

LVV- and VV-hemorphins: comparative levels in rat tissues

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Abstract Screening of hemorphins in extracts of rat lung, brain, heart and spleen was carried out. The threshold for detection of hemorphins was 0.01 nmol for spleen and 0.05 nmol for other tissues. Both the content and the composition of hemorphins differed significantly in the tissues analyzed. Heart and lung extracts were rich in these peptides, the content of the most abundant components reaching 16–44 nmol/g of tissue. In contrast, spleen and brain contained much lower amounts of hemorphins, i.e. about 0.3–2.6 nmol/g of tissue. The most represented hemorphin in lung, heart and brain was VV-hemorphin-5, while the content of other members of the hemorphin family depended significantly on the tissue analyzed: lung extract was also rich in LVV-hemorphin-5, heart contained similar amounts of LVV-hemorphin-7 and LVV-hemorphin-5 and brain of LVV-hemorphin-6. In contrast, the hemorphin family in spleen was represented mainly by C-terminally shortened VV-hemorphins, i.e. VV-hemorphin-4 and VV-hemorphin-3. The levels of hemorphins in all cases were sufficient to activate the opioid receptors of the respective tissues.

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Key words: Hemorphin; Hemoglobin fragment; Isolation; Composition; Tissue level; Rat

1. Introduction

In 1986 Brantl et al. found that proteolytic treatment of hemoglobin in vitro gives rise to peptides, called hemorphin-4 (β -globin 35–38) and hemorphin-5 (β -globin 35–39) and endowed with opioid-like activity [1,2]. Endogenous hemorphin-related peptides were isolated from brain [3–7], blood plasma [8], cerebrospinal fluid [9], spinal cord [10] and the supernatants of primary cultures of red bone marrow cells [11] and erythrocytes [12]. The members of that family of peptides are generated by proteolytic degradation of the (32–41) segment of the β -chain of hemoglobin. Being active in three basic opioid tests: inhibition of opioid ligand binding to brain membranes [4,5,13–15], induction of naloxone-dependent analgesia in rats in vivo [2,16] and contractile activity at the guinea pig ileum (GPI) [1,4,5,13,15], hemorphins are considered non-classical opioid peptides. Other effects related to the opioid nature of hemorphins are also reflected in the literature, such as coronar-constrictory [17], antitumor [18] and immunoregulatory activities [19], as well as inhibition of angiotensin-converting enzyme [15,20,21]. At the same time, the binding affinities of hemorphins to opioid receptors are much lower than those of classical opioid peptides. However,

we suggested that the overall effect of hemorphins on the opioid receptor system could be comparable with the action of classical opiates, enkephalins and endorphins, due to the relatively high content of these hemoglobin fragments in the tissue [7,23]. In order to obtain quantitative data related to that concept we measured in this work the levels of hemorphins in rat lung, heart, brain and spleen.

2. Materials and methods

2.1. Preparation of rat tissue extracts

Rat tissues were stored in liquid nitrogen. 4–6 g of brain, lung, heart and spleen tissues were homogenized in a Potter homogenizer in 20–30 ml of 10% acetic acid. The obtained mixture was centrifuged at $1500\times g$ for 15 min. All procedures were carried out within 17–18 min at 4°C. Supernatants were lyophilized.

2.2. Peptide synthesis

LVV-hemorphin-7, LVV-hemorphin-5 and VV-hemorphin-5 were synthesized on solid phase using the Boc technique in the Laboratory of Peptide Chemistry of our institute and characterized by nuclear magnetic resonance and mass spectrometry analysis.

2.3. Size exclusion chromatography

The size exclusion fractionation was performed using the liquid chromatography set (Pharmacia/LKB, Sweden) on a Sephadex G-25sf column (2.5×85 cm) equilibrated with 0.1 M acetic acid. The mixture of standard samples (1 mg of LVV-hemorphin-7; 2 mg of LVV-hemorphin-5; 0.5 mg of VV-hemorphin-5) or 200 mg of the lyophilized preparation of each tissue were dissolved in 12 ml of 0.1 M acetic acid and subjected to separation in conditions described in the legend to Fig. 1. The obtained fractions were lyophilized.

2.4. Reverse phase HPLC

The fractions obtained after size exclusion chromatography and the peptide standards were separated on a Nucleosil C₈ 120/5 μ (4.0×250 mm) cartridge equilibrated in buffer A (0.1% TFA in water). The elution was performed with a linear gradient of acetonitrile from 0 to 60% of buffer B (0.1% TFA, 80% acetonitrile in water) for 60 min at 0.75 ml/min.

The collected fractions were rechromatographed on a Nucleosil C₈ 120/5 μ (4.0×250 mm) cartridge equilibrated in 0.05 M ammonium-acetate buffer (pH 4.5). The elution was performed with a linear gradient of acetonitrile from 30 to 50% of buffer C (0.05 M ammonium-acetate buffer (pH 4.5), 80% acetonitrile in water) for 20 min at 0.75 ml/min. The elution profiles were recorded using the digitizer (Ampersend, Russia) at 226 nm. The absorbance values for Figs. 2 and 3 correspond to the ratio $1800\text{ mV}=2.56\text{ AUFS}$. Analysis of the elution profiles was performed using the MultiChrom-Spectr program, version 2.67 (Ampersend, Russia).

Elution zones corresponding to hemorphin standards were examined by UV spectrum analysis at wavelengths 273 nm and 288 nm using a multichannel photoarray detector (model 991, Waters). The peaks with a Tyr/Trp absorbance ratio characteristic for hemorphins (2:1) were identified as described in [22].

The content of hemorphins was calculated from sequencing data, using the peak area normalization method by means of the MultiChrom-Spectr program (version 2.67) and supported by UV spectrum analysis.

Reproducibility of the data presented in this paper was ensured by matching the elution profiles in five independent experiments. In sev-

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eral cases sequence analysis was used to verify the reproducibility of the results.

The monitoring of the losses of peptide material was carried out using the hemorphin standards at each separation stage. The total losses of hemorphins were less than 5%.

2.5. Peptide sequencing and sequence identification

Amino acid sequences were determined by means of a gas phase sequencer (Model 477A, Applied Biosystems). Identification of the established sequences was performed using PIR data bank version 38.

3. Results

Rat tissues were treated using a standard procedure under conditions ensuring minimal loss of original peptides [24,25]. The lyophilisates were fractionated on the Sephadex G-25sf column and the fractions corresponding to the elution zone of standard hemorphin mixtures (see below) were collected and lyophilized (Fig. 1). The yields of the material in the fractions obtained by separation of lung, heart, brain and spleen extracts were, respectively, 22.6, 14.6, 22.7, and 65.3 mg/g of tissue.

The conditions for size exclusion fractionation were selected using the mixture of synthetic preparations of LVV-hemorphin-7, LVV-hemorphin-5 and VV-hemorphin-5. Using these standards the elution zone of hemorphins was determined (Fig. 1). Based on the results of screening bovine brain extract, we expected the above mentioned tissues to contain, at least, LVV-hemorphin-7, LVV-hemorphin-5, LVV-hemorphin-4, VV-hemorphin-7 and VV-hemorphin-5 [7,24]. The chromatographic procedure developed for isolation of these hemorphins was applied for analysis of rat brain extract (Figs. 1A and 2A). The peaks of components corresponding to the zone of elution of hemorphins were subjected to three-dimen-

sional spectral analysis and the peaks with a 2:1 Tyr/Trp absorbance ratio were isolated and sequenced. Because of poor resolution of the substances corresponding to the shaded peaks in elution buffer containing 0.1% TFA, an additional purification stage was required. The non-resolved peaks were rechromatographed using 0.05 M ammonium-acetate buffer (pH 4.5) in conditions given in the legend to Fig. 2. As seen from Fig. 3, a resolution sufficient for sequencing analysis was achieved. As a result, the amino acid sequences of 10 hemorphins were established (Table 1). The chromatographic procedure developed was applied for identifying and quantifying hemorphins in rat lung, heart and spleen extracts. The fractions obtained after size exclusion chromatography (Fig. 1B–D) were subjected to further separation on Nucleosil C₈ 120/5 μ (Fig. 2B–D) and the peaks with a 2:1 Tyr/Trp absorbance ratio were isolated as described above and sequenced.

The data on composition and content of hemorphins in rat tissues are summarized in Table 1. Ten hemorphins were found in the rat brain, lung and heart and four in the spleen. Additionally, three fragments of functional proteins with a Tyr/Trp ratio similar to that of hemorphins were identified: the fragment (309–322) of glyceraldehyde phosphate dehydrogenase (ISWYDNEYGYSNRV) was isolated from the brain extract (Fig. 2A, peak I) [24]; the fragment (12–25) of the α -chain of hemoglobin (NCWGKIGGHGGEYGE) and the fragment (13–23) of carbonate dehydratase I (GPDNWSKLYPI) (Fig. 2D, peaks II and III, respectively) were isolated from spleen.

As seen from Table 1, both the content and the composition of hemorphins differ significantly in the tissues analyzed. Heart and lung extracts were rich in hemorphins, the contents of the most abundant components being, respectively, 16 and 44 nmol/g of tissue. In contrast, spleen and brain contained

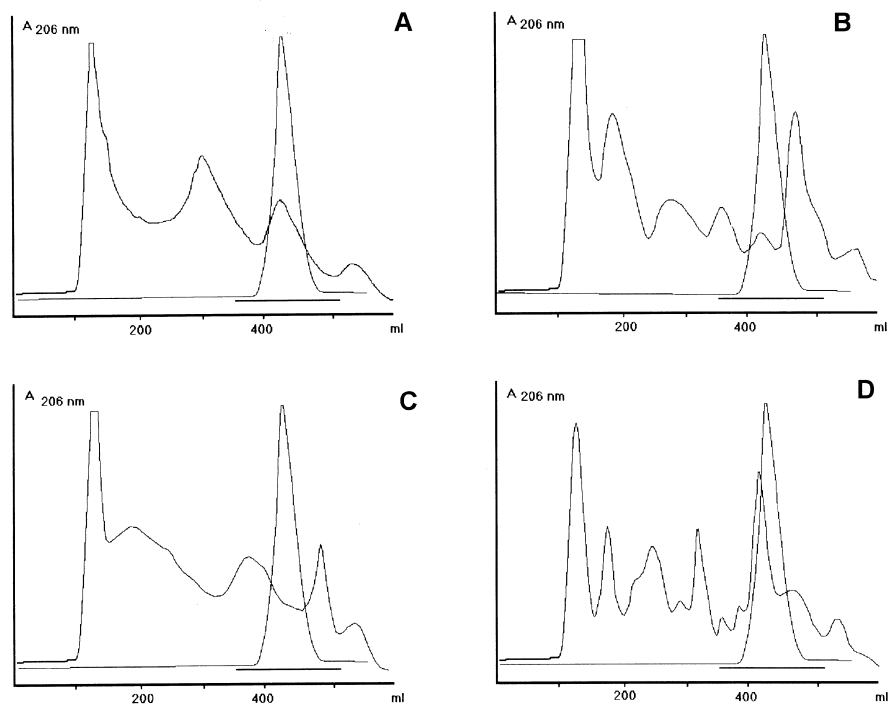


Fig. 1. Size exclusion chromatography of rat tissue extracts on a Sephadex G-25sf (2.5×85 cm) column equilibrated with 0.1 M acetic acid. The collected fractions are marked. Elution profile of standard mixture of hemorphins is shown. A: brain; B: heart; C: lung; D: spleen.

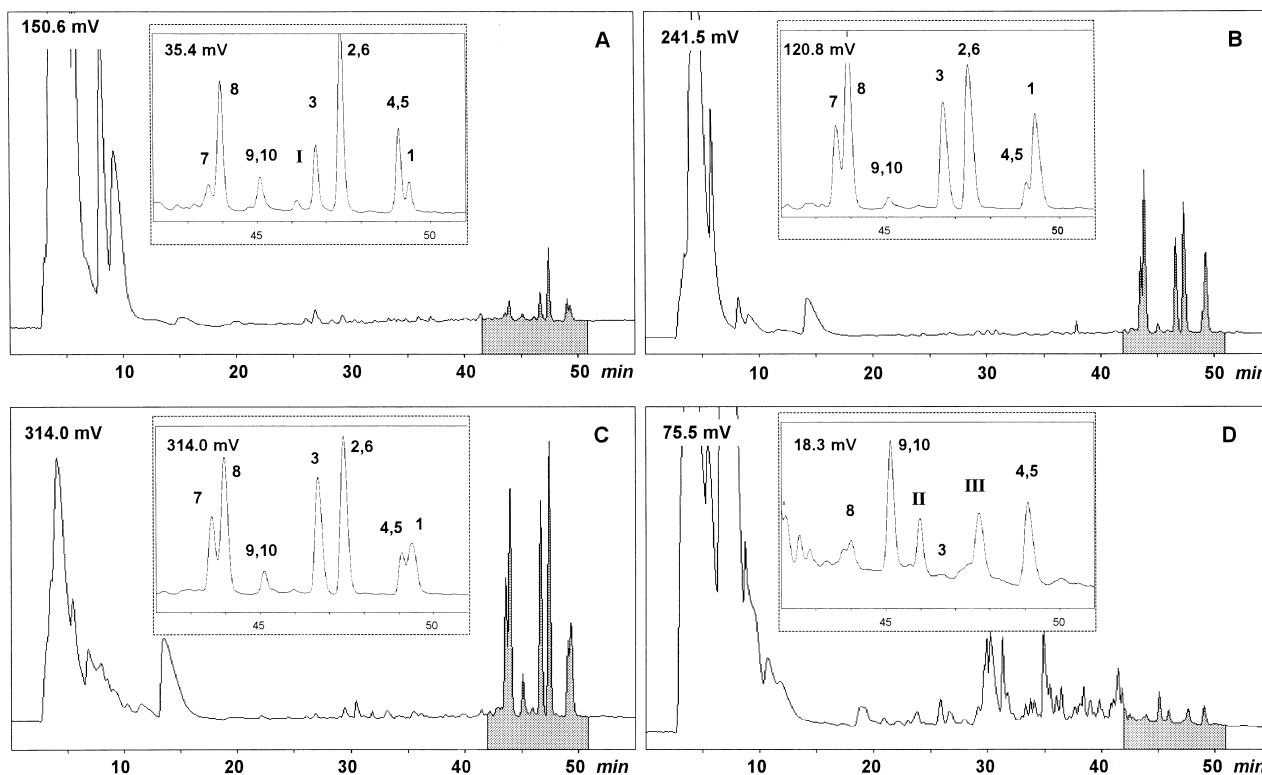


Fig. 2. RP-HPLC of peptide fractions isolated from rat tissue extracts (Fig. 1) on a Nucleosil C₈ 120/5 μ (4.0 \times 250 mm) cartridge equilibrated with buffer A (0.1% TFA in water) in a linear gradient of acetonitrile. Shading marks the elution zone of hemorphins. A: brain; B: heart; C: lung; D: spleen. Insertions show the fragments of elution zones of hemorphins. The numbers correspond to peptides represented in Table 1.

much lower amounts of hemorphins, the total amounts of all studied components being, respectively, only 0.35 and 2.6 nmol (per g of tissue). The peptide represented maximally in lung, heart and brain was valorphin (VV-hemorphin-5), while the content of other members of the hemorphin family de-

pended significantly on the tissue analyzed: lung extract was also rich in LVV-hemorphin-5 (85% of the valorphin level), heart contained similar amounts of LVV-hemorphin-7 and LVV-hemorphin-5 (69% of the valorphin level) and brain of LVV-hemorphin-6 (73% of the valorphin level). In contrast,

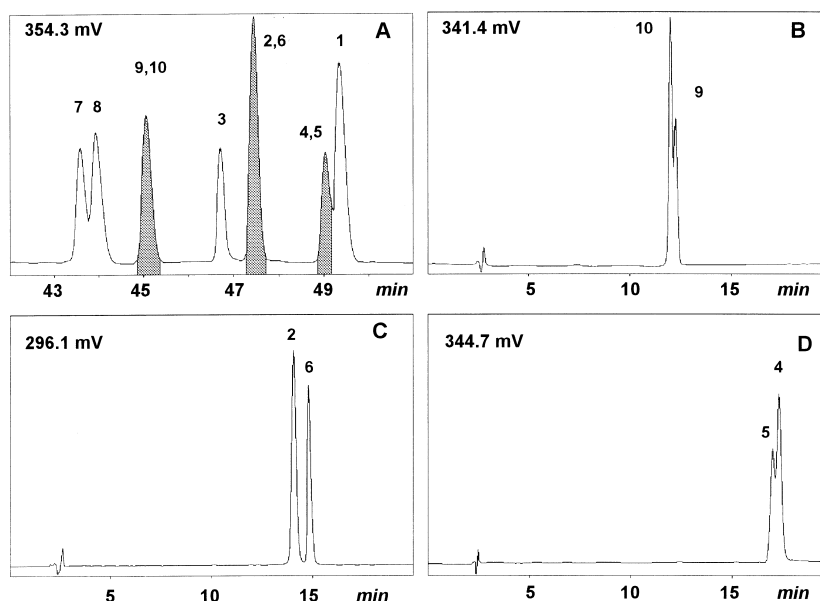


Fig. 3. Reverse phase rechromatography of hemorphins on a Nucleosil C₈ 120/5 μ (4.0 \times 250 mm) cartridge in a linear gradient of acetonitrile. A: Fragment of the elution profile obtained after separation of the prepared mixture of hemorphins isolated from rat tissues. The column was equilibrated with 0.1% TFA. Shading marks the elution zones of non-resolved hemorphins. B–D: Rechromatography of non-resolved hemorphins on the column equilibrated with 0.05 M ammonium-acetate buffer (pH 4.5) (substances 4, 5; 2, 6; and 9, 10, respectively). The numbers correspond to peptides represented in Table 1.

Table 1
Content of LVV- and VV-hemorphins in the extracts of rat tissues

No.	Peptide	Name	Retention time (min)	Tissue level (nmol/g)			
				Spleen	Brain	Heart	Lung
1	LVVYPWTQRY	LVV-hemorphin-7	49.25 ± 0.04	< 0.01	0.6 ± 0.05	11.0 ± 0.5	17.0 ± 1.0
2	LVVYPWTQR	LVV-hemorphin-6	47.39 ± 0.05	< 0.01	1.9 ± 0.1	8.0 ± 0.5	23.0 ± 1.0
3	LVVYPWTQ	LVV-hemorphin-5	46.66 ± 0.04	0.02 ± 0.01	1.7 ± 0.1	11.0 ± 0.5	38.0 ± 2.0
4	LVVYPWT	LVV-hemorphin-4	49.01 ± 0.11	0.15 ± 0.01	1.6 ± 0.1	2.5 ± 0.5	13.0 ± 0.5
5	LVVYPW	LVV-hemorphin-3	49.12 ± 0.08	< 0.01	0.35 ± 0.03	< 0.05	< 0.05
6	VVYPWTQRY	VV-hemorphin-7	47.45 ± 0.06	< 0.01	0.9 ± 0.05	4.0 ± 0.2	4.0 ± 0.05
7	VVYPWTQR	VV-hemorphin-6	45.58 ± 0.08	< 0.01	0.4 ± 0.04	9.0 ± 0.1	12.0 ± 0.5
8	VVYPWTQ	VV-hemorphin-5 (valorphin)	43.94 ± 0.04	0.02 ± 0.01	2.6 ± 0.1	16.0 ± 0.4	44.0 ± 1.2
9	VVYPWT	VV-hemorphin-4	45.09 ± 0.07	0.35 ± 0.02	0.25 ± 0.03	0.25 ± 0.1	7.0 ± 0.5
10	VVYPW	VV-hemorphin-3	45.21 ± 0.08	0.25 ± 0.02	0.25 ± 0.03	< 0.05	< 0.05

the hemorphin family in spleen was represented mainly by C-terminally shortened VV-hemorphins, i.e. VV-hemorphin-4 and VV-hemorphin-3, their contents were, respectively, 17- and 12-fold higher than that of valorphin.

4. Discussion

The data presented in Table 1 show that LVV-hemorphin-7, LVV-hemorphin-5 and VV-hemorphin-5 (valorphin) are present in lung and heart tissues in amounts sufficient for manifestation of their opioid activity, which requires 10–100 μM of these peptides [4,5,13–15]. The levels of LVV-hemorphin-6, LVV-hemorphin-4 and VV-hemorphin-6 in lung also corresponded to their binding affinity to opioid receptors. We suggested earlier that the classical regulatory peptide and the fragment of a functional protein might act at the same receptor and the hormone-induced signal can be controlled by the level of proteolytic activity in the tissue. In this case, the high content of hemorphins might compensate for the relatively low binding parameters of these peptides. For example, the binding constants of hemorphins maximally presented in tissue extracts with μ -opioid receptors are higher by several orders of magnitude than for Leu-enkephalin, (respectively, 1–50 μM [22] and 0.03–0.04 μM [4]). However, the overall content of hemorphins in lung, heart and brain tissues, as seen from Table 1, is approximately, 3–70 nmol/g compared to 0.02–0.2 nmol/g in the case of Leu-enkephalin in brain [26]. Since members of the hemorphin family have been shown to induce cytotoxicity and to decrease proliferation of tumor cells at very low concentrations (10^{-6} – 10^{-13} M) [18], as compared with the concentrations needed for manifesting the opioid effects, the content of less abundant hemorphins in tissues might be quite sufficient for inducing of cytotoxic and antiproliferative effects, characteristic for ‘classical’ ligands of opioid receptors [27] or the opioid-like peptides casomorphins [28].

The data obtained allow us to compare the composition of hemorphins in the examined tissues with that of biological fluids. In contrast to tissues, human blood plasma and cerebrospinal fluid were shown to contain very low amounts of hemorphins (respectively, less than 2.0 pmol/ml [8] and less than 3.5 pmol/ml [9]). The content of hemorphin-7 increases 3–4-fold in human blood plasma after marathon running [8] and LVV-hemorphin-7 increases in cerebrospinal fluid up to 115–300 pmol/ml during cerebrovascular bleeding [9]. The level of that peptide was also shown to rise in the plasma of patients undergoing hemofiltration [29]. In other words,

changes of hemorphin content in biological fluids are associated with alterations in the organism state. Rough estimation shows that the scale of these changes (up to 0.3 μM) might be sufficient for induction of opioid-like effects of hemorphins in vivo.

Recently we found that human erythrocytes release a set of hemorphin-related peptides different from that detected in tissue extracts [12]. Supernatant of human erythrocytes contained hemorphin-7, as well as V-hemorphin-7, V-hemorphin-5 and V-hemorphin-3, which have not been identified earlier. At the same time, LVV- and VV-hemorphins were not detected in the supernatant. These findings indicate that there must be more than one mechanism of generation of hemorphins in vivo, i.e. in addition to production by erythrocytes, proteolytic enzymes of tissues should be involved in formation of these peptides. For instance, macrophages were shown to produce VV-hemorphin-7 from hemoglobin in vitro [30].

Significant differences in the patterns of hemorphins in different biological sources might be related to variations in the spectra of their activity in vivo. In any case, we believe that the effects of hemorphins overlap with the functions of ‘classical’ opioid peptides: from influence on physiological processes in the tissues or the whole organism to regulation of the activity of immune cells or elimination of tumor cells.

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