Journal of Chromatography, 397 (1987) 279–285
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1162

DETERMINATION OF THYMOSIN β_4 IN HUMAN BLOOD CELLS AND SERUM

EWALD HANNAPPEL* and MICHAEL VAN KAMPEN
Institut für Physiologische Chemie, Universität Erlangen-Nürnberg, Fahrstrasse 17, D-8520 Erlangen (F.R.G.)

SUMMARY

A simple one-step procedure for the determination of thymosin β_4 in whole blood, various blood corpuscles, plasma and serum by reversed-phase high-performance liquid chromatography (HPLC) is described. The blood cells were isolated by a Ficoll density gradient technique. The isolated cells were lysed and deproteinized with perchloric acid in the presence of an internal standard. The supernatant solution was neutralized by potassium hydroxide solution and thymosin β_4 was measured by HPLC without further manipulations. The following amounts (fg per cell) were determined: polymorphonuclear leukocytes (409), mononuclear leukocytes (267) and platelets (22.3). Erythrocytes contained no thymosin β_4. The average concentration of thymosin β_4 in an extract of whole blood was 16.3 μg/ml. The concentration in plasma was less than 1% of that value. However, if serum was prepared and not immediately removed from the clotted blood, the concentration of the peptide increased from 0.04 to 2.1 μg/ml within 24 h. The biological significance of the findings is discussed.

INTRODUCTION

Thymosin β_4 is a polypeptide of molecular weight 4963-dalton which was originally isolated[1] from thymosin fraction 5 as a thymic hormone[2] that modifies the immune response. The biological importance of the peptide is emphasized by the fact that its amino acid sequence is conserved in the animal kingdom from humans to amphibia[3] and that it is often accompanied by a highly homologous peptide[4]. It has been reported that thymosin β_4 induces the expression of terminal deoxynucleotidyl transferase[2] and stimulates the secretion of luteinizing hormone-releasing factor[5]. Thymosin β_4 inhibits the migration of macrophages *in vitro*, as reported by Thurman *et al.*[6]. We have shown that thymosin β_4 is present in extrathymic tissues in fairly large amounts[7,8] and that it is synthesized but not secreted by various cell lines[9]. These results raise some doubts about a hormonal function of thymosin β_4. However, Naylor *et al.*[10] have recently reported high concentrations of thymosin β_4 in human serum, and this would support the assumption that the peptide has hormonal functions if the peptide is specifically released from a gland or cells.

0021-9673/87/$03.50

As we knew from our studies[9] on the biosynthesis of thymosin β_4 that Epstein–Barr virus-transformed human B-cell lines contain large amounts of this peptide, we considered that possibly non-transformed human blood cells might also contain thymosin β_4. If blood cells contain the peptide, it would be important to exclude the possibility that those cells lyse during the preparation of serum and thereby cause the appearance of the peptide in serum. Therefore, we determined the presence and concentration of thymosin β_4 in whole blood and blood corpuscles and in plasma and serum by a simple one-step high-performance liquid chromatographic (HPLC) procedure.

EXPERIMENTAL

Materials

Reagents were obtained from the following sources: Macrodex (6% dextran 60 in isotonic NaCl) from Knoll (Ludwigshafen, F.R.G.), Ficoll-Paque from Pharmacia (Uppsala, Sweden), thrombin from Boehringer (Mannheim, F.R.G.) and fluorescamine and phenylalanylphenylalanine (Phe_2) from Serva (Heidelberg, F.R.G.). All other chemicals were of high-quality commercial grade. Solvents used for HPLC were distilled over ninhydrin.

Methods

Thymosin β_4 and β_9 standard. Thymosin β_4 and β_9 were isolated from calf thymus as described elsewhere[8]. Their concentrations were determined by amino acid analyses. The peptide analyser was standardized by injecting a mixture of 1.4 μg of thymosin β_4 and 0.4 μg of Phe_2.

Whole blood, plasma, erythrocytes, polymorpohonuclear leukocytes (PMN), mononuclear leukocytes (MNL) and platelets. This set of specimens were isolated from peripheral venous blood of 21- to 35-year-old female and male volunteers.

Whole blood (25 ml) was gently mixed with 4 ml of Macrodex, containing 20 mg of K_2EDTA. An aliquot (2–3 ml) of this mixture (M) was used for the preparation of plasma.

Plasma was obtained after centrifugation at 14 500 *g* for 10 min at 4°C. A defined amount of Phe_2 was mixed with 9.14 *M* perchloric acid (PCA) so that the final concentration of PCA was 0.4 *M* after addition of whole blood or plasma.

The remaining mixture (M) was incubated for 30–60 min at room temperature in an inverted plastic syringe. During this time, erythrocytes sedimented faster than leukocytes, and a whitish upper phase was performed. This phase was enriched in leukocytes ("leukocyte-enriched fraction") whereas the lower, red phase contained most of the erythrocytes. The two phases were removed from thee syringe. The erythrocytes were still contaminated by leukocytes and platelets. These contaminants were removed by several washes with PBS and low-speed centrifugation.

The leukocyte-enriched fraction was diluted with an equal volume of 154 m*M* sodium chloride–3 m*M* EDTA–20 mM HEPES (pH 7.4) (SEH buffer) and layered on a Ficoll-Pague cushion. After centrifugation at 400 *g* for 30 min at 20°C, the interphase, which contained MNL and platelets[11], was transferred into a centrifuge tube filled with 40 ml of SEH buffer. The MNL were sedimented at 60 *g* for 10 min at room temperature. The pellet was resuspended in 50 ml of SEH buffer and the

centrifugation was repeated. The combined supernatant solutions contained platelets, whereas the pellet was less contaminated by platelets. In some instances, the platelets were removed by treatment with thrombin. After 1 h at room temperature, the clotted platelets were removed. The purified MNL were contamined by four platelets per ten MNL. This must be compared with the physiological ratio of 120 platelets per MNL in peripheral blood.

For the preparation of platelets, the combined supernatant solutions of the 60 *g* centrifugations were centrifuged at 100 *g* for 10 min. The supernatant solution was transferred into a new plastic centrifuge tube, and the platelets were sedimented at 1100 *g* within 10 min. The pellet of the Ficoll-Paque centrifugation contained mainly PMN, which were contaminated by erythrocytes. The erythrocytes were removed by lysis with 5 ml of 155 m*M* ammonium chloride–10 m*M* HEPES (pH 7.0) for 10 min at 37°C with gentle shaking. The PMN were washed several times with SEH buffer.

Preparation of serum. Whole blood (2 ml) without anticoagulants was kept at room temperature for 1, 8 and 24 h. Thereafter, the serum was removed by centrifugation at 14500 *g* for 5 min at 4°C. The serum was treated further as described in the following section.

Determination of thymosin β_4. Erythrocytes, MNL, PMN and platelets were suspended in SEH buffer and counted. Volumes of 1–2 ml of whole blood, plasma or serum were used for the determination. Perchloric acid (9.14 *M*) was added to the sample to a final concentration of 0.4 *M*. The concentrated PCA contained a defined amount of Phe_2, which served as an internal standard. The amount of Phe_2 was chosen in such a way that the area of the Phe_2 peak was similar to the expected area of the thymosin β_4 peak in the HPLC separation. In order to facilitate lysis of the cells, the sample was sonicated for a few seconds. After 30 min on ice, the sample was centrifuged at 14500 *g* for 5 min at 4°C. The supernatant solution was titrated with 0.2 *M* potassium hydroxide solution against bromthymol blue as indicator. Subsequently, pyridine and acetic acid were added to final concentrations of 0.11 and 0.076 *M*, respectively. The sample was kept on ice for a further 30 min and insoluble potassium perchlorate was removed by low-speed centrifugation.

An aliquot of the sample was analysed by HPLC, essentially as described elsewhere[8]. However, as we used an Altex IP column instead of an ODS column for analyses in the reversed-phase mode, it was possible to omit the lithium perchlorate from the buffer. The column was kept at 56°C. The peptides were eluted with a gradient of 1-propanol (0–40%) in 0.11 *M* pyridine–0.076 *M* acetic acid and detected by post-column derivatization with fluorescamine[12].

Chromatographic equipment. The Altex IP column (Ultrasphere, 5 μm, 250 × 4.6 mm I.D.) was purchased from Beckman (Munich, F.R.G.). The chromatographic conditions were controlled by a Beckman 420 system. The eluted compounds were derivatized with fluorescamine and detected by fluorescence using a Kratos (Berlin, F.R.G.) FS 950 fluorimeter with 365-nm excitation from an FSA 110 lamp, no excitation filter, and an FSA 427 emission filter with cut-off at 440 nm. The fluorimeter was connected to a dual-channel chart recorder and an integrator.

RESULTS AND DISCUSSION

The concentration of thymosin β_4 was determined in whole blood, plasma,

serum, erythrocytes and mononuclear and polymorphonuclear leukocytes of 21- to 35-year-old volunteers. We have already shown[9] that Epstein–Barr virus-transformed human B-cell lines contain and synthesize large amounts of thymosin β_4. However, it is not possible from the outset to conclude from our data on cell lines that non-transformed cells also contain the peptide. The question of the presence of thymosin β_4 in blood and in various blood corpuscles became important after Naylor *et al.*[10] had reported surprisingly high concentrations of the peptide in human serum. A simple one-step procedure for the determination of thymosin β_4, based on the treatment of the specimen with PCA, was developed for the determination of thymosin β_4 in small tissue samples[8]. Basically, the same procedure was used for the determination of thymosin β_4 in the specimen obtained from human blood. The different types of blood cells were separated by a Ficoll-Paque technique. In order to prove that in the case of thymosin β_4 it is possible to lyse and extract cells with 0.4 *M* PCA, we added to $1.3 \cdot 10^8$ platelets not only PCA and the usual internal standard (Phe_2) but additionally 5.5 μg of thymosin β_9. The amino acid sequences[13] of thymosin β_4 and β_9 are identical in 32 out of 41 positions. The recovery of the added thymosin β_9 was 92%. This indicated that the peptide was neither precipitated by PCA nor trapped by precipitated protein.

Thymosin β_4 from the different samples was identified by its retention time and chromatography in mixtures with authentic thymosin β_4, isolated from calf thymus. An additional chemical proof of the identity of the peptide was obtained from the oxidation of samples from the different specimens by 3% hydrogen peroxide. The area of the thymosin β_4 sulphoxide peak that formed was identical with the area of the original thymosin β_4 of the non-oxidized sample. Thymosin β_4 sulphoxide was eluted about 5 min earlier than thymosin β_4. Therefore, an accidental overlap of the thymosin β_4 peak with other fluorescent substances is very unlikely.

Typical HPLC profiles of the PCA-treated whole blood, plasma, MNL, PMN and platelets are shown in Fig. 1. In all instances, except in plasma, there is a large peak of thymosin β_4 at 42.3 min. The concentration of thymosin β_4 in whole blood ranges from 12.0 to 19.5 μg/ml (Table I). Most of this peptide is restricted to the various blood corpuscles, because only 0.026–0.387 μg/ml is found in plasma. The blood corpuscles with the highest concentration of thymosin β_4 are PMN, which contain an average of 409 fg of thymosin β_4. MNL contain 267 fg of thymosin β_4. Although MNL are a mixture of human B-cells, T-cells and monocytes, this value agrees well with the reported[9] content of thymosin β_4 in murine macrophages. Murine macrophages, which were extracted with guanidine hydrochloride, contain 274 fg. Platelets contain between 6.9 and 31.7 fg of thymosin β_4. The only type of blood cells studied so far that do not contain measurable amount of thymosin β_4 are erythrocytes (Table I). If one chromatographs an extract from $3 \cdot 10^9$ erythrocytes, one obtains only a very minute peak at the retention time of thymosin β_4. However, it is not poossible to convert this peak by oxidation with H_2O_2 to thymosin β_4 sulphoxide unequivocally. Therefore, it cannot be ruled out that the peak is not due to thymosin β_4. Another possibility is that the purified erythrocytes still contain some leukocytes or platelets. The concentration of thymosin β_4 is less than 90 ng per $3 \cdot 10^9$ erythrocytes. According to our results (Table I), 90 ng of thymosin β_4 are equivalent to a contamination of the $3 \cdot 10^9$ erythrocytes by either $250 \cdot 10^3$ leukocytes or $4.1 \cdot 10^6$ platelets.

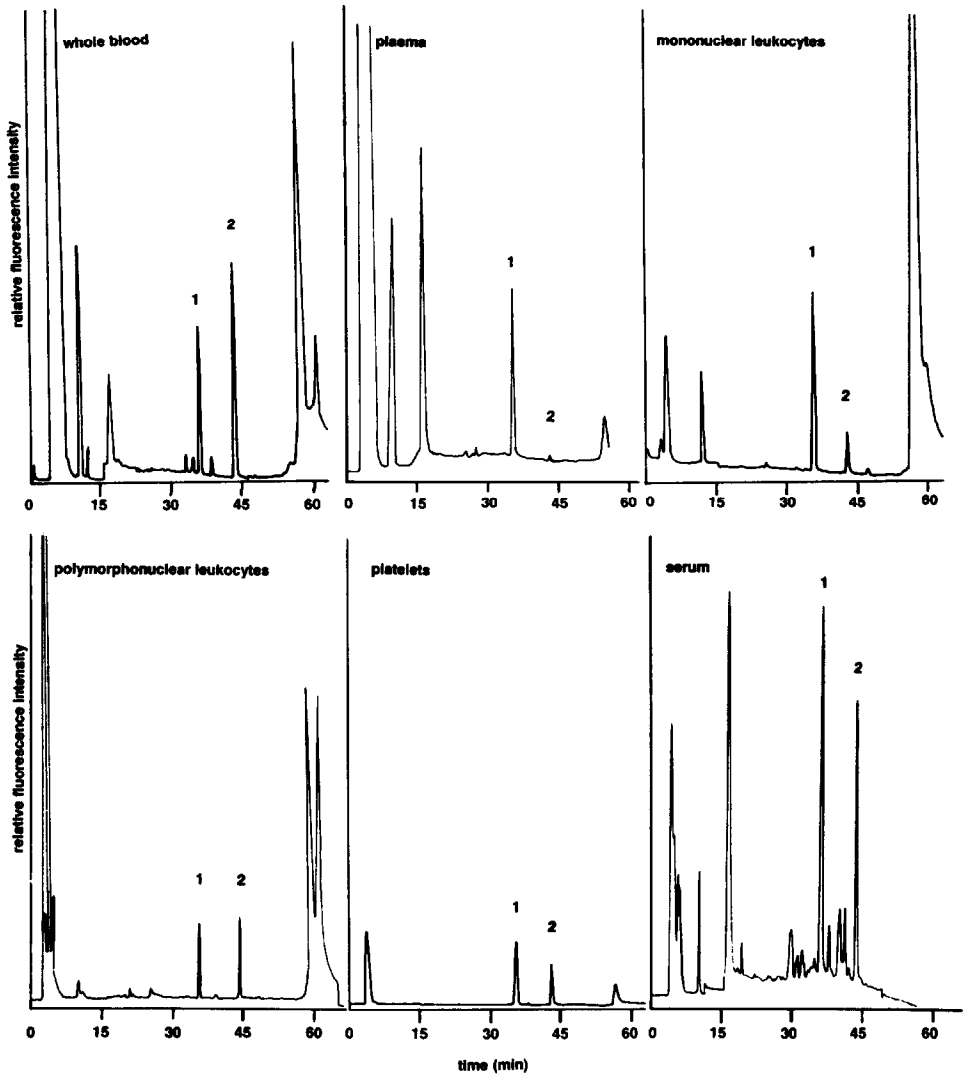

Fig. 1. HPLC profiles of whole blood, plasma and various blood corpuscles extracted with perchloric acid. After neutralization by potassium hydroxide, the samples were separated by reversed-phase HPLC. The amounts injected correspond to 0.1 ml of whole blood, 0.5 ml of plasma, $5 \cdot 10^6$ mononuclear leukocytes, $3 \cdot 10^6$ polymorphonuclear leukocytes, $60 \cdot 10^6$ platelets and 0.5 ml of serum. The serum was removed from the clotted blood after 24 h. Chromatographic conditions: column temperature, 56°C; flow-rate, 0.75 ml/min; buffer, 0.11 *M* pyridine–0.076 *M* acetic acid. The peptides were eluted with a linear gradient from 0 to 15% 1-propanol in 45 min, followed by a second gradient from 15 to 40% 1-propanol in 5 min. Peaks: 1 = Phe_2; 2 = thymosin β_4.

As mammalian erythrocytes do not contain a nucleus, we assumed that there might be a link between thymosin β_4 content and the presence of a nucleus. However, two facts weigh against this: (a) platelets, which, like erythrocytes, do not contain a nucleus, possess thymosin β_4, and (b) avian erythrocytes, which possess a nucleus,

TABLE I

CONCENTRATION OF THYMOSIN β_4 IN WHOLE BLOOD, PLASMA AND BLOOD CORPUSCLES OF 21- TO 35-YEAR-OLD HUMAN SUBJECTS

The specimens were deproteinized with perchloric acid and thymosin β_4 was determined by HPLC.

Specimen	*Thymosin β_4*	
	$x \pm$ S.E.M.(n)	*Range*
Whole blood	16.3 ± 0.8* (7)	12.0–19.5*
Plasma	0.134 ± 0.028* (13)	0.026–0.387*
Erythrocytes	<0.03**	
MNL	267 ± 21** (9)	183–380**
PMN	409 ± 24** (12)	269–564**
Platelets	22.3 ± 2.3** (14)	6.9–31.7**

* μg/ml.
** fg per cell.

do not contain thymosin β_4[14]. The presence of thymosin β_4 in chicken bursa of Fabricius has been described by Erickson-Viitanen *et al.*[3].

MNL contain 4%, PMN 10% and platelets 42% of the thymosin β_4 of whole blood if $2.7 \cdot 10^6$ MNL, $4.2 \cdot 10^6$ PMN and $310 \cdot 10^6$ platelets/ml are assumed. Taken all together, we can account for 50–60% of the thymosin β_4 in whole blood. The fact that we cannot reach 90 or 100% may be a result of the procedure for isolating the blood cells. During isolation, we may have lost a certain subpopulation of cells. We have found that the content of thymosin β_4 varies, for example, with the size of the platelets isolated (data not shown).

Naylor *et al.*[10] reported levels of thymosin β_4 ranging from 0.45 to 1.1 μg/ml, which are significantly higher than the concentrations in plasma, which range from 0.026 to 0.387 μg/ml (Table I). As Naylor *et al.* used serum for their determination, we also prepared serum and compared the concentrations of thymosin β_4 in serum and plasma. Even if the serum is removed from contact with the coagulated cell column as soon as after 1 h, the concentration in the serum is 0.25 μg/ml, compared with 0.04 μg/ml of plasma in samples from the same donor. After 8 and 24 h, the concentrations reached 1.1 and 2.1 μg/ml in serum, respectively. It can be concluded from our results that (a) thymosin β_4 appears in serum if the clotted blood is not removed as soon as possible and (b) thymosin β_4 is fairly stable in serum.

It is possible to assess very roughly the minimal intracellular concentration of thymosin β_4 of the different blood corpuscles from their volume and thymosin β_4 content. The concentrations of the peptide are 0.18, 0.20 and 0.27 m*M* in MNL, PMN and platelets, respectively. For this calculation, we assumed intracellular volumes of 270, 450 and 16 fl for MNL, PMN and platelets, respectively. No corrections were made for the nuclear volumes of MNL and PMN. The intracellular concentration of thymosin β_4 is fairly high, which supports the assumption of Goodall *et al.*[15] that thymosin β_4 may function as a structural element, perhaps as a component of the cytoskeleton. On the other hand, thymosin β_4 might have an additional function after appearing extracellularly either by specific secretion or just by death or lysis of cells. The second function might be the inhibition of the migration of macrophages.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft, grant Ha 1148/3. The technical assistance of M. Schindlbeck and M. Retzer is gratefully recognized. The authors thank Professor K. Brand for his support and M. Hergenhahn for stylistic advice.

REFERENCES

1 A. L. Goldstein, F. D. Salter and A. White, *Proc. Natl. Acad. Sci. USA*, 56 (1966) 1010.
2 T. L. K. Low and A. L. Goldstein, *Proc. Natl. Acad. Sci. USA*, 78 (1981) 1162.
3 S. Erickson- Viitanen, S. Rugieri, P. Natalini and B. L. Horecker, *Arch. Biochem. Biophys.*, 221 (1983) 570.
4 B. L. Horecker, S. Erickson-Viitanen and E. Hannappel, *Methods Enzymol.*, 116 (1985) 265.
5 R. W. Rebar, A. Miyaka, T. L. K. Low and A. L. Goldstein, *Science*, 214 (1981) 669.
6 G. B. Thurman, C. Seals, T. L. K. Low and A. L. Goldstein, *J. Biol. Response Modif.*, 3 (1984) 160.
7 E. Hannappel, G. J. Xu, J. Morgan, J. Hempstead and B. L. Horecker, *Proc. Natl. Acad. Sci. USA*, 79 (1982) 2172.
8 E. Hannappel, *Anal. Biochem.*, 156 (1986) 390.
9 E. Hannappel and W. Leibold, *Arch. Biochem. Biopohys.*, 240 (1985) 236.
10 P. H. Naylor, J. E. McClure, B. L. Spangelo, T. L. K. Low and A. L. Goldstein, *Immunopharmacology*, 7 (1984) 9.
11 A. Boyum, *Scand. J. Clin. Lab. Invest.*, 21, Suppl. (1968) 97.
12 S. Stein and J. Moschera, *Methods Enzymol.*, 79 (1981) 7.
13 E. Hannappel, S. Davoust and B. L. Horecker, *Proc. Natl. Acad. Sci. USA*, 79 (1982) 1708.
14 E. Hannappel and W. Leibold, unpublished results.
15 G. J. Goodall, J. I. Morgan and B. L. Horecker, *Arch. Biochem. Biophys.*, 221 (1983) 598.