

Chapter 24

LC–MS/MS Determination of 25-Hydroxyvitamin D in Human Serum Based on Covalent Organic Frameworks Magnetic Solid-Phase Extraction Materials



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Abstract This paper established a method for the test on 25-hydroxy vitamin D2/D3 in human serum based on a novel covalent organic material-based magnetic solid-phase extraction-liquid chromatography tandem mass spectrometry. At the same time, an automated pre-processing platform was developed that can process samples quickly, efficiently, and with multiple throughputs, reducing manual operations and errors, and providing a fully automatic sample pre-processing system that can realize large-scale sample detection in clinical laboratories. Experiments have investigated the effects of different materials and elution solvents on the extraction efficiency of the target. Under the best conditions, 25-hydroxyvitamin D2/D3 has a good linearity in the linear range, and the sensitivity, precision, and accuracy can all satisfy the testing requirements of clinical biological samples. This method is of great significance for clinical laboratories to improve testing efficiency.

24.1 Introduction

Vitamin D is a fat-soluble vitamin necessary for body metabolism, which not only affects calcium and phosphorus metabolism but also has a wide range of physiological effects. It is an essential substance for maintaining human health, cell growth, and development and is closely related to many diseases [1–3]. 25-hydroxy vitamin D, including 25-hydroxy vitamin D2 (25-OH VD2) and 25-hydroxy vitamin D3 (25-OH VD3), is one of the main metabolic forms of vitamin D in the body [4, 5]. Because

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of its long half-life and stable existence, it is considered to be a marker of vitamin D nutritional level [6]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the detection of vitamin D has the characteristics of strong specificity and high accuracy and is considered to be the “gold standard” assay for evaluating its nutritional status [7–10]. However, due to the complex sample pre-processing operation steps and different analyte pre-processing procedures, the detection throughput is low, which limits its application in the detection of large quantities of samples in clinical laboratories [11–13]. To alleviate the pressure of labor, space, and equipment investment caused by the increasing demand for clinical testing, and to reduce the quality risk caused by manual operation, it is urgent to improve the efficiency of LC-MS/MS method for detecting serum 25-hydroxy vitamin D.

Magnetic-solid phase extraction (M-SPE) based on magnetic micro-nanoparticles is a dispersive solid-phase extraction technology that uses magnetic or magnetizable materials as the adsorbent matrix [14–16]. Compared with the traditional solid-phase extraction (SPE) technology, this kind of micro-nanoparticles can be completely exposed to and fully contacted with the system to be tested, so it can absorb and extract the substance to be tested from the system to be tested in a short time, realizing the high-fold enrichment of trace compounds in the sample. In addition, the magnetic particles can be easily separated and collected from the system to be tested by an external magnetic field, which eliminates the complicated pumps, valves, pipelines, and other components of the traditional automatic SPE system and avoids tedious filtration or centrifugation process [17–19].

In this experiment, covalent organic frameworks based on magnetic nanospheres (MCOFs) with different pore diameters were selected. This material is independently developed and synthesized by the research group of Teacher Tang Anna in Nankai University, which can design the pore size of the substance based on the size of the target analyte molecule, so as to achieve maximum efficiency of adsorption and extraction of the substance to be measured. At the same time, the automatic pretreatment platform-the magnetic solid-phase extraction instrument can be designed with a variety of fluxes. It can be extracted by a single tube or 8×96 samples, and its operation is simple and fast. The extraction time of 8×96 samples is less than 20 min, which greatly improves the efficiency of the experiment. The automatic processing of the whole process of extraction of the test substance reduces the quality risk caused by manual operation. Using LC-MS/MS to achieve the quantitative detection of 25-hydroxyvitamin D in human serum greatly improves the detection efficiency of the sample.

24.2 Materials and Method

24.2.1 *Synthesis of Dual-Porous Magnetic Covalent Organic Composite Materials*

In the experiment, Fe_3O_4 nanoparticles were synthesized by the solvothermal reduction method, and then the amino-functionalized magnetic silicon nanospheres ($\text{Fe}_3\text{O}_4@\text{SiO}_2\text{-NH}_2$) were synthesized. Finally, 4,4',4'',4'''-(ethene-1,1,2,2-tetrayl) tetraaniline (ETTA) and [1,1'-biphenyl]-4,4'-dicarbaldehyde (BPDA) monomers were synthesized by solvothermal method to synthesize heterosporous MCOFs materials modified with COFs materials [20].

24.2.2 *Synthesis of Dual-Porous Magnetic Covalent Organic Composite Materials*

Using MCOFs as a carrier, using the π bond, hydrophobic effect, and pore retention provided by the surface material of MCOFs in an aqueous environment to absorb 25-hydroxy vitamin D, and destroy the force in an organic environment, making MCOFs and 25-hydroxy vitamin D to be separated [21–23], and then the entire extraction and purification process of 25-hydroxy vitamin D is realized by moving MCOFs or transferring liquid. The specific experimental process is as follows: adding MCOFs suspension (100 μl MCOFs solution + 300 μl activation solution) to the first row of the 96 deep well plates; adding 100 μl serum sample, 180 μl methanol, 20 μl saturated zinc sulfate solution, and 200 μl sample diluent to the second row; adding 200 μl eluent to the third row and fourth row; add 100 μl eluent to the fifth row. Then the designed program was input on the solid-phase extraction instrument, and the whole experiment was completed at room temperature. After the program was finished, the eluent was injected into LC–MS/MS for testing.

24.2.3 *LC–MS/MS*

A Shimadzu liquid chromatography (Shimadzu LC-20AD) tandem triple-quadrupole mass spectrometer (AB Triple Quad 4000 Mass Spectrometry) was used for sample detection. This experiment adopted electrospray ionization (ESI), multiple reaction monitoring (MRM) for selective ion monitor, and the following mass spectrometry detection conditions were applied: Atomizing gas (Gas1), 70 psi; Ionspray voltage (IS), 5000 V; Curtain gas (CUR), 40 psi; Collision gas (CAD), 3 psi; Ion source temperature (TEM), 500 °C. The transition pairs of each compound and its internal standard were as follows: 25-OH VD3, 401.4 \rightarrow 365.4, 25-OH VD2, 413.3 \rightarrow 337.4,

d6-25-OH VD3, 407.4 \rightarrow 371.4, d6-25-OH VD2, 419.3 \rightarrow 355.4. Chromatographic separation was performed on a C18 column (Phenomenex Company, 50 \times 3 mm, 2.6 μ m) at 30 $^{\circ}$ C. Solution A was 0.05% acetic acid in methanol, and solution B was 0.05% acetic acid in ultra-pure water. The gradient elution program was as follows: 80% A at 0 min and then held for 0.7 min; 80% A at 1.1 min; 97% A at 1.1 min and then held for 1.2 min for column equilibration. For LC analysis, 10 μ L extract was injected, and the flow rate was maintained at 0.6 mL min $^{-1}$. A representative spectrum of 25-hydroxy vitamin D is shown in Fig. 24.1.

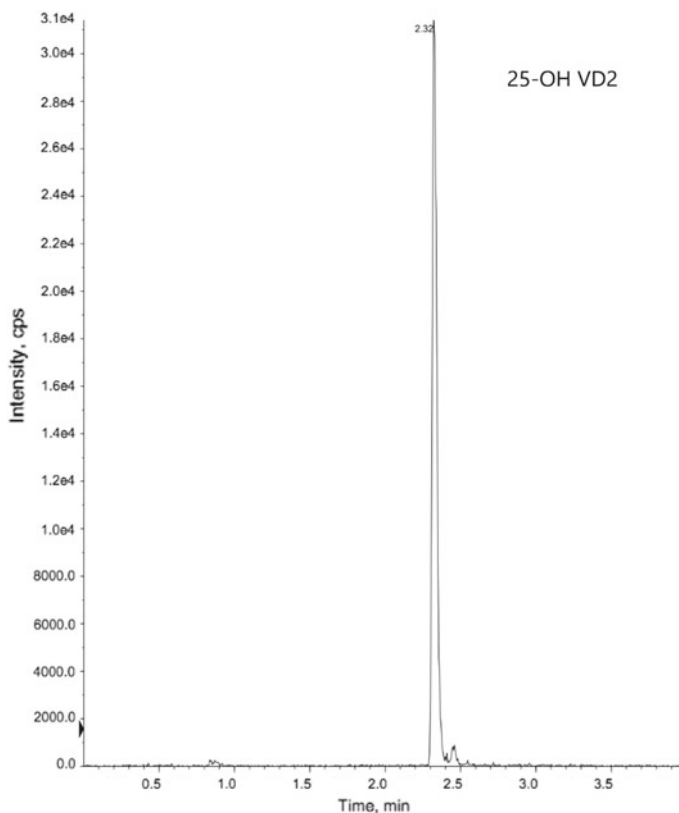


Fig. 24.1 LC-MS/MS representative chromatogram of 25-hydroxy vitamin D

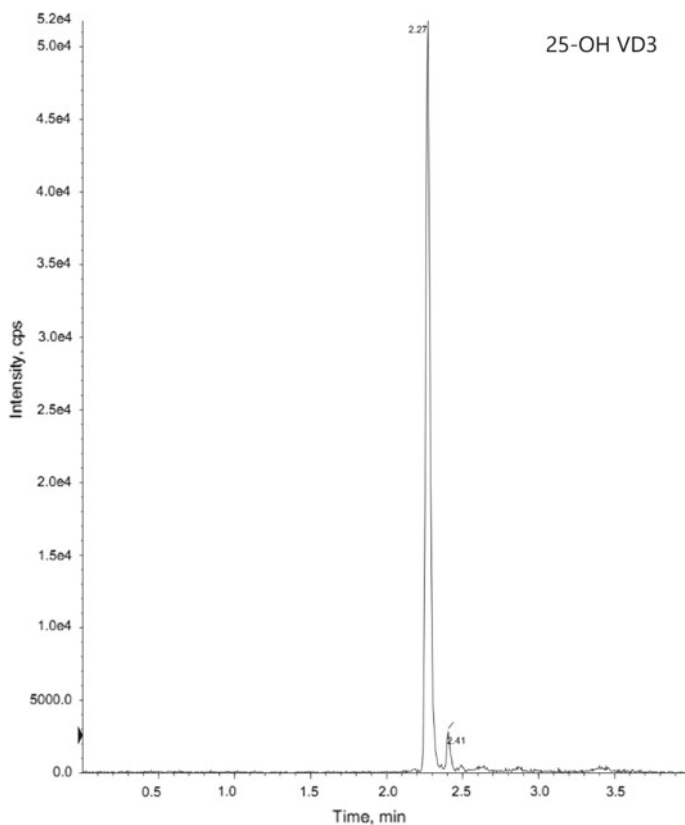


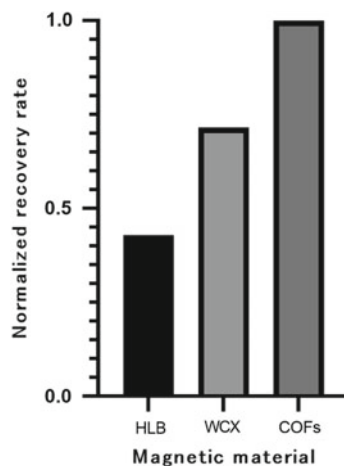
Fig. 24.1 (continued)

24.3 Results and Discussion

24.3.1 Methodological Evaluation

The detection linear ranges of 25-OH VD2 and 25-OH VD3 were 0.5–50.0 ng/mL and 1.0–100.0 ng/mL, respectively, and the linear correlation coefficients (r^2) obtained were all greater than 0.99; both the inter-day and intra-day precisions were less than 15% and the recoveries in the 80–120% range.

Fig. 24.2 The extraction recovery rate of three magnetic materials: HLB, WCX, and COFs to 25-hydroxyvitamin D in human serum, normalized based on the recovery rate of COFs material



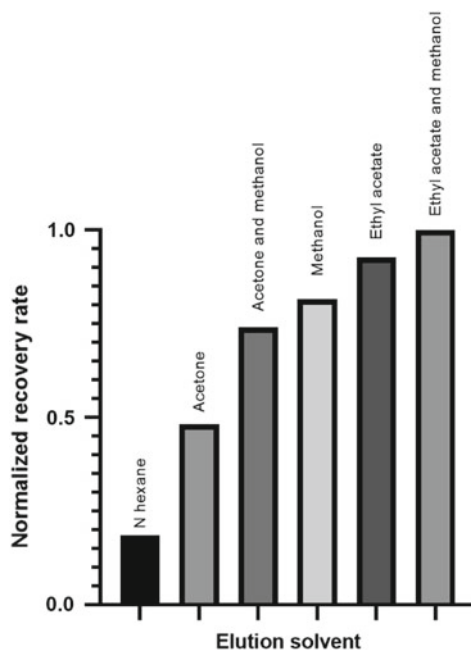
24.3.2 Material Selection

This study compared the extraction recovery rate of 25-hydroxyvitamin D from human serum by three magnetic solid-phase extraction materials of hydrophilic-lipophile balance (HLB), weak cation exchange (WCX), and COFs. The extraction performance of various materials was investigated, and the comparison result is shown in Fig. 24.2. ChemBio 3D software was used to calculate the size of 25-OH VD2 molecule as 0.67×1.69 nm and the size of 25-OH VD3 molecule as 0.58×1.69 nm. Based on the molecular size of 25-hydroxy vitamin D, the heteropore was independently developed and synthesized by the cooperating unit COFs magnetic material, and the size of the magnetic core was about 200 nm. After the COFs monomer was wrapped, the size of the magnetic particles was about 300–400 nm, and the size of the double pores was 3.2 and 1.3 nm, which can effectively intercept 25-hydroxy vitamin D molecules, and remove the protein and other organic macromolecules in the serum. Therefore, the extraction capacity of this COFs magnetic material for 25-hydroxyvitamin D in human serum is stronger than that of the other two commercial extraction materials.

24.3.3 Optimization of Eluent

The experiment was compared with methanol, n-hexane, acetone, ethyl acetate, ethyl acetate methanol, and acetone methanol solution as eluents. The experimental results are shown in Fig. 24.3. When ethyl acetate methanol solution is used as the eluent, the recovery rate is the highest. 25-hydroxy vitamin D is weakly polar, n-hexane is a non-polar solvent (polarity is 0.06), ethyl acetate and acetone are weakly polar solvents (polarity are 4.3 and 5.4, respectively), and methanol is a polar solvent (polarity

Fig. 24.3 Comparison of the extraction recovery rate of 25-hydroxyvitamin D in human serum with various eluents, normalized based on the recovery rate of ethyl acetate methanol solvent



is 0.06) [24]. According to the principle of similar mutual solubility, the polarity of the mixture of ethyl acetate and methanol is more suitable for the extraction of 25-hydroxy vitamin D, so the elution capacity is the strongest.

24.4 Conclusion

This experiment explored a new type of magnetic covalent organic framework material with different pore diameters for the extraction and recovery of 25-hydroxyvitamin D in serum. Combined with an automated pre-processing platform for magnetic solid-phase extraction, it can avoid the possibility of large differences between different batches and different experimenters due to manual intervention, which can improve throughput, consistency, and versatility. At the same time, the selected COFs magnetic materials can be designed with different pore sizes according to the molecular size of the target analyte to maximize the extraction of the target analyte.

Vitamins in the human body are essential nutrients for the human body and play a vital role in the physiology and pathology of the human body. However, since the current common clinical testing methods can only detect one to two specific vitamins, it is impossible to detect the content of multiple vitamins in the sample at the same time. In addition, the content of vitamins in the serum is widely distributed, such as the content of vitamin K that is below the nanogram level, and the content of

vitamin E that is at the microgram level. Therefore, it is very challenging to require a method that can simultaneously efficiently and accurately quantify a variety of fat-soluble vitamins in human serum. To solve this problem, this study also explored the simultaneous extraction of fat-soluble vitamins A, D, E, and K in human serum with COF magnetic materials, and the initial results have been achieved. In the future, the laboratory will work with cooperators to design a variety of COFs magnetic materials for the analysis of other organic substances such as water-soluble vitamins and hormones in the human body, combined with an automated pre-processing platform, to better serve the analysis of clinical samples.

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