CHAPTER 2
MATERIALS AND METHODS

This chapter involves preparations of anhydrobarakol hydrochloride from fresh young leaves and flowers of *Cassia siamea* Lamk. The chemical structure of the bioactive substance is then confirmed prior to insertion of iodine atoms into the molecule by iodination reaction. The [^{125}I]anhydrobarakol hydrochloride is then prepared and used as the radioligand to demonstrate barakol binding sites in rat brain by *in vitro* receptor autoradiographic technique. All detailed methodologies is described in a step-wise manner as follows;

1. Preparation of anhydrobarakol hydrochloride

1.1 Plant materials

Fresh young leaves and flowers of Khi-lek (*Cassia siamea* Lamk.) were obtained from the market in Saraburi, Thailand in April 1995. The plant materials were identified and confirmed by comparison with the herbarium specimens in the Botany Section, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand and the Standard of Acean Herbal Medicine, Jakata, Indonesia as reference.

1.2 Extraction and purification of anhydrobarakol hydrochloride

Ten kilograms of fresh young leaves and flowers of *Cassia siamea* Lamk. were blended into small pieces by blender and mixed with sulfuric acid (0.5%, AR Grade,

BDH chemical, UK). The mixture was then heated until boiling and left at room temperature for 30-60 minutes prior to filtration. The supernatant was alkalinized with sodium bicarbonate (1N, AR Grade, Merck, Germany), and adjusted to pH 7-8 prior to extraction with chloroform (1 volume of supernatant:2 volume of chloroform, AR Grade, Merck, Germany). The chloroform extract was evaporated under reduced pressure down to the volume of 200-300 milliliters, then, mixed with the equal volume of cold distilled water ($4^{\circ}C$) and shaked vigorously until precipitation of the yellow lemon needled-shape crystals. The solution was stored in the refrigerator for 20-30 minutes for complete precipitation. The crystal was separated by filtration and purified by recrystallization in acetic acid (5%, AR Grade, Merck, Germany) then, neutralized with strong ammonium hydroxide solution (AR Grade, Merck, Germany). The crystal was filtrated and dissolved in few drops of ethanol (AR Grade, Merck, Germany). The concentrated hydrochloric acid (AR Grade, Merck, Germany) was added later. The presumed dark green needle-shaped crystals of anhydrobarakol hydrochloride was slowly precipitated after administration of hydrochloric acid (concentrated). Purification of the crystal was accomplished by recrystallized it in absolute ethanol (Diagram 1).

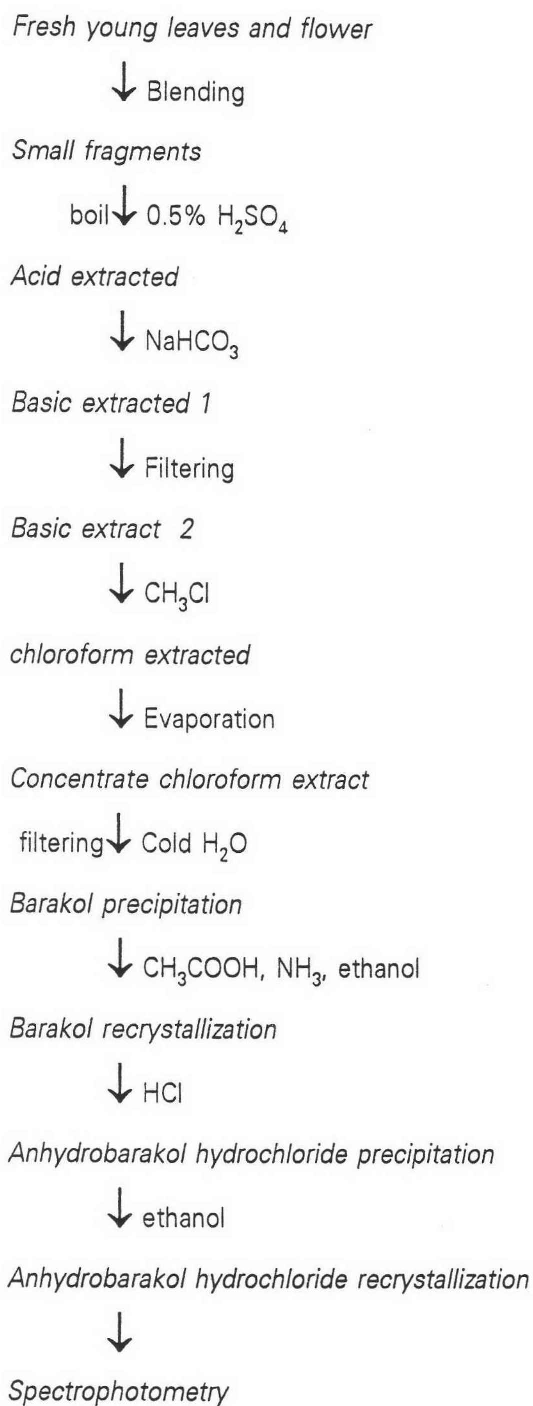

Diagram 1 : Flow-chart diagram demonstrating a step-wise preparations of anhydrobarakol hydrochloride.

1.3 Identification the chemical structure of anhydrobarakol hydrochloride

The chemical structure of the presumed anhydrobarakol hydrochloride obtained from 1.2 was verified by the following procedures.

1.3.1 Melting points

Melting points were determined by Buchi melting point apparatus.

1.3.2 Infrared spectrum

Infrared spectrum were registered in KBr pallets with Jasco IR-700 spectrophotometer.

1.3.3 Ultraviolet spectrum

Ultraviolet spectrum were determined in ethanol on a Shimadzu UV-160 spectrophotometer.

1.3.4 NMR spectrum

^{1}H-NMR spectrum were recorded on a Cryomagnetic for spectroscopy BZH 200/52 (200 MHz) spectrometer, using tetramethylsilane (TMS) as the internal standard.

1.3.5 Mass spectrum

Mass spectrum were measured with JEOL JMS-DX 300 mass spectrometer.

The results obtained from each procedured were compared to those of previous works (Bycroft,1970 and Kaokeaw,1993 : Table 2).

2. Iodination reaction of anhydrobarakol hydrochloride in macroscale.

The chloramine-T method is the most widely used for the radioiodination of small mass of protein and other substances to yield high specific radioactivities and is a good tracer in receptor autoradiography technique. (Anwar, 1993).

2.1 Preparation of [I]anhydrobarakol hydrochloride.

Twenty milliliters of chloramine-T solution (1 mg/ml in HCl pH 2, AR Grade, Merck, Germany) was added to a mixture of anhydrobarakol hydrochloride (25 ml, 2 mg/ml), sodium iodide (10 ml, 1 mg/ml, AR Grade, Merck, Germany) and HCl (100 ml, pH 2), until the color of solution changed from the pale yellow to the light brown (Kaokeaw, 1993). The brown precipitation was appeared after shaking for 2-3 minutes at room temperature.The reaction was left for 30 minute for complete precipitation, then filtered and kept in desiccator until used. Rf value of the precipitation was then demonstrate on thin-layer chromatography (TLC) plate coated with siliga gel. The mobile phase was the mixture of chloroform and ethyl acetate (ratio 6:4, AR Grade, Merck, Germany). The precipitation was also dissolved in chloroform and separated by column chromatography packed with siliga gel (70-230 mesh, Merck, Germany). A mixture of chloroform and ethyl acetate (6:4) was employed as the solvent system. Collected fraction was evaporated under reduce pressure using Buchi rotary evaporator until the yellow-brown crystalline was precipitated. The substance was purified by recrystallization with absolute ethanol (Diagram 2).

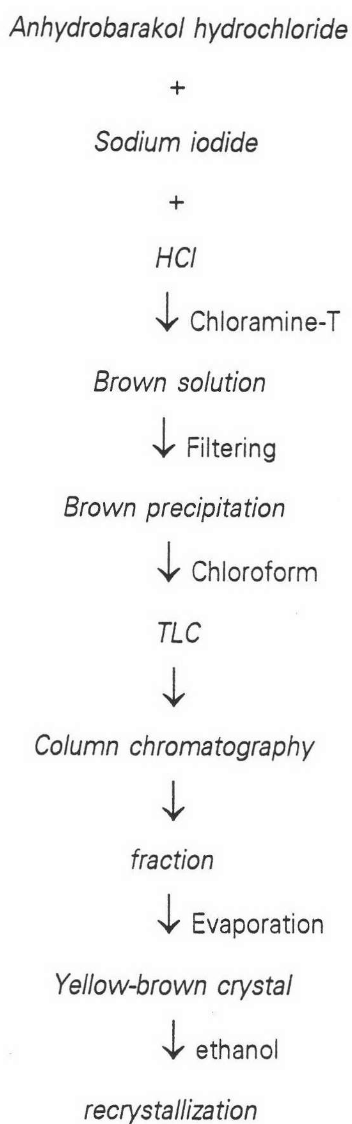

Diagram 2 : Flow-chart diagram demonstrating a step-wise preparations of iodination reaction of anhydrobarakol hydrochloride in macroscale.

2.2 Identification of chemical structure of [I]anhydrobarakol hydrochloride.

The chemical structure of the presumed anhydrobarakol hydrochloride obtained from 1.2 was verified by the following procedures.

2.2.1 Melting point

Melting points were determined on a Buchi melting point apparatus.

2.2.2 Infrared spectrum

Infrared spectrum were registered in KBr pallets with Jasco IR-700 spectrophotometer.

2.2.3 Ultraviolet spectrum

Ultraviolet spectrum were determined in ethanol on a Shimadzu UV-160 spectrophotometer.

2.2.4 NMR spectrum

^{1}H-NMR spectrum were recorded on a Cryomagnetic for spectroscopy BZH 200/52 (200 MHz) spectrometer, using tetramethylsilane (TMS) as the internal standard.

2.2.5 Mass spectrum

Mass spectrum were measured with JEOL JMS-DX 300 mass spectrometer.

The results from each procedure were compared with those of previous work (Kaokeaw,1993 :Table 3).

3. Preparation of [^{125}I]anhydrobarakol hydrochloride

Retention time of chloramine-T (CAT), sodium iodide (NaI), cold anhydrobarakol hydrochloride ([I]anhydrobarakol hydrochloride), anhydrobarakol hydrochloride standards and hot anhydrobarakol hydrochloride ([^{125}I]anhydrobarakol hydrochloride) were elucidated with reverse phase column (Ultracarb 5 ODS (20) 250*4.6 mm, 5 μm particle; Phenomenex, USA).

- The solvent system is a mixture of acetronitrile and distilled water (CH_3CN:H_2O 70:30, HPLC Grade). The flow-rate was set at 1.0 ml/min. The HPLC profiles was determined with a UV detector at 254 nm. Fraction were collected with a Fraction collector (Retriever 500, ISCO, USA).

3.1 Preparation of chloramine-T standard in microscale.

Chloramine-T (1 mg/ml) was dissolved in solvent composition of acetronitrile and distilled water (CH_3CN:H_2O, 70:30), then filtered prior to injection at the volume 60 μl through reverse phase column of HPLC system to demonstrate its peak and retention time.

3.2 Preparation of sodium iodide standard in microscale.

Sodium iodide (1 mg/ml) was dissolved in solvent composition of acetronitrile and distilled water (CH_3CN:H_2O, 70:30), then filtered prior to injection at 60 μl through reverse phase column of HPLC system to demonstrate its peak and retention time.

3.3 Preparation of [I]anhydrobarakol hydrochloride standard in microscale.

[I]anhydrobarakol hydrochloride from column chromatography (2 mg/ml) was dissolved in solvent composition of acetronitrile and distilled water ($CH_3CN:H_2O$, 70:30),then filtered prior to injection at 60 μl through reverse phase column of HPLC system to demonstrate its peak and retention time.

3.4 Preparation of anhydrobarakol hydrochloride standard in microscale.

Anhydrobarakol hydrochloride (2 mg/ml) was dissolved in solvent composition of acetronitrile and distilled water ($CH_3CN:H_2O$, 70:30), then filtered prior to injected at 60 μl through reverse phase column of HPLC system to demonstrate its peak and retention time.

3.5 Preparation of cold anhydrobarakol hydrochloride in microscale.

Eighty microlitres of chloramine-T (1mg/ml) was added to a mixture containing anhydrobarakol hydrochloride (100 μl, 2 mg/ml) and sodium iodide (40 μl, 2 mg/ml) in a sealed vial. The mixture was gently stirring by tip of volumetric pipette. The reaction was allowed to proceed at room temperature until light brown precipitation was appeared. Acetonitrile and distilled water (70:30, 1 ml) was later added to dissolved the precipitation. Then, it was filtered and injected at the volume of 60 μl through reverse phase column of HPLC system.

3.6 Identification of the product [I]anhydrobarakol hydrochloride peak

Confirmation of the product [I]anhydrobarakol hydrochloride peak obtained from the reaction in 3.5 was undertaken by the "spike technique". Sixty microlitres of the

[I]anhydrobarakol hydrochloride complex from column chromatography (1mg/ml) was add to 60 μl of the reaction mixture prepared in 3.5. Then, 60 μl of solution was injected through the reverse phase column of HPLC system to demonstrate different peak of substances.

3.7 Preparation of [^{125}I]anhydrobarakol hydrochloride.

This preparation was similar to the reaction in 3.5, but using radioactive sodium iodide (Na^{125}I) for substitution iodine atom into structure of anhydrobarakol hydrochloride. In brief, eighty microlitres of chloramine-T (1mg/ml) was added to a mixture containing anhydrobarakol hydrochloride (100 μl, 2 mg/ml), sodium iodide (20 μl, 2 mg/ml), and sodium [^{125}I]iodide (20 μl, specific activity 17 Ci/mg; 100 mCi/ml), Amersham, England). The mixture was then gently stirring by tip of volumetric pipette. The reaction was allowed to proceed at room temperature until light brown precipitate was appeared. Acetonitrite and distilled water (70:30, 1 ml) was later added to dissolved the precipitation. Then, it was filtered and injected at the volume of 60 μl through reverse phase column of HPLC system for isolation the products, [^{125}I] anhydrobarakol hydrochloride from the anhydrobarakol hydrochloride and a small amount of free sodium[^{125}I]iodide. A solvent system composition of acetonitrite and distilled water(70:30). A Ultracarb 5 ODS (20) column (250*4.6, 5 μm particle; Phenomenex, USA) was used for separation. The flow-rate was 1.0 ml/min. This elute was passed the UV detectors. Fraction of [^{125}I] anhydrobarakol hydrochloride were collected with a Fraction collector (Retriever 500,ISCO), measured the radioactivity with gamma counter (Mini-Assay type 6.20, Morgan) and kept at 4 °C for receptor autoradiographic studies (Diagram 3).

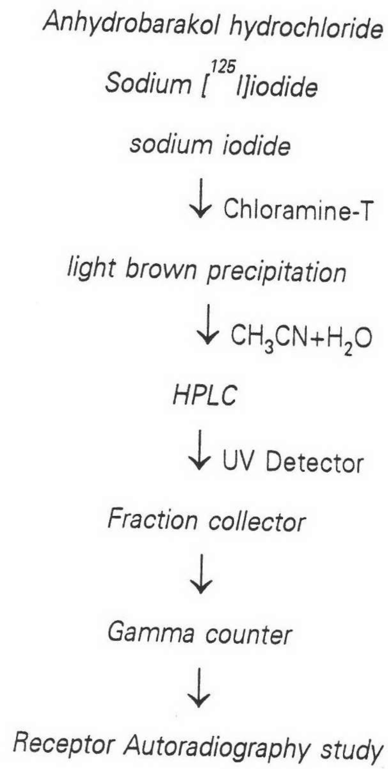

Diagram 3 : Flow-chart diagram demonstrating a step-wise preparations of [^{125}I]anhydrobarakol hydrochloride

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<u>4. Demonstration and localization of the $[^{125}I]$anhydrobarakol hydrochloride binding sites in rat brain by in vitro receptor autoradiography technique</u>

4.1 Animal

Twenty adult male Sprague Dawley rats weighing between 280-300 gm were kept on a 12 hour light / 12 hour dark cycle and had free access to food and water until sacrificed.

4.2 Subbed-slide preparation

Glass microscopic slides were washed in a mixture of gracial acetic acid and 95% alcohol (1:1). Dry in oven, and dipping the slides into a freshly prepared warm aqueous gelatin solution (0.3 g chromium potassium sulfate, 3 g gelatin, and 500 ml distilled water). The slides were dried and stored in slide box until used in sectioning.

4.3 Tissue preparation

Animals were anaesthetized with chloroform, and heart was exposed. Then, blood was flushed out by perfusion of normal saline (0.9%) through left ventricle. The perfusion was followed immediately by phosphate buffer saline containing 0.1% paraformaldehyde (pH 7.4). The brain was immediately dissected out and place on aluminium foil container floating on liquid nitrogen until the brain was frozen.The frozen brain was then mounted on cryostat microtome chucks with embedding medium at -20°C. Serial coronal sections (20 μm thick) were cut and thaw-mounted on cool gelatin-coated glass slide. Alternate sections were kept for the nonspecific

binding investigation and cresyl violet staining. All sections were kept at least 3 days in a sealed slide box at -70°C until use.

4.4 Demonstration of specific binding sites with [^{125}I]anhydrobarakol hydrochloride

For labeling of binding sites, section was warmed to room temperature and preincubated for 20 minute (25°C) in Tris-HCl (50 µM, pH 7.4), containing 0.2 M Tris, 0.1 N HCl, then incubated in the same buffer containing [^{125}I]anhydrobarakol hydrochloride (10^{-7} M) for 45 minute, at 21-25°C. Alternate sections were co-incubated with anhydrobarakol hydrochloride (10^{-3} M) for identification of nonspecific binding. After incubation, the slides were washed three times in the same buffer (4 °C, 2 minute each), then dipped in ice-cold (4 °C) distilled water to eliminate residual buffer. The slide sections were then dried under a stream of cold air.

Dried sections and Microscales standard (Amersham, England) were placed in Hypercassettes and Hyperfilm was placed on their upper surface. Then, the cassettes was closed and sealed tightly and kept in the dark at 4 °C for 24-48 hours. The film was removed out of the cassettes in the dark room and then developed with Kodak developer D-19 for 2 minute at 20°C then dipped in 5% acetic acid and fixed in Kodak rapid fixer for 10 minute at 20°C (Diagram 4). Alternate sections were stained with cresyl violet for histological reference and the binding sites were localized using the rat brain atlas of Paxinos and Watson (1982) as reference.

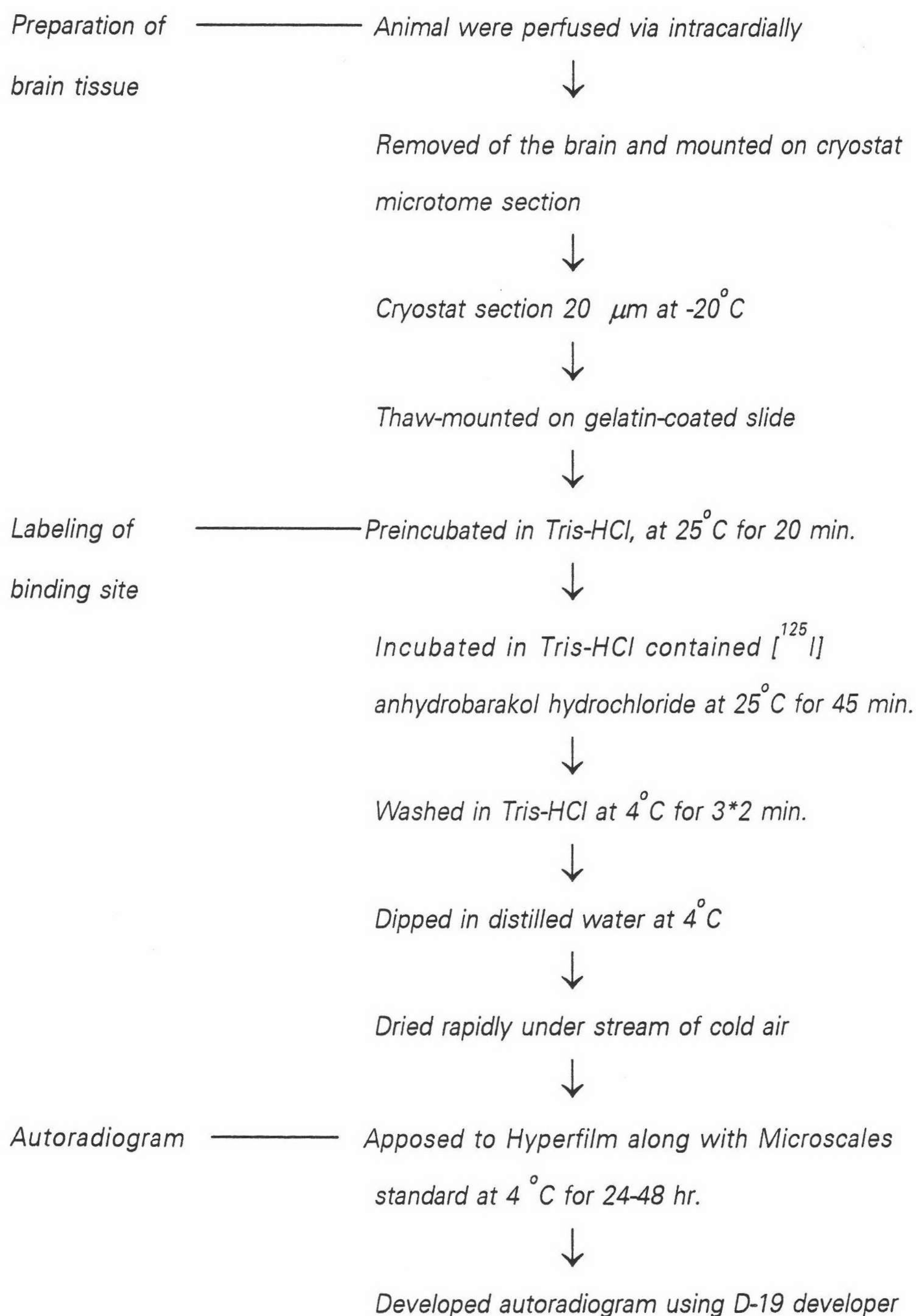

Diagram 4 : Flow-chart diagram demonstrating a step-wise preparations of autoradiograms from rat brain section.