Korean J. Food Sci. An.
Vol. 32, No. 5, pp. 571~577(2012)
DOI http://dx.do.org/10.5851/kosfa.2012.32.5.571

ARTICLE

Development of Vitamin D Determination in Infant Formula by Column-Switching HPLC with UV Detector

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Abstract

This study was carried out to develop an analytical method for the determination of vitamin D in infant formula. Vitamin D was determined by column-switching high-performance liquid chromatography (HPLC) equipped with a reversed phase column and UV detector after saponification and extraction of the formula with an organic solvent. A pre-separation column (C_8), focusing column (C_{18}), analytical column (C_{18}) and UV-Vis detector (254 nm) were used. The limits of detection (LOD) and the limits of quantification (LOQ) for vitamin D were estimated to be 1.51 μg/kg and 4.95 μg/kg, respectively. The linearity, recovery, precision and accuracy of the analytical method for vitamin D were evaluated through the application of a SRM (Standard Reference Material) 1846 (National Institute of Standard & Technology, USA). The linearity of this method was calculated with a value of the coefficient of determination (r^2) ≥0.9999. The recovery of vitamin D was 85.20±3.00%. The intra-assay precision for vitamin D was between 1.68±0.03% and 5.75±0.33%, and the inter-assay precision for vitamin D ranged from 1.73±0.03% to 2.96±0.09%. The intra-assay accuracy for vitamin D was between 100.03±2.77% and 102.01±0.59%, and the inter-assay accuracy for vitamin D ranged from 99.00±1.53% to 102.01±3.04%. The proposed method is optimal for the separation and quantification of vitamin D from infant formula.

Key words: vitamin D, column-switching high-performance liquid chromatography, UV detection, infant formula

Introduction

Infant formula consists of various ingredients, including inorganic matter and vitamins to supply the nutritional requirements of infants and children (Codex, 2007). Infant formula is the only processed foodstuff that entirely satisfies the nutritional requirements of infants during the first months of life until the introduction of appropriate complementary feeding (The commission of the European communities, 2006). Among the nutritional ingredients in infant formula, the vitamin D is very low levels of fat-soluble vitamin (Heudi *et al*., 2004). Vitmain D is one of the most important essential bioregulators of the Ca^{2+} and phosphate metabolism in higher animals (Friedrich, 1988). Vitamin D and its hydroxyl metabolites play significant roles as hormonal regulators of calcium metabolism and are associated primarily with bone health (Bell *et al*., 1979). Vitamin D deficiency leads to rickets in children and osteomalacia in adults (Friedrich, 1988) and the quantification of vitamin D is widely used as a means of clinical testing for several pathophysiological states (e.g., parathyroid gland disorders, renal failure, vitamin-dependent rickets and sarcoidosis) (Donald and Gerhard, 1992). For this reason, the accurate determination of low levels of vitamin D in infant formula is crucial to safeguard the health of infants and children.

Traditionally, the existing procedures for vitamin D determination include colorimetric method (Gharbo and Gosser, 1974; Hassan, 1980), high-performance liquid chromatographic method with UV detection (AOAC, 2005; KFDA, 2009) and mass spectrometry (Heudi *et al*., 2004; Kamao *et al*., 2007; Soldin *et al*., 2009). Complex matrix sample, such as infant formula, requires a highly complicated sample preparation methods to remove interference for detection of low level vitamin D. The high-

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performance liquid chromatographic (HPLC) method by KFDA (2009) and AOAC (2005) consists of two steps to remove interference in infant formula. Vitamin D fractions are collected from interfering substances on a cleanup column, and then an analytical procedure is carried out. However, this procedure is complicated and requires a long period of time; therefore, it is not suitable for handling a lot of samples. Recently, column-switching high performance liquid chromatographic method is used to eliminate the complicated sample cleanup step in sample preparation. Column-switching method is a rapid automated online extraction procedure and is successfully used to identify a wide range of analytes in complicated samples (Brunetto *et al*., 2004; Christians *et al*., 2000; Hartmann *et al*., 2001; Knebel *et al*., 2000). This study aims to develop vitamin D detection method in infant formula by column-switching high performance liquid chromatography with a rapid automated online extraction procedure without complicated sample cleanup step.

Materials and Methods

Reagents and materials

The experiments were performed using infant formula SRM (Standard Reference Material) 1849 (National Institute of Standard & Technology, USA), which contains 117.00±11.00 μg/kg of vitamin D. Sixteen kinds of commercial infant formula samples were collected from different markets in Daejeon, Korea. These samples were kept at room temperature. All reagents and solvents used were of HPLC or analytical reagent grade. The vitamin D standard was purchased from Sigma (USA). Water, methanol, absolute ethanol and hexane were obtained from E. Merck (Germany). Pyrogallol was supplied from Samchun Pure Chemical (Korea). Potassium hydroxide and sodium sulfate were purchased from Junsei Chemical (Japan). High-purity water was obtained through an Easy Pure system (Barnstead, USA).

Standard solutions

A stock solution (1000 mg/L) of the vitamin D standard was prepared in a 100 mL volumetric flask by dissolving 0.1 g of vitamin D in 100 mL of methanol. Working standards were prepared daily for analysis by diluting the vitamin D stock standard solution in a solution of methanol:ethanol:H_2O (74.7:8.3:17, v/v) to the desired range of 10-100 μg/L.

Sample preparation

Sample preparation, except for the dissolving step in water, sample volume and final elute solution volume, was performed by the KFDA official method (KFDA, 2009). The sample preparation procedure was optimized by improving a previous extraction procedure with organic solvents. 1 g of infant formula (5 g liquid sample) was weighed accurately and transferred into a 250 mL brown round-bottom flask. After the addition of 3 mL of water, the flasks were shaken enough to dissolve the formula. 40 mL of 10% pyrogallol:ethanol (1:10, w/v) was added, and the flasks were shaken slowly. 10 mL of KOH:H_2O (9:1, w/v) was added, and the samples were refluxed for 30 min in a steam bath at 90°C. The samples were cooled rapidly under running H_2O. After the addition of 50 mL of hexane, the flasks were shaken vigorously three times for 10 min each.

The collected hexane extract liquid was transferred to brown separatory funnels with 100 mL of 1 N KOH. The funnels were shaken vigorously for 15 s. They were allowed to stand until both layers were clear, and then the aqueous layer was drained. 40 mL of 0.5 N KOH was added, and the mixture was shaken vigorously for 15 s. The funnels were allowed to stand until both layers were clear, and the aqueous layer was then drained. The remaining fraction was washed with 50 mL portions of H_2O until the last washing was neutral to phenolphthalein. The last few drops of H_2O were drained, and the remaining sample was dehydrated by sodium sulfate. The hexane layer was collected in a 250 mL brown round-bottom flask and evaporated to dryness under a vacuum by swirling in a water bath at ≤40°C. The residue was dissolved immediately in 5 mL of MeOH and filtrated by a PTFE filter. A 210 μL aliquot of this solution was then ready to be injected into the column-switching HPLC system.

Instruments

The experiments were performed using a liquid chromatographic Shiseido NANO SPACE SI-2 system (Shiseido, Japan) connected to a Shiseido UV-Vis detector and with SMC-21 software for instrument control and data collection and processing. Detection was carried out at 254 nm. Injections were made with a 100 μL loop. For column-switching purposes, a column-switching six-port valve (Shiseido, Japan) controlled by the SMC-21 software was used, along with an additional pump 3001 (Shiseido, Japan) to deliver the extraction mobile phase.

Column-switching HPLC system

Pre-separation step

At this step, a volume of 30 μL of 1 mg/L of a vitamin D standard solution was injected onto the pre-separation column using the pre-separation mobile phase. The switching valve position is load position. While the endogenous compounds were flushed to waste, the analytes were retained by the hydrophobic inner surface of the pre-separation column. The pre-separation efficiency and retention time were considered with eight different mobile phases and three types of columns for establishing the optimum condition. The reverse phase C_8 (Capcell-pak MF, 4.6×150 mm, 5 μm), C_{18} (Sunfire, 4.6×150 mm, 3.5 μm) and C_{18} (ODP2 HP-4D, 4.6×150 mm, 5 μm) columns were tested. The mobile phase composition was tested with methanol:ethanol:H_2O (74.7:8.3:17, v/v), methanol:H_2O (90:10, v/v), methanol:H_2O (80:20, v/v), acetonitrile:H_2O (90:10, v/v), acetonitrile:H_2O (85:15, v/v), methanol:H_2O (75:25, v/v), methanol:H_2O (70:30, v/v) and acetonitrile:H_2O (80:20, v/v).

Focusing step

A 1 mg/L vitamin D standard solution was injected onto a pre-separation column to determine the switching time 30 s before and after the vitamin D peak. While determining the switching time, the switching valve was in the inject position, and the pre-separation column, focusing column were connected in series. At the focusing step, the reverse phase C_{18} (Capcell-pak UG120V, 2.0×35 mm, 5 μm) column was used in focusing column and ethanol:methanol (10:90, v/v) was used in mobile phase.

Analytical step

After switching time is setted, the switching valve was changed back to the load position to recondition the pre-separation column with the pre-separation mobile phase to be ready for the next injection. In the meantime, the analytes were separated in the analytical column under isocratic mode elution and detected by the UV-Vis detector at 254 nm. The analytical column efficiency was considered with two different mobile phases and a column for establishing the optimum condition. The reverse phase C_{18} (Capcell-pak UG120V, 4.6×250 mm, 5 μm) column was tested. The two different mobile phases were methylene chloride:methanol (10:90, v/v) and ethanol:methanol (10:90, v/v).

Validation

The method validation were evaluated in terms of linearity, LOD, LOQ, accuracy, precision, recovery and inter-laboratory test according to ICH harmonized tripartite guideline, IUPAC technical report and AOAC guideline (AOAC Committee report, 1989; ICH Expert Working Group, 2005; Michael *et al*., 2002). The linearity was evaluated by the coefficient of determination (r^2) and was calculated for three consecutive standard curves. The limits of detection (LOD) and quantification (LOQ) were determined by serial dilutions of vitamin D solutions to obtain signal/noise ratios of approximately 3:1 for LOD and approximately 10:1 for LOQ. To evaluated accuracy and precision, samples spiked with three levels concentrations of vitamin D (10, 40 and 80 μg/L). The accuracy was determined by comparing the theoretical concentrations of vitamin D standards and those obtained by chromatographic analysis. This was expressed as a percentage of the mean of the quantitative values of the reference samples, was calculated as (contentdetermined in spiked sample-contentdetermined in unspiked sample)/spiked content×100. The precision, which was calculated as the relative standard deviation (R.S.D., %), was obtained at three levels: intra-(within 24 h) and inter-day (for three consecutive days) precision. The recovery rates were calculated by comparing the theoretical concentrations of vitamin D in SRM 1846 and those obtained by chromatographic analysis. To evaluate inter-laboratory tests of this method, a recovery test was performed by three laboratories. Sixteen kinds of commercial infant formula were collected from different markets in Daejeon, Korea. These samples were kept at room temperature. The pre-separated samples were analyzed by HPLC with a switching valve. Every experiments for the method validation were analyzed in three replicates.

Results and Discussion

Column-switching HPLC system optimization

Pre-separation step

Eight different mobile phases and three types of columns were tested. The reverse phase C_8 (Capcell-pak MF, 4.6×150 mm, 5 μm) column and methanol:ethanol:H_2O (74.7:8.3:17, v/v) were selected as the optimum conditions for the pre-separation step. A working mobile phase of methanol:ethanol:H_2O (74.7:8.3:17, v/v) elevated the retention time to 12 min, providing high-resolution separation of vitamin D from endogenous compounds. The separated

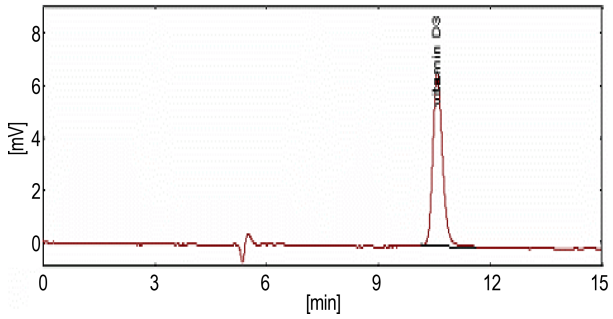


Fig. 1. Chromatogram of vitamin D standard in mobile phase methanol:ethanol:H_2O (74.7:8.3:17, v/v).

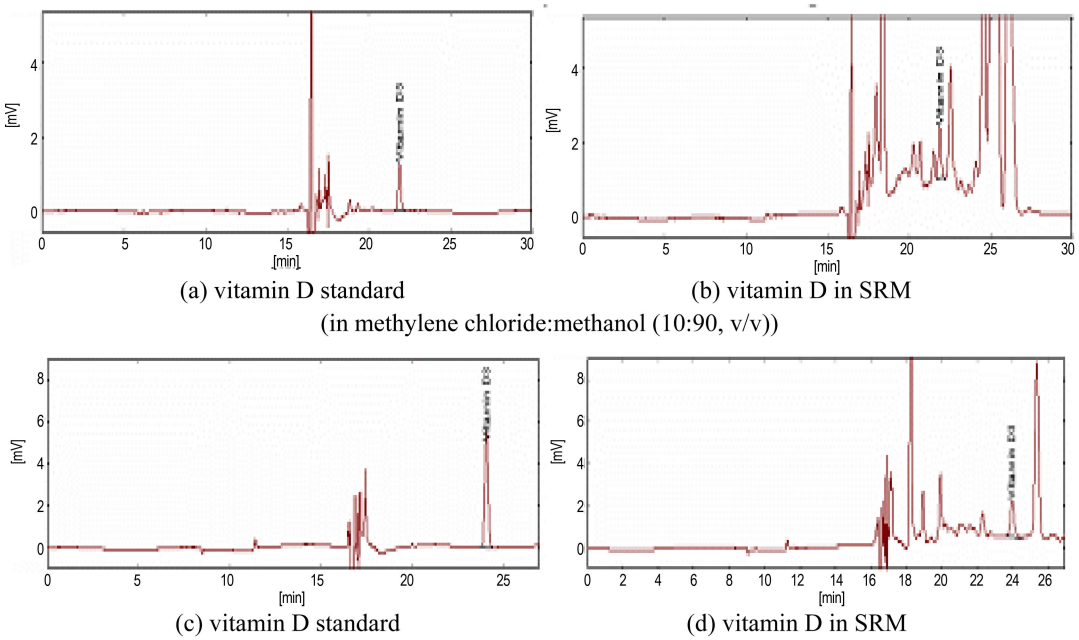
vitamin D peak was sharp and symmetrical, as shown in Fig. 1. Seven different mobile phases except methanol :ethanol:H_2O (74.7:8.3:17, v/v) did not present adequate responses for vitamin D quantitation and provided unsuitable retention times for the active substance. This result shows that the eluent comprising only with methanol or acetonitrile plus H_2O is not sufficient to separate and detect vitamin D in the test solution. Ethanol showed great efficiency to synergize for separating in the column and detect with UV.

Focusing and analytical step

At the focusing step, the reverse phase C_{18} (Capcell-pak UG120V, 2.0×35 mm, 5 μm) column was used in focusing column and ethanol:methanol (10:90, v/v) was used in mobile phase. At the analytical step, two different mobile phases were tested. A reverse phase C_{18} (Capcell-pak UG120V, 4.6×250 mm, 5 μm) column was tested in methylene chloride:methanol (10:90, v/v) and ethanol:methanol (10:90, v/v). The reverse phase C_{18} (Capcell-pak UG120V, 4.6×250 mm, 5 μm) column had a high separate efficiency in an ethanol:methanol (10:90, v/v) mobile phase. The separated vitamin D peak was sharp and symmetrical, and this condition avoided interferences from equipment noise. The reverse phase C_{18} column and an ethanol:methanol (10:90, v/v) mobile phase were determined to be the optimum conditions for the analytical step (Fig. 2). This result shows that the polarity of ethanol was more efficient to separate vitamin D in infant formula than methylen chloride.

Method Validation

The method validation were evaluated according to ICH harmonized tripartite guideline, IUPAC technical report and AOAC guideline (AOAC Committee report, 1989; ICH Expert Working Group, 2005; Michael *et al*., 2002). The linearity of this method was calculated with a value of the coefficient of determination (r^2) ≥0.9999. The LOD is defined as the lowest active substance concentration that can be determined by the method. By contrast, the LOQ is the concentration of the sample that is obtained with adequate precision and accuracy. An estimation of the limits can be achieved by determination of the signal/noise ratios at 3:1 (LOD) and 10:1 (LOQ). For the new HPLC method for vitamin D determination, the LOD and LOQ values were 1.51 and 4.95 μg/kg, respectively (Table



(a) vitamin D standard (b) vitamin D in SRM
(in methylene chloride:methanol (10:90, v/v))

(c) vitamin D standard (d) vitamin D in SRM
(in EtOH:MeOH (10:90, v/v))

Fig. 2.Chromatograms of vitamin D standard and SRM in various mobile phases.

Table 1. Analysis instrument conditions of vitamin D by column-switching HPLC systmems

Parameter	Condition
Column	Pre-separation column: Capcell-pak MF C_8 (4.6×150 mm, 5 μm) Focusing column: Capcell-pak UG120V C_{18} (2.0×35 mm, 5 μm) Analytical column: Capcell-pak UG 120V C_{18} (4.6×250 mm, 5 μm)
Flow rate	Pre-separation column: 300 μL/min Focusing/Analytical column: 500 μL/min
Oven temperature	Focusing column: room temperature Pre-separation/Analytical column: 40°C
Mobile phase	Pre-separation column: methanol:ethanol:H_2O (74.7:8.3:17, v/v) Focusing/Analytical column: methanol:ethanol (90:10, v/v)
Injection volume	210 μL
Detection	UV 254 nm
Dissolving solution	Methanol 5 mL
Sampling volume	1 g

3). As shown in Table 2, the new HPLC method provided adequate accuracy (99.00±1.53-102.01±3.04%) for vitamin D quantitation from infant formula. To calculate the precision, intra- and inter-day tests were performed and the results are expressed as the relative standard deviation (R.S.D., %) in terms of the area ratio of vitamin D. The low R.S.D. values (1.68±0.03-5.75±0.33%) revealed the appropriate precision of this new method. The experiments were performed using infant formula SRM 1846 for the recovery test. Infant formula SRM 1846 contains 117.00±11.00 μg/kg of vitamin D. Three replicates of each SRM 1846 sample were used. Within each analytical series, three calibration standards covering the expected concentration range and three SRM 1846 samples were processed. The recovery rate was calculated by determining the percentage difference between the theoretical concentrations of vitamin D in SRM 1846 and those obtained by chromatographic analysis. As shown in Table 4, an adequate recovery rate for vitamin D was obtained with the new HPLC method and KFDA official method (KFDA, 2009). The results were obtained by two methods were 85.20±3.00 and 84.09±2.58%, respectively. The values have no significant difference ($p > 0.05$) at the 95% of confidence level by the Student's *t*-test. To evaluate the inter-laboratory tests of this method, a recovery test was performed by three laboratories. Infant formula SRM 1846 containing 117.00±11.00 μg/kg of vitamin D was used as the Certified Reference Material (CRM). Table 5 shows the results of the validation of the inter-laboratory test using this method. The recovery rates were in the range of 85.20±3.00%-89.13±2.17% and the result shows that the method is satisfied for inspection of vitamin D in infant formula.

Table 2. Accuracy and precision of vitamin D determination

Content (μg/kg)	Intraday (n=3)		Interday (n=3)	
	Precision (CV, %)	Accuracy (%)	Precision (CV, %)	Accuracy (%)
10	1.68±0.03	101.00±2.31	1.73±0.03	99.00±1.53
40	3.13±0.10	100.03±2.77	2.80±0.08	102.01±3.04
80	5.75±0.33	102.01±0.59	2.96±0.09	101.02±0.50

Table 3. Detective and quantitative limits of vitamin D

Element	Switching system method	
	LOD (μg/kg)	LOQ (μg/kg)
Vitamin D	1.51	4.95

Vitamin D analysis in infant formula

The results of the analysis of 16 kinds of commercial infant formula are shown in Table 6. The pre-separated samples were analyzed by HPLC with a switching valve.

Table 4. Recovery rate of vitamin D by two methods

Analysis method	Content (μg/kg)	Recovery (%)	Standard content (μg/kg)
New method	99.69±3.51	85.20±3.00	117.00±11.00
KFDA method (2009)	98.38±3.02	84.09±2.58	

Table 5. Inter-laboratory recoveries of vitamin D determination from three laboratories by column-switching HPLC with the UV detection method

Laboratory	Content (μg/kg)	Reference content (μg/kg)	Recovery (%)
A	99.69±3.51	117.00±11.00	85.20±3.00
B	104.28±2.54	117.00±11.00	89.13±2.17
C	103.35±2.41	117.00±11.00	88.33±2.06

Table 6. Determination of vitamin D content in 16 kinds of commercial infant formula

Sample No.	Content (μg/kg)	Sample No.	Content (μg/kg)
1	100.10±0.08	9	107.10±0.02
2	102.00±0.25	10	105.10±0.04
3	100.10±0.01	11	91.10±0.06
4	99.00±0.18	12	95.10±0.47
5	106.10±0.52	13	95.10±0.53
6	105.00±0.14	14	101.10±0.03
7	105.10±0.01	15	108.10±0.32
8	99.00±0.06	16	103.10±0.28

The new HPLC vitamin D method was applied to vitamin D analysis. The vitamin D content was determined by comparing the concentrations of the standard vitamin D and those obtained by chromatographic analysis. By comparison to the concentration on the label of the commercial infant formula, the vitamin D was adequately analyzed by new chromotographic analysis. Also, all of the results were in the range of CODEX standard (Codex, 2007).

Discussion

The newly developed method provides for good resolution in the determination of vitamin D with a column-switching HPLC system and UV-Vis detection. The improved pre-separation and instrument analysis gave acceptable and reproducible recoveries. The new HPLC method for vitamin D determination described in the present study was validated by linearity, sufficient accuracy, recovery, precision, and low values of limits of detection and quantification, thereby confirming the sensitivity of this analytical tool. This new HPLC method for vitamin D determination allows for a low-cost mobile phase and high specificity. In addition to these advantages, the new analytical method allowed for automation of the pre-separation and focusing procedures by the HPLC system. Therefore, this new analytical method should provide a time-saving advantage in comparison to other previously used methods and is particularly attractive for the determination of vitamin D in infant formula.

Acknowledgements

The work performed in this study was supported the by Korea Food and Drug Administration research project (KFDA 09081KFDA088) in 2009. We would like to thank Dr. Jayoung Jeong, Dr. Leem Dong-gil and Mr. Taehyung Yoon of KFDA nutrition and functional food research team. We also thank Prof. Kyong-Su Kim of Chosun university, for helping as project partner.

References

1. AOAC Committee report (1989) Guidelines for collaborative study of procedure to validate characteristics of a method of analysis. *J. Assoc. Off. Anal. Chem*. **72**, 694-704.
2. AOAC (2005) Official Methods of Analysis. 18th ed, Association of Official Analytical Chemists, Gaithersburg, Maryland, USA. **50**, pp. 5-6.
3. Bell, N. H., Stern, P. H., Panther, E., Sinha, T. K. and DeLuca, H. F. (1979) Evidence that increased circulating 1α,25-dihydroxyvitamin D is the probable cause for abnormal calcium metabolism in sarcoidosis. *J. Clin. Invest*. **64**, 218.
4. Brunetto, M. R., Obando, M. A., Gallignani, M., Alarcon, O. M., Nieto, E., Salinas, R., J. Burguera, L., and Burguera, M. (2004) HPLC determination of Vitamin D3 and its metabolite in human plasma with on-line sample cleanup. *Talanta* **64**, 1364-1370.
5. Christians, U. and Jacobsen, W., and Serkova, N. (2000) Automated, fast and sensitive quantification of drugs in blood by liquid chromatography-mass spectrometry with on-line extraction: immunosuppressants. *J. Chromatogr. B* **748**, 41-53.
6. Codex (2007) Standard for infant formula and formulas for special medical purposes intended for infants. *Codex Stan*. 72-1981.
7. Donald, W. S. and Gerhard, G. (1992) The kidney: Physiology and physiopathology. 2nd ed, Raven Press, New York.
8. Friedrich F. (1988) Vitamin D. In: Vitamins. Walter De Gruyter, Berlin, p. 143.
9. Gharbo, S. A. and Gosser, L. A. (1974) A stable and sensitive colorimetric method for the determination of ergocalciferol (vitamin D2) by using trifluoroacetic acid. *Analyst*. **99**, 222-224.
10. Hartmann, S., Froescheis, O., Ringenbach, F., Wyss, R., Bucheli, F., Bischof, S., Bausch, J., and Wiegand, U. W. (2001) Determination of retinol and retinyl esters in human plasma by high-performance liquid chromatography with automated column switching and ultraviolet detection. *J. Chromatogr. B* **751**, 265-275.
11. Hassan, S. S. (1980) Colorimetric determination of vitamin D2 (calciferol). *Methods Enzymol*. **67**, 323-326.
12. Heudi, O., Trisconi, M. J., and Blake, C. J. (2004) Simultaneous quantification of Vitamins A, D3 and E in fortified infant formulae by liquid chromatography–mass spectrometry. *J. Chromatogr. A* **1022**, 115-123.
13. ICH Expert Working Group (2005) Validation of analytical procedures: text and methodology Q2(R1). *ICH Harmonised tripartite guideline* **Step 4**, 1-18.
14. Kamao, M., Tsugawa, N., and Suhara, Y. (2007) Quantification of fat-soluble vitamins in human breast milk by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B* **859**, 192-200.
15. KFDA (Korea Food and Drug Administration) (2009) Food

Standards Codes. Korean Foods Industry Association, Seoul, Korea. pp. 745-747.
16. Knebel, N.G., Grieb. S., and Leisenheimer, S. (2000) Determination of retigabine and its acetyl metabolite in biological matrices by on-line solid-phase extraction (column switching) liquid chromatography with tandem mass spectrometry. *J. Chromatogr. B* **748**, 97-111.
17. Michael, T., Stephen. R. E., and ROGER, W. (2002) Harmonized guidelines for singlelaboratory validation of methods of analysis (IUPAC technical report). *Pure Appl. Chem*. **74**, 835-855.
18. Soldin, O. P., Sharma, H., and Husted, L. (2009) Pediatric reference intervals for aldosterone, 17α-hydroxyprogesterone, dehydroepiandrosterone, testosterone and 25-hydroxy vitamin D3 using tandem mass spectrometry. *Clin. Biochem.* **42**, 823-827.
19. The commission of the European communities. (2006) Commission directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending directive 1999/21/EC. Official Journal of the European Union. Available from: http://eur-lex.europa.eu/LexUriServ/site/en/oj/2006/l_401/l_40120061230en00010033.pdf. Accessed Feb. 27, 2012.

(Received 2012.3.5/Revised 1st 2012.4.24, 2nd 2012.8.7/Accepted 2012.9.4)