Supporting Information

A Fluidic Isolation-Assisted Homogeneous-Flow-Pressure Chip-Solid Phase Extraction-Mass Spectrometry System for Online Dynamic Monitoring of 25-Hydroxyvitamin D_3 Biotransformation in Cells

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1. Chemicals and Reagents

The $25(OH)D_3$ and PTAD were purchased from Sigma-Aldrich (Saint Louis, USA), ketoconazole was bought from Aladdin (Shanghai, China), $24,25(OH)_2D_3$ and d_6-$24,25(OH)_2D_3$ were obtained from Toronto research chemicals (North York, Canada). Trizol was purchased from Invitrogen (California, USA). DNase I and RNase Inhibitor were bought from Fermentas (USA). 2×Ex TapMix was obtained from biological engineering Co., Ltd (Dalian, China). Agarose was bought from Biowest (Spain). Eva green was purchased from Biotium (California, USA). 50x TAE was obtained from Bioneer (Korea). Isopropanol, chloroform, formaldehyde, and ethanol were from Beijing chemical reagent company (Beijing, China). LC-MS grade water, methanol, formic acid, and ammonium formate were from Fisher Scientific (Fairlawn, NJ).

2. Manufacture the Microchip

The photo mask was designed six cell culture channels to satisfy the experimental variables and make high throughput detection of metabolites (Figure S1). This microchip was fabricated by soft lithography and replica molding techniques[1]. An SU-8 2050 negative photoresist (Microchem, USA) was spin-coated on a cleaned silicon wafer. After soft bake in the oven and cooling down to room temperature, the wafer was covered with the photo mask and exposed to UV light for 2 min. The silicon mold was developed by developing solution (Microchem, USA). A 10:1 weight mixture of PDMS prepolymer and curing agent (Sylgard184, Dow corning) was then poured onto the silicon mold and baked in the oven at 65 °C for 2 h. Then the PDMS was peeled from the wafer mold, and connection holes were punched before the PDMS replica was irreversibly sealed with glass slides by oxygen plasma treatment (PDC-32G, Harrick Plasma, Ithaca, NY).

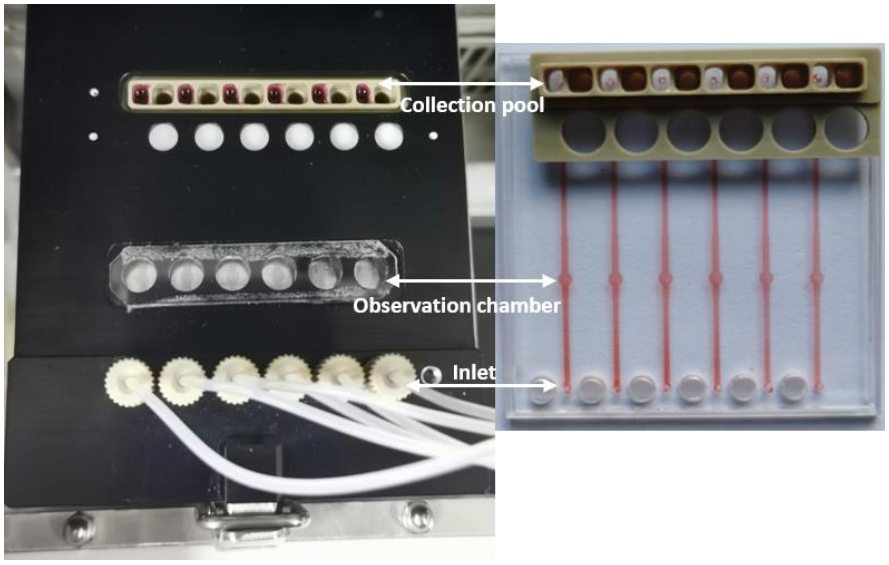

Figure S1. Photograph of the microfluidic device (left) and a design of microchip (right).

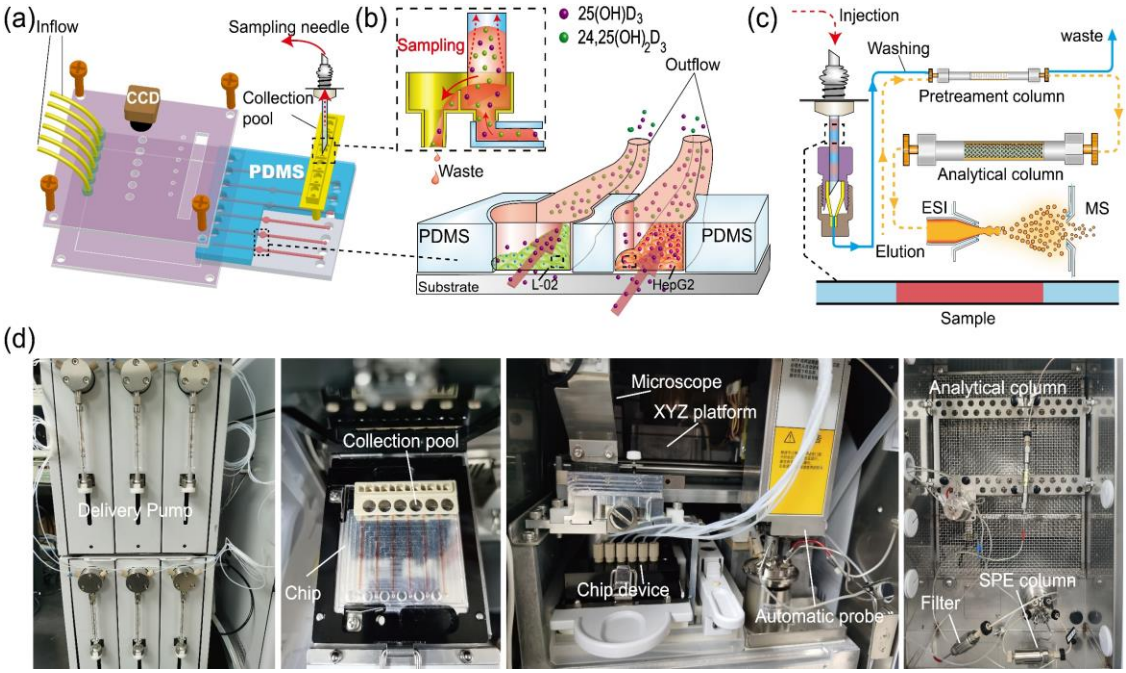

Figure S2. Schematic of 25(OH)D_3 biotransformation in human liver cells (a) Microfluidic device and a diagram indicating the fluid flow. (b) 25(OH)D_3 biotransformation of human hepatocytes (L-02 cells) and hepatoma cells (HepG2 cells) on microchip. (c) Online extraction of metabolites by switching "wash" and "elute" system based on SPE-MS. (d) Photograph of the experimental instrument.

3. Simulation by Comsol Multiphysics Software

We applied a turbulent flow module of Comsol Multiphysics (COMSOL 5.3a, MA) in numerical simulating the flow pressure in the culture chamber and SPE column. We constructed a model with incompressible fluids, open boundaries, and surface slip condition. The flow was selected as water, and all boundary liquid outside the flowing liquid were defined as open (under atmospheric pressure). The simulation is performed at steady state. The rules for conditions were set same to experimental data.

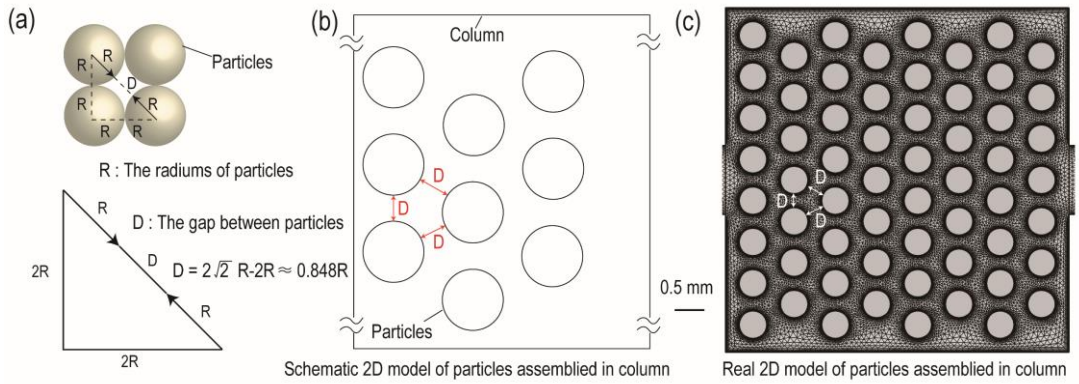

Figure S3. 2D modeling of numerical simulation for particles assembly in column. (a) The calculation of the gap between particles. (b) The schematic particles assembly in column for simulation. (c) The real 2D model of particles assembly in column.

4. High Flow Pressure Induced by Column Impact on Cells

In order to illustrate the necessary of homogenous-flow-pressure Chip-SPE-MS, we explored the effect of high flow pressure caused by the column on cell behavior (Figure S4). In the conventional Chip-SPE-MS system, there are huge diversity of the flow pressure in cell culture channels between sample loading and elution, where the cell culture channels are connected to column (particle diameter 50μm) for sample loading and without connecting to column when sample elution.

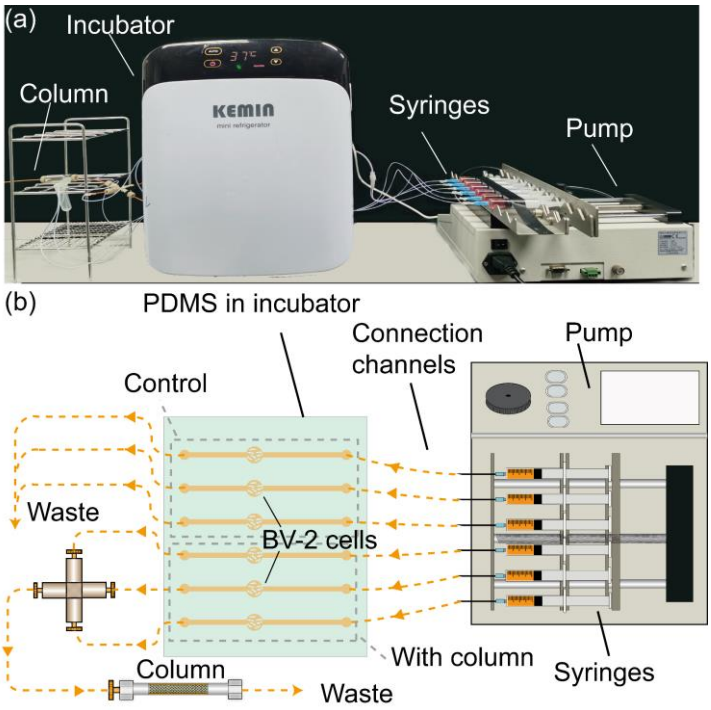

Figure S4. High flow pressure induced by column impact on BV-2 cells. (a) Construction of experiments for investigating high flow pressure induced by column impact on BV-2 cells. (b) The schematic diagram for devices connection. The cell culture channels of PDMS in the "with column" group are directly connected to column, while the ones in the control group are connected to waste reservoir.

The BV2 microglia were selected as the object to response the high flow pressure induced by column due to its sensitivity to the local microenvironment[2,3]. The BV-2 microglia have a characteristic form, called "resting state", composed of long branches for surveying the local microenvironment, while they change into "activated" phenotype with amoeboid or spherical shapes after physical and chemical stimulation[4,5].

The experiments results (Figure S5) noted that there are distinctions of BV-2 microglia morphology. The proportion of "activated" phenotype BV-2 microglia in the control group results was much less than the "with column" group results. The results showed that the percentage of activated BV-2 microglia was 86.7% in the "with column"

group, while it was to 46.8% in the control group, resulting from high flow pressure induced by column facilitated the BV-2 microglia transformation into the “activated” phenotype.

In consequence, the flow pressure change between sample loading and elution in conventional Chip-SPE-MS system should not be ignored due to pressure alteration affecting the phenotype and relative behaviors of cell. The homogeneous-flow-pressure Chip-SPE-MS system is necessary for online precisely determining cellular pharmacokinetics.

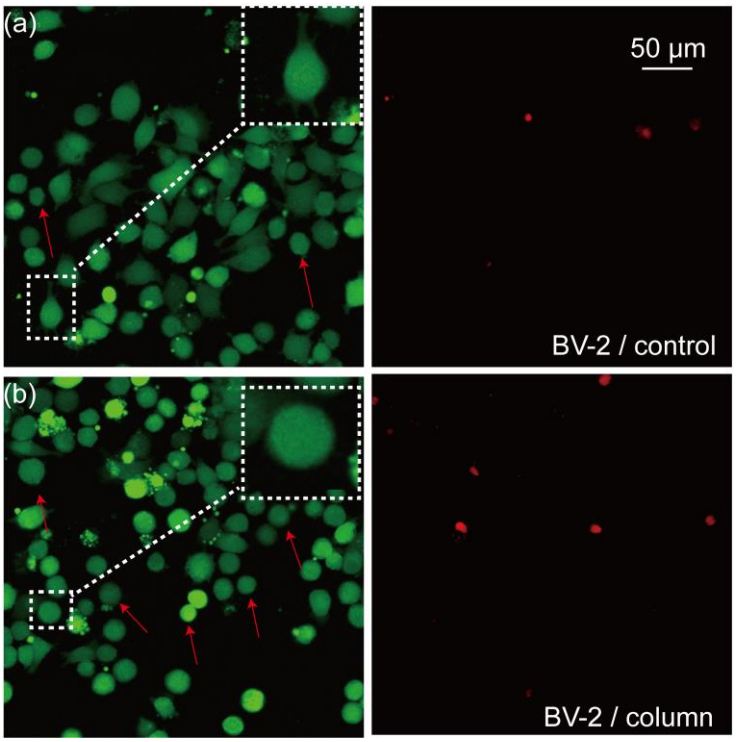

Figure S5. BV-2 microglia response to the high flow pressure induced by column. Images of BV-2 microglia with live /dead reagent staining from (a) control group and (b) “with column” group (green: live cells, red: dead cells).

5. Optimization of Online Analysis Conditions

Firstly, we stored 10 mmol/L stocks of $25(OH)D_3$, $24,25(OH)_2D_3$, d_6-$24,25(OH)_2D_3$ in DMSO at −80 °C, then the $25(OH)_2D_3$ and $24,25(OH)_2D_3$ were spiked at 50nmol/L each to MEM with d_6-$24,25(OH)_2D_3$, 5% FBS and 1% antibiotics solution. 2μL of the samples were then loaded. The washing line was washed with one of the following concentrations of MeOH: 10%, 40%, or 70%. The absorbed analytes were eluted with 85% MeOH. The elution line was washed with 10% MeOH, the absorbed analytes were then eluted in one of the following concentrations of MeOH: 90%, 95% or 100%. This result of this optimized condition was used for recovering vitamin D metabolites.

The online SPE was used Shim-pack MAYI-ODS column (4.6mm i.d.×10mm, particle diameter 50μm, Shimadzu) and separation used a C18 column (2.1mm i.d.×50 mm, particle diameter 3μm, Shimadzu). The washing mobile phase (blue flow in Figure S2c) containing 0.5mM ammonium formate and 0.1% formic acid in methanol/water was held at 1mL/min for the first 1 min for eliminating salt and protein, and then quickly decreased to 0.35mL/min over 1 s and kept for 4 min. Meanwhile the elution mobile phase (yellow flow in Figure S2c) consisted of (A) 0.5mM ammonium formate and 0.1% formic acid in water and (B) 0.1% formic acid in methanol and used the following gradient program: 0-1min 85% B, 2-4.25min, 90/95/100% B, 4.25-5min 85% B. It maintains at 0.35mL/min. One minute later, the valve was switched from the precolumn to the separation channel. The entire analysis cycle was estimated to be 5 min for each sample from injection to column washing.

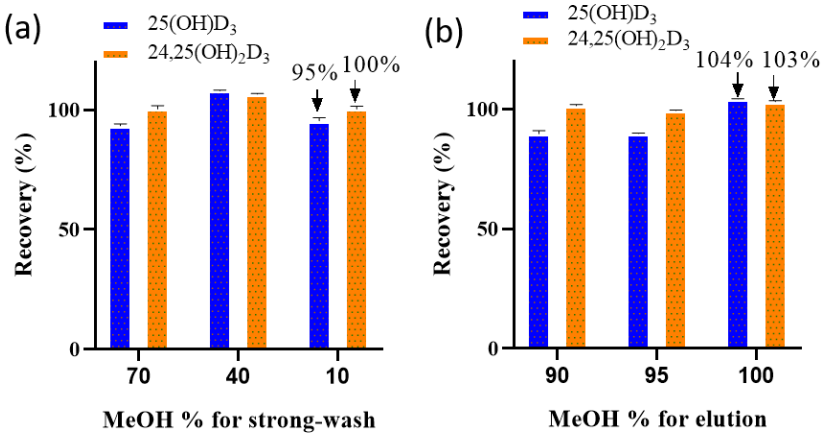

Figure S6. Optimization of online extraction conditions. (a) Optimization of the washing solvent: online SPE column was washed with 10%, 40%, 70% MeOH. (b) Optimization of the elution solvent: online SPE column eluted with a range of 90-100% MeOH. Data are presented as mean at least three replicate experiments.

6. Derivatization of 25(OH)D_3 Metabolites

After incubating with 10μmol/L 25(OH)D_3 solution for 40 h base on microfluidic chip, extracellular medium samples were filtered through the 0.45μm membrane, and subjected to SPE using Agela Technologies Cleanert PEP-2 (60mg/mL) cartridge according to the manufacturer's protocol, then transferred to an amber glass. The derivatization regent PTAD (1mg/mL) in acetonitrile, was added to the glass, and left at room temperature for 1 h to complete the reaction.

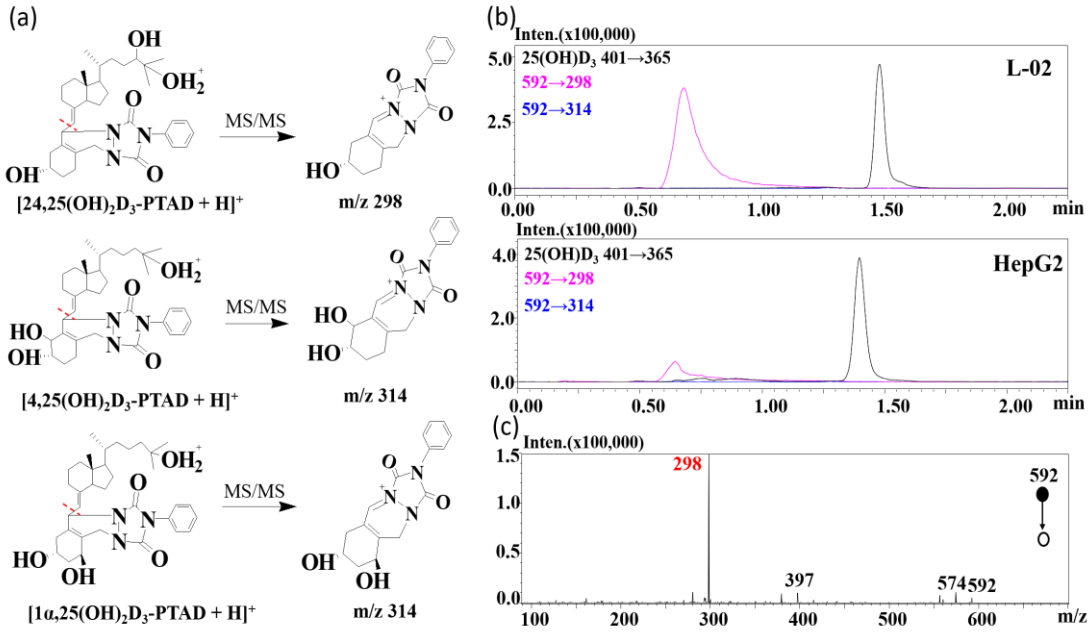

Figure S7. Investigation of 25(OH)D_3 metabolites in L-02 cells and HepG2 cells. (a) Schematic diagram of mass spectrometry product ions of the derivatized 25(OH)D_3 metabolites. (b) Chromatograms of the derivatized 25(OH)D_3 metabolites. (c) Product ion mass spectra of the derivatized 25(OH)D_3 metabolites. The product ion spectra were acquired from the dominant $[M\text{-}PTAD+H]^+$ precursor ion.

7. Quantitative Analysis of Target Compounds

A working standard solution was prepared from each stock solution by dilution with culture medium containing MEM, 5% FBS and 1% antibiotics solution, yielding concentrations of 24,25(OH)$_2$D$_3$ ranging from 5 to 100 nM (5, 20, 50, 80 and 100nM), concentration of 25(OH)D$_3$ ranging from 50 to 10000 nM (50, 100, 500, 1000, 5000, 10000nM). The solution of an isotopically labelled internal standard, d$_6$-24,25(OH)$_2$D$_3$ was prepared using the same procedure and added to working standard solution at a concentration of 30nM. Calibration curves were constructed by plotting the peak area ratios of each target compound and isotope versus the corresponding concentrations and fitting the data using linear regression.

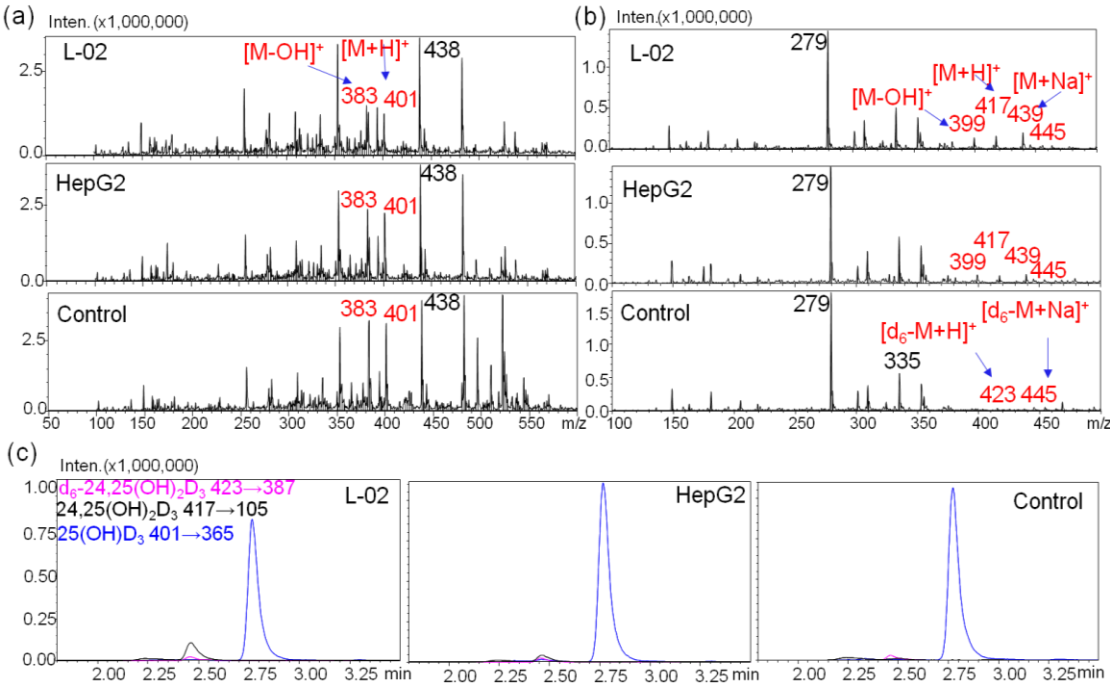

Figure S8. Monitoring of 25(OH)D$_3$ metabolites. Mass spectra of (a) 25(OH)D$_3$ and (b) 24,25(OH)$_2$D$_3$, d$_6$-24,25(OH)$_2$D$_3$ of the experiment implemented by L-02 cells, HepG2 cells and no cell being incubated with 25(OH)D$_3$, respectively. (c) Ion chromatographic peaks of metabolic compound 24,25(OH)$_2$D$_3$, internal standard compound d$_6$-24,25(OH)$_2$D$_3$, and unoxidized compound 25(OH)D$_3$ from both cell metabolism was eluted at 2.484, 2.486, and 2.766 min, respectively.

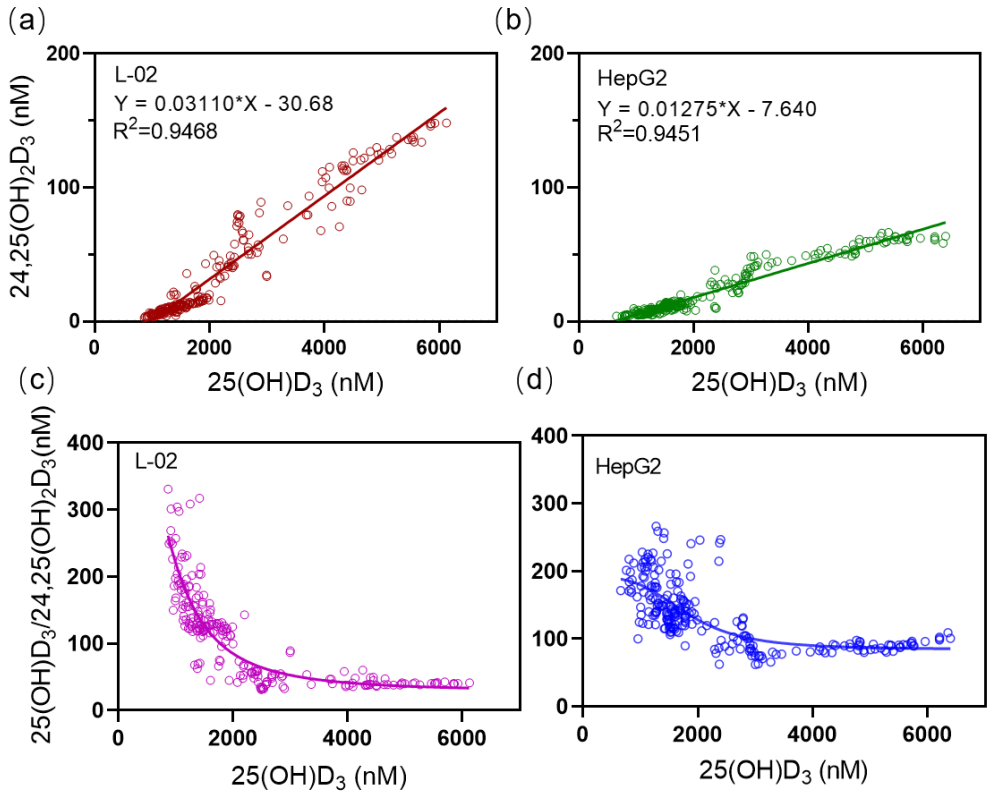

Figure S9. Correlation between metabolic $24,25(OH)_2D_3$ and unoxidized $25(OH)D_3$ in both L-02 cells and HepG2 cells. (a) and (b) the concentration of $25(OH)D_3$ correlated with the concentration of $24,25(OH)_2D_3$ using linear regression. (c) and (d) correlation between the ratio of $25(OH)D_3$ /$24,25(OH)_2D_3$, it was estimated using the Nonlinear Sigmoidal fitting.

8. Electrophoresis of PCR Products

1μL cDNA template, 12.5μL 2×Ex TaqMixl and 0.35μL of each of the forward and reverse primers (Tianyihuiyuan Biotechnology Co., Ltd, Beijing, China) were added per PCR tube. Finally, double distilled water (ddH2O) was added to each tube for a final reaction volume of 25 μL. Especially, one tube without cDNA template was used as a negative control. PCR system was performed using a standard thermocycler ((ABI, USA). The PCR conditions were as follows, initial denaturation (95 °C for 5 minutes), followed by 45 cycles of 15 s at 95 °C and 30 s at 65 °C. PCR products were removed from the thermocycler and maintained at room temperature for a few minutes before loading on the agarose gel.

0.45g agarose gels were dissolved using 4.5ml 10×MOPS buffer and 39.5ml DEPC by heating the solution in a microwave oven for 2–3 minutes. 1mL formaldehyde was added to the melted agarose, and it was immediately poured on gel tray. High concentration agarose gels should be poured rapidly as the gel solidifies quickly. The gel tray was placed on electrophoresis system (TAKARA, Japan) and the electrophoresis chamber filled with TAE buffer. 25 μL of PCR product was loaded into each well and electrophoresis was performed for 20 minutes. Our power supply was set to 120 V. Gel images were acquired using a regular gel UV analyzer (Biotech, Korea).

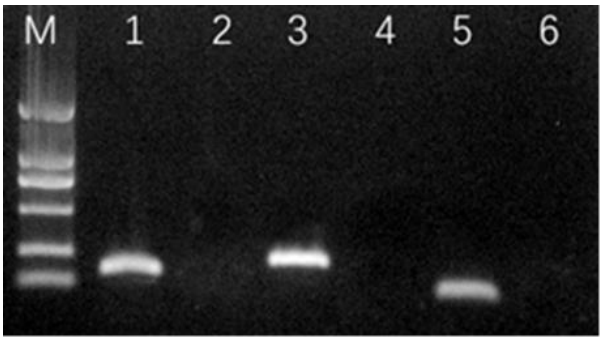

Figure S10. PCR products with cDNA template (1: β-actin; 3: CYP24A1; 5: CYP3A4) and without cDNA template (2: β-actin negative control; 4: CYP24A1 negative control; 6: CYP3A4 negative control) were detected by agarose gel electrophoresis for 2 min. (M: DL2000).

Table S1. List of sequence of different primer sets used to amplify β-actin, CYP24A1 and CYP3A4 genes.

Gene targeted	Primer sequence(5'to3')	Amplicon size
β-actin	F：TCTGGCACCACACCTTCTACAATG	170bp
	R：GGATAGCACAGCCTGGATAGCAA	
CYP24A1	F：CTCGGACTCTTGACAAGGCAACAG	225bp
	R：GCTCTGCTAATCGGCGACCAATG	
CYP3A4	F：TCATTGCTGTCTCCAACCTTCACC	101bp
	R：GCTTCCCGCCTCAGATTTCTCAC	

Table S2. Performance of two homogeneous-flow-pressure Chip-SPE-MS systems.

Systems	LOD (μM)	Time resolution (min.)	Throughput	Maximum working pressure	Reference
"Pneumatically actuated valves" fluidic isolation	1.4	30	1 channel	≦2.5 Psi	[6]
"Automatic probe" fluidic isolation	0.00254	5	⩾6 channels	⩾42 Psi	This work

Movie S1. Real-time imaging of L-02 cells incubated with 1μM 25(OH)D_3 at flow rate 15μL/h for 24 h.

Movie S2. Real-time imaging of HepG2 cells incubated with 1μM 25(OH)D_3 at flow rate 15μL/h for 24 h.

References

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