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Kinetics of appearance of hemorphins from bovine hemoglobin peptic hydrolysates by a direct coupling of reversed-phase high-performance liquid chromatography and electrospray ionization mass spectrometry

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Abstract

Reversed-phase high-performance liquid chromatography coupled with electrospray ionization mass spectrometry was used to improve the preparation of three opioid peptides (Leu–Val–Val–hemorphin-7, Val–Val–hemorphin-7 and Val–Val–hemorphin-4) resulting from bovine hemoglobin peptic hydrolysates. Optimal conditions for the preparation of these peptides were determined thanks to their kinetic studies of appearance in the course of peptic hydrolyses as a function of degree of hydrolysis of hemoglobin. We propose a low degree of hydrolysis (3%) to prepare Leu–Val–Val–hemorphin-7, a mean degree of hydrolysis (11%) to prepare Val–Val–hemorphin-7 and a high degree of hydrolysis (21%) to prepare Val–Val–hemorphin-4. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Hemorphins are a new class of opioid peptides derived from the blood protein hemoglobin. These peptides were isolated and purified in vivo in biological fluids [1–8], and in vitro from enzymatically treated hemoglobin [9–12].

A number of hemorphin peptides were obtained from a peptic bovine hemoglobin at pH 2. The two first hemorphins obtained were hemorphin-4 (Tyr–Pro–Trp–Thr) and hemorphin-5 (Tyr–Pro–Trp–Thr–Gln). These amino acid sequences correspond to the 34–37 and 34–38 fragments of the β-chain of hemoglobin [9]. We have isolated and characterized two other opioïd peptides [10]: LVV-hemorphin-7 (Leu–Val–Val–Tyr–Pro–Trp–Thr–Gln–Arg–Phe) and VV-hemorphin-7 (Val–Val–Tyr–Pro–Trp–Thr–Gln–Arg–Phe). These peptides correspond respectively to the sequence 31–40 and 32–40 of β-chain. Recently, Zhao et al. [11] also isolated other opioïd peptides from this hydrolysate: LVV-hemorphin-5 (Leu–Val–Val–Tyr–Pro–Trp–Thr–Gln) and VV-

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hemorphin-5 (Val–Val–Tyr–Pro–Trp–Thr–Gln), corresponding respectively to the sequences 31–38 and 32–38 of β-chain. More recently, we also isolated an other opioïd peptide by hydrolysing hemoglobin in its native state at pH 4.5 [13]: VV-hemorphin-4 (Val–Val–Tyr–Pro–Trp–Thr) corresponding to the sequence 32–37 of β-chain.

The purification of peptides from a bovine hemoglobin hydrolysate was first performed by a combination of low- or high-pressure size-exclusion (SEC) with reversed-phase (RP) HPLC [14,15]. This proved to be a useful strategy for the fractionation of such a mixture. Volatile buffers were employed in both HPLC techniques in order to get an easy recovery of peptides for further applications. Amino acid analysis by the Picotag method [16] and fast atom bombardment (FAB) mass spectrometry confirmed the purity and allowed accurate molecular weights to be determined for isolated peptides.

Progress in HPLC photodiode array detection technology have made possible not only to carry out the fly-real time spectral scanning during the chromatographic process, but also to obtain derivative spectra simultaneously [17–23]. We recently reported a method of isolation of these active peptides using one step by HPLC [24]. Then, the molecular weight and primary structure were determined in second step by mass spectrometry.

However, procedures for isolation and identification of peptides with opioid-like activity remain laborious and time-consuming. So, we propose direct coupling of HPLC and electrospray mass spectrometry (ESI–MS) which became one of the most important techniques of the analysis of proteins and peptides mixtures in the last few years [25]. This technique is widely used in the analysis of mixtures containing limited numbers of peptides, e.g., enzymatic digests of protein, to confirm primary structures [26–32]. Other reports describe the analysis of complex biological samples which contain a large number of different peptides [33,34].

The aim of the study presented here was to propose a method of isolation and identification of three active peptides (LVV-hemorphin-7, VV-hemorphin-7 and VV-hemorphin-4) using one step during the hydrolysis of bovine hemoglobin by a direct coupling of RP-HPLC and ESI–MS. In order to find ideal conditions for the preparation of these active peptides, we have applied this method to the kinetic of appearance of these peptides in vitro, in our peptic hydrolysate as a function of the degree of hydrolysis of hemoglobin.

2. Experimental

2.1. Materials

All common chemicals and solvents were of analytical grade from commercial sources. Bovine hemoglobin and pig pepsin were purchased from Sigma. Acetonitrile was of HPLC grade. All aqueous HPLC eluents were degased with helium.

2.2. Hydrolysate preparation

Hemoglobin at 5% (w/v) was digested at 23°C in 0.1 *M* sodium acetate buffer, pH 4.5, in the presence of urea (5.3 *M*), by pig pepsin (EC 3.4.23.1) ($1\cdot10^6$ Anson units; Sigma), with a substrate-to-enzyme ratio of 11:1 (w/w). The enzymatic hydrolysis was stopped at different degrees of hydrolysis by the addition of disodium tetraborate (0.32 *M*, pH 12.7) to yield a final pH of 10.

2.3. Determination of the degree of hydrolysis of hemoglobin

Degree of hydrolysis is defined as the number of peptide bonds cleaved to the total number of peptide bonds in hemoglobin. The cleavage of peptide bonds during hydrolysis was quantified by the trinitrobenzene sulfonate method (20). The corrected degree of hydrolysis (DHc) is defined as the ratio of the number of cleaved peptide bonds (determined by the trinitrobenzenesulphonate method) to the total number of peptide bonds of hemoglobin effectively hydrolysed. The proportion of non-hydrolysed hemoglobin was deduced from the determination of its peak area by HPLC.

2.4. Direct coupling of RP-HPLC and ESI–MS

All MS measurements were carried out in the positive ion mode on a Quattro II triple quadrupole instrument (Micromass, Altrincham, UK) equipped

with an electrospray ion source. The nebulizer and drying gases were delivered at flow-rates of 0.3 and 6.7 $l\ min^{-1}$, respectively whereas the source temperature was maintained at 110°C. A mixture of polypropylene glycol (PPG) was used to calibrate the quadrupole mass spectrometer.

Separations were performed on a Hewlett-Packard 1100 HPLC system equipped with a Zorbax C_3 column (200×2.1 mm, 5 μm) directly coupled to the UV detector. Elution was carried out at a flow-rate of $0.170 \cdot 10^{-3}$ $l\ min^{-1}$ with a water–acetonitrile gradient. The A solvent was 0.1% trifluoroacetic (TFA) in water and the B solvent was 0.1% TFA in water–acetonitrile (40:60). The gradient was 0–67% B in 30 min and then 87% by 35 min. Detection was effected at 215 nm. Quadrupole mass spectrometer was scanned from m/z 400 to 1750 with a scan duration of 3.2 s and a scan delay of 0.1 s. The cone voltage was maintained at 60 V and the capillary and high-voltage electrode potentials were at 3.2 kV and 0.82 kV, respectively. An aliquot of pepsic hydrolysate corresponding to 55 pmoles of hemoglobin was injected for HPLC–MS analysis.

3. Results and discussion

The peptic hydrolysis of bovine hemoglobin was performed for 72 h at 23°C in 0.1 M sodium acetate buffer at pH 4.5. In order to follow on-line kinetic of appearance of three active peptides (LVV-hemorphin-7, VV-hemorphin-7 and VV-hemorphin-4) in a peptic hydrolysate as a function of the degree of hydrolysis of hemoglobin, RP-HPLC was coupled with ESI–MS. This complex hydrolysate was analysed speedily, and with moderate ease, without resorting to off-line separation of components or derivatization procedures. The power of the technique derives from the ability to determine both the retention time (UV chromatogram) and the molecular mass of the eluting components [total ion current (TIC) chromatogram]. Fig. 1 shows HPLC–MS chromatograms obtained from the analysis of the peptic hydrolysate of bovine hemoglobin at different corrected degrees of hydrolysis (DHc) (1.5, 3, 9, 11, 15, 17, 19, 21%) corresponding respectively to 30 s, 2.5 min, 1, 3, 10, 24, 48 and 72 h respectively. The excellent correspondence of the UV chromatogram to the TIC chromatogram in the HPLC–ESI–MS data acquired over the range mentioned before each peptic hydrolysate, allows reliable correlation of retention times of hemorphins with their corresponding mass spectra. In general, the differences observed were variations in relative peak intensities that could be attributed to the specificities of each of the two detectors: the UV detector was sensitive to the number of chromophores absorbing at 215 nm while the mass spectrometer was sensitive to differences in desorption/ionization efficiency for each hemorphin.

LVV-hemorphin-7, which was eluted at retention time of 30.80 min with a molecular cation $(MH)^+$ of M_r 1309, was detected at a DHc of 1.5% (Fig. 1a). The concentration of this hemorphin increases in the course of the hydrolysis before decreasing and completely disappearing at DHc of 15% (Fig. 1b–d). VV-Hemorphin-7 which was eluted at a retention time of 28.90 min with a molecular cation $(MH)^+$ of M_r 1195.7 was detected at a DHc of 9% (Fig. 1c). The concentration of this active peptide increases up to a DHc of 11% (Fig. 1d), then decreases to disappear completely at a DHc of 21% (Fig. 1e–h). These kinetic evolutions indicate that the peptic hydrolysis of LVV-hemorphin-7 gives rise to VV-hemorphin-7. As discussed previously [23], at pH 4.5, LVV-hemorphin-7 disappears while the concentration of VV-hemorphin-7 increases. Other authors [35,36] have also reported the same phenomenon at pH 2 and assumed that LVV-hemorphin-7 is a precursor of VV-hemorphin-7.

VV-Hemorphin-4 was late detected during peptic hydrolysis of hemoglobin at a DHc of 15% (Fig. 1e). It eluted at a retention time of 27.00 min with a molecular cation $(MH)^+$ of M_r 763.4. Its concentration increased during the hydrolysis and was not cleaved by pepsin even after 72 h of hydrolysis (Fig. 1f–h), whereas, LVV-hemorphin-7 and VV-hemorphin-7 were cleaved by pepsin. Previously [13], we supposed that LVV-hemorphin-7 and VV-hemorphin-7 were intermediate peptides, whereas VV-hemorphin-4 was a final peptide. This hypothesis is confirmed by a direct coupling of RP-HPLC and ESI–MS. These results also show that the concentration of VV-hemorphin-7 decreases while the concentration of VV-hemorphin-4 increases. So, VV-hemorphin-7 is a precursor of VV-hemorphin-4 and the

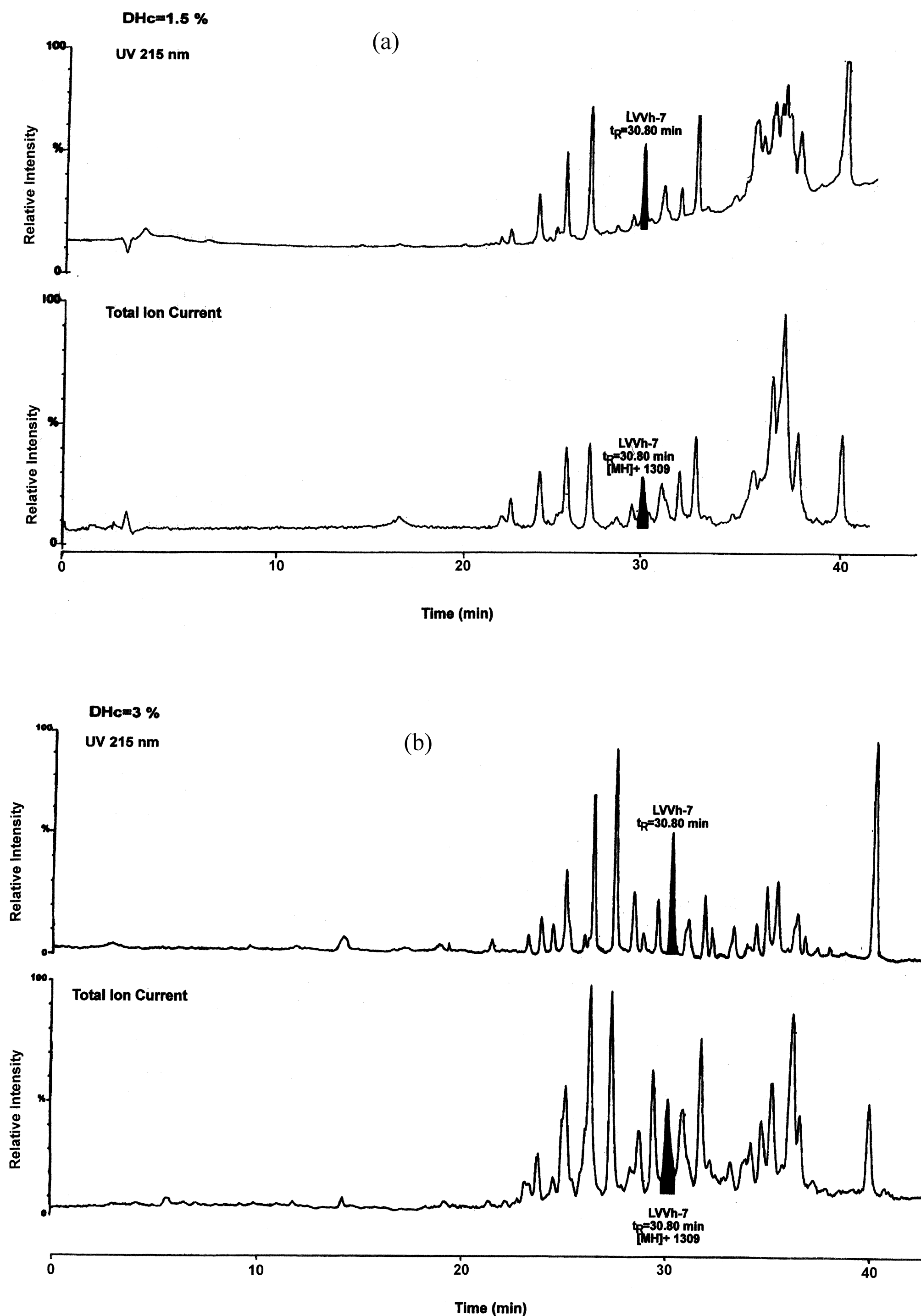
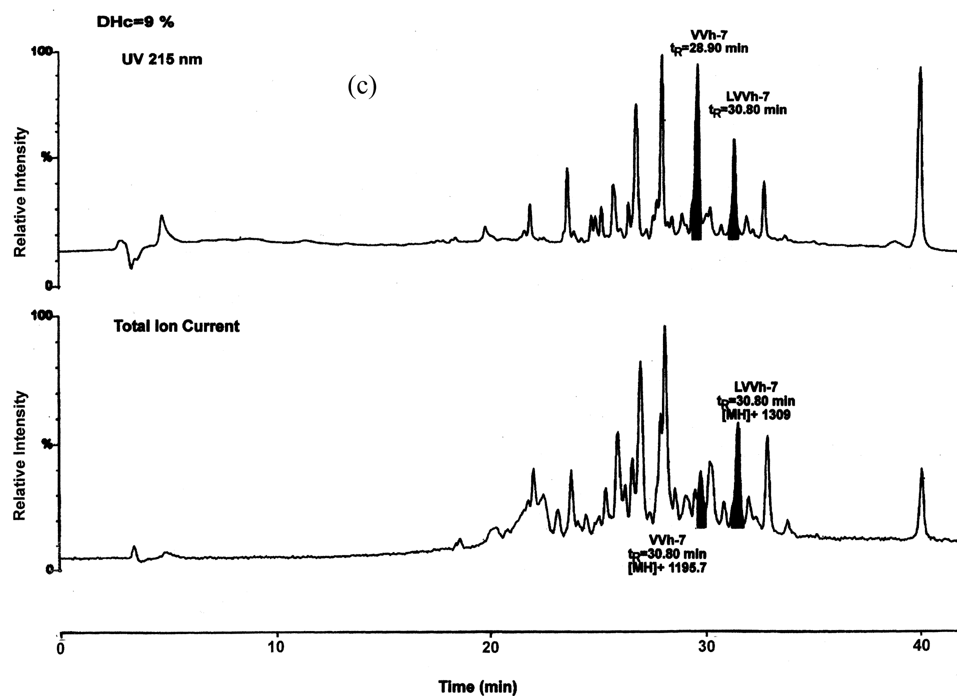
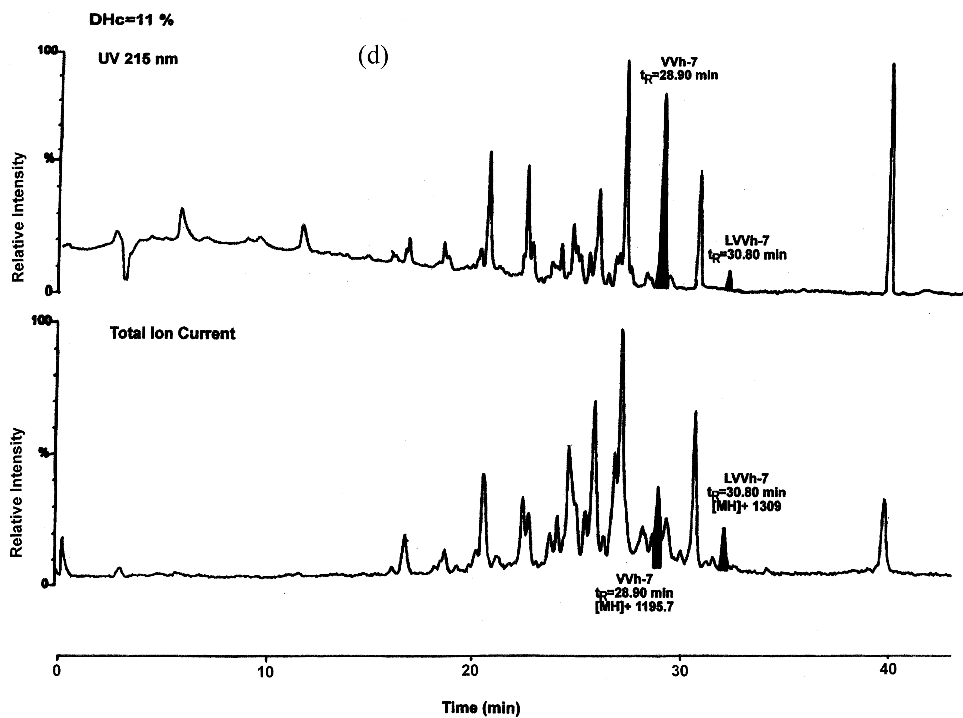


Fig. 1. Kinetics of appearance of hemorphins (Leu–Val–Val–hemorphin-7, Val–Val– hemorphin-7 and Val–Val–hemorphin-4) from bovine hemoglobin peptic hydrolysate at different degrees of hydrolysis (DHc) by a direct coupling RP-HPLC and ESI–MS. The peptic hydrolysate was separated on a Zorbax C_3 column (200×2.1 mm, 5 μm), flow-rate: $0.170 \cdot 10^{-3}$ l min^{-1}, monitoring wavelength: 215 nm, separate mass range TIC (total ion current) profiles at scan ranges from m/z 400 to 1750. (a) DHc = 1.5%, (b) DHc = 3%, (c) DHc = 9%, (d) DHc = 11%, (e) DHc = 15%, (f) DHc = 17%, (g) DHc = 19%, (h) DHc = 21%.





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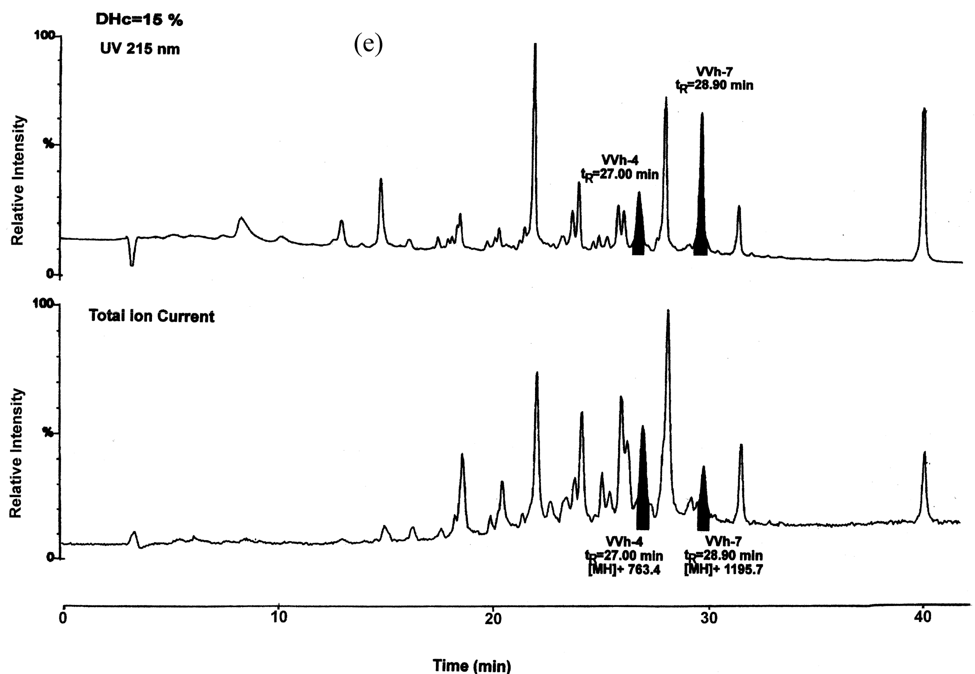

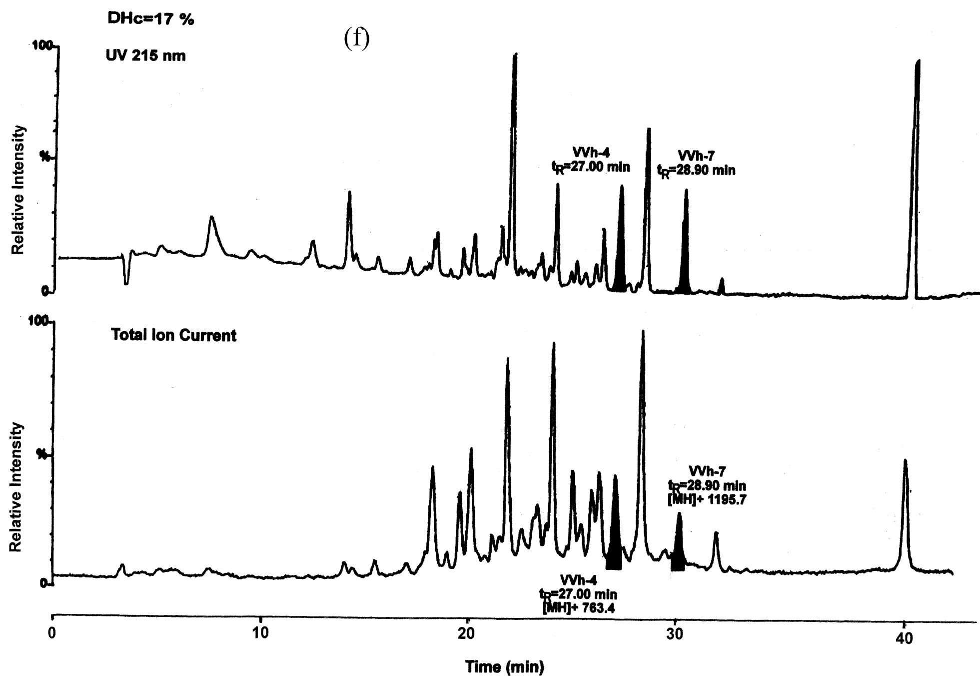

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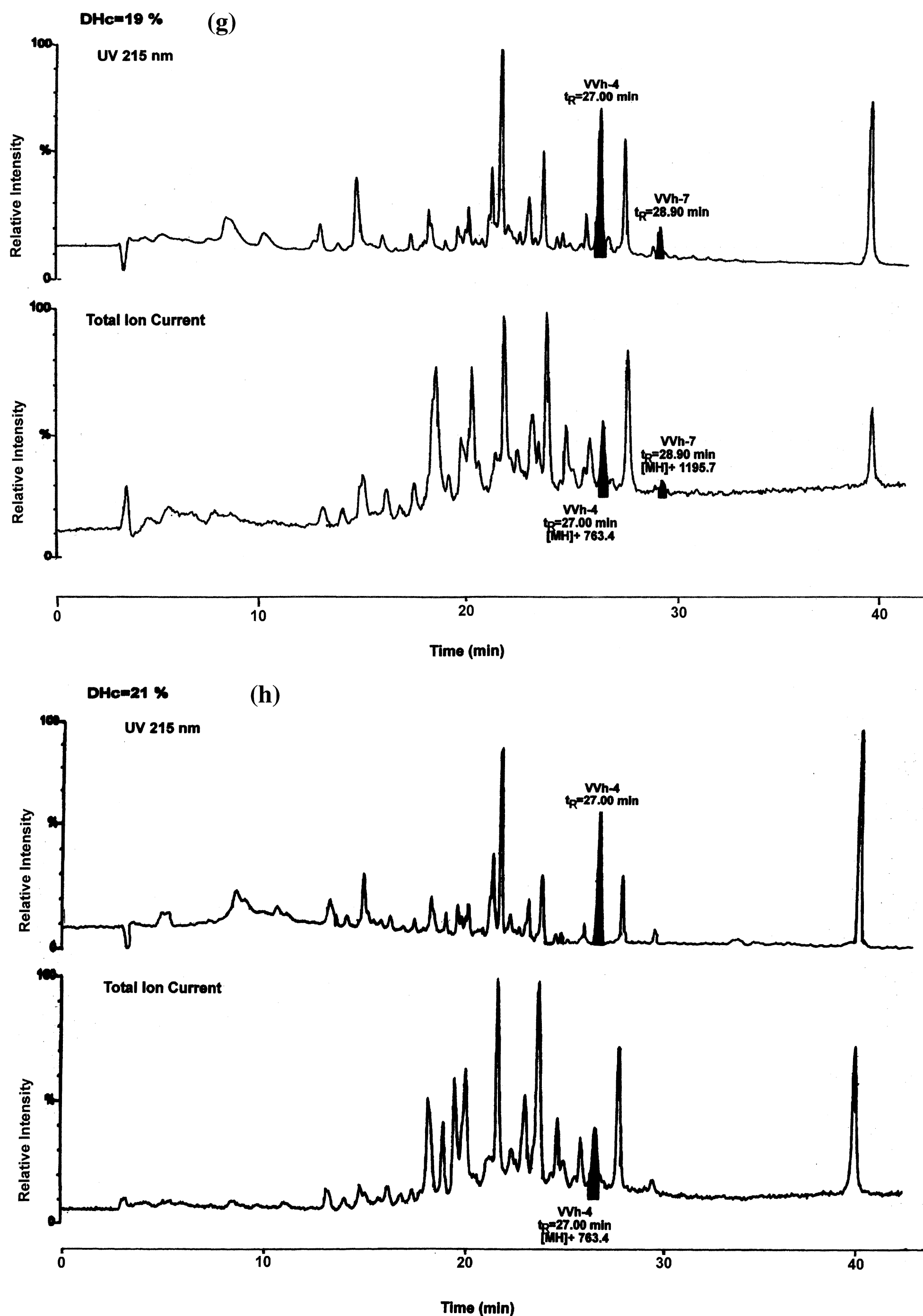

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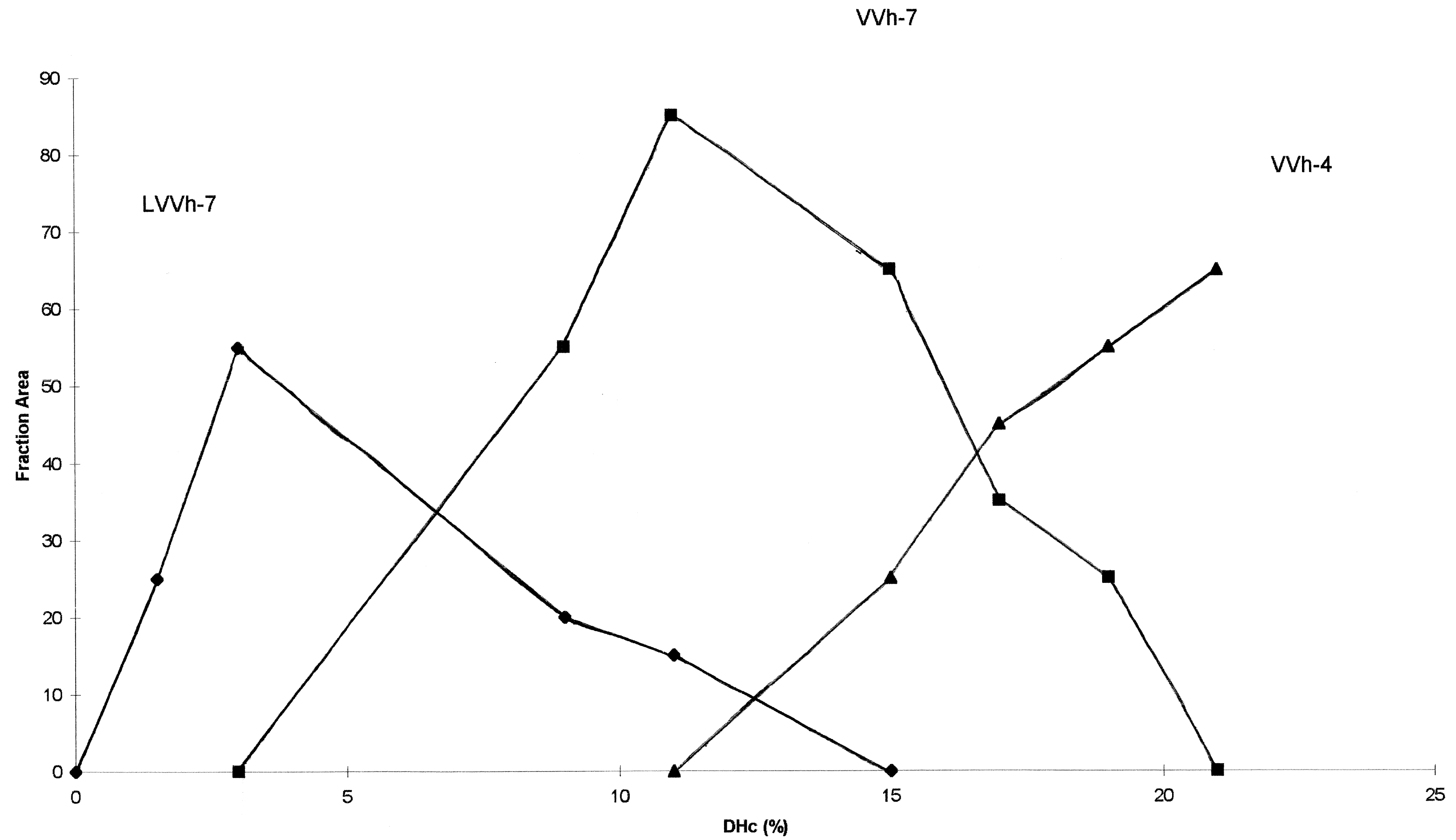

Fig. 2. Comparison of kinetics of appearance of Leu–Val–Val–hemorphin-7, Val–Val–hemorphin-7 and Val–Val–hemorphin-4 as a function of degree of hydrolysis of hemoglobin.

three hemorphins are produced successively during the hydrolysis of the β-chain of bovine hemoglobin by pepsin at pH 4.5: LVV-hemorphin-7 is a precursor of VV-hemorphin-7 which is a precursor of VV-hemorphin-4. Zhao et al. [11] showed that LVV-hemorphin-7 and VV-hemorphin-7 are precursors of LVV-hemorphin-5 β(31–38) and VV-hemorphin-5 β(32–38) at pH 2, however they did not detect the formation of VV-hemorphin-4. In our case, we have observed the generation of VV-hemorphin-4 but not the formation of LVV-hemorphin-5 and VV-hemorphin-5 at pH 4.5. These results evidence that the selectivity of the hemoglobin peptic hydrolysis is different at pH 4.5 and pH 2.

Optimal conditions for the preparation of LVV-hemorphin-7, VV-hemorphin-7 and VV-hemorphin-4 were determined thanks to the kinetic studies of appearance of these peptides in the course of peptic hydrolyses as a function of the corrected degree of hydrolysis (DHc) of hemoglobin.The appearance of these three hemorphins during the degradation of bovine hemoglobin is resumed in the Fig. 2. We propose a low DHc (3%) to prepare LVV-hemorphin-7, a mean DHc (11%) to prepare VV-hemorphin-7 and a high DHc (21%) to prepare VV-hemorphin-4.

In this study, we developed a rapid technique using a direct coupling of RP-HPLC and ESI–MS for isolation and identification of three hemorphins produced during the hydrolysis of bovine hemoglobin (LVV-hemorphin-7, VV-hemorphin-7 and VV-hemorphin-4). This method is a way to identify these active peptides without a preliminary extraction or disruption step and allows to reduce their time of purification. This technique is a good analytical separation and sensitive detection and can be achieved with mass spectrometric detection. The use of HPLC–MS for the characterization of active peptides can be a very powerful bioanalytical technique. Moreover, the search for known peptides by their molecular mass is a reliable method to guide peptide purification.

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