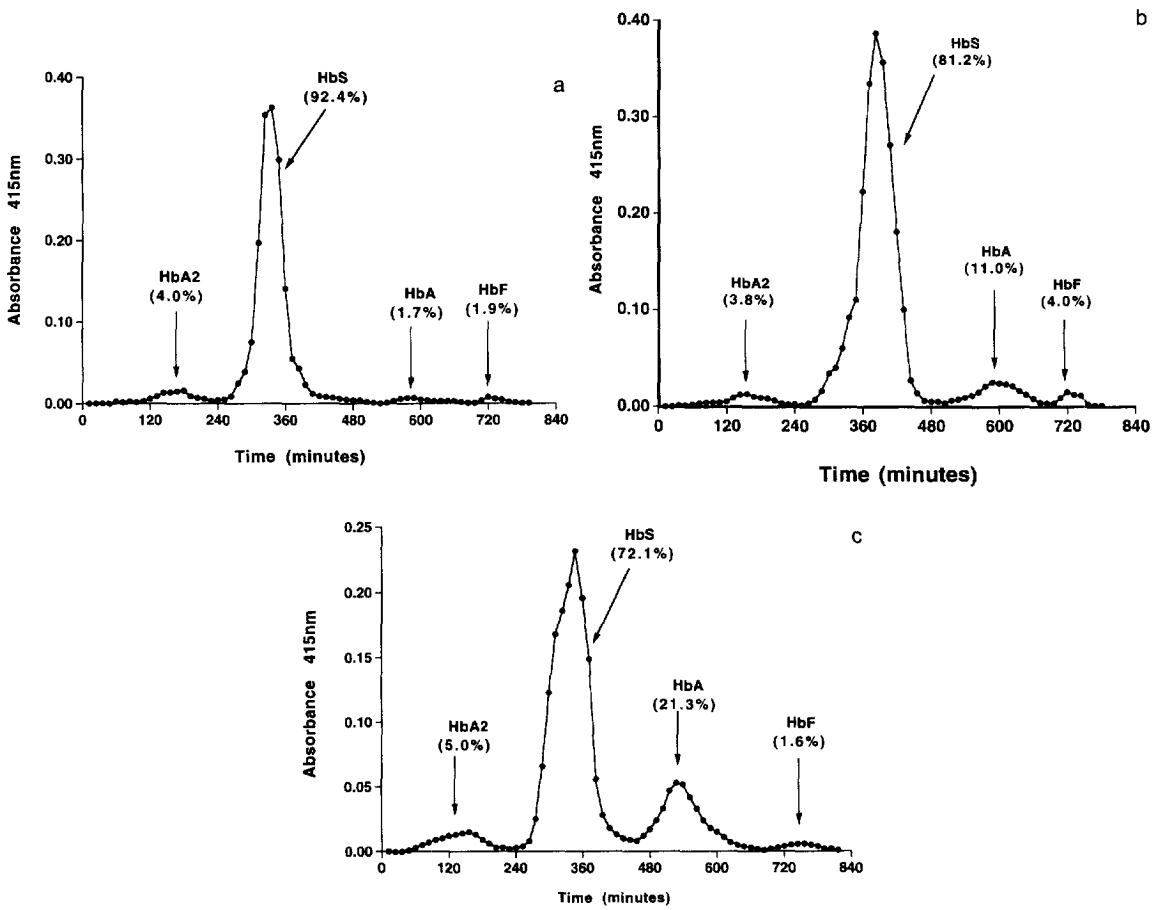


Fig. 2. Illustrative chromatograms in (a) patient 55 with 1.7% HbA, (b) patient 38 with 11.0% HbA and (c) patient 68 with 21.3% HbA.

Table 1
HbA levels in sickle cell-$\beta^+$ thalassemia syndromes (ranked in order of mean HbA within molecular genotypes)

| Patient no. | HbA level | | | | $HbA_2$ Mean (SD) | HbF Mean (SD) |
|---|---|---|---|---|---|---|
| | Run 1 | Run 2 | Run 3 | Mean (SD) | | |
| *Molecular basis −29 A→G* | | | | | | |
| 1 | 8.9 | 7.0 | 12.3 | 9.4(2.7) | 5.2(0.5) | 2.1(0.1) |
| 2 | 11.3 | 12.2 | | 11.8(0.6) | 5.1(0.4) | 17.4(2.4) |
| 3 | 16.5 | 12.9 | 13.3 | 14.2(2.0) | 4.7(0.8) | 19.5(1.2) |
| 4 | 14.3 | 16.5 | | 15.4(1.6) | 3.6(0.4) | 0.6(0.2) |
| 5 | 15.6 | 15.7 | 18.4 | 16.6(1.6) | 4.5(0.5) | 4.0(0.4) |
| 6 | 18.0 | 15.1 | | 16.6(2.1) | 4.1(1.1) | 8.2(5.6) |
| 7 | 16.5 | 17.3 | | 16.9(0.6) | 4.6(0.5) | 10.1(2.3) |
| 8 | 17.5 | 18.1 | | 17.8(0.4) | 5.8(0.3) | 5.8(0.3) |
| 9 | 15.2 | 20.6 | | 17.9(3.8) | 4.7(0.6) | 8.1(2.2) |
| 10 | 18.4 | 17.4 | | 17.9(0.9) | 4.8(0.9) | 5.9(0.3) |
| 11 | 17.6 | 18.1 | | 17.9(0.4) | 6.2(0.3) | 8.8(2.0) |
| 12 | 17.0 | 19.4 | | 18.2(1.7) | 7.1(3.4) | 2.9(1.3) |
| 13 | 19.4 | 17.1 | | 18.5(1.6) | 5.8(0.9) | 3.5(0.6) |
| 14 | 19.4 | 17.6 | | 18.5(1.3) | 6.8(0.5) | 6.1(1.6) |
| 15 | 19.1 | 18.0 | | 18.6(0.8) | 5.5(1.7) | 16.2(0.4) |
| 16 | 18.9 | 18.2 | 18.9 | 18.7(0.4) | 4.9(0.9) | 12.2(2.0) |
| 17 | 20.7 | 17.8 | 18.2 | 18.9(1.6) | 7.8(1.9) | 7.0(0.3) |
| 18 | 19.5 | 18.8 | | 19.2(0.5) | 4.5(0.1) | 4.5(0.4) |
| 19 | 18.6 | 20.2 | | 19.4(1.0) | 4.8(0.1) | 7.6(0.8) |
| 20 | 21.3 | 18.2 | | 19.8(2.2) | 7.1(2.5) | 5.8(0.8) |
| 21 | 22.1 | 18.8 | 19.6 | 20.2(2.0) | 3.0(0.7)[a] | 3.3(0.7) |
| 22 | 19.4 | 21.2 | | 20.3(1.3) | 4.1(0.1) | 13.4(0.3) |
| 23 | 19.8 | 22.7 | | 21.3(2.1) | 3.7(1.5) | 3.0(0.3) |
| 24 | 22.3 | 21.9 | | 21.3(1.1) | 7.3(0.3) | 7.4(3.2) |
| 25 | 19.4 | 20.5 | 24.3 | 21.4(2.6) | 3.4(1.7)[a] | 6.5(1.4) |
| 26 | 21.8 | 21.2 | | 21.5(0.4) | 5.8(0.4) | 7.3(0.8) |
| 27 | 22.1 | 20.9 | | 21.5(0.8) | 3.6(0.1) | 18.9(0.2) |
| 28 | 22.3 | 21.9 | | 22.1(0.3) | 5.2(0.3) | 3.8(0) |
| 29 | 23.2 | 21.2 | | 22.2(1.4) | 7.8(1.9) | 7.0(0.3) |
| 30 | 22.1 | 21.6 | 23.2 | 22.3(0.8) | 4.4(1.0) | 12.3(2.5) |
| 31 | 21.8 | 23.3 | | 22.6(1.0) | 5.9(0.3) | 6.5(3.0) |
| 32 | 22.9 | 22.5 | | 22.7(0.3) | 5.2(0) | 5.8(3.3) |
| 33 | 23.1 | 23.0 | | 23.1(0.1) | 5.0(1.6) | 2.7(0.6) |
| 34 | 23.9 | 21.9 | 24.1 | 23.3(1.2) | 5.3(0.8) | 1.8(1.9) |
| 35 | 23.8 | 25.8 | | 24.8(1.4) | 3.2(0.9) | 7.3(4.8) |
| 36 | 26.3 | 24.6 | | 25.5(1.2) | 3.8(3.0) | 4.1(2.7) |
| 37 | 26.8 | 31.7 | 24.0 | 27.9(3.9) | 3.6(0.5) | 0.7(0.1) |
| *Molecular basis −88 C→T* | | | | | | |
| 38 | 8.2 | 11.0 | | 9.6(2.0) | 3.7(0.2) | 4.3(0.4) |
| 39 | 12.7 | 10.8 | | 11.8(1.3) | 6.6(1.0) | 3.1(1.1) |
| 40 | 14.3 | 16.1 | | 15.2(1.3) | 5.6(1.5) | 11.6(2.2) |
| 41 | 14.2 | 16.5 | | 15.4(1.6) | 5.5(0.4) | 11.3(1.0) |
| 42 | 15.5 | 17.8 | | 16.7(1.6) | 3.0(1.3)[a] | 5.4(0.8) |
| 43 | 16.6 | 18.1 | 17.4 | 17.4(0.8) | 6.2(0.7) | 6.3(0.8) |
| 44 | 17.0 | 17.9 | | 17.5(0.6) | 5.6(0.3) | 8.8(0.6) |
| 45 | 19.2 | 18.0 | | 18.6(0.8) | 4.4(3.3) | 6.6(6.1) |
| 46 | 19.1 | 20.2 | 19.3 | 19.5(0.5) | 5.8(0.9) | 3.5(0.4) |
| 47 | 22.0 | 19.2 | | 20.6(2.0) | 5.0(0.1) | 4.1(0.6) |
| 48 | 21.4 | 24.2 | | 22.8(2.0) | 7.6(0.8) | 7.2(3.1) |
| 49 | 22.1 | 25.6 | | 23.9(2.5) | 7.3(0.7) | 3.5(0.6) |
| 50 | 27.9 | 27.9 | 26.9 | 27.6(0.6) | 4.0(0.1) | 11.9(1.0) |

Table 1. Continued

| Patient no. | HbA level | | | | $HbA_2$ Mean (SD) | HbF Mean (SD) |
|---|---|---|---|---|---|---|
| | Run 1 | Run 2 | Run 3 | Mean (SD) | | |
| 51 | 34.0 | 33.2 | | 33.6(0.6) | 4.4(0.2) | 1.5(0.4) |
| *Molecular basis IVS1-5 G→C* | | | | | | |
| 52 | 1.1 | 0.9 | 1.4 | 1.1(0.3) | 7.1(0.5) | 2.3(0.5) |
| 53 | 1.4 | 3.2 | | 2.3(1.3) | 4.6(1.0) | 4.9(0.9) |
| 54 | 1.8 | 3.9 | 1.5 | 2.4(1.3) | 2.8(0.3)[a] | 11.2(3.2) |
| 55 | 1.7 | 3.8 | 2.8 | 2.8(1.1) | 5.7(2.1) | 3.2(1.8) |
| 56 | 3.1 | 3.0 | | 3.1(0.1) | | |
| 57 | 3.1 | 3.8 | 3.4 | 3.4(0.4) | 5.4(1.0) | 1.6(0.5) |
| 58 | 4.7 | 5.8 | | 5.3(0.8) | 5.6(0.7) | 2.8(1.1) |
| 59 | 6.9 | 6.4 | | 6.7(0.4) | 2.9(0.1)[a] | 4.6(2.0) |
| 60 | 6.8 | 6.9 | | 6.9(0.1) | 8.3(0.1) | 15.4(0.3) |
| 61 | 9.5 | 6.1 | 6.0 | 7.2(2.0) | 6.9(1.0) | 11.6 (3.4) |
| *Molecular basis poly-A T→C* | | | | | | |
| 62 | 13.9 | 15.9 | 13.9 | 14.5(1.3) | 4.4(0.2) | 0.9(0.1) |
| 63 | 13.8 | 16.4 | 15.9 | 15.4(1.4) | 3.1(0.3)[a] | 2.9(0.2) |
| 64 | 14.7 | 16.3 | | 15.5(1.1) | 5.6(0.5) | 5.5(3.6) |
| 65 | 16.6 | 16.0 | | 16.3(0.4) | 4.4(0.4) | 1.4(0.3) |
| 66 | 17.1 | 19.7 | 18.4 | 18.4(1.3) | 5.1(0.4) | 3.1(0.9) |
| 67 | 21.3 | 19.4 | | 20.4(1.3) | 5.3(0.6) | 8.0(0.5) |
| 68 | 22.1 | 21.3 | 20.1 | 21.2(1.0) | 5.4(2.5) | 2.2(0.9) |
| 69 | 26.8 | 24.8 | | 25.8(1.4) | 6.2(0.9) | 5.5(1.1) |
| 70 | 25.5 | 26.1 | 27.2 | 26.3(0.9) | 4.5(0.3) | 7.8(1.5) |
| *Molecular basis C-24 T→A* | | | | | | |
| 71 | 21.5 | 21.1 | 21.3 | 21.3(0.2) | 3.7(0.6) | 2.1(0.4) |
| 72 | 21.9 | 21.9 | 21.5 | 21.8(0.2) | 4.9(2.1) | 13.5(2.1) |
| *Molecular basis IVS2-654 T→C* | | | | | | |
| 73 | 7.2 | 7.7 | | 7.5(0.4) | 9.9(0.4) | 3.9(1.8) |
| *Molecular basis unknown* | | | | | | |
| 74 | 5.6 | 3.1 | | 4.4(2.1) | 4.8(0.1) | 4.8(0.1) |
| 75 | 16.8 | 16.9 | 16.8 | 16.8(0.1) | 5.4(0.1) | 3.9(1.1) |
| 76 | 18.6 | 19.7 | | 19.2(0.8) | 4.3(0.1) | 7.7(0) |
| 77 | 27.3 | 26.8 | | 27.1(0.4) | 4.4(0.1) | 4.9(0.3) |
| 78 | 35.7 | 35.1 | | 35.4(0.4) | 5.1(1.2) | 1.5(1.7) |

[a]These patients had $HbA_2$ levels below the diagnostic range on chromatography but had levels above 3.5% on quantitation by elution after electrophoresis on cellulose acetate, which is used as the 'gold standard' for diagnosis.

HbF in a sample from a newborn AA subject appeared adequate and a sample from a non-transfused patient with SS disease, who, by definition, had no HbA, confirmed the absence of a band in the position of HbA on the chromatogram. Examples of chromatographic separations on patients with low, moderate, and high levels of HbA are shown in Fig. 2.

The results of two or more runs using the same hemolysates from 78 patients with sickle cell-$\beta^+$ thalassemia, ranked according to the molecular basis of the $\beta^+$ thalassemia gene, show wide variability in HbA levels between genotypes (Table 1). The mean difference between duplicate runs was 0.2% (95% confidence intervals −0.7, 2.1) and 95% of the between-run differences were 4.6% or less.

The molecular heterogeneity of sickle cell-$\beta^+$ thalassemia syndromes in Jamaica provides a variety of HbA levels on which to test techniques for estimating the amount of HbA. Most common are the −29 and −88 substitutions, characteristic of patients of West African ancestry, which are general-

ly associated with HbA levels of between 15–25%. Most values fell within the expected range, although it is noteworthy that the values for five subjects were consistently below this level, with mean values of between 9.4–14.2%, respectively. The IVS 1-5 G→C mutation, which was always of Indian origin, produced a sickle cell-$\beta^+$ thalassemia syndrome that gave rise to very low levels of HbA (1.1–7.2%). The other groups were small, nine subjects predominantly of West African origin with the poly-A T→C mutation had intermediate levels (14.5–26.3%), two subjects with the C-24 T→A mutation had moderately high levels (21.3–21.8%) and a single subject with an IVS2-654 T→C mutation had a level of 7.5% HbA. The factors contributing to the wide range of HbA levels in the −29 and −88 mutations are beyond the scope of this paper. The chromatographic method described is a simple, cheap technique that is capable of distinguishing HbA levels in the major forms of sickle cell-$\beta^+$ thalassemia in Jamaica.

## Acknowledgments

We thank Dr. T.H.J. Huisman of Augusta, Georgia for values obtained by HPLC. I.S. was partially sponsored by The Government of the Bahamas. Materials and equipment were provided by the Medical Research Council Laboratories (Jamaica), University of the West Indies, Kingston Jamaica.

## References

[1] P. Basset, J. Chromatogr. 227 (1982) 267.
[2] S.E. Antonarakis, S.H. Irkin, T.U. Cheng, A.F. Scott, J.P. Sexton, S.P. Trusko, S. Charache, H.H. Kazazian, Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 1154.
[3] S.H. Orkin, S.E. Antonarakis, H.H. Kazazian, J. Biol. Chem. 259 (1984) 8679.
[4] A.M. Dozy, T.H.J. Huisman, J. Chromatogr. 40 (1968) 62.
[5] E.C. Abraham, A. Reese, M. Stallings, T.H.J. Huisman, Hemoglobin 1 (1976) 27.
[6] G.D. Efremov, T.H.J. Huisman, J. Chromatogr. 89 (1984) 197.
[7] J.B. Wilson, M.E. Headlee, T.H.J. Huisman, J. Lab. Clin. Med. 102 (1983) 176.
[8] G.R. Serjeant, M.T. Ashcroft, B.E. Serjeant, P.F. Milner, Br. J. Haematol. 24 (1978) 19.