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Reversed-phase high-performance liquid chromatography coupled with second-order derivative spectroscopy for the quantitation of aromatic amino acids in peptides: application to hemorphins

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Abstract

The characterization of aromatic amino acid-containing peptides in biological fluids or protein hydrolysates is commonly achieved using classical size-exclusion (SE) and reversed-phase (RP) high-performance liquid chromatography (HPLC) coupled with direct ultraviolet (UV) spectrometry. Here, a non-destructive quantitative determination of aromatic amino acids in peptides is developed using second-order derivative spectra obtained during RP-HPLC coupled with photodiode array detection. In this method, the free aromatic amino acids were used as standards. Sensitivity and accuracy were verified using some peptides, including bioactive hemorphins. The method was applied to determine the amounts of hemorphins present in a complex peptic bovine hemoglobin hydrolysate.

Keywords: Hemorphins; Amino acids; Peptides

1. Introduction

Usually, the analysis of aromatic amino acids in peptides and proteins by acidic hydrolysis is a destructive process, especially for tryptophan [1]. The colorimetric methods used for the direct quantitation of aromatic amino acids are sample- and time consuming [2]. The direct spectrophotometric (UV) determination of tryptophan and tyrosine, although much studied, presents many difficulties [3]. For example, very pure peptides, proteins or standards must be used, and nevertheless the error for tryptophan still exceeds 10% and simultaneous determination of tyrosine is often impossible. Other physicochemical methods for the determination of tryptophan and tyrosine, e.g. magnetic circular dichroism, present similar problems and are tedious, complicated, sensitive to reaction conditions and require heavy equipment and techniques [4].

Recently, a high-performance liquid chromatographic (HPLC) method with photodiode array detection was described which allowed the real-time spectral analysis of peptides [5]. Although UV–Vis spectrophotometers are able to generate wavelength scans of peptides and proteins, their

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useful application is limited to fairly pure samples. Diode array detection systems, coupled with computer equipment, allow the almost instantaneous acquisition of UV–Vis spectra at any time during a chromatographic separation. Real-time spectral analysis is extremely helpful in the resolution of complex mixtures of peptides. Any peak occurring during an HPLC chromatography can be scanned. These spectral data are automatically processed to yield second-order derivative spectra, which permits the identification of aromatic amino acids present in peptides [6–8].

The determination of the ratio between the aromatic amino acids in peptides was reported in our previous study [9]. It was only applied to the qualitative determination of hemorphins, bioactive peptides originally obtained by Brantl et al. [10] from bovine blood. More recently, these peptides were isolated from human pituitary gland [11] and blood plasma [12]. It has been demonstrated that some of them were able to exhibit opioid activity [13] and to inhibit angiotensin-converting enzyme (ACE) activity [14]. Thus, since a possible role for hemorphins as natural occurring bioactive peptides has been suggested their rapid quantitation could be of great interest. However, their isolation and characterization from hemoglobin hydrolysates is often time-consuming and destructive [15]. This paper firstly describes a rapid, non-destructive and quantitative method to determine any aromatic amino acid in a peptide using RP-HPLC. This method was then applied to the quantitative determination of hemorphins in a total bovine hemoglobin peptic hydrolysate [16].

2. Experimental

2.1. Materials

All common chemicals and solvents were of analytical grade from commercial sources. Tryptophan, tyrosine and phenylalanine were purchased from Sigma. Amino acid standard H (AASH) was obtained from Pierce. The peptide fragments of tuna myoglobin M1: ADF, M2: YTTMGG, and M3: LKCWGPVEA, were kindly provided by a colleague from our laboratory.

2.2. Hemoglobin hydrolysate, LVV-hemorphin-7 and VV-hemorphin-7 preparation

Bovine hemoglobin hydrolysate was obtained at pilot-plant scale by peptic proteolysis in an ultrafiltration reactor as previously described [15]. The active fraction FVII was prepared by size-exclusion HPLC using a TSK G2000 SWG column (600 × 19 mm I.D.) and RP-HPLC using a Delta Pak C_{18} column (300 × 19 mm I.D.) in order to obtain LVV-hemorphin-7 (LVVYPWTQRF) and VV-hemorphin-7 (VVYPWTQRF) [17].

2.3. HPLC system

The liquid chromatographic system consisted of a Waters 600E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 computer. Millennium software was used to plot, acquire and treat the chromatographic data.

2.4. Mobile phase for Delta Pak C_{18} column (300 × 3.9 mm I.D.)

The mobile phase consisted of 10 m*M* ammonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. The flow-rate was 1.5 ml/min. Samples dissolved in buffer A, were filtered through 0.22 μm filters before injection. The gradient applied was 0–40% B in 40 min for free aromatic amino acids and peptide analysis. For bovine hemoglobin peptic hydrolysate the gradient applied was first 0–20% B in 40 min and then 20–40% B in 20 min.

Tryptophan, tyrosine, phenylalanine, AASH, VV-hemorphin-7, LVV-hemorphin-7, peptides M1, M2, M3 and bovine hemoglobin peptic hydrolysate were chromatographed at room temperature on the Delta Pak C_{18} column under the conditions described above. On-line instantaneous UV absorbance spectral scans were per-

formed between 200 nm and 300 nm at a rate of one spectrum/s. The resolution was 1.2 nm. Chromatographic analyses were completed using Millennium software.

2.5. Amino acid analysis

Amino acids were analyzed using a Waters Picotag Work Station. Peptide hydrolysis was achieved with constant-boiling HCl containing 1% phenol, for 24 h at 110°C. Pre-column derivatization of amino acids with phenylisothiocyanate and HPLC separation of the derivatized amino acids on a Waters RP-Picotag column (150×3.9 mm I.D.) were performed according to Bidlingmeyer et al. [18]. The detection wavelength was 254 nm and the flow-rate 1 ml/min.

3. Results and discussion

3.1. Linearity and sensitivity

Phe, Tyr and Trp were separately loaded on a Delta Pak C_{18} column and their absorbance spectra were monitored with the photodiode array detector and processed to yield second-order derivative spectra (Fig. 1). As described previously [9], the secondary derivative spectra of each eluted aromatic amino acid indicated that the specific wavelengths of the most prominent minima for Phe, Tyr and Trp were 258.5 nm, 283.5 nm and 289.5 nm, respectively. The amplitudes at these wavelengths were defined as X (Phe), Y (Tyr) and Z (Trp) [9]. It is well known that derivative spectroscopy offers the advantage of sharper spectral features when compared to conventional absorbance spectroscopy [8]. Thus, a peak shoulder present in a zero-order derivative spectrum can be transformed into a peak minimum in the second-order derivative spectrum. Furthermore, overlapping bands can be transformed into resolved bands. Thus, we may speculate about the proportions of the amounts of Phe, Tyr, Trp and the values of X, Y, and Z. In order to investigate this suggestion, increasing amounts of the three aromatic amino acids were successively injected on the RP-HPLC system

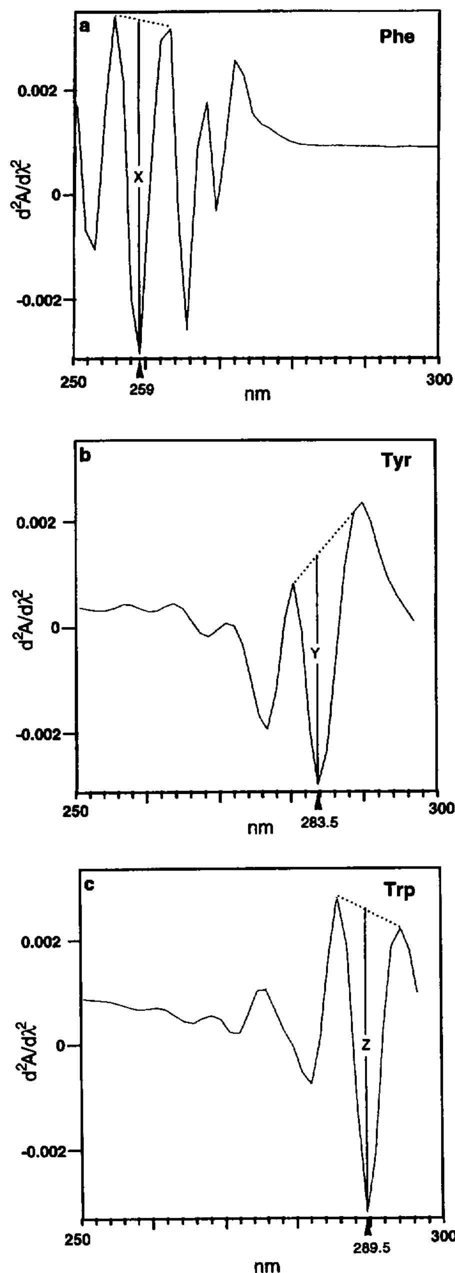

Fig. 1. Secondary derivative spectra of free aromatic amino acids. (a) Phenylalanine, (b) tyrosine, (c) tryptophane. X, Y and Z: amplitudes at the major minima wavelength; Y': amplitude at the second minimum for tryptophan (283.5 nm).

under the conditions described in the Experimental section. Fig. 2 shows the response pattern between the amplitudes for Phe, Tyr and

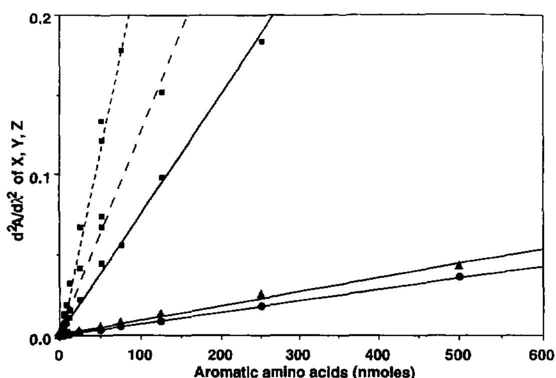

Fig. 2. Linear plots of second-order derivative absorbances obtained from the analysis on a C_{18} Delta Pak column of free aromatic amino acids. (●) Phe at 258.5 nm, (▲) Tyr at 283.5 nm, (■) Trp at 289.5 nm. Acetonitrile gradient: (solid line) 1%/min, (---) 3%/min, (— —) 6%/min.

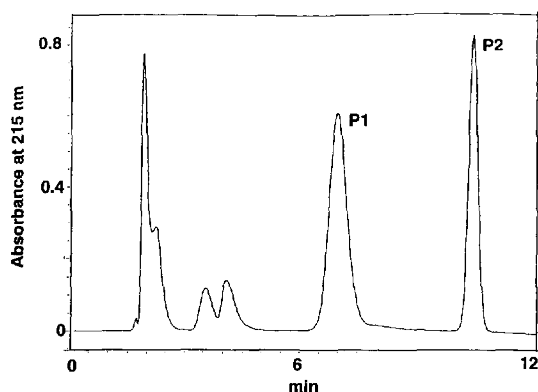

Fig. 3. Chromatographic profile of AASH on a C_{18} Delta Pak column under the conditions described in the Experimental section. Peaks P1 and P2 were identified as Tyr and Phe, respectively.

Trp and their relative quantities. The experimental equations deduced from these curves were the following:

Phe: $y = 7.2 \cdot 10^{-5}x + 4.3 \cdot 10^{-5}$, $r = 1.00$

Tyr: $y = 0.9 \cdot 10^{-4}x + 5.9 \cdot 10^{-4}$, $r = 0.99$

Trp: $y = 7.5 \cdot 10^{-4}x + 1.5 \cdot 10^{-3}$, $r = 0.99$

The good linearities between 2.5 and 500 nmoles for Phe, 0.75 and 250 nmoles for Tyr, and 0.075 and 250 nmoles for Trp were attested by the correlation factor (near 1)This indicated that the minimal quantities that could be determined by this method were as low as 2.5, 0.75 and 0.075 nmoles for Phe, Tyr and Trp, respectively. The relationship between the slopes ($7.2 \cdot 10^{-5}$, $0.9 \cdot 10^{-4}$ and $7.5 \cdot 10^{-4}$ for Phe, Tyr and Trp) appeared to be dependent on the relative values of the molar extinction coefficient ϵ varying in the order: $\epsilon_{Phe} < \epsilon_{Tyr} < \epsilon_{Trp}$. As far as the slope of the acetonitrile gradient is concerned, a marked effect can be noted as illustrated for Trp (Fig. 2). Thus, the gradient composition is an essential parameter, which should be optimized prior to the determination of the aromatic amino acids by this method.

3.2. Experiment with an amino acid mixture

AASH, a mixture of 17 amino acids (without Asn, Gln and Trp), was loaded on the Delta Pak C_{18} column (Fig. 3). Peaks 1 and 2 were identified to contain Tyr (P1) and Phe (P2) by their second-order derivative spectra (not shown). We can not observe 17 peaks since some amino acids have only a weak absorbance at 215 nm. Increasing amounts of AASH and individual aromatic amino acids were chromatographed separately under the same conditions. The responses between the secondary derivative amplitude and the amounts of the aromatic amino acids corresponded to the following equations:

Phe: $y = 7.2 \cdot 10^{-5}x + 2.2 \cdot 10^{-5}$, $r = 1.00$

Tyr: $y = 1.0 \cdot 10^{-4}x + 4.8 \cdot 10^{-5}$, $r = 1.00$

Thus, whether these amino acids were alone or in a mixture (AASH), identical results were obtained. So, using this method, quantitative analysis of aromatic amino acids in a mixture was efficiently achieved.

3.3. Validation using peptides of known composition

Three peptides, M1, M2 and M3, were chromatographed as described above. Each peptide contained only one aromatic amino acid. Analysis of their second-order derivative spectra between 240 nm and 300 nm gave the same spectral characteristics as the free corresponding aromatic amino acids (not shown). Measurement of the

amplitude at their specific wavelength resulted in the following experimental equations:

Phe: $Y = 7.5 \cdot 10^{-5}x + 3.3 \cdot 10^{-5}$, $r = 0.99$

Tyr: $y = 1.0 \cdot 10^{-4}x + 2.1 \cdot 10^{-5}$, $r = 0.99$

Trp: $y = 8.0 \cdot 10^{-4}x + 8.7 \cdot 10^{-4}$, $r = 0.99$

We can observe that the response patterns of these peptides were very similar to that of free aromatic amino acids. So, a calibration curve achieved with free aromatic amino acids allowed the quantitative determination of aromatic amino acids in peptides. Furthermore this method could permit the determination of peptide amount when the amino acid composition is known.

3.4. Validation by the hemorphins

As previously described [9,14,17], the biologically active peptides VV- and LVV-hemorphin-7 contain all three aromatic amino acids. Thus, these peptides present a good model for the investigation of the relation between the aromatic amino acid content and second-order derivative spectroscopy. Different amounts of VV-hemorphin-7 and LVV-hemorphin-7 were analyzed on the RP-HPLC system and the results were compared with the results for the free corresponding aromatic amino acids. Analysis of the second-order derivative spectra for VV-hemorphin-7 gave the following experimental equations:

Phe: $Y = 7.0 \cdot 10^{-5}x + 1.6 \cdot 10^{-5}$, $r = 0.99$

Tyr: $y = 1.0 \cdot 10^{-4}x + 1.3 \cdot 10^{-4}$, $r = 0.99$

Trp: $y = 7.9 \cdot 10^{-4}x + 9.2 \cdot 10^{-4}$, $r = 0.99$

Amplitudes for Phe and Trp were directly determined by simple measurement at 258.5 and 289.5; however, the amplitude for tyrosine must be calculated according to Ref. [14]. It is known that when tyrosine and tryptophan coexist in the same peptide, the primary minimum for tyrosine (at 283.5 nm) and the secondary minimum for tryptophan (at 282 nm) overlap. This overlap could make the identification of tyrosine in the presence of tryptophan difficult [10,12,13]. In a previous study [14] we have shown that the amplitude at 283.5 nm was simply additional and that the presence of tyrosine could be easily proved. In the present work, we show that, whether the aromatic amino acids are free or included in a peptidic chain, the response patterns are identical. Thus, since one mole of VV-hemorphin-7 contains one mole of each aromatic amino acid any of them could be used as a standard in order to quantitate the amount of hemorphin. Similar results were obtained with LVV-hemorphin-7 (not shown). Thus, the method could be extended to the quantitative determination of aromatic amino acids in any peptide.

3.5. Quantitation of hemorphins in a total peptic hydrolysate of bovine hemoglobin

Fig. 4A shows the chromatographic profile of the total bovine hemoglobin hydrolysate. Peaks 1 and 2 were identified as VV-hemorphin-7 and LVV-hemorphin-7 respectively, using the UV spectra comparison method [19]. This result was confirmed by amino acid composition analysis. Figs. 4B and 4C exhibit the second-order derivative spectra which allowed to measure the amounts of Phe, Tyr and Trp (Table 1). The amounts of VV-hemorphin-7 and LVV-hemorphin-7 were found to be 34.6 μg and 7 μg respectively, which represent 3.46% (w/w) and 0.7% (w/w) of the total hydrolysate. Table 2 shows the collected amounts of hemorphins from the total hydrolysate obtained by the usual purification steps (size-exclusion HPLC and RP-HPLC) [16]. From 5 g of total hydrolysate 156 mg (3.12%, w/w) of VV-hemorphin-7 and 37 mg (0.7%, w/w) of LVV-hemorphin-7 were recovered. Thus these results are in very good agreement with the on-line determination of hemorphins described in the present paper.

4. Conclusion

We have introduced a new approach for the quantitative real-time determination of aromatic amino acids in peptides. This determination was demonstrated to be very accurate and non-destructive and could be carried out during the chromatographic separation process. It is con-

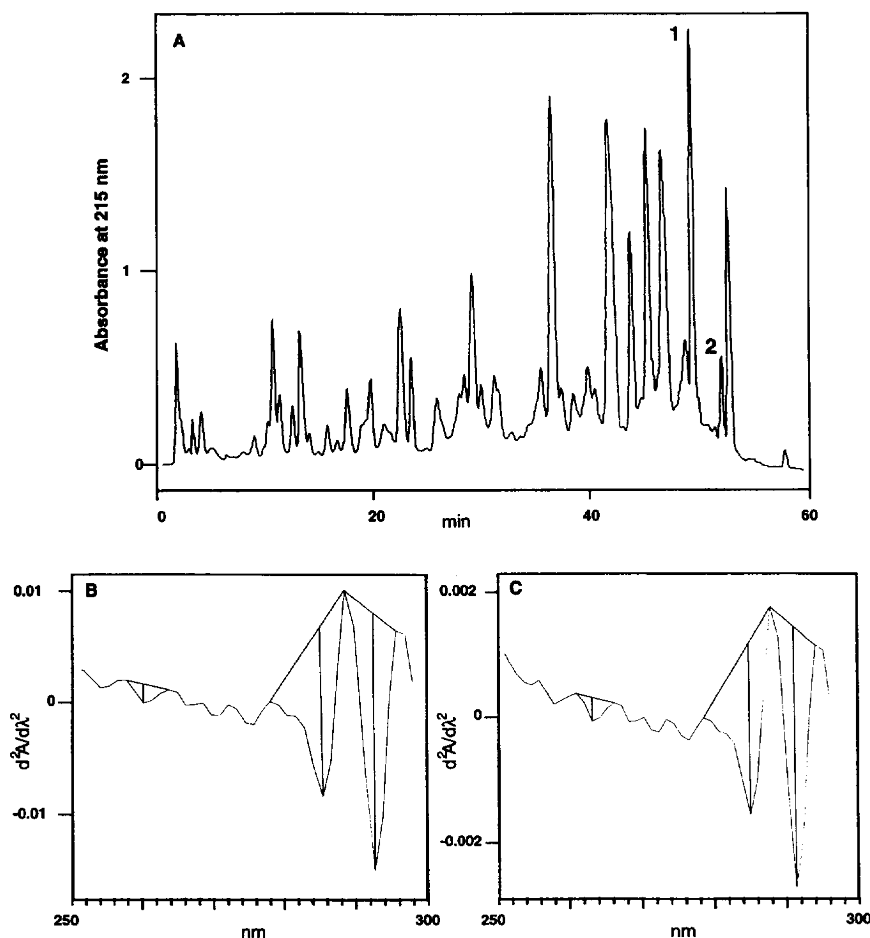

Fig. 4. (A) Chromatographic profile of a total peptic bovine hemoglobin hydrolysate in a RP-HPLC system under the conditions described in the Experimental section. Second derivative spectra at the top of: (B), peak 1 corresponding to VV-hemorphin-7 and (C), peak 2 corresponding to LVV-hemorphin-7 for the determination of the amounts of hemorphins-7 in total hydrolysate.

Table 1
Quantitative determination of hemorphins in a peptic bovine hemoglobin hydrolysate using the UV spectra comparison method; comparison with amino acid composition analysis

	VV-hemorphin	LVV-hemorphin
Phe	26.8 n*M*	5.3 n*M*
Tyr	29.3 n*M*	5.4 n*M*
Trp	29.0 n*M*	5.4 n*M*
Amounts of hemorphins determined by A.A.A	29 n*M* or 34.6 μg	5.4 n*M* or 7.0 μg
Percentage in total hydrolysate	3.5%	0.7%

ceivable that such an approach would facilitate the research and quantitation of biologically active peptides from physiological fluids. Furthermore, this procedure should improve the on-line monitoring of definite peptides released during protein enzymatic hydrolysis.

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Table 2
Quantitative determination of hemorphins from the peptic bovine hemoglobin hydrolysate after classical purification steps

Purification steps	Fraction	Amount (mg)	Percentage (%)
Total hydrolysate		5000	100
TSK G2000 SWG	F VII	294	5.9
Delta Pak C_{18}	VV-hemorphin-7	156	3.1
	LVV-hemorphin-7	37	0.7

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