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Short Communication

Chromatographic separation of PTAD-derivatized 25-hydroxyvitamin D_3 and its C-3 epimer from human serum and murine skin

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ABSTRACT

The detection of 25-hydroxyvitamin D at low levels in biological samples is facilitated by the use of chemical derivatization with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) in concert with liquid chromatography–tandem mass spectrometry (LC–MS/MS). This mode of analysis is notably hampered by chromatographic co-elution of 25-hydroxyvitamin D_3 (25OHD_3) and its C-3 epimer (C3epi). The objective of this work was to improve upon current LC–MS/MS methods used for the analysis of PTAD-derivatized 25-hydroxyvitamin D_3 by resolving it from C3epi. Additionally, the applicability of this method in human serum and murine skin was investigated. C18 columns of increasing length and varying particle sizes were assessed for performance using a mixed standard of PTAD-derivatized 25OHD_3 and C3epi. Serum samples were processed using solid phase extraction, and skin was powdered and extracted for lipophilic compounds. The samples were derivatized with PTAD and subsequently analyzed using isotope dilution LC–MS/MS with atmospheric pressure chemical ionization operated in positive mode. Near baseline resolution of PTAD-25OHD_3 from PTAD-C3epi was achieved on a 250 mm C18 column with 3 μm sized particles. This separation allowed for detection and quantification of both metabolites in serum and skin samples. PTAD-C3epi represented a significant confounding analyte in all samples, and comprised up to 20% of the status measurement in skin. This method is a significant improvement on the chromatography of PTAD-derivatized vitamin D metabolites that could greatly influence the assessment of vitamin D status and C3epi biology in low abundance samples.

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1. Introduction

Determination of vitamin D status, serum 25-hydroxyvitamin D (25OHD), has been a notable challenge in clinical and research laboratories, as there are inconsistencies between methods used for measurement and between laboratories performing the assays [1]. Among modern methods for 25OHD measurement, high performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS) is the gold standard, albeit this method is not devoid of challenges [2]. Perhaps the most notable challenge in HPLC–MS/MS analysis of vitamin D status is the presence of 3-epi-25-hydroxyvitamin D (C3epi), which behaves similarly to 25OHD with respect to both MS/MS fragmentation and chromatography. The biological activity of C3epi is known to be less than that of native 25OHD, though the exact bioequivalence is still unknown [3]. Thus, resolution of these two compounds is essential for proper measurement of vitamin D status. Once only of concern as a false positive in infants less than one year of age [4], recent work has shown that C3epi is also present in the serum of adults at significant levels [5,6]. The presence of this metabolite in biological samples aside from serum has also been left largely unexplored.

In order to increase the sensitivity of HPLC–MS/MS methods, several groups have begun employing chemical derivatization with Cookson-type reagents. These compounds are 4-substituted 1,2,4-triazoline-3,5-diones that react with conjugated diene systems in a Diels–Alder fashion [7]. A number of Cookson-type reagents have been suggested for use in vitamin D analysis [8], but the most prevalent is commercially available 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD). The chromatography of these compounds is notably challenging because (*R*) and (*S*) isomers of each metabolite are created as a result of the derivatization process [9]. To our knowledge, no chromatographic method that adequately separates PTAD-derivatized C3epi from 25OHD has been published.

The objective of this work was to develop an HPLC–MS/MS method that allows for sensitive and selective measurement of PTAD-derivatized 25OHD_3 and C3epi in serum as well as murine

Abbreviation: PTAD, 4-phenyl-1,2,4-triazole-3,5-dione.

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skin as an example extrahematic matrix. This will allow further study of vitamin D status in biological samples containing low amounts of vitamin D metabolites.

2. Materials and methods

2.1. Biological samples

All mice used in this work were housed in a vivarium at The Ohio State University according to standards established by the American Association for Accreditation of Laboratory Animal Care, and procedures were approved by the appropriate Institutional Animal Care Utilization Committee. These male Skh-1 mice were fed modified AIN-93G diets containing 25, 150, or 1000 IU vitamin D_3 provided with water *ad libitum* for 29 weeks. Human serum and pooled umbilical cord serum were acquired from Innovative Research (Novi, MI).

2.2. Chemicals

Optima grade water, acetonitrile, methanol, hexane, HPLC grade dichloromethane, and 99% formic acid were obtained from Fisher Scientific (Pittsburgh, PA). $25OHD_3$ was obtained from Isosciences (King of Prussia, PA) and d3-$25OHD_3$, C3epi, and PTAD were obtained from Sigma Aldrich (St. Louis, MO).

2.3. Sample preparation

Mixed standards of $25OHD_3$ and C3epi ranging in concentration from 7.80 to 250 nM were spiked with 20 μL of a d3-$25OHD_3$ solution (200 nM in acetonitrile), dried, and set aside. Skin samples (0.30 g) were frozen with liquid nitrogen, crushed to a fine powder using a Cellcrusher (Cellcrusher Ltd.; Cork, Ireland), and transferred to glass vials. The powdered skin was then suspended in 1 mL of water, spiked with 10 μL of the d3-$25OHD_3$ solution, allowed to equilibrate for 15 min, and extracted for lipophilic compounds as previously described [10,11]. Briefly, 1 mL of ethanol containing 0.1% butylated hydroxytoluene and 5 mL of 5:1 hexane/dichloromethane were added to the skin samples, and the mixture was probe sonicated (Branson Ultrasonics; Danbury, CT) for 30 s. The homogenized solutions were centrifuged at 2000 × *g* for 5 min, and the upper organic layer was decanted into clean glass vials. The extraction was repeated two more times, with the addition of 5 mL 5:1 hexane/dicholormethane, and the pooled organic layers were dried under a stream of nitrogen gas.

Serum samples were extracted using Phree™ SPE cartridges (1 mL tubes; Phenomenex, Torrance, CA) according to the Phenomenex technical protocol [12] with minor changes. First, 200 μL of serum was deposited into an SPE cartridge, spiked with 20 μL of the d3-$25OHD_3$ solution, and allowed to equilibrate for 15 min. Then, 500 μL of an 85:15 acetonitrile/methanol solution was added to the cartridge, vortexed for 30 s, allowed to rest for 1 min, and eluted under vacuum (−50 kPa) for 2 min. The extraction was repeated once more and the pooled eluents were dried under a stream of nitrogen gas.

Dried extracts and standards were derivatized with PTAD according to Lipkie et al. [13]. Briefly, 100 μL of a 2 mg/mL PTAD solution was added to the extracts, which were then vortexed for 10 min. An additional 100 μL of PTAD was added to the solution and mixing was repeated for another 10 min. The reaction was quenched by adding 20 μL of water and vortexing for 5 min, and the reaction mixture was dried under nitrogen gas. Skin extracts were reconstituted with 100 μL of acetonitrile, and centrifuged at 21,130 × *g* for 2 min prior to analysis. Serum extracts were reconstituted with 200 μL acetonitrile and passed through 0.22 μm, 4 mm nylon filters (W.R. Grace; Deerfield, IL) before injection (10 μL) onto the HPLC–MS/MS system.

2.4. Method development and biological sample analysis

Details of the HPLC columns which were evaluated for the resolution of PTAD-C3epi and PTAD-$25OHD_3$ are summarized in Table 1. Each column was evaluated using a mixed standard of PTAD-derivatized $25OHD_3$ (100 nM) and C3epi (100 nM) with a binary mobile phase consisting of 0.1% (v/v) aqueous formic acid solution (A) and acetonitrile with 0.1% (v/v) formic acid (B) on an Agilent 1200 series HPLC (Santa Clara, CA). Mobile phase composition was adjusted for the varying column geometries. Eluent from the HPLC was directed to a QTRAP 5500 mass spectrometer (AB Sciex; Framingham, MA) equipped with atmospheric pressure chemical ionization (APCI) operated in positive ion mode. MS parameters were as follows: source temperature, 450 °C; collision energy, 25 eV; dwell time, 280 ms; curtain gas, 30 psi; ion source gas, 60 psi; declustering potential, 185 V; entrance potential, 10 V; collision cell exit potential, 11 V. PTAD-$25OHD_3$ and PTAD-C3epi were monitored from 558.3 > 298.3 *m*/*z*, and PTAD-d3-$25OHD_3$ was monitored from 561.3 > 301.3 *m*/*z*.

Vitamin D metabolites from biological samples were separated using a Luna C18 reversed phase column (4.6 mm × 250 mm, 3 μm; Phenomenex) maintained at 40 °C. Optimal separation was achieved using an isocratic flow of 29% A for 3.5 min with an immediate switch to 20% A for 3 min, followed by a column wash with 100% B for 2 min and reconditioning at initial conditions for 3.5 min. The injection needle was rinsed with acetonitrile before each injection to minimize carryover. Eluent from the HPLC was directed to the QTRAP system described above.

Peaks were integrated in Analyst 1.5.1 (AB Sciex, Framingham, MA). Areas of both PTAD-$25OHD_3$ isomers were summed and used for quantification. Isotope dilution methodology was utilized for quantification, thus analyte concentrations were calculated based on the ratio of analyte peak area to that of the labeled internal standard.

3. Results and discussion

Varying the dimensions of the C18 columns greatly influenced the separation of PTAD-derivatized $25OHD_3$ and C3epi. As column length was increased above 50 mm, three peaks were clearly observed (Fig. 1). Peak identities were confirmed using separate solutions of PTAD-derivatized $25OHD_3$ and C3epi standards. 6(*R*)-PTAD-$25OHD_3$ and 6(*S*)-PTAD-$25OHD_3$ were resolved and present in an approximately 1:4 ratio, as has been described previously [9]. Both 6(*R*)-PTAD-C3epi and 6(*S*)-PTAD-C3epi co-eluted as a single peak between the two PTAD-$25OHD_3$ diasteriomers. The best resolution of PTAD-C3epi from 6(*S*)-PTAD-$25OHD_3$ was achieved with the 4.6 mm × 250 mm Luna C18 column with a 3 μm particle size.

Table 1
Details of evaluated HPLC columns.

Column name	Particle size (μm)	Dimensions (mm × mm)	Manufacturer
Zorbax extend UHPLC C18 HT	1.8	2.1 × 50	Agilent[a]
Symmetry C18	3.5	4.6 × 75	Waters[b]
Eclipse XDB-C18	5.0	4.6 × 150	Agilent
Luna C18(2)	5.0	4.6 × 250	Phenomenex[c]
Luna C18(2)	3.0	4.6 × 250	Phenomenex

[a] Santa Clara, CA.
[b] Milford, MA.
[c] Torrance, CA.

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