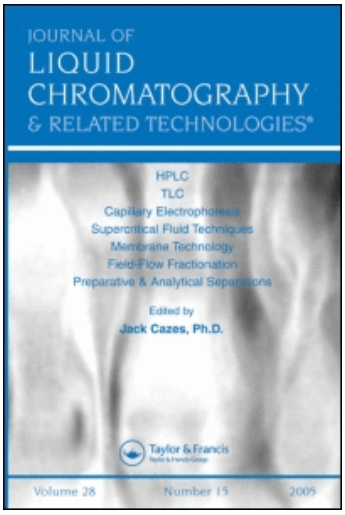

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Purification of 25-Hydroxycholecalciferol from Irradiation of Cholesta-5,7-Diol by High-Speed Countercurrent Chromatography

Q. Du[a]; T. Y. Zhang[b]; Y. Ito[c]

[a] Tea Research Institute Chinese Academy of Agricultural Sciences Hangzhou, Zhejiang, The People's Republic of China [b] Beijing Institute of New Technology Application, Beijing, The People's Republic of China [c] Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute National Institutes of Health, Bethesda, Maryland

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PURIFICATION OF 25-HYDROXY-CHOLECALCIFEROL FROM IRRADIATION OF CHOLESTA-5,7-DIOL BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

QIZHEN DU[1], TIAN-YOU ZHANG[2], AND YOICHIRO ITO[3]

[1]*Tea Research Institute*
Chinese Academy of Agricultural Sciences
Hangzhou, Zhejiang 310008
The People's Republic of China
[2]*Beijing Institute of New Technology Application*
Beijing, 100035
The People's Republic of China
[3]*Laboratory of Biophysical Chemistry*
National Heart, Lung, and Blood Institute
National Institutes of Health
Bethesda, Maryland 20892

ABSTRACT

High-speed countercurrent chromatography (HSCCC) was used for the separation of 25-hydroxycholecalciferol (25-HCC) from a post-irradiation mixture of cholesta-5,7-diene-3ß,25-diol. With a two-phase solvent system composed of hexane/ethyl acetate/methanol/water (5:1:5:1, v/v/v/v), 500 mg of reaction mixture containing 245 mg of 25-HCC was separated with 207 mg of the product obtained at a purity of 97.7%. The separation was completed within 2 hours.

INTRODUCTION

25-Hydroxycholecalciferol (25-HCC) is a major circulating metabolite of vitamin D_3. This metabolite was shown to be more potent than vitamin D_3 itself for curing rickets and is able to stimulate calcium transport faster than a similar amount of vitamin D_3 (1). The conventional method of synthesizing 25-HCC utilizes radiation of cholesta-5,7-diene-3ß,25-diol (Fig. 1), followed by purification of the products by silicic acid column chromatography (2-4). However, we found this technique results in considerable loss of the product on the silicic acid column. In order to alleviate this problem, we used high-speed countercurrent chromatography (HSCCC) because it eliminates a sample loss from this source (5).

EXPERIMENTAL

Apparatus

HSCCC experiments were performed using a coil planet centrifuge equipped with a multilayer coil separation column that was designed and fabricated at the Beijing Institute of New Technology Application, Beijing, P. R. China. The multilayer coil was prepared by winding a 1.6mm ID PTFE (polytetrafluoroethylene) tube coaxially

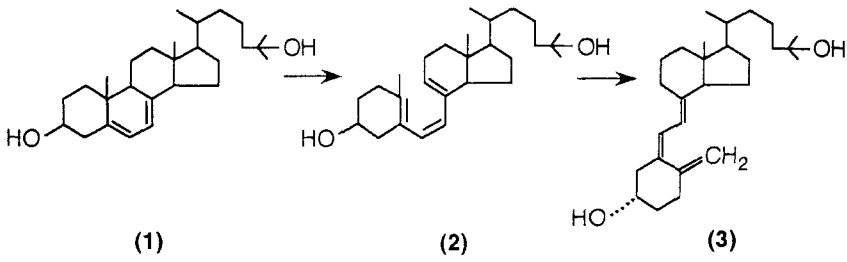

Figure 1. Irradiation reaction of cholesta-5,7-diene-3ß,25-diol. Compound 1: cholesta-5,7-diene-3ß,25-diol; Compound 2: 25-hydroxycholecalciferol (25-HCC); Compound 3: 25-hydroxyprecholecalciferol.

onto the column holder hub. The total column capacity measured 230 ml. The HSCCC centrifuge was rotated at 800 rpm with an 8cm revolution radius. The system was equipped with an FMI pump (Zhejiang Instrument Factory, Hangzhou, P.R. China), a variable wave-length UV detector (UV-752, Shanghai Analytical Instrument Factory, Shanghai, P.R. China), a recorder and a sample injection valve.

Reagents

All organic solvents were of analytical grade and purchased from Shanghai Chemical Factory, Shanghai, P.R. China. Cholesta-5,7-diene-3ß,25-diol and standard 25-hydroxycholecalciferol (25-HCC) were gifts of Hangzhou Mingsheng Medicine Factory, Hangzhou, P.R. China.

Irradiation of Cholesta-5,7-diene-3ß,25-diol

In each treatment, 1000 mg of cholesta-5,7-diene-3ß,25-diol dissolved in 1L of diethyl ether was irradiated in a double-walled, water-cooled jacketed quartz immersion well. A high-pressure quartz mercury vapor lamp, Model 1000 (Yixin Optics Instrument Factory, Yixin, P.R. China), was lit 1 minute before placing the solution in the immersion well. During 1.5 h of irradiation the ether was constantly flushed and stirred with nitrogen. The reaction solution was then evaporated to dryness in a rotary evaporator at room temperature.

HSCCC Procedure

The HSCCC experiment was performed with a two-phase solvent system composed of hexane/ethyl acetate/methanol/water (5:1:5:1, v/v/v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use. In each separation, the multilayer coil column was first entirely filled with the upper stationary phase. Then the lower mobile phase was pumped into the inlet of the column at a flow rate of 2.0 ml/min, while the apparatus was rotated at 800 rpm. After the mobile phase front emerged and the two phases had established

hydrodynamic equilibrium in the column, the sample solution, containing 500 mg of the reaction mixture in 10 ml of the mobile phase, was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 280 nm and the fractions collected (Fig. 3).

HPLC Analysis

HPLC analysis of the reaction mixture and CCC fractions were performed with Waters HPLC equipment (Waters Associates/Millipore Chromatography Co., Milford, MA, USA) consisting of a Model 510 pump, a Model 717 auto-injector, a Model 996 PDA detector and a Millennium 2010 data processor. HPLC separations were performed on a μ-Bondapak C_{18} column, 0.46 x 25 cm ID. (Waters Associates). The mobile phase, composed of methanol-water (90:10, v/v), was isocratically eluted at 1 ml/min and the effluent was monitored at 280 nm.

RESULTS AND DISCUSSION

HPLC analysis of the post-radiation reaction mixture (Fig. 2) shows five major components three of which are: 25-HCC (R.T.: 7.08 min)(49.0% of the total), cholesta-

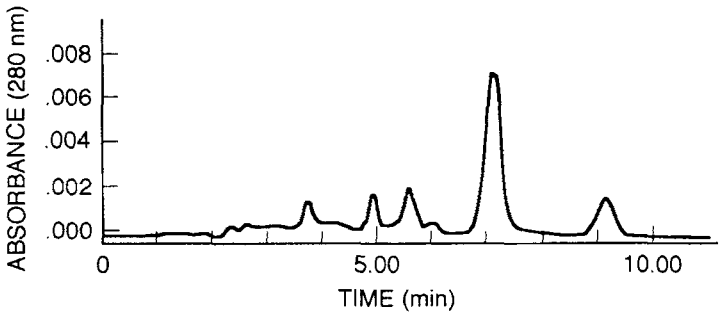

Figure 2. HPLC analysis of the reaction mixture. Retention time: cholesta-5,7-diene-3ß,25-diol (Compound 1): 5.57 min; 25-HCC (Compound 2): 7.08 min; and 25-hydroxyprecholecalciferol (Compound 3): 9.12 min.

5,7-diene-3ß,25-diol (R.T.: 5.57 min) and 25-hydroxyprecholecalciferol (R.T.: 9.12 min). The HSCCC separation of the reaction mixture is shown in Fig. 3. A 500mg amount of the sample was separated into peaks A, B and C in a relatively short elution time of 110 min. The fractions corresponding to peak B contained 25-HCC as confirmed by the HPLC analysis with the standard sample. Peak B fractions were combined, dehydrated by $MgSO_4$, and evaporated to dryness in rotatory evaporator. The 25-HCC present in the residue was extracted with dichloromethane which was in turn evaporated in vacuum. This yielded a net amount of 204 mg of 25-HCC of 97.7% purity as determined by HPLC analysis.

Separation of the reaction mixture by silicic acid column chromatography performed according to the method described in refs. 2-4 resulted in 148mg of the product

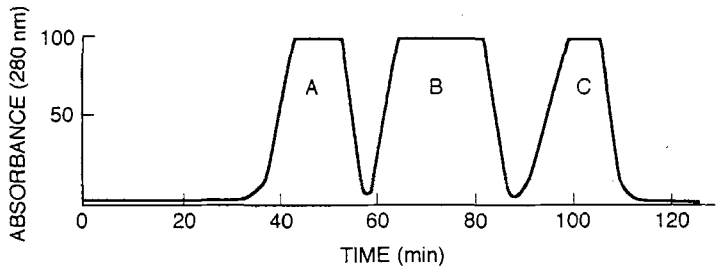

Figure 3. HSCCC separation of the reaction mixture. Peak B corresponds to 25-HCC (Compound 2) and peak C to 25-hydroxyprecholecalciferol (Compound 3).

and the purity of the product was 90-94 % as determined by HPLC. This necessitated recrystallization of the products with additional sample loss to raise the purity to the 97%, requirement of the Federal Drugs Administration of P.R. China. In the present HSCCC method, the loss of the product is no more than 17.5% of the total (HPLC) while purity of the product was over 97%.

We believe that pharmacy-scale production can be realized by the use of multiple sets of HSCCC instruments, since an adult daily dose of 25-HCC is only 20 micrograms (6).

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