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Fast separation of 25-hydroxyvitamin D_3 from its C3-epimer in human serum by liquid chromatography-tandem mass spectrometry showing variable C3-epimer prevalence in infants and adults

J.M.W. van den OUWELAND, A.M. BEIJERS and H. van DAAL

Measurement of 25-hydroxyvitamin D (25(OH)D) is accepted as a reliable clinical indicator of the vitamin D status in humans, which is important in the diagnosis of vitamin D deficiency and for monitoring supplementation therapy. The number of laboratories that use liquid chromatography-tandem mass spectrometry (LC-MS/MS) for measurement of 25(OH) D is steadily increasing. LC-MS/MS allows measurement of both 25(OH)D3 and 25(OH)D2 independently with high sensitivity. A limitation of most LC-MS/MS methods is the potential interference from co-eluting isomeric compounds having identical elemental composition but different structure, leading to overestimation of true 25(OH)D concentrations. It was recently shown that 25(OH)D3 is metabolized through the C3-epimerization pathway like 1,25(OH)2D3 and 24,25(OH)2D3 (1). However, the biological relevance of 3-epi-25(OH)D remains to be elucidated. The C3-epimer has received attention from its detection in 23% of infant sera less than 1 year old with 3-epi-25(OH) D contributing 9-61% of the total 25(OH)D by using a modified LC-MS/MS method which partially separated 25(OH)D from its C3-epimer (2). There was an inverse relationship between patient age in days and the percentage of 3-epi-25(OH)D. The 3-epi-25(OH) D metabolite was not detected in children from 1-18 years of age or in adults. This led the authors to postulate that C3-epimerisation originates from immature vitamin D metabolism. A limitation of their study was that 3-epi-25(OH)D was only partially separated from the major 25(OH)D peak which did not allow them to quantify low (<8.5%) levels of 3-epi-25(OH)D. It was recently shown that by using techniques achieving baseline separation between the 25(OH)D3 and epi-25(OH)D3, the C3-epimer can also be detected in sera from adults, albeit at much lower concentrations when compared to infants (3,4). To separate 3-epi-25(OH) D from 25(OH)D, current procedures require lengthy chromatographic run times varying from 12 to 40 min (5), which makes these methods unsuitable for clinical laboratories that must deal with increasing numbers of vitamin D requests. We describe a modification of an established LC-MS/MS method for measurement of 25(OH)D3 and 25(OH)D2 (6) that allows fast separation of 25(OH)D3 from 3-epi-25(OH)D3 in human serum.

Methods

Sample preparation, assay calibration, and instrument operation were carried out as described (6), with minor modifications. 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 were from Sigma Aldrich (Zwijndrecht, The Netherlands), and the internal standard (IS) 26,27-hexadeuterium labelled 25(OH)D_3 was from Synthetica AS (Oslo, Norway). Six-point 25(OH) D_3 and 25(OH)D_2 calibration curves (range 25-545 nmol/L) were constructed from 25(OH)D_3 and 25(OH)D_2 stock solutions prepared in PBS containing 60 g/L albumin. Calibrators, controls and patient sera were treated with sodium hydroxide to release vitamin D metabolites from the binding protein before protein precipitation. Subsequent off-line solid-phase extraction was followed by chromatographic separation performed by use of penta-fluorophenyl-propyl (PFP) column (ACQUITY, CSH fluoro-phenyl 1.7 µm, 2.1x100 mm, Waters). Mobile phases A and B consisted of 1 ml/L formic acid in ammonium acetate (2 mmol/L), and 3 ml/L formic acid in methanol, respectively. A flow rate of 0.35 ml/min was used, with reduction to 0.30 mL/min in the final step, by using a gradient to 85% B (0-5 min), 85% B rinse (5.0 to 5.3 min), and reversion to 50% B (5.3-5.4 min), followed by 50% B (5.4 to 6.5 min). Detection was by registration *m/z* transitions 401.5→159.2 for 25(OH) D_3 and 3-epi-25(OH)D_3, 413.4→159.2 for 25(OH)D_2 and 407.5→159.2 for the IS. The percentage 3-epi-25(OH)D_3 was calculated relative to the total 25(OH) D content. Inter-assay coefficients of variation for 3 concentrations of 25(OH)D_3 control sera (39, 92, and 127 nmol/L; Chromsystems)(n=4) were 4.2%, 3.5%, and 2.8%, respectively. We further investigated the prevalence of the 3-epi-25(OH)D_3 in leftover serum samples from infants (<1 year of age, n=51), children (1-10 years of age, n=74), and adults (>18 years of age, n=104). The samples were treated in agreement with local ethics guidelines.

Klinisch Chemisch Laboratorium, Canisius-Wilhelmina Ziekenhuis, Nijmegen

E-mail: j.v.d.ouweland@cwz.nl

Afkortingen: 25(OH)D: 25-hydroxyvitamin D; LC-MS/MS: liquid chromatography-tandem mass spectrometry; PFP: pentafluorophenylpropyl; IS: internal standard

Results and discussion

25(OH)D_3 and IS eluted at about 4.32 min, with 3-epi-25(OH)D_3 eluting at 4.42 min, with near base-line separation from 25(OH)D_3 (figure 1). 25(OH)D_2 eluted at 4.42 min (result not shown). 25(OH)D_3 concentrations ranged from 4.3 to 300 nmol/L, with no relevant concentrations of 25(OH)D_2. We could detect the presence of 3-epi-25(OH)D_3 in all sera from infants and children and in 75% of sera from adults (table 1). The mean (median; range) percentages were 11.1% (9.3%;2.3%-49.2%) in infants, 6.2% (5.7%; 2.5%-20.0%) in children, and 3.5% (3.1%; <2%-10.6%) in adults. No correlation was found between the relative content of 3-epi-25(OH)D_3 and the absolute amount of 25(OH)D_3. Percentages of 3-epi-25(OH)D_3 exceeding 10% were mainly found in 18 (39%) of 46 infants < 3 months of age, consistent with previous findings (2), although higher percentages (10%-20%) were found in 4 (5.4%) of 74 children and 1 (1.0%) of 104 adults as well, confirming recent findings of considerable amounts of 3-epi-25(OH)D_3 in adults (4).

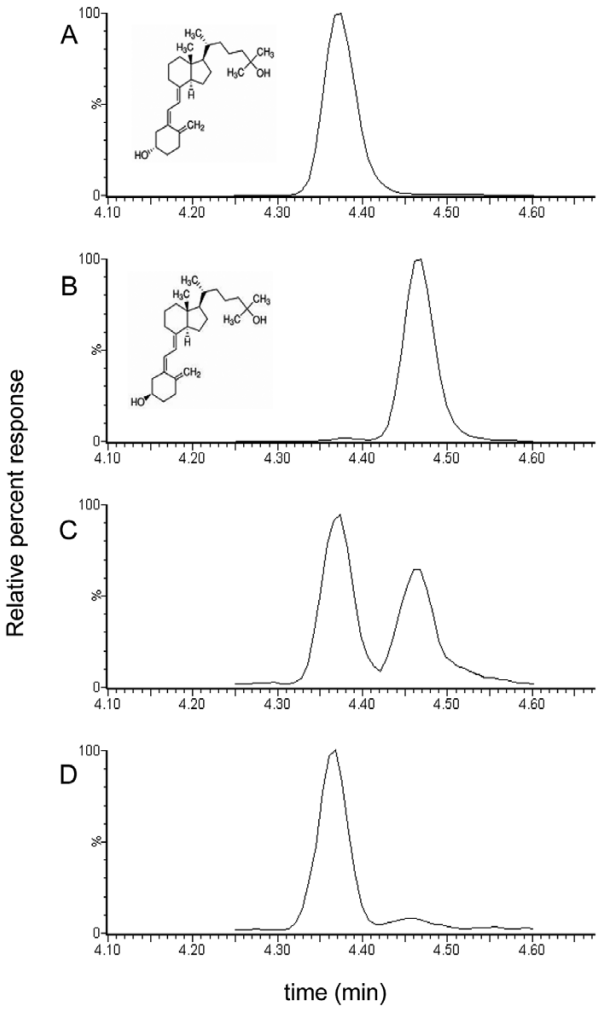

Figure 1. Chromatographic separation of 3-epi-25(OH)D_3 from 25(OH)D_3

LC-MS/MS chromatograms of injection of pure standards 25-(OH)D_3 (a) and 3-epi-25(OH)D_3 (b), as well as examples of an infant serum sample (c) with 25(OH)D_3 concentration of 68.5 nmol/L and 44.6% percentage of 3-epi-25-(OH)D_3, and of an adult serum sample (d) with 25(OH)D_3 concentration of 79.3 nmol/L and 4.7% percentage 3-epi-25-(OH)D_3. Note the difference in orientation of the C3-hydroxylgroup between 3-epi-25(OH)D_3 (α-position) and 25(OH)D_3 (β-position).

Table 1. 3-epi-25(OH)D_3 prevalence in different age categories

		25(OH)D_3 (nmol/L)			3-epi-25(OH)D_3 (% of total 25(OH)D_3)		
Group	n	mean	median	range	mean	median	range
< 1yr	51	64.7	62.1	17.1-300.0	11.1	9.3	2.3-49.2
1-10 yr	74	73.2	73.1	30.5-113.2	6.2	5.7	2.5-20.0
>18 yr	104	47.7	43.8	4.3-163.5	3.5	3.1	<2*-10.6

* In 26 of 104 adult sera (25%) 3-epi-25(OH)D_3 could not be detected (<2%).

Measurement of adult sera with detectable C3-epimer (n=78) for total 25(OH)D (25(OH)D_3 including 3-epi-25(OH)D_3) by the standard UPLC-MS/MS (6) and the new LC-MS/MS showed good comparability (Passing and Bablock (P&B) regression: Fluoro-phenyl UPLC-MS/MS = 0.98 (95% Confidence Interval (CI): 0.95-1.01) x C18 UPLC-MS/MS + 0.31 (95% CI:-0.73-1.55); r= 0.994). By exclusion of 3-epi-25(OH)D_3 the new LC-MS/MS method gives a mean 4% lower concentration for 25(OH)D_3 compared with our previous method, for which we used C-18 as stationary phase (P&B regression: Fluoro-phenyl UPLC-MS/MS = 0.94 (95% CI: 0.92-0.97) x C18 UPLC-MS/MS + 0.31 (95% CI:-0.42-0.96); r= 0.995, n=104). The co-measurement of 3-epi-25(OH)D is likely to contribute to the positive bias of many current LC-MS/MS assays compared to the NIST candidate reference measurement procedure (5, 7, 8). Evidently, further investigations are needed to elucidate the biological significance of the 3-epi-25(OH)D metabolites, the conditions that favor C3 epimerization of 25(OH)D, and to what extent separate reporting of 3-epi-25(OH)D_3 might be of clinical relevance.

Conclusion

In conclusion, the presence of 3-epi-25(OH)D_3 in nearly all human sera necessitates the use of an LC-MS/MS method that separates 3-epi-25(OH)D_3 from 25(OH)D_3 for accurate detection of 25(OH)D_3. By using a PFP column, 25(OH)D_3 and the 3-epi-25(OH)D_3 can be separated within a total run time of 6.5 min, making this method fast and attractive for routine measurement of 25(OH)D in clinical laboratories.

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Development and validation of a testosterone assay using liquid chromatography tandem mass spectrometry without derivatization

H.H. van ROSSUM[1], J.D. FAIX[2] and R.Z. SHI[2]

Analysis of testosterone is helpful for investigation of several conditions such as hypogonadism or limited testis function in man, hirsutism hyperandrogenism or polycystic ovarian syndrome in women, and early or late onset of puberty in boys. Unfortunately the analytical performance of the commonly used testosterone immunoassays is limited in terms of sensitivity and specificity for analysis of low testosterone concentrations that are normally found in females and children. Since improved sensitivity and specificity in the low testosterone concentration range has been reported for testosterone assays using liquid chromatography tandem mass spectrometry (LC-MS/MS), we intended to set-up such an assay (1-5). Our goal was to develop a method applicable for routine testing, and although methods that utilize testosterone derivatization generally result in even higher testosterone assay sensitivity, we choose to avoid derivatization in order to simplify sample preparation.

Methods

Sample preparation

Patient samples were obtained by venous phlebotomy using serum BD vacutainer coagulation tubes and serum was obtained after centrifugation.

50 µL of internal standard solution (2000 ng/dL d3-testosterone in methanol; Sigma-Aldrich) was added to 200 µL of quality control, calibrator or patient sample and incubated for 20 minutes at room temperature (RT). Next, samples were extracted for 30 minutes at RT using 1 mL of methyl t-butyl ether. After transferring the organic phase to new vials and evaporation of the solvent, the residue was reconstituted in 150 µL water-methanol solution (1:1 v/v).

LC-MS/MS

LC was run on a Shimazu HPLC system consisting of two pumps, auto-sampler and column oven. 30 µL of sample was injected and separation was performed using a Kinetex reverse phase C18 column (2.6 µm, 100 x 3 mm, Phenomenex) kept at 40˚C. The flow-rate was kept constant at 0.45 mL/min and 30% mobile phase A (0.1% formic acid in water) and 70% mobile phase B (0.1% formic acid in methanol) was used as starting liquid phase condition. After 1 minute, mobile phase B was increased linearly to 95% in 2 minutes and left at 95% for another 1.5 minutes. Thereafter the system was reset at starting condition and allowed to equilibrate for 2 minutes. The total run time was 5.5 minutes.

MS/MS analyses were performed on an API 5000 (AB Sciex). Positive mode electrospray ionization (ESI, Turbospray) was applied. The ion-source settings were: Curtain gas 40, CAD 9, GS1 50, GS2 50, Temperature 650˚C and ion source 3500 V.

Sample analysis was performed using multiple reaction monitoring (MRM) with a dwell time of 50 ms. The 289.4/97.1 and 289.4/109.1 transitions were used to monitor testosterone and the 292.4/97.0 and 292.4/109.2 transitions for d3-testosterone. The first was used as IS for all testosterone concentration calculations. N_2 was used as collision gas and declustering potential, entrance potential, cell entrance potential and collision cell exit potential settings were optimized for each transition.

Clinical chemistry and Hematology laboratory, Bronovo Hospital, The Hague [1] and Department of Pathology, Stanford University[2], Palo Alto, CA

E-mail: hhvrossum@bronovo.nl