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Brief Communication: Research Report

A Sensitive Method for Determination 1,25-Dihydroxyvitamin D3 in Human Brain using Ultra-Pressure Liquid Chromatography Tandem Mass Spectrometry

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A B S T R A C T

The hormonally active form of vitamin D, 1,25-Dihydroxyvitamin D3 [1,25$(OH)_2D_3$], has been associated with neuroprotective effects in the brain, but has been difficult to measure in human brain tissue because of its low concentration. The aim of this study was to develop and validate a sensitive method to quantify 1,25$(OH)_2D_3$ in the human brain. Prior to analysis by the LC-MS/MS, the samples were derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione. The method showed good linearity of 1,25$(OH)_2D_3$ over the physiological range ($R^2 = 0.9998$). The limit of detection was 2.5 pg/g, >10 times lower than the previously reported limit of detection. The average 1,25$(OH)_2D_3$ concentrations in 3 regions of human brain tissue samples were: anterior watershed 30.7 pg/g; mid-temporal cortex 19.2 pg/g; and cerebellum 18.5 pg/g. This validated method to quantify 1,25$(OH)_2D_3$ in human brain tissue can be applied to obtain information about its presence in various regions of the human brain associated with neurodegenerative diseases.

Keywords: 1,25-dihydroxyvitamin D3, brain, QTRAP, Vitamin D, neurodegenerative diseases

Introduction

Vitamin D is an essential fat-soluble nutrient that is classically known for its role in maintaining calcium homeostasis and skeletal health [1]. Vitamin D can be obtained in the diet or synthesized cutaneously in the presence of UVB light. It is hydroxylated by the liver to produce 25-hydroxyvitamin D3 [25$(OH)D_3$], which is the primary circulating form of vitamin D. Through a tightly regulated series of feedback loops, 25$(OH)D_3$ is further hydroxylated to produce 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$], which is the hormonally active form that can bind to the nuclear vitamin D receptor (VDR) to activate gene expression [2]. Several tissues in the body are capable of hydroxylating 25$(OH)D_3$ to 1,25$(OH)_2D_3$ and also express the VDR, including brain tissue [3]. It has been suggested that vitamin D is mechanistically linked to neurodegenerative diseases [4]. Most of the studies that have associated vitamin D with neurodegenerative diseases and cognitive decline have relied on circulating of 25$(OH)D_3$ as the indicator of vitamin D status [5]. However, it is not clear if the 25$(OH)D_3$ measured in circulation reflects vitamin D in the brain. We recently reported that 25$(OH)D_3$ in human brain tissue was associated with postmortem cognitive status but was not associated with Alzheimer's disease pathology [6]. Unfortunately, the 1,25$(OH)_2D_3$, using our original assay [7], was below the assay lower limit of detection (LOD) in 58% of the samples, and therefore excluded from our data analysis. Because 1,25$(OH)_2D_3$ is the active form of vitamin D, and we still do not have the exact mechanisms underlying the role of vitamin D in the brain, it is critical to be able to measure this active form. Indeed, we posit that the role of vitamin D in neurodegenerative diseases can only be elucidated by quantifying 1,25$(OH)_2D_3$, in addition to 25$(OH)D_3$, in the human brain, which requires greater assay sensitivity. Here we report on the development and validation of a mass spectrometry assay

Abbreviations: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D3; 25$(OH)D_3$, 25-hydroxyvitamin D3; AWS, anterior watershed; CR, cerebellum; IS, internal standard; LOD, limit of detection; MT, middle temporal cortex; PTAD, 4-phenyl-1,2,4-triazoline-3,5-dione; RSD, relative standard deviation; SPE, solid phase extraction; VDR, vitamin D receptor.

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with improved sensitivity, with an LOD 10 times lower than that of the previous assay, to quantify $1,25(OH)_2D_3$ in postmortem human brain tissue. This assay will enable future research into the role of $1,25(OH)_2D_3$ in the human brain and its potential relevance to neurodegenerative diseases.

Methods

Chemicals, regents, and standards

All solvents used for working solutions and chromatography were of LC-MS grade (Thermo Scientific). All the stock solutions and working standards were prepared every 6 mo and stored at −80°C. The $1,25(OH)_2D_3$ and 1,25-dihydroxyvitamin D3-(d6) [d6–$1,25(OH)_2D_3$ as internal standard (IS)] were obtained from IsoScience. The calibration standards were prepared in methanol. For sample derivatization, 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD; 0.25 mg/mL) was prepared in acetonitrile.

Samples and clinical application

One human cadaver brain obtained from a 54-y-old woman donor through the National Development and Research Institutes was utilized for method validation, which has been described previously [7]. The cortex was homogenized and aliquoted for use as brain controls (blank), then stored at −80°C. Quality controls (low and high) were freshly prepared by taking 0.1 g of brain control and spiking it with a specific amount of $1,25(OH)_2D_3$.

Postmortem brain tissue samples, stored at −80°C for no longer than 6 y, were obtained from 153 participants in the RUSH Memory and Aging project [6,8]. During autopsy and brain dissection, the tissues were promptly frozen and maintained in a frozen state throughout the dissection process without thawing [9]. The $1,25(OH)_2D_3$ concentrations were measured in the following brain regions: anterior watershed (AWS), middle temporal cortex (MT), and cerebellum (CR).

Preparation of brain samples and analyses

Brain sample preparation of 0.1 g and liquid–liquid extractions with methylene chloride:methanol (1:1) were used, as described previously [7]. Silica solid phase extraction (SPE) columns (Agilent) and a Vac-Elute SPS 24 manifold rack were used for SPE, in which each sample was reconstituted in 1 mL of 4% isopropanol in hexane and transferred into the SPE columns, then washed with 9 mL 4% isopropanol in hexane and 6 mL 6% isopropanol in hexane, and eluted with 4.5 mL of 25% isopropanol in hexane to be dried under N_2 gas at 60°C. For the derivatization, 200 μL of PTAD solution (0.25 mg/mL) were added to each dried sample, vortexed, and subsequently left to be stored in dark place for 1 h at room temperature. Derivatized samples were dried and reconstituted in 100 μL of 20 mM methylamine, vortexed for 2–3 min, then centrifuged at 16,300 × *g* for 5 min at 4 °C. The supernatant was pipetted into vials with glass inserts to be analyzed with the LC-MS/MS system.

Quadrupole ion trap instrumentations and conditions

The LC-MS/MS system comprised an Agilent 1290 Infinity Series coupled to a Sciex 7500 QTRAP tandem mass spectrometer. The chromatographic separation column was an Agilent Eclipse Plus C18 (50 mm × 1.2 mm, 1.8 μm). Mobile phase A was 4 mM methylamine in water with 0.1% formic acid, and mobile phase B was 4 mM methylamine in methanol with 0.1% formic acid. The gradient program operated with a flow of 0.1 mL/min: 0−24 min, 65% B; 0.2 mL/min flow from 24.1 to 30.0 min, gradient at 100% B; 0.2 mL/min flow at 30.1 min, 62% B; and then 0.1 mL/min flow to 35min, 62% B. Methylamine was added into the mobile phase as additive to enhance assay sensitivity after the PTAD derivatization [10]. The QTRAP settings were as follows: ion source, positive electrospray ionization; temperature, 500°C; ion source gas 1: 50 psi; gas 2: 50 psi; curtain gas: 40 psi; ion spray voltage, 4900; entrance potential: 2 V; collision energy: 30V; collision cell exit potential: 20 V. The multiple reaction monitoring transitions (m/z) used for $1,25(OH)_2D_3$ and d6-$1,25(OH)_2D_3$ (IS) were 623.4/314.1 and 629.4/314.1, respectively. Data were collected using Sciex OS.

Validation experiments

Linearity was established for $1,25(OH)_2D_3$ using serial dilutions of the calibration standard to concentrations ranging from 1.25 to 400 pg/mL. The LOD for $1,25(OH)_2D_3$ was determined by spiking human brain with serially diluted vitamin D standards. Further validation with intra-assay and inter-assay precision were characterized with relative standard deviations (RSD) for the targeted concentrations of 10 and 80 pg/g in human brain. The inter-assay variability was determined by repeating the same procedure on 4 consecutive days. The accuracy was calculated as the percentage of nominal concentration [(measured concentration-blank brain concentration)/(nominal concentration) ×100%]. d6–$1,25(OH)_2D_3$ as IS was added to spiked brain samples to evaluate the IS-normalization extraction recovery.

Quantification

Respective response factors of the $1,25(OH)_2D_3$ from the sample and from the IS [d6-$1,25(OH)_2D_3$] were used to quantify $1,25(OH)_2D_3$, as described previously [7].

Statistical analysis

Linearity, slope, and regression coefficients were determined by linear regression using Microsoft Excel. Spearman's correlation of $1,25(OH)_2D_3$ concentrations in different brain sessions was calculated using SPSS (IBM, version 29).

Results

This method for successfully measuring $1,25(OH)_2D_3$ in human brain demonstrates linearity between 1.25 and 400 pg/mL with an R^2 value of 0.9998. The LOD was 2.5 pg/g, which is more sensitive than the previous LOD of 25 pg/g [7]. The precision and accuracy of $1,25(OH)_2D_3$ in spiked human brain are shown in Table 1. The precision of these measurements is characterized with an intra-assay variability for the targeted brain concentration of 10 and 80 pg/g as RSDs of 4.0% and 6.7%, respectively. As for the inter-assay variability, the RSDs for the targeted brain concentration of 10 and 80 pg/g were 13.6% and 6.0%, respectively. The accuracy of spiked brain samples was slightly over 100%, ranged from 101.1% to 108.5%. The extraction recovery, as shown in Table 1, indicates that this method yields high recovery rates for brain samples. The resulting multiple reaction monitoring chromatographs of the

TABLE 1
Precision, accuracy, and recovery of $1,25(OH)_2D_3$ in spiked human brain.

Spiked level	Intra-day		Inter-day		Recovery	
	Precision RSD (%)	Accuracy (%)	Precision RSD (%)	Accuracy (%)	% ($n = 8$)	RSD (%)
Brain 10 (pg/g)	4.0	104.7	13.6	108.5	51.0	9.3
Brain 80 (pg/g)	6.7	102.5	6.0	101.1	53.8	14.5

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D3; RSD, relative standard deviation.

derivatized $1,25(OH)_2D_3$ with PTAD are shown in Figure 1. Studies have shown that the double peak on the chromatogram is the result of the Diels–Alder reaction with derivatization reagent PTAD, reacting with the *s-cis-diene* moiety from both the α and β sides and forming *6S* and *6R* epimers [11].

According to the previously published method, there are certain challenges in isolating one vitamin D metabolite, the $1,25(OH)_2D_3$, from brain samples because of the differences in polarity among the vitamin D metabolites analyzed [12,13]. The polarity difference makes it impossible to extract all of the vitamin D3 metabolites using the same solvent. Of all the vitamin D metabolites, $1,25(OH)_2D_3$ has the greatest polarity because of the additional hydroxyl group. This difference in polarity allows for its extraction through SPE, with mobile phases of varying polarity. The specific silica column used in the SPE is polar, allowing for the $1,25(OH)_2D_3$ to bind to silica particles and other less polar vitamin D metabolites to wash through. SPE has been successfully applied for measuring $1,25(OH)_2D_3$ in plasma [14]. Using the sequential washes of 4% and then 6% isopropanol in hexane allows for selective isolation of $1,25(OH)_2D_3$ to be bound to the column. The last eluant is collected with the greatest polar concentration of 25% isopropanol in hexane. This specific sequence of polar mobile phases was found to yield the greatest recovery of $1,25(OH)_2D_3$.

The method was used to analyze 459 human brain samples, from 153 postmortem brains obtained from participants in the RUSH Memory and Aging project study. All 3 analyzed brain regions ($n = 153$), AWS, MT, and CR, measured $1,25(OH)_2D_3$ with the respective concentrations 30.7 ± 15.2 pg/g, 19.2 ± 9.3 pg/g, and 18.5 ± 8.6 pg/g (Figure 2). These correlations of $1,25(OH)_2D_3$ concentrations among the 3 regions were the following: MT and CR Spearman $r = 0.70$, MT and AWS Spearman $r = 0.55$. CR and AWS Spearman $r = 0.45$; all $P \leq 0.005$. The method's LOD of 2.5 pg/g contributed to the quantification $1,25(OH)_2D_3$ in all brain samples analyzed (Figure 2). Compared with the previous method, which was unable to detect $1,25(OH)_2D_3$ in 58% of brain samples below the LOD of 25 pg/g, this assay could detect

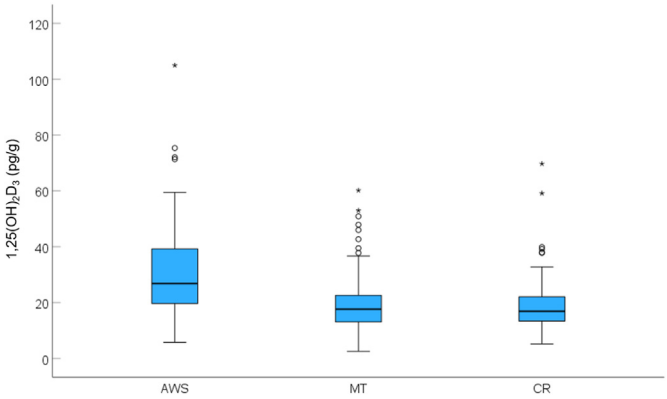

FIGURE 2. The box and whisker plots of $1,25(OH)_2D_3$ concentrations (pg/g) for brain regions AWS, MT, and CR, as measured by the LC-MS/MS ($n = 153$). $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D3; AWS, anterior watershed; CR, cerebellum; MT, middle temporal cortex.

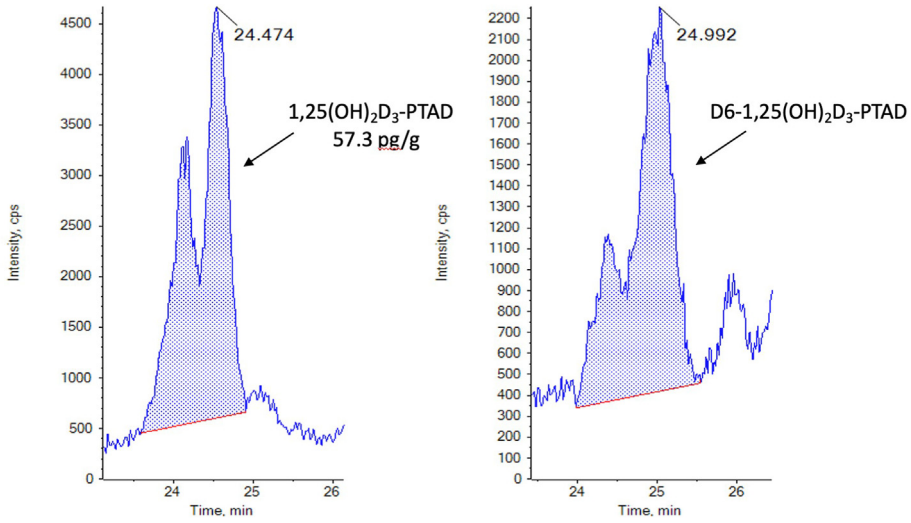

FIGURE 1. The representative MRM chromatograms for the derivatized $1,25(OH)_2D_3$-PTAD and labeled D6-$1,25(OH)_2D_3$-PTAD from the anterior watershed (AWS) region of the 1 human brain obtained from RUSH Memory and Aging project. $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D3; MRM, multiple reaction monitoring; PTAD, 4-phenyl-1,2,4-triazoline-3,5-dione.

$1,25(OH)_2D_3$ in all brain samples and with greater linearity over the concentration range evaluated [7].

Discussion

Here we present a successful modification to a prior validated method of measuring vitamin D metabolites in human brain tissue that now allows us to quantify $1,25(OH)_2D_3$, the hormonally active form of vitamin D. This assay can detect $1,25(OH)_2D_3$ in multiple human brain regions. Accurate measurement of $1,25(OH)_2D_3$ in human brain tissue will allow us to better elucidate the mechanism underlying associations between vitamin D status and cognitive function in older adults. Because of the small sample size available for assay development, we were not powered to further analyze the associations between $1,25(OH)_2D_3$ and cognitive or neuropathological outcomes. However, now that we have a validated assay, this will be an important future direction for this research. Future research is also needed to elucidate the factors that contribute to the variability in human brain $1,25(OH)_2D_3$ concentrations. For example, the brain $1,25(OH)_2D_3$ concentrations may be attributed to variations in VDR and/or 1-alpha hydroxylase expression, because the VDR and the 1-alpha hydroxylase [crucial for synthesizing $1,25(OH)_2D_3$] have been identified within the adult cadaver human brain, as well as in both neurons and glial cells [15].

There are few studies using similar methods that can measure $1,25(OH)_2D_3$ in mammalian brain tissues, because brain tissue is ~60% lipids, and the composition of these lipids is very complex. One recent method using LC-MS/MS with an online extraction to measure $1,25(OH)_2D_3$ in the rodent brain, with a limit of quantification of 12.5 pg/g [16]. However, the majority of methodologies that use LC-MS/MS to measure $1,25(OH)_2D_3$ in human plasma samples have higher LOD ranges from 20 to 210 pg/mL [5, 17]. The SPE step provided additional purification of the brain samples, enhancing the selectivity of the assay. Approximately 500 samples were analyzed with quality controls inter-assay coefficient of variation of 7%. This indicates that the assay is robust and suitable for large-scale studies. Overall, our method was successfully used for the determination of $1,25(OH)_2D_3$ in the human brain, with detection of $1,25(OH)_2D_3$ in all samples because of the improved LOD. With this method, research into the role of the $1,25(OH)_2D_3$ in the human brain can be further elucidated. In conclusion, this validated method can be further applied and coupled with other methods that measure vitamin D metabolites to bring further insight into the role of vitamin D and its metabolites in the brain and neurodegenerative diseases.

Author contributions

The authors' responsibilities were as follows – AX, GGD, XF: designed the study; AX, XF: conducted the laboratory analyses; MKS: analyzed the data; AX, GGD, XF, MKS, SLB, JAS: wrote the manuscript; XF, SLB: are responsible for final content; and all authors: read and approved the final manuscript.

Conflict of interest

MKS is an Academic Editor for Current Developments in Nutrition. She was not involved in the journal's evaluation of this manuscript.

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Data availability

Data described in the manuscript and analytic code will be made available upon request.

References

[1] S. Christakos, P. Dhawan, A. Verstuyf, L. Verlinden, G. Carmeliet, Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects, Physiol. Rev. 96 (1) (2016) 365–408, https://doi.org/10.1152/physrev.00014.2015.

[2] D.W. Eyles, Vitamin D: brain and behavior, JBMR Plus 5 (1) (2021) e10419, https://doi.org/10.1002/jbm4.10419.

[3] M.F. Holick, M.B. Clark, The photobiogenesis and metabolism of vitamin D, Fed, Proc. 37 (12) (1978) 2567–2574.

[4] D. Gezen-Ak, E. Dursun, Vitamin D, a secosteroid hormone and its multifunctional receptor, vitamin D receptor, in Alzheimer's type neurodegeneration, J. Alzheimers Dis. 95 (2023) 1273–1299, https://doi.org/10.3233/JAD-230214.

[5] B.C. DeFelice, T.L. Pedersen, H. Shorrosh, R.K. Johnson, J.A. Seifert, J.M. Norris, et al., Utilizing cooled liquid chromatography and chemical derivatization to separate and quantify C3-epimers of 25-hydroxy vitamin D and low abundant 1α,25(OH)2D3: application in a pediatric population, J. Steroid Biochem. Mol. Biol. 197 (2020) 105519, https://doi.org/10.1016/j.jsbmb.2019.105519.

[6] M.K. Shea, K. Barger, B. Dawson-Hughes, S.E. Leurgans, X. Fu, B.D. James, et al., Brain vitamin D forms, cognitive decline, and neuropathology in community-dwelling older adults, Alzheimers Dement 19 (6) (2023) 2389–2396, https://doi.org/10.1002/alz.12836.

[7] X. Fu, G.G. Dolnikowski, W.B. Patterson, B. Dawson-Hughes, T. Zheng, M.C. Morris, et al., Determination of vitamin D and its metabolites in human brain using an ultra-pressure LC–tandem mass spectra method, Curr. Dev. Nutr. 3 (7) (2019) nzz074, https://doi.org/10.1093/cdn/nzz074.

[8] D.A. Bennett, J.A. Schneider, A.S. Buchman, L.L. Barnes, P.A. Boyle, R.S. Wilson, Overview and findings from the RUSH memory and aging project, Curr. Alzheimer Res. 9 (6) (2012) 646–663, https://doi.org/10.2174/156720512801322663.

[9] X. Fu, M.K. Shea, G.G. Dolnikowski, W.B. Patterson, B. Dawson-Hughes, T.M. Holland, et al., Vitamin D and vitamin K concentrations in human brain tissue are influenced by freezer storage time: the memory and aging project, J. Nutr. 151 (1) (2021) 104–108, https://doi.org/10.1093/jn/nxaa336.

[10] B.A. Stanley, J.P. Dai, A. Xu, E.J. Battaglioli, R.T. Wilson, A sensitive and cost-effective LC–MS-MS method for determination of 1α,25-Dihydroxyvitamin D3 in human plasma, LCGC Supplements 13 (Special Issues) (2015).

[11] P. Schorr, C.S. Stokes, D.A. Volmer, Improved quantitative LC-MS/MS analysis of vitamin D metabolites in serum after one-pot double derivatization, J. Pharm. Biomed. Anal. 234 (2023) 115522, https://doi.org/10.1016/j.jpba.2023.115522.

[12] Y. Xue, X. He, H.-D. Li, Y. Deng, M. Yan, H.-L. Cai, et al., Simultaneous quantification of 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 in rats shows strong correlations between serum and brain tissue levels, Int. J. Endocrinol. 2015 (2015) 296531, https://doi.org/10.1155/2015/296531.

[13] S. Zelzer, A. Meinitzer, M. Herrmann, W. Goessler, D. Enko, A novel method for the determination of vitamin D metabolites assessed at the blood-cerebrospinal fluid barrier, Biomolecules 11 (9) (2021) 1288, https://doi.org/10.3390/biom11091288.

[14] A. Mena-Bravo, F. Priego-Capote, M.D.L. de Castro, Study of blood collection and sample preparation for analysis of vitamin D and its metabolites by liquid chromatography–tandem mass spectrometry, Anal. Chim. Acta. 879 (2015) 69–76, https://doi.org/10.1016/j.aca.2015.03.012.

[15] D.W. Eyles, S. Smith, R. Kinobe, M. Hewison, J.J. McGrath, Distribution of the vitamin D receptor and 1 alpha-hydroxylase in human brain,

J. Chem. Neuroanat. 29 (1) (2005) 21–30, https://doi.org/10.1016/j.jchemneu.2004.08.006.

[16] A.J. Stephenson, B. Hunter, P.N. Shaw, N.S.A. Kassim, R. Trengove, R. Takechi, et al., A highly sensitive LC-MS/MS method for quantitative determination of 7 vitamin D metabolites in mouse brain tissue, Anal. Bioanal. Chem. 415 (7) (2023) 1357–1369, https://doi.org/10.1007/s00216-023-04527-8.

[17] S. Xu, R. Ni, L. Lv, R. Chen, Y. Chen, F. Huang, et al., Simultaneous determination of vitamin D metabolites 25(OH)D3 and 1α,25(OH)2D3 in human plasma using liquid chromatography tandem mass spectrometry, J. Mass Spectrom Adv. Clin. Lab. 24 (2022) 65–79, https://doi.org/10.1016/j.jmsacl.2022.04.001.