ELSEVIER

Journal of Chromatography B, 754 (2001) 461–466

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of growth hormone-releasing hexapeptide by reversed-phase high-performance liquid chromatography with electrochemical detection

Sung Jin Choi[a], Hee-Yong Lee[a], Sang Beam Kim[a], Jeong-Han Kim[b], Seung Seok Lee[c], Sun Dong Yoo[c], Kang Choon Lee[c], Hye Suk Lee[a,*]

[a]*Bioanalysis Laboratory, College of Pharmacy, Wonkwang University, Iksan 570-549, South Korea*
[b]*School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, South Korea*
[c]*College of Pharmacy, SungKyunKwan University, Suwon 440-746, South Korea*

Received 17 August 2000; received in revised form 27 November 2000; accepted 4 January 2001

Abstract

A novel HPLC method with electrochemical detection is described for the determination of a growth-hormone-releasing hexapeptide (GHRP-6). HPLC conditions, such as the column, mobile phase, and oxidation potential, were optimized for sensitivity and selectivity of analysis. GHRP-6 was separated on a reversed-phase CN column with 37% acetonitrile in 100 m*M* phosphate buffer (pH 7.0) as the mobile phase. The optimum electrochemical oxidation signal was obtained at 0.85 V vs. Ag/AgCl in a glassy carbon working electrode due to two electroactive tryptophans and a histidine residue. Solid-phase extraction using octadecyl cartridges was optimized for sample cleanup of GHRP-6 from serum samples and the method was successfully applied over the concentration range of 5 to 100 ng/ml of analyte.

Keywords: Growth-hormone-releasing peptide

1. Introduction

GHRP-6 (His–D-Trp–Ala–Trp–D-Phe–Lys-NH_2) is a synthetic growth-hormone-releasing hexapeptide that works specifically in animals and humans after intravenous, subcutaneous, intranasal and even oral administration. This peptide has the characteristics of being small, stable, soluble and of low toxicity, as well as being quick and cheap to synthesize. As a result, it became a gold standard that all non-classic growth-hormone secretagogues can be compared with (reviewed in Refs. [1–3]).

For the determination of GHRP-6 in biological fluids and preparations, a few studies have been reported that used reversed-phase HPLC methods with UV [4–6], mass spectrometry [6,7], flow radiochemical detection [7] or fluorescence detection [8]. A HPLC method with fluorescence detection following pre-column derivatization with fluorescamine [8] was used for the determination of plasma GHRP-6 determination and to characterize the pharmacokinetics of GHRP-6 in rats and dogs [7], but it

*Correspondence author. Tel.: +82-63-850-6817; fax: +82-63-850-7309.
E-mail address: hslee@wonkwang.ac.kr (H. Suk Lee).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00030-5

suffered from a lack of routine applicability, due to the high cost of reagents and equipment, i.e., the need for an autosampler with sophisticated pre-column derivatization capabilities and a gradient pump. However, the measurement of pharmacological activity, i.e., growth-hormone levels using a radioimmunoassay, has generally been used in the pharmacokinetic disposition study of GHRP-6 [9–11].

A highly sensitive, reproducible and selective analytical method is therefore needed for the determination of GHRP-6 in biological samples. Electrochemical detection has been demonstrated to be a reliable, sensitive and specific method for the determination of salmon calcitonin [12], microcystins [13] and neuropeptides [14–18] containing the electroactive amino acids, tyrosine, tryptophan or arginine. This paper describes a novel HPLC method with electrochemical detection for the analysis of tryptophan-containing GHRP-6. The effects of various stationary phases and mobile-phase components on the separation and detection of GHRP-6 were examined to maximize the electrochemical detection of GHRP-6. The applicability of this method to the determination of GHRP-6 from serum samples was limited without sample pretreatment and, therefore, solid-phase extraction using the universal octadecyl cartridge was described as the sample cleanup procedure, so that reasonable recovery of GHRP-6 could be obtained and impurities in the serum samples could be removed.

2. Experimental

2.1. Materials

GHRP-6 and GHRP-4 (internal standard) were obtained from SmithKline Beecham (King of Prussia, PA, USA) and Sigma (St. Louis, MO, USA), respectively. Stock solutions of GHRP-6 and GHRP-4 were prepared in methanol (1 mg/ml) and stored at −20°C. HPLC-grade methanol and acetonitrile were obtained from Burdick and Jackson (Muskegon, MI, USA). HPLC-grade trifluoroacetic acid (TFA) and other analytical-grade reagents were from Sigma. Various bonded-phase cartridges (500 mg, 3 ml) were obtained from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Chromatographic system

The chromatographic system consisted of a Nanospace SI-2 system (Shiseido, Tokyo, Japan), an amperometric detector with a glassy carbon working electrode, a degasser and an autosampler. Data acquisition and processing were accomplished by DS Chrom-2 (Seoul, Korea).

2.3. Evaluation of chromatographic parameters

A variety of columns were evaluated for the optimization of analyte separation. They included an Alltima C_{18} (250×4.6 mm I.D., 5 μm), an Alltima CN (250×4.6 mm I.D., 5 μm), a Luna C_{18} (250×4.6 mm I.D., 5 μm), an Ultracarb ODS (250×4.6 mm I.D., 5 μm), a μBondapak CN (150×3.9 mm I.D., 10 μm) and a Capcellpak phenyl (250×4.6 mm I.D., 5 μm) column. All columns, with the exception of the μBondapak CN column, were of the end-capped type. The mobile phase was 37% acetonitrile in 100 m*M* potassium phosphate buffer (pH 7.0), which was eluted at a flow-rate of 1.0 ml/min.

The retention times for unretained peaks were determined in each column by injecting 2 μl of 0.1 *M* $NaNO_3$ dissolved in the mobile phase. Asymmetry was assessed at 5% of peak height.

2.4. Hydrodynamic voltammetry

GHRP-6 standard (1 μg/ml mobile phase) was analyzed in duplicate and the oxidation current for GHRP-6 was recorded stepwise at each applied potential from 0.95 to 0.50 V. The plot of peak area vs. oxidation potential for GHRP-6 was determined.

2.5. Sample cleanup with solid-phase extraction

To determine the ideal adsorbent and eluent for the pretreatment of GHRP-6 from serum samples, the extraction recovery was evaluated using commercial solid-phase extraction cartridges, such as octadecyl, octyl, cyano, amino and phenyl bonded-phase. Each cartridge was washed with methanol and equilibrated with 10 m*M* potassium phosphate buffer

(pH 7.0) before sample loading. A 2-ml volume of GHRP-6 in phosphate buffer (pH 7.0; 1 μg/ml) was passed through each of the cartridges and the filtrate was collected. GHRP-6 was eluted with methanol or 0.5% TFA in methanol (2 ml). Both filtrate and eluate were analyzed by HPLC.

2.6. Analysis of GHRP-6 from serum samples

ODS cartridges were washed with methanol and water, followed by equilibration with 10 m*M* potassium phosphate buffer (pH 7.0). Serum samples (1 ml) that were fortified to concentrations of 5, 20, 50 and 100 ng/ml were passed through an ODS cartridge, washed with 2 ml of 10 m*M* potassium phosphate buffer (pH 7.0) and 2 ml of methanol, to remove serum impurities. GHRP-6 was eluted with 2 ml of 0.5% TFA in methanol and the eluate was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 50 μl of internal standard solution (GHRP-4 in mobile phase, 0.25 μg/ml) and GHRP-6 was separated on an Alltima CN column that was protected by an Alltima CN guard column (7.5×4.6 mm I.D., 5 μm). The flow rate of the mobile phase (37% acetonitrile in 100 m*M* potassium phosphate buffer, pH 7.0) was 0.8 ml/min. The injection volume was 20 μl and the column temperature was maintained at 25°C. The glassy carbon working electrode was operated at 0.85 V vs. a Ag/AgCl reference electrode.

3. Results and discussion

3.1. Chromatography

The efficiencies of the various bonded stationary phases, including octadecyl, phenyl and cyano phases, in the separation of GHRP-6 were evaluated. As shown in Table 1, the polarity of the columns correlated with the retention strengths of the columns for GHRP-6. Polar cyano columns had higher capacity factors and better peak-shapes than octadecyl and phenyl columns, which suggests that there is interaction between the two polar amino-acid residues (histidine and lysine) of GHRP-6 and the polar cyano group. Alltima CN had a higher retention strength and a lower asymmetry factor than

Table 1
Efficiency of bonded-phase columns in the separation of GHRP-6

Column	Capacity factor	Asymmetry factor
Alltima C_{18}	0.57	2.64
Luna C_{18}	0.47	2.49
Ultracarb ODS	0.59	2.51
Capcellpak phenyl	0.79	1.38
μBondapak CN	1.08	1.50
Alltima CN	2.58	1.13

Mobile phase: acetonitrile–100 m*M* potassium phosphate buffer, pH 7.0 (37:63, v/v). Flow-rate: 1 ml/min. Detection: electrochemical oxidation at 0.85 V.

μBondapak CN due to its higher carbon load and end-capping, and, therefore, Alltima CN was chosen as the stationary phase of choice for the determination of GHRP-6.

As the optimum mobile phase for the separation and the ideal mobile phase for detection may be different, it may be necessary to compromise in order to obtain an appropriate level of sensitivity and selectivity. The retention times and peak areas (oxidation signal) of GHRP-6 were found to increase when the pH of the mobile-phase buffer was increased from pH 5 to pH 9 (Fig. 1). According to previous reports for the basic peptide, salmon calcitonin [12,19], this phenomenon might result from some ionized silanol groups of the stationary phase interacting with basic amino acids (histidine and

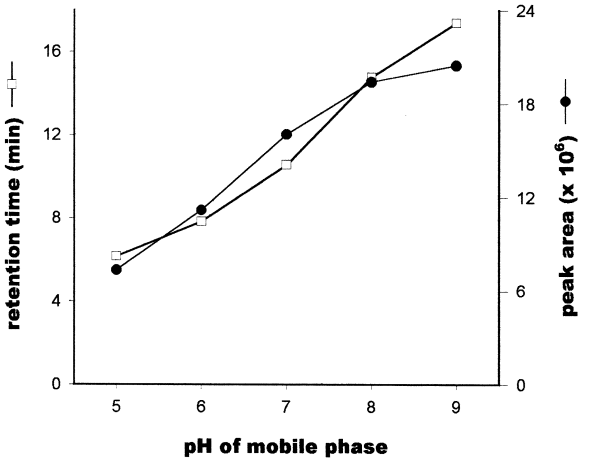

Fig. 1. Effect of pH on the retention time and peak area of GHRP-6. Column, Alltima CN (250×4.6 mm I.D., 5 μm); mobile phase, 37% acetonitrile in 100 m*M* potassium phosphate buffer; flow-rate, 0.8 ml/min; electrochemical detection (0.85 V); sample volume, 20 μl.

lysine). Maximum sensitivity for GHRP-6 was obtained at pH values above 8.0, however, the pH of the mobile phase was adjusted to pH 7.0, because the silica-based Alltima CN column should not be used at pH values above 7.0. Buffer-type and concentration have been shown to be important factors in electrochemical detection as well as in the separation of peptides [12–18]. A phosphate-based mobile phase was chosen because it resulted in better peak-shape and a lower detection limit for GHRP-6 than acetate-based mobile phases. When the concentration of phosphate buffer in the mobile phase was increased from 20 to 200 m*M*, the retention time of GHRP-6 decreased, but the peak area of GHRP-6 reached a maximum at a concentration of 50 m*M* (Fig. 2). A 100-m*M* phosphate buffer was chosen for GHRP-6 to have the optimum retention and sensitivity. Increasing the percentage of acetonitrile in the mobile phase, from 30 to 45%, resulted in a decrease in the retention time of GHRP-6, from 17.7 to 4.8 min.

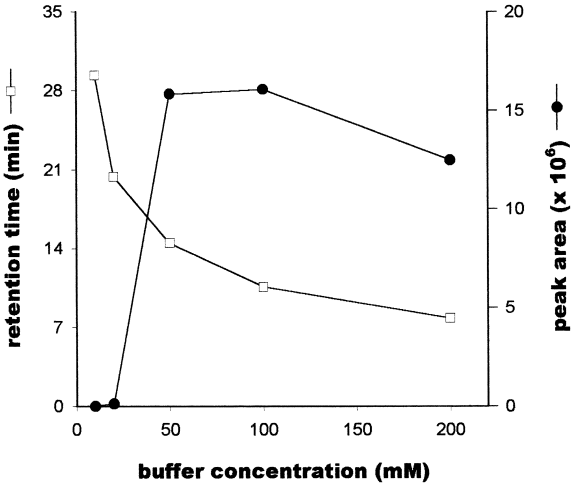

Fig. 2. Effect of the concentration of the mobile-phase buffer on the retention time and peak area of GHRP-6. Column, Alltima CN (250×4.6 mm I.D., 5 μm); mobile phase, 37% acetonitrile in potassium phosphate buffer, pH 7.0; flow-rate, 0.8 ml/min; electrochemical detection (0.85 V); sample volume, 20 μl.

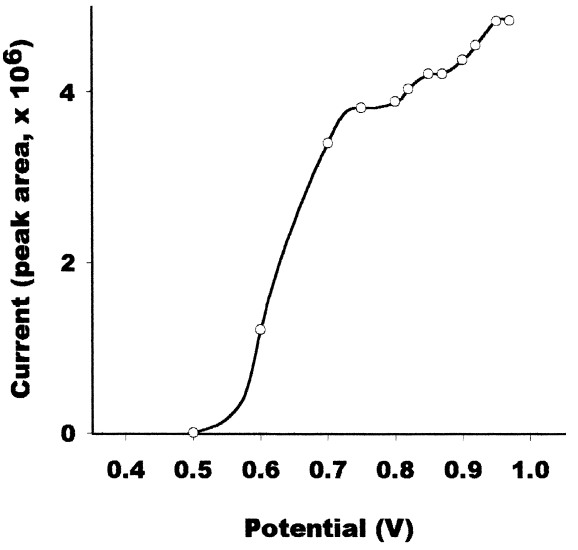

Fig. 3. Hydrodynamic voltammogram of GHRP-6. Chromatographic conditions, other than oxidation potential, were the same as in Fig. 2.

3.2. Electrochemical oxidation of GHRP-6

A hydrodynamic voltammogram was generated to determine the optimum oxidation potential of GHRP-6. As shown in Fig. 3, the oxidation signal of GHRP-6 increased as the applied potential was increased from 0.5 to 0.95 V. However, 0.85 V was chosen as the working potential because the stabilization time, baseline noise and electrode passivation over time increased at higher potentials (≥0.9 V). Internal standard was necessarily used in order to remove the system error caused by electrode passivation. The electrode began to be slowly passivated after running overnight under these conditions and had to be polished every day when the external standard method was used. The molar electrochemical oxidation response of GHRP-6 was about 1.3 times (peak-area-based calculation) stronger than that of the internal standard GHRP-4 (Trp–Ala–Trp–Phe-NH_2). This result suggested the participation of other amino-acid residues, as well as tryptophan, in the electrochemical oxidation of GHRP-6. The participation of histidine in the electrochemical reaction of GHRP-6 was proved by determining the electrochemical oxidation signals of amino-acid residues, i.e., alanine, histidine, phenylalanine and lysine. These results lead to the conclusion that oxidative amperometric detection is a sensitive and reliable method for the determination of GHRP-6 containing the two electroactive residues, tryptophan and histidine. A typical chromatogram of GHRP-6 and GHRP-4 in a standard solution is shown in Fig. 4a,

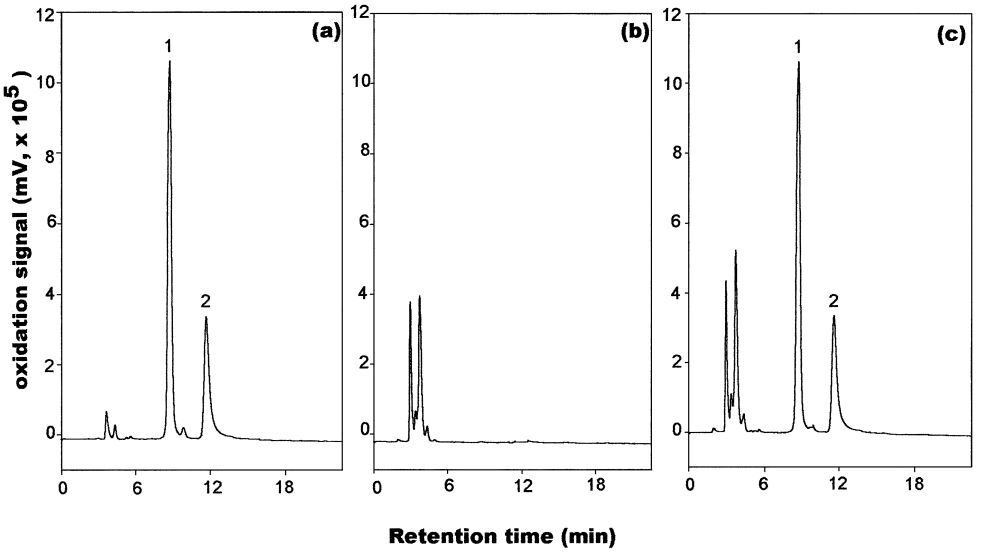

Fig. 4. Chromatograms of GHRP-6 with electrochemical detection. (a) GHRP-6 standard (1 μg/ml), (b) blank human serum, and (c) serum sample containing GHRP-6 (50 ng/ml). Peaks: 1=GHRP-6; 2=GHRP-4.

with the retention times of GHRP-6 and GHRP-4 at 9.1 and 11.9 min, respectively.

3.3. Solid-phase extraction

Sample cleanup steps are extremely important in the HPLC assay with electrochemical detection. Solid-phase extraction using a weak cation-exchange cartridge was previously reported for the extraction of GHRP-6 from plasma [8], but there were no systematic studies on the optimization of solid-phase extraction for GHRP-6. The efficiency of widely used bonded-phase adsorbents and eluents on GHRP-6 recovery was evaluated (Table 2). Octadecyl, octyl, amino, cyano and phenyl cartridges showed good adsorptivity for GHRP-6, while desorption of GHRP-6 was maximum from octadecyl cartridges. Polar bonded-phases, such as cyano- and amino-, showed stronger retention of GHRP-6 due to polar lysine and histidine residues, in accordance with the result obtained using the corresponding analytical column. A solution containing 0.5% TFA in methanol (2 ml) was sufficient for the quantitative elution of GHRP-6 from octadecyl adsorbents (Table 2).

Table 2
Adsorptivity and desorptivity of adsorbents (500 mg) for GHRP-6

Adsorbents	Percent recovery of GHRP-6		
	Filtrate	Methanol eluate	0.5% TFA in methanol eluate
Octadecyl	N.D.	N.D.	85.7
Octyl	N.D.	0.8	55.7
Cyano	N.D.	11.3	30.1
Amino	N.D.	24.7	15.3
Phenyl	N.D.	0.8	65.7

N.D.=not detected.

Fig. 4b–c shows typical chromatograms obtained from blank human serum and from serum containing GHRP-6 (50 ng/ml). The electrochemical determination of GHRP-6 was not affected by other electrochemically active interferences.

3.4. Method validation

The absolute mean recovery of GHRP-6 from serum samples was quantitative (86.6±2.2%) over the concentration range 5–100 ng/ml. The calibration curve of peak-area ratios against the concentrations of GHRP-6 in serum was linear over the range of 2 to 100 ng/ml, with a weighted correlation coefficient of 0.999 (±0.001). The limit of detection (LOD), defined as the concentration of GHRP-6 resulting in a signal-to-noise ratio of three, was 2 ng/ml, using 1 ml of serum, which is comparable to the value obtained by fluorescence detection (4.4 ng/ml using 0.5 ml of plasma) after fluorescamine

Table 3
Reproducibility of GHRP-6 in human serum samples ($n=6$)

Concentration added (ng/ml)	Concentration found±S.D. (ng/ml) (C.V., %)	
	Intra-day	Inter-day
5	5.1±0.3 (5.0)	5.2±0.4 (5.4)
20	19.8±1.1 (3.8)	20.2±1.2 (4.5)
50	52.4±2.0 (3.8)	50.1±2.5 (3.3)
100	100.6±3.8 (2.8)	101.2±4.9 (3.4)

derivatization [8]. Therefore, the present method has the advantage that derivatization is not necessary, which saves analytical time and cost, in addition to having a good LOD.

The intra- and inter-day precision and accuracy of the assay are shown in Table 3. The concentrations of GHRP-6 were found to deviate within a narrow range, from −1.0 to 4.8%, of the theoretical concentrations in the spiked serum samples. The relative standard deviation was less than 5.4%, which suggests that the assay is both precise and accurate.

In conclusion, this paper describes for the first time the isocratic reversed-phase HPLC analysis with amperometric detection of GHRP-6. It was also proved that solid-phase extraction using an octadecyl cartridge is an effective way of cleaning up GHRP-6 from serum samples. This method is likely to be applicable to the determination of other GHRP homologues containing oxidizable residues, like tryptophan.

Acknowledgements

This study was supported by the Korean Science Engineering Foundation Grant KOSEF 97-04-03-12-01-3.

References

[1] V. Locatelli, A. Torselio, Pharmacol. Res. 36 (1997) 415.
[2] F.F. Casanueva, C. Dieguez, Trends Endocrinol. Metab. 10 (1999) 30.
[3] F. Carmanni, E. Ghigo, E. Arvat, Front. Neuroendocrinol. 19 (1998) 47.
[4] P.M. Reardon, C.H. Gochoco, K.L. Audus, G. Wilson, P.L. Smith, Pharm. Res. 10 (1993) 553.
[5] I.S. Ha, B.H. Woo, J.T. Lee, T.S. Kang, K.K. Tak, C.K. Oh, K.C. Lee, Int. J. Pharm. 144 (1996) 91.
[6] H.Y. Cheng, L.L. Davis, M.J. Huddieston, S.A. Carr, Anal. Chem. 67 (1995) 4053.
[7] C.B. Davis, C.S. Crysler, V.K. Boppana, K.L. Fong, G.L. Joseph, J.J. Urbanski, R.A. Macia, G.R. Rhodes, Drug Metab. Dispos. 22 (1994) 90.
[8] V.K. Boppana, C. Miller-Stein, J.F. Politowski, G.R. Rhodes, J. Chromatogr. 548 (1991) 319.
[9] C.Y. Bowers, D.K. Alster, J.M. Frentz, J. Clin. Endocrinol. Metab. 74 (1992) 292.
[10] J.K. Vora, G.A. Christensen, M.J. Reginato, R.A. Macia, C.H. Gochoco, C.K. Oh, J. Control. Release 24 (1993) 193.
[11] A. Giustina, A.R. Bussi, R. Deghenghi, B. Imbimbo, M. licini, C. Poiesi, W.B. Wehrenberg, J. Endocrinol. 146 (1995) 227.
[12] H.S. Lee, S.J. Choi, H.M. Lee, C.K. Jeong, S.B. Kim, J.T. Lee, S.D. Yoo, P.P. DeLuca, K.C. Lee, Chromatographia 50 (1999) 701.
[13] J. Meriluto, B. Kincaid, M.R. Smyth, M. Wasberg, J. Chromatogr. A 810 (1998) 226.
[14] G.W. Benett, M.P. Brazell, C.A. Marsden, Life Sci. 29 (1981) 1001.
[15] L.H. Fleming, N.C. Reynolds, J. Liq. Chromatogr. 7 (1984) 793.
[16] R. Dawson Jr., J.P. Steves, J.F. Lorden, S. Oparil, Peptides 6 (1985) 1173.
[17] Y. Sagara, Y. Okatani, S. Yamanaka, T. Kiriyama, J. Chromatogr. 431 (1988) 170.
[18] W. Ding, H. Veening, R.M. Van Effen, J. Chromatogr. 526 (1990) 355.
[19] I.H. Lee, S. Pollack, S.H. Hsu, J.R. Miksic, J. Chromatogr. Sci. 29 (1991) 136.