Research Paper

Highly-sensitive analysis of vitamin D_3 metabolites by liquid chromatography/electrochemistry/electrospray ionization-mass spectrometry

Hajime Mizuno[1], Yuta Kobayashi[1,2], Jun Zhe Min[1,3], Toshimasa Toyo'oka[1], Kenichiro Todoroki[1]*

[1] Laboratory of Analytical and Bio-Analytical Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52−1 Yada, Suruga-ku, Shizuoka 422−8526, Japan
[2] Present address: Kyoto R&D center, Maruho Co., Ltd., 92 Chudoji Awatacho, Shimogyo-ku, Kyoto 600−8815, Japan
[3] Present address: Pharmaceutical Analysis, College of Pharmacy, Yanbian University, Yanji 133002, Jilin Province, China

Abstract A highly-sensitive analytical method using a liquid chromatography (LC)/electrochemistry (EC)/electrospray ionization (ESI)-mass spectrometry (MS) is developed to analyze vitamin D_3 (VD_3) and its active metabolites, 25-hydroxyvitamin D_3 ($25(OH)D_3$) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in blood. LC/ESI-MS, which is used for the general sensitive analysis of biological compounds, has a low ionization efficiency for low polarity molecules such as VD_3 metabolites. The LC/EC/ESI-MS method, which connects the electrochemical detector (ECD) between the LC and ESI-MS, enables simultaneous and sensitive detection of VD_3 and its metabolites. The sample separated by LC is oxidized by an electrochemical cell, then the oxidant is ionized by ESI and detected by a mass spectrometer. Based on the results of analyzing VD_3 metabolites, ion peaks of the oxidized products generated by EC are detected, and their peak intensities are 10 times or greater than the unoxidized metabolites. This proposed method for the VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ analysis achieves the low detection limit of 1 nmol/L, 1 nmol/L, and 20 nmol/L, respectively, a good linearity ($r^2 > 0.9903$), and a good repeatability within < 10% (n=5). Analysis of serum samples spiked with VD_3 metabolites detect their oxidants peaks with high sensitivity. This result indicates that this LC/EC/ESI-MS method is also useful for biological sample analysis and allows for quantification of trace amounts of endogenous fat-soluble metabolites other than vitamin D_3 metabolites.

Key words: Vitamin D_3, 25-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3, electrochemistry, electrospray ionization, LC/EC/MS

Introduction

Vitamin D_2 (VD_2) and vitamin D_3 (VD_3) are some of the fat-soluble vitamins and essential nutrients. VD_2 is synthesized by plants, whereas VD_3 is synthesized by exposure to UV (sunlight) in the epidermis of animals. The active metabolites, 25-hydroxyvitamin D_3 ($25(OH)D_3$) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), are obtained by the hydroxylation reaction from VD_2 and VD_3. These metabolites play a significant role in the calcium homeostasis and are useful biomarkers for bone metabolic diseases[1]. Furthermore, the metabolites deficiency has been linked to various diseases such as diabetes[2], cancer[3], autoimmunity[4], and cardiovascular[5]. In order to quantify the concentrations of VD_3 and its metabolites in the human blood sample, a highly-sensitive analytical method is required. However, liquid chromatography (LC)/electrospray (ESI)-mass spectrometry (MS), which is used for general sensitivity analysis of biological compounds, has a low ionization efficiency for low polarity molecules such as VD_3 metabolites. To increase the sensitivity, atmospheric pressure chemical ionization (APCI), which is a suitable ionization technique for nonpolar molecules, has been used for ioniza-

* **Corresponding author**
Kenichiro Todoroki
Laboratory of Analytical and Bio-Analytical Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52−1 Yada, Suruga-ku, Shizuoka 422−8526, Japan
Tel: +81−54−264−5656, Fax: +81−54−264−5654
E-mail: todoroki@u-shizuoka-ken.ac.jp
Received: January 15, 2020. Accepted: March 12, 2020.
Epub April 16, 2020.
DOI: 10.24508/mms.2020.06.004

tion during the VD_3 metabolites analysis[5–8]. In addition, Ogawa *et al.* reported that the ionization efficiency of ESI was improved by labeling VD_3 metabolites with a functional cationic reagent (DAPTAD: 4-(4′-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione) by a derivatization reaction[9,10]. However, APCI is limited to low polar (fat-soluble) substances and the derivatization is limited to target substances, and is not suitable for comprehensive molecular analyses.

On the other hand, an electrochemical detector (ECD) has been used as a detector for the LC analysis of vitamin D and its metabolites[11]. In this study, we developed an LC/EC/ESI-MS method which enables the simultaneous and sensitive detection of VD_3 and its metabolites by ESI-MS analysis combined with electrochemical oxidation. Analytical methods in which an ECD is connected to a mass spectrometer have been reported before[12,13], however, most of them focused on the analysis of drug metabolites by EC oxidation[14,15] and the reduction of disulfide bonds in proteins and peptides[16,17]. This is the first study to improve the mass spectrometric detection sensitivity of VD_3 metabolites using an EC oxidation reaction. To detect EC oxidized fat-soluble metabolites by ESI-MS, a coulometric cell capable of oxidizing almost all molecules passing through the electrodes was used. Since porous graphite is used for the cell electrode, a highly reproducible oxidation reaction is possible even at high flow rates during the sample analysis[13]. The sample separated by LC is oxidized by an electrochemical cell, then the oxidant is ionized by ESI and detected by a mass spectrometer. This method not only enables the highly-sensitive analysis of fatty and low polarity molecules without any pretreatment such as derivatization, but also the simultaneous analysis of polar molecules as the normal ESI-MS is possible when the ECD voltage is turned off.

Materials and Methods

Materials

L-Menthol and Vitamin D_3 was purchased from the Tokyo Chemical Institute (Tokyo, Japan). 25-Hydroxy vitamin D_3(calcidiol) and 1,25-dihydroxyvitamin D_3(calcitriol) were purchased from Toronto Research Chemicals (North York, ON, Canada) and Cayman Chemical Company (Ann Arbor, MI, USA), respectively. LC/MS grade acetonitrile (MeCN; Optima LC/MS), formic acid (FA; Optima LC/MS), and ammonium formate (Optima LC/MS) were purchased from Fisher Scientific (Waltham, MA, USA). Methanol of LC/MS grade was purchased from the Kanto Kagaku Co. (Tokyo, Japan). All other reagents and solvents were of analytical reagent grade. Water (H_2O) was purified using a PURELAB flex ultrapure water purification system (ELGA LabWater, High Wycombe, UK).

Sample preparation

Standard solutions of VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ were made by dissolving in acetonitrile to 1 μmol/L each. The serum samples were prepared by dissolving 5 pmol of VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ in 50 μL of human pooled serum (L-Consera I EX, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). To remove proteins in the serum, 450 μL of acetonitrile was added, and centrifugation for 10 min (10,000 *g*, 5˚C) was performed. The supernatant was evaporated using a centrifugal evaporator, and the residue was redissolved with 50 μL of acetonitrile to 1 μM of VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ each.

LC/EC/ESI-MS analysis

The LC/EC/ESI-MS analysis was performed by connection to an EC detector (CoulArray Detector, Model 5600A, Dionex) between the LC and ESI-MS. The schematic image of this method is shown in Fig. 1. In order to separate the analytes, the LC conditions were described as follows: the column was an ACQUITY UPLC BEH C18 (particle size 1.7 μm, 2.1 mm I.D. × 100 mm, Waters, Milford, MA, USA) at the flow rate at 0.2 mL/min and column temperature of 40˚C. The LC was isocratic elution and the mobile phase used 100 mmol/L formic acid and 5 mmol/L ammonium formate in 90% acetonitrile. The eluted analytes from the column were oxidized by the EC. The applied voltage to the EC analytical cell was 1,300 mV. The autosampler temperature and injection volume were 10˚C and 5 μL, respectively. To prevent a high voltage affect in the EC cell, the grounding wire was connected between the ESI ion source and EC cell. The oxidized analytes were ionized and detected by a high-resolution mass spectrometer (Q Exactive, Thermo Fisher Scientific, Waltham, MA, USA). The MS conditions were as follows: ESI positive ionization, spray voltage; 3.5 kV, sheath gas flow rate; 10 (arbitrary units (au)), auxiliary gas flow rate; 10 au, capillary temperature; 350˚C, heater temperature; 300˚C, s-lens RF level; 50%, resolution power; 70,000, and scan range; *m/z* 200–3,000. Mass accuracy of detecting ion peaks was within

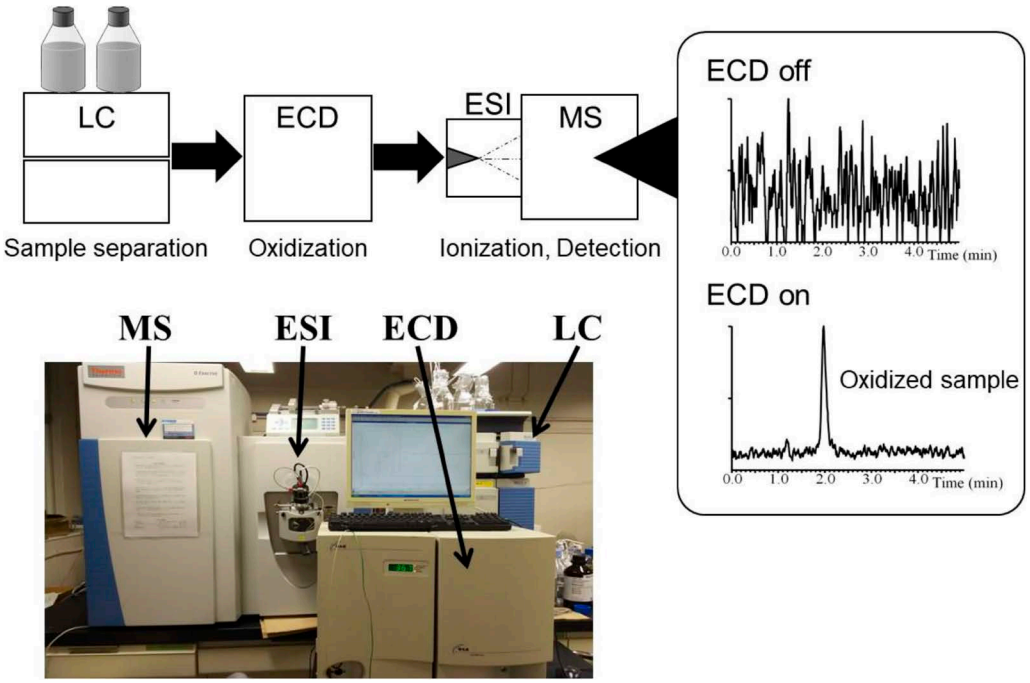

Fig. 1. The schematic image and photo of LC/EC/ESI-MS used in this study. An electrochemical reaction cell (EC cell) is connected between the LC and MS ion source.

3 ppm. The MS/MS spectra (*m/z* 50−500) were obtained by a data dependent acquisition program in the mass spectrometer. The normalized collision energy (NCE) and the resolution power were 20% and 35,000, respectively. In order to preserve the mass accuracy, the mass spectrometer was calibrated using a calibration solution containing n-butylamine, caffeine, MRFA, and Ultramark 1621 before the analysis.

Data analysis

Monitoring of the EC cell and control of the applied voltage was performed using the Coul Array Data Station Program (version 3.06, Dionex) software. The obtained LC/EC/ESI-MS data were evaluated using Xcalibur software (Thermo Fisher Scientific). The Xcalibur analyzed the exact *m/z* values and the ion chromatograms of the VD_3 metabolites and their oxidant ion peaks detected from the obtained data.

Results and Discussion

Optimization of EC oxidation conditions

In order to oxidize VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ in the EC analytical cell, the acetonitrile, formic acid, and ammonium formate concentrations of the mobile phase and the voltage applied to the EC cell were optimized. Since $25(OH)D_3$ and $1,25(OH)_2D_3$ are both metabolites of VD_3 and their basic chemical structures were almost the same, this reaction optimization used only the VD_3 standard. Fig. 1S shows a plot of the detected peak height of the protonated VD_3 oxidant ion (*m/z* 383.3313) for each acetonitrile, formic acid, ammonium formate concentration and applied the EC voltage conditions. For connection to a mass spectrometer, volatile ammonium formate was used as the salt additive[13]. From the maximum peak intensity in each figure, the optimal values for each concentration in the mobile phase were determined as follows: 90% acetonitrile, 5 mmol/L ammonium formate, and 100 mmol/L formic acid. The oxidized VD_3 ion peak at *m/z* 383.3313 showed the maximum intensity values when 1,300 mV or higher was applied in the EC cell. Based on these results, the selected applied voltage of the EC cell was 1,300 mV in this study.

LC/EC/ESI-MS analysis for VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$

In order to confirm the improved sensitivity of the VD_3 metabolites, each 1 μmol/L of VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ was analyzed by the LC/EC/ESI-MS using the optimized conditions. Figs. 2(a) and (b) show the extracted ion chromatograms of the protonated VD_3 ion at *m/z* 385.3470 and VD_3 oxidant ion at *m/z* 383.3313. When no voltage was applied to the EC cell, only a peak at *m/z* 385.3470 was detected at 17.5 min. However, after a volt-

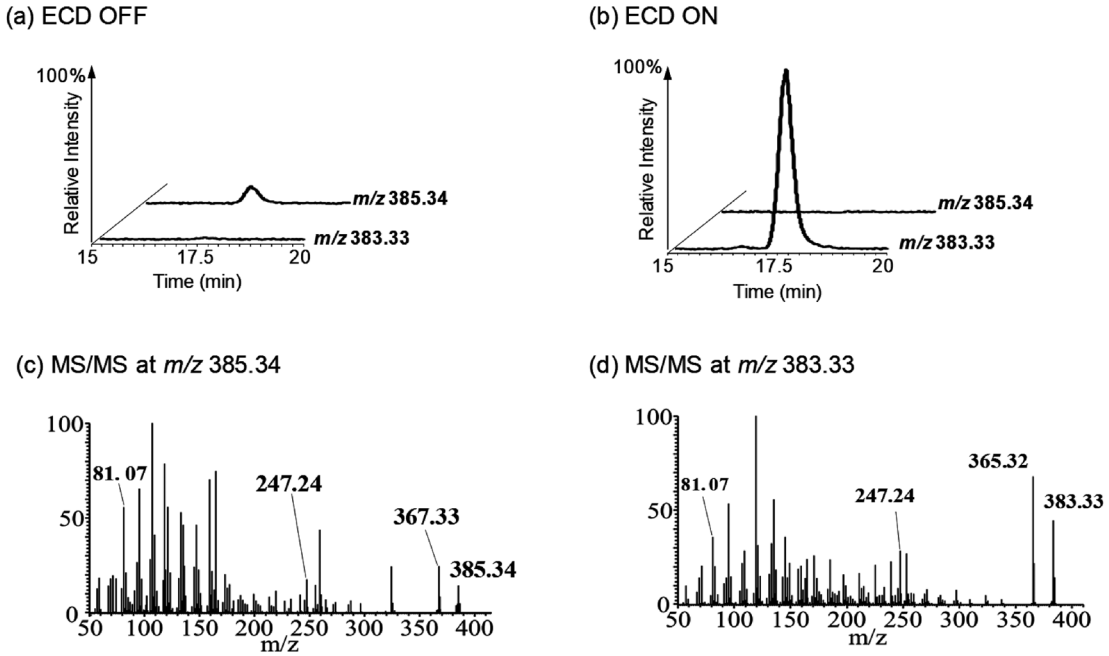

Fig. 2. Extracted ion chromatograms of VD_3 (*m/z* 385.34) and oxidized VD_3 (*m/z* 383.33).
(a): No applied EC voltage, and (b): applied EC voltage (1,300 mV). The MS/MS fragment spectra of VD_3 (c) and oxidized VD_3 (d). Each precursor ion is *m/z* 385.34 (c) and *m/z* 383.33 (d), respectively.

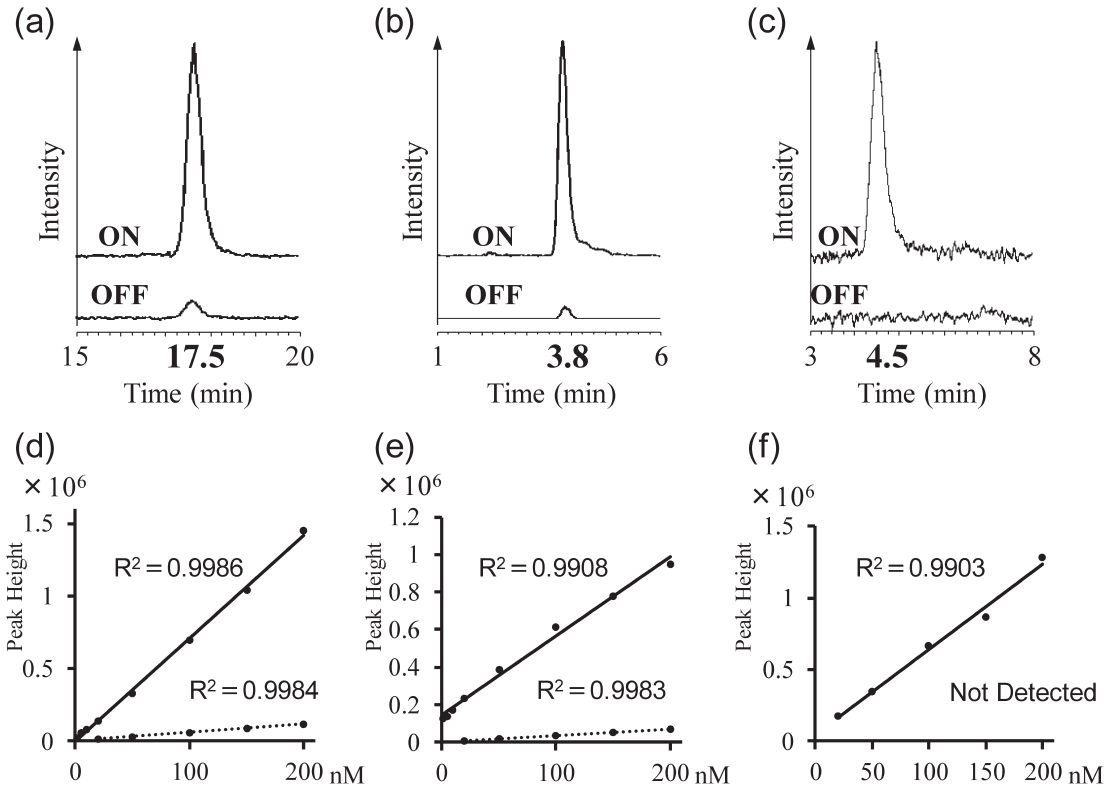

Fig. 3. Extracted ion chromatograms of VD_3 (a), $25(OH)D_3$ (b), and $1,25(OH)_2D_3$ (c).
Each upper chromatogram is no applied EC voltage and the lower chromatogram is the applied EC voltage (1,300 mV). Obtained calibration curves of VD_3 (c), $25(OH)D_3$ (d), and $1,25(OH)_2D_3$ (e). The solid lines show the calibration curves for the EC oxidants, and the dotted lines show the unchanged forms.

age (1,300 mV) was applied to the EC cell, the peak at *m/z* 385.3470 disappeared and a new ion peak at *m/z* 383.3313 appeared at 17.5 min. The mass difference between the two peaks was 2.0157 Da, which was the same as the *m/z* value of exactly two hydrogen atoms being removed from the ion at *m/z* 385.3470. Figs. 2(c) and (d) show the MS/MS fragment spectra of both of these ions. The ion at *m/z* 385.3470 had the same fragment pattern as VD_3. A similar fragment pattern was obtained for the ion at *m/z* 383.3313. Based on these results, it was found that the ion at *m/z* 383.3313 was an oxidized form of VD_3.

Figs. 3(a)−(c) show the ion chromatograms obtained by analyzing the VD_3, $25(OH)D_3$ and $1,25(OH)_2D_3$ standard samples. When a voltage was applied to the EC cell, ion peaks of *m/z* 399.3262 and *m/z* 415.3211, which were the oxidized forms of $25(OH)D_3$ and $1,25(OH)_2D_3$, were detected at 3.8 min and 4.5 min, respectively. Furthermore, $25(OH)D_3$ and $1,25(OH)_2D_3$ were similarly oxidized by EC to VD_3 since almost no peaks at *m/z* 401.3405 and *m/z* 417.3362 were detected in the Fig. 3 chromatogram. Comparing the native forms of the detected ion peak intensities with the oxidized forms, all the oxidative VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ ion peaks were detected at more than 10 times higher. These results suggested that the sensitivity of the ESI-MS detection was improved by improving the proton affinity of VD_3, and its metabolites were dehydrogenated by the EC[13,18]. Each calibration curve of VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ was obtained by analysis of the calibration standard solutions (n=5) with concentrations ranging from 0.5 to 200 nmol/L (0.5, 1, 2, 5, 10, 150, and 200 pmol/L) which were prepared by diluting the stock solutions. Peak heights were used for making the calibration curves. The relative standard deviations (RSD) of the peak heights at each concentration of VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ were all 10% or less. The limit of detections (LODs) were determined from the signal to noise ratio of 3 to 20. The calibration curves as shown in Figs. 3(d)−(f) showed that VD_3 and its metabolite oxidants were good with R^2 values of 0.9986 (oxidized VD_3), 0.9908 (oxidized $25(OH)D_3$ and 0.9903 (oxidized $1,25(OH)_2D_3$). Their LODs were 1 nmol/L (oxidized VD_3), 1 nmol/L (oxidized $25(OH)D_3$), and 20 nmol/L (oxidized $1,25(OH)_2D_3$), respectively. In the combination method of DAPTAD derivatization and SRM, the detection sensitivity of $25(OH)D_3$ and $1,25(OH)_2D_3$ was about 30 times higher in this method[9,10]. The derivatization method is suitable for highly sensitive analysis of VD_3 and its metabolites, however cannot analyze other metabolites other than VD_3 simultaneously. In order to achieve comprehensive metabolite analysis, it is necessary to analyze intact metabolites with high sensitivity. Table 1 shows a comparison of the LOD values of the VD_3 metabolites obtained in this study with the general method using the LC/APCI/tandem quadrupole MS and selected reaction monitoring (SRM)[6−8]. It was clarified that the sensitivity was dramatically improved by oxidizing with the EC and ionizing with the ESI compared to the conventional method. Furthermore, it was close to the LODs in

Table 1. LOD values of VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ by LC/EC/ESI-MS and other previous methods

Ionization method	LOD (nmol/L)		
	VD_3	$25(OH)D_3$	$1,25(OH)_2D_3$
ESI	20	20	ND
EC/ESI	1	1	20
APCI/tandem MS[a]	60[6]	2.8[7]−3.8[8]	1.2[7]−3.8[8]

a: APCI data were obtained by QQQ-SRM. LOD values of VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ obtained by LC/EC/ESI-MS and other previous methods[6−8].

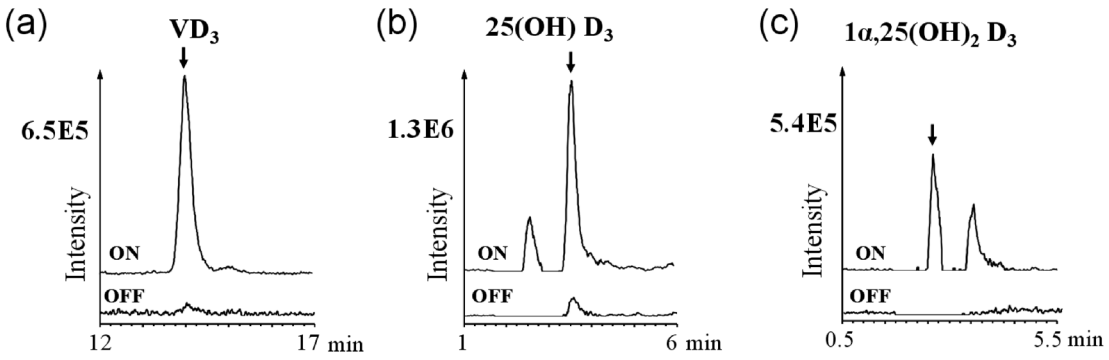

Fig. 4. Extracted ion chromatograms of VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ in the serum added samples.
Each upper chromatogram is no applied EC voltage and the lower chromatogram is the applied EC voltage (1,300 mV).

the target analysis by SRM using APCI. This result indicates that metabolites other than VD_3 can be simultaneously detected with a detection sensitivity comparable to the QQQ using SRM mode because a full scan measurement is performed in this study. Furthermore, this method enables simultaneous detection of various metabolites other than the targeted VD_3-related metabolites. The blood vitamin D_3 concentration is the range of 50 to 125 nmol/L for $25(OH)D_3$, and the clinical test reference value for vitamin D deficiency is 30 nmol/L or less as serum concentration of $25(OH)D_3$[2]. The method developed in this study has sufficient sensitivity to detect these reference values, confirming that it is a very useful analytical method. However, this method is not sensitive enough for the clinical application of $1,25(OH)_2D_3$ analysis, and further analytical improvement such as SRM detection is needed.

Analysis of the serum samples with spiked VD_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ standards using this proposed method revealed that these oxidized ion peaks were detected at a higher intensity than the native forms (Fig. 4). This result shows that this method can detect VD_3 and its metabolites with high sensitivity even in matrix components such as biological samples.

Conclusion

In this study, we developed a highly-sensitive analytical method for VD_3 metabolites by using the combination LC/ESI-MS with electrochemical oxidation. Based on the results of analyzing the VD_3 metabolites using this proposed method, the ion peaks of the oxidized products generated by EC were detected, and their peak intensities were 10 times or higher than the unoxidized metabolites. The LODs of the VD_3 metabolites in the LC/EC/ESI-MS were 1 nmol/L for VD_3 and $25(OH)D_3$, and 20 nmol/L for $1,25(OH)_2D_3$. Their R^2 values were 0.9903 or greater indicating good linearity. The serum samples analysis to which the VD_3 metabolites were spiked was able to detect these oxidized peaks with high sensitivity. This result indicates that this method is also useful for biological sample analysis.

Based on these results, it was possible for the sensitive and rapid analysis of VD_3 metabolites by this proposed method. It is expected that this method not only enables the quantification of trace amounts of endogenous fat-soluble metabolites other than the VD_3 metabolites, but can also be applied to the comprehensive analysis of biological components.

Acknowledgment

This work was supported by JSPS KAKENHI under grant number 16K21276 (to H.M.).

Conflict of Interest

The authors have no conflicts of interest directly relevant to the content of this article.

References

1) Prentice A, Goldberg GR, Schoenmakers I: Vitamin D across the lifecycle: Physiology and biomarkers. *Am J Clin Nutr* 88(2): 500s−506s, 2008.
2) Holick MF: Vitamin D deficiency. *N Engl J Med* 357(3): 266−281, 2007.
3) Moukayed M, Grant WB: The roles of UVB and vitamin D in reducing risk of cancer incidence and mortality: A review of the epidemiology, clinical trials, and mechanisms. *Rev Endocr Metab Disord* 18(2): 167−182, 2017.
4) Illescas-Montes R, Melguizo-Rodriguez L, Ruiz C, Costela-Ruiz VJ: Vitamin D and autoimmune diseases. *Life Sci* 233: 116744, 2019.
5) Adela R, Borkar RM, Bhandi MM, Vishwakarma G, Reddy PN, et al: Lower vitamin D metabolites levels were associated with increased coronary artery diseases in Type 2 diabetes patients in India. *Sci Rep* 6: 37593, 2016.
6) Byrdwell WC: Quadruple parallel mass spectrometry for analysis of vitamin D and triacylglycerols in a dietary supplement. *J Chromatogr A* 1320: 48−65, 2013.
7) Fabregat-Cabello N, Farre-Segura J, Huyghebaert L, Peeters S, Le Goff C, et al: A fast and simple method for simultaneous measurements of 25(OH)D, 24,25(OH)2D and the Vitamin D Metabolite Ratio (VMR) in serum samples by LC-MS/MS. *Clin Chim Acta* 473: 116−123, 2017.
8) Hagenhoff S, Hayen H: LC/MS analysis of vitamin D metabolites by dielectric barrier discharge ionization and a comparison with electrospray ionization and atmospheric pressure chemical ionization. *Anal Bioanal Chem* 410(20): 4905−4911, 2018.
9) Ogawa S, Ooki S, Shinoda K, Higashi T: Analysis of urinary vitamin D(3) metabolites by liquid chromatography/tandem mass spectrometry with ESI-enhancing and stable isotope-coded derivatization. *Anal Bioanal Chem* 406(26): 6647−6654, 2014.

10) Ishige T, Satoh M, Ogawa S, Nishimura M, Matsushita K, et al: Improved sensitivity of serum/plasma 1alpha,25-dihydroxyvitamin D quantification by DAPTAD derivatization. *Clin Chim Acta* 473: 173−179, 2017.
11) Masuda S, Okano T, Kamao M, Kanedai Y, Kobayashi T: A novel high-performance liquid chromatographic assay for vitamin D metabolites using a coulometric electrochemical detector. *J Pharm Biomed Anal* 15(9−10): 1497−1502, 1997.
12) Oberacher H, Pitterl F, Erb R, Plattner S: Mass spectrometric methods for monitoring redox processes in electrochemical cells. *Mass Spectrom Rev* 34(1): 64−92, 2015.
13) Faber H, Vogel M, Karst U: Electrochemistry/mass spectrometry as a tool in metabolism studies-a review. *Anal Chim Acta* 834: 9−21, 2014.
14) Szultka-Mlynska M, Buszewski B: Electrochemistry-mass spectrometry for in-vitro determination of selected chemotherapeutics and their electrochemical products in comparison to in-vivo approach. *Talanta* 160: 694−703, 2016.
15) Mielczarek P, Smoluch M, Kotlinska JH, Labuz K, Gotszalk T, et al: Electrochemical generation of selegiline metabolites coupled to mass spectrometry. *J Chromatogr A* 1389: 96−103, 2015.
16) Cramer CN, Haselmann KF, Olsen JV, Nielsen PK: Disulfide linkage characterization of disulfide bond-containing proteins and peptides by reducing electrochemistry and mass spectrometry. *Anal Chem* 88(3): 1585−1592, 2016.
17) Switzar L, Nicolardi S, Rutten JW, Oberstein SA, Aartsma-Rus A, et al: In-depth characterization of protein disulfide bonds by online liquid chromatography−electrochemistry−mass Spectrometry. *J Am Soc Mass Spectrom* 27(1): 50−58, 2016.
18) Simon H, Wehe CA, Pajaziti B, Heinrich L, Karst U: Simultaneous electrochemical oxidation/ionization of a selenoxanthene revealed by on-line electrospray-high resolution mass spectrometry. *Electrochimica Acta* 111: 324−331, 2013.