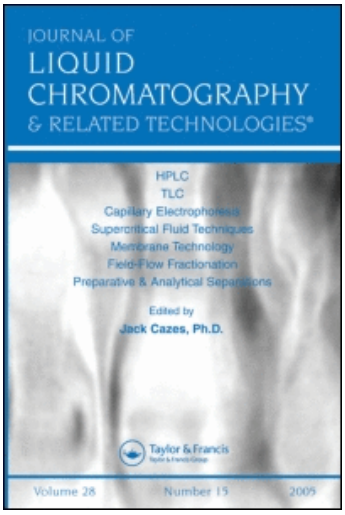

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MAGNOLOL INHIBITS ARACHIDONIC ACID-INDUCED RABBIT PLATELET SEROTONIN RELEASE, MEASURE BY MICROBORE HPLC WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

Serotonin (5-hydroxytryptamine; 5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were determined simultaneously in rabbit platelet by a sensitive microbore high-performance liquid chromatographic method with electrochemical detection. The detection limit of 5-HT in rabbit platelet suspension was 0.1 ng/mL. To evaluate the 5 - HT release from platelet suspension, the aggregating agent arachidonic acid 30 and 100 μM were added and the 5-HT was elevated from

basal level 2.96±0.89 ng/mL to 154.72±28.03 and 246.77±24.38 ng/mL, respectively. The results demonstrated that magnolol inhibits arachidonic acid-induced 5-HT release from platelet suspension significantly.

INTRODUCTION

Magnolol is the major phenolic constituents of Magnolia bark.[1,2] The bark of Magnolia has been used as a folk medicine in China for the relief of fever, headache, anxiety, diarrhea and stroke.[3]

Recent studies indicate that magnolol inhibits intracellular calcium mobilization in platelets,[4] relaxes vascular smooth muscle,[5] antihemostatic, antithrombotic effects,[6] inhibits collagen-induced platelet 5-HT release[7] and modulatory effects of brain 5-HT release.[8]

In the present study, we have examined the inhibitory effects of magnolol on arachidonic acid-stimulated 5-HT release from platelet by a sensitive microbore HPLC/ECD system.[9]

MATERIALS AND METHOD

Materials

The methanol extract of magnolia bark was partitioned between water and chloroform. The chloroform layer was separated repeatedly by column chromatography on silica gel, magnolol (Fig. 1) was then extracted.[1] Identification and purity were compared with authentic compound by [^{13}C]NMR (Bruker, Germany) and HPLC, coupled with photodiode-array detection.[10] 5-HT and arachidonic acid, were purchased from Sigma Chemical (St. Louis, MO, USA). HPLC reagents and buffer reagents were obtained from E. Merck (Darmstadt, Germany). Thrice-deionized water (Millipore Corp., Bedford, MA, USA) was used for all preparations.

Platelet Suspension

Rabbit blood collected from the marginal ear vein, with one sixth volume of acid-citrate-dextrose was mixed. The blood was centrifuged by swinging centrifugation at 200 g for 15 min at room temperature. The upper platelet rich

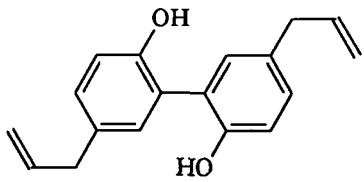

Figure. 1. Chemical structure of magnolol isolated from the bark of *Magnolia officinali.*

plasma was mixed with 2 mM EDTA and centrifuged at 1,000 g for 12 min. The supernatant was discarded and platelet pellet was suspended in Ca^{++}-free Tyrode's buffer (136.89 mM NaCl; 2.68 mM KCl; 2 mM $MgCl_2$ 0.33 mM NaH_2PO_4; 5 mM glucose, 10 mM HEPES; 4-(2-hydroxyethyl)-piperazine-1-ethane-sulfonic acid) with 0.35 % bovine serum albumin, heparin (50 unit/mL) and apyrase (1 unit/mL). After 20 min incubation at 37°C, the washed platelet pellet was resuspended in Tyrode's buffer containing 1 mM calcium and the cell concentration was adjusted to around 1 x 10^8 platelets/mL. The reaction of drug treatment was terminated after 3 min by mixing the sample with one-fifth volume of 0.05 mM EDTA in ice. After centrifugation at 10,000 g for 3 min, the supernatant was filtered through a 0.2 μm membrane filter. Aliquot (10 μL) of the filtrate was directly injected onto the HPLC apparatus[11] for analysis.

Apparatus and Chromatography

The HPLC/ECD system consisted of a syringe pump (ISCO, Lincoln, NE, USA) at flow rate 0.06 mL/min for 5-HT analysis (Fig. 2). Samples were separated using a reverse phase C_{18} SepStik microbore column (150 x 1 mm; 5 μm; BAS, West Lafayette, IN, USA) fitted with a microbore guard column (14 x 1 mm; 5 μm; BAS). The injection volume was configured with a 10 μL sample loop. The mobile phase consisted of 110 mL acetonitrile, 2.08 mM sodium 1-octanesulfonate, 13.48 mM monosodium dihydrogen orthophosphate, 56.59 mM sodium citrate, 0.027 mM EDTA, and 1 mL diethylamine. The final volume of the mobile phase was added to 1 liter of triple-deionized water. The solution was adjusted to pH 3.0 by orthophosphoric acid. The mixture was filtered with a 0.22 μm Millipore membrane. 5-HT and 5-HIAA were detected using amperometric detector (BAS-4C) coupled to a glassy carbon working electrode and referenced to a Ag/AgCl electrode at +0.6 V. Output from the ECD was amplified and recorded using Waters Millennium 2010 software.[12]

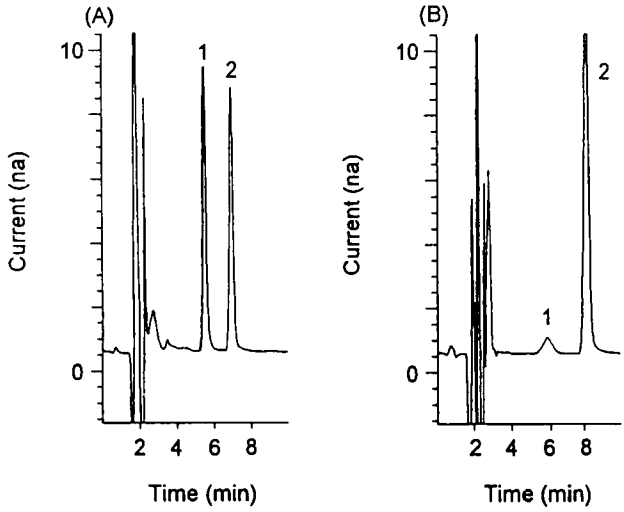

Figure 2. Typical chromatograms of (A) standard mixture of 5-HT (50 ng/mL) and 5-HIAA (50 ng/mL) (B) arachidonic acid (30 μM)-induced rabbit platelet 5-HT (155.86 ng/mL) release.

Statistical analysis

All results are expressed as the mean±S.E.M. from four experiments. Statistical analysis was performed by Student t test with the level of significance set at $p<0.01$.

RESULTS AND DISCUSSION

Peaks detection limit of 5-HT and 5-HIAA were about 0.1 ng/mL at a signal-to-noise ratio of 4. Fig. 2 (A) shows the typical chromatograms of a standard mixture containing 50 ng/mL of 5-HT and 5-HIAA. The retention times were 5.5 and 7.0 min, respectively. Fig. 2 (B) shows the arachidonic acid (30 μM) induced 5-HT (155.86 ng/mL) release.

The calibration curve was linear for 5-HT (range 1 to 200 ng/mL). The reproducibility of the method can be defined by examining both intra- and inter-assay variabilities. The intra-assay coefficients of variation (C.V.s) for 5-HT at concentrations of 10 and 50 ng/mL were 2.45 and 1.87%, respectively, the inter-assay C.V.s for 5-HT at the same concentrations were 5.09 and

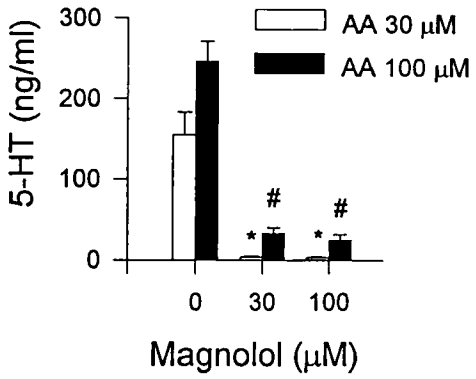

Figure 3. Arachidonic acid-induced 5-HT release from platelet suspension (n=4). * $p<0.01$ vs arachidonic acid (30 μM). # $p<0.01$ vs arachidonic acid (100 μM).

2.61%, respectively. Peaks detection limit of 5-HT and 5-HIAA were about 0.1 ng/mL at a signal-to-noise ratio of 4.

Using challenge doses of arachidonic acid 30 and 100 μM, the levels of platelet 5-HT increased from the basal value 2.96±0.89 ng/mL to 154.72±28.03 and 246.77±24.38 ng/mL, respectively. The antiplatelet agent magnolol dose-dependently inhibited arachidonic acid induced 5-HT release from rabbit platelet suspension. Fig. 3 shows that arachidonic acid (30 or 100 μM) induced 5-HT release from rabbit platelet suspension and magnolol inhibited arachidonic acid-induced 5-HT release.

Regardless, this HPLC/ECD system uses commercially available microbore column. The microbore column increase sensitivity through a decrease in band broadening that results in sharper peaks. Furthermore, the low flow rate (0.06 mL/min) is also a considerable advantage to the environment because of the organic solvent present in the mobile phase.[11]

The major activation of platelets induced arachidonic acid is due to the formation of thromboxane A_2.[13,14] Moreover, the thromboxane A_2 triggered secretion is mediated by increase intracellular calcium.[15] In addition, intracellular calcium increase has been regarded as the final common pathway for the platelet shape change, secretion and aggregation.[16]

Teng et al.[4] demonstrated that the mechanism of the antiplatelet agent, magnolol, was due to an inhibitory effect on thromboxane formation and

intracellular calcium mobilization. In the present study, we promote the above results that magnolol inhibits arachidonic acid-induced 5-HT release.

Serotonin displays complex properties on vascular tissues, producing constriction in venules and dilatation in arterioles. However, the relationship between serotonin content and platelet function in initiation and maintenance of vascular function are poorly understood.[17] The rapid measurement of platelet 5-HT by microbore HPLC/ECD system may be a useful tool in this area of investigation.

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