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Development of Analytical Method of Biotin in Complex Drugs and Dietary Supplements Using HPLC-UV

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ABSTRACT – Recently, Korean Food and Drug Administration (KFDA) has focused on developing quality control guidelines for all commercial products in Korea to enforce regulations, improve the quality control, and protect consumers by developing prevalently used and efficient analytical tools to determine and quantify target compounds. Because the Korean Pharmacopeia (KP) presents microbiological assays for biotin, which is laborious and time-consuming, this study is focused on applying HPLC-UV to detect and quantify biotin in complex drugs and dietary supplements like multi-vitamin. Biotin in complex drugs was extracted from methanol and analyzed using mobile phase with 10 mM potassium phosphate (monobasic, pH=3.0) in distilled water and acetonitrile. Gradient condition was used to successfully detect and quantify biotin within 20 minutes. Validation result for linearity was significant that average r^2 was 0.999 (n=3) and its relative standard deviation (RSD) was 0.0578% which was less than 2%. Using this method, quantification of biotin in complex drugs was completed successfully and recovery tests were finished that recovery percentage greater than 95% with relative standard deviation less than 2%.

Key words – Biotin, High performance liquid chromatography, Validation

Biotin is water-soluble vitamin B-complex and also called vitamin H. Its chemical structure consists of tetrahydroimidizalone ring fused with a tetrahydrothiophene ring where valeric acid is attached to the tetrahydrothiophene ring (Fig. 1). Also, its major biological function is known as coenzyme for cell growth, the production of fatty acids, and the metabolism of fats and amino acids (Nojiri et al., 1998; Yomota and Ohnishi, 2007). Moreover, biotin is daily required as other vitamins participates in gluconeogenesis. Thus, its deficiency could be serious and even fatal (Aboul-Enein et al., 2004; Watanabe et al., 2005).

Recently, biotin has been included in various commercial products such as complex drugs and dietary supplements like multi-vitamin. Therefore, solid and efficient method should be established in order to control and enforce regulations of products and protect consumers. However, since microbiological assays of biotin, the typical analytical method of biotin introduced in the Korean Pharmacopeia, requires laborious and time-consuming works by growing and incubating cells; The Korean Food and Drug Administration (KFDA) is currently searching prevalent, fast, efficient and accurate analytical methods to determine biotin. In this aspect, KFDA focuses on high performance liquid chromatography with an ultraviolet detector (HPLC-UV), a fast and accurate qualitative and quantitative analytical tool widely available in common analytical laboratories. Even though there are other methods developed to analyze biotin using HPLC-UV (Chen et al., 2009; Durstl et al, 1975; Ekpe and Hazen, 1998; Nandhasri et al., 1991; Nelson et al., 2006), most of them require complicated derivatization steps (Desbene et al., 1983) to prepare samples and its low yield due to incomplete reaction causes further problem with accurate determination of biotin in samples (Wilbur et al., 2000).

Here, we report an easy and efficient method to quantify biotin in various products accurately using HPLC-UV without derizatization steps. Since its good analytical performance was confirmed, it will potentially become a quality control guideline for commercial products including biotin, and will provide low economic cost and simple procedures for easier approach to be performed in common laboratories.

Material and Experimental

Biotin standard solutions

Biotin standard solution was prepared by dissolving biotin standard (Sigma Aldrich, MO, USA) in methanol (Duksan Chemicals, Ansan, Korea). Five different concentrations were prepared by making 120, 135, 150, 165, and 180 μg/mL (ppm).

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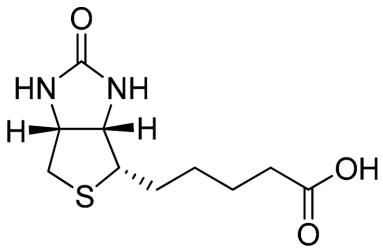

Figure 1. Chemical structure of biotin

All solvents used were of HPLC grade.

Samples preparation

Samples used for recovery tests were referred to as Drug A (500 μg/tablet), B (165 μg/tablet) and C (300 μg/tablet). All samples were multi-vitamins tablets and twenty of them were weighted and grounded. Twenty tablets for each drug were weighted and finely grounded. Then, the average amount was obtained and dissolved in methanol to make 150, 165, and 180 ppm. All solutions were sonicated for 30 minutes (min.) and centrifuged for 10 min. at 14,000 rpm. Finally, supernatants were filtered using an Advantec membrane filter (0.45 μm, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) before injection to HPLC.

Method validation protocol for assays and impurities in tablets by HPLC

The validation was carried out properly based on a protocol recommended by KFDA (The United States Pharmacopeia, 2002).

HPLC instrument and conditions

10 μL of a sample was introduced into a Phenomenex Luna analytical column (C18, 150 × 4.6 mm, 5 μm) via Phenomenex Security Guard column (C18, 3.0 × 4.0 mm) by a Waters 717 auto-sampler. Compounds in samples were separated by a mobile phase program between mobile phase A [10 mM potassium phosphate (mono basic) in water (pH=3.0)] and the mobile phase B (acetonitrile) at a flow rate of 1.0 mL/min. using Waters 510 pump. Mobile phase B was started from 5% for 5 min. to 85% over 15 min. at a linear gradient and then back to 5% for 10 min. for equilibrium. Eluted compounds from the analytical column were observed at 205 nm through a Waters 486 UV detector and the column temperature was kept at 25°C.

Results and Discussion

The purpose of this study was to develop a new analytical method for biotin in order to utilize in administrative performance. Since many precedents have not fully satisfied the ultimate goal of their usage (Miwa et al., 2000), it was urgent to establish an efficient and widely available method to detect and quantify biotin in complex drugs. HPLC-UV was the best method because it was the most extensively used analytical instrument that provided accurate results with high sensitivity (Aboul-Enein et al., 2004). The importance of this study is to provide simple procedure to prepare samples to run experiment within 30 min. and therefore, build a bridge that connects scientific research and practical application of analytical chemistry for administrative usage.

UV absorption wavelength to detect biotin was set at 205 nm. Because biotin lacks chromophore, it does not show high sensitivity to UV absorption (Miwa et al., 1985). There are previously studied methods that support the weakness of UV absorption by running additional derivatization in order to give stronger chromophore to biotin. However, many of them cause problems because performing reactions might produce low yield of finally derivatized products or even no product at all (Miwa et al., 2000) and that will not accomplish the goal to accurately quantify biotin in complex drug. Therefore, it was determined to use UV detector without following precedents but instead the lowest possible wavelength was chosen that could still detect the target compound.

The mobile phase used was 10 mM of potassium phosphate in water with pH of 3.0 and acetonitrile. The phosphate buffer and pH control in aqueous mobile phase increase selectivity and sensitivity of biotin peak. Methanol was not suitable for this study because its UV cutoff is higher than 205 nm (Armstrong and Carey, 1982), and so acetonitrile was used as organic mobile phase. Since developed analytical method should be applicable to commercial products, such as complex drugs, the gradient mobile phase condition instead of the isocratic condition was used for the clear separation of biotin from other components and their complete elution from a column in a short analytical time.

In order to validate the analytical method, a validation protocol recommended by KFDA was followed (The United States Pharmacopeia., 2002). It introduced three parts of the experiment: linearity, precision, and accuracy/system suitability and in order to prove the linearity, five different concentrations were required, usually 80, 90, 100, 110 and 120% of standard solution. In this study, 150 ppm was determined 100%. Required relative standard deviation (RSD) in the protocol is less than 2% in the case of three trials and the RSD for linearity in this study satisfied the requirement (5.78×10^{-2}%). Then, precision was examined followed by system validation, intra-day validation, and inter-day validation. System valida-

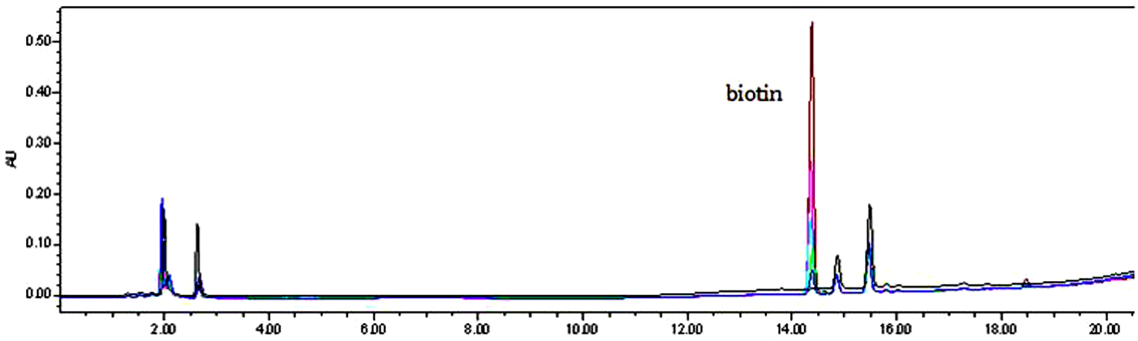

Figure 2. Overlaid HPLC chromatograms of biotin standard

Table I. *Slope, y-intercept, and r^2 of calibration curves generated by biotin standard solutions*

Trial	Slope	y Intercept	r^2
1	4.83×10^{3}	-7.23×10^{4}	9.99×10^{-1}
2	5.04×10^{3}	-1.02×10^{4}	9.99×10^{-1}
3	4.51×10^{3}	-2.34×10^{4}	9.98×10^{-1}
Average	4.79×10^{3}	-3.53×10^{4}	9.99×10^{-1}
STDEV[1]	2.63×10^{2}	3.27×10^{4}	5.77×10^{-4}
		RSD[2](%)	5.78×10^{-2}

[1]STDEV: standard deviation
[2]RSD: relative standard deviation

Table II. *System validation (n=6) of the HPLC- UV method using a biotin standard solution (150 ppm)*

Trial	Peak Area
1	6.76×10^{5}
2	6.76×10^{5}
3	6.71×10^{5}
4	6.73×10^{5}
5	6.75×10^{5}
6	6.66×10^{5}
Average	6.73×10^{5}
STDEV	3.92×10^{3}
RSD(%)	5.83×10^{-1}

[1]STDEV: standard deviation
[2] RSD: relative standard deviation

tion requires six trials and intra- and inter-day validation need three trials. Results for system validation, intra-, and inter-day RSD values were 5.83×10^{-2}%, 9.72×10^{-2}%, and 4.37×10^{-1}%, respectively. Finally, accuracy/system suitability test was done to confirm the accuracy of the method and applied to commercial complex drugs. As a result, the recovery percentages were calculated greater than 95% with RSD less than 2%.

Linearity

The biotin standard peak was observed at 14.4 min (Fig. 2) and its peak area from each biotin standard solution run was used for generating calibration curves and testing linearity. In each trial, standard solutions with five different concentrations were analyzed and total three trials were performed. As a result, the average value of r^2 greater than 0.999 and RSD less than 2% were obtained (Table I).

Precision and accuracy/system suitability

As a part of the validation of the analytical method, its repro-

Table III. *Intra-day validation (n=3) of the HPLC-UV method using biotin standard solutions*

Trial	Peak Area					RSD[2] (%) for each set
	120 ppm	135 ppm	150 ppm	165 ppm	180 ppm	
1	5.11×10^{5}	5.76×10^{5}	6.51×10^{5}	7.26×10^{5}	7.98×10^{5}	1.28
2	5.02×10^{5}	5.80×10^{5}	6.57×10^{5}	7.20×10^{5}	8.09×10^{5}	1.25
3	5.14×10^{5}	5.90×10^{5}	6.54×10^{5}	7.23×10^{5}	7.86×10^{5}	4.98×10^{-1}
Average	5.09×10^{5}	5.82×10^{5}	6.54×10^{5}	7.23×10^{5}	7.98×10^{5}	3.85×10^{-1}
STDEV[1]	6.50×10^{3}	7.26×10^{3}	3.26×10^{3}	2.79×10^{3}	1.16×10^{4}	1.45
RSD[2](%)	1.28	1.25	4.97×10^{-1}	3.85×10^{-1}	1.45	9.72×10^{-1}

[1]STDEV: standard deviation
[2]RSD: relative standard deviation

Table IV. *Inter-day validation (n=3) of the HPLC-UV method using biotin standard solutions*

	Peak Area		
	120 ppm	150 ppm	180 ppm
Day 1	5.09×10^5	6.54×10^5	7.98×10^5
Day 2	5.10×10^5	6.53×10^5	8.02×10^5
Day 3	5.05×10^5	6.50×10^5	8.05×10^5
Average	5.08×10^5	6.52×10^5	8.01×10^5
STDEV[1]	2.31×10^3	1.94×10^3	3.51×10^3
RSD[2](%)	4.55×10^{-1}	2.97×10^{-1}	4.37×10^{-1}

[1]STDEV: standard deviation
[2]RSD: relative standard deviation

ducibility was examined, first. System validation was performed by injecting a biotin standard solution of 150 ppm six times consecutively and each area was compared to obtain standard deviation and RSD. As presented in Table II, the RSD for system validation was confirmed as 0.583%, which was less than 2%. Further, intra-day validation was carried out by repeating one set of five different concentrations for three times and each RSD value was calculated to verify intra-day repeatability. As shown in the Table III, the average RSD value was 0.972%. In this test, RSD for each set was gained by comparing each concentration with its corresponding concentration obtained from calibration curve equation. Lower RSD values (the average RSD for each set was 0.972%) imply better accuracy of our method. Finally, inter-day validation was performed by running three different concentrations per day for three consecutive days. Table IV illuminates the areas for different concentrations with RSDs.

Recovery test was examined to determine the accuracy of the developed method and the system suitability for the analysis of biotin in commercial products and three complex tablet drugs were used to evaluate recovery and reproducibility of the method. Each drug was diluted and extracted using methanol to make 150, 165 and 180 ppm and each sample (n=3) was membrane filtered to remove impurities. Satisfactory ranges for recovery test were from 95% to 105% and RSD value less than 2% (The United States Pharmacopeia., 2002). Biotin peaks for all three drugs appeared at same retention time as Fig. 3 shows. Table V summarizes recovery percentages for drug A, B and C. It was confirmed that all data accomplished desired ranges of results. Since our precision and accuracy

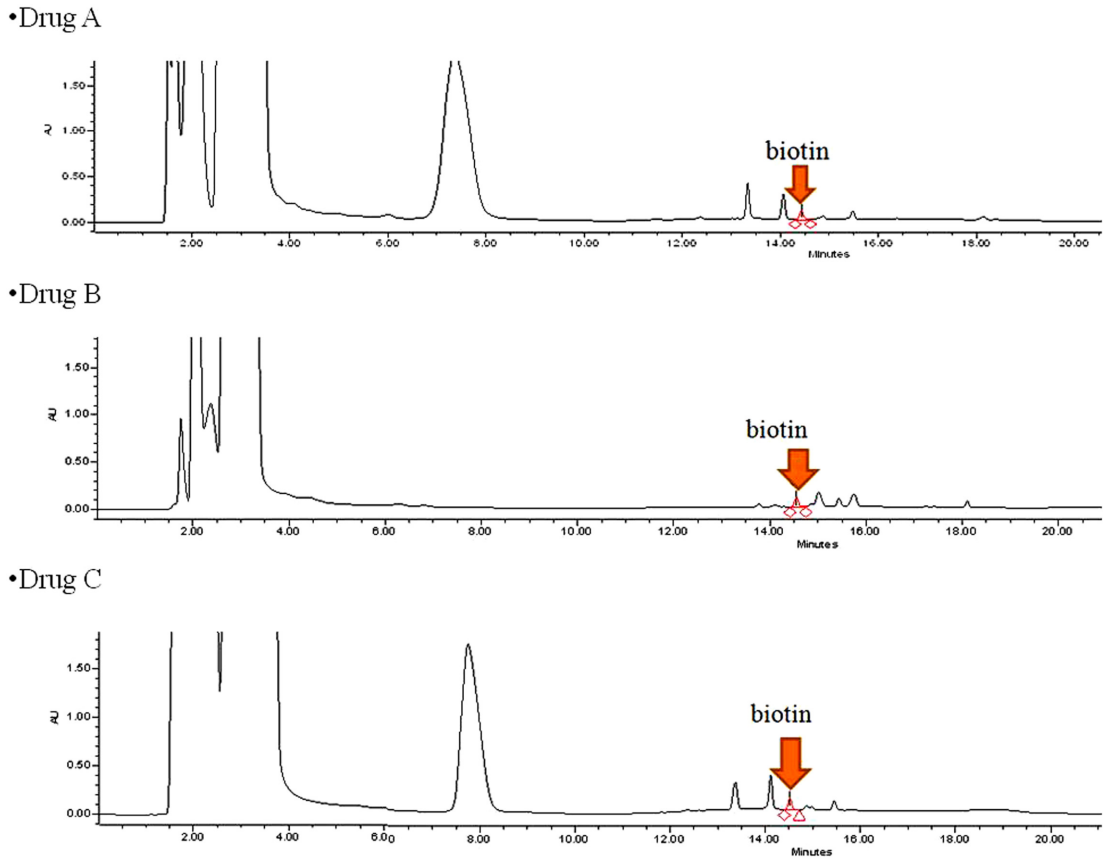

Figure 3. HPLC chromatograms for complex drug A, B, and C

Table V. *Recovery tests for drug A, B and C (n=3) using the HPLC-UV method*

	Averaged recovery (%)	Averaged calibration recovery (%)
Drug A		
150 ppm	95.9	96.4
RSD[1] (%)	1.02	92.6×10^{-2}
165 ppm	95.2	95.4
RSD (%)	81.2×10^{-2}	74.2×10^{-2}
180 ppm	95.1	95.6
RSD (%)	69.6×10^{-2}	64.0×10^{-2}
Drug B		
150 ppm	98.0	98.3
RSD (%)	1.61	1.46
165 ppm	95.2	95.3
RSD (%)	1.34	1.23
180 ppm	97.1	97.4
RSD (%)	1.70	1.57
Drug C		
150 ppm	96.9	97.3
RSD (%)	6.71×10^{-1}	6.08×10^{-1}
165 ppm	96.0	96.1
RSD (%)	6.83×10^{-1}	6.24×10^{-1}
180 ppm	96.2	96.6
RSD (%)	2.06×10^{-1}	1.90×10^{-1}

[1]RSD: relative standard deviation

results without using any internal standard satisfied the KFDA recommendation (RSD values less than 2% and recovery values between 95 and 105%), further studies employing any internal standard were not tried.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were scrutinized to determine the lowest quantity of biotin that could be discriminated and the lowest quantity of biotin that can be obtained quantitatively, respectively. These numerical values were calculated using following equation 1 and 2. σ represents the SD of intercept from the linearity calibration curve. As a result, the values of LOD were 22.5 ppm and LOQ was 68.3 ppm.

$$LOD = 3.33\times\frac{\sigma}{slope} \quad \text{(Eq. 1)}$$

$$LOQ = 10\times\frac{\sigma}{slope} \quad \text{(Eq. 2)}$$

Conclusions

In this study, a solid analytical method for biotin was developed and successfully completed while adhereing to the method validation protocol set by the HPLC-UV. Therefore, this method could potentially become a quality control guideline that could provide low economic cost and simple procedures for easier approach to be performed in common laboratories.

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