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Modifier Effects on Supercritical CO_2 Extraction Efficiency of Cephalotaxine from *Cephalotaxus wilsoniana* Leaves

Young Hae Choi[1], Jinwoong Kim[1], Jin Yeol Kim[2], Seung Nam Joung[2], and Ki-Pung Yoo[2] and Yuan Shiun Chang[3]

[1]College of Pharmacy, Seoul National University, Seoul 151-742, Korea, [2]Department of Chemical Engineering, Sogang University, Seoul 121-742, Korea, and [3]China Medical College, Taichung, Taiwan

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The effects of modifiers such as methanol, water, diethylamine in methanol (10 v/v %), and diethylamine in water (10 v/v %) were investigated at three different concentrations (1, 5, and 10 v/v %) of the modifiers in supercritical CO_2 (SC-CO_2) in order to enhance the supercritical fluid extraction (SFE) efficiency of cephalotaxine from *Cephalotaxus wilsoniana* leaves. Among the modifiers employed, methanol basified with diethylamine was found to greatly enhance the extraction efficiency relative to any other modifiers employed. The results suggest that cephalotaxine in plant matrices may be readily changed from SC-CO_2-insoluble salt to SC-CO_2-soluble free base by basified modifiers. In addition, SC-CO_2 modified with basified methanol could enhance the extraction efficiency of cephalotaxine more than 30% when compared to the conventional organic solvent extraction.

Key words: Supercritical CO_2, Cephalotaxine, *Cephalotaxus wilsoniana*, Basified modifier

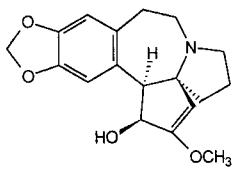
INTRODUCTION

Supercritical fluid extraction (SFE) is a form of extraction where the usual liquid solvent has been replaced with a substance above critical point (McHugh and Krukonis, 1994; Stahl *et al.*, 1988). Among a wide variety of supercritical fluids, carbon dioxide has been the most commonly employed due to its comparatively low critical temperature (31.1°C) and pressure (7.4 MPa) together with its another advantages such as environmental acceptance and non toxicity to human health (McHugh and Krukonis, 1994). In particular, supercritical carbon dioxide has taken the place of the conventional organic solvent in the extraction of natural products as an alternative technique. Recently, there have been reported a number of its application to natural products and in some cases SFE has been found to have an advantage over conventional organic solvent extraction in an economic aspect (Bevan and Marshall, 1994; Jarvis and Morgan, 1997; Modey *et al.*, 1996).

Alkaloids have been regarded as important target compounds for SFE because of their diverse and intense biological activities. However, most alkaloids are too polar to be sufficiently extracted by pure SC-CO_2. Therefore, the alternative SFE methods including the addition of polar solvents in SC-CO_2 (Bicchi *et al.*, 1991; Janicot *et al.*, 1990; Schaeffer *et al.*, 1989) and replacement of SC-CO_2 with other supercritical fluids such as N_2O (Queckenberg and Frahm, 1994; Stahl and Willing, 1978) and CHF_3 (Stahl *et al.*, 1988) have been considered to enhance SFE efficiencies of alkaloids. However, both the limitation of percentage of modifiers added and the environmental hazard of the alternative supercritical solvents have led to developing another SFE methods. Recently, we have performed SFE of some alkaloids, e. g., hyoscyamine and scopolamine from *Scopolia japonica* (Choi *et al.*, 1999a) and methyl ephedrine, norephedrine, ephedrine, and pseudoephedrine from *Ephedra sinica* (Choi *et al.*,1999b). These reports showed that basified modifier would dramatically increase SFE yields of target alkaloids relative to neutral ones, because the basified modifiers could change the alkaloids from salts in plant tissues to free bases which were freely soluble in SC-CO_2.

In this study, cephalotaxine (Fig. 1), the major antitumor alkaloid of *Cephalotaxus species* (Powell *et al.*, 1972) was extracted using pure SC-CO_2 or modified ones with several solvents and their efficiencies were

Correspondence to: Jinwoong Kim, College of Pharmacy, Seoul National University, Shilim-Dong, Kwanak-Gu, San 56-1, Seoul 151-742, Korea
E-mail: jwkim@snu.ac.kr



Cephalotaxine

Fig. 1. Chemical structure of cephalotaxine

compared with that of the conventional organic solvent extraction. On the basis of these results, SFE would be considered as a novel extraction method for alkaloids to substitute the conventional organic solvent extraction.

MATERIALS AND METHODS

Chemicals and reagents

Cephalotaxine was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and ethanolamine from Aldrich Co. (Milwaukee, WIS, U.S.A.). HPLC grade acetonitrile and deionized water, diethylamine (99%), ammonia water (28%), methanol, chloroform, and hydrochloroic acid were purchased from Duksan Chemical Co. (Yongin, Kyungki-Do, Korea). Carbon dio-xide (99.9%) of Seoul Gas Co. (Seoul, Korea) was employed.

Plant material

The leaves of *Cephalotaxus wilsoniana* Hayata were collected in Taiwan in August 1997 and identified by one of the authors (Y. S. Chang). The plant materials were dried at 40°C for 3 days in a vacuum oven. They were pulverized and sieved to be under 0.71 mm of particle size.

Organic solvent extraction

Dried and powdered *C. wilsoniana* leaves (500 mg) was extracted for 1 h in an ultrasonic apparatus with methanol (100 mL each, 3 times) and the methanol extract was evaporated *in vacuo*. The residue was dissolved in chloroform (30 mL) and extracted with aqueous HCl (0.5 N, 15 mL each, 3 times). The pH of the aqueous phase was adjusted to 9.0 with ammonia water and alkaloids from the solution were extracted with chloroform (15 mL each, 3 times). The combined chloroform extract was concentrated, and the residue was dissolved in 1 mL of methanol for HPLC analysis.

Supercritical fluid extraction

SFE was performed on an Isco supercritical fluid extractor, model SFX 3560 equipped with an Isco 260 D syringe pump (Lincoln, NE, U.S.A.) using pure carbon dioxide in the pressure range of 10.2-34.0 MPa, respectively. In each experiment, 500 mg of *C. wilsoniana* leaves was loaded into an extraction cell (57 mm × 20 mm i.d., Isco). The temperature of the restrictor was kept at 80°C. For a static extraction, SC-CO_2 and the plant material in extraction vessel were left for 15 min. Then, dynamic extraction was performed with 1.0 mL/min of flow rate. The total amount of consumed CO_2 was 50 mL at each condition.

As a next step, the modifiers such as methanol, water, diethylamine in methanol (10%, v/v), and diethylamine in water (10%, v/v) were continuously added into the extraction cell at the concentrations of the modifiers in SC-CO_2, 1, 5, and 10% (v/v) through another syringe pump at 80°C and 34.0 MPa. The static time, flow rate of dynamic extraction, and restrictor temperature were kept at 15 min, 1.0 mL/min, and 80°C, respectively. All SFE extracts were purified with the acid-base extraction according to the organic solvent extraction and dissolved in 1 mL of methanol for HPLC analyse.

HPLC analysis of cephalotaxine

The HPLC instrument was consisted of a Hitachi L-7100 pump, an L-7420 UV detector fixed at 298 nm and a D-7500 integrator (Hitachi, Tokyo, Japan). The sample solution was injected through 20 µl loop. A C_{18} column (250 × 4.6 mm, S-5 µm, YMC Inc., Kyoto, Japan) with a C_{18} guard column (15 × 3.2 mm S-7 µm, Cobert Associates, St. Louis, MO, USA) was eluted isocratically with a mixture of acetonitrile-water (45:55) containing 0.1% ethanolamine at a flow rate of 1.0 ml/min.

RESULTS AND DISCUSSION

It was reported that *C. wilsoniana* contained cephalotaxine, cephalotaxinone, acetyl cephalotaxine, demethyl cephalotaxine, epicephalotaxine, wilsonine, epiwilsonine, harringtonine, and homoharringtonine (Chiu and Chang, 1992). In particular, cephalotaxine, together with harring-

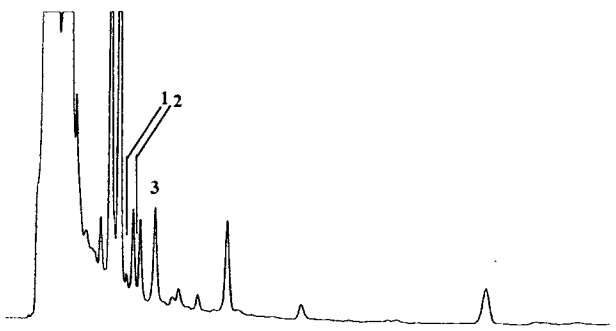


Fig. 2. HPLC chromatogram of methanol extract of *C. wilsoniana* leaves. 1; harringtonine region, 2; homoharringtonine region, 3; cephalotaxine. The protocols employed are described in the Materials and Methods section.

tonine and homoharringtonine, showed intensive antitumor activity (Powell *et al.*, 1972). The percent content of cephalotaxine in *C. wilsoniana* leaves employed in this study was found to be 0.0022% through the conventional methanol extraction followed by acid-base purification of alkaloids. However, the contents of harringtonine and homoharringtonine were found to be only trace amount as shown in Fig. 2. Thus, SFE efficiency was evaluated only for the extraction of cephalotaxine.

As a first attempt, pure SC-CO_2 was used to extract cephalotaxine from *C. wilsoniana* leaves in a wide rage of temperature (40-80°C) and pressure (10.2-34.0 MPa). However, pure SC-CO_2 could not extract cephalotaxine at any experimental condition. It was also found that some alkaloids such as hyoscyamine, scopolamine, methylephedrine, norephedrine, ephedrine, and pseudoephedrine were not extracted at all from plant materials by pure SC-CO_2 in spite of their high solubilities in SC-CO_2 (Choi *et al.*, 1999a; 1999b). It was possibly due to the fact that the alkaloids did not exist as free bases but salt forms conjugated with organic acids in plant tissues.

Accordingly, modifiers were added in SC-CO_2 to enhance the yields of cephalotaxine. The most common modifier used in SFE is methanol because of its high solvent polarity parameter which can greatly increase the polarity of carbon dioxide (Lochmuller and Mink, 1987; 1989; Fahmy *et al.*, 1993). Water was chosen as another modifier because the alkaloidal salts were freely soluble in water as well as methanol. Moreover, the addition of water into carbon dioxide has been reported to improve the extraction yield of some alkaloids (Janicot *et al.*, 1990). Methanol or water as a modifier was added into the extractor at the concentration levels of 1, 5 and 10% (v/v), respectively. However, the addition of methanol or water at any volume percent did not greatly increase the extraction yields of cephalotaxine from *C. wilsoniana* leaves compared to pure SC-CO_2. The extraction yields obtained by SC-CO_2 modified with methanol or water were merely less than 20% of the conventional organic solvent extraction. In our previous reports for SFE of alkaloids, basified modifiers instead of neat methanol or water could dramatically increase SFE yields of target alkaloids because they could enhance both the solubilities of target alkaloids and desorption from a matrix (Choi *et al.*, 1999a; 1999b). Therefore, basified modifiers were used in order to increase the SFE yield of cephalotaxine. As basified modifiers, diethylamine was added to methanol or water (10% v/v), respectively. The percent SFE yields of cephalotaxine when compared to the conventional organic solvent extraction are shown in Fig. 3. As expected, diethyl-amine in methanol as a modifier showed the highest yield of cephalotaxine than any other modifiers employed. It was dramatically increased to four times compared to that of the other modifiers employed. In addition, this modifier could obtain 30% excessive yield relative to the organic solvent extraction. While diethylamine in methanol as a modifier yielded a great enhancement of the extractability of cephalotaxine, diethylamine in water did not show any significant influence on the extractability. It was reported that water was not miscible as methanol in CO_2 (Jackson *et al.*, 1995). This immiscibility of water resulted in the low yield relative to basified methanol, though it was also basified modifier.

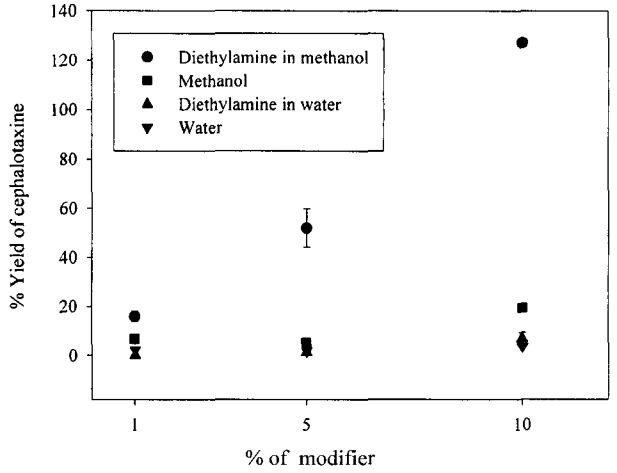


Fig. 3. Relative extraction yields (%) of cephalotaxine from *C. wilsoniana* leaves by SC-CO_2 added by modifiers when compared to methanol extraction

SC-CO_2 extraction has been paid attention as a novel prospective extraction method for natural products because it could be environmental acceptable, nontoxic to human heath, selective to a target compound, and economic which could not be expected by any conventional organic solvent extraction method. Regardless of numerous advantages of SFE as an alternative extraction method, the polarity and salt form of alkaloids in plant tissue have brought about difficulty in the application of SFE to these compounds. However, the SFE method using basified modifiers developed in this study exhibit that SFE could be applied to the extraction of alkaloids from plant materials.

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