의학석사 학위논문

액체크로마토그래피질량분석기를 이용한 인체 혈청 비오틴 정량법과 multivitamin 복용과 신기능이 biotin 농도에 미치는 영향 연구

Quantitative Measurement of Biotin by LC-MS/MS in Human Serum and the Effect of Multivitamin and Renal Function on Biotin Concentration

2019년 2월

서울대학교 대학원
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지도교수 송 상 훈

이 논문을 의학석사 학위논문으로 제출함

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송 다 영

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2019년 1월

위 원 장 _____ (인)

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위 원 _____ (인)

ABSTRACT

Quantitative Measurement of Biotin by LC-MS/MS in Human Serum and the Effect of Multivitamin and Renal Function on Biotin Concentration

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Background: Biotin, also known as vitamin B7 or vitamin H is water-soluble vitamin which acts as coenzyme of metabolism. Its deficiency is known to be rare, however, excess of biotin from exogenous intake have been reported to make interference on several analytical systems using biotin-streptavidin immunoassay. This study is for setting up quantitation of serum biotin using liquid chromatography tandem-mass spectrometry, and to measure the serum level of biotin in patients with chronic kidney disease (CKD) and general population and predicting its potential effect on immunoassay.

Methods: Biotin spiked drug free serums and patient samples were separated and detected by liquid chromatography tandem-mass spectrometry on positive electrospray ionization mode. Analytical performance of the developed

method was analyzed based on standard protocols. Serum biotin levels from patients with chronic kidney disease, and those under health checkup were compared.

Results: Precision of low- and high-concentration controls showed coefficient of variations (%) of less than 10%. Linearity was satisfied with first degree polynomial model. Lower limit of quantification was 3 ng/mL. Ionization suppression was observed in serum in matrix effect evaluation. Biotin concentration decreased regardless of storage temperature and number of storage days. Biotin concentrations in more than 98% of samples exceeded the 10 ng/mL which is the lowest known threshold for biotin interference in frequently used immunoassay system. In the non-dialysis CKD group, the patients who were administered the multivitamin containing biotin 300 μg had a higher biotin concentration than those who were not (29.9 ± 11.0 ng/mL on multivitamin taking group, 23.7 ± 9.3 ng/mL on multivitamin not taking group, and 26.0 ± 9.1 ng/mL on normal control, P = 0.011).

Conclusion: This method is eligible for detect the level of biotin level which is able to evaluate the biotin interference. Clinical laboratorians should be alerted of Korean's overt high concentration of biotin and its possible interference.

Keywords: Biotin, Human serum, Liquid chromatography, Tandem mass spectrometry, Interference

Student number: 2017-28827

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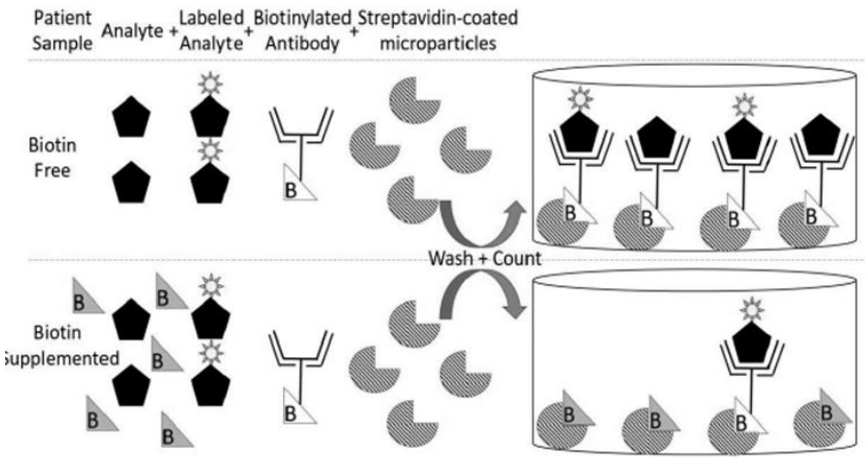
INTRODUCTION

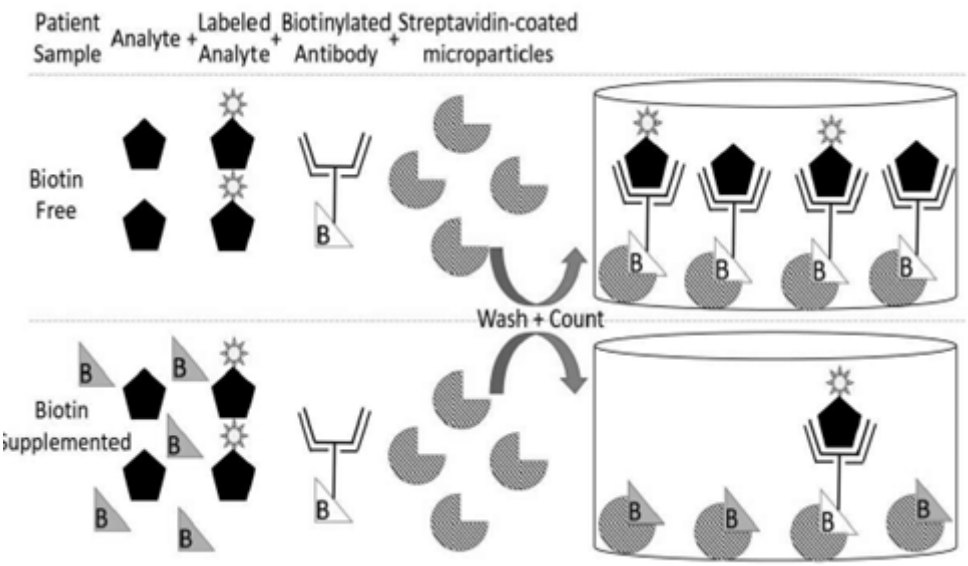
Biotin, also known as vitamin B7 or vitamin H, is water-soluble vitamin. It acts like an enzyme co-factor of carboxyl units and fixes carbon dioxide (1). The process occurs mainly when it bounds to protein or polypeptides. Its deficiency is known to be rare, however skin problems (e.g. dry skin, seborrheic dermatitis, rashes, and fungal infection) and neuromuscular diseases (e.g. neuralgia, myalgia, lassitude, and somnolence) could occur in deficiency status (2). The adequate daily supplement has been estimated to be 30 μg/day, and the blood concentration range from 0.05 to 0.83 ng/mL(3). Nevertheless, high-dose (5 mg-10 mg) of pills have been consumed as 'cosmetic' purpose, because they have been believed to make hairs, nails, and skin stronger (4). In Korea, the dietary supplements market has grown 17.2% over the previous year and reached 3.8 trillion won in 2018. And vitamins take a large part of the market, multivitamins (9.6%) and single vitamin (6.7%) were the 3rd and 4th highest respectively (5). Also, high-dose intake has been evaluated and used for treatment for multiple sclerosis, propionic acidemia, and biotinidase deficiency (5 mg -300 mg per day) (6, 7).

Biotin is considered to be non-toxic, but it is currently causing interference in most antibody based analytical systems. Most immunoassay systems are using biotinylated antibodies and analogues, which strongly binds to streptavidin. That is because biotin can covalently couple to various target materials including low-molecular weight antigens, polypeptides, and proteins and does not affect biological and antigenic activity of materials. Furthermore, streptavidin is exceptionally stable and biotin-binding activity maintains despite harsh reaction and extensive derivatizations (8). There are two main types of clinical immunoassay methods, which are sandwich and competitive immunoassay. Generally, in sandwich methods, exogenous biotin

competes with the binding of labeled complexes to solid phase and eventually reduces the signal and the result. In competitive immunoassays that use streptavidin/biotin, exogenous biotin competes with the labeled analyte reagent and inversely obtained signal intensity is much reduced and results in falsely elevated values, respectively (Figure 1) (9).



(A)



(B)

Figure 1. Biotin mechanism of interference. (A) Sandwich immunoassay (B) Competitive immunoassay

Biotin serum concentration that is higher than 10 ng/mL to 50 ng/mL have been reported to have considerable effect on most immunoassay systems using biotinylated assay (9-11). The affected immunoassay systems included Roche Elecsys®, Ortho Vitros®, Siemens Dimension® and Centaur®, Beckman Coulter Access®/DXI®, and Abbott Architect i2000® which are the most frequently used systems. Particularly, in some cases, incorrect results of thyroid function test including thyroid stimulating hormone, free thyroxine, and free triiodothyronine were mistaken for hyperthyroidism (12). The analytes associated with reproductive endocrinology such as luteinizing hormone, follicle stimulating hormone, estradiol, progesterone, prolactin, and sex hormone-binding globulin are also known to be affected by the biotin interference (9). Furthermore, a patient who ingested high-dose biotin was reported to expire as a result of the falsely-low troponin result (13). The biotin interference may result in waste of time and money, misdiagnosis of patient, and fatal outcome in the worst cases. The chances of the biotin interference are not considered very low. In one study, both hormonal and non-hormonal test results of individuals who took 10 mg/day for 1 week were affected in 27% of biotinylated sandwich immunoassays and in 63% of competitive immunoassays (14).

Usually biotin absorption and its renal excretion are so fast, its half-life is 8 to 16 hours (15, 16). However, patients with decreased renal function will excrete the biotin inefficiently and serum biotin levels are known to be elevated (17, 18). Elevated concentrations of biotin and its metabolites were associated with muscle cramp of hemodialysis patient (18-20). In Seoul National University Hospital, some multivitamins contain biotin between 22.5 μg and 300 μg. The multivitamin containing 300 μg of biotin, Nephvita tab., has been prescribed for chronic kidney disease patients.

Despite recent increasing interest in biotin interference on clinical laboratory tests in western countries, data such as serum level of biotin in

patients and general population and the degree of interference on laboratory tests are rarely reported in Korea. In addition, in order to evaluate the biotin interference, the biotin level in serum or plasma must be quantified along with the inter-system comparison. However, it is unavailable to assess the biotin interference without non-biotinylated assay. Also, it is difficult to measure the biotin level, since there is no biotin quantitative kit validated enough for use in clinical laboratories in Korea. Therefore, the aim of this study was to develop a reliable quantitation method for biotin in serum using liquid chromatography tandem-mass spectrometry (LC-MS/MS), and to measure the serum level of biotin in patients with chronic kidney disease (CKD) and general population and predicting its potential effect on immunoassay.

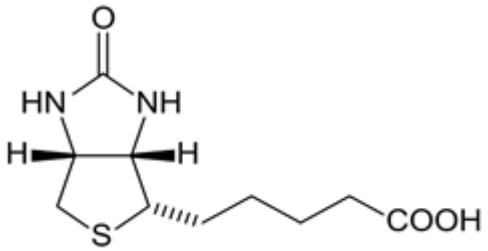
METHODS

This study was approved by the Institutional Review Board (IRB) of the Seoul National University Hospital of Korea. (1810-080-980). Leftover serum samples were used and thus written informed consent was exempted.

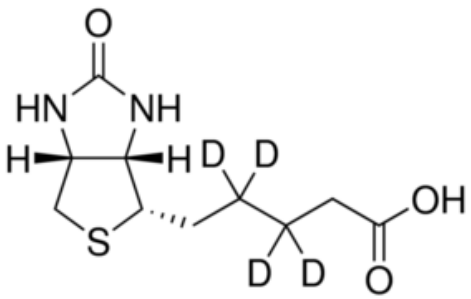
Analytical systems

Chemicals

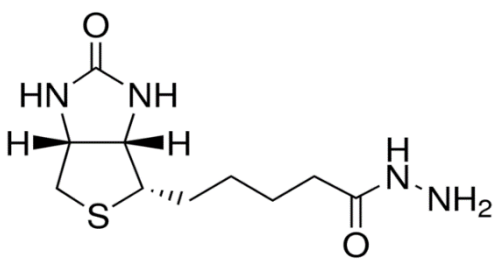
Biotin and (+)-Biotin hydrazide was purchased from Sigma-Aldrich (Sigma-Aldrich, St Louis, MO, USA) and Biotin-[d4] was purchased from (Cambridge Isotope Laboratories, Tewksbury, MA, USA) (Figure 2) (21, 22). The biotin-[d4] orders from several companies had rejected due to lack of inventory, so a ready-to-use (+)-Biotin hydrazide with similar chemical structure and molecular weight to biotin was tested first. Mobile phases were made of liquid chromatography-mass spectrometry (LC-MS) grade acetonitrile (ACN) (Baker HPLC analyzed, Mallinckrodt Baker BV, Deventer, Netherlands) and formic acid (FA) (Samchun Pure Chemical Co. Ltd., Korea). Ammonia hydroxide (Sigma-Aldrich) and ACN (Baker HPLC analyzed, Mallinckrodt Baker BV) were used to dissolve the stock. Drug free serum (Bio-Rad Laboratories, Anaheim, CA, USA) and charcoal stripped patient samples were used for evaluation of matrix effect.



(A) Biotin



(B) (+)-Biotin hydrazide



(C) Biotin-[d4]

Figure 2. Structure of biotin, (+)-Biotin hydrazide, and biotin-[d4]

Standard solution and sample preparation

Biotin as standard and (+) - biotin hydrazide and biotin- [d4] as internal standard (IS) were used in the study. Stock solutions (1 mg/mL) of biotin, (+)-biotin hydrazide, and biotin-[d4] were dissolved in ammonium hydroxide. Stock solutions were diluted with ACN for working solutions. Biotin working solutions were made up into 1, 10, 100, 500, 1,000, 5,000, and 10,000 ng/mL. The (+)-biotin hydrazide and biotin-[d4] were prepared at 100 ng/mL. These stock and working solutions were prepared in ACN and then aliquoted and stored at -20℃, except for the working solutions in use.

Total of 100 μL calibration samples were prepared by spiking biotin in different concentrations in ACN. Quality control materials (of low and high concentration) were made prepared by appropriate amount of spiking biotin into charcoal stripped patient samples. The concentrations of quality control materials were 7 and 30 ng/mL.

Total of 100 μL serum samples were placed in 1.5 mL Eppendorf tubes. Then, 400 μL of 100 ng/mL IS dissolved in ACN was added for extraction and centrifuged at 13,200 g for 10 mins. Nitrogen gas was used to evaporate the 200 μL of extracted supernatant. Finally, the residue was reconstituted in 100 μL of 3% ACN (97% DW and 3% ACN) and measured on LC-MS/MS.

Liquid chromatography and mass spectrometry

LC separation of biotin was performed on Poroshell 120 EC-C18 column 3 × 50 mm, 2.7 um (Agilent Technologies, Santa Clara, CA, USA) and Ascentis® Express F5, 2.7 Micron HPLC Column 2.1 mm × 100 mm, 2.7 μm (Sigma-Aldrich) at 40.0℃ using 1200 series infinity system (Agilent Technologies) and 6490 Mass Spectrometer (Agilent Technologies) in positive ionization mode. This study used mobile phase A composed of 0.1% formic acid in distilled water (DW) and mobile phase B composed of 0.1% formic acid in ACN. The mobile phases were used with 6 minutes or 10 minutes analytical gradient method. The constant flow of 0.3 mL / min was applied to the Poroshell 120 EC-C18 column and the 0.25 mL / min was applied to the Ascentis® Express F5 column (Table 1). Total 2 μL of sample were injected into the LC-MS/MS system.

The ion source parameters were as follows; 200℃ gas temperature; 16 l/min gas flow rate; 30 psi nebulizer; 3.5 kV electrospray voltage; 200℃ sheath gas temperature, 11 l/min sheath gas flow rate Agilent Jet Stream ion mode. To reduce the large noise signal of the 6 minutes gradient method, different multiple reaction monitoring (MRM) transitions of biotin and internal standard were applied to the 10 minutes gradient method. Details on MS parameters of retention times, *m/z* transition on MRM, fragmentation voltages, and collision energies (CEs) are given in Table 2.

Table 1. Mobile phase gradient parameters for biotin elution with 6 minutes and 10 minutes gradient

6 minutes gradient method			10 minutes gradient method		
Time (min)	Mobile phase A (%)	Mobile phase B (%)	Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.1	80	20	0.3	97	3
			0.31	70	30
2	5	95	2	70	30
			2.1	3	97
4.1	80	20	7	3	97
			7.1	97	3

Table 2. MS/MS conditions and MRM transitions of biotin with different internal standards

	Compound	Retention time (min)	MRM (*m/z*)	Fragmentation voltage	Collision energy
6 minutes gradient method	Biotin (quantitation)	1.15	245.1 → 227.1	380	11
	Biotin (qualification)	1.15	245.1 → 123.1	380	18
	(+)-Biotin hydrazide	0.98	259.1 → 224.1	380	20
10 minutes gradient method	Biotin (quantitation)	3.35	245.1 → 97.1	380	27
	Biotin (qualification)	3.35	245.1 → 123.1	380	28
	Biotin-[d4]	3.35	249.1 → 97.1	380	28

Abbreviation: MRM, multiple reaction monitoring

Analytical performance evaluation

Precision

The precision was evaluated by analyzing the 4 replicates of 7 ng/mL and 30 ng/mL biotin spiked charcoal stripped serum for 6 days. The within-run precision test was repeated 10 times for each concentration. The mean and coefficients of variations (CVs) of each concentration were calculated.

Linearity

The linearity was evaluated according to the method described in CLSI EP 6-A (23). Biotin spiked ACN samples with concentration from 0, 1, 5, 10, 50, 100, 500 ng/mL were measured. The most suitable regression model was evaluated to be linear. If the curve of more than quadratic form is more suitable model, linearity is evaluated by calculating whether the difference from linear formula is within 10% of allowable range.

Lower limit of quantification (LLOQ)

The lower limit of quantitation was investigated by measuring 3 replicates of each 3 ng/mL, 5 ng/mL, 7 ng/mL, 10 ng/mL, 12 ng/mL, and 15 ng/mL biotin spiked ACN. LLOQ was defined as the lowest concentration with a signal-to-noise (S/N) ratio of >10.0.

Matrix effect, recovery, and process efficiency

The matrix effect was evaluated by comparing 5 replicates of 5 ng/mL biotin spiked ACN, charcoal stripped blank serum, and DFS by the method

as described earlier (24). Since biotin is an endogenous material, ACN was used as control and matrix effects were evaluated in blank serum and drug free serum. In brief, the biotin level of blank serum and DFS were measured in two sets; biotin spike before extraction and postextraction. Matrix effect expressed as the ratio of the mean peak area of the biotin spiked postextraction to the mean peak area of the biotin spiked ACN multiplied by 100. Recovery calculated as the ratio of the mean peak area of the biotin spiked before extraction to the mean peak area of the biotin spiked postextraction multiplied by 100. Process efficiency expressed as the ratio of the mean peak area of biotin spiked before extraction to the mean peak area of the biotin spiked ACN multiplied by 100.

Stability

The stability of biotin in serum was evaluated by comparing the biotin concentration of random 3 patient samples on day 0, day 1, day 2, and day 3. Samples were stored in three different temperatures at room temperature for 1 day and at 4℃ and -20℃ for 3 days. The biotin concentrations of day 0 samples and stored samples on day 1 to 3 were measured by LC-MS/MS.

Patient serum sample analysis

To find the patient groups with high serum biotin concentrations, samples from patients with reduced renal function and those who took biotin supplementation were collected and compared with normal controls. Due to concern of biotin degradation, residual serum samples were collected in a single day. First set of samples were selected from patients who have been prescribed Nephvita tab. (Dongindang Pharm, Siheung-si, Korea), which contains 300 ug biotin. The second set of samples were from patients with estimated glomerular filtration rate (eGFR) less than 60 mL/min/1.73m^2 calculated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation and without biotin supplement intake history. Normal controls were selected from patients who had visited the health promotion center for medical checkups. After screening the medical records, patient samples were newly numbered. Collected samples were immediately prepared or frozen at -20℃ and thawed for the analysis.

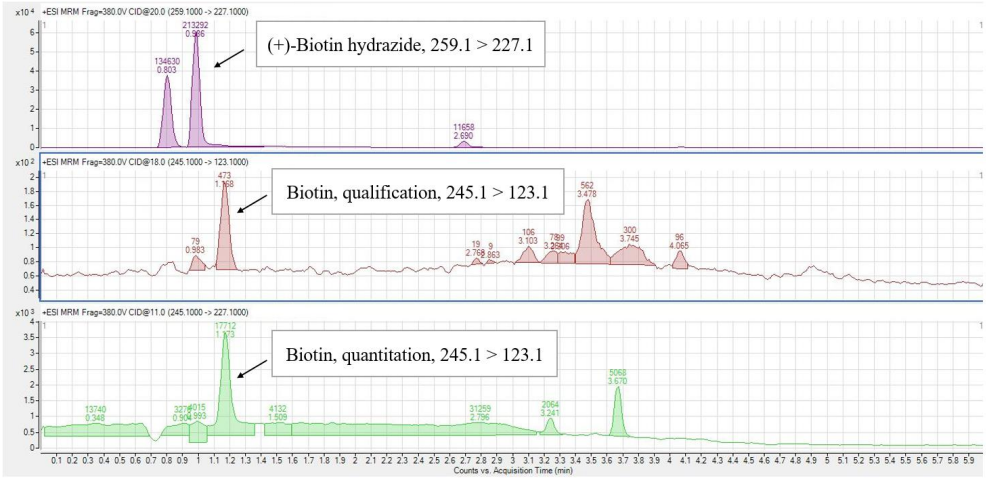
Statistical analysis

Cbstat version 5.1.2 (www.cbstat.com) was used for precision and linearity test evaluation. Within-run, between-run, and within laboratory CV (%) were calculated for precision. For the linearity evaluation, polynomial regression was performed and first-order model compared to second- and third-order model to figure out whether the first-order is the best fit or not by using *t*-test. The *t*-test and ANOVA were performed for the biotin serum level comparison of patient subgroups on R software (http://www.r-project.org) was used. *P* value less than 0.05 was considered statistically significant.

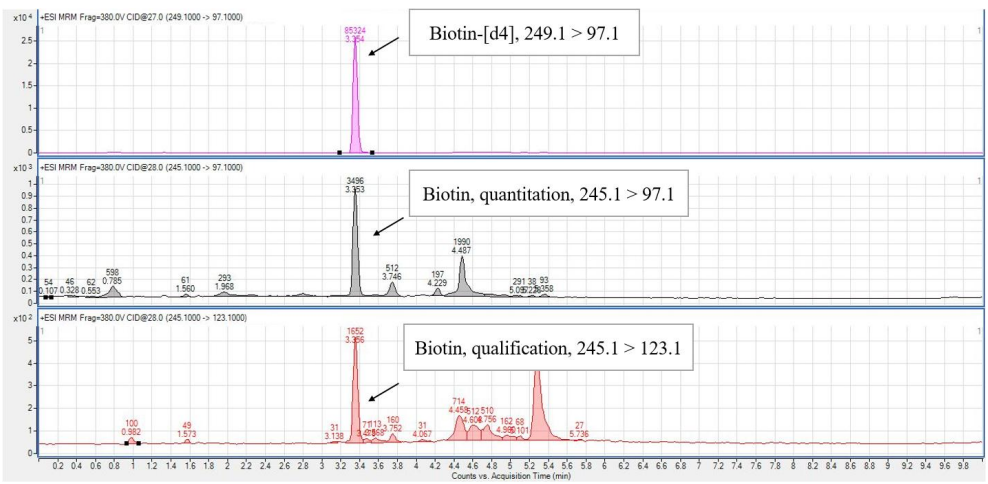
RESULTS

Method development

To develop the optimal LC-MS/MS conditions, several factors including IS compounds, MRM transitions, column types, mobile phase types, gradient and time, and flow rates were changed. The (+)-Biotin hydrazide was not eligible for IS of biotin because the high level of noise signal induced by (+)-Biotin hydrazide in the blank specimen, which lowered the signal to noise ratio of the biotin and consequently interfered with biotin quantification, and finally biotin-[d4] was selected as the IS. At MRM transition of 245.1>227.1, the noise signal was high at around 200, making accurate measurements difficult at low biotin level. To reduce the high noise signal, the MRM *m/z* transition of biotin and biotin-[d4] quantitation were selected 245.1>97.1 and 249.1>97.1, respectively. As a result, the noise signal was decreased to around 50 at this monitored MRM transition. Initially, separation was performed using a Poroshell 120 EC-C18 column, but it was difficult to obtain stable results due to the high interference peaks. After testing the various columns, the Ascentis® Express F5 column was the least affected by interference peaks. To detect the biotin more efficiently and to eliminate the full measure in one test cycle, the time of each test cycle was increased to 10 minutes and the flow rate was reduced to 0.25 mL/min (Figure 3).



(A) 6 minutes gradient method



(B) 10 minutes gradient method

Figure 3. Representative selected reaction monitoring chromatograms of biotin and the IS: (A) calibration sample of 6 minutes gradient method (10 ng/mL biotin) with using (+)-Biotin hydrazide as internal standard and the Poroshell 120 EC-C18 column; (B) calibration sample of 10 minutes gradient method (10 ng/mL biotin) with using the Ascentis® Express F5 column and biotin-[d4] as internal standard.

Precision

The precision result is shown in Table 3. All of within-run, between-run, and within-laboratory CVs (%) were less than 10% at two concentrations.

Table 3. Precision of biotin measurement using LC-MS/MS

	7 ng/mL biotin spiked blank serum	30 ng/mL biotin spiked blank serum
Mean (ng/mL)	7.5	29.0
CV (%)		
Within-run	4.9	1.3
Between-run	7.4	8.8
Within laboratory	8.9	8.9

Abbreviation: CV, coefficient of variation

Linearity

At linear regression, the linear equation was y = 0.0028x + 0.0035 and its R^2 was more than 0.99. When the nonlinearity was evaluated through the comparison with second and third polynomial curve, first degree polynomial model was the best fit (P = 0.108 and 0.899 on second and third polynomial curve, respectively). Excellent linearity was confirmed with a difference of less than 5% in all evaluation concentrations except 1 and 5 ng/mL concentration (Figure 4).

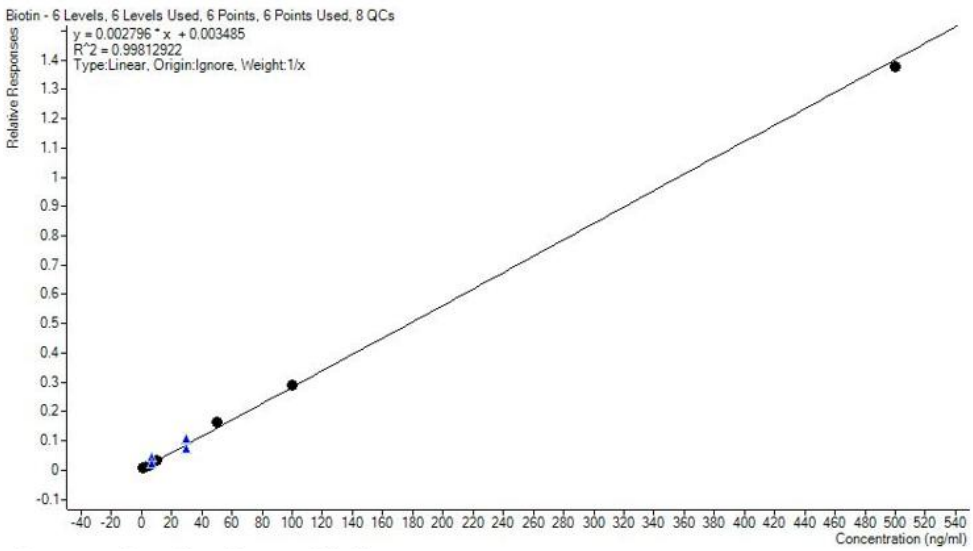


Figure 4. Linearity of serum biotin

LLOQ evaluation

LLOQ evaluation is shown on Table 4. As S/N ratio of 3 ng/mL biotin exceeded 10, 3 ng/mL was confirmed as LLOQ.

Table 4. The lower limit of quantification evaluation of serum biotin

Biotin concentrations (ng/mL)	Mean	CV (%)	S/N ratio mean (range)
3	2.2	2.0	19.7 (17.3-23.7)
5	3.5	2.7	19.2 (17.0-23.2)
7	4.8	0.2	38.3 (28.0-56.2)
10	6.8	2.2	98.4 (53.1-130.9)
12	8.6	1.3	43.3 (37.5-48.8)
15	11.5	1.4	47.0 (41.6-51.3)

Abbreviations: LLOQ, lower limit of quantitation; S/N, signal-to-noise.

Matrix effect, recovery, and process efficiency

The matrix effect, recovery, and process efficiency were evaluated by comparing 5 ng/mL biotin spiked ACN, blank serum, and DFS (Table 5). The matrix effect was higher in DFS than in blank serum, but the degree of the effect was acceptable. In contrast, recovery rate was higher in DFS than in blank serum. Finally, the process efficiency was more than 80% in both blank serum and DFS and the two types of matrix showed similar performance.

Table 5. Matrix effect evaluation of serum biotin

	Mean peak area					Matrix effect (%)		Recovery (%)		Process efficiency (%)	
5 ng/mL biotin spiked	ACN	Blank serum spiked after extraction	DFS spiked after extraction	Blank serum spiked before extraction	DFS spiked before extraction	Blank serum	DFS	Blank serum	DFS	Blank serum	DFS
	4.7	4.6	4.4	3.9	4.1	97.5	93.5	84.4	91.5	82.3	85.6

Abbreviation: DFS, drug free serum

Stability

The biotin concentrations decreased regardless of storage temperature and number of storage days (mean biotin values 33.1 ± 1.5 ng/mL on day 0, 16.1 ± 3.3 ng/mL on day 1, 25.4 ± 2.7 ng/mL on day 2, and 21.5 ± 2.1 ng/mL on day 3, $P < 0.001$). The biotin concentrations when stored in room temperature, 4℃, and -20℃ were 23.2 ± 10.9 ng/mL, 20.3 ± 3.8 ng/mL, and 22.6 ± 4.5 ng/mL, respectively, and were not statistically significant ($P = 0.639$).

Comparison of the biotin level patients administered the Nephvita tab., patients with decreased eGFR, and normal controls

A total of 298 samples of biotin level were analyzed by the LC-MS/MS method. Patients were divided into 3 groups and 117 patients were administered the Nephvita tab, 85 patients with decreased eGFR and 96 with normal control groups (Table 6). About 88% of patients in 'Nephvita group' and the 'decreased eGFR group' were CKD patients and their eGFRs which were estimated by CKD-EPI calculation were 26.1 ± 9.0 and 11.0 ± 4.5, respectively. The distribution of biotin serum concentration was centered between 15 and 35 ng/mL, and 98.3% (293/298) of samples had biotin concentrations of more than 10 ng/mL (Figure 5). The serum biotin levels between the 3 groups did not show any statistically significant differences (P = 0.085). The causes of CKD were divided into diabetes (DM), glomerulonephritis (GN), hypertension (HTN) and polycystic kidney disease (PKD). Patients whose cause of CKD were not listed in the medical record were classified as unknown. The other CKD causes group was composed of patients with systemic lupus erythematosus, multiple myeloma, IgG4-related disease, septic AKI, urinary tract infection, hepatitis-B-associated glomerulonephritis, neurogenic bladder, and megaureter. Eight patients in the Nephvita group and 11 patients in the reduced eGFR group had acute kidney injury (AKI). A total of 1 of 8 patients in the Nephvita group and 3 of 11 patients in the reduced eGFR group were AKI on CKD status. Sixty-eight (58.1%) patients in the Nephvita group and 34 (40.0%) patients in the reduced eGFR group have received the hemodialysis (HD). Eleven (9.4%) patients in the Nephvita group and 1 (1.2%) patient in the reduced eGFR group have received the peritoneal dialysis hemodialysis (PD).

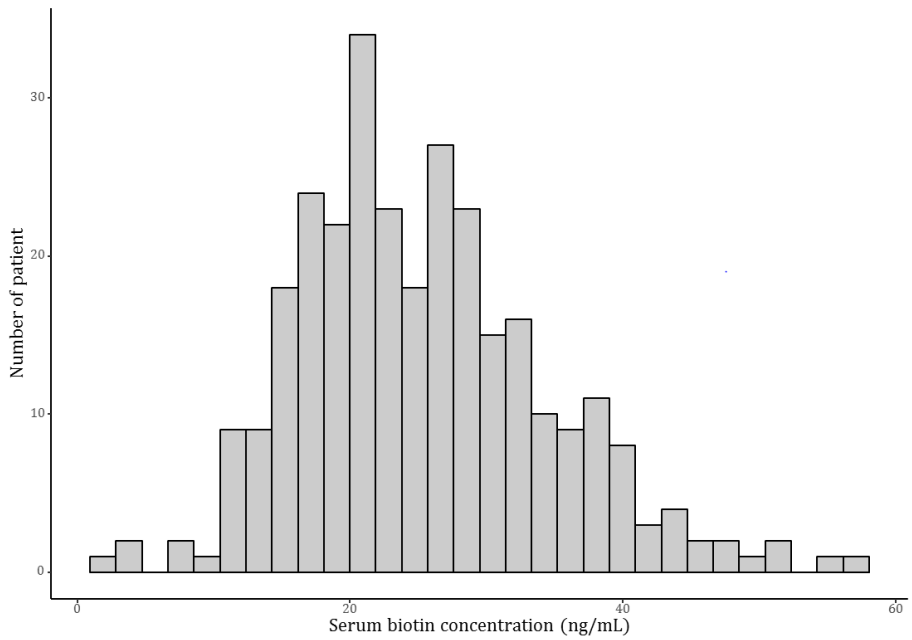


Figure 5. Distribution of serum biotin level in 298 patient samples

Table 6. Patient demographics and serum biotin level

	Nephvita administered	Reduced eGFR	Normal controls	*P*
	(N=117)	(N=85)	(N=96)	
Age	60.6 ± 15.8	62.8 ± 12.4	58.3 ± 12.3	0.086
Sex				0.028
M	62 (53.0%)	57 (67.1%)	46 (47.9%)	
F	55 (47.0%)	28 (32.9%)	50 (52.1%)	
CKD-EPI				
	16.5 ± 21.4	1.0 ± 4.5	91.4 ± 14.6	< 0.001
Biotin	26.1 ± 9.0	23.5 ± 9.4	26.0 ± 9.1	0.085
CKD stage				
1	2 (1.7%)	0 (0%)	55 (57.3%)	
2	3 (2.6%)	0 (0%)	41 (42.7%)	
3	13 (11.1%)	0 (0%)	0 (0%)	
4	10 (8.5%)	23 (27.1%)	0 (0%)	
5	89 (76.1%)	62 (72.9%)	0 (0%)	
CKD				< 0.001
Yes	104 (88.9%)	75 (88.2%)	0 (0%)	
No	13 (11.1%)	10 (11.8%)	96 (100.0%)	
CKD cause				0.180
DM	28 (37.3%)	34 (32.7%)		
Unknown	25 (33.3%)	22 (21.2%)		
GN	11 (14.7%)	22 (21.2%)		
HTN	2 (2.7%)	8 (7.7%)		
PKD	3 (4.0%)	10 (9.6%)		
Other	6 (8.0%)	8 (7.7%)		
AKI				0.002
Yes	8 (6.8%)	11 (12.9%)	0 (0%)	
No	109 (93.2%)	74 (87.1%)	96 (100.0%)	

HD				< 0.001
Yes	68 (58.1%)	34 (40.0%)	0 (0%)	
No	49 (41.9%)	51 (60.0%)	96 (100.0%)	
PD				0.001
Yes	11 (9.4%)	1 (1.2%)	0 (0%)	
No	106 (90.6%)	84 (98.8%)	96 (100.0%)	

Abbreviations: eGFR, estimated glomerular filtration rate; CKD, chronic kidney disease; CKD-EPI, the Chronic Kidney Disease Epidemiology Collaboration; DM, diabetes; GN, glomerulonephritis; HTN, hypertension; PKD, polycystic kidney disease; AKI, acute kidney injury; HD, hemodialysis; PD, peritoneal dialysis.

In the CKD group, there was no significant difference in biotin concentration between the patients who took the Nephvita and those who did not (Nephvita taking group = 25.63 ± 8.53 ng/mL, Nephvita not taking group = 23.4 ± 9.6 ng/mL, P = 0.110). There was no difference in biotin concentration between the dialysis and non-dialysis CKD group (dialysis group = 23.9 ± 8.2 ng/mL, non-dialysis group 26.1 ± 10.2 ng/mL, P = 0.144). On the other hand, in the non-dialysis CKD group, the patients who were administered the Nephvita had a significantly higher biotin concentration than patients group who were not administered the Nephviata and normal control (29.9 ± 11.0 ng/mL on Nephvita taking group, 23.7 ± 9.3 ng/mL on Nephvita not taking group, and 26.0 ± 9.1 ng/mL on normal control, P = 0.011).

There were total of 21 patients (7.0%) with biotin concentration of 40 ng/mL or higher. Nine of 21 were in the Nephvita group, 6 were in the reduced eFGR group, and 6 were in the normal control group. Sixteen of 21 patients were male which meant that only approximately a third were female, but no other common clinical history was found.

DISCUSSION

This study was performed to demonstrate the methods for measuring levels of biotin in human serum using LC-MS/MS method within a clinical laboratory. Although high biotin levels have been reported as a considerable interference cause, most of the case studies noticed the interference through comparison of the results via different analytical systems (12, 25) and/or by discrepancy with clinical feature. High biotin levels induce the interference on the biotin-streptavidin based analysis not on the other immunoassay. However, even the analyses target the same measurand and using biotin-streptavidin based method, it is known that interference occurs depending on the different analytical system. There are a few ways to prove the high biotin level and interference. Piketty et al. reported the biotin precipitation method using enriched streptavidin (26). Direct biotin quantitation is the basic hypothesis-proving clue, and it is reported that about 7% of outpatient samples were over the 10 ng/mL which is the lowest known threshold (10 ng/mL) for biotin interference in Roche Diagnostics immunoassay tests. There are a few biotin immunoassay kits (e.g. Immundiagnostik, GmbH, Bensheim, Germany), they are only applicable for research use and not available in Korea. It measures not only biotin but also biotin metabolite. The specific and quantifiable method like LC-MS/MS is required for the direct measurement the biotin.

In method development, this study chose a different MRM *m/z* transition from the previous report. Most reports selected the biotin MRM *m/z* 245.1>227.1 and biotin-[d4] MRM *m/z* 249.1>231.1 with an 18 molecular weight difference. Considering that the 18 molecular weight is H_2O, the different MRM *m/z* transitions were selected to avoid detecting the common molecule subtraction for the reduction of noise, which were actually effective in reducing noise. In future studies, changing the extraction process

should be studied in order to detect lower concentration of biotin.

In method validation, precision showed acceptable CV (%) values, which were less than 15%. The linearity was also satisfied. LLOQ was confirmed as 3 ng/mL. Biotin concentration decreased regardless of storage temperature and number of storage days. Therefore, for accurate determination of serum biotin concentration, analysis on the day of sample collection is important. Accuracy at lower than 1 ng/mL biotin level should be improved for detect biotin deficiency, even though apparent matrix effect was observed in our experiment and the other report (18). This method is expected to be able to measure the high biotin level, which makes the interference on several analytical systems using biotin-streptavidin immunoassay.

The reason for the choice of patient groups was to test the hypothesis that patients who take supplements containing biotin are more likely to have higher biotin concentration of their blood, and patients with reduced eGFR assumed that their blood concentration would be high due to the slow biotin elimination. Also, since it was not known about the distribution of biotin concentration in Korea and the effect of the clinical laboratory according to the high concentration biotin, the biotin concentration of normal control was measured. In our hospital, a high dose of biotin has not been prescribed, but multivitamin, Nephvita (which contains biotin 300 μg), has been used for nephropathy patients. Unlike the previous report that serum biotin and its metabolite levels in nephropathic patients on hemodialysis are higher than in normal controls, patients with HD did not show statistically significant difference with normal controls (19, 20). On the other hand, comparing the reduced eGFR group who were not receiving HD with the patients who were administered the Nephvita, the second group had the higher levels of serum biotin. This is likely to be due to the slower elimination of biotin, while the higher intake of biotin at higher doses than Nephvita may lead to higher concentrations. In the measured samples in this study, no one seemed

to take mega-dose of biotin within two or three days of sample measurement. However, most of the samples (98.3%, 293/298) had levels higher than 10 ng/mL, which could result in interference of the frequently used diagnostics systems. The result showed much higher percentage comparing to the previous result (7.4%) which was carried out for visitors in emergency department in a U.S hospital (27). Biotin is known to be widely contained in egg yolk, livers and some vegetables (28). The overall hyperbiotinemia of Koreans could be induced by the diet, although the exact reason of hyperbiotinemia still remains unknown. The difficulty of harmonization with the previously reported methods could be the reason of overt hyperbiotinemia. It is difficult to compare with other laboratory results because to my knowledge, there is no other clinical laboratory measuring the serum biotin concentration using LC-MS/MS in Korea. The comparison of this method with the commercial biotin quantitation kit should be studied in the future. Clinical laboratorians should be alerted of Koreans' high concentration of biotin to consider the situation that may cause the interference.

Lowering the LLOQ may be necessary to detect biotin deficiency caused by malnutrition of newborn or congenital diseases such as biotinidase deficiency, multiple carboxylase deficiency, and holocarboxylase synthetase deficiency. A degree of neuropathy progression and biotin concentration is an interesting subject. A significant reduction of biotin in cerebrospinal fluid and serum was reported in patients with multiple sclerosis in a previous study (29). Other neuropathies such as diabetes or dialysis related peripheral neuropathy and their correlation with biotin concentration could be investigated in the future. A reference interval of biotin in Korean could be calculated from serum samples of Korean National Health and Nutrition Examination Survey by this method.

In conclusion, the LC-MS/MS method for biotin quantitation has been

developed and it is applicable for detecting the evaluation of serum biotin and for analyzing samples with possible interference. Our patients had relatively high biotin levels. It is important to note that patients with decreased renal function may have elevated serum biotin levels if high-dose biotin supplement is administered, and supplemental drug history should be investigated if the interference is suspected on frequently used analytical systems using biotin-streptavidin immunoassay.

REFERENCES

1. Pacheco-Alvarez D, Solórzano-Vargas RS, Del Río AL. Biotin in metabolism and its relationship to human disease. Archives of medical research. 2002;33(5):439-47.
2. Biotin Deficiency: Medscape; [updated Oct 22, 2018. Available from: https://emedicine.medscape.com/article/984803-clinical.
3. LabCorp. LabCorp Vitamin B7 (Biotin) test catalog entry Available from: https://www.labcorp.com/test-menu/36691/vitamin-bsub7-sub
4. Clerico A, Plebani M. Biotin interference on immunoassay methods: sporadic cases or hidden epidemic? Clin Chem Lab Med. 2017;55(6):777-9.
5. Available from: http://www.foodnews.co.kr/news/articleView.html?idxno=66552.
6. Chun KY. Biotin Interference in Diagnostic Tests. Clin Chem. 2017;63(2):619-20.
7. Sedel F, Papeix C, Bellanger A, Touitou V, Lebrun-Frenay C, Galanaud D, et al. High doses of biotin in chronic progressive multiple sclerosis: a pilot study. Mult Scler Relat Disord. 2015;4(2):159-69.
8. Diamandis EP, Christopoulos TK. The biotin-(strept) avidin system: principles and applications in biotechnology. Clinical chemistry. 1991;37(5):625-36.
9. Samarasinghe S, Meah F, Singh V, Basit A, Emanuele N, Emanuele MA, et al. Biotin Interference with Routine Clinical Immunoassays: Understand the Causes and Mitigate the Risks. Endocr Pract. 2017;23(8):989-98.
10. Roche Diagnostics. Elecsys T3 package insert, 2017-10, V 2.0 English.
11. Kwok JS, Chan IH, Chan MH. Biotin interference on TSH and free thyroid hormone measurement. Pathology. 2012;44(3):278-80.
12. Elston MS, Sehgal S, Du Toit S, Yarndley T, Conaglen JV. Factitious Graves' Disease Due to Biotin Immunoassay Interference-A Case and Review of the Literature. J Clin Endocrinol Metab. 2016;101(9):3251-5.
13. The FDA Warns that Biotin May Interfere with Lab Tests: FDA Safety Communication 2017, updated November 28, 2017.; Available from: https://www.fda.gov/medicaldevices/safety/alertsandnotices/ucm586505.htm.
14. Li D, Radulescu A, Shrestha RT, Root M, Karger AB, Killeen AA, et al. Association of Biotin Ingestion With Performance of Hormone and Nonhormone

Assays in Healthy Adults. JAMA. 2017;318(12):1150-60.
15. Peyro Saint Paul L, Debruyne D, Bernard D, Mock DM, Defer GL. Pharmacokinetics and pharmacodynamics of MD1003 (high-dose biotin) in the treatment of progressive multiple sclerosis. Expert Opin Drug Metab Toxicol. 2016;12(3):327-44.
16. Mardach R, Zempleni J, Wolf B, Cannon MJ, Jennings ML, Cress S, et al. Biotin dependency due to a defect in biotin transport. The Journal of clinical investigation. 2002;109(12):1617-23.
17. Livaniou E, Evangelatos GP, Ithakissios DS, Yatzidis H, Koutsicos DC. Serum biotin levels in patients undergoing chronic hemodialysis. Nephron. 1987;46(3):331-2.
18. Yagi S, Nishizawa M, Ando I, Oguma S, Sato E, Imai Y, et al. A simple and rapid ultra-high-performance liquid chromatography-tandem mass spectrometry method to determine plasma biotin in hemodialysis patients. Biomed Chromatogr. 2016;30(8):1285-90.
19. Oguma S, Ando I, Hirose T, Totsune K, Sekino H, Sato H, et al. Biotin Ameliorates Muscle Cramps of Hemodialysis Patients: A Prospective Trial. The Tohoku Journal of Experimental Medicine. 2012;227(3):217-23.
20. Fujiwara M, Ando I, Yagi S, Nishizawa M, Oguma S, Satoh K, et al. Plasma Levels of Biotin Metabolites Are Elevated in Hemodialysis Patients with Cramps. Tohoku J Exp Med. 2016;239(4):263-7.
21. Merck Index. 11th ed. 1244 p.
22. Available from: https://www.sigmaaldrich.com/catalog/product/sial/14403?lang=ko&region=KR.
23. CLSI. Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline. CLSI document EP06-A. Wayne, PA: Clinical and Laboratory Standards Institute; 2003.
24. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem. 2003;75(13):3019-30.
25. Meany DL, Jan de Beur SM, Bill MJ, Sokoll LJ. A case of renal osteodystrophy with unexpected serum intact parathyroid hormone concentrations. Clin Chem. 2009;55(9):1737-9.

26. Piketty ML, Prie D, Sedel F, Bernard D, Hercend C, Chanson P, et al. High-dose biotin therapy leading to false biochemical endocrine profiles: validation of a simple method to overcome biotin interference. Clin Chem Lab Med. 2017;55(6):817-25.
27. Katzman BM, Lueke AJ, Donato LJ, Jaffe AS, Baumann NA. Prevalence of biotin supplement usage in outpatients and plasma biotin concentrations in patients presenting to the emergency department. Clin Biochem. 2018.
28. Zempleni J, Kuroishi T. Biotin. Advances in Nutrition. 2012;3(2):213-4.
29. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem. 2003;75(13):3019-30.

초록

액체크로마토그래피질량분석기를 이용한 인체 혈청 비오틴 정량법과 multivitamin 복용과 신기능이 biotin 농도에 미치는 영향 연구

배경: 비오틴은 비타민 B7 혹은 비타민 H로 알려진 수용성 비타민으로, 대사작용의 coenzyme으로 작용한다. 비오틴 결핍은 매우 드문 것으로 알려져 있으나, 비오틴 제제의 고용량 섭취로 인한 비오틴-스트렙타비딘 면역법을 쓰는 여러 장비에서 간섭현상을 일으키는 것으로 알려져 있다. 이 논문은 액체크로마토그래피질량분석기를 이용한 혈청 비오틴 정량법을 설정하고, 신기능 저하 환자와 건강검진군의 혈청 비오틴 농도를 측정하여 비오틴 농도가 면역측정법에 미칠 수 있는 잠재적인 영향을 예측하고자 한다.

방법: 특정 설정 농도의 비오틴를 첨가한 acetonitrile과 공혈청, 그리고 환자 혈청 검체를 양성 이온 모드로 전기분무이온화하여 분리, 검출하였다.

결과: 검사일 간 변동 계수는 10% 미만, 검사실 내 변동 계수는 15% 미만, 반복성시험에서 변동 계수는 5% 미만으로 정밀도는 양호하였다. 선형성은 일차식을 만족하였고 R^2값은 0.99 이상으로 우수했다. 정량 한계는 3 ng/mL이었으며, 기질 효과 검사에서 이온 억제 현상을 보였고, 비오틴 농도는 4℃와 -20℃ 조건 모두에서 초기 농도보다 보관 농도가 감소하였다. 분석 검체 중 98% 이상이 Roche사의 면역측정법에서 간섭현상을 일으킬 수 있는 최소 농도인 10 ng/mL를 초과하였다. 투석치료를

하지 않는 만성신질환군에서 biotin 300 μg을 포함한 멀티비타민을 복용한 군이 복용하지 않은 군보다 biotin 농도가 유의하게 높았다 (멀티비타민복용군 29.9 ± 11.0 ng/mL, 멀티비타민 비복용군 23.7 ± 9.3 ng/mL, 정상대조군 26.0 ± 9.1, P = 0.011).

결론: 이 정량법은 간섭현상을 일으킬 수 있는 고농도의 비오틴을 측정할 수 있다. 진단검사의학과의 사람들은 한국인의 높은 biotin 농도와 이로 인한 간섭현상이 일어날 수 있는 가능성에 대해 주지할 것을 권고한다.

주요어: 비오틴, 인체 혈청, 액체크로마토그래피질량분석기, 간섭현상
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