Original Article

การตรวจสอบความถูกต้องของวิธีวิเคราะห์ Recombinant Human Growth Hormone โดยวิธี HPLC ซึ่งใช้เครื่องตรวจวัด UV และ Fluorescence

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บทคัดย่อ

บทนำ: Recombinant human growth hormone เป็นผลิตภัณฑ์ที่ได้จากเทคโนโลยีชีวภาพ ซึ่งอาจมีความแปรปรวนของขนาดโมเลกุลโปรตีน ความบริสุทธิ์และปริมาณในแต่ละรุ่นการผลิต การควบคุมคุณภาพจึงมีความสำคัญต่อการพิสูจน์เอกลักษณ์ การประเมินประสิทธิภาพ ความปลอดภัยและมาตรฐานของผลิตภัณฑ์ วิธีวิเคราะห์หาปริมาณตามตำรายา คือ HPLC-UV แบบ single point method ซึ่งต้องใช้ปริมาณสารในการวิเคราะห์สูง การวิจัยนี้จึงมีวัตถุประสงค์เพื่อพัฒนาวิธีวิเคราะห์ Growth hormone ที่มีความไว มีประสิทธิภาพและประหยัดค่าใช้จ่าย **วิธีดำเนินการวิจัย:** ทำการวิเคราะห์สารโดยหลักการ HPLC-size exclusion chromatography (SEC) โดยใช้คอลัมน์ TSKgel G2000SW$_{XL}$ และมีเฟสเคลื่อนที่เป็น 0.063 M phosphate buffer solution : 2-propanol (97:3) อัตราการไหลเท่ากับ 0.6 mL/min ใช้เครื่องตรวจวัดเป็น UV ที่ความยาวคลื่น 214 nm และ fluorescence ที่ความยาวคลื่นกระตุ้น 275 nm และความยาวคลื่นคายแสง 337 nm **ผลการวิจัย:** วิธี HPLC-UV Retention time ของ Growth hormone เท่ากับ 14.51 นาที ปริมาณต่ำสุดของการตรวจวิเคราะห์เชิงปริมาณเท่ากับ 10 µg/ml สัมประสิทธิ์สหสัมพันธ์ของสมการเส้นตรงเท่ากับ 0.9973 วิธี HPLC-fluorescence มีค่า Retention time เท่ากับ 14.77 นาที ปริมาณต่ำสุดของการตรวจวิเคราะห์เชิงปริมาณเท่ากับ 1.5 µg/ml สัมประสิทธิ์สหสัมพันธ์ของสมการเส้นตรงเท่ากับ 0.9998 ทั้งสองวิธีมีความถูกต้องอยู่ในช่วง 98.10-102.49% และมีความแม่นยำภายในวันและระหว่างวันไม่เกิน 2.55% **สรุปผลการวิจัย:** วิธีวิเคราะห์ที่พัฒนาขึ้นมีความเป็นเส้นตรง ถูกต้อง แม่นยำ และมีความไว วิธีวิเคราะห์ด้วย HPLC-fluorescence เพิ่มความไวในการวิเคราะห์ซึ่งใช้ความเข้มข้นของ Growth hormone ต่ำลง

คำสำคัญ: โซมาโทรปิน, , โครมาโทกราฟีแบบแยกตามขนาดโมเลกุล, ความจำเพาะ, ความไว, ความคงสภาพ
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Method Validation of Recombinant Human Growth Hormone by HPLC with UV and Fluorescence Detectors

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Abstract

Introduction: Recombinant human growth hormone is a biotechnological product that there may be variations in the protein size, purity, or yield of an individual batch. Quality control of the products is important for identification, efficacy, safety, and standardization. The pharmacopeial assay of growth hormone was completed by high-performance liquid chromatography with a UV detector (HPLC-UV) using the single point method. Since this method requires a high amount of substance for analysis, the purpose of our study is to develop a sensitive, efficient, and low cost assay for determining growth hormone. **Methods:** The analytical principle was based on HPLC-size exclusion chromatography (SEC) by using a TSKgel G2000SW$_{XL}$ column and 0.063 M phosphate buffer solution: 2-propanol (97:3) as the mobile phase. The flow rate in the experiments was 0.6 mL/min , while UV detection at 214 nm and fluorescence detection with the excitation at 275 nm and the emission at 337 nm were used. **Results:** For HPLC-UV, the retention time of growth hormone was 14.51 minutes and the limit of quantitation (LOQ) was found to be 10 µg/ml. The correlation coefficient of the calibration curve was 0.9973. For HPLC-fluorescence, the retention time of growth hormone was achieved at 14.77 min and the LOQ was 1.5 µg/ml. The calibration curve was linear with a correlation coefficient of 0.9998. For accuracy, the recovery of both methods was found to be within 98.10-102.49%. While the intra-day and inter-day precisions were measured not more than 2.55%. **Conclusions:** The developed methods were linear, accurate, precise, and sensitive. The HPLC-fluorescence improves sensitivity requiring a lower concentration of growth hormone.

Keywords: Somatropin, , Size-exclusion chromatography, Specificity, Sensitivity, Stability
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Introduction

Recombinant human growth hormone (somatropin or somatotropin) is a biotechnological protein used in the treatment of growth failure, somatropin deficiency syndrome, and Turner's syndrome. (Arato and Yamaguchi, 2011) The pharmacological function of growth hormone is responsible for cell proliferation and growth promotion (Lacy et al., 2010-2011; Levarski et al., 2014). Human growth hormone is composed of 4 helices containing 191 amino acids and 2 disulfide bonds. Growth hormone is an unstable mononer whose degradation pathways occur through oxidation, deamidation, and aggregation (Cholewinski et al, 1996; Mulinacci et al., 2013). The oxidation occurs at the position of methionine 14 and 125. The deamidation occurs at the position of asparagine 149 and 152 but asparagine 149 is more approachable because the neighbor of this amino acid shows less steric hindrance. The aggregation of the growth hormone occurs via covalent bonding, electrostatic interactions, and hydrophobic interactions. Heating, freezing, and shaking can accelerate its aggregation process (Cholewinski et al., 1996). The aggregated forms include dimers and the higher molecular weight. The dimeric form is less bioactive than the monomeric form (Charrier and Martal; 1988). Dimers should not interfere the quantification of the growth hormone; therefore, an analytical method to distinguish the monomers and dimers of human growth hormone is crucial to investigate the quality during manufacture, distribution, and storage.

Various methods to detect and quantify growth hormone have been reported such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Cholewinski et al., 1996), reverse-phase high performance liquid chromatography (RP-HPLC) (Varcheh et al., 2004; Yilmaz et al., 2012A), size-exclusion chromatography (SEC) (Mulinacci et al., 2013), spectrofluorometry (Yilmaz et al., 2012A; Yilmaz et al., 2012B), mass spectrophotometry, and capillary electrophoresis (CE) (Catai et al., 2007; Haselberg et al., 2011). SDS-PAGE can separate proteins based on different sizes but in the presence of sodium dodecyl sulphate, the proteins are in the monomeric form. Gradient RP-HPLC can separate the deamidated and the oxidized forms from growth hormone (Varcheh et al., 2004). CE can separate mono- and dideamidated forms of growth hormone (Cholewinski et al., 1996). Spectrofluorometry can sensitively analyze growth hormone since growth hormone contains fluorescent amino acids such as tryptophan, phenylalanine, and tyrosine. However, these methods are unable to distinguish growth hormone in the monomeric and dimeric forms. Size-exclusion chromatography is an advantageous separation technique based on the different sizes of molecules because it can separate the active form as the monomers and the less bioactive form as the dimers of the growth hormone (Cholewinski et al., 1996; Girard and Mousseau, 1999). The pharmacopeial methods for the assay of human growth hormone in the United State Pharmacopeia 36 and British Pharmacopoeia 2013 are performed by a single point method based on HPLC-SEC with UV detection (BP Commission, 2013; USP Convention, 2013). However, these

methods require highly concentrated sample. The combination of SEC and fluorescence detector could offer a specific and sensitive method in determining growth hormone (Hong et al., 2012). The objective of this study was to develop a sensitive analytical method to improve the sensitivity by using the UV detector and the fluorescence detector with lower concentrations of growth hormone. The methods were then validated to demonstrate the linearity, accuracy, precision, sensitivity, and specificity. The stability of the growth hormone solutions at room temperature and in the ice bath was studied by HPLC-UV to determine the suitable storage condition and time for the prepared solutions during the analysis.

Methods and materials

Chemicals

Recombinant human growth hormone with lot number 201303109 (Genheal®, China) was purchased from Zuellig Pharma Ltd., Thailand. Anhydrous disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate dihydrate (Univar®, Australia), sodium azide (Sigma-Aldrich®, Germany) were reagent grade. 2-Propanol (J.T. Baker®, USA) was HPLC grade. Ultrapure water was obtained freshly from the water purifier (Maxima®, England) used for all solutions in the experiments.

Diluent, mobile phase, and storage solution

The diluent for growth hormone was 0.025 M phosphate buffer pH 7.0 prepared by dissolving 0.3276 g of sodium dihydrogen orthophosphate dihydrate and 0.4117 g of anhydrous disodium hydrogen orthophosphate in 200 mL of ultrapure water and adjusting the pH to 7.0 by adding phosphoric acid and/or sodium hydroxide solution.

The mobile phase was composed of 0.063 M phosphate buffer solution: 2-propanol (97:3). A 0.063 M phosphate buffer was prepared by dissolving 4.9611 g of sodium dihydrogen orthophosphate dihydrate and 6.2178 g of anhydrous disodium hydrogen orthophosphate in 1200 mL of ultrapure water. Phosphoric acid and/or sodium hydroxide solution were then added in the solution to adjust the pH to be 7.0 by using a pH meter (Orion® model 320, USA). The buffer solution and 2-propanol were filtered through 0.45 µm Nylon membrane filter (Filtrex®, USA) in a vacuum filter set (Favorit®, England). Then, 970 mL of the 0.063 M phosphate buffer solution and 30 mL of 2-propanol measured by cylinders were mixed to obtain the mobile phase. The mobile phase was degassed for 30 minutes in a sonicator (Power Sonic® 410, Korea). In order to prolong the lifetime of the column, the mobile phase was run for 60 minutes to clean the remaining components and the storage solution was run for 30 minutes after the end of the analysis. The storage solution consisted of 0.05%w/v sodium azide in 0.025 M phosphate buffer pH 7.0. The cleaning and storage solutions were filtered through 0.45 µm Nylon membrane filter and sonicated for 30 minutes.

Instrumentation and HPLC conditions

The analysis was performed on a Perkin Elmer® Series 200 HPLC system consisting of vacuum degasser (PerkinElmer®, USA), HPLC

pump (PerkinElmer®, USA), 20-µL fixed loop injector, UV detector (PerkinElmer®, USA), and fluorescence detector (PerkinElmer®, USA). The separating column was TSK gel G2000SW$_{XL}$ (7.8 mm I.D. x 30 cm, 5 µm; Tosoh®, Japan). The flow rate of the mobile phase was set at 0.6 mL/min. The injection volume was 20 µL. The UV wavelength was 214 nm for the UV detection; and the excitation wavelength was 275 nm and the emission wavelength was 337 nm for the fluorescence detection. The software of Turbochrom® Workstation system (PerkinElmer®, USA) was used to record and interpret all HPLC chromatograms

Standard solutions

One vial of Genheal® contains 1.6 mg or 4 international units of the recombinant human growth hormone. In order to measure the weight of the whole powder in the vial, we weighed the vial before and after transferring the powder into another glass container. The difference of the weights was the weight of the powder. The powder of Genheal® which was equivalent to 1.6 mg was accurately weighed and diluted in the diluent to obtain 100 µg/mL for the UV method and the powder which was equivalent to 0.5 mg was accurately weighed and diluted in the diluent to obtain 25 µg/mL for the fluorescence method. The stock solutions were filtered through 0.45 µm Nylon membrane filter. Then, 2.0, 3.0, 4.0 mL of the stock standard solution were then transferred into 5 mL-volumetric flasks and 3.0 mL of the stock standard solution was transferred into a 10 mL-volumetric flask and diluted in the diluent to obtain 30, 40, 60, 80 µg/mL for the UV detection. For the fluorescence detection, 1.0, 2.0, 3.0, 4.0 mL of the stock standard solution were then transferred into 5 mL-volumetric flasks and dissolved in the diluent to obtain 5, 10, 15, 20 µg/mL. All solutions including the stock solutions were used as the standard solutions. The solutions were mixed carefully by hand in order to prevent protein precipitation. The solutions were stored and analyzed at room temperature within six hours after preparation.

System suitability testing

The system suitability of the HPLC systems was assessed from 6 injections of the solution of growth hormone at 60 µg/mL for HPLC-UV and 15 µg/mL for HPLC-fluorescence. The middle concentrations of the calibration curves were chosen to assess the system suitability. The parameters used in the system suitability testing included number of theoretical plates (N), tailing factors (T), retention times, and peak areas. The calculations were based on equations in the United States Pharmacopeia 36.

Method validation

Linearity and range

The standard solutions at 30, 40, 60, 80, 100 µg/mL were injected into the HPLC-UV system and the standard solutions at 5, 10, 15, 20, 25 µg/mL were injected into the HPLC-fluorescence system. When the chromatograms were obtained, the peak areas of growth hormone and the concentrations were plotted as $y = mx + c$; y = peak area, x = concentration in µg/mL, m = slope, and c = y-intercept to obtain the regression equation. The correlation coefficient (R) was calculated to determine the linearity of the calibration curve.

Accuracy

The Genheal® powder equivalent to 1.6 mg was accurately weighed and diluted into 100 µg/mL of growth hormone. The stock solution was then diluted to be 30 and 60 µg/mL for the HPLC-UV method. The Genheal® powder equivalent to 0.5 mg was accurately weighed and diluted into 25 µg/mL of growth hormone. The stock solution was then diluted to be 5 and 15 µg/mL for the HPLC-fluorescence method. The growth hormone solutions at three known concentrations were prepared and injected into the HPLC systems. The accuracy of the UV method was tested at 30, 60, and 100 µg/mL while the accuracy of the fluorescence method was tested at 5, 15, and 25 µg/mL. The peak areas of growth hormone obtained from the chromatograms were converted into the found amounts. The accuracy was expressed in the percentage of recovery which was found amount x 100/ added amount.

Precision

The Genheal® powder equivalent to 1.6 mg was accurately weighed and diluted into 100 µg/mL of growth hormone. The stock solution was then diluted to be 30 and 60 µg/mL of growth hormone for the HPLC-UV method. The Genheal® powder equivalent to 0.5 mg was accurately weighed and diluted into 25 µg/mL of growth hormone. The stock solution was then diluted to be 5 and 15 µg/mL of growth hormone for the HPLC-fluorescence method. Intra-day precisions were tested at three concentrations with nine determinations in the same day while inter-day precisions were tested at one middle concentration at six determinations for three different days. The relative standard deviations of three determinations at each concentration and six determinations at the middle concentration were calculated for intra-day and inter-day precisions, respectively.

Sensitivity

The signal-to-noise ratios at 3:1 and 10:1 were used to determine limit of detection (LOD) and limit of quantitation (LOQ), respectively. The serial dilution was started from the 30 µg/mL standard solution for the UV detection and from the 5 µg/mL standard solution for the fluorescence detection. The diluted solutions were then injected into the HPLC system to obtain chromatograms. The peak heights of signal and noise were measured to calculate the signal-to-noise ratios.

Specificity

Two 1.5 mL-microcentrifuge tubes containing the Genheal® powder were placed in an oven at 50°C for 24 hours to force the degradation of growth hormone. Then, the Genheal® powder in one tube was diluted in the diluent to create a concentration about 100 µg/mL for HPLC-UV and the Genheal® powder in the other tube was diluted in the diluent to create a concentration about 25 µg/mL for HPLC-fluorescence. The solutions were filtered through 0.45 µm Nylon membrane filter and the solutions were injected into the systems of HPLC-UV and HPLC-fluorescence to obtain chromatograms.

Stability

The Genheal® powder equivalent to 1.6 mg was weighed and diluted into 100 µg/mL of growth hormone. The stock solution was then diluted to 60 µg/mL of growth hormone. The

solution was filtered through 0.45 µm Nylon membrane filter into two 15 mL-microcentrifuge tubes. One tube was kept at room temperature and the other tube was kept in an ice bath. The solutions were injected every hour for 8 hours into the HPLC-UV system to monitor the peak areas of growth hormone. The stability of growth hormone in each condition was reported as a graph between time as the x-axis and the percentages of drug remaining as the y-axis.

Results

System suitability testing

The system suitability testing was demonstrated by six injections of the solutions of growth hormone into HPLC-UV and HPLC-fluorescence. The average retention time of growth hormone in the HPLC-UV system was 14.51 minutes while the average retention time in the system of HPLC-fluorescence was 14.77 minutes (Figure 1). The relative standard deviation of peak areas was 0.38% for HPLC-UV and 3.77% for HPLC-fluorescence. The number of theoretical plates involving the separating units in the column was 10494.40 plates with HPLC-UV and 5520.38 plates with HPLC-fluorescence. The tailing factors of the peaks in both HPLC-UV and HPLC-fluorescence were 0.86 and 0.88, respectively (Table 1).

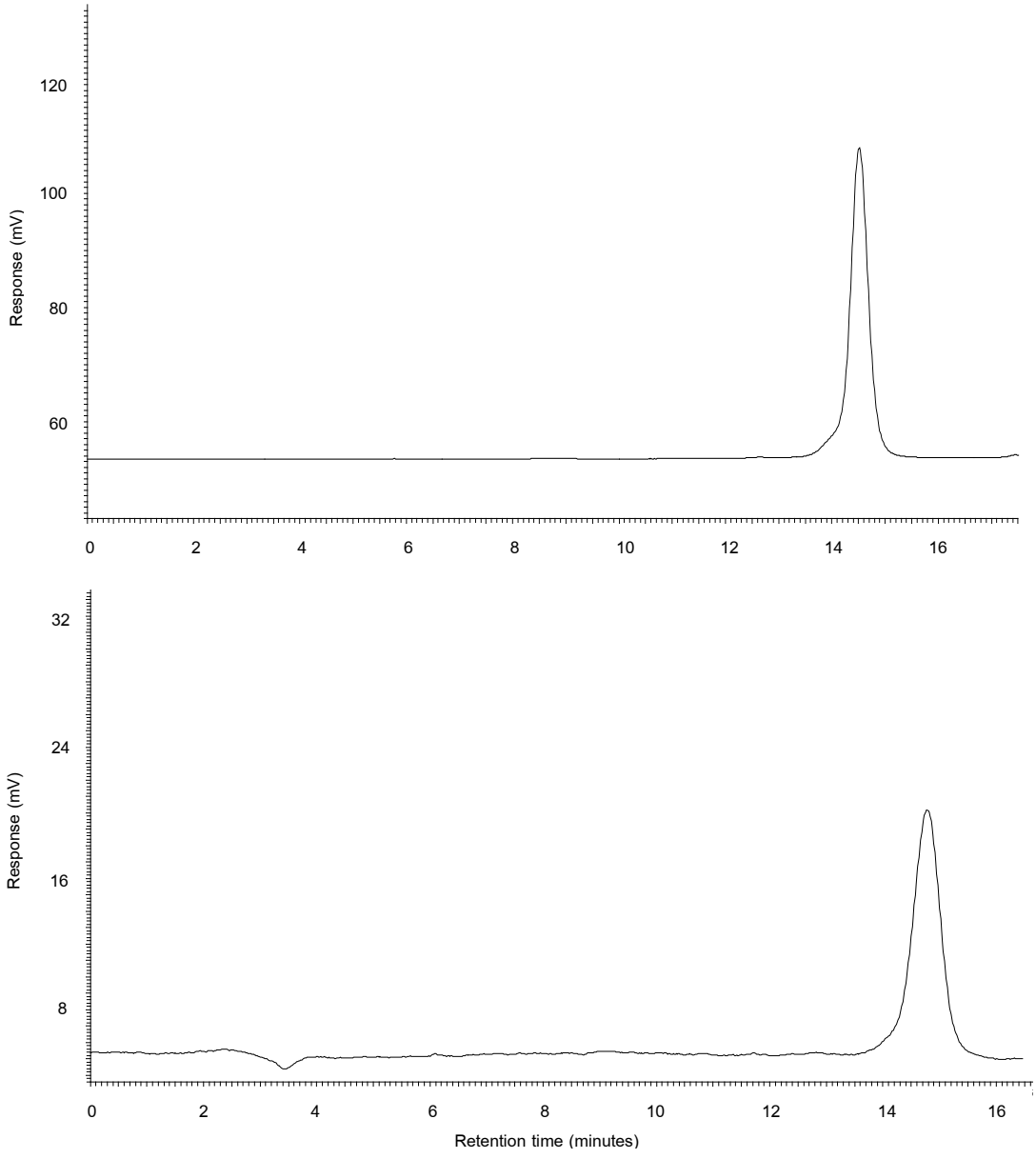

Figure 1 The HPLC chromatograms at the concentration of 60 µg/mL of growth hormone by HPLC-UV (upper) and at the concentration of 15 µg/mL of growth hormone by HPLC-fluorescence (lower). The average retention times of growth hormone were 14.51 and 14.77 minutes for HPLC-UV and HPLC-fluorescence, respectively.

Table 1 The parameters used in system suitability testing for growth hormone by HPLC-UV and HPLC-fluorescence (n=6)

Parameters in system suitability testing	HPLC-UV Mean (%RSD)	HPLC-Fluorescence Mean (%RSD)
Number of theoretical plates (N)	10494.40 (0.09)	5520.38 (30.09)
Tailing factor (T)	0.86 (3.93)	0.88 (11.43)
Repeatability		
- Retention time (min)	14.51 (0.05)	14.77 (0.10)
- Peak area (μV.s)	1396006.76 (0.38)	543242.56 (3.77)

Method validation

Linearity and range

The linearity of the HPLC-UV was in the range of 30-100 μg/mL of growth hormone. The linear regression equation was y = 20452.25x – 41790.18 with a correlation coefficient of 0.9973. The HPLC-fluorescence demonstrated an advantage to analyze growth hormone at lower concentrations; therefore the linearity in the HPLC-fluorescence was in the lower range of 5-25 μg/mL of growth hormone. The linear regression equation was y = 38002.42x + 4119.41 with a correlation coefficient of 0.9998 (Table 2).

Table 2 Results of limit of detection, limit of quantitation, linearity, and range of the HPLC-UV and HPLC-fluorescence methods for growth hormone analysis

Parameters in method validation	HPLC-UV	HPLC-Fluorescence
Limit of detection (μg/mL)	1	0.9
Limit of quantitation (μg/mL)	10	1.5
Linear regression equation	y = 20452.25x – 41790.18	y = 38002.42x + 4119.41
Correlation coefficient	0.9973	0.9998
Concentration range (μg/mL)	30-100	5-25

Accuracy

The percentage of recovery was calculated to investigate the accuracy of the methods. The added amounts and the found amounts were used to calculate the percentage of recovery at the lowest, middle, and highest concentrations of the calibration curve by the HPLC-UV and HPLC-fluorescence methods. The accuracy of the HPLC-UV was found to be in the range of 99.41-101.02%and the accuracy of the HPLC-fluorescence was found to be in the range of 98.10-102.49% (Table 3).

Precision

The intra-day precisions of the methods were studied at the lowest, middle, and highest concentrations of the calibration curves within a day and the inter-day precisions were determined at the middle concentrations on three different days. The intra-day precisions of the HPLC-UV method at the concentration of 30, 60, and 100 µg/mL were 0.35-1.71%, while the intra-day precisions of the HPLC-fluorescence method at the concentration of 5, 15, and 25 µg/mL were 0.96-2.14%. The inter-day precision at the concentration of 60 µg/mL was 2.55% for the HPLC-UV method and at the concentration of 15 µg/mL was 1.53% for HPLC-fluorescence method (Table 3).

Table 3 Results of accuracy, intra-day precisions and inter-day precisions of the HPLC-UV and HPLC-fluorescence methods

Methods	Concentration of growth hormone (µg/mL)	% average recovery (n = 3)	%RSD	
			Intra-day precision (n = 3)	Inter-day precision (n = 6)
HPLC-UV	30	101.02	1.71	
	60	100.72	1.57	2.55
	100	99.41	0.35	
HPLC-Fluorescence	5	101.83	1.42	
	15	98.10	0.96	1.53
	25	102.49	2.14	

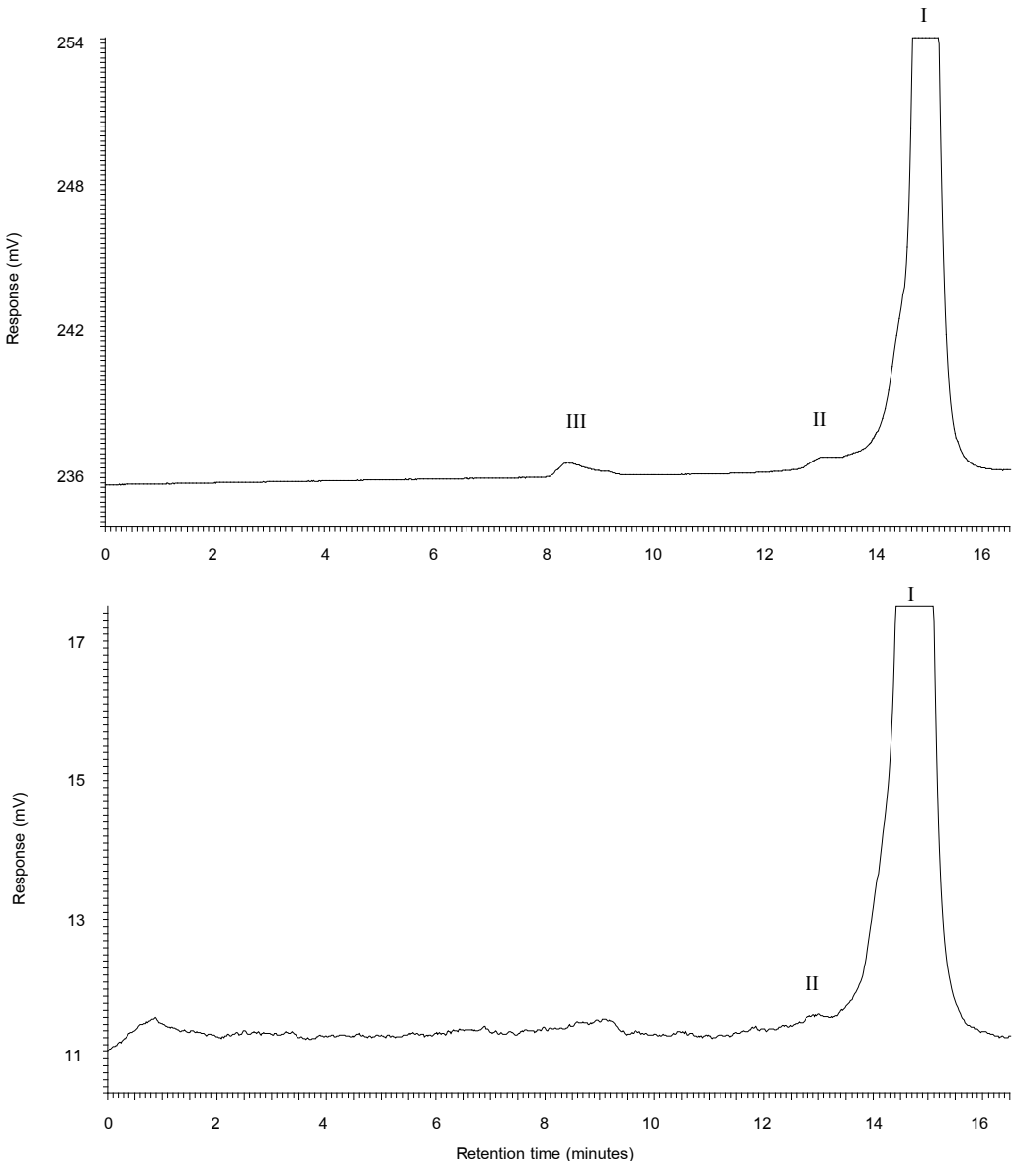

Figure 2 The HPLC chromatograms of the degraded growth hormone, the growth hormone powder was previously placed in an oven at 50°C for 24 hours. The solutions were prepared at the concentrations about 100 µg/mL of growth hormone for HPLC-UV (upper) and 25 µg/mL of growth hormone for HPLC-fluorescence (lower). The scales of y-axis were reduced so the peaks of growth hormone were partially shown. In HPLC-UV, the retention times of growth hormone (I), dimers (II), and the higher molecular weight (III) were 14.95, 13.15, and 8.44 minutes. In HPLC-fluorescence, the retention times of growth hormone (I) and dimers (II) were 14.75 and 13.01 minutes.

Sensitivity

The sensitivity of the methods included LOD which was the lowest amount of growth hormone to be detected and LOQ which was the lowest amount of growth hormone to be quantified. The signal-to-noise ratio was applied to determine LOD and LOQ of the methods. The LOD at 3:1 and LOQ at 10:1 signal-to-noise ratios in the HPLC-UV were determined to be 1 and 10 µg/mL and in the HPLC-fluorescence were 0.9 and 1.5 µg/mL (Table 2).

Specificity

The degradation of growth hormone was performed by placing the growth hormone powder in an oven at 50°C for 24 hours. The degradation products included its dimers, and the higher molecular weight.

The relative retention times of the dimers and the higher molecular weight were 0.9 and 0.65 of the growth hormone (BP Commission, 2013; USP convention, 2013). The HPLC-UV chromatogram displays the peaks of growth hormone, dimers, and the higher molecular weight at 14.95, 13.15, and 8.44 minutes while the HPLC-fluorescence displays the peaks of growth hormone and dimers at 14.75 and 13.01 minutes (Figure 2).

Stability

The stability of the growth hormone solutions at the concentration of 60 µg/mL was studied at room temperature and in the ice bath by the HPLC-UV method. The percentages of drug remaining at room temperature were calculated to be 100.00, 99.48, 99.70, 99.41, 99.42, 98.85, 98.00, 98.14, 97.93 percents at one-hour intervals. The percentages of drug remaining in the ice bath were 100.00, 100.20, 99.84, 99.51, 98.26, 99.11, 98.34, 97.89, 96.73 percents at one-hour intervals (Figure 3).

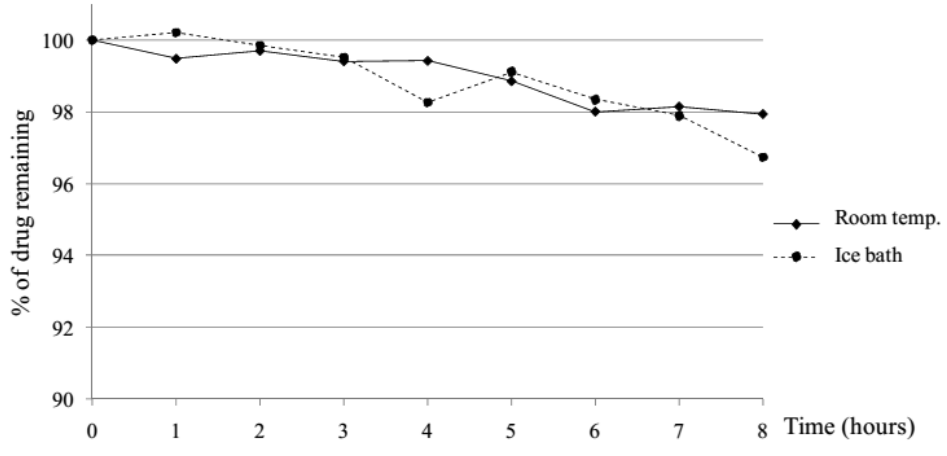

Figure 3 The stability data of the growth hormone solutions at the concentration of 60 µg/mL kept at room temperature (solid line) and the ice bath (dashed line), the solutions were injected into the HPLC-UV system every hour for 8 hours.

Discussion and conclusion

Spectrofluorometry has been shown to improve the sensitivity for determining growth hormone (Yilmaz et al., 2012A); however, this method has not been reported to distinguish its dimers from growth hormone. The HPLC-SEC is the official method for the assay of growth hormone in the pharmacopeias (BP Commission, 2013; USP convention, 2013). It can separate the higher molecular weight, dimers, and growth hormone. However, the official methods in USP 36 and BP 2013 require the concentration at 1 mg/mL of growth hormone. The presented method incorporated the HPLC-SEC with the fluorescence detector to increase the sensitivity and maintain the specificity of the analysis. The UV detector was also chosen in this study because of its availability in pharmaceutical industries; however, the concentration range of growth hormone in the HPLC-UV system was reduced from the official methods. The range and the detector were changed from the official methods; therefore, the developed methods were validated for linearity, accuracy, precision, sensitivity, and specificity.

For system suitability testing, the relative standard deviations based on peak areas of growth hormone in HPLC-UV and HPLC-fluorescence were found to be 0.38% and 3.77% (Table 3). The fluorescence detector displayed more variation of the peak areas than the UV detector so the HPLC-fluorescence system could cause less consistent results than the HPLC-UV system. It would be recommended that a constant intensity of the light source should be created by warming the lamp for 30 minutes before every analysis. The HPLC-UV system contained the average number of theoretical plates to be 10494.40 which was higher than the HPLC-fluorescence system. The HPLC-fluorescence system contained the average number of theoretical plates to be 5520.38. The lower number of theoretical plates in HPLC-fluorescence was caused by its broader peaks according to the longer tubing from the column to the detector.

The linearity and range of the developed methods were determined by the correlation coefficients of the linear regression equations which were 0.9973 and 0.9998 for HPLC-UV and HPLC-fluorescence. The concentrations of growth hormone were studied in the range of 30-100 and 5-25 µg/mL for the HPLC-UV and HPLC-fluorescence, respectively. Both methods exhibited acceptable linear regression equations because the correlation coefficients were at least of 0.995. The calibration curves plotted between the concentrations and peak areas displayed a more reliable correlation than the ones plotted between concentrations and peak heights. The peak shapes of growth hormone could be variable among injections; therefore, using peak areas demonstrated more reproducible results than using peak heights in the calibration curves.

The accuracy was within 99.41-101.02 % for HPLC-UV and 98.10-102.49% for HPLC-fluorescence. The intra-day precisions of HPLC-UV were in the range of 0.35-1.71% and the intra-day precisions of HPLC-fluorescence were in the range of 0.96-2.14%. Thus, the HPLC-UV system was found to provide more accurate and precise results than HPLC-fluorescence when the analysis

was performed on the same day. The intra-day and inter-day precisions of HPLC-UV tested at the concentration of 60 µg/mL were 1.57% and 2.55% while the intra-day and inter-day precisions of HPLC-fluorescence tested at the concentration of 15 µg/mL were 0.96% and 1.53% (Table 3). At the middle concentrations of the calibration curves, HPLC-fluorescence was found be more precise than HPLC-UV and the inter-day precisions displayed more variations than the intra-day precisions in both methods.

The sensitivity of HPLC-UV and HPLC-fluorescence was investigated by signal-to-noise ratios. The LOD and LOQ of the HPLC-UV method were 1 and 10 µg/mL and the LOD and LOQ of the HPLC-fluorescence method were 0.9 and 1.5 µg/mL (Table 2). In this study, the sensitivity of the HPLC-UV and the HPLC-fluorescence was compared by the same determination methods, and it was found that the HPLC-fluorescence exhibited better LOQ than the HPLC-UV so HPLC-fluorescence required less concentration and reduced the cost of growth hormone in the analysis.

The specificity was studied by placing the growth hormone powder in an oven at 50°C for 24 hours to force the degradation. Then, the growth hormone powder was diluted and injected into the HPLC systems. The HPLC-UV and HPLC-fluorescence in this study were shown to discriminate the 22 kD-monomers and 45 kD-dimers of growth hormone. The molecular weight of the dimers is higher than the molecular weight of the monomers so the dimers were eluted before the monomers. The peak of the dimers can be identified by the relative retention time at 0.9. In HPLC-UV system, the peaks of dimers and monomers were 13.15 and 14.95 minutes. In HPLC-fluorescence system, the peaks of dimers and monomers were 13.01 and 14.75 minutes (Figure 2). The SEC in both systems could distinguish the monomers and the dimers of growth hormone even though the SEC could not entirely separate the monomers and dimers of growth hormone. The monomers and dimers of growth hormone were detectable by the UV detection at 214 nm and the fluorescence detection with the excitation wavelength at 275 nm and the emission wavelength at 337 nm (Figure 2). Since dimers could absorb and emit at the same wavelengths as the monomers, UV detection at 214 nm or fluorescence detection with the excitation wavelength at 275 nm and the emission wavelength at 337 nm without SEC would be unable to specifically quantify the active form of growth hormone. It was noticeable that incorporating SEC to separate growth hormone as the therapeutic drug and dimers as its impurity by different sizes in HPLC-UV and HPLC-fluorescence systems could increase the specificity of the analysis.

The stability of growth hormone is essential to define a condition to keep the prepared solution during the analysis since the official methods do not include this information in the monograph (BP Commission, 2013; USP convention, 2013). The stability of growth hormone in the solutions by spectrofluorometry and RP-HPLC with the UV detection showed that the growth hormone solutions were stable at room

temperature and in a refrigerator at 4°C for 24 hours (Yilmaz et al*.,* 2012A). However, the stability data by the HPLC-SEC with the UV detection of the growth hormone solutions kept at room temperature and in the ice bath revealed that growth hormone was degraded in both conditions in this study. It was shown that the ice bath did not prevent the degradation of growth hormone (Figure 3). It would be recommended that the growth hormone solution should be freshly prepared because the percentage of drug remaining gradually reduced as time passed. If the prepared solutions could not be analyzed immediately, it would be recommended to analyze them within six hours since the percentages of drug remaining were within 98.00 percents during the first six hours (Figure 3).

In conclusions, the HPLC-UV and HPLC-fluorescence based on size-exclusion chromatography were validated and exhibited appropriate linearity, accuracy, precision, and sensitivity for the determination of growth hormone. The HPLC-UV and HPLC-fluorescence methods in this study required a lower concentration of growth hormone in the range of 30-100 µg/mL for HPLC-UV and 5-25 µg/mL for HPLC-fluorescence than the official methods which require 1 mg/mL of growth hormone. These methods could be routinely and economically applied in the pharmaceutical quality control.

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