Separation and Purification Technology 65 (2009) 275–281

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

Supercritical fluid extraction of monoamine oxidase inhibitor from antler velvet

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ARTICLE INFO

Article history:
Received 23 June 2008
Received in revised form 24 October 2008
Accepted 25 October 2008

Keywords:
Monoamine oxidase inhibitor
Antler velvet
Supercritical fluid extraction
Supercritical carbon dioxide

ABSTRACT

Supercritical fluid extraction (SFE) of the monoamine oxidase (MAO) inhibitor from antler velvet with CO_2 was explored. The effect of different parameters, such as particle size, the kind of co-solvent, extraction temperature and pressure, on the extraction yield (*Y*) and the total inhibitory activity (TI) on MAO-B were investigated using three-level orthogonal array design. The experimental results show that when the absolute ethanol as co-solvent was used and the particle size was 120 μm, the extraction temperature was 70 °C, the extraction pressure was 30 MPa, the extract yield reached 3.58% and the TI of the extract was 3319.13 U/g. Evaluation of the inhibitory activity of extract on MAO indicated that the extracts had strong inhibitory effects on MAO-B, but had slight effects on MAO-A. The MAO-B activity was inhibited by 93.77% at the concentration of 278.15 mg/L, which was much higher than that of water extract and ethanol extracts. The compositions of estradiol, uracil, hypoxanthine, *p*-hydroxybenzaldehyde and phospholipids in the SFE extracts were identified, which were reported to have the inhibitory effect on MAO.

1. Introduction

Antler velvet is unossified horn cut from male deer, which belongs to the Cervidae. It is generally termed "Lu rong" in China, and "Nokyong" in Korea, "Tokujo" in Japan or "Pantui" in Russia [1]. Antler velvet has been used in traditional Chinese medicines for over 2000 years. It has long been recognized as one of the most effective and powerful invigorants for strengthening the kidney, sexual-reinforcing, and prolonging life [2,3]. Recently, there were many studies on the components and pharmacological effects of antler velvet. Many researches made it evident that antler velvet and its extracts have some inhibitory activities on arthritis [1,4–6], anti-inflammatory effects [7], anti-stress activities [8], inhibitory action on monoamine oxidase B [9–11], and anti-aging activities [12–14], etc. The compositions of estradiol, uracil, hypoxanthine, *p*-hydroxybenzaldehyde and phospholipids in antler velvet were reported to have the inhibitory effect on monoamine oxidase (MAO).

MAO is an important enzyme in the metabolism of a wide range of monoamine neurotransmitters such as noradrenaline, dopamine, and serotonin (5-HT). Two different forms, MAO-A and MAO-B, have been identified, which play key roles in monoamine neurotransmitter metabolism and involve in many neuropsychiatry disorders [15,16]. Inhibition of MAO can effectively prevent depression and various oxidative stresses in the brain [17]. The compositions which have the function of inhibiting the activity on MAO were called MAO inhibitors in this paper. Some MAO inhibitors appeared to be effective to prevent and treat neural disease, such as Parkinson's disease or Alzheimer's disease (AD) [18]. However, some MAO inhibitors were reported to have severe adverse effects [19]. Thus, it caused great interest to find new MAO inhibitors without severe side-effects from natural product [15,19,20].

Previous studies on pharmacological effects of antler velvet were generally performed by using its water extract or organic solvent extract. These extracts were obtained commonly by solvent extraction or boiling water [4,8–13]. These processes can spend long extraction times and consume large quantities of solvents, especially they can result in the loss or degradation of active components. These disadvantages can be avoided by using the supercritical fluid extraction (SFE). Compared with liquid extraction, SFE is relatively rapid because of the low viscosities and high diffusivities associated with supercritical fluids. The extraction can be selective to some extent by controlling the density of the medium and the extracted material is easily recovered by simply depressurizing, allowing the supercritical fluid to return to gas phase and evaporate leaving no or little solvent residues. However, none of investigations has been reports about the extraction of the monoamine oxidase inhibitor by supercritical CO_2.

In this paper, supercritical fluid extraction (SFE) of the monoamine oxidase (MAO) inhibitor from antler velvet with CO_2 was explored and optimized. Evaluation of the inhibitory activity of the SFE extract on MAO was made, and the compositions

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doi:10.1016/j.seppur.2008.10.036

of estradiol, uracil, hypoxanthine, *p*-hydroxybenzaldehyde and phospholipids in the SFE extracts were also identified which are considered to have the inhibitory effect on MAO.

2. Experimental methods

2.1. Chemicals and raw materials

Antler velvet (*Cervus nippon* TEMMINCK var. *mantchuricus Swinhoe*) was provided and identified by Tianjin Shencao pharmaceutical zoo breeding Co. Ltd. Kunming rats (30 ± 5 g), 7 weeks old, were supplied by Beijing Experimental Zoo Centre.

Carbon dioxide (99.99% pure) supplied in a cylinder was purchased from Liu Fang Gas Co. (Tianjin, China). Serotonin creatinine sulfate complex (5-HT, 99.0%), Benzylamine hydrochloride (BA, 99.0%), Sphingomyelin (SPH, 98.0%) were obtained from Sigma Chemical Co. (USA). The standard samples of estradiol (E_2), uracil (U), hypoxanthine (HX) and *p*-Hydroxybenzaldehyde (PHBD), all with purity more than 99.0% were obtained from Sigma Chemical Co. (USA). Sphingomyelin (SPH) with purity of 98.0% was acquired from Sigma Chemical Co. (USA). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) all were bought from Fluka Co. (Germany). Butyl acetate and cyclohexane were HPLC grades (99.0%) obtained from Guangfu chemical reagent Co. (Tianjin, China).

2.2. Supercritical fluid extraction procedure

Supercritical CO_2 extraction was experimentally performed with Spe-ed SFE instrument (Applied Separations Inc., Allenton, PA, USA), shown schematically in Fig. 1. Antler velvet powder or its mixture with co-solvent were weighed and packed into the extraction column (32 cm^3 capacity) (6). The ratio of co-solvent to antler velvet powder was 1:1 (v/m). Liquid CO_2 was pressurized with a high-pressure pump (3) and then charged into the extraction column (6) to desired pressure. The pressure was controlled to an accuracy of about 2% over the measuring range. The extraction column was heated through an oven (7) and its temperature was indicated and controlled by the thermocouple (12) within ± 1 °C. The extraction process started after the column hold equilibration for 20 min at working conditions. The supercritical CO_2 with dissolved compounds passed through a heated micrometer valve (9), and was subsequently expanded to ambient pressure at room temperature. The flow-rate of CO_2 was controlled at 1.0 L/min (ambient temperature and pressure) in this study. A calibrated wet-test meter (11) at known temperature and pressure measured the total amounts of CO_2.

The SFE extracts were precipitated in a 20 mL collecting vial (10). Collected extracts were dried under vacuum equipment (ZK-30 (BS), Huabei Apparatus Co., Ltd., Tianjin, China). Finally, the total extract yield (defined as extract mass in per unit mass antler velvet material) was calculated. The MAO activity and the chemical compositions in extract were also analyzed.

2.3. MAO activity assays

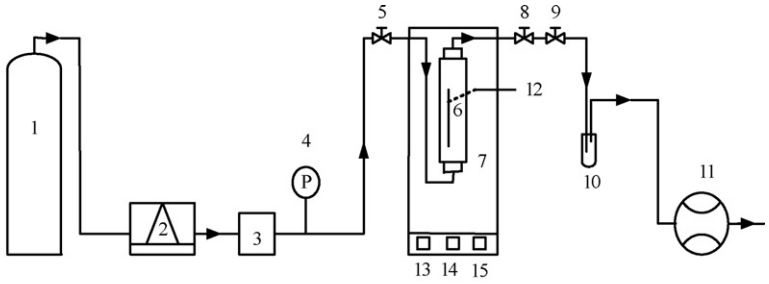
An in vitro assay was designed and reported to measure the inhibitory activity of the extract on MAO-A and MAO-B [21].

2.3.1. Preparation of the source of MAO activity

Rat brain mitochondrial fraction as a source of MAO activity was prepared. Male Kunming rats were killed by stunning and decapitation. The head was dissected and the brain tissue was removed. Then the brain tissue was homogenized on ice in 10 vol. of 100 mmol/L sodium phosphate buffer (PBS, pH 7.4) with 0.32 mol/L sucrose. The mixture was centrifuged (TGL-16M, Xiangyi Centrifuge Instrument Co., Ltd., Hunan, China, $600 \times g$, 10 min, 4 °C) immediately. The precipitation fraction was removed, and the supernatant liquid was continued to centrifuge ($15{,}000 \times g$, 10 min, 4 °C). Abandoning the supernatant this time, the solids were suspended in the same buffer and centrifuged ($15{,}000 \times g$, 10 min, 4 °C) again. The final solids were suspended in the above PBS solution. The protein concentration of brain mitochondrial extract was diluted with the same PBS to 1 mg/mL. Protein concentration was estimated by the Lowry method which uses bovine serum albumin as the standard [22]. The brain mitochondrial extract was stored at −20 °C.

2.3.2. MAO activity assays with UV spectrophotometer method

MAO activity was assessed by UV spectrophotometer method. Serotonin creatinine sulfate complex (5-HT) and Benzylamine hydrochloride (BA) were diluted as specific substrates for MAO-



1 CO_2 cylinder 2 Liquid-cooled bath 3 Gas booster pump
4 Pressure gauge 5 In-let valve 6 Extraction column
7 Constant temperature oven 8 Out-let valve 9 Micrometer valve
10 Collecting vial 11 Wet-test meter 12 Thermocouple
13 Oven temperature indicator 14 Column temperature indicator 15 Micrometer valve temperature indicator

Fig. 1. Schematic diagram of the experimental apparatus.