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Validation and comparison of a rapid liquid chromatography tandem mass spectrometry method for serum 25OHD with the efficiency of separating 3-*epi* 25OHD$_3$

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ABSTRACT

Objectives: To develop a rapid liquid chromatography tandem mass spectrometry (LC-MS/MS) method with ability to separate 3-*epi* 25OHD$_3$ (EPI-LC-MS/MS) from 25OHD$_3$, and evaluate the effects of 3-*epi* 25OHD$_3$ on routine LC-MS/MS that cannot separate 3-*epi* 25OHD$_3$ (NEPI-LC-MS/MS).

Design and methods: Performance of the newly built EPI-LC-MS/MS was validated, and 982 samples were analyzed and compared by the two methods.

Results: Both methods showed a linearity coefficient correlation exceeding 0.999 in the 6.25–500 nmol/L range for 25OHD$_2$ and 25OHD$_3$. Moreover, they showed a between run coefficient variation (CV) and total CV of < 5% for 25OHD$_2$ and 25OHD$_3$. The results of the accuracy test showed that the bias was below 6.19% in the absence of 3-*epi* 25OHD$_3$. Comparison of the 25OHD results obtained by the two methods for 982 patients (age 1-100 years) revealed excellent clinical agreement (Cohen's kappa = 0.875) and correlation (R2 = 0.973). Among the 982 patients, only 73 patients had 3-epi 25OHD$_3$ (> 6.25 nmol/L); out of these 73 patients, the 3-*epi* 25OHD$_3$ level in 58 patients was between 6.25 and 12.5 nmol/L. In patients with < 375 nmol/L 25OHD (25OHD2 + 25OHD3), only 8 had 3-epi 25OHD$_3$ levels exceeding 12.5 nmol/L (range: 13.3 - 27.5 nmol/L). Among samples containing 3-*epi* 25OHD$_3$, only three were separated into different 25OHD-deficiency groups using the above methods.

Conclusion: A rapid and precise EPI-LC-MS/MS method for measuring 25OHD with efficient separation of 3-*epi* 25OHD$_3$ was developed. Our results showed that 3-epi 25OHD$_3$ had little effect on the routinely used NEPI-LC-MS/MS.

In the last few years, the interest in 25-hydroxyvitamin D (25OHD) has increased because of its skeletal and non-skeletal effects. It has been reported that people in all age groups suffer from 25OHD deficiency [1,2], which has led to a massive increase in vitamin D testing. Automated immunoassays for measuring 25OHD are advantageous because they are rapid and simple. However, their applicability may be limited because of the cross-reactivity of antibodies and nonequimolar recognition of 25OHD$_2$ and 25OHD$_3$—the two main types of 25OHD that are believed to have different efficacies [3,4]. Various laboratories are using LC-MS/MS methods to determine both 25OHD$_2$ and 25OHD$_3$. These methods are advantageous for the accurate measurement and research of 25OHD$_2$ and 25OHD$_3$. However, few laboratories use LC-MS/MS to measure 25OHD and to separate 3-*epi* 25OHD$_3$—an epimer of 25OHD$_3$ whose bioactivity is still unclear [5–7]. The current methods available for separating 3-*epi* 25OHD$_3$ require a long analysis time [8–10], which decreases the clinical detection efficiency. However, it has been reported that most people have 3-*epi* 25OHD$_3$, especially infants [11,12]. However, the effects of 3-*epi* 25OHD$_3$ on routine LC-MS/MS assays for vitamin D have not been determined completely and they are still controversial [10,13]. Moreover, no data is available on 3-*epi* 25OHD$_3$ in China, and the amount of 3-*epi* 25OHD$_3$ in Chinese people is unclear. In this study, we developed and validated a rapid LC-MS/MS method that can separate 3-*epi* 25OHD$_3$ (called EPI-LC-MS/MS), and compared the obtained results to those derived using a routine LC-MS/MS method that cannot separate 3-*epi* 25OHD$_3$ (called NEPI-LC-MS/MS). We also analyzed the necessity to separate 3-*epi* 25OHD$_3$ by measuring vitamin D levels in 982 patients using both methods.

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This work demonstrates the first evaluation of 3-*epi* 25OHD$_3$ levels in Chinese patients who requested vitamin D tests.

1. Materials and methods

1.1. Chemicals

25OHD$_2$, 25OHD$_3$, 3-*epi* 25OHD$_3$, [^{2}H]$_3$-25OHD$_2$ (internal standard for 25OHD$_2$), [^{2}H]$_3$-25OHD$_3$ (internal standard for 25OHD$_3$ and 3-*epi* 25OHD$_3$), and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard reference materials SRM 2972 and SRM 972a were purchased from the National Institute of Standards and Technology (NIST). Organic solvents, hexane and methanol (HPLC-grade), were obtained from Fisher Scientific (Pittsburgh, PA, USA). $ZnSO_4$ was purchased from China National Medicines Corporation Ltd. (Beijing, China).

1.2. Preparation of calibrators

Mixed calibration standards were prepared at concentrations of 6.25/6.25, 12.5/12.5, 25/25, 50/50, 125/125, 250/250, and 500/500 nmol/L (25OHD$_2$/25OHD$_3$) (conversion: SI to traditional units - 1 nmol/L ≈ 0.4 ng/mL) in methanol. They were certified using NIST SRM2972. All calibrators were treated as samples in each batch.

1.3. Serum samples

From April to August 2014, 982 fresh serum samples were collected from leftover samples from patients that came in to the Department of Laboratory Medicine of Peking Union Medical College Hospital for 25OHD tests. Fasting blood samples were taken by venipuncture into tubes containing a clot activator. The serum was isolated and stored at 4 °C and analyzed once a week using both LC-MS/MS methods. This study was reviewed and approved by the Ethics Committee of Peking Union Medical College Hospital.

1.4. Sample preparation

The sample preparation protocol for EPI-LC-MS/MS was the same as that for NEPI-LC-MS/MS, which was described previously [2]. Generally, serum samples, calibrators, and controls were first equilibrated at room temperature, and then 180 μL of each sample was precisely transferred to a 5 mL glass tube using a micropipettor. Next, 50 μL of internal standards (including 250 nmol/L [^{2}H]$_3$-25OHD$_2$ and [^{2}H]$_3$-25OHD$_3$) was delivered with 450 μL methanol into a glass tube using an automatic diluter (Hamilton, Reno, NV, USA). Then the solution was shaken on a mechanical shaker at 1500 rpm for 3 min. Then, 40 μL of 1 mol/L $ZnSO_4$ and 1 mL of hexane was added to each tube, and the tubes were vortexed on the mechanical shaker at 1500 rpm for 15 min. The solution was centrifuged at 1200 *g* for 10 min. Subsequently, 800 μL of the supernatant was transferred to a 2 mL vial and evaporated to dryness under nitrogen at room temperature. The residue was then reconstituted in 200 μL methanol/water (70:30) and loaded into the LC-MS/MS system.

1.5. LC-MS/MS analysis

The LC-MS/MS methods used in this work used a Waters ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) in tandem with an AB Sciex 4000 QTrap system (Sciex Applied Biosystems, Foster City, CA, USA).

The routinely used NEPI-LC-MS/MS chromatographic separation was performed using a Waters ACQUITY UPLC® BEH Phenyl column (2.1 mm × 100 mm, 1.7 μm). Mobile phase A consisted of methanol and phase B comprised water with 0.1% formic acid. The gradient was as follows: 0–0.1 min, 60% A; 0.1–2.0 min, 75% A; 2.0–3.0 min, 98% A; 3.0–3.1 min, 60% A; and 3.1–4.5 min, 60% A. At a flow rate of 0.4 mL/min, this method cannot separate 3-*epi* 25OHD from 25OHD$_3$.

The newly built EPI-LC-MS/MS that can separate 3-*epi* 25OHD$_3$ from 25OHD$_3$ was comprised of a Phenomenex Kinetex PFP analytical column (100 × 3.0 mm, 2.6 μm) with methanol as mobile phase A and 0.1% formic acid in water as mobile phase B. The gradient was: 0–0.2 min, 40% A; 0.2–0.3 min, 40%–75% A; 0.3–4.5 min, 84%

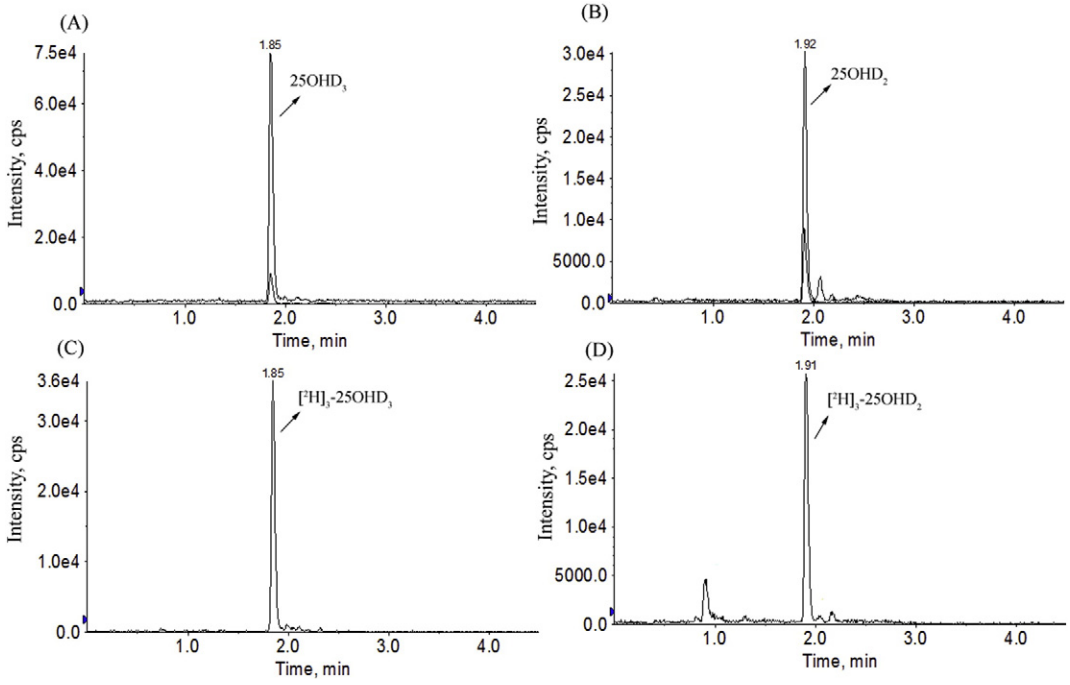

Fig. 1. Representative chromatography of standard solution after extraction as analyzed by NEPI-LC-MS/MS. A, B, C, and D are representative chromatography of 25OHD$_3$ (≈50 nmol/L), 25OHD$_2$ (≈50 nmol/L), [^{2}H]$_3$-25OHD$_3$ (≈25 nmol/L) (internal standard), and [^{2}H]$_3$-25OHD$_2$ (≈25 nmol/L)(internal standard), respectively.