CHAPTER 4
DISCUSSION

The main purpose of this study was to elucidate reliable procedures for preparation of [^{125}I]anhydrobarakol hydrochloride. Then the radioligand was employed in the preliminary study for detection and localization of specific binding sites to the anhydrobarakol hydrochloride.

Purified bioactive substance prepared from the fresh young leaves and flower of *Cassia siamea* Lamk. was obtained as a lemon yellow needle-shaped crystal. This substance was unstable, thus, it was converted to the stable form of anhydronium salt as a dark green compound. The physical and spectrophotometric characteristics of the substance were shown ; mp. 205 $^{\circ}$C (dec.); UV λ_{max} (EtOH) nm (log ε): 241 (4.8) and 398 (4.54) ; IR ν_{max} (KBr) 3445, 1670, 1589, and 1271 cm^{-1} ; ^{1}H-NMR (D_2O): δ 7.03 (2H,d), 6.89 (1H,b), 6.86 (1H,t), 2.71 (3H,s) and 2.48 (3H,s) ; MS, m/z (rel.): M^+ 216 (3), 214 (100), 186 (158), 115 (11), 89 (11), 63 (9), and 51 (9). These data were similar to those of anhydrobarakol hydrochloride in previous reports (Bycroft, 1970 and Kaokeaw, 1993). Thus, this confirmed that the dark-green compound prepared in this experiment was anhydrobarakol hydrochloride.

Iodination compound was obtained as yellow brown precipitation. Its melting point was shown at 220 $^{\circ}$C (dec.), while the spectroscopic characteristics were evaluated in detailed; UV λ_{max} (EtOH) nm (log ε): 246 (4.87) and 384 (4.29); IR ν_{max} (KBr) 3046, 1667, 1564 and 1527 cm^{-1} ; ^{1}H-NMR ($CDCl_3$) : δ 6.69 (1H,d) 6.16 (1H,d), 2.49 (3H,s), 2.33 (3H,d) and MS, m/z (rel.): M^+ 467 (14.8), 466 (100), 438 (10), 339 (11), 311 (16), 184 (12), 169 (13) and 43 (17). These data were similar to those of

[I]anhydrobarakol hydrochloride in previous reports (Kaokeaw, 1993). Thus, this confirmed that brown precipitation prepared in this experiment was [I]anhydrobarakol hydrochloride.

At microscale preparation, the [I]anhydrobarakol hydrochloride was also observed as a light brown precipitation. Separation of the compound from chloramine-T, sodium iodide and anhydrobarakol hydrochloride was achieved by HPLC. Four major peaks were observed at retention time 2.608, 3.714, 4.297, and 5.994 minute postinjection. The peak at retention time 4.297 minute was appeared to be of [I]anhydrobarakol hydrochloride, while those of 2.608 and 5.994 minute were chloramine-T and anhydrobarakol hydrochloride. However, the peak at retention time 4.297 minute was confirm to be [I]anhydrobarakol hydrochloride by the spike preparation. The [^{125}I]anhydrobarakol hydrochloride obtained from the reaction was also shown at the peak with retention time 4.275 minute. Thus, this confirm that fraction collected at this retention time is [^{125}I]anhydrobarakol hydrochloride. This was also confirmed by the highest radioactivity of this fraction.

[I]anhydrobarakol hydrochloride is the anhydrobarakol hydrochloride molecule with two iodine atoms at ortho positions.The compound has also been elucidated to have depressive effect in previous report (70%, Kaokaew, 1993). Thus it is quite reasonable to use this [^{125}I]anhydrobarakol hydrochloride as a radioligand to demonstrate and localize of barakol binding sites.

In this experiment, binding sites were clearly demonstrated as dense grain density on the Hyperfilm after using [^{125}I]anhydrobarakol hydrochloride (10^{-7} M) as radioligand. These binding sites were uniformly demonstrated in the caudate putamen

from the rostral level until the level of rostral part of thalamic nuclei, accumben nucleus, cerebral cortex from the level of frontoparietal cortex to the level of striate cortex and entorhinal cortex, hippocampus, thalamic nuclei from the rostral to the caudal level, granule cell layer of cerebellum along its entire length, inferior colliculus and substantia nigra. Dark grain density observed in these areas was clearly contrasted to that of background ones. Likewise, the dark grain density in the observed areas was reduced sharply to the level of background density in the experiment using radioligand containing cold ligand (anhydrobarakol hydrochloride 10^{-3} M). These evidences suggested that those areas contain specific binding sites of the anhydrobarakol hydrochloride. (Penney, 1981; Carlson, 1992; Palacious, 1981; Negro, 1995; Yogoyama, 1995).

Iodine-125 (^{125}I) was employed as radioisotope for labeling the anhydrobarakol hydrochloride due to the fact that the preparation reaction was convenient (Sonoda, 1970) and had high energy to demonstrate the grain density on the film. Because of the high energies of the γ-particles emission from [^{125}I], thus there is no a gray/white quenching effect and also has higher specific activity than tritium with resultant shorter exposure time (Kuhar, 1985). The ^{3}H-labeled radioligand always produces gray/white quenching effect due to its low β-emission. However, with iodine-125, infinite thickness is experimentary much greater and variations in section thickness, including those due to microtome errors, will affect the density of the autoradiogram. Thus, section thickness becomes a consideration when using iodine-125 (Kuhar, 1986). Moreover, the ^{125}I radioligand could be used in clinical brain imaging application with single photon emission computerized tomography (SPEC) (Brucke, 1988 ; Kung, 1988). Localizations of anhydrobarakol hydrochloride binding sites demonstrated in this study was a unique pattern. These were not comparable to any of those previous known

receptors such as dopamine D_1, D_2 , GABA and serotonin. Dopamine-D_1 receptors have been clearly demonstrated in substantia nigra pars reticularta, caudate-putamen, accumbens nucleus (Staley,1994), olfactory tubercle amygdaloid nucleus, subthalamic nucleus, molecular layer of dentate gyrus, superior colliculus and frontoparietal cortex (Dawson, 1985; Staley, 1994 and Martres, 1985a). Dopamine-D_2 receptors have been demonstrated in substantia nigra pars compacta, parietal cortex, cerebellum (Martres, 1985b), caudate-putamen, accumbens nucleus and olfactory tubercle (David, 1992; Lahti, 1993 and Malmberg, 1993). Serotonin receptors have been demonstrated in subiculum, superior colliculus, dentate gyrus, substantia nigra, raphe nuclei, frontal cortex, occipital cortex and locus ceruleus (Biegon, 1981; Meltzer, 1991). GABA receptors have been demonstrated in cerebral cortex, globus pallidus, thalamus, hypothalamus, granular cell layer of cerebellum, hippocampus and substantia nigra pars reticularta (Unnerstall, 1981; Palacious, 1981 and Penney, 1981).

Background density obseved in the Hyperfilms used in this study was rather high when compared to that of the Ultrofilm. Hyperfilm was chosen in this study due to the fact thay we were not able to obtain the Ultrofilm from the domestic and oversea markets.

In this study, the blockade of total binding sites of [^{125}I]anhydrobarakol hydrochloride was achived after addition of 10^{-3} M of cold ligand. The concentration of the cold ligand was rather high when compared to those previous reports using different radioligands (Przedborski, 1991). This may indicate specific properties of [^{125}I]anhydrobarakol hydrochloride since this ligand was prepared for the first time and no previous data ever reported on this matter. Likewise, the radioligand is structurally different from the cold ligand due to the two iodine atoms in the molecule. This

supported by less depressive activity of the [I]anhydrobarakol hydrochloride than that the cold ligand.

In this study only approximate K_d value was undertaken by varying different concentration of hot ligand containing one concentration of cold ligand. This study revealed the figure at 10^{-6} M. However, this data need to study in more details in order to obtain the appropriate concentration of hot ligand used in the *in vitro* autoradiographic study.

Quality of the autoradiogram presented in this study was not satisfactory since there were no proper equipments for producing them. Actually, we need image analyzer for better quality autoradiogram.

This study is able to demonstrate the binding sites and their localization of anhydrobarakol hydrochloride. The results seems to favored the idea that these anhydrobarakol hydrochloride may stimulate its depressive effect via binding sites which are not the same as dopamine D_1, D_2, serotonin, and GABA receptors.