# Reversed-phase HPLC study of low-molecular-weight peptides in human blood extracts

*Yu. L. Polyakova,[a]* K. Row,[a] and O. G. Larionov[b]*

*[a]Center for Advanced Bioseparation Technology and Department of Chemical Engineering, Inha University, 253 Yonghyun-Dong, Nam-Ku, Incheon 402-751, Korea. E-mail: ylc@mail.ru*

*[b]N. N. Semenov Institute of Physical Chemistry, Russian Academy of Sciences, 31 Leninsky prosp., 119991 Moscow, Russian Federation. Fax: +7 (095) 952 5308*

Peptides in human blood extracts were separated and identified by HPLC. The processes of peptide formation under energy deficient conditions, which develop during blood storage, were studied.

**Key words:** peptides, liquid chromatography, human blood.

The problems of cell death provoke substantial interest, because they are related to aging of organisms, pathological changes of tissues and organs, regeneration, and carcinogenesis.[1—3]

Mammal erythrocytes, in particular, human erythrocytes are cells unique in their way because their apoptosis (cell death) program may be switched on even at the maturing state.[4] This program is implemented during functioning of erythrocytes.

Having entered the bloodstream, erythrocytes are gradually disintegrated while performing their functions. The rates and mechanisms of disintegration cannot be defined as mere wearing of the cell caused by the lack of protein renovation and intensification of denaturation changes. The presence of diverse types of reception on erythrocytes and the possible endocrine[5] and, perhaps, also the autocrine activity of erythrocytic cells prompt the idea of execution of a definite disintegration program whose mechanism depends on the conditions of functioning.

This communication presents a chromatographic study of the extracts of peptides from human blood carried out within the framework of the research into degradation of human haemoglobin. About 200 endogenous haemoglobin fragments have now been isolated from various sources, about 50 of these peptides being biologically active both *in vivo* and *in vitro*.[6—8] Meanwhile, the formation of biologically active fragments of this protein has not been adequately studied. The analysis of the peptide composition of the erythrocyte lysate carried out in a known work[9] has shown that red blood cells contain substantial amounts of haemoglobin fragments comprising mainly about 30 amino acid residues.

The purpose of this study is chromatographic separation and identification of peptides in extracts obtained from fresh blood and from blood stored for 3, 5, and 7 days and analysis of peptide formation processes under energy deficiency arising during incubation of the whole blood.

## Experimental

The chromatographic experiment was carried out at ~20 °C on a Waters 600S liquid chromatograph with a Waters 2487 spectrophotometric detector ($\lambda$ = 254 nm). A 150.0×4.6 mm Althech steel column with silica gel (5 μm) with grafted octadecyl groups was used. The mobile phase was prepared using acetonitrile (chromatographic grade) and doubly distilled water. The elution rate was 1 mL $min^{-1}$ and the sample volume was 10 μm. The results of the chromatographic analysis were processed by the Millenium software[32] (Waters). As a result of the study, we chose the optimal mobile phase and the gradient elution mode for the separation of peptides. In order to suppress peptide ionization, $CF_3COOH$ was added to water (0.1% v/v).

| Time /min | The composition of the mobile phase (% v/v) | |
|---|---|---|
| | $H_2O$ + $CF_3COOH$ (0.1% v/v) | MeCN |
| 0—5 | 100 | 0 |
| 5—10 | 90 | 10 |
| 10—15 | 50 | 50 |
| 15—20 | 0 | 100 |

Identification was carried out using commercial peptides (Aldrich, Sigma). The samples were prepared by reported procedures[8,9] using Sigma commercial chemicals (analytically pure).

The study was performed with donor whole blood either fresh or stored for 3, 5, or 7 days at 283—285 K. The erythrocyte

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 2, pp. 449—452, February, 2005.

1066-5285/05/5402-0458 

lysate was prepared in the following way. Erythrocytes were washed three times with a cold 0.154 *M* solution of NaCl (283 K) and centrifuged (600 g) on a refrigerator centrifuge (Micro 17M+, Korea) for 10 min at 277 K, and the supernatant liquid was discarded. The suspension of erythrocytes was subjected to deproteinization with a 15% aqueous solution of $CCl_3COOH$ (1 : 3, v/v). The mixture was centrifuged (1000 g) for 15 min at 298 K and the supernatant liquid (below referred to as the extract) was separated. The resulting extracts were analyzed by reversed-phase HPLC.

Processing of the results of the chromatographic experiment was carried out by mathematical statistics techniques.[10] The relative error of a single measurement did not exceed 5%.

The retention factors of the peptides ($k$) were calculated from the formula

$$k = (t_r - t_m)/t_m,$$

where $t_r$ is the peptide retention time, $t_m$ is the retention time of a nonsorbed component; $t_m$ was determined from the retention time of sodium nitrite.

## Results and Discussion

Chromatography is often used to study peptides. Numerous chromatographic procedures for proteins and peptides have been reported in the literature.[11,12] However, theoretical grounds for the separation of complex compounds such as those of the peptide origin have not yet been developed. The known classical models of chromatography are designed for idealized cases and prove to be of low use in practice. In most cases, when peptides and proteins are chromatographed, one has to be guided by general recommendations and empirical considerations.[13,14]

The key problem in our chromatographic study was to attain reproducibility of the results. Therefore, considerable attention was paid to sample preparation, because the resulting extracts were to be free from proteins and high-molecular-weight peptides.

The chromatograms of the peptide extracts obtained from blood stored for different periods of time are shown in Fig. 1, *a* and *b*. It can readily be seen that the composition and gradient of the mobile phase chosen for the experiment allow one to analyze peptides over an acceptable time and provide a satisfactory separation. The gradient was formed by increasing the elution strength and decreasing the polarity of the mobile phase during the analysis, which was attained by increasing the content of acetonitrile. Therefore, peptides were apparently eluted from the column in the order of increasing hydrophobicity.

During storage, the whole blood experiences oxygen deficiency, *i.e.*, energy deficiency. Our goal was to trace the changes in the qualitative and quantitative composition of the peptide extracts obtained from blood stored for different periods and thus to collect information on the energy dependence of peptide formation processes. The dynamics of this process is shown in Fig. 2. It can be seen that the contents of some peptides change during storage.

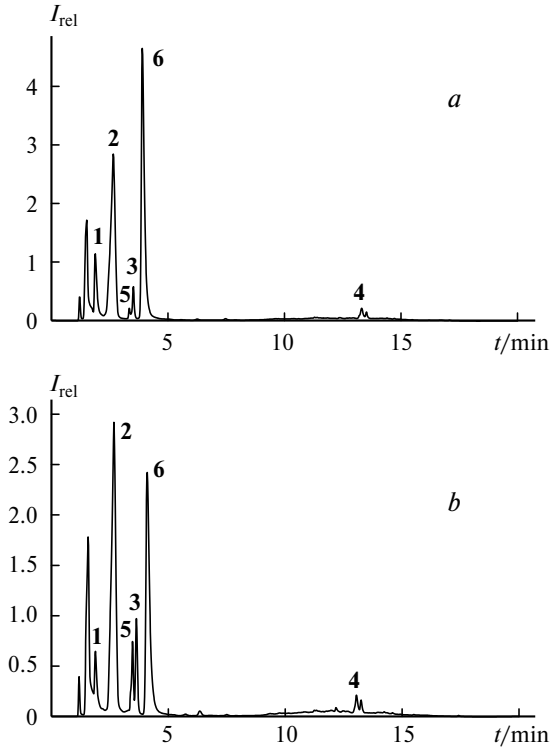


**Fig. 1.** HPLC data for the extract of peptides obtained from the blood stored for 3 (*a*) and 7 days (*b*): Thr-Ser-Leu-Tyr-Arg (**1**), Val-His-Leu-Thr-Arg-Glu-Glu-Lys-Ser-Ala-Val (**2**), Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-His-Arg-Arg-Tyr-His (**3**), Ala-Leu-Trp-Gly-Lys-Val-Asn-Val (**4**), Val-Val-Leu-Ser-Pro-Ala-Asp-Lys-Thr-Asn-Val (**5**) and Val-His-Leu-Thr-Pro-Gly-Glu-Lys-Ser-Ala-Val (**6**).

We identified six peptides, four of which were missing from the extracts prepared from fresh blood samples. The identification was based on the retention times of reference compounds. The chromatogram of an artificial mixture of peptides of known structures is presented in Fig. 3. The retention factors and the structures of the identified peptides are listed in Table 1. It can be seen that some of these compounds possess biological activities or are fragments of haemoglobin α- or β-chains.[5,15]

The peptides identified in extracts obtained from fresh blood were Ala-Leu-Trp-Gly-Lys-Val-Asn-Val (**4**) and Val-Val-Leu-Ser-Pro-Ala-Asp-Lys-Thr-Asn-Val (**5**). The latter was also found in extracts from incubated blood (for 3, 5, and 7 days), its amount being higher for longer storage periods. The other peptide (**4**) was found in blood extracts only after 3- and 7-day storage and its content decreased with time.

Peptides **1** (Thr-Ser-Leu-Tyr-Arg) and **2** (Val-His-Leu-Thr-Arg-Glu-Glu-Lys-Ser-Ala-Val) were found in blood extracts stored for 3, 5, and 7 days. The amount of

**Table 1.** Structure, retention factors ($k$), and biological properties of the identified peptides

| Peptide | Incubation time/days | $k$ | Biological activity |
|---|---|---|---|
| Thr-Ser-Leu-Tyr-Arg (**1**) | 3 | 0.27 | Fragment of the |
| **1** | 5 | 0.26 | α-chain of human |
| **1** | 7 | 0.26 | haemoglobin |
| Val-His-Leu-Thr-Arg-Glu-Glu-Lys-Ser-Ala-Val (**2**) | 3 | 0.71 | Human growth |
| **2** | 5 | 0.71 | hormone factor |
| **2** | 7 | 0.72 | |
| Val-His-Leu-Thr-Pro-Gly-Glu-Lys-Ser-Ala-Val (**6**) | 3 | 1.21 | — |
| **6** | 7 | 1.21 | |
| Ala-Leu-Trp-Gly-Lys-Val-Asn-Val (**4**) | —* | 6.62 | Fragment of the |
| **4** | 3 | 6.62 | β-chain of human |
| **4** | 7 | 6.63 | haemoglobin |
| Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-His-Arg-Arg-Tyr-His (**3**) | 3 | 1.68 | — |
| | 7 | 1.69 | |
| Val-Val-Leu-Ser-Pro-Ala-Asp-Lys-Thr-Asn-Val (**5**) | —* | 1.06 | Hemopoietic |
| **5** | 3 | 1.05 | activity |
| **5** | 5 | 1.05 | |
| **5** | 7 | 1.05 | |

* Fresh blood.

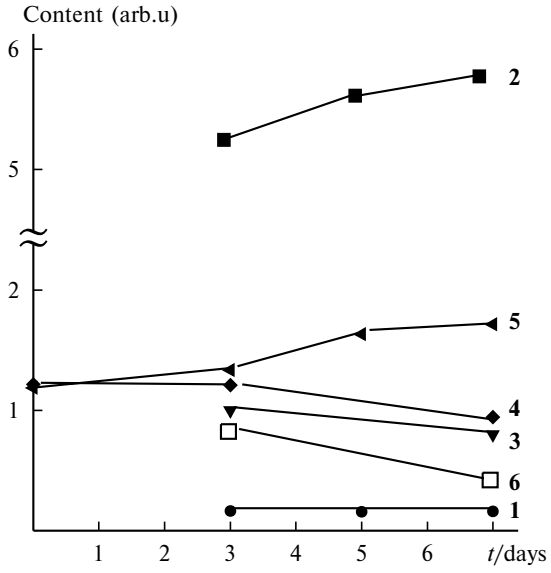


**Fig. 2.** Dynamics of excretion of low-molecular-weight peptides **1—6** with cells of a survival culture of erythrocytes.

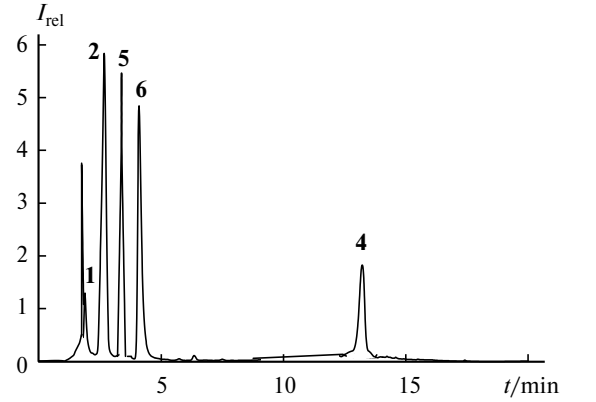


**Fig. 3.** HPLC data for an artificial mixture of standards of peptides **1**, **2**, **4—6**.

peptide **2** increased during storage, while the content of pentapeptide **1** remained virtually unchanged.

The blood stored for 3 and 7 days was found to contain two peptides that were missing from fresh blood and from the 5-day-storage blood, namely, Val-His-Leu-Thr-Pro-Gly-Glu-Lys-Ser-Ala-Val (**6**) and Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-His-Arg-Arg-Tyr-His (**3**), their concentration in the extracts decreasing.

Peptides **3**, **4**, and **6** were not detected in the blood stored for 5 days but they can be found qualitatively in the 7-day storage blood. Currently we cannot interpret this fact, because this requires further research.

The fact that the extracts obtained from incubated blood contained four peptides that were absent from fresh blood extracts points to an energy-dependent (depending on the oxygen concentration in blood) nature of the formation of low-molecular-weight peptides by erythrocyte culture cells. The change in the amount of the material in the samples taking place during incubation attests to a specific character of formation of some peptides.

## References

1. V. P. Skulachev, *Biokhimiya*, 1999, **64**, 1679 [*Biochemistry (Moscow)*, 1999, **64** (Engl. Transl.)].

2. V. D. Samuilov, A. V. Oleskin, and E. M. Logunova, *Biokhimiya*, 2000, **65**, 1029 [*Biochemistry (Moscow)*, 2000, **65** (Engl. Transl.)].
3. S. Ya. Proskuryakov, V. L. Gobai, and A. G. Konoplyannikov, *Biokhimiya*, 2002, **67**, 467 [*Biochemistry (Moscow)*, 2002, **67** (Engl. Transl.)].
4. E. F. Lushnikov and V. M. Zagrebin, *Arkhiv patologii* [*Pathology Archive*], 1987, **49**, 84 (in Russian).
5. A. A. Karelin, M. M. Filippova, O. M. Yatskin, E. Yu. Blishchenko, I. V. Nazimov, and V. T. Ivanov, *Bioorgan. Khim.*, 1998, **24**, 271 [*Russ. J. Bioorg. Chem.*, 1998, **24** (Engl. Transl.)].
6. L. A. Fonina, S. A. Gur´yanov, I. V. Nazimov, O. G. Yanovskii, L. A. Zakharova, A. A. Mikhailova, and R. V. Petrov, *Dokl. Akad. Nauk SSSR*, 1991, **319**, 755 [*Dokl. Chem.*, 1991 (Engl. Transl.)].
7. R. Kh. Ziganshin, V. I. Sviryaev, B. V. Vas´kovskii, I. I. Mikhaleva, V. T. Ivanov, Yu. M. Kokoz, and A. E. Alekseev, *Bioorgan. Khim.*, 1994, **20**, 899 [*Russ. J. Bioorg. Chem.*, 1994, **20** (Engl. Transl.)].
8. V. T. Ivanov, A. A. Karelin, I. I. Mikhaleva, B. V. Vas´kovskii, V. I. Sviryaev, and I. V. Nazimov, *Bioorgan. Khim.*, 1992, **18**, 1271 [*Russ. J. Bioorg. Chem.*, 1992, **18** (Engl. Transl.)].
9. A. A. Karelin, M. M. Philippova, and V. T. Ivanov, *Peptides*, 1995, **16**, 693.
10. A. K. Charykov, *Matematicheskaya obrabotka rezul´tatov khimicheskogo analiza* [*Mathematical Processing of the Results of Chemical Analysis*], Khimiya, Moscow, 1984, 168 (in Russian).
11. J. C. Lindon, J. K. Nicholson, and I. D. Wilson, *J. Chromatogr. B: Biomedical Appl.*, 2000, **748**, 233.
12. J. Zukowski, M. Pawlowska, M. Nagatkina, and D. W. Armstrong, *J. Chromatogr.*, 1993, **629**, 169.
13. J. Florance, A. Galdes, Z. Konteatis, Z. Kosarych, K. Langer, and C. Martucci, *J. Chromatogr.*, 1987, **414**, 313.
14. C. T. Mant and R. S. Hodges, *High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis, and Conformation*, CRC Press, Boca Raton, 1991, 938.
15. A. Bairoch and R. Apweiler, *J. Mol. Med.*, 1997, **75**, 312.

*Received April 28, 2003;*
*in revised form October 15, 2003*