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Peptide content determination of crude synthetic peptides by reversed-phase liquid chromatography and nitrogen-specific detection with a chemiluminescent nitrogen detector

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Abstract

The need for a rapid and accurate method to assess peptide content is driven by the recent surge in demand for pharmaceutical-grade synthetic peptides manufactured by solid-phase chemistry. Routinely, the purity of synthetic peptides is analyzed by reversed-phase HPLC with UV detection, and peptide content is typically determined from amino acid analysis of synthetic peptides. Recently, we developed a new method to assess the peptide content of synthetic peptides in one simple technique: simultaneous reversed-phase HPLC–chemiluminescent nitrogen detection and UV detection. In this paper we present the simplicity of this method and its universal application to analyzing crude synthetic peptides produced by solid-phase synthesis, an important first step in manufacturing bioactive peptides.

Keywords: Chemiluminescence nitrogen detection; Detection, LC; Peptides

1. Introduction

Reversed-phase HPLC is routinely used with either a C_8 or C_{18} stationary phase for purification and analyses of both natural and synthetic peptides [1–6]. Peptide mapping is typically accomplished by UV detection. However, quantitation of eluted peptides is not possible by UV detection without pure analytical standards because of the differential UV absorption properties between peptides with aromatic functional groups and those containing primarily non-aromatic amino acid residues. Recently, a novel RP-HPLC–UV/nitrogen-specific detection method was reported [7]. HPLC–chemiluminescent nitrogen detection (CLND) demonstrated direct quantitation (without the use of each individual analytical standard for peak identification) of peptides isolated from casein hydrolysate. The dual detection system showed important advantages over stand-alone UV detection.

HPLC–CLND was also used by Bizanek et al. for direct peptide content determination to assess purity of both crude and purified synthetic C-peptides produced by solid-phase peptide synthesis (SPPS) [8].

In this paper, RP-HPLC–CLND was used to rapidly determine the peptide content of 16 crude synthetic peptides produced by SPPS. The UV channel in our dual detection system was used to

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monitor the typical UV-active components in the cleavage mixtures. Peptides were chromatographed using standard RP-HPLC column and solvents.

2. Experimental

2.1. Apparatus

Peptides were synthesized on an Advanced ChemTech Automated Peptide Synthesizer Model 200 (Lowsville, KY, USA) as reported in [8]. Each of the 16 crude peptides (Table 1) were then analyzed by RP-HPLC for peptide content. All HPLC mobile phases were filtered through a Gelman Sciences (Ann Arbor, MI, USA) filter with a pore size of 0.45 μm. RP-HPLC was performed on a LC10 System consisting of two Model LC-10AS pumps, SIL-10A autoinjector, SCL-10A system controller, SPD-10A UV–Vis detector, and a dual channel CR501 Chromatopac integrator purchased from Shimadzu Scientific Instruments (Columbia, MD, USA) and a Model 7000 HPLC–CLND nitrogen-specific detector from Antek Instruments (Houston, TX, USA). Symmetry C_{18} analytical HPLC column from Waters (Milford, MA, USA) was used and the eluate passed through a GC capillary splitter from SGE (Austin, TX, USA) to split the mobile phase flow to the two detectors.

2.2. Reagents and standards

HPLC peptide standard mixture (approx. 0.125 mg Gly–Tyr and approx. 0.5 mg each of Val–Tyr–Val, methionine enkephalin, leucine enkephalin, and angiotensin II) were purchased from Sigma (St. Louis, MO, USA). HPLC-grade methanol and isopropanol reagents were acquired from EM Science (Wakefield, RI, USA). Water for HPLC mobile phase was obtained from a Mega-Pure System MP-12A from Barnstead Thermolyne (Dubuque, IA, USA).

2.3. Standard preparation and analytical method

A reference peptide mixture containing five peptides was dissolved in 2 ml of 0.05% TFA (from Sigma) in water. Sequential partial filled injections (10, 20, 30, and 40 μl of the peptide standard solution) were made into a 50 μl sample injection loop of the HPLC system. Peptides were analyzed by RP-HPLC (150 mm×3.9 mm I.D., 300 Å pore size, 5 μm particle size) Symmetry C_{18} column at 25°C.

Table 1
Peptide content determination of sixteen crude peptide cleavage mixtures by reversed-phase HPLC–chemiluminescent nitrogen detection analyses (cleavages from the solid phase synthesis resins)

Peptide	M_r	Amount injected (μl)	Measured (μg N)	Calculated (μg N)	Peptide content (%)
Xenospin	979	20	2.95	3.71	79.5
Neuromedin N	746	30	2.82	3.94	71.5
Leucopyrokinin	931	25	2.26	4.51	50.1
Delicious Peptide	847	30	2.01	4.95	40.6
Thymopentin	934	20	1.93	3.20	60.3
PTH (1–34)	4118	20	1.82	3.74	48.7
Dermorphin	803	20	2.25	2.79	80.7
C-Peptide	3617	20	0.86	3.79	22.7
Allatostatin I	1336	15	1.49	2.26	65.9
Allatostatin II	1067	20	2.33	4.02	57.3
Allatostatin III	899	20	2.61	3.11	83.9
Allatostatin IV	969	20	1.90	3.48	54.6
Kinetensin	1172	15	1.63	3.04	53.6
Eledoisin	1188	20	2.00	3.06	65.4
Kassinin	1335	20	1.13	3.14	36.0
β-Casomorphin	1124	20	1.71	2.24	79.8

Note: Parathyroid hormone (PTH) (1–34) human, C-peptide=insulin chain C and β-Casomorphin, human.

Mobile phases: 0.05% TFA in water (A) and 80% alcohol mixture (methanol–isopropanol–water, 2:2:1, v/v/v) with 0.04% TFA (B), flow-rate of 500 μl/min, and a linear gradient elution: 5% to 50% B in 30 min with a 5 min hold at 50% B was used. The flow was split after the column using the SGE capillary splitter where 100 μl/min was directed to CLND and 400 μl/min to UV detection. Crude peptides were lyophilized to dryness after solid-phase syntheses, collected and dissolved in mobile phase A at 1 mg/ml concentration. CLND conditions: 1050°C pyrolysis temperature, photomultiplier tube voltage 700, range ×25, and detector output of 1 V; UV condition: 220 nm.

3. Results and discussion

Simultaneous RP-HPLC–CLND and UV detection at 220 nm is presented for the analysis of pure and crude synthetic peptides. CLND provided direct quantitation of peptide content of several crude synthetic peptides obtained from an automated solid-phase peptide synthesis as previously reported in [8]. Some of these peptides have important biological functions like human parathyroid hormone (PTH) (1–34), which is currently under investigation for therapeutic use in the treatment of osteoporosis. Chromatograms of other peptides such as xenospin and dermorphin are also presented to show the usefulness of the dual detection method. The peptide content is very important when measuring its biological activity. Using the nitrogen-specific capability of the CLND, the analyst can readily measure the peptide content of any peptide since the detector is not chromophore dependent but rather nitrogen dependent. Results can be obtained at stages before extensive and time-consuming production of analytical standards of novel peptides. Another advantage for using CLND is that it provides a cost-effective approach for the production of synthetic peptides and other important nitrogen-containing molecules.

The CLND response factors were calculated using the HPLC reference standard (Gly–Tyr, Val–Tyr–Val, methionine enkephalin, angiotensin II, and leucine enkephalin) mixture and the average nitrogen response of the five peptides was used to determine the peptide content of 16 crude synthetic peptides (Table 1). These peptides were the product of the cleavages from the solid-phase synthesis resins. The peptide content as determined by CLND ranged between 83.9% (for allatostatin III) to 22.7% (for C-peptide). The C-peptide is a relatively large peptide (Table 1) and does not contain any aromatic amino acid residues. The results of the dual CLND/UV detection is discussed in detail in [8].

Simultaneous CLND and UV detection (at 220 nm) profiles for the crude xenospin peptide cleavage mixture are shown in Fig. 1. Xenospin (peak 2) contains one aromatic UV chromophore, Trp. Both detectors showed similar profiles however, the CLND tracing showed an additional nitrogen-con-

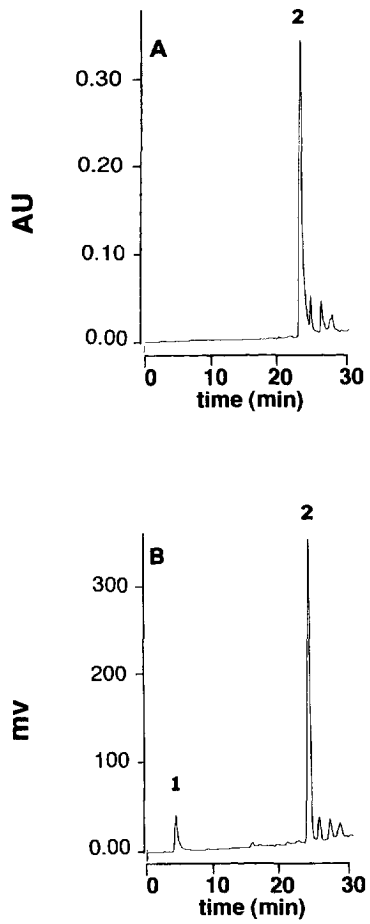

Fig. 1. RP-HPLC chromatograms of crude xenospin peptide cleavage mixture. Peak: 1=nitrogen containing impurity, 2= xenospin. (A) UV detection at 220 nm, (B) HPLC–CLND.

taining component (peak 1) which was not found by UV detection. The peptide content of xenospin was found to be 79.5% by CLND.

Dual detection of the crude PTH (1–34) peptide cleavage mixture is elucidated in Fig. 2A (by UV) and Fig. 2B (by CLND). Note that PTH (1–34) analyzed in this study is a relatively large peptide (M_r=4118) and contains two aromatic UV chromophores, Trp and Phe. Much lower peptide content (48.7%) of the synthesized peptide (peak 2) was determined by the CLND than by UV detection which accounted for 60% the UV chromophores in the crude sample. The CLND also clearly shows a large early-eluting nitrogenous component (peak 1) which was not observed by the UV detector. Fig. 2C is the CLND profile of the purified peptide product.

Dermorphin has three aromatic UV chromophores, 2 Tyr and Phe. It is a peptide approximately 1/5 the M_r of PTH (1–34). The UV (Fig. 3A, chromophore dependent) response for dermorphin was 90.4% based on peak area counts and was much larger relative to the CLND response of Fig. 3B The peptide content of dermorphin by CLND was in fact 80.7% in the crude peptide cleavage mixture.

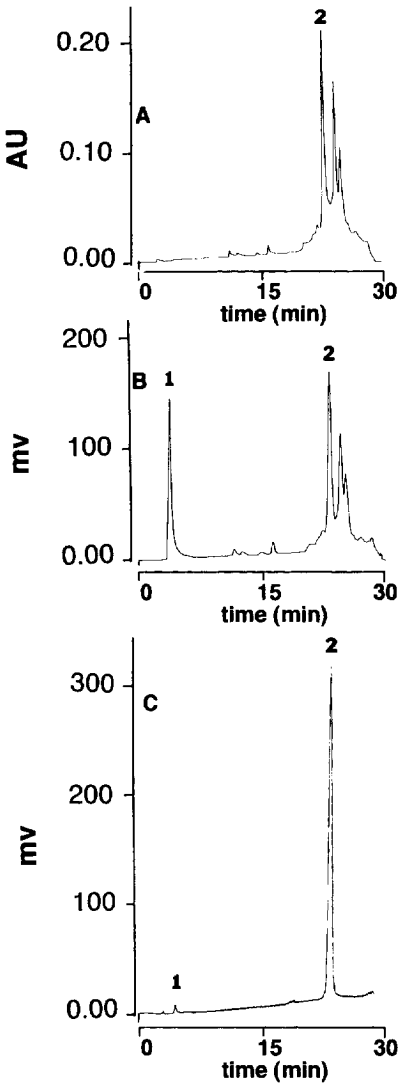

Fig. 2. RP-HPLC chromatograms of crude and purified PTH (1–34) peptide cleavage mixture. Peak: 1=nitrogen-containing impurity, 2=PTH (1–34) human. (A) UV detection at 220 nm of crude peptide, (B) HPLC–CLND of crude peptide, (C) HPLC–CLND of purified PTH (1–34) peptide.

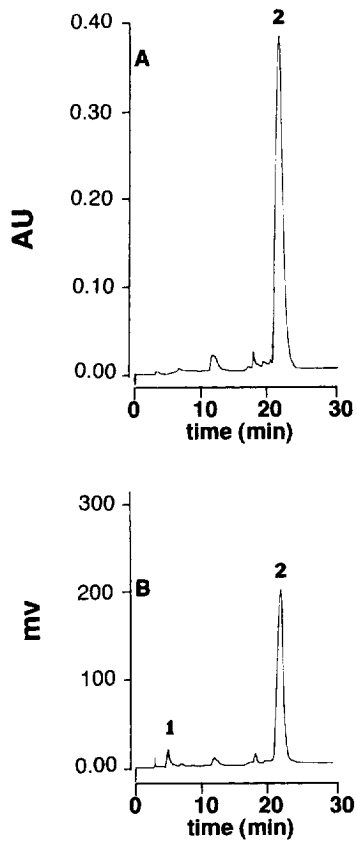

Fig. 3. RP-HPLC chromatograms of crude dermorphin peptide cleavage mixture. Peak: 1=nitrogen-containing impurity, 2= dermorphin. (A) UV detection at 220 nm, (B) HPLC–CLND.

4. Conclusion

A new method for determining the peptide content of crude peptides in cleavage mixtures after solid-phase peptide syntheses using HPLC–CLND is demonstrated. This was first demonstrated using a crude and purified C-peptides [8]. Prior to that report, there was no means to accurately determine the peptide content in any crude synthetic peptide mixture. Traditionally, peptides were purified first, then peptide content was usually determined by amino acid analysis or by micro-Kjeldahl method. The peptide content determination for process control at the crude mixture stage by amino acid analysis only provided the total peptide content of the mixture. The significance of utilizing HPLC–CLND is that peptide content can now be determined chromatographically in crude peptide mixtures. Furthermore, the on-line nitrogen content determination of other nitrogen containing compounds of natural or synthetic origin in crude mixtures can also be accomplished using this technique.

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