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# THE BASIS OF ANALGESIC ACTIVITY OF FOUR CHINESE HERBS

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# ABSTRACT

This study investigate the effects of four Chinese herbs for their potential therapeutic use as analgesics. It involved screening for inhibitors of enkephalin-hydrolysing enzymes, especially selective NEP inhibitors, mixed NEP/APN inhibitors and mixed NEP/ACE inhibitors. NEP was the key enzyme in the screening systems.

Four Chinese herbs Yan (*Corydalis yanhusuo T.Wang*); Gou (*Nauclea sinensis Oliv.*); Qiang (*Notopterygium incisum Ting ex H. T. Chang*); and Chuan (*Ligusticum chuanxiong Hort.*) were tested for their inhibitory effects on NEP activity measured in a rat kidney extract. Aqueous extracts of Gou and Qiang showed the significant inhibitory activity on NEP but results with organic extracts were not significant. Gou showed the strongest inhibitory activity on NEP. It was found that Gou also showed an inhibitory activity on the other two enkephalin-hydrolysing enzymes, APN and ACE in the microsomal mixture.

A rat-brain opiate-receptor preparation (section 5.2.1) was used in ligand binding experiments with competition for the receptor sites between the aqueous Gou extract and tritium-labelled naloxone. For very small quantities of Gou there was a lowering of naloxone binding showing the antagonistical action of Gou. At higher concentrations ($\geq$ 4 mg/ml of Gou) there was a tendency for naloxone binding to increase demonstrating potential agonistic activity.

To test whether one compound in Gou had inhibitory activity on the three enzymes, or several compounds acting together, the aqueous extract of Gou was separated by HPLC. The HPLC-eluted fractions were pooled into seven parts and collected. The seven eluates were assayed for their effects on NEP proteolysis of $^{3}$H(leu)-enkephalin. Only six eluates showed inhibitory activity on NEP. The three single compounds already fractionated by others (rhynchophylline, mitraphylline and poteropodine) were shown to correspond in the Pool 7. An unexpectedly large number of active fractions suggest that many active compounds are present in Gou. It can be concluded that of the four herbs tested Gou (*Nauclea sinensis Oliv*) offers considerable potential as a novel analgesic agent.

# DECLARATION

The work described in this dissertation was carried out in the School of Biomolecular Science, Liverpool John Moores University from October 1994 to May 1999. Unless otherwise stated it is the original work of the author. This dissertation has not been submitted, in whole or in part, for any other degree at this or any other university.

B. C. Liu

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Lastly but certainly not the least, to my family, my daughter Monwei Gao and my husband Huanggen Gao, I bet you're glad this is over finally!!!

# ABBREVIATIONS

| | |
|---|---|
| A(nm) | Absorbance or Absorption at some wavelength (nm) |
| ACE | Angiotensin-converting enzyme |
| A° | Angstron unit ( $10^{-8}$ cm) |
| APN | Aminopeptidase N (APN E.C. 3.4.24.11.2) |
| APS | Aminopeptidase S |
| ANP | Atrial natriuretic peptide |
| Bes | Bestatin |
| BFC | Buffer control |
| BSA | Bovine serum albumin |
| CALLA | Common acute lymphocytic leukemia antigen |
| CCK | Cholecystokinin |
| CCK-A | Cholecystokinin A |
| CCK-B | Cholecystokinin B |
| cDNA | Complementary DNA |
| Cap | Captopril |
| CE/E | Enzyme in a given mixture of rat kidney crude microsomal fractions |
| Chuan | Chuanxiong (*Ligusticum chuanxiong Hort.*) |
| Cn | Chuanxiong undiluted (neat) |
| CPM | Counts per minute |
| °C | centigrade |
| Dap | Dipeptidyl aminopeptidase |
| DNA | Deoxyribonucleic acid |
| DPM | Disintegrations per minute |
| EDTA | Ethylenediamine tetraacetic acid |
| Gou | Gouteng (*Uncaria rhynchophylla. Miq . Jackson*) |
| Gn | Gouteng undiluted (neat) |
| Gwext | Gou water (aqueous) extract |
| HIC | Hydrophobic interaction chromatography |
| HPLC | High performance liquid chromatography |
| Hr | Hour |
| $IC_{50}$ | 50% of inhibitory concentration |
| i.p. | Intraperitoneally |

| | |
|---|---|
| IR | Infra red spectrum |
| i.v. | Intra venous |
| kDa | kilo Dalton |
| $K_D$ | Equilibrium dissociation constant |
| Ki | Equilibrium inhibition constant |
| McAb | Monoclonal antibody |
| MIC | Minimal inhibitory concentration |
| mg | milli gram |
| ml | milli liter |
| min | Minutes |
| mM | Millimolar |
| nM | nano molar |
| pmoles | $10^{-12}$ moles |
| Mw | Molecular weight |
| nm | Nanometre |
| NEP | Neutral endopeptidase (E.C. 3.4.24.11) |
| OD | Optical density |
| P | Probability |
| PCT | Proximal convoluted tubule |
| PBQ | Phenyl-1,4-benzo quinone |
| Qiang | Qianghuo (*Notopterygium incisum Ting ex H. T. Chang*) |
| Qn | Qianghuo undiluted (neat) |
| Qwext | Qiang water (aqueous) extract |
| RAS | Renin angiotensin aldosterone system |
| RPM | Revolutions per minute |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| TCM | Traditional chinese medicine |
| TCHM | Traditional chinese herbal medicine |
| TCA | Trichloroacetic acid |
| Thio | Thiorphan |
| TFA | Trifluroacetic acid |
| TLC | Thin layer chromatography |
| Tm | Melting temperature |

| | |
|---|---|
| Tris | Tri-hydroxymethyl methylamine |
| UV | Ultra violet |
| µl | $10^{-6}$ liter |
| µM | $10^{-6}$ molar |
| Yan | Yan hu suo (*Corydalis yanhusuo T.Wang*) |
| Yn | Yan hu suo undiluted (neat) |
| % | percent |

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# CHAPTER I

# INTRODUCTION

## 1.1 Research Background

It has been known that the endogenous opioid peptides Met- and Leu-enkephalin are rapidly inactivated *in vivo*, by neutral endopeptidase (NEP E.C.3.4.24.11) (Malfroy *et al.*, 1978; Guyon *et al.*, 1979) and aminopeptidase N (APN E.C 3.4.11.2) (Hambrook *et al.*, 1976; Hersh, 1986); a further enkephalin inactivator is angiotensin-converting enzymes (ACE) (Waksman *et al.*, 1984; Giros *et al.*, 1986). In recent years the inhibition of enkephalin metabolism has been extensively investigated with the aim of testing the hypothesis that increasing the level of endogenous enkephalins in neuronal pathways implicated in pain transmission could lead to a "physiological" analgesia free of the major side effects of morphine (Roques and Fournie-Zaluski, 1986; Chipkin., 1986). These hypotheses have been demonstrated in experiments and the relationship among the NEP/APN versus enkephalin and analgesia can be briefly described in the following diagram (**Figure 1.1**).

According to **Figure 1.1** inhibitors of NEP and APN increase the level of endogenous enkephalin, and potent physiological analgesic responses should be produced without major side effects in all animals in pain for which morphine is normally prescribed (Fournie-zaliski *et al.*, 1984; Claude *et al.*, 1984; Nobel *et al.*, 1991, 1992, 1997; Chen *et al.*, 1998). In addition, specific inhibitors of NEP have been found to have the following uses as: (1) antidiarrheal without constipation; (2) treatment for acute cholecystitis; (3) diuretic and natri uretic (protection of atrial natriuretic peptide) and (4) antidepressant (Seymour *et al.*, 1995; Roques, 1993).

**Figure 1.1 Schematic Representation of the Spinal Localization of NEP in a Model of "Neuropeptide Extended Synaptic Area" (Roques *et al.*, 1993).**

**The enkephalins, released from the spinal interneurons, interact presynaptically with opioid receptors located on the afferent fiber terminals to modulate peptide secretion (SP, CGRP). The concentration of the peptides at the receptor level is dependent on both their passive dilution in the extended synaptic volume and the efficiency of the inactivation processes ensured by peptidases, such as NEP, mainly located on the enkephalin neurones. The fast enkephalin-induced responses observed in electrophysiological experiments could correspond to the release of the opioid peptide into a classical synaptic area at the cell body level of spinothalamic neurons.**

Based the above background many scientists are interested in screening the inhibitors of the enkephalin-hydrolying enzymes (including NEP, APN and ACE) and have obtained much knowledge in this field. French scientists are concentrating on biosynthesis of the enkephalin-like compounds which are sensitive to NEP, APN, DAP and ACE (Adjroud, 1995; Nobel *et al.*, 1991, 1992, 1997). So far they have developed a family of analgesics devoid of opioid side effects (Chen *et al.*, 1998; Nobel *et al.*, 1997), but no compound has actually been applied clinically. Japanese scientists are engaged in screening the natural inhibitors of these enzymes from micro-organisms (Kojima, 1990; Akiyama *et al.*, 1998; Otani, 1991, 1992; Tsurumi, 1995; Kimura, 1990; Tsuru *et al.*, 1992). However, far from satisfactory results were obtained. As one kind of natural resource, plants are also worth investigating but so far no papers have been published. Traditional Chinese Herbs (TCH) have been used as analgesics for thousands of years (Yang *et al.*, 1985). Even the most widely used analgesic---morphine, originates from a plant. Considering the above facts and the close relationship between NEP inhibitors and analgesics, it might be possible to find new analgesics by use of Chinese herbs clinically. This effect may be mediated by altering the metabolism of endogenous enkephalin, by inhibiting NEP and APN, thus making analgeia possible free of induced physical and psychic dependence.

## 1.2 Nociception, Pain, Analgesia and Drug Addiction

### 1.2.1 Nociception, Pain and Their Relationship

Nociception refers to the reception of signals in the central nervous system evoked by activation of specialized sensory receptors (nociceptors) that provide information about tissue damage. Pain is the perception of an aversive or unpleasant sensation that originates from a specific region of the body. Not all noxious stimuli that activate nociceptors necessarily cause an experience of pain (Jessel and Kelly, 1991). The nociceptors can be activated by mechanical, thermal, or chemical stimuli. Tissue damage can also sensitize nociceptors. All perception of pain involves an abstraction and elaboration of sensory inputs. Usually local pain can be sensed when the

nociceptive pathways are damaged, but pain can also be modulated by the balance of activity between nociceptive and other afferent inputs.

Pain is a highly complex perception. More than any other modality it is influenced by emotions and the environment. Because it is so dependent on experience, and therefore varies from person to person, pain is a difficult clinical problem. Moreover, the current understanding of the anatomy and physiology of specific pain circuits is still fragmentary. Nevertheless, recent advances in understanding the basic physiology of pain mechanisms have led to some effective pain therapies.

First, the finding that the balance of activity in small and large fibers is important in pain transmission led to the use of dorsal column stimulation and transcutaneous electrical nerve stimulation for certain types of peripheral pain. Second, the experimental finding that stimulation of specific sites in the brain stem produces profound analgesia may eventually lead to better ways of controlling pain by activating endogenous pain modulatory systems. Third, the discovery that opiates applied directly to the spinal cord exert potent analgesic effects has led to the use of intrathecal and epidural administration of opiates for certain conditions. Finally the unravelling of the neurotransmitter systems underlying endogenous pain control circuits may provide a more rational basis for drug therapies in a variety of pain syndromes (Jessel and Kelly, 1991).

### 1.2.2 Analgesia and Whole Animal Tests for the Analgesia

Pain is an unpleasant sensory experience associated with actual or potential tissue damage. It may be acute or chronic in character; Both types are customarily treated by the administration of drugs known as analgesics. Analgesics used to treat pain are often classified into two categories: the narcotics that bind to opioid receptors, and the non-narcotics that lack an affinity for such receptors but work via other mechanisms (Tyler, 1980). The investigation of analgesics is a long-term project, and the research advances are relevant to our understanding of endogenous opioid systems in the human brain. So far the most direct tests of analgesic action are those carried out on whole animals. The following are typical tests in this category.

## Writhing Test

The writhing test was derived from that of Hendershot and Forsaith (1959). Mice received i.p.(intra peritoneally) 0.1 ml of a freshly prepared solution of PBQ (Phenyl-1,4-benzo quinone) per 10 g of body weight. 0.02% of PBQ was dissolved in 5% ethanol obtained by prior dissolution of PBQ in ethanol followed by addition of distilled water: the solution was kept out of the light and in a water bath set at 40°C. The injection produced the typical writhing reaction, which is characterized by contractions of the abdominal musculature followed by extension of the hind limbs. The mice were placed in individual transparent containers and the number of writhes per animal was counted during a 10-min period starting 10 min after the injection. The mean number of writhes for each treatment group was statistically analyzed with ANOVA, followed by Dunnett's t-test or Newman-Keuls test for multiple comparisons.

## Tail Flick Test

The antinociceptive responses were determined by measuring the time required to respond to a painful radiating thermal stimulus, according to the method of D'Amour and Smith (1941). The rat was restricted so that the radiant heat source was focused onto the base of the tail. An automated tail-flick analgesymeter (Apelex, France) was used. The cut-off time was set at 15 seconds. For each rat, three determinations were carried out before drug injection (control latency). The tail-flick latency responses were expressed as a percentage of analgesia calculated by: percent analgesia = (test latency-control latency)/ (cut-off time-control latency) $\times 100$. The intensity of the thermal stimulus was adjusted to obtain a control latency between 4 and 6 seconds. Results were analyzed with ANOVA, followed by the Newman-Keuls test.

**Hot Plate Test**

The test was based on that described by Eddy and Leimbach (1953). A glass cylinder (16 cm diameter) was used to keep the mouse at 55±0.5°C using a thermoregulated water-circulating pump. The latency period until the mouse jumped was registered by a means of a stopwatch (cut-off time 240 sec). Dose-response curves were established by expressing the data as a percent analgesia using the following equation; percent analgesia = (test latency - control latency) (cut-off time - control latency) × 100.

Statistical analysis was carried out with ANOVA followed by the Dunnett's t-test or the Newman-Keuls test for multiple comparisons.

In this thesis the opioid receptor-binding assays were carried out so that the correlative mechanism between the analgesic activity of target herbs and their inhibitory activity on NEP/APN/ACE were to be clarified. In detail highly sensitive bioassays, antagonised by naloxone, a powerful morphine antagonist, provided the detection system.

### 1.2.3 The Investigation of Analgesics and Drug Dependence

Many of the analgesics applied clinically were found to induce drug dependence. The term "drug dependence" was defined as a state arising from repeated administration of a drug on a periodic or continuous basis and included both psychic and physical dependence (Warburton, 1975; Cooper *et al.*, 1986).

According to the definition, the dependence characteristics varied with the drug involved so that there would be drug dependence of the morphine type, of the amphetamine type, of the cannabis type, of the barbiturate type etc..

## Opiate (Morphine) Dependence

Opiates have long been widely recognised as excellent analgesics, but with disadvantageous side effects such as physical dependence, appetite suppression, respiratory depression, and other behavioural effects that have limited their use. It has been shown that these effects are mediated through multiple receptors in the central nervous system (Akil *et al.*, 1984; Watkins and Mayer, 1982; Chao *et al.*, 1996; Wang *et al.*, 1998; Abdulla and Smith, 1998). Medically, opiates have been used as analgesics and also they produce a sensation of euphoria and calmness in the patient. Recently Leventhal *et al.* (1998) found that opiates can induce hyperphagia and this effect was also mediated by distinct receptors.

The principal active ingredient of opium is the alkaloid morphine, and the effects of morphine led to the synthesis of heroin and over 400 other derivatives, but the search has not revealed a compound superior to morphine in terms of analgesia and low-dependence properties, although recently a synthesized compound--RB120 is possibly a break through (Nobel *et al.*, 1997). Opioid dependence is often considered to be a long lasting form of neuronal plasticity. The molecular mechanism through which chronic opiate treatment induces states of tolerance and dependence in target neurons remains unknown. Recent studies have improved our knowledge of the molecular events linking transient activation of membrane receptors to genomic responses (Condorelli *et al.*, 1994). A group of genes are believed to participate in the long-term plastic neuronal changes. Both activator protein-1 (AP-1) and nuclear factor KB (NF-KB) transcription factor complex is composed of dimers of c-fos and c-jun proto-oncogene products or closely related proteins (Chuang *et al.*, 1995; Leventhal *et al.*, 1998). Similarly the heterodimeric NF-KB complex is composed of two DNA-binding subunits p50 and p65, which share structural homology with the c-rel proto-oncogene product. Earlier studies have demonstrated that opiate treatment can stimulate brain region-specific expression of the fos and jun genes (Couceyro and Douglass, 1995; Hayard *et al.*, 1990; Liu *et al.*, 1994) increasing evidence suggests that NF-KB is also an inducible transcription factor present in the brain (Bakalkin *et al.*, 1993; Guerrini *et al.*, 1995; Kaltschimidt *et al.*, 1993, 1995). However, very

little is known about its (i.e.NF-KB) role in the neuronal plasticity associated with the administration of drugs of abuse (Hou *et al.*, 1996).

Dependence on these drugs develops even though the relief of pain is not involved, and this process seems to fall into two stages, the initiation phase and the physical dependence stage. The first dose of an opiate gives the subject an elevated mood with the amount of euphoria depending on the dose administered and the route of administration. The effects with other opiates are similar to those with perhaps more intense euphoria with heroin and less after other opiates like codeine, meperidine and methadone (Binz, 1985; Oswald, 1968; Lindesmith, 1970).

## Amphetamine Dependence

The amphetamines are a group of drugs similar to epinephrine and they are used to control asthma. Amphetamine users report that the drug gives the feeling of excitement and omnipotence. The following account of the subjective experience of amphetamine comes from a paper by Gioscia (1972).

According to the report of Gioscia (1972) the typical behavioural manifestations of amphetamine psychosis are stereotyped searching and examining movements (Ellenwood, 1967). Amphetamine psychosis is thought to be rare in Britain.

Morgan (1991) investigated the biochemical consequences of injecting amphetamine into chicks every 12 hr for 5 days. This procedure resulted in increased activity of tyrosine hydroxylase, the synthesizing enzyme for norepinephrine, and choline acetylase, the synthesizing enzyme for acetylcholine in the brain stem of the chick. Thus, the euphoria seen in some patients after withdrawal from amphetamine may be due to the increased capacity of the nervous system for synthesizing norepinephrine as a result of the induction of tyrosine hydroxylase.

## Abuse of Cannabis Derivatives

The major "versions" of Indian Hemp (*Cannabis sativa*) are marijiuana and hashish, the latter being the stronger product because it is derived from the resin of the female plant. Both types are usually smoked, but marijuana is rolled into cigarettes ('reefers') while hashish is always smoked in a pipe. The active ingredient which gives hashish and marijuana their particular properties is tetrahydrocannabinol. In the United States cannabis derivatives are the most frequently used illicit drugs (Warburton, 1968).

It is only recently that the tetrahydrocannabinols have become available for research purposes (Mechoulam, 1970). Analysis of the brain chemistry has shown that 45 min after low doses of the drug there was a depletion of norepinephrine in the mouse brain (Holtzman, 1969; Dalton, 1968). Higher doses showed much less spontaneous activity. Injections of comparable doses in monkeys produced increased responses in continuous avoidance situations with the low doses, like amphetamine, but decreased responses in the later phases with higher doses (Scheckel, 1968; Dalton, 1968). It seems reasonable to suggest that the depletion of norepinephrine reflected the release of this transmitter by tetrahydrocannabinol, and it is this release, occurring in the median forebrain bundle, that produces the euphoric effects. So the behavioural effects of tetrahydrocannabinol are not the same as those of amphetamine (Warburton, 1968).

## Barbiturate Abuse

The extent of barbiturate abuse is larger than the incidence of opiate abuse, but it has not been subjected to the same intensive study. The mechanism underlying barbiturate tolerance is not certain, but Seevers and Deneau (1963) have shown that there is markedly increased activity of the hepatic enzymes after chronic injections, suggesting that barbiturates are metabolized quicker in tolerant animals. In Warburton (1968) it was found that the recovery rate of tolerant and non-tolerant rats was not significantly different, suggesting that increased enzyme activity did not play a part in tolerance effect obtained after single doses, and that the effects were

probably due to changes in the sensitivity of the central nervous system. The nature of this change is unclear at the present time.

## Alcoholism

Research suggests that the mechanism of tolerance or physical dependence are very similar to those found after chronic barbiturate use. Alcohol increased the release of norepinephrine in the brain stem (Gursey and Olsen, 1959, 1960).

Ideal analgesics which would not lead to both psychic and physical dependence have been investigated for thousands of years. No such analgesics have been obtained so far. RB101 was reported to be such an analgesic (Honore *et al.*, 1997; Nobel *et al.*, 1992) but is only slightly active after oral administration. Recently (Nobel *et al.*, 1997) the more active compound---RB120 (RB101 derivative) was synthesized and was selected for a complete study. After oral administration, in various assays commonly used to select analgesics (mouse hot plate test, rat tail-flick test, electrical stimulation of the tail in rats, paw pressure test on inflamed paws in rats, acetic acid-induced writhing test and the formalin test in mice), RB120 induced potent dose-dependent antinociceptive responses in all these tests after oral administration. The differences between RB101 and RB120 in antinociceptive effects in the various assays are probably related to the amount of enkephalins and to the efficiency of peptidase inactivation in particular brain regions implicated in the control of a given nociceptive input. The goal of discovering orally active analgesics endowed with a potency similar to that of morphine but devoid of its major side-effects, seems now to have been reached with inhibitors of the enzymes NEP and APN. However RB120 still awaits clinical trials.

## 1.3 Opioid System : Opioid Peptides and Receptors

### 1.3.1 Opioid Peptides

In this thesis we screened Chinese herbs with the potential to be inhibitors of enkephalin-hydrolysing enzymes so that the endogenous opioid peptide (enkephalin) could be kept at high level *in vivo*. As endogenous opioid peptides and their receptors are located at key points in the pain modulatory system of the brain (as shown in **Figure 1.1**), the role of the opioid system is discussed.

Two advances have greatly increased our understanding of the role of opioid systems in the modulation of nociception and pain perception. First was the demonstration by Solomon Snyder and Candace Pert ( Jessel and Kelly, 1991), and independently by Lars Terenius (1973) and by Simon (1973), that morphine and related alkaloids exert their physiological actions by binding to specific membrane receptors. Hughes and Kosterlitz (1975) also found that the brain contains endogenous opioid peptides.

There are three classes of endogenous opioid peptides. The first, identified by Hughes and Kosterlitz (1979), are the enkephalins, two small peptides isolated from pig brain. The second class, discovered by Smythe and Chao Ho Li (Jessel and Kelly, 1991), belongs to the proopiomelanocortin (POMC) family (Cooper *et al.*, 1980). The POMC precursor is expressed in the pituitary, and its peptide products are released into the blood stream in response to stress. The third class, discovered by Avram Goldstein and colleagues (Jessel and Kelly, 1991), belongs to the dynorphin family. Many other peptides with opioid activity have now been discovered and are shown in **Table 1.1**.

**Table 1.1 Amino Acid Sequences of Endogenous Opioid Peptides**

| Name | Amino acid sequence |
|---|---|
| Leucine-enkephalin | **Tyr-Gly-Gly-Phe**-Leu-OH |
| Methionine-enkephalin | **Tyr-Gly-Gly-Phe**-Met-OH |
| β-Endorphin | **Tyr-Gly-Gly-Phe**-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-leu-Phe-Lys-Asn-Ala-Ile-Val-Lys-Asn- Ala-His-Lys-Gly-Gln-OH |
| Dynorphin | **Tyr-Gly-Gly-Phe**-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH |
| α-Neoendorphin | **Tyr-Gly-Gly-Phe**-Leu-Arg-lys-Tyr-pro-Lys |

**Table 1.1** suggests that all of the discovered opioid peptides contain the sequence of **Tyr-Gly-Gly-Phe**. Morphine and the opioid peptides bind to distinct subclasses of opiate receptors that have been defined on the basis of their ligand binding properties. There are three major classes of opiate alkaloids, such as morphine, which are potent agonists of the mu receptor. The endogenous enkephalins are active at both the mu and kappa receptors. Each class of receptor is widely distributed throughout the central nervous system, suggesting that endogenous opioid systems are involved in physiological functions other than pain modulation. High levels of mu receptors are found in the periaqueductal gray region and in the superficial dorsal horn of the spinal cord, coincident with the distribution of enkephalin-containing neurones (Jessel and Kelly, 1991). Functional opioid systems are located in several regions of the brain involved in modulating nociception. Opiate antagonists that are used clinically, such as naloxone, are structural analogs of morphine and consequently are most effective in antagonizing opiate actions at mu receptors. There is a good correlation between analgesic potency and agonist affinity at the mu receptor. This is not surprising since the mu receptor was originally defined by its affinity for analgesic compounds. In experimental studies kappa agonists suppress nociceptive responses after noxious mechanical stimulation, where as mu agonists are most effective in analgesic tests that use noxious thermal stimuli. Different classes of opiate receptors may therefore be involved in modulating the activity of different classes of pain.

Each of the endogenous opioids derives from one of three genes that encode the large polyprotein precursors of the physiologically active peptides. These three genes are the POMC, proenkephalin and prodynorphin genes (**Figure 1.2**).

**Figure 1.2 The POMC Gene Structure (Cooper and Martin, 1980)**

**A. Proopiomelanocortin (POMC) is so named because it gives rise to β-endorphin(β-endo), melanocyte-stimulating hormone (MSH) adrenocortico-tropic hormone (ACTH), and corticotropin-like intermediate lobe peptide (CLIP);**

**B. Proenkephalin (pro-enk) gives rise to multiple copies of met-enkephalin (ME), a leucine-enkephalin (LE) and several extended enkephalins including ME-Arg-Leu (ME-RGL), ME-Arg-Phe (ME-RF), and peptidases E, F, and peptides E is further broken down into a family of large enkephalins that appear to be the most potent analgesic fragments derived from proenkephalin;**

**C. Prodynorphin (Pro-dyn) gives rise to dynorphin (dyno), which contains the LE sequence, and neoendorphin(α-neo-endo).**

Although the anatomical distributions of the peptides encoded by the three opioid genes differ, members of each family are located at sites associated with the processing or modulation of nociception. Enkephalin- and dynorphin-containing neuronal cell bodies and nerve terminals are found in periaqueductal gray matter and retroventral medulla and in the dorsal horn of the spinal cord, particularly in laminae I and II. In contrast, β-endorphin has a more restricted distribution and is confined primarily to neurons in the hypothalamus that send projections to the periaqueductal gray region and to noradrenergic nuclei in the brain stem.

### 1.3.2 Distributions of Opioid Peptides

Studies on the distribution of these peptides and their precursors in the central nervous system by direct assay and by immunohistochemistry have shown that the enkephalins are widely distributed in discrete pathways and that the β-endorphin system is found in the brain stem, periaqeductal grey matter, locus ceruleus, and reticular formation (Enna and Yammura, 1980; Bloom *et al*., 1978; Waston *et al*., 1978). It is also present in certain areas such as the corpus striatum, cudate, and globus pallidus. In the spinal cord, laminae I and II contain high levels of enkephalin but very little β-endorphin (**Figure 1.3**).

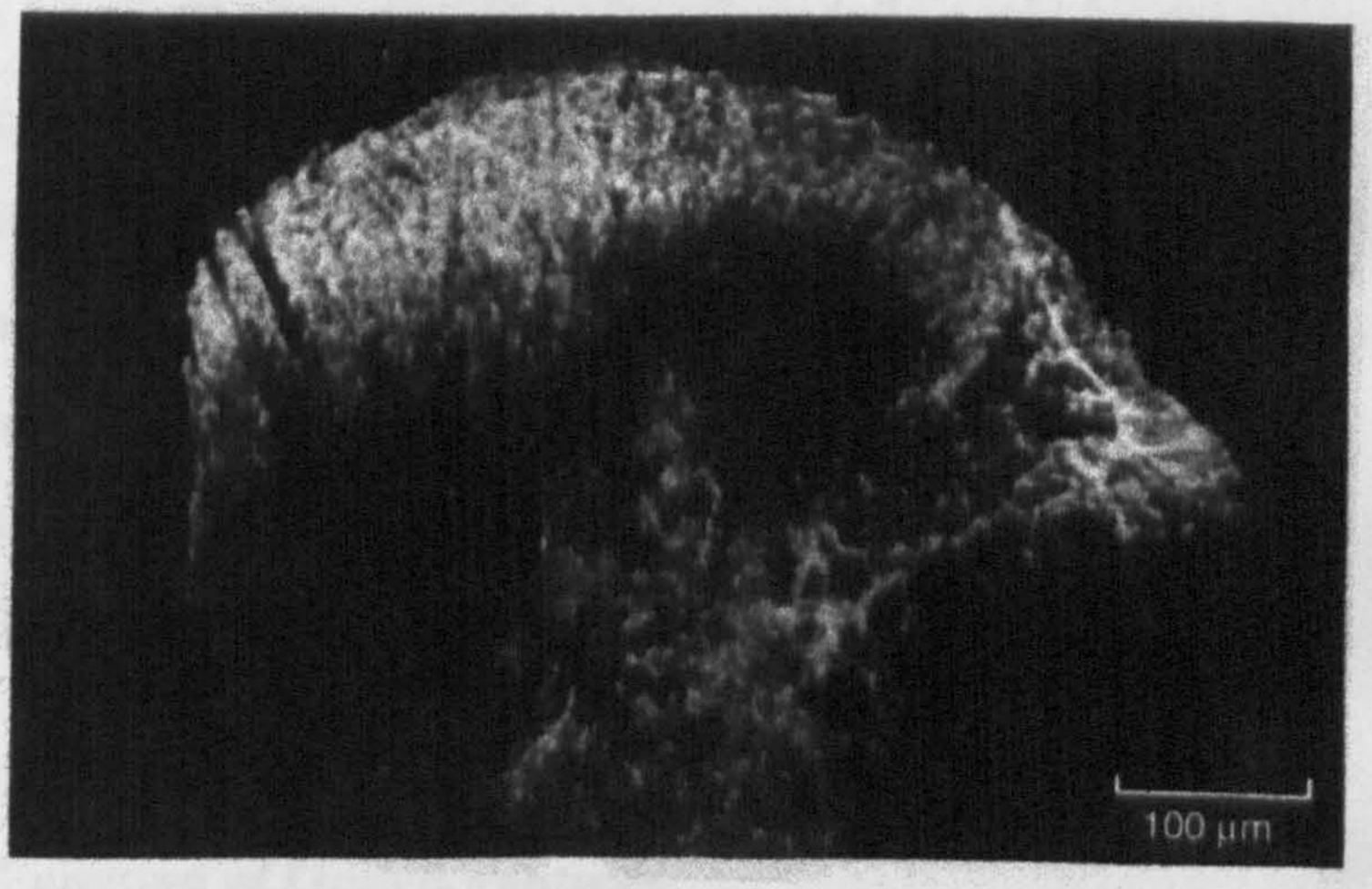



**Figure 1.3 The Localization of Enkephalin in the Brain**

**Enkephalin is localized in interneurons concentrated in the superficial dorsal horn, in the same region as afferent terminals containing substance P.**

The globus pallidus, in fact, seems to be the area richest in enkephalin. Enkephalins have been detected in amacrine cells of the retina and there is a rich enkephalinergic innervation of the gastrointestinal tract, emphasizing that they, too, are found in many different regions of the nervous system and particularly in those regions concerned with sensory transmission, endocrine control, respiration, motor activity, and behaviour (Stell *et al*., 1980). Enkephalin-containing neurons are often short, intrinsic interneurons, such as those found in the substantia gelatinosa of the spinal cord (Iversen *et al*., 1978).

Enkephalins have a very short half-life in blood because of the action of peptidases, and they have not been thought to function as circulating agents. Instead, attention is concentrated on their likely neurotransmitter role. Enkephalins exist in nerve fibers and nerve-cell bodies in sympathetic ganglia of rat and guinea pig and in the adrenal medulla endocrine cells. They appear to be present throughout the gastro-intestinal tract of animals, including humans, with the myenteric plexus containing the highest concentrations (Iversen *et al.*, 1978).

Enkephalins have been detected in normal human blood (14-140 pg/ml) and human cerebrospinal fluid (5-29 pg/ml) by employing a specific radioimmunoassay for met-enkephalin. The high content of met-enkephalin in adrenal medulla and adrenal venous blood suggests that the adrenal medulla could be the source of the peptide found circulating in the blood (Rees, 1981; Clement *et al.*, 1980). Opioid peptides met-(enkephalin), leu-(enkephalin) and β-endorphin are distributed in the brain and can bind with receptor glycoproteins existing on the surface of each neuronal cell thus producing analgesia. These glycoproteins are commonly called 'opioid receptors'. Some natural chemicals such as naloxone and morphine which have similar biological activities to the endogenous opioid peptides, also act as analgesics.

### 1.3.3 Function of Opioid Peptides

Enkephalins, methionine (Met)-enkephalin and leucine (Leu)-enkephalin, are the natural analgesics of the body, first isolated from pig brain by Hughes and Kosterlitz (1975). **Figure 1.4** shows some important endogenous opioid peptides. CCK stimulate the gut to release enkephalin. Enkephalins exert their analgesic effects by acting on one or more of the three known opioid receptors; mu, delta and kappa. Binding of enkephalins to these receptors causes inhibition of the activity of nociceptive (pain sensitive) neurons, abundant in the grey matter of the brain. Another receptor thought to exist is the sigma receptor. This one is of particular interest as it appears that drugs of abuse can bind to it ( Roques *et al.*, 1984).

**Figure 1.4 Hypothetical Model of the Interactions Between CCK, CCK-A, CCK-B and the Receptors ( including the opioid system via δ- opioid and μ opioid receptors)** (Benoliel J.J. *et al.*, 1991)

## 1.4 The Opiate System: Opioid Receptors

### 1.4.1 Classification of Opioid Receptors

Opioid receptors are classified as indicated in table 1.2.

**Table 1.2 Classification of Opioid Receptors**

### 1.4.2 The Interactions of Opioid Peptide and Receptors

The brain contains at least three separate opioid receptor subcategories, mu, delta, and kappa. Current evidence suggests that each of the three large protein precursors, pro-opiomelanocortin, proenkephalin, and prodynorphin, produces opioid peptides that interact differentially with mu, delta, and kappa receptors respectively. However, this is in no way exclusive. For instance, enkephalins that appear to interact predominantly with delta receptors can also interact with mu receptors mediating analgesia. It also seems that mu and delta receptors have an equal affinity for β-endorphin, which has a strong analgesic action. In fact, β-endorphin which has a strong analgesic action could well exert its actions through its own specific category of receptor (epsilon receptors).

Opioid receptors in brain cells have been extensively studied and the gene sequences have been molecularly cloned (Reisine *et al.*, 1993; Kong *et al.*, 1993). Three major classes of opioid receptors have been defined according to their ligand binding affinities: mu for morphine, kappa for dynorphin and delta for enkephalin specificity (Atcheson, 1994; Bzdega, and Chin *et al.*, 1993; Kozak,1994).

Although they are each synthesized from different genes with distinct chromosomal locations ( mu for chromosomal 10, δ for chromosomal 4, κ for chromosomal 1), the three classes of opioid receptors all belong to the superfamily of G protein-coupled receptors which possess seven transmembrane domains. The three receptors have been successfully expressed in human lymphocytes (delta receptor); in human and monkey immune cells (mμ receptor) and in human and monkey lymphocytes (kappa receptor) (Chuang *et al.*, 1991, 1993, 1994, 1995).

### 1.4.3 Opioid Receptor-type Specific Opiates

During the past years, considerable experimental and theoretical effort has focused on the design of receptor-type opiates for the purpose of elucidating the physiological roles of various opioid receptor types and of developing improved opioid analgesics that have minimal side effects (Chao *et al.*, Fan *et al.*, 1998; Yu

and Xie, 1998; Wang *et al.*, 1998; Wenzlaff, 1998; Foxx *et al.*, 1998; Abdulla, *et al.*, 1998; Singh *et al.*, 1998). For example Pilar *et al* (1996) used one strain of mouse, CXBK, which is deficient in supraspinal receptors $\mu_1$R and exhibits a poor response to morphine. They used this special strain of mice to screen for agents which produce antagonism or agonism action on $\mu_2$ receptor ($\mu_2$ R)-ligand binding reaction. Agents for agonism and antagonism other types of opioid receptors (including subtype receptors) have also been studied. As a whole the prototype agonists at the opioid receptors include: morphine, [D-Ala$^2$, Me Phe$^4$, Gly-ol$^5$]-enkephalin (DAMGO) for μR; dynorphin, U-50 488H for κR; D-Pen$^2$, D-Pen$^5$-enkephalin (DPDPE), ICI 174864 for δR (Hemendra *et al.*, 1996).

### 1.4.4 Opioid Receptor Binding Assay

Based on an understanding of the endogenous or exogenous opioid peptide-receptor correlation, receptor binding assays were designed and applied to study the mechanism of some exogenous or endogenous analgesics (opioid peptide).

Normally, there are three ways to study the interactions of analgesics with cells. The first procedure is to determine the biological response of an intact isolated organ, such as the guinea pig ilium, to applied agonists or antagonists. The disadvantage of this procedure is that one is obviously enmeshed in a cascade of events begining with transport, distribution, and metabolism of the agent before it even interacts with a receptor and ranging through an unknown multiplicity of steps before the final biological response of the tissue is measured. Thus, although studies with agonists may be interpretable, it is not difficult to envisage problems when antagonists are employed since these compounds may be competing at a different level from receptor binding. Despite the usual problem of a nonlinear relationship between receptor occupancy and biological response, this approach has yielded a considerable amount of information. The second approach to study receptors is by measuring ligand binding to a homogenate or slice preparation. This technique has become more feasible with a high affinity for the receptor (Gilean and Kosterlitz *et al.*, 1980). The third procedure is applied to the site selective opioid receptor ligands to establish subtype receptor binding assay. The latter is suited to study the

molecular mechanism of some specified agonist or antagonist. The detailed procedure of subtype opioid receptor binding assay was described by Thom *et al.* (1996) and Shen *et al.* (1995).

## 1.5 Enkephalin-Hydrolysing Enzymes

The following sections will concentrate on introducing some of the basic concepts underlying the investigation of the inhibitors of the three enkephalin-hydrolysing enzymes: NEP, APN and ACE.

### 1.5.1 Metallopeptidases

Metallopeptidases comprise a large group of enzymes most of which contain zinc. All the Zn-metallopeptidases have similarities in their active sites and in their respective mechanisms of action. Based on the sequence and structural similarities, Hooper (1994) classified the Zn-metallopeptidases into the five families shown in **Table 1.3.**

**Table 1.3** shows that all the zinc metallopeptidases contain a HExxH or like peptide active site, where xx are amino acids such as LI, FL. FG. IG etc.. The specificity of the Zn-metallopeptidases is essentially ensured by Van der Waals and ionic interactions between their S2, S1, S1′ and S2′ subsites and the lateral chains of the corresponding P2, P1, P1′ and P2′ moieties of the substrate.

## Table 1.3 The Family of Metallopeptidases and the Active Sequence

(see **Appendix.1** for the single-letter code for amino acids)

| Enzyme | | Amino acid sequence |
|---|---|---|
| **1 Gluzincins:** | | **HExxH.....E** |
| Neutral endopeptidase (NEP) | | VIG**HEITH**GF |
| Rat thimet oligopeptidase | | **HEFGH**VMHQLCSQAEFAMFSGTHVE |
| Aminopeptidase N (APN) | | **VIAHELAH**QWFG |
| Angiotensin-converting enzyme (ACE) | | |
| (N-terminal domain) | | **HEMGH**IQYYLQYKDLPVSLRRGANP |
| (C-terminal domain) | | **HEMGH**IQYFMQYKDLPVALREGANP |
| Endothelin-converting enzyme (ECE) | | **VVGHELTH**AF |
| Thermolysin (TLN) | | **HELTHA**VTDYAGLIYQNESGAINE |
| Clostridial neurotoxins: | | |
| Botulinum serotype | A | **HELIHAGH**RLYG |
| | B | **HELIHVLH**GLYG |
| | $C_1$ | **HELNHAMH**NLYG |
| | D | **HELTHSLH**QLYG |
| | E | **HELIHSLH**GLYG |
| Tetanus toxin | | **HELIH**VLHGLYG |
| **2 Metzincins:** | | **HEbxHxbGbxH.....M** |
| Crayfish astacin | | **HELMHAIGFYHEHTRM**DRDNYVTIN |
| Mouse meprin ( $\alpha$-chain) | | **HEILHALGFFHEQSRTDRD**DYVNIW |
| Rat meprin ($\beta$-chain) | | **HEFLHALGFWHEQSRADRD**DYITIV |
| Human 72-kDa gelatinase | | **HEFGH**AM**GLEHS**QDPGAL**MAPIY**-T |
| Human stromelysin | | **HEIGHS**LGLFHSANTEALMYPLYHS |
| **3 Inverzincins:** | | **HxxEH** |
| Human insulinase | | **HFCEH**MLFLGTKKYPKENEVSQFLS |
| E. coli proteaseIII (pitrilysin) | | **HYLEH**MSLHGSKKYPQADSLAEYLK |
| **4 Carboxypeptidase** | | **FHTYSE** |
| **5 DD-Carboxypeptidase** | | **HxH** |

Specificity is also determined by several well-positioned hydrogen bonds between the donor and the acceptor groups of the bound molecule and the polar residues of the peptidases (seen in **Figure 1.5**).

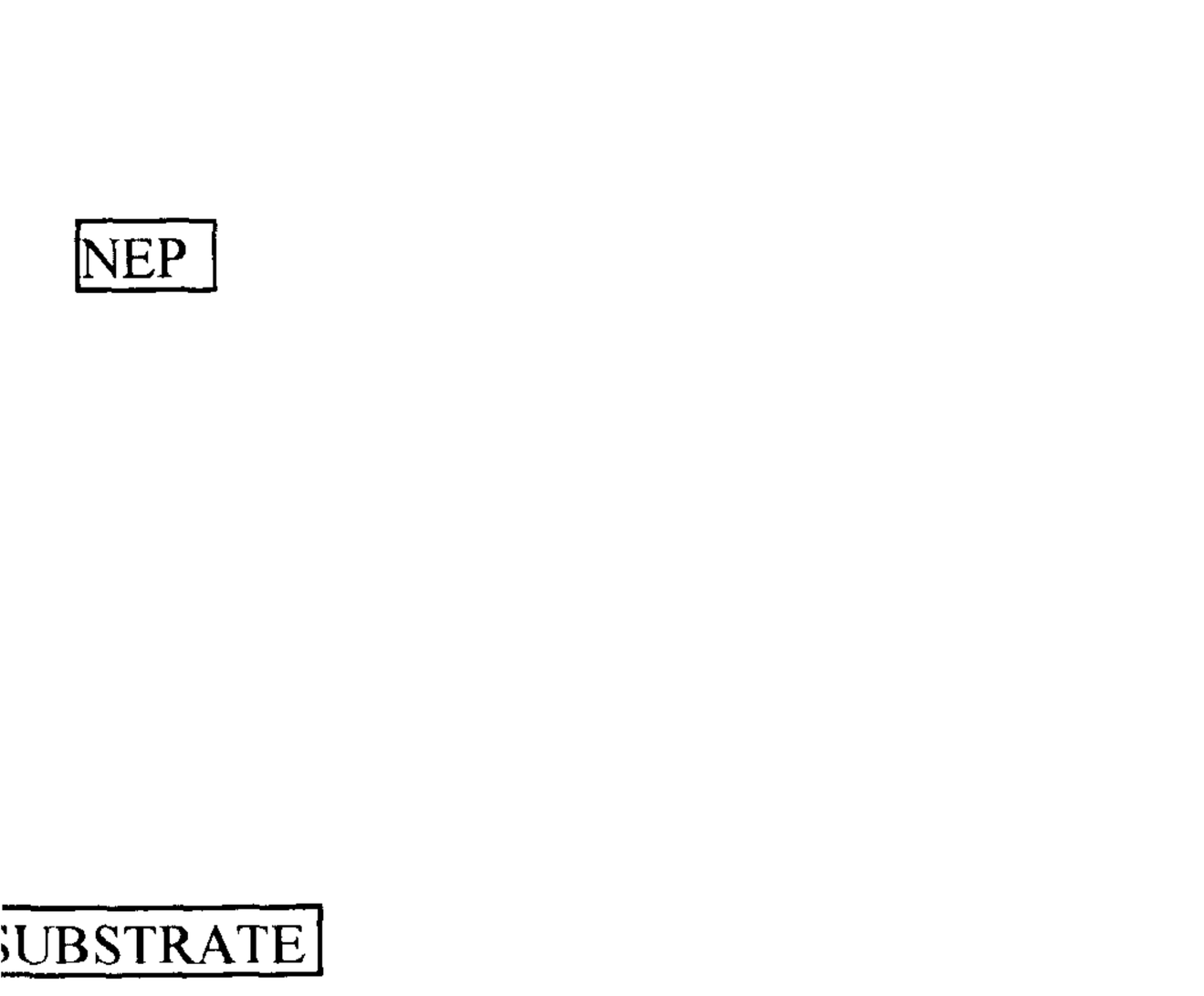



**Figure 1.5 Schematic Representation of the Binding of Substrates to the Active Site of NEP** (Roques, 1993)

Enkephalin-hydrolysing enzymes are the enzymes that cleave either methionine$^5$- or leucine$^5$-enkephalin (ME or LE), they also act on other biomolecules such as endorphin, dynorphin and bradykinin. NEP was the first enzyme found to be able to degrade enkephalin in the human brain, therefore it is also called enkephalinase. It is now clear that APN, DAP and ACE as well as NEP have recognition sites on enkephalin and should also be called enkephalin-hydrolysing enzymes (seen in **Figure 1.6**).

**Figure 1.6 Recognition Sites of Enkephalin-hydrolysing Enzymes**

(Claude *et al*, 1984)

### 1.5.2 Neutral Endopeptidase ( NEP, EC 3.4.24.11)

**Neutral endopeptidase 24.11 (NEP)** is the most important enzyme which was studied in this project. It acts on several natural substrates *in vivo*, such as enkephalins, endorphins, dynorphins etc.. All of these substrates have similar amino acid sequences in the region of their active site (Jessel and Kelly, 1991). Enkephalin or one of its analogues are usually used to establish the enzyme assay which allows selective inhibitors or mixed inhibitors of NEP/APN and NEP/ACE to be tested.

**Location and Distribution of NEP**

NEP is particularly abundant in the membranes of kidney, the lymph nodes and the placenta. It is found also at lower concentrations in lung, testis, prostate, fibroblasts, neutrophils, chondrocytes in articular cartilage, exocrine glands, various epithelial and endocrine glands and in various epithelial and endocrine cells (Sales *et al.*, 1991). It is also found in eye and gut brush border.

Several groups have studied the distribution of NEP in the spinal central nervous system (CNS) and peripheral organs. The first precise localization of NEP in the CNS was obtained by quantitative autoradiography using the tritiated inhibitor

[$^3$H]HACBO-Gly, which selectively interacts with the peptidase with a high affinity ($K_D$ = 0.5 nM) (Waksman *et al.*, 1984, 1985b, 1986a). [$^3$H]HACBO-Gly-binding sites were found to be discretely distributed in rat brain, with the highest concentrations in the choroid plexus, substantia nigra, caudate putmen, globus pallidus, olfactory tubercle, nucleus accumbens, and the substantia gelatinosa of the spinal cord. Moderate binding levels were found in the amygdana, the interpeduncular nucleus, the molecular layer of the cerebellum, the periaqueductal gray matter, and the hippocampus.

NEP was also found to be discretely distributed in the spinal cord and meninges, high levels of NEP were found in rat and pig choroid plexus, the enzyme uniquely located on the brush border of the apical surface. The distribution of NEP on peripheral tissue was also observed. A soluble form of NEP has been found in various human physiological fluids such as plasma, cerebrospinal fluid, amniotic fluid, and seminal plasma (Spillantini *et al.*, 1990) and its concentration seems to increase during inflammatory processes in the synovial fluid (Appelboom *et al.*, 1991), in sarcoidosis, or in adult respiratory distress syndrome (Johnson *et al.*, 1985).

## Substrate Specificity of NEP

NEP has a broad substrate selectivity and can cleave various of the short linear or cyclic peptides such as endothelin (Fagny *et al.*, 1991) or the ANP (Atrial Natri-uretic Peptide) (Stephenson and Kenny, 1987a), as well as polypeptides of intermediate or long length, such as the insulin-chain (approximately 3000 daltons) (Kerr and Kenny, 1974b) and interleukin-α1 (17000 daltons) (Pierart *et al.*, 1988) (**Figure 1.7**). However, it sometimes acts more efficiently as a dipeptidyl carboxypeptidase than as a true NEP. Other than the enkephalins and Met-enkephalin-Arg-Phe, NEP shows little activity toward other opioid peptides. All of these peptides are characterized by the N-terminal sequence of Met or Leu-enkephalin, but the efficiency of NEP in cleaving their $Gly^3$-$Phe^4$ bonds is exquisitely sensitive to the length of the amino acid sequence added at the COOH terminus (Turner *et al.*, 1987). Thus dynorphin 1-9, dynorphin 1-13, α- and β- neoendorphin, and β-endorphin are poor substrates, suggesting a conformationally related hindered

access of the enzyme-sensitive bonds to the active site. This is an important observation because it indicates that the "opioid" pharmacological effects induced by NEP inhibitors are mainly due to the protection of the two endogenous enkephalins and perhaps partially to the protection of the extended hepatapeptide, met-enkephalin-Arg-Phe. As we mentioned for NEP, APN also has a broad specificity for its proteolytic action. (Mcdonald and Barrett, 1986), although hydrophobic residues, preferentially aromatic, in the $NH_2$-terminal position are more rapidly removed. The $S_1'$ and $S_2'$ subsites of APN also seem to prefer hydrophobic residues (Hermandez, *et al.*, 1988; Xie, *et al.*, 1989a).

**Figure 1.7 The Relative Substrate Selectivity of NEP and the Efficiency of Hydrolysis of Some Neuropeptides by NEP (Kenny, 1993)**

## Biochemical and Biophysical Properties of NEP

**NEP** is a neutral glycoprotein which consists of a single polypeptide chain with a molecular weight of approximately 90 kDa (Roques *et al.*, 1993). It is a Zn-metallopeptidase and is located in many mammalian tissues. eg. kidney, brain and intestine. NEP is particularly abundant in the proximal convoluted tubule (PCT), located in the cortical region of the kidney (Booth, 1974; Corbeil, 1989). This is why the rat kidney was used to prepare the NEP sample in this thesis.

## Molecular Biology of NEP

NEP has a short $NH_2$-terminal cytoplasmic domain (27 amino acids), followed by a 23-residue hydrophobic domain, which anchors the protein in the plasma membrane, and a large c-terminal extracellular domain that contains the active site (**Figure 1.8 and 1.9**). NEP contains a consensus sequence VxxHExxH, which has been found in numerous other Zn-endopeptidases, such as human collagenase and human ACE (Soubrier *et al.*, 1988).

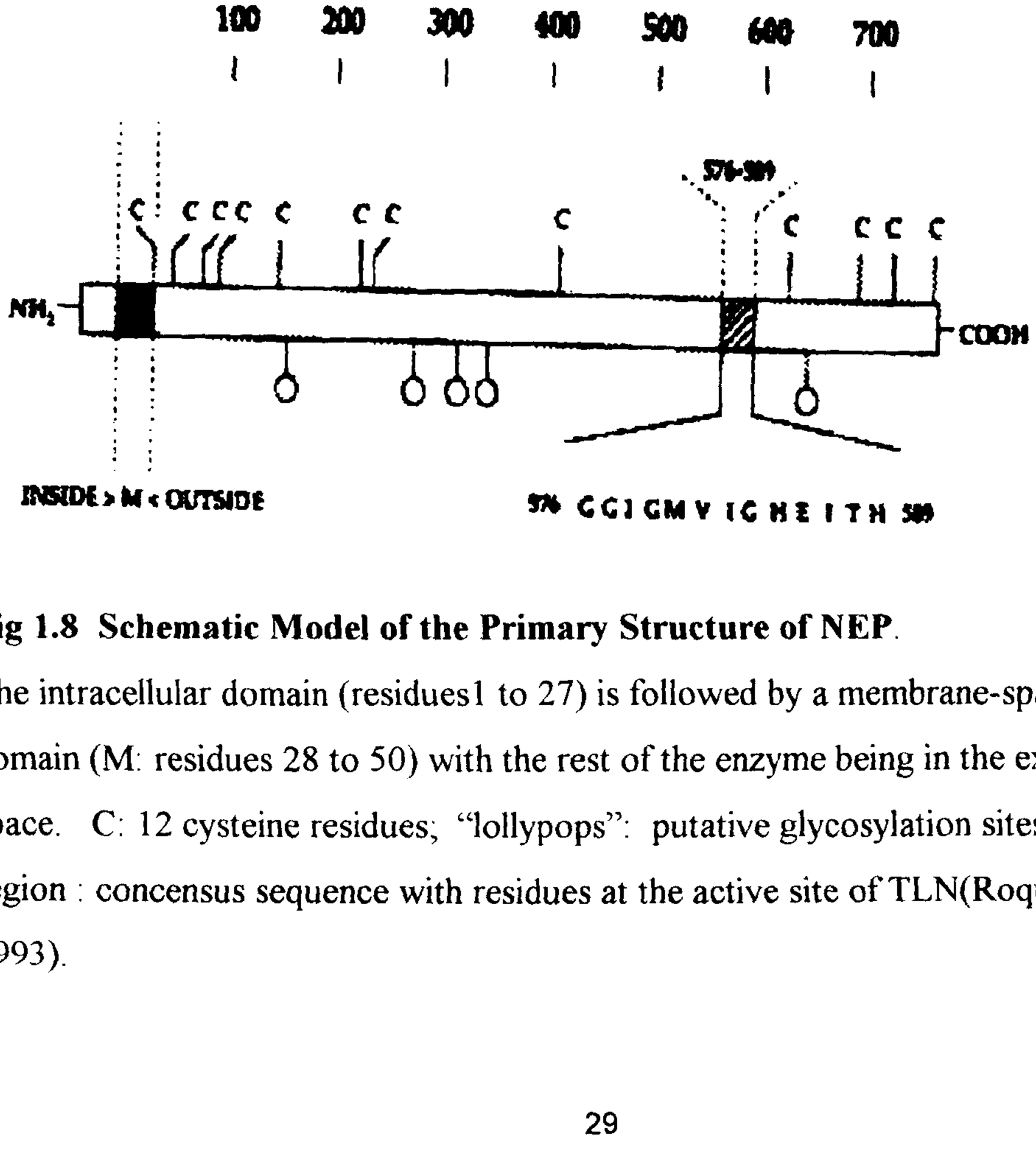



**Fig 1.8 Schematic Model of the Primary Structure of NEP**.

The intracellular domain (residues1 to 27) is followed by a membrane-spanning domain (M: residues 28 to 50) with the rest of the enzyme being in the extracellular space. C: 12 cysteine residues; "lollypops": putative glycosylation sites; hatched region : concensus sequence with residues at the active site of TLN(Roques *et al.*, 1993).

**Figure 1.9 A Schematic Representation of the Binding of the NEP Inhibitor, Retrothiorphan, to the Active Site of the Enzyme, With the Sulphydryl Group of the Inhibitor Pointing Toward the Zn Atom ( Roques *et al*, 1993).**

Site-directed mutagenesis studies have confirmed the proposed roles of $Glu^{584}$ in catalysis (Devault *et al*., 1988b), $His^{583}$, and $His^{587}$, and $Glu^{646}$ in Zn-binding (Devault *et al*., 1988a; Le Moual *et al*., 1991), $Arg^{747}$ (Beaumont *et al*., 1991) and $Arg^{102}$ (Bateman *et al*., 1989; Beaumont *et al*., 1991, 1992) in ligand binding, and $His^{711}$ which may be involved in transition state binding (Bateman *et al*., 1990). $Asn^{542}$ and $Ala^{543}$ are probably involved in substrate binding (Benchetrit *et al*., 1988). All of these residues except $Arg^{102}$ have their homologues in the active site of TLN (Matthew, 1988), and the binding mode of retrothiorphan is taken from data obtained by crystallization of the inhibitor with this enzyme (Roderick *et al*., 1989).

The cDNA clone encoding human NEP in a λgt 10 library was isolated from human placenta by Malfroy *et al* (1988). The complete 742 amino acid sequence of human NEP was obtained. The human enzyme displays a high homology with rat and rabbit NEP. Like the rat and rabbit enzyme, human NEP contains a single N-terminal transmembrane region and its carboxyl-terminus, is located extracellularly. The detailed amino acid sequences of NEP may be found in Appendix. 1.

## Mechanism of Action

NEP usually acts on $NH_2$-amino sites of enkephalin. The enkephalins are degraded by APN, DAP, NEP and ACE. It is found that the inactivation of enkephalin takes place as follows!

Tyr-Gly-Gly-Phe-Leu $\xrightarrow{\text{APN}}$ Tyr + Gly-Gly-Phe-Leu

Gly-Gly-Phe-Leu $\xrightarrow{\text{NEP}}$ Gly-Gly + Phe-Leu

Based the above mechanism, many assays (Roques, 1982; Chipkin, 1986) were established to study the enkephalin-hydrolysing enzymes and their inhibitors.

## Physiological Function of NEP

NEP has many important physiological functions. In brain it appears to participate in the inactivation of enkephalins. The reason for the presence of NEP in the kidney is that it degrades peptides and proteins in the PCT, which are filtered by the glomerulus, such as Atrial Natriuretic Peptides (ANPS) (Soleilhac, 1992; Seymour, 1995). NEP is also seen to have an important role in the inactivation of peptide hormones. Recently NEP (Jane, 1988; Pierart *et al*, 1988) was found to be one of the many proteins used in immunology and leukemia research as lymphocyte markers where it was designated as CALLA (CD10) (Delikat, *et al*., 1994; Letarte *et al*., 1988).

### 1.5.3 Aminopeptidase (APN) and Angiotensin-Converting Enzymes (ACE)

Besides NEP, there exist the metalloenzymes APN (aminopeptidase) and ACE (angiotensin-converting enzyme) which are also enkephalin-hydrolysing enzymes. They take part in the degradation of enkephalin and in that way they mediate the metabolism of the opioid system. Therefore it is necessary to include these enzymes when screening for the inhibitors of enkephalin-hydrolysing enzymes. In this project NEP was chosen as the first target enzyme and the NEP inhibitor was screened. Following this, the positive NEP inhibitors were assayed further for their activity on

APN and ACE.

## 1.6 Inhibitors of Neutral Endopeptidase 24.11 (NEP), Aminopeptidase (APN) and Angiotensin-Converting Enzymes (ACE)

Since the discovery of the peptidases, research on inhibitors commenced. So far thiorphan is the only commercially used NEP inhibitor. The best APN inhibitor is bestatin and ACE inhibitor is captopril (Roques *et al.*, 1993). Until today scientists have obtained selective inhibitors for NEP(Roques *et al.*, 1993) or mixed inhibitors for NEP/APN, NEP/ACE (Roques *et al.*, 1993). Based on today's knowledge the above inhibitors are believed to have following pharmaceutical applications:

**a Selective NEP inhibitors**

Antidiarrhea agents without constipation (Roques, 1993),

Treatment of acute cholecystitis (Wilkins, 1993),

Diuretic and natriuretic agents (Protection of Atrial Natriuretic Peptide, ANP), (Krulan *et al.*, 1993; Seymour, 1995 and Robl *et al.*, 1996; Kenny, 1993)

New antidepressants (Real *et al.*, 1995);

**b Mixed NEP/APN inhibitors**

Analgesics devoid of tolerance and dependence (Protection of endogenous enkephalins) (Chen *et al.*, 1998);

**c Mixed NEP/ACE inhibitors**

New antihypertensive agents (Gros *et al.*, 1991; Roques *et al.*, 1993).

In nature, NEP inhibitors have other special applications besides use as analgesics. So far all of the reported NEP inhibitors were obtained by one of the following three methods: (1) Biosynthesis; (2) Isolation from micro-organisms; (3) Isolation from plants (herbs).

### 1.6.1 Biosynthetic Inhibitors of NEP

All the biosynthetic inhibitors are designed by using enkephalin as a model as shown in **Figure 1.10** (see also **Figure 1.5**). Modification of the inhibitors increases the reaction specificity and lowers the minimal inhibitory concentrations (MIC).

**Figure 1.10 Proposed Model For the Interaction of Enkephalins and Inhibitors With the Active Site of "Enkephalinase---enkephalin hydrolysing enzymes"** ( Schwartz *et al.*, 1982).

## The Classification of Biosynthetic Inhibitors of NEP

All the biosynthetic inhibitors of NEP have the general structure of X-$AA_1$-$AA_2$, where X= the zinc-complexing ligand and $AA_1$, $AA_2$ = different amino acids or amino acid-type structures. $AA_1$ is hydrophobic, uncharged, and can be large since cyclohexyl and biphenyl moieties are acceptable. But $AA_1$ is preferentially small with stereochemistry. Furthermore, a free carboxylic acid seems to enhance potency. Finally the bond between $AA_1$ and $AA_2$ can be a retro-isomer but insertions of extra atoms leads to a decrease in potency. The Zn-complexing ligand, X, can be of several classes. Thus, mercapto, carboxyalkyl, phosphoryl and hydroxamates are all useful. Moreover proximity to $AA_1$ is important because carbonyl insertions here lead to decreases in activity. According to the difference between X/$AA_1$/$AA_2$, the biosynthetic inhibitors can be divided into following four types (Chipkin, 1986).

## (1) Thio Inhibitors of NEP

Extensive studies of enkephalin analogues and dipeptides have shown that the specificity of NEP for targeting the dipeptide is essentially ensured by the $S_1'$ subsite (see **Figure 1.5**), which interacts preferentially with aromatic or large hydrophobic moieties. The $S_2'$ subsite has poor specificity for specific types of amino acid; proline at the $S_2'$ subsite leads to poor inhibition of NEP activity (Fournie-Zaluski *et al.*, 1979, 1981a). The above observations were used to design one class of inhibitor, the thio inhibitors (**Table1.4**)

**Table 1.4 The Thio Inhibitors of NEP**

| Drug | $IC_{50}$ or $K_i$ (nM) vs. | | References |
|---|---|---|---|
| | NEP | ACE | |
| Thiorphan** | | | |
| HS-$CH_2$CH-($CH_2$-ϕ)CO-Gly | 4.7; 1.4 | 147; 480 | Roques *et al.*, 1980 |
| Retro-thiorphan | | | |
| HS-$CH_2$-CH-($CH_2$-ϕ)-NHCO-$CH_2$-COOH | 6 | ≥10,000 | Roques *et al.*, 1983 |
| Modified Phe-Leu derivatives | | | |
| HS-$CH_2$-CH($CH_2$-ϕ)-NHCO-Leu | 10 | 37 | Gordon *et al.*, 1983 |
| Mercaptoacetyl-Leu-Phe | 15 | 1000 | Altstein *et al.*, 1983 |
| Modified butyric acid derivatives | | | |
| HS-$CH_2$-CH($CH_2$-ϕ)-CONH-CH($CO_2$H)-($CH_2$)$_2$$SCH_3$ | 9.1 | - | Bindra US 4329 495 |

** Clinically used inhibitors; $IC_{50}$ and Ki value are constants for inhibition of proteolytic action of NEP and ACE. They are defined in the list of abbreviations.

In **Table1.4** the first described synthetic potent NEP inhibitor is Thiorphan (HS-$CH_2$-CH($CH_2$-ϕ)-CONH-$CH_2$-COOH, N[(R,S) (3-mercapto-2-benzyl-propanoyl)] -glycine (Ki = 4 nM) (Roques *et al.*, 1980). Thiorphan is about 50-fold more potent in inhibiting NEP activity than ACE activity. However, its two enationmers (R and S)

have the same inhibitory potency toward NEP but not for ACE (S-isomer =140 nM; R-isomer = 860 nM), indicating large differences in the stereochemical requirements for optimal interactions in the active sites of the two enzymes.

## (2) Carboxyl Inhibitors of NEP

Introduction of a carboxyalkyl group at the $NH_2$-terminus of the dipeptide Phe-Leu resulted in carboxyl-derived inhibitors of NEP and various N-carboxyl alkyl-dipeptide inhibitors were synthesized. The significant increase in potency obtained with these compounds and the influence of the chirality of the side chains suggested the existence of a $S_1$ subsite that could exhibit some selectivity for the NEP recognition site. However, nonspecific Van der Waals interactions and bidentate coordination of the carboxyl group are more probable explanations for the specificity of the carboxyl inhibitors (Roques *et al.*, 1982a, b; Elliot *et al.*, 1985; Ksander *et al.*, 1995). Three carboxyl NEP inhibitors have so far been extensively studied, and the following compounds were already biosynthesized (Seen in the **Table1.5**).

**Table 1.5 Carboxyalkyl Inhibitors of NEP**

| Drug | $IC_{50}$ or $K_i$ vs | | Refernces |
|---|---|---|---|
| | NEP | ACE | |
| Phe-Leu derivatives | | | |
| HOOCCH($CH_2$-ϕ)CO-Leu | 0.7 μM | >1000 μM | Zaluski *et al.*, 1982 |
| HOOCC$H_2$CH($CH_2$-ϕ)CO-Leu | 0.8 μM | 20 μM | Zaluski *et al.*, 1983 |
| HOOC-$CH_2$-$CH_2$-NH-Phe-Leu | 11 μM | >100 μM | Zaluski *et al.*, |
| Phe-βAla derivatives | | | |
| ϕ-$CH_2$-CH($CO_2$H)-Phe-βAla | 20 nM | >100,000 nM | Mumford *et al.*, 1982 |
| ϕ-$CH_2$-CH($CO_2$H)-Phe-Ala | 300 nM | 1850 nM | Mumford *et al.*, 1982 |
| N-Carboxyphenylethyl derivatives | | | |
| $CO_2$H-CH($CH_2$-ϕ)-Phe-PABA | 71nM | - | Murthy *et al.*, 1984 |

NB: $IC_{50}$ and Ki see Table1.4

### (3) Bidentate Inhibitors (Hydroxamate) of NEP

A detailed analysis of NEP inhibition by $NH_2$-terminal hydroxamates was performed by Bouboutou *et al.* (1984), using a series of four novel dipeptide analogues. This study showed that (a) hydroxamates were more efficient than N-formal-N-hydroxyamino derivatives; (b) a study of the insertion of a methylene spacer between the Zn-chelating group and the benzyl-bearing carbon increased the inhibitory potency for NEP proteolysis of the molecules; and (c) all of the inhibitors have poor affinities for ACE ($IC_{50}$ >10,000 nM). Studies using HACBO-Gly (Fournie-Zaluski *et al.*, 1985; Xie, *et al.*, 1989a, b) have shown that the absolute configuration of the P1' residue (see **Figure 1.5**), as well as the size and the hydrophobicity of the P2' residue, does not greatly influence NEP recognition by the inhibitor. However, these two parameters have played an important role in the design of selective or mixed inhibitors of NEP, APN, and DAP; The retroinversion of the amide bond in dipeptide hydroxamates led to very efficient NEP inhibitors with compounds containing a methylene spacer between the hydroxamate group and the carbon bearing the P1' benzyl side chain (Fournie-Zaluski *et al.*, 1989). The first retro-

HACBO-Gly is a highly potent and selective NEP inhibitor with a concentration of 0.5 nM. An increase in NEP affinity was also obtained by monosubstitution of the P2' residue. Thus, the inhibitor (S, S) HONH-CO-CCH (CH24)-NHCO-CH2-CH (CH3)-COOH (JFH19), is more than 100- and 2000-fold more selective for NEP ($IC_{50}$ approximately 0.15 nM) than for APN and DAP, respectively (Fournie-Zaluski *et al.*, 1989). The replacement of Gly in retro-HACBO-Gly by a highly hydrophobic aromatic moiety in RB104 led to a large increase in NEP affinity. [$^{125}$I]RB104 is more potent than RB101 ($K_D$ = 0.03 nM), a property that has been used directly. But RB104 can not pass through the blood brain barrier.

Hydroxamaic acid derivatives have a general structure: OH-NHCO-X-Ala-Gly, where X=Phe, Leu or Gly. These compounds potently inhibited NEP (seen in the **Table1.6**).

**Table 1.6 Bidentate Inhibitors of NEP**

| Drug | $IC_{50}$ or $K_i$ vs | | References |
|---|---|---|---|
| | NEP | ACE | |
| Ala-Gly derivatives | | | |
| OH-NHCO-Phe-Gly | 8.4 nM | - | Hudigin *et al.*, 1981 |
| OH-NHCO-Leu-Ala-Gly | 3.1 nM | - | Hudigin *et al.*, 1981 |
| OH-NHCO-Gly-Ala-Gly | 41 μM | - | Hudigin *et al.*, 1981 |
| BOC *-Hydroxamates | | | |
| BOC-Leu-NHOH | 40 nM | - | Blumberg *et al.*, 1981 |
| BOC-Phe-NHOH | 200 nM | - | Blumberg *et al.*, 1981 |

* BOC = benzyloxycarbonyl

## (4) Phosphorous-containing Inhibitors of NEP

Another interesting series of inhibitors are the phosphorus-containing dipeptides, among which is the natural competitive inhibitor of NEP, phosphoramidon, produced by Streptomyces tanashiensis; This compound was initially described as a TLN inhibitor (Umezawa, 1972). Phosphoramidon [rhamnosyl-O-P(O)(OH)-Leu-Trp] is a potent NEP inhibitor ($IC_{50}$ = 2-5 nM). Based on the hypothesized similarity of thermolysin and NEP, the phosphorus containing complexing ligands were designed (Kukkola *et al.*, 1995) and various potent phosphorylated inhibitors of NEP have been described (Altstein *et al.*, 1982; Garcialopez *et al.*, 1985). More recently, phosphoramidon derivatives have also been reported (Elliot *et al.*, 1985). The other phosphorus-containing complexing ligands were tried and newer NEP inhibitors were made. The inhibitors are given in **Table 1.7**. However, these compounds, although potentially able to interact with the Sl, S1', and S2' subsites of NEP (see **Figure 1.5**), are less efficient than the phosphyl dipeptide. Nevertheless, this series of potential inhibitors deserves further investigation.

**Table 1.7 Phosphoryl-Based Enkaphalinase Inhibitors**

| Drug | $IC_{50}$ or $K_i$ vs | | References |
|---|---|---|---|
| | Enka'se | ACE | |
| Natural compounds | | | |
| Rhamnosyl-O-P(O)(OH)-Leu-Trp | 1.1 nM | 150 μM | Altstein *et al.*, 1983 |
| Phosphoryl compounds | | | |
| $PO(OH)_2$-Leu-Phe | 0.3 nM | 1 μM | Malfroy *et al.*, 1982 |
| Phosphoamidates | | | |
| $CH_3$-CONH-$CH_2$-$PO_2$-$Na^1$-Phe-Met | 140 nM | 3.4 μM | Elliott *et al.*, 1985 |
| Phosphonic acids | | | |
| $PO(OH)_2$-$CH_2$-CH($CH_2$-ϕ)CO-βAla | 20 nM | >100 μM | Chipkin, 1986 |
| $PO(OH)_2$-CH($CH_2$-ϕ)CO-Leu l | 30 nM | - | Wilkinson *et al.*, EP75334 |

Present attempts for chemists are to find a compound with high affinity both to NEP and APN, but so far this has not been successful (Chen *et al.*, 1998). Roques and his colleagues (1995) obtained one compound (RB101)---N-{(R,S)-2-benzyl-3[(S)(2-amino-4-methylthio) butyl dithio]-1-ox- opropyl}-L-phenylalanine benzyl ester which showed dual inhibitory activity on both NEP and APN. Periodic clinical trials demonstrated that RB101 gave a strong analgesic response without inducing antinociceptive tolerance nor cross-tolerance with morphine. RB101 is also active in the tail-flick and tail-electric stimulation tests in rats. The pain-alleviating effect of RB101 was suppressed by naloxone, RB101 therefore is the first compound allowing analgesia to be critically evaluated, and the side effects assessed for treatments which elevate the extracellular level of enkephalins as opposed to analgesia produced by morphine. RB101 easily crossed the blood-barrier and so the above properties made RB101 become interesting for treating pain. Unfortunately, it is only slightly active after oral administration. Based on above facts Chen *et al.* (1998) designed dual competitive inhibitors of both enzymes with Ki values in the nanomolar range. These have been obtained by selecting R-1, R-2, R-3 determinants in aminophosphophilic-containing inhibitors: NH2-CH(R-1) P(O)-(OH)CH2-CH(R-2) CONH-CH(R-3)COOH. For optimal recognition of the two enkephalin inactivating enzymes, active site peculiarities being determined by site-directed mutagenesis, have been taken into account. The best inhibitors were ten times more potent than dual inhibitors described before in alleviating acute and inflammatory nociceptive stimuli in mice, thus providing a basis for the development of a family of analgesics devoid of opioid side effects. Nobel *et al.* (1997) also chemically modified RB101 and a new compound--RB120 was obtained. RB120 has already passed through all the pre-clinical trials and showed excellent analgesic activity without major side effects. It still awaits clinical trial.

### 1.6.2 Inhibitors of Peptidases From Micro-Organisms (See Table 1.8)

In addition to the biosynthetic compounds described above, inhibitors of enkephalin-hydrolysing enzymes can also be isolated either from micro-organisms or from plants as well as animals and insects. The Japanese have done lot of work in screening new

NEP inhibitors from Streptomyces (Kojima, 1990; Otani, 1991; Tsurumi, 1995) and other micro-organisms (Kimura, 1990; Tsuru *et al.*, 1992; Akiyama, *et al.*, 1998).

So far they have made significant progress but without marketable products. The inhibitors isolated from micro-organisms are summarised as given in **Table 1.8**, but the work has not really yet been effective.

**Table 1.8 Inhibitors of Enkephalin-hydrolysing Enzymes From Micro-organisms**

| Inhibitors | Producing strains | Activity |
|---|---|---|
| Staurosporine | Steptomyces staurosporeus | Anti-fungi, anti-amnesic effect |
| WS75624 A and B | Saccharothrix sp. No. 75624 | Inhibition of NEP, ECE (Endothelin-converting enzyme) and collagenase |
| Metalloprotease inhibitor (SMPI) | Streptomyces nigrescens (SMPI) | Inhibition NEP isolated from Bacillus subtilis var. amylosacchriticus |
| Streptomyces subtilisin inhibitor (SSI) | | Inhibition of trypsin activity |
| Fluostatins A and B | Streptomyces sp. TA-3391 | Inhibition of DAP (dipeptidyl peptidase III, EC 3.4.14.4) |

## 1.7 Research Advances of Chinese Herbs For Use As Analgesics

### 1.7.1 Introduction

Herbal medicine, acupuncture, moxibustion, and massage are the four major constituent parts of traditional Chinese medicine (TCM). Although acupuncture is well known in many western countries (Jarret, 1995; Sher, 1998), Chinese herbal medicine, is less well known in the west (Zhu *et al.*, 1995; Berry and Tinorua, 1993). However in recent years it has become increasingly popular with its application in treating dermatological diseases such as psoriasis (Koo and Arain, 1998), atopic dermatitis (Rustin *et al.*, 1994; Li, 1995), and atopic eczema (Galloway *et al.*, 1991; Atherton *et al.*, 1990; Allen *et al.*, Hollman *et al.*, Harper *et al.*, Davies *et al.*, 1990; Latchman *et al.*, 1994, 1996; Kirby, 1997). Some biotechnology companies are beginning to explore the potential of ancient remedies such as Chinese herbs in the hunt for sources of new therapies (Mack, 1997). However, systematic study of Chinese herbs in the western world still has a long way to go.

In China systematic study of Chinese herbs has been made for about twenty years (Yang, 1998). Most of the work has concentrated on isolating single compounds from herbal crude extracts. Less work has been done on pharmacolo-gical applications and many compounds have been purified without assaying their pharmacological effects. Even less work has been done on the molecular pharmacology of Chinese herbal extracts and so it is now of great interest to study the molecular pharmacological activity of the traditionally-used Chinese herbs especially for herbs which have already been well characterised. According to traditional Chinese-herbal medicine (TCHM), quite lot of Chinese herbs are found to be useful as: analgesics (Yang, 1985, 1986; Sher, 1998), treatments with diuretic and antidepressant activity (Chan, 1995; Li, *et al.*, 1994), treatments of nephrotic syndrome (Jiang, 1994) and treatments of neurological disorders (Ding and He, 1986). Such functions suggest that it should be possible to find some inhibitors of NEP, NEP/APN or NEP/ACE from Chinese herbs.

### 1.7.2 The Use of Chinese Herbs as Analgesics and Anaesthetics

The following is a list of Chinese herbs currently used as analgesics or anaesthetics in clinics;

Aconitum carmichaeli Debx; Aconitum brachypodum Diels; Aconitum szechenyianum Gay; Daphne giraldii Nitsche; Rhododendron molle (Bl) G. Don; Hyoscyamus niger; Datura metel L.; Corydalis decumbens (thumb) Pers; Alangium chinese (Lour) Rehd; Zanthoxylum nitidum (Roxb) Dc; Cynanchum paniculatum (Bunge) K. Schum; Hemsleya amabilis Diels; Uncaria rhynchophylla (Miq.) Jackson; Notopterygium incisum Ting; Ligusticum chuanxiong Hort; Corydalis bulbosa De; Corydalis Yahusuo T. Wang.

Many of these herbs have already been used as analgesics for thousands of years. And indeed the best clinically-used analgesic, morphine, originated from herbs. On the other hand, Chinese scientists have done much work in the last 20 years on screening new analgesics free of major side effects of morphine. Yang and his colleagues (1985) have successfully investigated the effects of three hundred cases of Chinese herbs on drug addiction *in vivo*. They used whole-animal tests to investigate the analgesic activity of twenty herbs, combined with data from treating opium and heroin addicts in Hong Kong. The results of three hundred cases were analysed and evaluated. The effects of Chinese herbs on withdrawal behaviour in morphine-addicted rats were also investigated. Four herbs (Qiang huo, Gou teng, Chuan xiong, Yan husuo) were found to reduce the three main withdrawal symptoms significantly. The effect of the above four herbs in combination on the morphine-withdrawal signs were also compared with that of endogenous opiate-like peptides (endorphin, enkephalin, dynorphin) and acupuncture. The analgesic effects of the above herbs were also studied in mice where in the writhing test (a standard analgesic experiment—see section of 1.2) they caused a reduction of the writhing sign. Tests on the contraction of guinea pig ileum showed that the inhibition of the enzymes caused by the herbs was not blocked by naloxone. This suggested that none of the above herbs contained morphine-like substances showing that the analgesic activity was by a different mechanism. Based on the study of Yang *et al* (1985), it was decided in this thesis to investigate the effects of the above four listed herbs on

enkephalin-hydrolysing enzymes (NEP, APN and ACE), so that the molecular mechanism for the analgesic activity of the above four herbs might be clarified and the active ingredients identified.

### 1.7.3 Brief Introduction For the Selected Four Chinese Herbs

The four selected Chinese herbs Yan, Gou, Qiang and Chuan have been clinically used as analgesics according to traditional Chinese herbal medicine (TCHM). Keys (1975) described the above four Chinese herbs in his book as follows and being quoted.

Descriptions of the crude drugs, as used in commence, are available in the Chinese Pharmacopoeia, and in each case reference is made to the relevant monograph. The crude drugs were checked for authenticity against these monographs by staff at the Institute of Medicines Plant Development, Chinese Academy of Science, Beijing, China.

**(1) <u>Yan hu suo (yan)</u>---the latin name is <u>CORYDALIS AMBIGUA Cham. et Schlecht. (Fumariaceae)</u> or <u>CORYDALIS YANHUSUO T. Wang</u> .** (Keys, 1975)

It is an herbaceous perennial, the stem erect, tender, 20 cm. high. Leaves alternate, compound, the last leaflet larger than the others and tridentate at the tip. Inflorescence a multifloral cluster; April-May. Sepals 2; Corolla irregular, with 4 erect, connivent petals, one of which is spurred, greenish violet; stamens 6; ovary 2-celled. Fruit an oblong, linear capsule. Siberia. Manchuria, Japan.

The root is officinal. It occurs as a small, flat tuber 17 cm. thick by 20 mm. in diameter, hard, ochre-yellow, the exterior covered with a thin, wrinkled cuticle, the interior light yellow, semi-transparent, cirrose. The taste is bitter. A large number of alkaloids have been isolated from the tube. It was prescibed as sedative, antispasmodic, analgesic in headache, gastralgia, menstrual colic. Dose 3-5 gm."

(2) "<u>Gou teng</u>---**the latin name is NAUCLEA SINENSIS Oliv. (Rubiaceae) or UNCARIA RHYNCHOPHYLLINE (MIQ.) JACKSON.** (Keys, 1975)

Rubiaceae is a shrub, the branches small, bearing compressed, curved spines occasionally. Leaves oval-elliptical, tip acute, base rounded, 10-14 cm. long by 5.0-7.5 cm. Wide. Inflorescence an axillary capitulum. Flowers white; Calyx in 5 segments; corolla infundibular, with 5 lobes which are much smaller than the tube; stamens 5; ovary 2 celled. Fruit a dry capsule. central China.

The stem and spines are officinal. The taste is bitter and astrigent. The drug contains the alkaloid rhynchophylline ($C_{22}H_{28}N_2O_4$; crystal; m.p. 216°; freely soluble in organic solvents except petroleum ether). Rhynchophylline lowers blood pressure and paralyzes sympathetic nerve endings.

Employed as sedative, antispasmodic in infantile nervous disorders. dose, 5-10 gm."

(3) **"Qiang huo--the latin name is NOTOPTERYGIUM INCISUM Ting ex H. T. Chang** (Yang *et al.*, 1993)

Root bitter, very fragrant. 1-25 essential oil. emmenagogue,

About 15 compounds were obtained. They are isoimperatorin, bergapten, cnidinin, notopterol, notoptol, nodakenin, bergaptol, decuroside etc.

Sedative, analgesic. dose, 4-11gm."

**(4) "Chuan xiong ---the latin name is LIGUSTICUM CHUANXIONG Hort.**

(Tsao *et al.*, 1983; He and Zhang, 1957)

Herbaceous, high 40-70 cm. Fragrant root. About 20 compounds were obtained from chuan xiong. They are 3-sec-butyl-6-isopropyl-2,5-piperazinedione; perlolyrine; chuanxingol; ferulic acid; chrysophannol ; sedanic acid etc. Sedative, analgesic. be effective in treating nephropathy, antihypertension."

### 1.7.4 Extraction of Active Ingredients From Chinese Herbs and Traditional Method of Studying Pharmacological Activity

The isolation and structure determination of pharmacologically active substances from Chinese herbs may be followed by determination of their pharmacological profile.

Standardising herbal extracts is problematical because the properties of herbal extracts depend on the season and location of growth and the herbal extraction method. Usually the herbal extracts contain quite a few components which may interact in their medicinal effects. To combat this, scientists have adopted the following strategies to study Chinese herbs (Chan, 1995; Zhu *et al*, 1995).

(1) Selection of the herbal prescriptions which have been used clinically to treat the target disease;
(2) Identification of target herb and establishment of all the decoction methods;
(3) The extraction of the active ingredients from crude drugs and clarification of their pharmacological activity.

## 1.8 Overall Objectives of The Thesis Work

The overall objectives of the work involved in the project of this study were as follows:

### (1) Design and utilization of an enkephalin-hydrolysing enzyme assay to screen NEP inhibitors from Chinese herbs

Although some laboratories (Nortier *et al.*, 1997; Kabanda, 1995) have reported use of NEP assay to monitor the proximal tubular injury induced by Chinese herbs *in vivo*, so far there are no reports of work to screen NEP inhibitors from Chinese herbs. Therefore, we believe that this laboratory is the first to use NEP assay as a primary model to screen selective NEP inhibitors from Chinese herbs. In this project,

the $^{3}$H(leu)-enkephalin was used as the competitive substrate to establish an NEP assay.

**(2) Design and utilization of opioid receptor binding assay to investigate the effect of target herbs on naloxone - opioid receptor reaction**

**(3) Partially purification of selected herbal extracts**

# CHAPTER II

# METHODS

General methods, such as enzyme preparation, enzyme assays, enzyme purification etc. are introduced in this chapter. However specific methods which apply to the contents of a particular chapter are introduced with that related chapter. Therefore, in particular, nuclease treatment of herbs is described in section 6.4 of Chapter 6 and buffer treatments of herbs are dealt with in section 4.5. The preparation of opiate binding receptors from rat-brain membrane is described in section 5.2.1, the experimental methods of opiate binding is described in section 5.2.2, the effect of Gou on ligand-receptor binding activity is described in section 5.4 .

## 2.1 Methods for the Extraction of Chinese Herbs

All the Chinese herbs studied in this project were either supplied by East & West Herbs LTD (in Kingham, Oxfordshire, U.K.) as powders, or dried pieces of the plants. In the case where whole pieces of plant were supplied, the plant was ground into a coarse powder by a pestle and mortar. For studies of the different effects of Chinese herbs it was necessary to solubilise or extract the active ingredients from the Chinese herbs by water extraction or by organic-solvent extraction. Before use for research all the Chinese herbs were identified by the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing, China.

### (a) Water Extraction

"Fire-water" extraction involves boiling the Chinese herbs in water to extract the active components. 3 grams of herbs were mixed with 100 ml of distilled water and this suspension was boiled for 3 hours to aid solubilisation. The extract was then filtred and the filtrate was concentrated to dryness in a microcentrifuge concentrator (HOWE GYRO VAP) and the dried extract was kept at 4°C for enzyme assay and opioid receptor-binding assay.

### (b) Organic Solvent Extraction

An amount of 100 mg of Chinese herbs was mixed with 10 ml of an organic solvent (methanol in this experiment). The mixture was centrifuged at 1000 g for 5 minutes

to remove any undissolved particles from solution. The supernatant was concentrated to dryness by the centrifugal concentrator (HOWE GYRO VAP) and the dried extract was kept at 4°C for further study.

## 2.2 Characterisation of the Chinese Herbs

### 2.2.1 Spectral Analyses of Gou Crude Extract

Dried extracts of herbs (Gou etc.) were dissolved in 50 mM of Tris-HCl buffer at pH 7.0 until the final concentration was 100 μg/ml. Tris-HCl buffer was used as a control and the absorption spectrum was measured by scanning between 200 nm and 900 nm.

### 2.2.2 The Fractionation of Gou By Sephadex G-10 Gel Filtration

Absolute ethanol was mixed with the Gou aqueous solution in the ratio of 1:1 to a final concentration of 50% ethanol. The solution was kept at room temperature for 10 minutes and centrifuged at 13, 000 rpm (Microcentaur, MSE) for 20 minutes. The pellet and the supernatant were separated and stored in the cold room for further study.

The prepared Sephadex G-10 column (NAP-10, length: 10 cm, diameter: 1 cm) was washed and the void volume was measured by means of blue dextran. The maximum loading volume was calculated and the 0.25 ml of Gou crude sample was applied onto the column and washed by 0.5 M Sodium Chloride. The elution fractions (200 μl / tube) were collected and their activities assayed.

### 2.2.3 Reversed-phase Preparative HPLC Analysis of Gou Crude Extracts

(1) The mobile phase was prepared for reversed-phase preparative HPLC analyses as follows: 500 ml of a solution of 0.07% trifluroacetic acid (TFA) in 100% acetonitrile was prepared and also a solution in distilled water of 0.07% of TFA.

(2) The sample preparation and running on the HPLC column (S5 $ODS_2$ , $C_{18}$ column) was done by taking a Gou solution at a concentration of 1 mg/ml and filtering it. The solution (50 µl) was then loaded onto the HPLC column to analyse the components of Gou. The flow rate was 1.0 ml/min. The gradient was 0 % to 100% acetonitrile over 60 minutes.

## 2.3 Enzyme Preparation, Purification and Characterisation

Ethical approval was obtained to use animals in this study.

### 2.3.1 Preparation of NEP Samples From a Rat Kidney Crude Microsomal Fraction of NEP

This preparation was essentially that of Booth and Kenny (1974). Twelve wistar rats were killed by the inhalation method ($CO_2$ for 5-10 minutes) and their kidneys were removed rapidly and chilled. The adipose tissue was removed and the kidneys were washed with 0.3 M sucrose, chopped finely, and filtered with cleaned gauze. The pellet left in the gauze was collected, dried and weighed.

The pellet was resuspended in 0.3 M sucrose (in the ratio of 1 gram to 9 mls) and homogenised until a fine suspension was obtained. This was centrifuged at 8000 g for 15 minutes at 4°C (Beckman Centaur JA10), the pellet was discarded and the supernatant was centrifuged at 26000 g for 2 hours at 4°C (Beckman Centaur JA20); The supernatant was removed and the pellet resuspended in one third of the homogenised initial volume of 50 mM Tris-HCl, pH 7.4 with 1% of Triton-X-100; This was then stirred at room temperature for 60 minutes before being centrifuged at 26000g for 90 minutes at 4°C. The supernatant, or "solubilised microsomal fraction" was retained and subsequently stored at 4°C.

### 2.3.2 Protein Determination of the Rat-Kidney Crude-Microsomal Fraction

Protein determination was carried out by using BSA (Bovine Serum Albumin) as the standard for the Lowry's assay and the HA (Human Albumin) for the Bradford

assay. The details of this are described in the section 3.4 (Bradford, 1976; Lowry *et al.*, 1951).

### 2.3.3 Bio-Bead Column Preparation

Bio-bead column preparation (SM-Z, BIO-RAD, Absorbent 100-200 meshes) was carried out following Vogel and Altsein (1977) with slight modification. A small amount of glass wool was pushed tightly into the necks of 12 pasteur pipettes, 12×80 mg of Bio-beads in 50 ml of ethanol was washed for 60 minutes then distributed equally between the pipettes, the Bio-beads were then washed with 10×1 ml of distilled water and were ready for use. After each use the column was washed with 2×1 ml of ethanol and 10×1 ml of distilled water.

### 2.3.4 Measurement of the Efficiency of $^{3}$H(leu)-enkephalin Binding to Bio-beads

20 µl of 5 nM $^{3}$H(leu)-enkephalin was loaded at the top of prepared columns of Bio-beads. The columns were then eluted twice with 1 ml of distilled water. Substances bound to the column were removed by eluting twice, each time with 1 ml of absolute ethanol (this was more hydrophobic than water and thus it removed substances bound to the Bio-beads). A volume of 1 ml of eluent was collected each time (for the two distilled water elutions and for the two alcohol elutions) directly into scintillation vials containing 4 ml of Ecoscint H. Scintillation counts were taken of the four 1 ml samples. Two separate 20 µl aliquots of 5 nM of $^{3}$H(leu)-enkephalin were also counted to act as controls.

### 2.3.5 Assay for $^{3}$H(leu)-enkephalin Hydrolysis of NEP

The assay for $^{3}$H(leu)-enkephalin hydrolysis was carried out as described by as Malfroy *et al* (1978) and Roques (1980), with some modifications:

The following mixture was prepared and incubated at 37°C for 30 minutes:

40 µl of diluted crude microsomal sample or eluate

or 50 mM of Tris-HCl buffer, pH 7.4;

20 µl of 200 nM non-isotopic (leu)-enkephalin;

20 µl of 5 nM $^{3}$H(leu)-enkephalin;

20 µl of 10 mM Bestatin (APN inhibitor);

20 µl of 10 µM Captopril (ACE inhibitor);

80 µl of 50 mM Tris-HCl at pH 7.4;

Final volume was 200 µl.

A volume of 50 µl of 0.1 M HCl was added immediately and the solutions were put into a 95°C water bath for 5 minutes to denature the protein and then centrifuged at 13000 rpm for 20 minutes (Microcentaur, MSE). 200 µl of supernatant was applied to the washed Bio-bead columns and eluted with 2×1 ml of distilled water. This was followed by 2×1 ml of absolute ethanol. 1 ml of the eluted solution was collected directly into scintillation vials with 4 ml of Ecoscint H before being counted.

### 2.3.6 The Purification And Identification of Enzymes By Hydrophobic-Interaction Chromatography (HIC) on Phenyl Agarose

The following method is according to Almenoff and Orlowski (1983).

The additional method of HIC purification involves the use of hydrophobic interactions in high salt. The binding buffer was 2 M ammonium sulphate in 20 mM potassium phosphate buffer, pH 7.0.

The column was pre-equilibrated with binding buffer at a flow rate of 0.5 ml/minute and 5 ml of kidney microsomal protein was loaded and run into the column. The protein was allowed to bind for 30 minutes.

The unbound protein was removed from the column by elution with binding buffer at 0.5 ml/min; 1 ml fractions were collected and all the unbound protein was removed (monitored at 280 nm).

A decreasing salt gradient was then applied to the column using a gradient former. The gradient was from 20 mM potassium phosphate pH 7.0 + 2.0 M $(NH_4)SO_4$ to 20 mM potassium phosphate buffer pH 7.0.

Following this gradient, 20 mM potassium phosphate buffer was run through until all the bound protein was eluted from the column.

1 ml fractions were collected through-out and their absorbance at $A_{280}$ nm taken to plot an elution profile (shown in Chapter 3).

### 2.3.7 Use of Polyacrylamide Gel Electrophoresis (PAGE) to Identify NEP

Based on the elution profile protein estimation was performed on selected peak and plateau fractions by the Bradford method and samples were run on SDS-PAGE to evaluate the protein content of the elution peaks.

The detailed method used is a variation on that used by Laemmli (1970)

Glass plates for electrophoresis was washed in detergent, rinsed in distilled water, sprayed in 95% ethanol and dried with a paper towel. For silver staining the plates should be soaked in nitric acid before use.

30.0 g of acrylamide was dissolved in water, the volume adjusted to 100 ml, and 5.0 g of decolourising charcoal added. This was left at room temperature for 2 hours. The solution was filtered and stored in a brown bottle. 1.0 g of methylene bisacrylamide was dissolved in water and the volume adjusted to 100 ml. This was stored at 4°C. 1.5 M of Tris-HCl pH 8.8, 1.0 M of Tris-HCl pH 6.8 and 10% SDS were prepared; Together with freshly prepared 10% ammonium persulphate (APS), an electrode buffer was prepared containing 28.8 g of glycine, 6.06 g of Tris base and 1.0 g of SDS, make up to 1000 ml with distilled water.

The sample buffer contained 0.77 g of dithiothreitol, 1.0 g of SDS, 4.0 ml of 1.0 M

Tris-HCl pH 6.8, 5.0 ml of glycerol and 0.2% bromophenol blue, adjusted to 50 ml with distilled water.

Solution 'A'which contained 16.66 ml of 30% acrylamide, 13.33 ml of 1.0 % methylene bis-acrylamide and 20.0 ml of distilled water. Solution 'B' which contained 12.5 ml of 1.0 M Tris-HCl pH 6.8, 1.0 ml of 10% SDS and 36.5 ml of distilled water. The resolving gel is prepared, as shown in **Table 2.1**

**Table2.1 Volume of Reagents for SDS PAGE Resolving Gel**

| | Percentage gel | | | | |
|---|---|---|---|---|---|
| | 5.0% | 10.0% | 12.5% | 17.5% | 20.0% |
| 30% acrylamide | 5.0 | 10.0 | 12.5 | 17.5 | 20.0 |
| 1% bisacrylamide | 7.8 | 3.9 | 3.1 | 2.2 | 2.0 |
| 1.5 M Tris pH 8.8 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 |
| 10% SDS | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Water | 9.2 | 8.1 | 6.4 | 2.3 | 0.0 |
| TEMED ( μl ) | 20.0 | 20.0 | 20.0 | 20.0 | 20.0 |
| 10% APS ( μl ) | 200 | 200 | 200 | 200 | 200 |

The stacking gel has been prepared by mixing 5.0 ml of solution 'A' , 5.0 ml of solution 'B', 0.01 ml of 10% APS and 10.0 μl of TEMED. The gel was run at 25.0 mA constant current, negative to positive.

The gel was stained in 0.03% Coomasie Brilliant Blue R-250, 45% methanol and 10% glacial acetic acid, destain in 25% methanol and 10% glacial acetic acid. The procedure of Silver staining was shown in Appendix 2.

## 2.4 Inhibition Experiments of Herbs on Enkephalin-hydrolysing Enzymes

A defined volume (0-80 μl) of herbal extracts or controls was mixed as follows to a final volume of 160 μl:

40 μl of rat kidney crude microsomal sample (1:20);

20 μl of 10 mM Bestatin;

20 μl of 10 μM Captopril;

80 μl of 50 mM Tris-HCl, pH 7.4;

The mixture was incubated at room temperature for 15 minutes and

20 μl of 200 nM non-isotopic (Leu)-enkephalin;

20 μl of 5 nM $^{3}$H(leu)-enkephalin;

were added. The solution was incubated at 37°C for a further 30 minutes. The enzyme was assayed as in section of 2.3.5. to determine the inhibition effects of the Chinese herbs on the enkephalin-hydrolysing enzymes.

## 2.5 Statistical Analyses

In this report the results of statistical calculations are presented as mean values ± standard deviations from at least three experiments. The data were analysed with an unpaired two-tailed Student's t-test. The statistical differences were considered significant according to detailed situation. P stands for "probability". The detailed principle and related information is given in the Appendix. 3.

# CHAPTER III

# THE DESIGN AND ESTABLISHMENT OF THE SCREENING MODEL

## Preparation And Partial Purification of NEP

# INTRODUCTION

## 3.1 Screening model

The first step for screening bioactive molecules is to design a efficient screening *in vitro* model such as enzyme assay. As discussed in Chapter 1, NEP has been implicated in pathways of analgesia, NEP has a broad substrate specificity and is a widely distributed enzyme (Gee *et al.*, 1985). In the nervous system, the enzyme has been implicated in the metabolism of the enkephalins which are hydrolysed at the $Gly^3$-$Phe^4$ bond with the release of Tyr-Gly-Gly. Because of its broad substrate specificity, the enzyme may also participate in the degradation of tachykinins (e.g. Substance P), neurotensin, cholecystokinin and other neuropeptides. One $Zn^{2+}$ is bound at the active site of the enzyme which is essential for its catalytic activity.

NEP hydrolyses peptide bonds involving the amino groups of hydrophobic residues, (X-Ywhere Y is Phe, Leu, Ile, Ala, Val, Tyr, or Trp). Recently (Hersh and Morihara, 1986), the optimum substrate sequence for NEP has been elucidated as,

| $P_3$ | $P_2$ | $P_1$ | $P'_1$ | $P'_2$ | $P'_3$ |
|---|---|---|---|---|---|
| Phe | Phe | Ala | Phe | Leu | Ala |
| Ala | | Gly | Leu | | |

Several specific inhibitors of the enzyme exist, of which the most widely used are Phosphoramidon and Thiorphan (For review see Turner *et al.*, 1987). The effective concentration of both inhibitors, where controls containing them are suggested, is 1 μM. In this thesis the Thiorphan was used as inhibitors of NEP, Bestatin was used to be inhibitor of APN and Captopril as that of ACE.

## 3.2 The Principle of the Assay Used for NEP

Three different kinds of NEP assay have been established in recent years and were widely used for different purposes.

They are (1) Radiometric assay; (2) HPLC assay; (3) Fluorimetric assays. Compared with the other two methods, the radiometric assay allowed the assay of

more samples at one time and was suited for large-scale screening of new medicines. Therefore in this study the radiometric assay was adopted to investigate the inhibitory effects of the selected four Chinese herbs on NEP and then on APN/ACE.

Radiometric Assay ( The actual method used is fully described in section 2.3. The discussion here relates to the principles behind this method only.)

The basic principle of NEP/APN/ACE degradation of the above substrate can be found in Chapter 1. The initial assays of enkephalin degradation employed radiometric methods and was reported by Malfroy (1978) and Vogel and Altstein (1977). Both thin layer chromatography (TLC) and polystyrene beads have been used to separate quantitatively intact enkephalins from the N-terminal tripeptide produced on incubation with NEP.

In the normal method used, the incubation mixture in Eppendorf tubes consists of [$^3$H-Leu$^5$]-enkephalin (10 nM final concentration), the enzyme source (50 µl) and buffer (50 mM Tris-HCl, pH 7.4) made up to a final volume of 100 µ1. After 30-60 minutes at 37°C the reaction is stopped by the addition of 25 µ1 of 0.2 M HC1.

In the thin layer chromatography method, aliquots of the acidified incubation mixture (40-100 µ1) are spotted on to silica-gel plates or sheets along with marker peptides. The chromatogram is developed with acetic acid: ethyl acetate: butanol: H20 (1:2:1:1) and then stained with ninhydrin to reveal the marker peptides (e.g. Tyr-Gly-Gly). The sample spots are scraped off or cut out and their radioactivity determined by liquid-scintillation (Harmar, 1989).

Another method of analysing the breakdown of enkephalin is based on the observation that in aqueous solutions the pentapeptides are quantitatively retained on polystyrene beads (Vogel and Altstein, 1977) but the radiolabelled reaction products (e.g. Tyr, Tyr-Gly, Tyr-Gly-Gly) are not. Aliquots of the acidified incubation mixture (75µl) are transferred to pasteur pippettes containing 60 mg polystyrene beads, previously washed with 6x2 ml ethanol and 10x2 ml $H_2O$. The [$^3$H]Tyr-Gly-Gly is directly eluted into scintillation vials with 2x2 ml $H_2O$, and the radioactivity

determined by liquid scintillation (Llorens *et al.*, 1982). The columns may be washed and stored in ethanol, and re-used several times.

[$^3$H-Leu$^5$]enkephalin is also a substrate for Aminopeptidase S (APS), dipeptidyl peptidases and peptidyl dipeptidases, and even Angiotensin-converting enzymes (ACE). To prevent the production of [$^3$H]Tyr, which is also not absorbed to Bio-beads, Puromycin (100 μM) or Bestatin (100 μM) or Amastatin (10 μM) are included as APS inhibitors and Captopril (10 μM) is used as an ACE inhibitor. A more selective susbtrate is [Tyrosyl-3,5-$^3$H-DpAla$^2$, Leu$^5$]enkephalin, which has been synthesized but is not available commercially (Llorens *et al.*, 1982).

The radiometric methods are generally less satisfactory than fluorimetric or HPLC assays, since substrate concentrations substantially below $K_m$ are employed. The methodology also led to anomalously low $K_m$ values for enkephalin hydrolysis (Malfroy *et al.*, 1978). However, the high sensitivity and operation scale means that the radiometric assay still has advantages for certain applications, for example, being used as a primary screening model to screen for novel medicines from natural resources.

## 3.3 The Preparation and Purification of NEP Samples

### 3.3.1 The Preparation of NEP Samples

The preparation procedure for this enkephalin-hydrolysing enzyme sample was as described in section 2.3.1 of chapter 2. In chapter 1 it was also shown that the NEP is widely distributed in many kinds of tissues but most concentrated in the kidney. This is the reason that the rat kidney was used to prepare enkephalin-hydrolysing enzyme samples in this study.

### 3.3.2 Use of Hydrophobic-Interaction Chromatography (HIC) to Purify The NEP

Hydrophobic-interaction-affinity chromatography relies on the fact that interaction occurs when the hydrophobic sites of proteins undergo differing degrees of hydrophobic interaction with an uncharged matrix containing hydrophobic groups. This method can provide a general systematic approach to the purification of both water-soluble and lipophilic proteins. In this thesis, hydrophobic phenyl-agarose was used to achieve resolution and purification of NEP from other enzymes. This method was according to Almenoff and Orlowski (1983). The detailed procedure is dealt in section 2.3.6 of chapter 2.

### 3.3.3 Use of Polyacrylamide Gel Electrophoresis (PAGE) to Identify NEP

At any pH other than at their isoelectric point, proteins carry a net electrical charge. If the charged proteins are placed in an electrical field they will migrate at a rate dependent on their charge density, i.e. the ratio of their charge to their molecular mass. Electrophoresis uses a semi-rigid porous matrix through which molecular sieving can occur, and so two molecules of similar charge density may be separated by a difference in their size. The extent of the molecular sieving is dependent on the pore size of the matrix, this can be reproducibly selected for the range of molecular weights under consideration (Hames and Rickwood, 1983).

Polyacrylamide gels have a working range of concentrations from 2.5% to 30%. As the concentration increases the effective pore size decreases. Pore size is varied by adjusting the proportion of cross-linker present in the gel, this is generally methylene bis-acrylamide. Pore size decreases as the concentration of methylene bis-acrylamide increases, reaching a minimum when the cross-linker reaches about 5% of the total acrylamide monomer present (Laemmli, 1970).

Polymerisation of the gel requires an activator in the form of free radicals, these are usually obtained from ammonium persulphate, but riboflavin can be used. Light causes the decomposition of riboflavin into the free radicals necessary to initiate poly-

merisation. The polymerisation process can be accelerated by the addition of a catalyst. This is usually N, N, N′, N′-tetramethyl ethylenediamine (TEMED), which catalyses the formation of the free radicals from ammonium persulphate which initiate polymerisation (Hames and Rickwood, 1983).

Polyacrylamide gels were prepared immediately before use from an acrylamide monomer ($CH_2$=CHCONH$_2$) copolymerised with a cross linking agent, N, N-methylenebisacrylmide [$CH_2$(NHCOCH=$CH_2$)2] in the presence of an initiator (ammonium persulphate) and a catalyst N, N, N', N', -tetramethyl ethylenediamine (TEMED). Polyacrylamide gel electrophoresis (PAGE) is usually carried out as slab gels sandwiched between glass plates. The gap between the plates is usually between 0.5 and 3.0 mm. A major advantage of this system is that several samples and standards can be run at the same time, therefore the results are comparable. For a higher degree of separation between proteins of similar molecular weight and charge density, systems such as Triton-acid-urea or two-dimensional gels ( Davie, 1982 ) may be run.

The majority of the buffering systems used are denaturing in nature and are designed to dissociate protein complexes into their constituent subunits. They are designed to give the proteins a similar overall charge so that they will be separated according to molecular weight. Exceptions to this do occur. Because of the basicity of histones, the overall negative charge induced by sodium dodecyl sulphate (SDS) is reduced so that the histones migrate at a slower rate, when compared with molecular weight markers, than their molecular weight would suggest.

In this study the SDS-PAGE was used to identify the NEP band of the pooled fractions. Both Coomassie brilliant blue and Silver were compared for staining the gel. The detailed procedure for SDS-PAGE was shown in section 2.3.7. and silver staining was given in Appendix 2.

# RESULTS AND DISCUSSION

## 3.4 Assay of Protein Concentration in the Rat Kidney Crude Microsomal Fraction

After obtaining the crude microsomal fraction prepared as in Chapter 2, the concentration of proteins was measured. In order to find a best method, two different methods were compared. All the results in this report were repeated at least three times.

### 3.4.1 The Lowry Method (Rosebrough *et al.*, 1951)

The Lowry method is based on measuring the content of tyrosine and tryptophan in a protein using Folin-Ciocalteau reagent as the dye. BSA (Bovine serum albumin) (10 mg/ml) was used as the reference to prepare a standard curve, from which, by comparison the concentration of protein in experimental samples could be calculated. The results were as shown in **Table 3.1** and in **Figure 3.1**

**Table 3.1 Result of The Lowry Method to Determine the Protein Concentration of the Rat-Kidney Crude Microsomal Fraction (see section 2.3.2)**

| The concentration of BSA (μg/ml) | $A_{650}$ |
|---|---|
| 0 | 0.000±0.000 |
| 100 | 0.173±0.008 |
| 200 | 0.305±0.004 |
| 300 | 0.454±0.018 |
| 400 | 0.563±0.006 |
| 500 | 0.657±0.005 |
| Crude microsomal fraction: | |
| neat | 1.861±0.013 |
| 1:10 | 0.508±0.015 |
| 1:100 | 0.044±0.004 |

Based on the data presented in **Table 3.1** the standard curve was plotted in **Figure 3.1**.

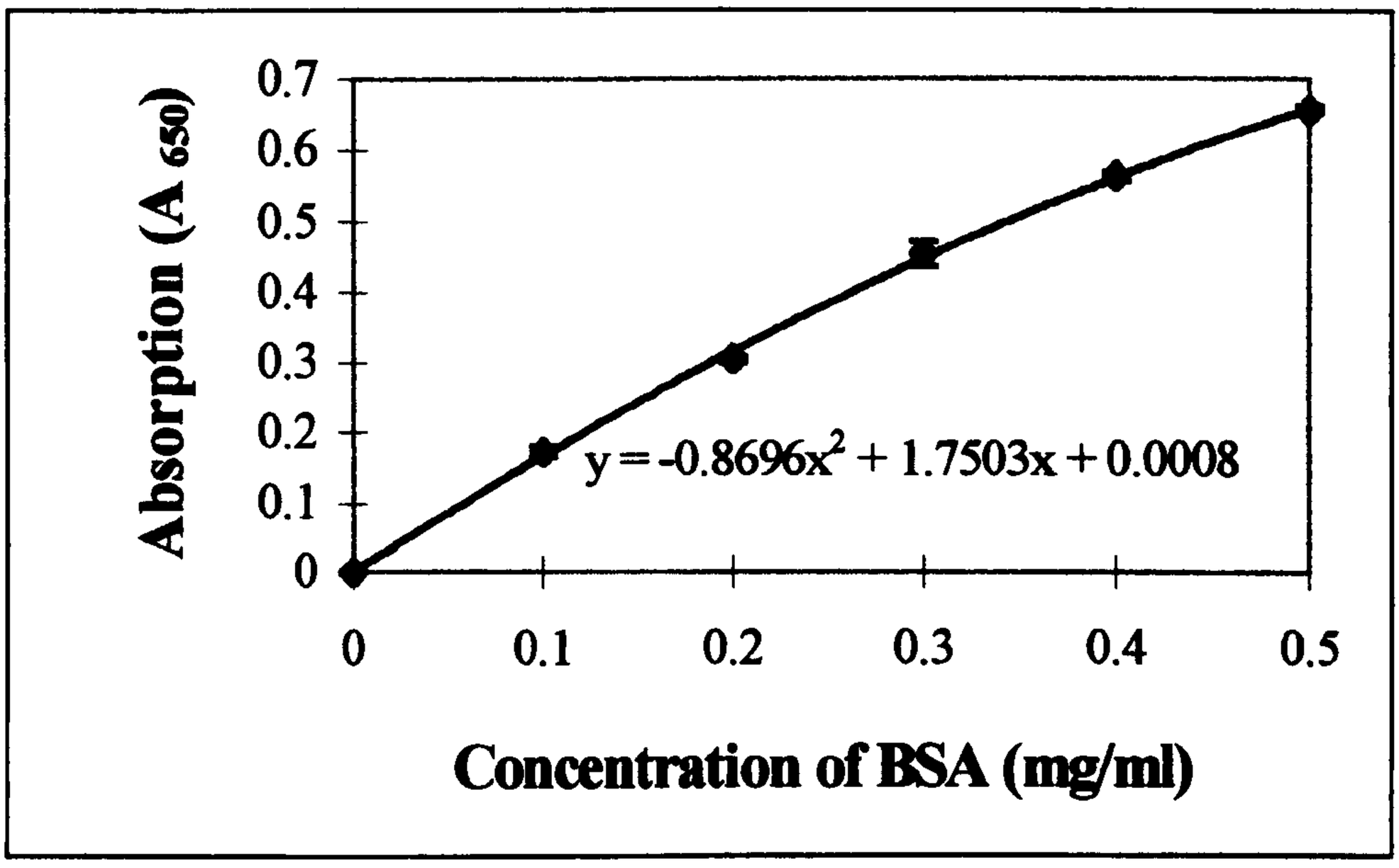



**Figure 3.1 The Standardisation Curve for the Lowry Method Used to Determine the Protein Concentration of the Rat Kidney Crude Microsomal Fraction**

From the standard curve in **Figure 3.1**, the $A_{650}$ of the rat kidney crude microsomal fraction could be read against the protein concentration, so that the protein content could be obtained. For dilution at 1:10 of rat kidney crude microsomal fraction, the reading taken from the **Figure 3.1** was 351 μg/ml, for 1:100 dilution the reading was 250 μg/ml. Then the concentration of protein in the crude microsomal fractions was calculated, which was 3.01±0.71 mg/ml.

### 3.4.2 The Bradford Method (Spector, 1977)

The difference between the Bradford and the Lowry's method is that Coomassie brilliant blue is used as the dye for the Bradford assay and the absorbance is measured at 595 nm. The dye-binding capacities can affect the $A_{595}$ value and finally affect the estimation of the protein concentration in the samples. However this method is easier and quicker to manipulate than the Lowry method. Therefore the protein concentration was also measured by the Bradford method so that the most appropriate method could be chosen in the following experiments. The sample contained 10-100 μg of protein in 0.1 ml and 5.0 ml of Coomassie brilliant blue G-

250 was added and mixed with protein sample quickly. After 5 minutes and before 1 hour, the absorbance at 595 nm was read against a reagent blank. Protein concentration was calculated by comparison with the results as presented in **Table 3.2** and in **Figure 3.2**.

**Table 3.2 Bradford Method Used to Determine the Protein Concentration of Crude Microsomal Fraction**

| The concentration of human albumin HA (mg/ml) | $A_{595}$ |
|---|---|
| 0.0 | 0.000±0.000 |
| 0.2 | 0.156±0.014 |
| 0.4 | 0.250±0.014 |
| 0.6 | 0.387±0.012 |
| 0.8 | 0.474±0.010 |
| 1.0 | 0.555±0.007 |
| Crude sample dilution: 1:5 | 0.445±0.010 |
| 1:10 | 0.219±0.008 |
| Tris-HCl buffer, (pH 7.4) control | 0.002±0.000 |

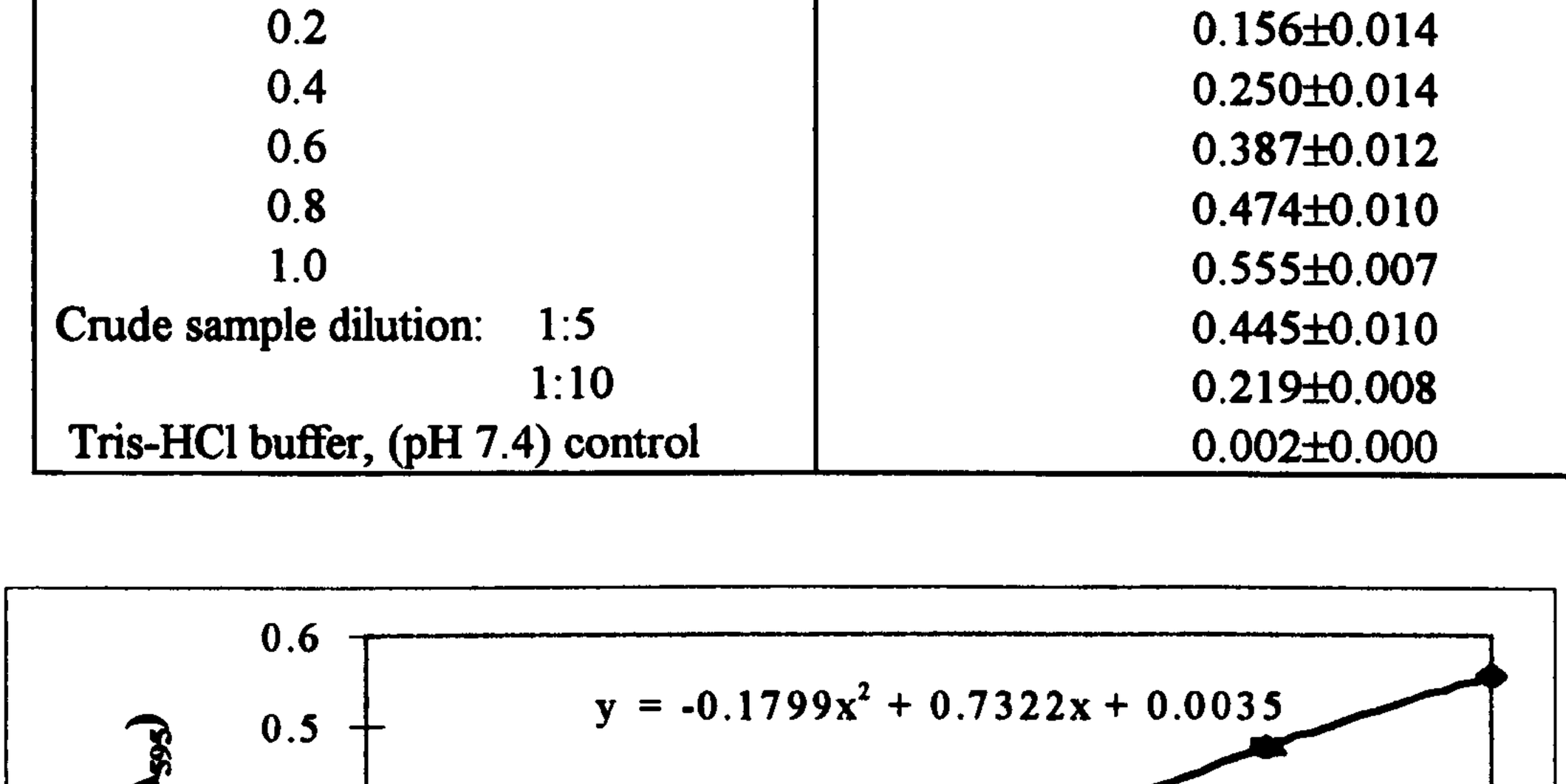



**Figure 3.2 The Standard Curve For the Bradford Method Used to Determine the Protein Concentration of the Crude Microsomal Fraction, prepared as section 2.3.2.**

Human Albumin (HA) stock: 10 mg/ml; The crude microsomal sample was tested at two dilutions: 1:5 and 1:10.

Based on the data in **Table 3.2** the standard curve was produced in **Figure 3.2**. The $A_{595}$ of the crude microsomal fraction could be read against the protein concentration so that the protein content of the samples could be obtained. The protein concentration from the Bradford assay of the crude microsomal fraction was calculated (by means of the equation in the figure) which is 0.7365 mg/ml at the dilution of 1:5 and 0.32 mg/ml at the dilution of 1:10, then the protein concentration in the undiluted crude microsomal fraction was 3.44±0.34 mg/ml. The statistical significance of difference between the Bradford and the Lowry was determined by Student's t-test. $P<0.05$ was considered as the criterion of significance (The detail of Student's t-test is described in Chapter 2 and Appendix. 3). This suggested that the two results had no statistical significance in their difference ($P>0.05$). The Bradford assay was easier to operate than the Lowry's assay and so it was the chosen method to measure the protein concentration in the following experiments.

### 3.4.3 The Stability Determination of the Rat Kidney Crude Microsomal Fraction

After the rat kidney crude microsomal fraction was stored in the cold room at 4°C for two months, some white precipitate was observed on the bottom of the sample tube. In order to determine if it was the inactivated enzyme and if this would affect the effective concentration of protein, the concentration of the protein in our sample was remeasured periodically by carrying out the protein assay at two-monthly intervals. The results were as shown in **Table 3.3**.

**Table 3.3 The Monitoring of the Stability of the Crude Microsomal Fraction (Bradford Method)**

| Dilution of sample | The concentration of protein (mg/ml) | | |
|---|---|---|---|
| | 1 day after prepared | 2 months after prepared | Significance (P) |
| 1:5 | 3.88 | 3.50 | $P < 0.01$ |
| 1:10 | 3.30 | 3.35 | $P < 0.01$ |

The results presented in **Table 3.3** indicated that the concentration of protein was still about 3.4±0.1 mg/ml ($P<0.01$). The NEP assay also showed that the enzyme

activity was stable enough (see in the section 3.5.3 in this chapter). These two results suggested that the white precipitate would not affect the NEP activity significantly and so the white precipitate could be neglected.

## 3.5 Assay of NEP Activity in the Crude Microsomal Fraction

### 3.5.1 The Binding Efficiency of the Column

After the crude microsomal fraction was obtained and the protein concentration was determined by the Bradford assay, the NEP assay had to be established. Firstly, the binding efficiency of the Bio-beads column was investigated so that the most efficient Bio-beads columns (the principles of the method was introduced in section 3.2 of this chapter) could be chosen for NEP assay. The results were as shown in **Table 3.4**.

**Table 3.4 The Binding Efficiency of Bio-beads Column**

| Number of columns | Total DPM | Binding efficiency ( % ) |
|---|---|---|
| 1 | 40082.2 | 87.0 (DPM) 90.5 (CPM) |
| 2 | 65352.2 | 93.2 (DPM) 92.5 (CPM) |
| $^{3}$H(leu)-enkephalin control | 13508.9±8239 | |
| Ecoscint H control | 29.9±2.8 | |

DPM: Disintegrations per minute

The binding efficiency of the randomly-selected two bio-beads columns were therefore 87% and 93.2%, giving an average binding efficiency of 90.1±4.4%. This suggested that the columns' binding efficiency was higher than 90%. Normally if the binding efficiencies of randomly-selected beads were above (85-90)%, the beads could be used to carry out the effective NEP assays.

### 3.5.2 Concentration Curve of the Crude Microsomal Fraction For 50% Hydrolysis of $^{3}$H(leu)-enkephalin

In order to establish a stable and reasonable NEP assay for primary screening of the Chinese herbs, the concentration of the crude microsomal fraction required to produce 50% hydrolysis of $^{3}$H(leu)-enkephalin needed to be investigated. The buffer

containing 50 mM of Tris-HCl pH 7.4 was used to dilute the crude microsomal sample according to the following ratios 1:5, 1:10, 1:20, 1:30, 1:40, 1:60, 1:100 then in each case the enzyme was assayed. The results were as shown in **Table 3.5** and in **Figure 3.3**.

**Table 3.5 Determination of the Concentration of the Crude Microsomal Fraction Required to Produce a 50% Hydrolysis of $^{3}$H(leu)-enkephalin**

| Dilution of enzyme sample | Conc. of protein in sample (μg/ml) | Total DPM | Specific DPM | Hydrolysis% of $^{3}$H(leu)-enkephalin |
|---|---|---|---|---|
| undiluted | 3400 | 68460.5 | 66269.76 | 96.8 |
| 1:5 | 680 | 79051.1 | 72647.96 | 91.9 |
| 1:10 | 340 | 89852.7 | 55169.55 | 61.4 |
| 1:20 | 170 | 85908.0 | 44414.44 | 51.7 |
| 1:30 | 113.3 | 84105.8 | 27166.17 | 32.3 |
| 1:40 | 85.0 | 93543.8 | 22076.34 | 23.6 |
| 1:60 | 56.7 | 82227.6 | 14389.83 | 17.5 |
| 1:100 | 34.0 | 81711.1 | 8334.53 | 10.2 |
| Tris-HCl buffer control | | 52311.3 | 4760.33 | 9.1 |
| Ecoscint H control | | 28.8±0.2 | | |

NB: HD% = (Specific DPM / Total DPM) X 100%; DPM means disintegrations per minute; Digestion was taken to completion in these experiments for which a digestion time of 30 minutes was considered sufficient.

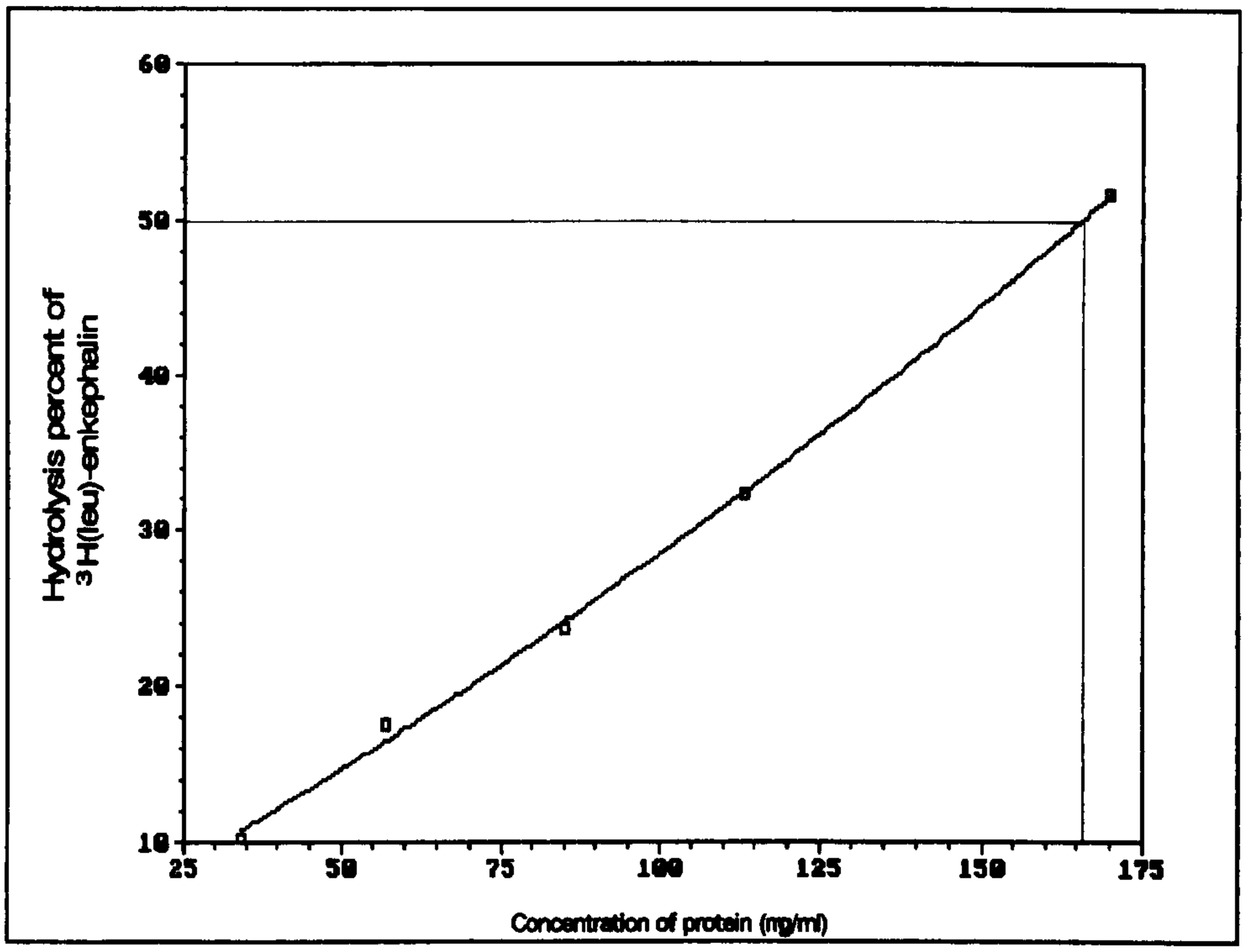



**Figure 3.3 Determination of the Concentration of the Crude Microsomal Fraction Required to Produce 50% Hydrolysis of $^{3}$H(leu)-enkephalin.**

Based on the data in **Table 3.5** the dilution curve was as plotted in **Figure 3.3**. The curve indicated that the optimum dilution for 50 % hydrolysis of $^{3}$H(leu)-enkephalin was 1:20. Since the original concentration of protein in the rat-kidney crude microsomal fraction was 3.4 mg/ml, the enzyme concentration required to produce 50% hydrolysis of $^{3}$H(leu)-enkephalin was therefore 165.6 μg /ml.

### 3.5.3 The Time Course for Hydrolysis of $^{3}$H(leu)-enkephalin by the Rat Kidney Crude Microsomal Fraction

The above experiment demonstrated that the concentration of the rat kidney crude microsomal fraction for 50% hydrolysis on $^{3}$H(leu)-enkephalin was 165.6 μg/ml. The time course for digestion of $^{3}$H(leu)-enkephalin by the rat-kidney crude microsomal fraction was then determined.

The reaction mixture ( 40 μl of crude microsomal fraction —protein concentration at 165.6 μg/ml, being mixed with 20 μl of 200 nM cold (leu)-enkephalin, 20 μl of 5 nM

$^{3}$H(leu)-enkephalin, 20 μl of 10 mM Bestatin, 20 μl of 10 μM Captopril and 80 μl of 50 mM Tris-HCl, pH7.4 ) was incubated at 37°C and aliquots of volume were taken out at 0, 10, 20, 30, 60, 90, 120, 180 minutes separately, and 50 μl of 0.1N HCl was added to stop the reaction for each aliquot. Each aliquot was denatured at 95°C for 15 minutes, and centrifuged at 13000 rpm for 20 minutes. The supernatant was applied to Bio-beads and counted for DPM(disintegration per minute) in a liquid scintillation counter. The results were as shown in **Table 3.6**. and as plotted in **Figure 3.4**.

**Table 3.6 Determination of the Time Course of $^{3}$H(leu)-enkephalin Hydrolysis for Crude Microsomal Fraction**

| Incubation time ( min ) | Hydrolysis % of $^{3}$H(leu)-enkephalin (HD%) | | | | |
|---|---|---|---|---|---|
| | Experiment 1 | | Experiment 2 | | Average |
| | DPM | HD% | DPM | HD% | HD% |
| 0 | 78231.5 | 4.3 | 78784.0 | 6.5 | 5.4±1.5 |
| 10 | 84999.4 | 21.5 | 89890.0 | 20.8 | 21.2±0.5 |
| 20 | 92246.4 | 35.7 | 90253.6 | 34.5 | 35.1±0.8 |
| 30 | 87490.2 | 47.9 | 91282.8 | 46.2 | 47.1±1.2 |
| 60 | 95414.4 | 68.7 | 96340.5 | 67.7 | 68.2±0.7 |
| 90 | 93514.8 | 82.1 | 94166.5 | 81.3 | 81.7±0.6 |
| 120 | 95026.9 | 88.1 | 99856.8 | 85.8 | 87.0±1.6 |
| 180 | 95515.0 | 93.8 | 100389.7 | 92.9 | 93.4±0.6 |
| BC* | 89322.8 | 7.4 | | | |
| Ecoscint H control | | | 30.0±0.3 | | |

NB: * BFC: 50 mM Tris-HCl (pH 7.4) control; HD%: Hydrolysis percent of $^{3}$H(leu)-enkephalin (see Table3.5 for definition)

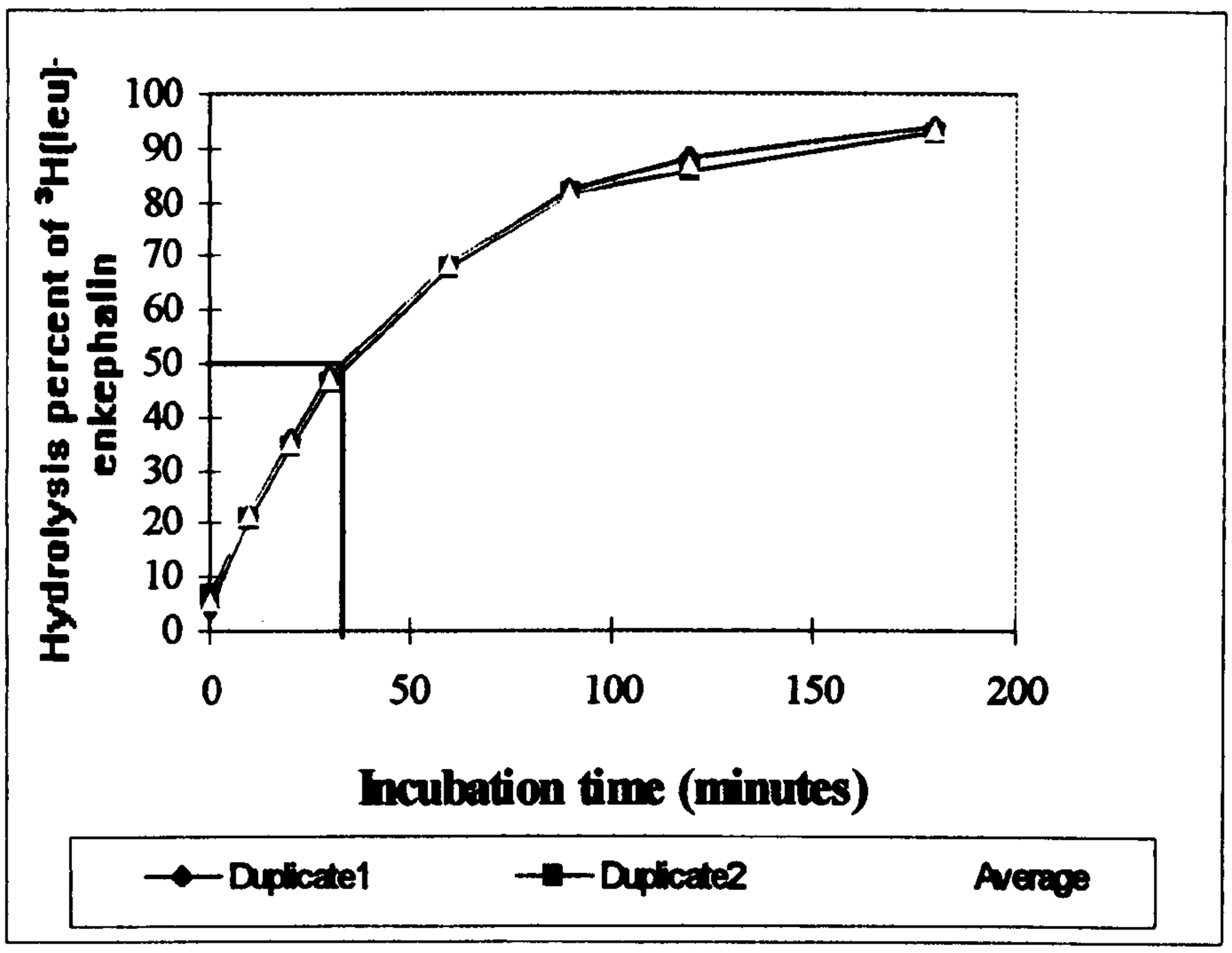



**Figure 3.4 Determination of the Time Course of $^{3}$H(leu)-enkephalin Hydrolysis For the Rat Kidney Crude Microsomal Fraction.**

The graph of the time course of the hydrolysis was produced from the data in **Table 3.6**. This indicated that for 50% hydrolysis at 1:20 dilution, the incubation time was 33.5 minutes. In the detailed experiments the incubation time was chosen to be 30 minutes. A t-test showed that 33.5 minutes incubation time was not significantly different from 30 minutes ($P > 0.05$).

Based on the above experiments the characterisation of the rat kidney crude microsomal extract was carried out. Up to this stage of the work an optimum enkephalin-hydrolysing enzyme assay was established as follows:

40 µl of crude microsomal fraction (protein concentration at 165.6 µg/ml) was mixed with: (1) 20 µl of 200 nM cold (leu)-enkephalin, (2) 20 µl of 5 nM $^{3}$H(leu)-enkephalin, (3) 20 µl of 10 mM Bestatin, (4) 20 µl of 10 µM Captopril. The solution was made up to 200 µl with 50 mM Tris-HCl buffer, pH7.4. The reaction mixture was incubated at 37°C for 30 minutes and digestion was stopped with 0.1 N HCl and denatured at 95°C for 15 minutes. The digestion solutions were then centrifuged at 13000 rpm for 20 minutes and the supernatants were applied to Bio-

beads and counted for DPM in the liquid scintillation counter. All the following screening experiments were carried out using the optimum assay (as described in this section).

### 3.5.4 Final Calculation of the Enzyme Activity of NEP in the Rat Kidney Crude Microsomal Fraction

In order to understand the enkephalin-hydrolysing enzyme assay better, the NEP activity was calculated as follows. According to the **Table 3.6,** 47.1% of 20.5 nM (leu)-enkephalin added into the incubation mixture, was hydrolysed in a total volume of 200 µl in 30 minutes.

Thus $20.5 \times 47.1\% = 9.656$ nM

$9.6555/5000 = 1.93 \times 10^{-3}$ nmols

$\therefore$ In 1 minute hydrolysed $1.93 \times 10^{-3} / 30 = 6.4 \times 10^{-5}$ nmoles/min

Thus $6.4 \times 10^{-5}$ nmoles/min of (leu)-enkephalin are hydrolysed by 40 µl of 1:20 dilution of the enzyme solution. Therefore using 1 ml of 1: 20 dilution of the enzyme solution, hydrolysis of $^{3}$H (leu)-enkephalin should be $6.4 \times 10^{-5}$ nmoles/min x 25 = 1.6 pmoles/min per ml of the crude microsomal fraction.

**Enzyme Activity**

One SI unit of enzyme activity is that amount of enzyme which will catalyse the conversion of 1 pmole of substrate under given assay conditions.

Thus 16 enzyme units/ml of 1: 20 dilution (From the dilution curve it could be found that the dilution was 20) of enzyme solution. Thus in original solution:

1.6 x 20 = 32 enzyme units/ml of original solution

Specific activity = units of enzyme activity/mg of protein

= 32 / 3.4 = 9.41 units of enzyme activity/mg of protein

Total activity = specific activity x total protein

As the volume of the obtained rat kidney crude microsomal fraction is 47.5 ml, the protein concentration is 3.4 mg/ml; Therefore the total activity = 9.41 x 47.5 x 3.4 = 1.52 x $10^3$ units.

Therefore under the experimental conditions, for 1 mg of protein in the crude microsomal fraction the specific activity of NEP is 9.41 units. Totally 1.52 $\times 10^3$ units of NEP was harvested. This was compatible with the reported results of Roques (1984).

## 3.6 The Purification of NEP By Hydrophobic-Interaction Chromatography

Purification of NEP was essential in order to study NEP activity quantitatively. Hydrophobic-Interaction Chromatography (HIC) was therefore chosen for partial purification of the enzymes.

The detailed HIC method for purification of NEP was described in section 2.3.6. Eluted fractions were collected in 1 ml aliquots in separate tubes for protein assay and enzyme assay.

### 3.6.1 Protein Assay

The collected fractions from the Hydrophobic-Interaction Chromatography (HIC) were measured at $OD_{280}$ by Ultra-violet absorption spectrophotometry from which the protein concentration was calculated. A plot of the fraction-tube number on the abscissa against the average protein concentration as ordinate, the elution curve, was made and was as shown in **Figure 3.5**.

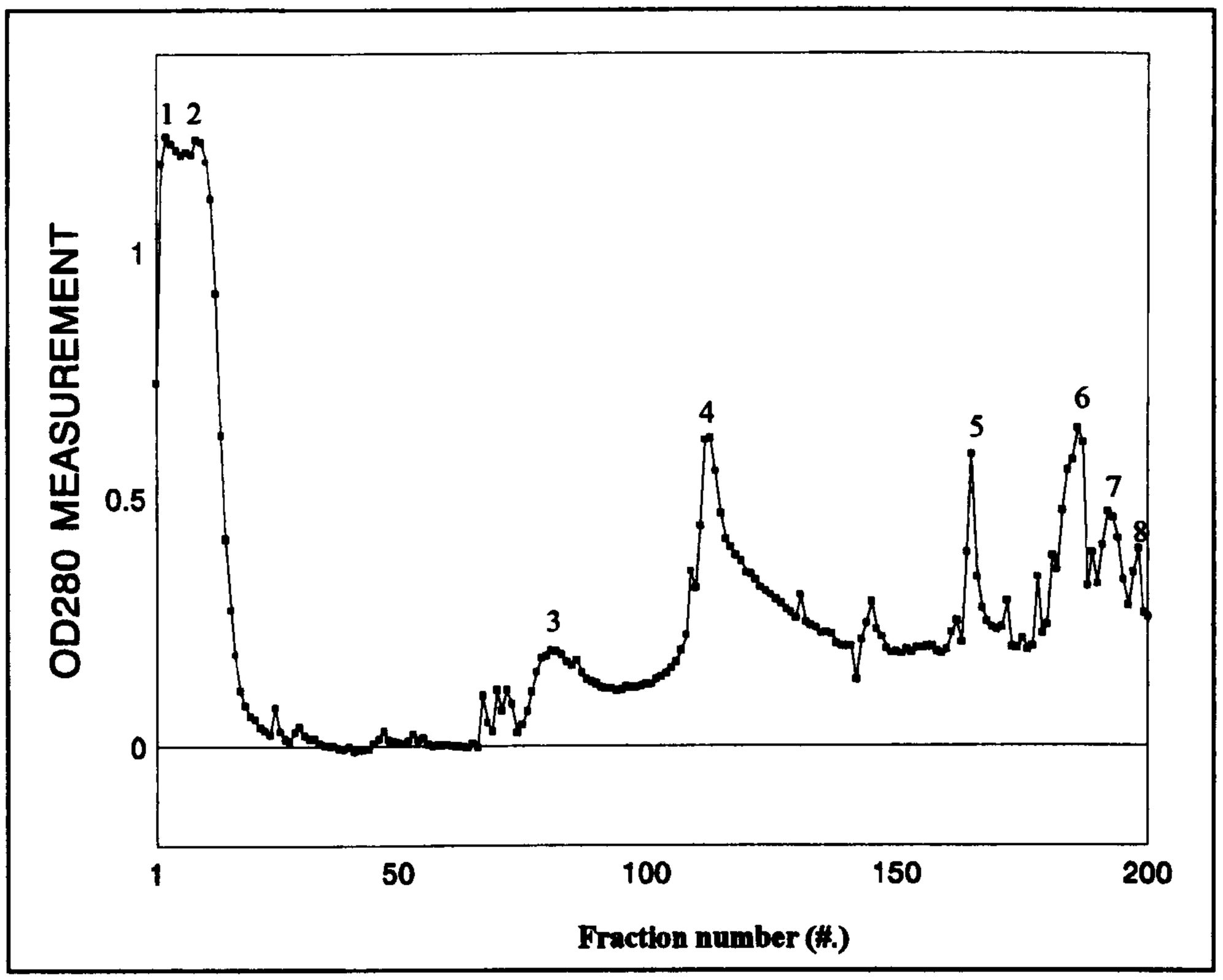



**Figure 3.5 Elution Profile For NEP from the HIC Purification Procedure**

The elution curve of **Figure 3.5** showed 8 peaks. They were in the positions: Peak 1: # 3, Peak 2: # 9, Peak 3: # 81, Peak 4: # 113, Peak 5: # 166, Peak 6: # 185, Peak 7: # 193, Peak 8: # 199, where # indicates fraction number.

### 3.6.2 Enzyme Assay

Based on the **Figure 3.5,** the enzyme activity was assayed for each of the selected five fractions, and the corresponding activity curve was calculated as plotted in **Figure 3.6**. The result presented in **Figure 3.6** suggested that the elution curve for protein ($OD_{280}$ peak) was compatible with the enzyme activity curve (hydrolysis percent peak). i.e. there is a strong correlation between the chromatographic peaks and the levels of enzyme activity. The fractions corresponding to the peaks were then pooled for further study.

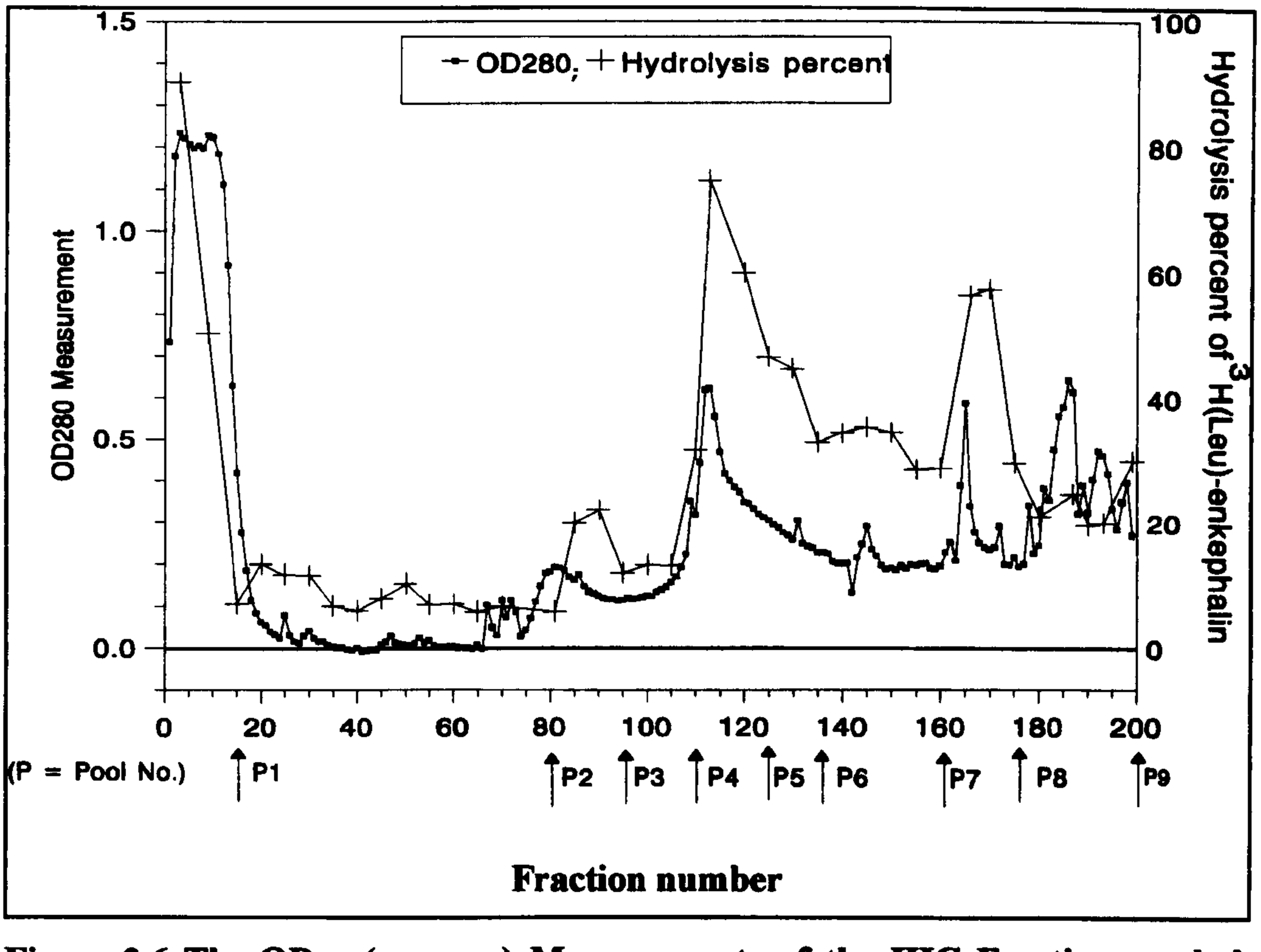



**Figure 3.6 The $OD_{280}$ (— • —) Measurements of the HIC Fractions and the Related Enzyme Activity as Indicated by the Percentage Hydrolysed (— + —).** Each tube contained a 1 ml elution fraction which was tested for $OD_{280}$ and enzyme activity.

### 3.6.3 Determination of the Protein Concentration and the Enzyme Activity in Pooled Fractions

By careful consideration of the two curves in **Figure 3.6** fractions were pooled so that the maximum yield of individual fractionated proteins were collected. The pooled fractions were taken as indicated in the following table.

| No. of Pools | Fraction numbers (#) | No. Pools | Fraction numbers (#) |
|---|---|---|---|
| Pool 1 | 1-15 | Pool 2 | 16-80 |
| Pool 3 | 81-95 | Pool 4 | 96-110 |
| Pool 5 | 111-125 | Pool 6 | 126-135 |
| Pool 7 | 136-160 | Pool 8 | 161-175 |
| Pool 9 | 176-200 | | |

Then the protein concentration and the enzyme activity in the pooled fractions were measured. The results were as shown in **Table 3.7**.

**Table 3.7 Determination of the Protein Concentration of Pooled Fractions and Related Enzyme Activity**

| Pooled fractions | Volume of pools (ml) | Protein conc. (mg/ml) | Total DPM | Hydrolysis% of $^{3}$H(leu)-enkephalin | Total NEP activity (unit) |
|---|---|---|---|---|---|
| Pool 1 | 10.0 | 0.900 | 26049.5 | 81.7 | 27.91 |
| Pool 2 | 47.0 | 0.048 | 28661.9 | 6.5 | 10.44 |
| Pool 3 | 11.0 | 0.125 | 26287.8 | 19.3 | 7.25 |
| Pool 4 | 12.0 | 0.142 | 23591.9 | 17.3 | 7.09 |
| Pool 5 | 10.2 | 0.510 | 22583.3 | 71.4 | 24.88 |
| Pool 6 | 12.0 | 0.307 | 24164.2 | 20.7 | 8.49 |
| Pool 7 | 17.0 | 0.291 | 25472.3 | 22.0 | 12.78 |
| Pool 8 | 14.5 | 0.152 | 21632.4 | 13.8 | 6.84 |
| Pool 9 | 14.6 | 0.188 | 23830.8 | 7.7 | 3.84 |
| Crude Enzyme sample (-20°C) | | | 26363.9 | 48.1 | 164.34 |
| Fresh enzyme sample (-4°C) | | | 20987.5 | 56.5 | 193.04 |

The data presented in **Table 3.7** was used to calculate the recovery percent of enzyme activity as follows;

The total activity of pooled fractions are:

$$\frac{20.5 \times \text{hydrolysis\% of substrate}}{5000 \times 30 \times \text{protein conc.}} \times 25 \times 10^{3} \times \text{V.of pools} \times \text{protein conc.}$$

Thus the total loading enzyme activity is: 164.34 units; The total recovery enzyme activity is: 109.52 units.

So, Recovery % of enzyme activity $= 109.52 \div 164.34 \times 100\ \%$

$= 66.6\ \%$

Therefore about 66.6 % of the enzyme activity was recovered.

### 3.6.4 Using Polyacrylamide Gel Electrophoresis (PAGE) to Analyse the Pooled Fractions

As shown in the SDS polyacrylamide gels in **Figure 3.7**, Pool 1 (P1), Pool 3 (P3), Pool 6 (P6) seemly contained the 90 kDa band as calculated by comparison with the standards run in the first alley. 90 kDa should be the band of NEP according to the work of Kenny *et al*. (1992). The gel profile of **Figure 3.7** showed that the NEP had not been purified completely. This suggested that HIC was useful but not good enough to purify the NEP completely. HIC still needed to be combined with other methods so that completely purified NEP could be obtained. The detailed procedures for obtaining completely purified NEP was described by Almenoff and Orlonski (1983). According to Roques (1993), affinity chromatography using monoclonal antibodies raised to the NEP would be the most efficient way to obtain purified NEP. In future this method will be considered for use if circumstances allow it.

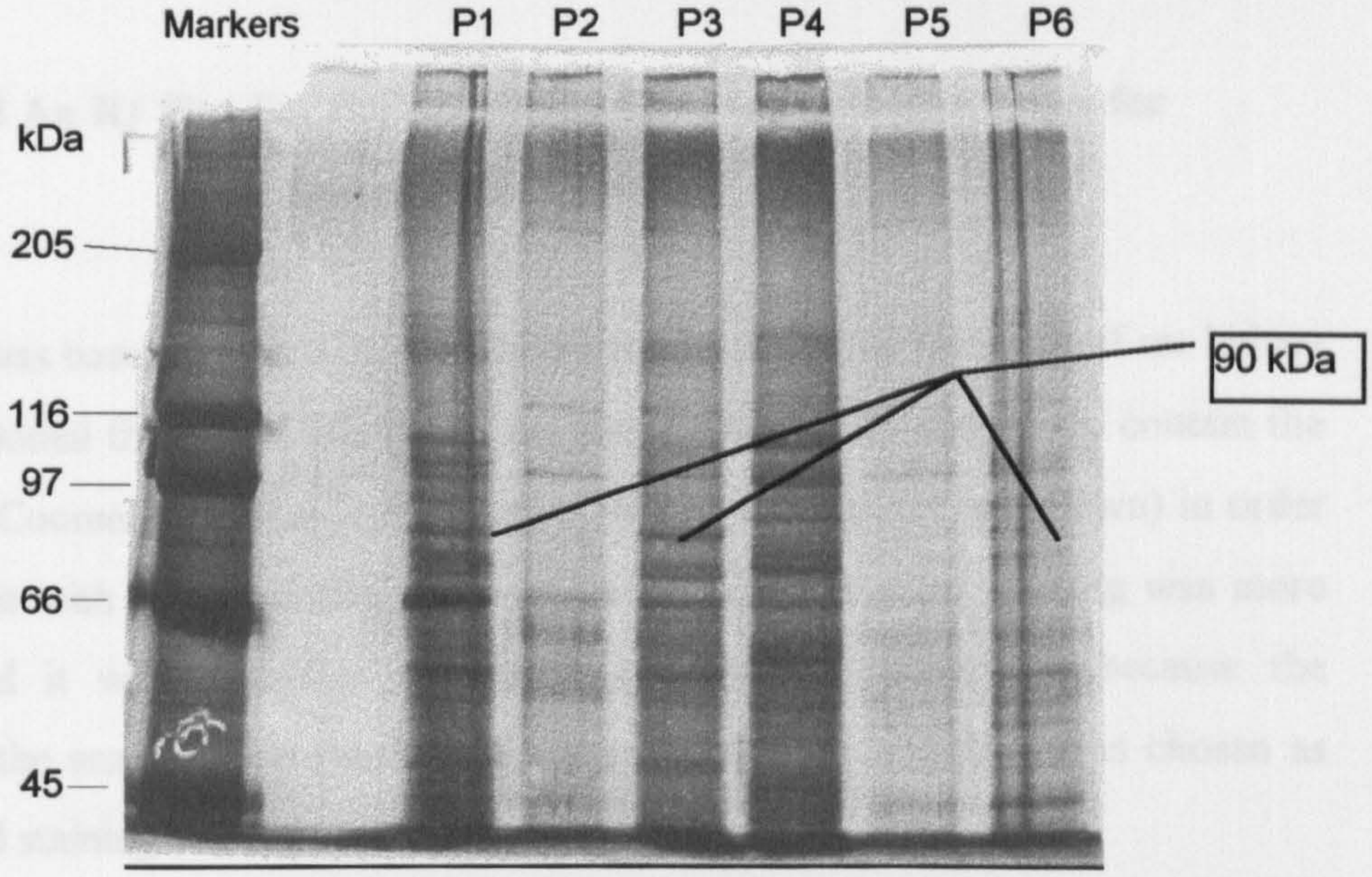



**Figure 3.7 Silver Stained SDS-acrylamide Gel of the Pooled Fractions of Rat Kidney Crude Microsomal Fractions, where P Denotes Pool Number.**

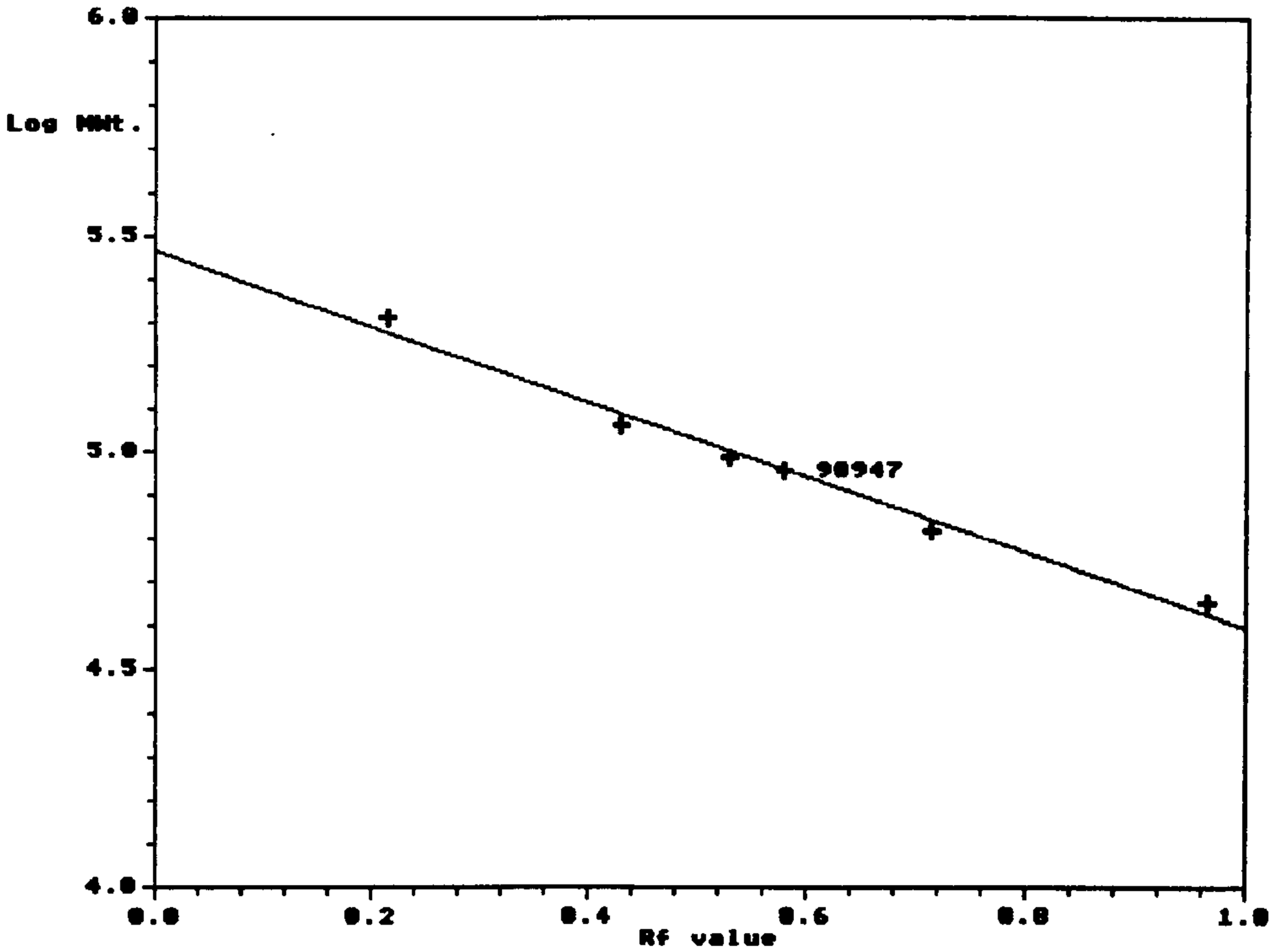



**Figure 3.8 An R*f* Plot For the Above Gel Showing a 90 kDa Value for the Indicated Band.**

**Figure 3.8** was based on the SDS-PAGE gel result of pooled fractions of rat kidney crude microsomal fractions. The result suggested that P1, P3 and P6 do contain the NEP band. Coomassie brilliant blue staining was also done (gel not shown) in order to compare it with silver staining. The result showed that silver staining was more sensitive and it was important under this experimental conditions because the amounts of the enzymes were quite limited, silver staining therefore was chosen as the preferred staining method.

## CONCLUSION AND SUMMARY

### 3.6.5 Conclusion

The hydrophobic-interaction chromatography (HIC) method proved to be useful to purify NEP from the crude rat-kidney fraction from the above experiments and the gels showed the presence of a 90 kDa band consistant with NEP but indicated that impurity proteins were also present. In future a more detailed purification method will be needed to isolate NEP. The McAb method is reported to be the most efficient

method to obtain completely purified NEP (Roques, 1993). The NEP enzyme did not fractionate in a unique fraction of the HIC column but was observed in several fractions. However in the total pooled fractions 66.6% of enzyme activity was recovered and the NEP was not observed in pools of fraction numbers 16-80, 96-110, 111-125 but was observed in pools of fractions 1-15, 81-95 and 126-135. Clearly fractions were not taken where there was 33.4% of the enzyme activity, however the above 33.4% of enzyme were not in the region which would expect to contain NEP.

## 3.7 Summary

At this stage of the work the enkephalin-hydrolysing enzymes assay had been established. According to the results in the **Figure 3.3** the optimum dilution for 50% hydrolysis of $^{3}$H(leu)-enkephalin was 1:20, the protein concentration was 165.6 μg/ml and the incubation time was 33.5 minutes.

In the experiments which are described in the later sections of this study, by changing the inhibitors of the three enkephalin-hydrolysing enzymes, the activity of three enkephalin-hydrolysing enzymes (NEP, APN, ACE) could be assayed separately. By using selective NEP inhibitors or mixed inhibitors of NEP/APN, APN/ACE, and NEP/ACE, the activity of specific enzymes or groups of enzymes could be screened. The protocol for the assays for the activities of the specific enzymes was as given in **Table 3.8**

**Table 3.8 The Protocols of the Assays for the Activities of the Specific Enzymes**

| Enzyme inhibited | Inhibitors' name and concentration | Remaining uninhibited enzymes therefore being assayed |
|---|---|---|
| NEP | Thiorphan (2.0 mM) | APN, ACE, DAP |
| APN, DAP | Bestatin (10 mM) | NEP, ACE |
| ACE | Captopril (10 μM) | NEP, APN, DAP |
| APN, DAP, ACE | Bestatin, Captopril | NEP |
| NEP, ACE | Thiorphan, Captopril | APN/DAP |
| NEP, APN, DAP | Thiorphan, Bestatin | ACE |
| NEP, APN, DAP, ACE | Thiorphan, Bestatin, Captopril | N/A |

# CHAPTER IV

# THE EFFECTIVE USE OF GOU AND QIANG AS INHIBITORS OF PROTEOLYTIC ENZYMES NEP, APN AND ACE IN THE CRUDE MICROSOMAL FRACTION PRIMARY SCREENING EXPERIMENTS

# INTRODUCTION

After the assay for proteolytic enzymes had been established and the partially purified NEP had been obtained as described in Chapter 3, Chinese herbs were assayed for their inhibitory effects on NEP. The crude microsomal fraction contained two other enzymes (APN and ACE) which also have proteolytic action on enkephalin. The standard inhibitors of enzymes other than NEP were therefore used to ensure that screening was specifically for NEP (see **Table 3.8** of chapter 3). The screening work was done on NEP first, then the effect of target herbs on APN or/and on ACE were investigated in subsequent experiments. All the results in this chapter were repeated at least three times.

# RESULTS AND DISCUSSION

## 4.1 The Optimum Inhibitory Concentration of Standard Inhibitors

In order to establish an optimum screening model, the optimum concentrations for inhibition of enkephalin proteolysis in **Table 4.1** had to be investigated first. Three concentrations of each inhibitor were assayed for their inhibitory activity on the rat kidney crude microsomal fraction. The results were as presented in **Table 4.1**. and the experiments were carried out as following steps: (1) Preparation of the preincubation mixture: Bestatin (final concentration: 1mM , 10mM, 100 mM) or Thiorphan (final concentration: 0.2 mM 2.0 mM, 20 mM) or Captopril (final concentration: 10μM, 1mM, 10 mM) was usually mixed with 40 μl of rat kidney crude microsomal fraction (diluted at 1:20) , then the 50 mM Tris-HCl buffer, pH7.4 was added until the final volume was 140 μl , preincubated at room temperature for 15 minutes; (2) NEP assay : The NEP assay was run by mixing above 140 μl solution with 20 μl of 200 nM cold (leu)-enkephalin, 20 μl of 5nM of $^{3}$H(leu)-enkephalin, and 20 μl of 10 mM Bestatin, then final volume (200 μl) solution were incubated at 37°C for 30 minutes, then 50 μl of 0.1 M HCl was added to stop the reaction immediately and inactivated at 95°C for 15 minutes. (3) The mixture was centrifuged at 13000 rpm for 20 minutes and the 200 μl of supernatants were loaded onto Bio-beads column and washed with 2 × 1 ml distilled water to elute and

followed with 2 × 1 ml absolute ethanol to elute, the eluates were counted using a liquid scintillation counter.

**Table 4.1 Determination of the Optimum Inhibitory Concentration Standard Inhibitors of NEP, APN and ACE in the Crude Microsomal Fraction**

| Classified group | | HD% | Enzyme inhibited | Enzyme active still |
|---|---|---|---|---|
| Bestatin (Bes) | 1 mM | 86.1 | APN | NEP, ACE |
| | 10 mM | 44.9 * | | |
| | 100 mM | 16.7 | | |
| Bes (10mM) + Captopril | 10 μM | 30.4 * | APN, ACE | NEP |
| | 100 μM | 28.2 | | |
| | 1 mM | 27.9 | | |
| | 10 mM | 16.4 | | |
| Bes (10 mM) + Thiorphan | 0.2 mM | 30.8* | APN, NEP | ACE |
| | 2.0 mM | 22.9 | | |
| | 20 mM | 13.0 | | |
| CE only | | 94.5 | None | NEP, NEP, ACE |
| BFC | | 6.7 | Buffer control | |

NB: BFC: buffer control; CE: Rat kidney crude microsomal sample

*: The optimum concentration under this experimental condition

HD% : Hydrolysis percent of $^{3}$H(leu)-enkephalin (see Table3.5 for definition)

The results presented in **Table 4.1** suggested that 10 mM of Bestatin, 10 μM of Captopril and 0.2 mM of Thiorphan could be considered as the optimum inhibitory concentrations under laboratory conditions for these studies. Firstly the concentration of Bestatin that led to 50% of $^{3}$H(leu)-enkephalin to be degraded by NEP was obtained, then the minimum inhibitory concentration (MIC) of Captopril and Thiorphan was assayed. Under these experimental conditions the 0.2 mM of Thiorphan could significantly decrease the hydrolysis of $^{3}$H(leu)-enkephalin and was used as the inhibitory concentration. 10 mM of Bestatin could significantly decrease the hydrolysis of $^{3}$H(leu)-enkephalin and was chosen to inhibit APN in the following screening experiments. While 10 μM of Captopril was used to inhibit ACE. By controlling the use of the three inhibitors, the activity of three enzymes (NEP, APN

and ACE) could be assayed. In this way the effects of Chinese herbs on NEP APN and or ACE individually could be investigated.

The 50 percent inhibitory concentration ($IC_{50}$) value of Thiorphan was reported by Roques (1980) to be 4.7±1.2 nM. Bestatin was reported to be 0.2 μM (Barclay, 1980) and Captopril was reported to be 1 μM (Ondetti, 1977, 1979). These results were obtained by assaying the rat kidney crude microsomal fraction, not the pure enzymes, and of course it also contained proteins other than NEP, APN and ACE. It is known that none of these other proteins act as peptidases against enkephalins but they will affect the calculation of the enzyme inhibitory concentration. Therefore the optimum concentration used in this experimental condition was much higher than the reported Minimum Inhibitory Concentration (MIC) of the three standard inhibitors (Roques, 1980). In fact a higher concentration of standard inhibitors was necessary in order to obtain complete inhibition of enzymes combinations (APN, ACE and DAP) so that the effect of the herbal extract on NEP could be properly investigated.

## 4.2 The Effect of Four Chinese Herbal Extracts on the Activity of NEP in the Crude Microsomal Fraction

Yang (1985) identified four herbs which were clinically found to have different degrees of analgesic activity on patients. They were: Yan; Gou; Chuan; and Qiang. The molecular mechanism for the analgesic activity of the above herbs has not yet been reported. In view of the relationship between NEP and opioid peptides (see Figure 1.1), it was worthwhile to test the activity of above four herbs on NEP in the crude microsomal extract. Both the aqueous and organic extracts (see section 2.1 of Chapter 2) of above four herbs were tested for their inhibitory activity on NEP *in vitro*. The results were as given in **Table 4.2** and plotted in **Figure 4.2**. The detailed experimental procedure was described in section 2.4. and section 2.3.5.

**Table 4.2 Determination of the Effects on the Percentage of Hydrolysis of $^{3}$H(leu)-enkephalin in the Presence of Four Herbs to Show Their Inhibition of NEP in the Crude Microsomal Fraction**

| Enzyme activity group | | Organic extract | Aqueous extract |
|---|---|---|---|
| | | HD% | HD% |
| NEP active (CB) | | 44.6 | 47.8 |
| NEP active (CB) plus: | neat | 28.5 | 31.7 |
| Yan | 1:10 | 45.6 | 37.9 |
| Gou | neat | 22.2 * | 6.0 *** |
| | 1:10 | 37.8 | 25.8 * |
| Qiang | neat | 23.7 * | 7.8 *** |
| | 1:10 | 38.7 | 39.4 |
| Chuan | neat | 32.9 | 15.3 ** |
| | 1:10 | 54.0 | 39.2 |
| Tris-HCl buffer control | | 5.8 | |

HD% means Hydrolysis% of $^{3}$H(leu)-enkephalin; CB means Captopril plus Bestatin which inhibit APN and ACE, leaving NEP active (see **Table 4.1**). Then the four selected herbal extracts were mixed with the NEP-active preparation (CB) and assayed for proteolysis of (leu)-enkehalin. Student's t-test was used to analyse the significant differences between the NEP only active (CB) preparation and other NEP active (CB) plus herbs preparation. Probability "P" was expressed as follows: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

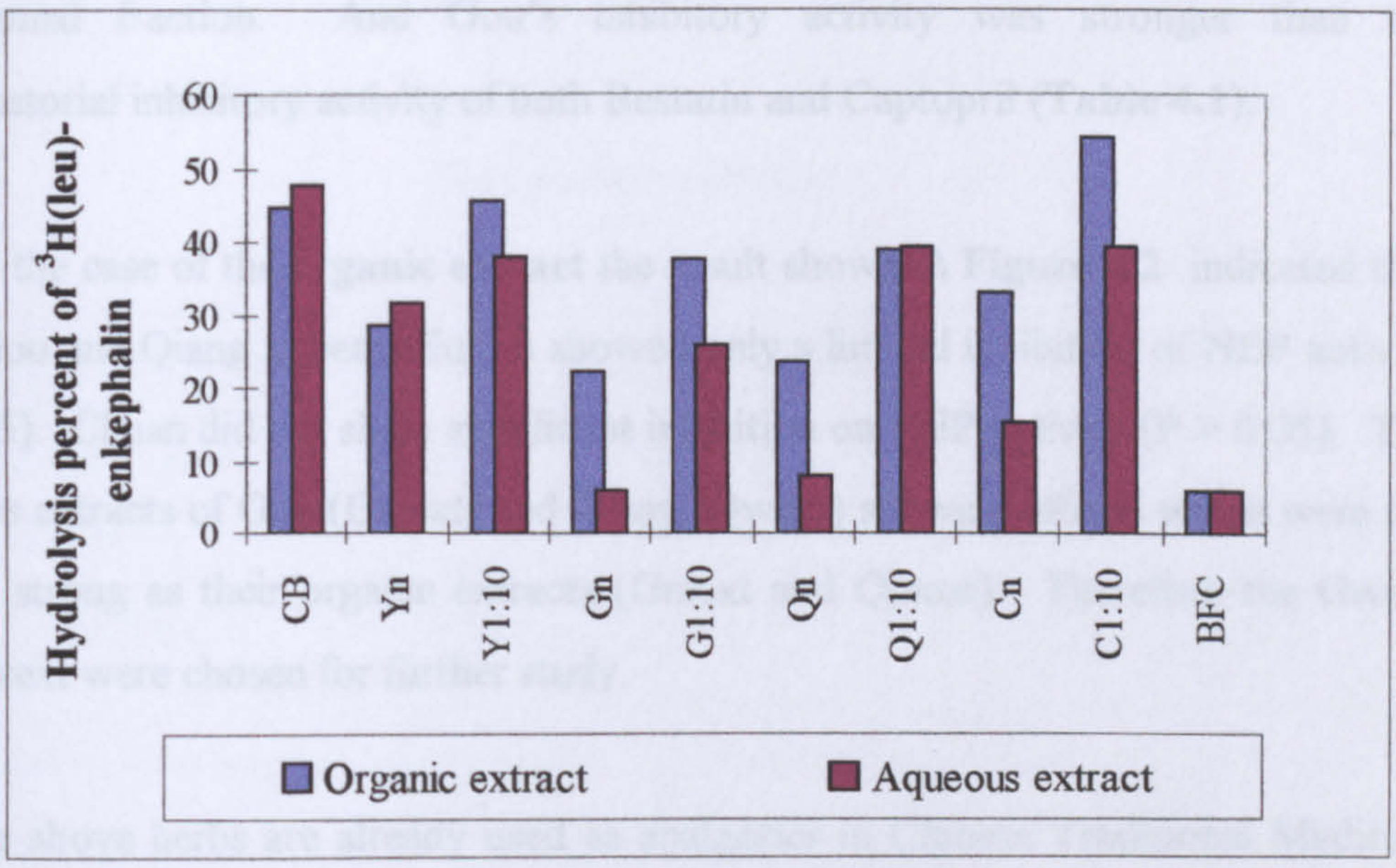



**Figure 4.2 Determination of the Influence of the Four Herbs on NEP Activity In the Rat Kidney Microsomal Fraction.**

CB: Captopril, Bestatin (NEP active) no herbs; BFC: Buffer Control; All herbal extracts were tested at 2 dilutions: neat (n) and 1:10. The herbs are Yan (Y), Gou (G), Qiang (Q) and Chuan (C).

The results presented in **Figure 4.2** indicated the following facts.

(1) For the **aqueous extract case**, the hydrolysis percent of $^{3}$H-(leu)-enkephalin in the rat kidney microsomal fraction was 47.8% in the case where NEP only was active without herbs (CB). All the digestion experiments with herbal extract present showed a lower hydrolysis percent of $^{3}$H-(leu)-enkephalin than was the case with NEP in the crude microsomal extract without herbal extracts. However only Gou, Qiang (undiluted only) and Chuan (undiluted only) showed statistically significant inhibition on NEP ( $P<0.05$). The undiluted Gou and Qiang decreased the hydrolysis of $^{3}$H-(leu)-enkephalin significantly ($P<0.001$). This suggested that the Gou aqueous extracts (Gwext) and Qiang aqueous extract (Qwext) had strong inhibitory activity on NEP activity in the crude microsomal fraction. Under experimental conditions the two standard inhibitors could reduce the hydrolysis percent to 16.4% (**Table 4.1**), However the undiluted Gwext could even reduce the hydrolysis percent to 6%. This suggested that Gwext showed the strongest inhibition on NEP activity from the crude

microsomal fraction. And Gou's inhibitory activity was stronger than the combinatorial inhibitory activity of both Bestatin and Captopril (**Table 4.1**).

(2) In the case of the **organic extract** the result shown in **Figure 4.2** indicated that Yan, Gou and Qiang at neat dilution showed only a limited inhibition of NEP activity ($P<0.05$). Chuan did not show significant inhibition on NEP activity ($P > 0.05$). The aqueous extracts of Gou (Gwext) and Qiang (Qwext) showed effects which were ten fold as strong as their organic extracts (Gmext and Qmext). Therefore the Gwext and Qwext were chosen for further study.

(3) The above herbs are already used as analgesics in Chinese Traditional Medicine (TCM), but they showed different inhibitory activity on NEP. It seems possible therefore that the above four herbs might have their analgesic activity by different mechanisms (see the description in section 1.7.3 of chapter 1).

## 4.3 Concentration Dependence of The Effect of Gwext And Qwext on The Activity of NEP in The Crude Microsomal Fraction

Based on the above results, the effects of Gwext and Qwext were studied further. In this experiment, four concentrations of Gwext and Qwext were observed for their effects on NEP. The results were as given in **Table 4.3** and graphed in **Figure 4.3**.

**Table 4.3 Determination of the Effect of Different Concentrations of Herb Aqueous Extracts on the Activity of NEP in the Crude Microsomal Fraction**

| Active enzyme group | | | HD% a | Inhibiting % b |
|---|---|---|---|---|
| NEP active (CB) | | | 45.1 | 62.5 |
| NEP active (CB) plus: | dilu-tion | Conc. (mg/ml) | | |
| Gwext | neat, | 40 | 8.2 | 81.8 *** |
| | 1:2 , | 20 | 9.7 | 78.5 *** |
| | 1:4, | 10 | 17.2 | 61.9 ** |
| | 1:8, | 5 | 27.5 | 39.0 * |
| Qwext | neat, | 40 | 15.4 | 65.9 ** |
| | 1:2, | 20 | 26.2 | 41.9 * |
| | 1:4, | 10 | 27.4 | 39.2 * |
| | 1:8, | 5 | 33.3 | 26.2 |
| Tris-HCl buffer control | | | 4.2 | |

a: HD% = Hydrolysis percent of $^{3}$H(leu)-enkephalin.

b: Inhibiting % = ( HD% of all the group minus HD% of CB group) divided by (HD% of CB group) × 100%. Ecoscint H control : 28.4± 0.2.

Student's t-test was used to do statistical analysis. Probability "P" was as follows. *: P<0.01; **: P<.001

**Table 4.3** indicated that the Gou aqueous extract (Gwext) decreased the hydrolysis percent (HD%) more significantly than Qiang aqueous extract (Qwext) while being added at the same concentrations in the range of 40-20 mg/ml. This suggested that Gwext was more worthy of investigation in the future.

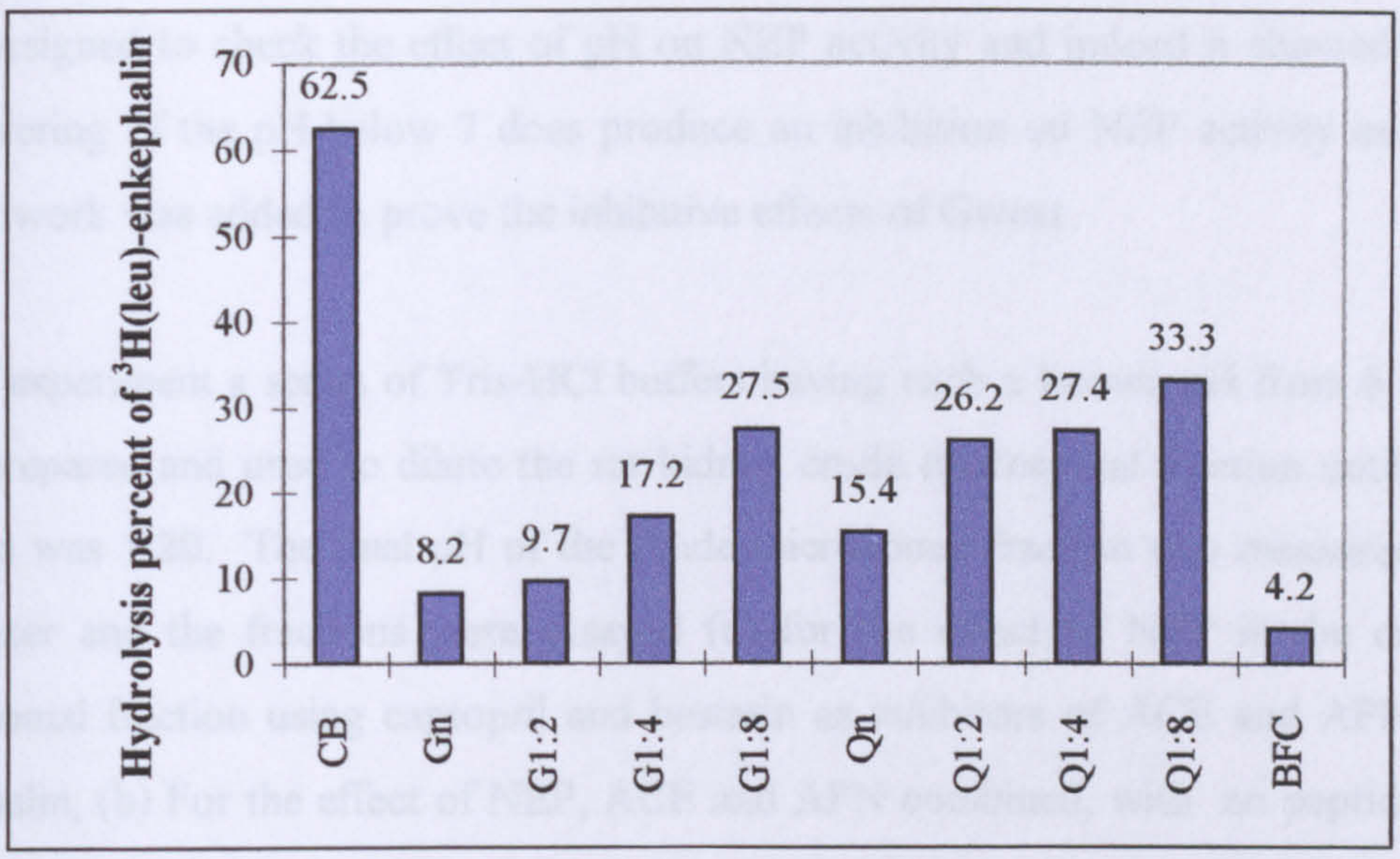



**Figure 4.3 Determination of the Effects of Different Concentrations of Herbs on the Activity of NEP in the Crude Microsomal Fraction**

CB: NEP active (Captopril and Bestatin were added) group without adding herbs ; BFC: buffer control; Captopril and Bestatin were presented in all samples to inhibit APN and ACE and without herbal extracts have CE; Gn: Gou undiluted (neat) group; G1:2, G1:4 and G1:8 : dilutions of Gou were 1:2, 1:4 and 1:8; Qn: Qiang undiluted (neat) group; Q1:2, Q1:4 and Q1:8: The dilutions of Qiang were 1:2, 1:4 and 1:8.

The more direct evidence was as presented in **Figure 4.3**. It demonstrated fully that the Gwext truly showed very strong inhibitory activity on NEP while being compared with the rat kidney crude microsomal fraction where no herbal extract was present, (student's t-test showed $P<0.001$). Qwext also showed strong inhibitory activity on NEP, but its inhibitory potency was not as strong as that of Gou. Considering the time limitation, Gwext was chosen for further study in the following experiments.

## 4.4 The Effect of pH on NEP in The Crude Microsomal Fraction

The results in **Table 4.3** showed that Gwext produced inhibition of NEP activity on $^{3}$H(leu)-enkephalin. However the pH of the Gwext solution was found to be lower than pH 7.0 and so it was possible that Gwext's inhibitory activity was due to a lowering of the pH, which inhibited the NEP activity. The following experiments

were designed to check the effect of pH on NEP activity and indeed it showed that the lowering of the pH below 7 does produce an inhibition on NEP activity and so further work was added to prove the inhibitive effects of Gwext.

In this experiment a series of Tris-HCl buffers having each a known pH from 6 to 9 were prepared and used to dilute the rat kidney crude microsomal fraction until the dilution was 1:20. The final pH of the crude microsomal fraction was measured by pH meter and the fractions were assayed (a) for the effect of NEP in the crude microsomal fraction using captopril and bestatin as inhibitors of ACE and APN on enkephalin; (b) For the effect of NEP, ACE and APN combined, with no peptidases inhibitors present. The results were as presented in **Table 4.4a** and graphically in **Figure 4.4.**

**Table 4.4a The Dependence on pH of the Hydrolysis of $^{3}$H(leu)-enkephalin By NEP in the Crude Microsomal Fraction**

| pH of enzyme solution | | On endopeptidase only (CB) | On NEP, APN and ACE (CE only)- no inhibitors |
|---|---|---|---|
| pH of 50 mM Tris-HCl buffer | Final pH of enzyme solution | HD % of $^{3}$H(leu)-enkephalin | HD% of $^{3}$H (leu)-enkephalin |
| 6 | 6.27 | 5.6 | 97.4 |
| 7 | 7.12 | 82.3 | 95.2 |
| 8 | 7.91 | 49.5 | 95.5 |
| 9 | 8.72 | 34.0 | 95.6 |
| 7.4 | 7.40 | 61.6 | 95.6 |
| BFC | | 61.5 | |

NB: BFC: Buffer Control.

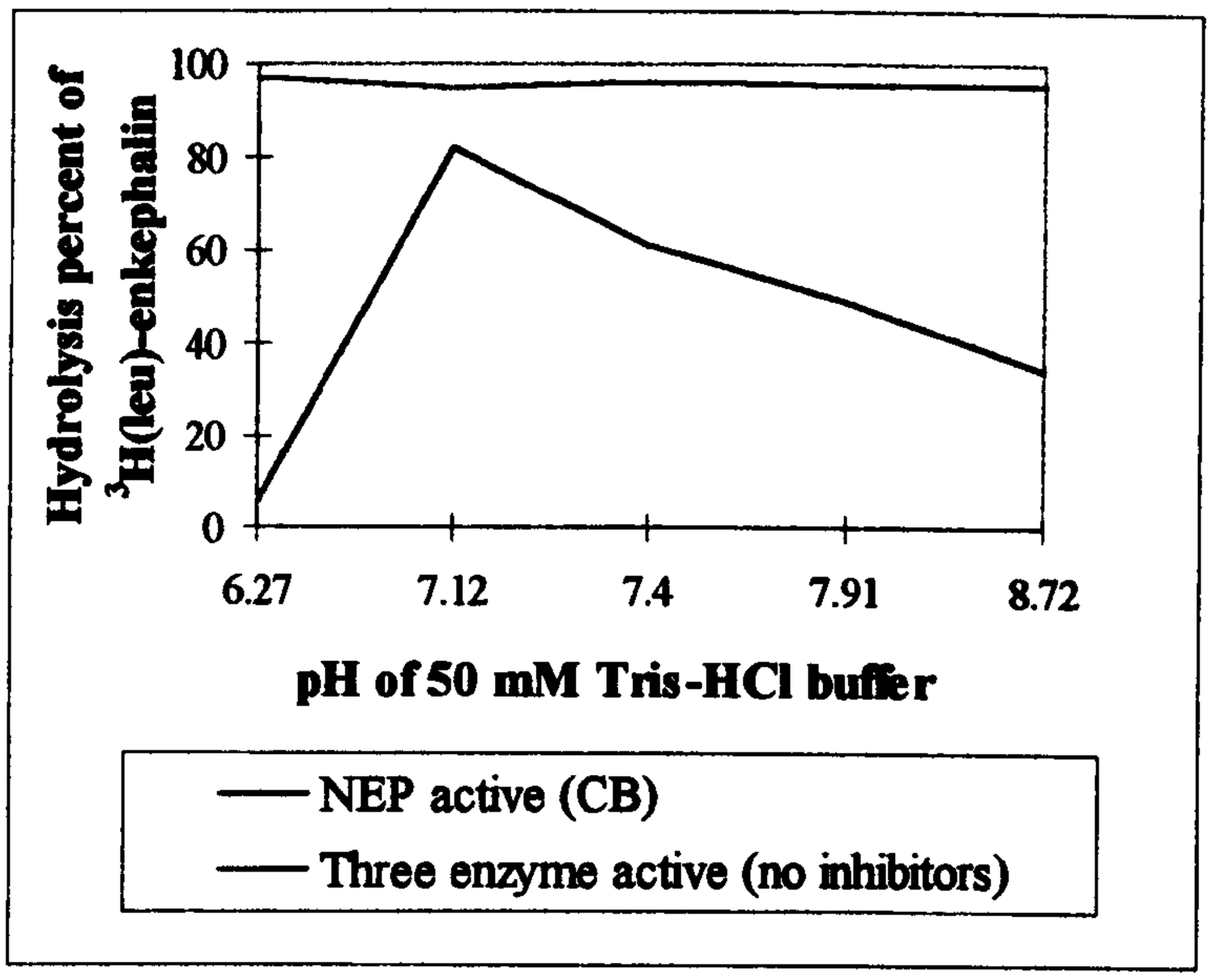



**Figure 4.4 The pH Curve of the Activity of NEP in the Crude Microsomal Fraction**

The buffers were 50 mM of Tris-HCl at the different pHs (undiluted between pH 6 and 9). The enzyme assay was the same as section 2.3.5 of chapter 2.

**Figure 4.4** indicated that pH seemed to have no inhibitory effect on the mixtures of the crude microsomal fraction (red curve), but showed inhibitory activity on NEP. The optimum pH of NEP was 7.12. Lower than 6.27 and higher than pH 8.0 would reduce the NEP activity. This result suggested that 50 mM of Tris-HCl buffer (pH 7.4) can be used as the solvent of enkephalin-hydrolysing assay. Our result about the optimum pH of NEP was compatible with that of Roques *et al.* (1990) who reported an optimum pH for NEP activity was 7.0.

## The Effect of pH on the inhibition of the NEP Hydrolysis of $^{3}$H(leu)-enkephalin by Gwext

Since Tris-HCl buffers could only buffer properly from pH 7.5-8.5, the results of **Table 4.4a** and **Figure 4.4** are reliable over the 7.5-8.5 pH range. The fall off below of pH 7.2 in enzyme activity (hydrolysis percent) could be due to the loss of the buffering effect of Tris ions. This means that pH might have some effect on NEP activity but not completely affect the activity of NEP. In order to find an optimum pH for the Gwext-NEP reaction, two specific pHs were chosen in order to study the

effect of Gwext on NEP activity. The stock enzyme sample was diluted by Tris-HCl buffer at either pH 7.4 or 8.0 until the dilution was 1:20 and then the Gwext's solution was mixed with following mixture:

20 μl of 10 μM Captopril (pH 7.0), 20 μl of Bestatin (pH 7.0), 40 μl of enzyme solution (1:20, pH 7.4 or 8.0), 40 μl of Tris-HCl buffer (pH 7.4 or 8.0).

Under these experimental conditions the maximum volume of the Gwext solution was 80 μl. Gwext solution was mixed with the crude microsomal fraction which was already diluted in 1:20 in buffer at two pHs for about 15 minutes and combined with above mixtures. The hydrolysis percent of $^{3}$H(leu)-enkephalin was measured and the results were as presented in **Table 4.4b**.

**Table 4.4b Determination of the Effect of pH on Gwext-NEP Reaction**

| Classified Group | Hydrolysis percent of $^{3}$H(leu)-enkephalin (HD%) |
|---|---|
| | |
| $E_{8.0}$+50 mM Tris-HCl, pH 8.0 | 45.1 |
| $E_{8.0}$+50 mM Tris-HCl, pH 7.4 | 51.7 |
| $E_{7.4}$+50 mM Tris-HCl, pH 8.0 | 49.3 |
| $E_{7.4}$+50 mM Tris-HCl, pH 7.4 | 56.7 |
| Gwext(100mg/ml)+$E_{8.0}$ | 10.4 |
| Gwext(100mg/ml)+$E_{7.4}$ | 10.3 |
| Buffer Control | 8.4 |

NB: $E_{8.0}$ : The stock crude enzyme sample (NEP active) was diluted to 1:20 by 50 mM Tris-HCl, pH 8.0; $E_{7.4}$ : The stock crude enzyme sample was diluted to 1:20 by 50 mM Tris-HCl, pH 7.4.

The result in **Table 4.4b** indicated that no matter what the pH of the buffer used, to dilute the enzyme, the hydrolysis percent of $^{3}$H-(leu)-enkephalin remained at 50% approximately and there was no significant difference while being compared with each other by means of Student's t-test ( see section 2.5 of Chapter 2 and Appendix. 3) over the range of pHs studied. The Gwext solution was pre-mixed with different buffer-diluted enzyme samples and then the activity of NEP was measured. The

result indicated that Gou still showed strong inhibitory activity on NEP no matter which buffer was used. This directly demonstrated that the pH of the Gou solution itself was definitely not the only influence on the activity of NEP. So primarily it was the active ingredients existing in the Gou solution that inactivated the NEP activity.

## 4.5 Selection of the Best Buffer to Carry Out the Inhibition of Proteolysis Experiments on Gou Aqueous Extract (Gwext)

In the experiments described earlier, the conditions were established for the hydrolysis-inhibition experiments on Gou and on the fractions of the components of the Gou aqueous extract (Gwext) involved in Tris-HCl buffer (a buffering range 7.4 to 8.5). However, all the work by others used pHs of 7.4, where the buffering capacity of Tris is not high. Therefore, other buffers for lower pHs were tried to dissolve up the powder in order to find a suitable buffer. 50 mM Tris-HCl buffer and 50 mM phosphate buffer pH at 7.0 and 7.4 were prepared and then samples of Gwext were dissolved in the buffer at a concentration of 100 mg/ml. The pH was measured to see how effective the buffer had been in stabilising the pH. The results were as shown in **Table 4.5a**.

**Table 4.5a Determination of the pH of Gou in Different Buffers.**

| Classified group | 50 mM Tris-HCl Buffer | | 50 mM Phosphate Buffer | |
|---|---|---|---|---|
| Original pH of buffer | 7.0 | 7.4 | 7.0 | 7.4 |
| pH of the Gwext solution (100mg/ml) | 5.4 | 6.8 | 6.7 | 7.3 |

The results presented in **Table 4.5a** indicated that phosphate buffer was more able to maintain its pH in the presence of Gwext than Tris-HCl buffer. It was necessary to consider the effect of buffer on NEP also, besides the effectiveness of buffering. The enzyme assay was done to check if Phosphate buffer was really suited to be Gwext's solvent and used in our reaction mixture. The results were as presented in **Table 4.5b**.

**Table 4.5b The Effect of Dissolved Gou in Different Buffers on the Crude Microsomal Fraction**

| Classified group | Hydrolysis percent of $^{3}$H(leu)-enkephalin( HD%) | | | |
|---|---|---|---|---|
| | 50 mM Tris-HCl buffer, pH 7.4 | | 50 mM Phosphate buffer, pH 7.4 | |
| | DPM | HD% | DPM | HD% |
| Buffer only | 58361.3 | 55.5 | 53579.9 | 38.9 |
| Gwext+buffer | 53775.6 | 27.9 | 54715.5 | 36.7 |
| Ecoscint H control | 160.0±2.0 | | | |

NB: The Gwext was diluted at 1: 16. The dilution of the enzyme sample: 1:20.

The results in **Table 4.5b** suggested that phosphate buffer itself had an inhibitory activity on NEP and so it was not suited to be a solvent for Gwext. Combining this result with the result presented in **Figure 4.4** and **Table 4.4b**, 50 mM of Tris-HCl buffer (pH 7.4) was still chosen to be used as the solvent of Gwext.

## 4.6 The Concentration Curve of Gwext

After the inhibitory activity of Gwext itself had been confirmed, the dilution curve of Gwext was then tested. The results were as given in **Table 4.6** and as a graph in **Figure 4.6**.

**Table 4.6 Determination of the Concentration Curve of Gwext For its Inhibition of the Hydrolysis of $^{3}$H(leu)-enkephalin in the Crude Microsomal Fraction**

| Classified group | | HD% of $^{3}$H(leu)-enkephalin | Inhibitory activity % |
|---|---|---|---|
| NEP active (CB) | | 50.1 | |
| NEP active (CB) plus : | Conc. of Gwext (mg/ml) | | |
| Gwext | 40 | 3.6 | 92.8 ** |
| | 20 | 6.8 | 86.4 ** |
| | 10 | 9.3 | 81.4 ** |
| | 5 | 19.6 | 60.9 * |
| | 2.5 | 29.0 | 42.1* |
| | 1.25 | 31.3 | 37.5 |
| | 0.625 | 46.7 | 6.8 |
| Buffer control | | 4.7 | |

NB: The inhibitory activity %= (HD% of CB –HD%of Gwext) /HD% of CB ×100%.

The concentration of stock solution of Gwext was 100 mg/ml; Student's t-test was used to analyse the statistically significant difference between each sample group and the CB group. The Probability " P " was shown as follows : * : $P< 0.01$; ** : $P< 0.001$. Ecoscint H control: $30.30 \pm 4.45$.

The results shown in **Table 4.6** indicated that Gwext significantly decreased the hydrolysis percent of $^{3}$H(leu)-enkephalin over a range of concentrations between 2.5 and 40 μg/ml. Gwext could not further significantly decrease the hydrolysis percent while the concentration was lower than 2.5 mg/ml ($P>0.05$). This suggested that the minimum inhibitory concentration (MIC) of Gwext was 2.5 mg/ml ($P< 0.01$).

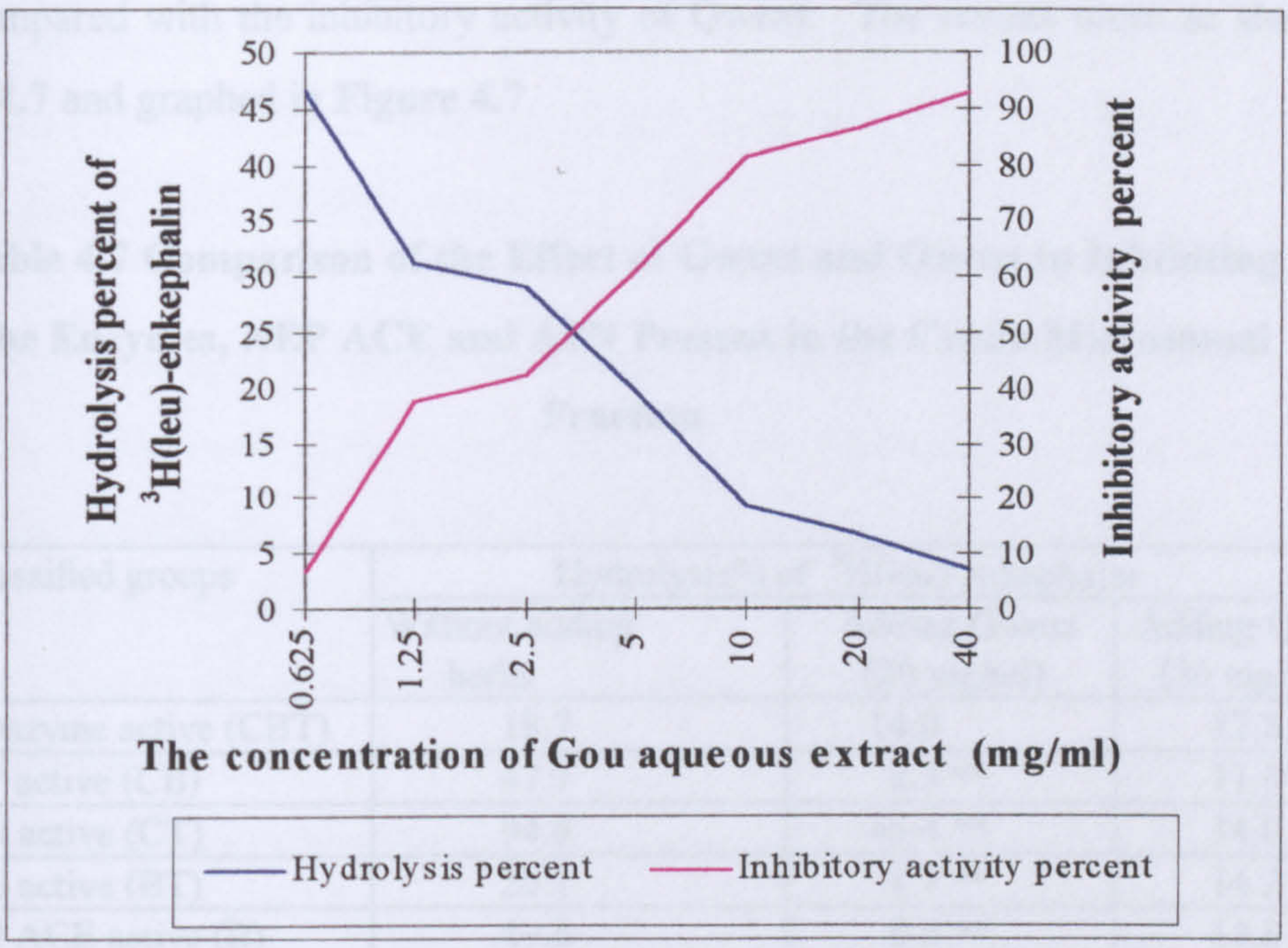



**Figure 4.6 The Concentration Curve of Enkephalin Hydrolysis by NEP in The Crude Microsomal Fraction in The Presence of Gwext**

**Figure 4.6** indicated that the larger the concentration of Gwext the stronger was the inhibition potency. While 0.625 mg/ml of Gwext only induced 6.8% inhibition. 40 mg/ml of Gwext could inhibit 96% of hydrolysis while compared with the CB group. The Inhibitory Concentration ($IC_{50}$ ) of Gwext was 2.7 mg/ml. Therefore the Minimum Inhibitory Concentration (MIC) of Gou was 2.5 mg/ml and $IC_{50}$ of Gwext was 2.7 mg/ml.

## 4.7 The Effect of Gwext on The Other Enzymes (ACE and APN) in The Crude Microsomal Fraction

It was known that there existed three enkephalin-hydrolysing enzymes in the crude microsomal fraction. They were: Aminopeptidase (APN), Angiotensin Converting Enzyme (ACE), and Neutral Endopeptidase 24.11 (NEP). By controlling the use of the three specific inhibitors of the above three enzymes the activity of each enzyme specifically could be studied (see **Table 4.1**). In order to analyse the mechanism of the inhibitory activity of Gwext, its effect on the above three enzymes was observed

and compared with the inhibitory activity of Qwext. The results were as shown in **Table 4.7** and graphed in **Figure 4.7**.

**Table 4.7 Comparison of the Effect of Gwext and Qwext in Inhibiting the Enzymes, NEP ACE and APN Present in the Crude Microsomal Fraction**

| Classified groups | Hydrolysis% of $^3$H(leu)-enkephalin | | |
|---|---|---|---|
| | Without adding herbs | Adding Gwext (20 mg/ml) | Adding Qwext (20 mg/ml) |
| No enzyme active (CBT) | 18.7 | 14.0 | 12.8 |
| NEP active (CB) | 47.7 | 8.3 ** | 11.0 ** |
| APN active (CT) | 94.6 | 46.4 ** | 34.0 ** |
| ACE active (BT) | 20.1 | 8.7 ** | 14.2 * |
| NEP ACE active (B) | 49.6 | 6.9 ** | 13.8 ** |
| NEP APN ACE active (E) | 92.0 | 17.2 ** | 14.9 ** |
| Buffer control | 5.8 | | |
| Ecoscint H Control | 37.09±2.40 | | |

NB: Student t-test was used to analyse the significant difference between the sample group and CB group. Probability P was shown as follows:

*: $P<0.01$; **: $P< 0.001$

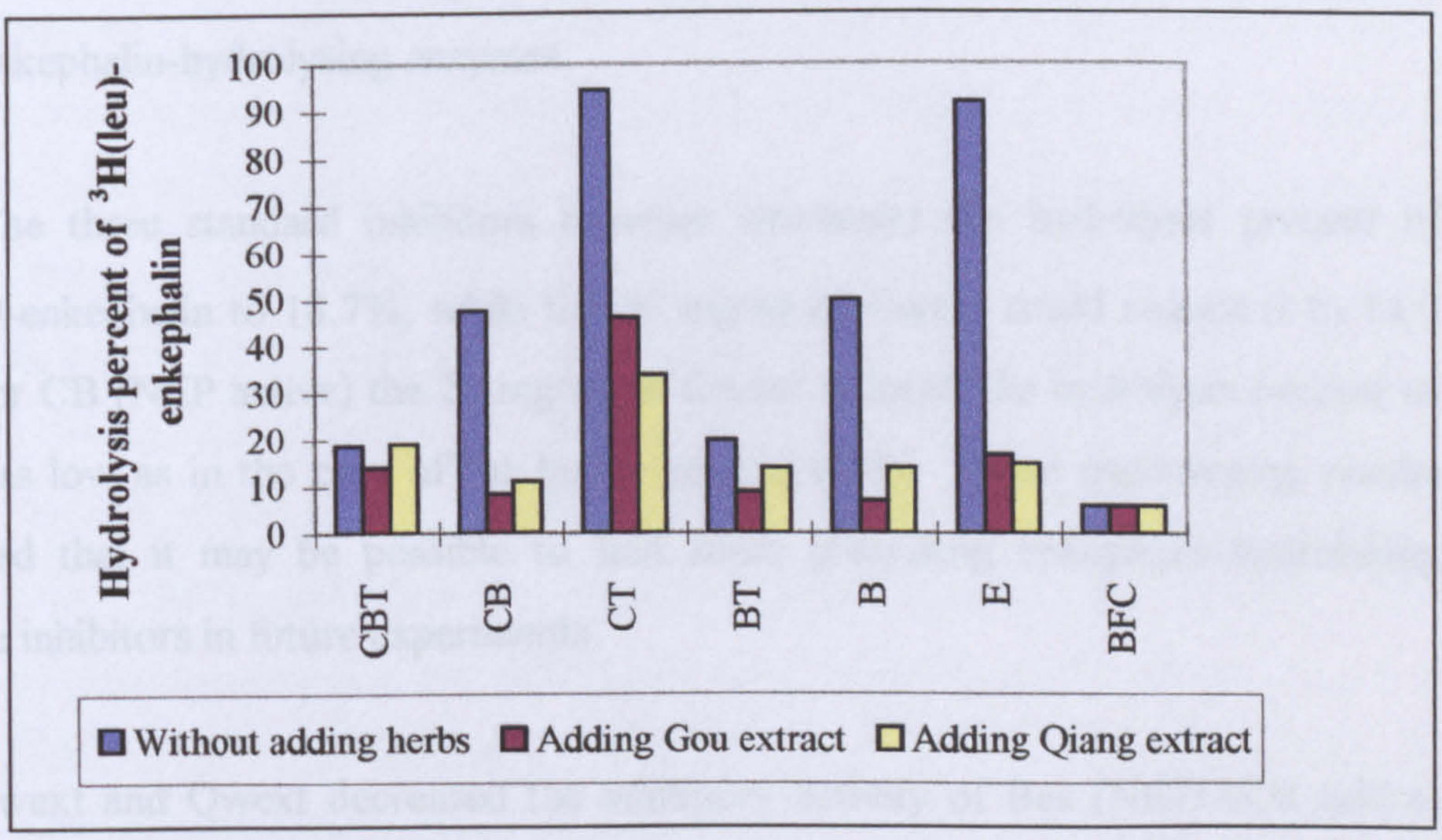



**Figure 4.7 Determination of the Effect of Gwext and Qwext on the Different Enzymes in the Crude Microsomal Fraction (mean of n=3)**

CBT: Cap+Bes+Thio (no enzyme active); CB: Cap+Bes( NEP active); CT: Cap+Thio (APN active); B: Bes only (NEP, ACE active); E: No inhibitors (Three enzyme active); BFC: Buffer control

The results presented in **Table 4.7** and **Figure 4.7** indicated the following four facts:

(1) Both Gwext and Qwext could decrease the hydroysis percent of $^{3}$H(leu)-enkephalin significantly, no matter whether one used CB enzyme (NEP active) or CT(APN active) or BT(ACE active) or B (NEP/ACE active) and even E (Three enzyme active in the crude microsomal fraction). This suggested that both Gwext and Qwext showed inhibitory activity on three enzymes but the mechanism seemed to be different, because Qwext showed lower inhibitory activity on ACE / NEP.

(2) Gwext and Qwext showed significant inhibitory activity on the crude microsomal fraction in this experiment. This result was of importance because it reminded us of the possible side effect of pH on NEP. The results presented in **Fig 4.4** indicated that the pH had no complete inhibitory activity on the crude microsomal fraction, or in other words on the total enzymes existing in the crude microsomal fraction mixture as pHs between 7.14 and 8.7. Also we had reason to believe that Gwext and Qwext really contained some active ingredients which showed inhibitory activity on all the three enkephalin-hydrolysing enzymes.

(3) The three standard inhibitors together decreased the hydrolysis percent of $^{3}$H(leu)-enkephalin to 18.7%, while the 20 mg/ml of Gwext could reduce it to 14.0 %. For CB (NEP active) the 20 mg/ml of Gwext reduced the hydrolysis percent to 8.3%, as low as in the case of the buffer control (5.8). These encouraging results indicated that it may be possible to find some promising enkephalin-hydrolysing enzyme inhibitors in future experiments.

(4) Gwext and Qwext decreased the inhibitory activity of Bes (NEP/ACE active) which suggested that Gwext and Qwext also behaved as the mixed inhibitors of NEP/ACE. These results provided evidence that it may be possible for us to develop new peptidase inhibitors. Therefore more experiments were needed with the intention of extracting new inhibitors of peptidases.

Indeed, the recent studies have shown that co-administration of selective ACE and NEP inhibitors is more effective in animal models of hypertension and congestive heart failure than treatment with either enzyme inhibitor given alone (Seymour *et al*, 1991). On the basis of this premise, a number of groups, have been active in the pursuit of a dual-acting ACE/NEP inhibitor (Gros *et al*, 1991; Roques *et al*, 1993; Trippodo *et al*., 1995; Fink *et al*., 1995). It was found that NEP can degrade the atriuretic peptide to mediate the blood pressure change (Krulan *et al*, 1993; Seymour, 1995 and Robl *et al*, 1996). Therefore the above results are of importance theoretically and practically.

(5) Gwext and Qwext have also been shown to be the triple inhibitors of NEP/APN and ACE. In the three enzyme active (E) group, the hydrolysis percent of $^{3}$H(leu)-enkehaplin decreased from 92.0% to 19.2% (Gwext case) and 14.9% (Qwext case) respectively. Qwext also showed stronger inhibitory activity than Gwext in this case. According to Chen *et al*. (1998), inhibition of APN and NEP involved in the inactivation of the opioid peptides enkephalin completely, produce potent physiological analgesic responses, without major side-effects, in all animal models of pain in which morphine is always active. Dual inhibitors of both enzymes could fill the gap between opioid analgesics, until now, the attempts to find a compound with high affinity both for NEP and APN have failed. They therefore have tried to design dual competitive inhibitors of both enzymes with K-1 values in the nanomolar range. So far the best inhibitors they obtained were 10 times more potent than the described dual inhibitors in alleviating acute inflammatory nociceptive stimuli in mice, thus providing a basis for the development of a family of analgesics devoid of opioid side effects. Nobel *et al*. (1997) also obtained a new compound---RB120 which has passed through all the pre-clinical trials and are now on clinical trials, according to Nobel *et al*. (1997), RB120 is the best reported dual inhibitor of NEP/APN. Our experiment showed that Gwext and Qwext were not only dual inhibitors but also triple inhibitors of NEP/APN/ACE. This suggested that Gwext and Qwext are promising to be investigated further. Of course Gou's MIC was 2.5 mg dried extract of Gou per ml, $IC_{50}$ was 2.7 mg dried extract per ml. The above data are far from nanomolar range, but as both Gwext and Qwext are mixtures of compounds at this stage, the individual compounds in Gwext and Qwext should have much lower MIC

and $IC_{50}$. On another hand the direct analgesic effect of Gwext and Qwext has not yet been investigated. Unfortunately because of limitation of time, only Gwext was further investigated in the following experiments.

## 4.8 The Inhibitory Activity of Gou on the Partially Purified Enzymes (pooled fractions) from the Crude Microsomal Fraction

In order to estimate the inhibitory potency of Gou on NEP, the effect of Gou on pooled fractions of the crude microsomal fractions (see section 3.7 of Chapter 3) further purified on Hydrophobic Interaction Column (HIC) was assayed. Some of these pooled fractions contained partially purified enzymes. All the pooled fractions were diluted at 1:2 and 40 µl of Gou was added into the reaction mixture before carrying out the enzyme assay. The results were as shown in **Table 4.8** and plotted in **Figure 4.8**.

**Table 4.8 The Inhibitory Activity of Gou on The Partially Purified Enzymes (pooled fractions) From The Crude Microsomal Fraction**

| Classified group | Hydrolysis percent of $^3$H(leu)-enkephalin |
|---|---|
| NEP active (Pool 1+CB) | 68.1 |
| NEP active (Pool 3+CB) | 12.5 |
| NEP active (Pool 6+CB) | 33.6 |
| NEP active ( CE+CB) | 64.1 |
| | |
| NEP active (Pool 1+CB+Gou) | 4.9 |
| NEP active (Pool 3+CB+Gou) | 3.7 |
| NEP active (Pool 6+CB+Gou) | 3.3 |
| NEP active (CE+CB+Gou) | 6.9 |
| Tris-HCl buffer control | 5.5 |
| Ecoscint H control | 36.08±8.8 (DPM) |

Note that the presence of Bestatin and Captopril (CB) as ussual leaves NEP only active in the fractions (see **Table 4.1**). All the pooled fractions were added into the reaction mixture at 40 µl. The concentration of the stock solution of Gou was 100 mg/ml. The ilution of the crude microsomal sample was 1:20 and 40 µl of this was used in the reaction mixture.

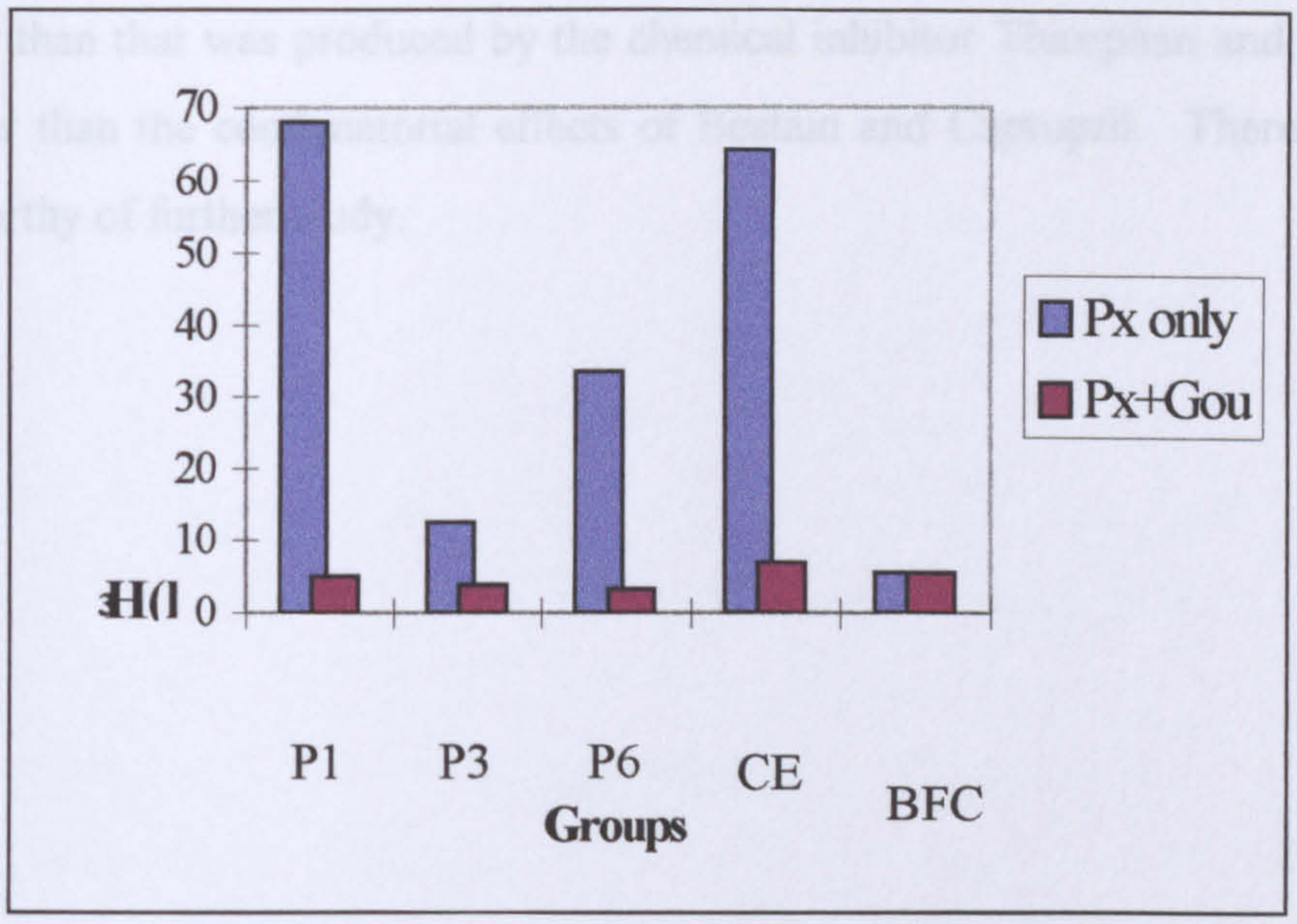



**Figure 4.8 The Inhibitory Activity of Gou on the Partially Purified Enzymes (pooled fractions).**

Px : Pool number; The dilution of Gou was 1:2 and that of the crude enzyme sample (CE) was diluted at 1:20; BFC was the buffer control. In **Figure 3.7** it was shown by PAGE that fractions P1, P3 and P6 contained a gel band (90 kDa band) corresponding to the molecular weight of NEP.

The above results showed that Gou had an inhibitory effect on the partially purified NEP. Because the fractions were pooled and in them the NEP was only partially purified, the above result was not enough to demonstrate the exact inhibition of the hydrolysing activity of NEP. Completely purified NEP was needed in order to obtain the direct evidence for the inhibitory activity of Gwext on NEP. However due to the limitation of time, such an experiment had to be left for the future.

## CONCLUSION AND SUMMMARY

Gwext was found to display strong inhibitory activity on NEP. Its minimum inhibitory concentration (MIC) was 2.5 mg dried extract of Gou per ml, and 50% inhibitory concentration ($IC_{50}$) was 2.7 mg dried extract of Gou per ml for a digestion time of 30 minutes. The inhibitory effect of Gwext was compared with standard inhibitors and it was found that Gwext in the experiment showed stronger inhibitory

activity than that was produced by the chemical inhibitor Thiorphan and it was even stronger than the combinatorial effects of Bestain and Captopril. Therefore Gwext was worthy of further study.

# CHAPTER V

# THE OPIOID RECEPTOR BINDING ASSAY

# INTRODUCTION

## 5.1 Review of the Principles of the Receptor Binding Assay

The receptor binding assays in this study concerned binding to the opiate-peptide receptor in rat brain. The study involved the isolation and characterisation of the receptor and the establishment of a sensitive and specific assay to determine the pharmacological specificity of the retention site of the receptor for binding of naloxone (Holt *et al.*, 1995). The investigation of the effect of some drugs on naloxone binding to the opiate-peptide receptor was then studied. By far the most widely used general method for opioid-receptor-binding assays involves the use of radiolabelled ligand. This inherently simple procedure, first described by Paton and Rang in 1965 has developed into a major activity in both basic research and in the pharmaceutical industry where such assays offer the prospect of a simple rapid screen for the evaluation of drugs which bind to a receptor. In this chapter the naloxone (morphine-antagonist) receptor-binding assay is established in order to study the effect of one Chinese Herb (Gou) on naloxone binding activity.

### 5.1.1 General Strategies for Isolation and Purification of Receptors

Normally receptor isolation and purification can be considered in three basic stages. Firstly the tissue is homogenized in a homogeniser (normally the Potter-Elvejhem type of homogeniser) and the homogenate processed by differential centrifugation to obtain the membranes; The homogenate is then treated with detergent in order to solubilize the receptor which is thirdly purified in an affinity-chromatography step. The successful purifications of the vertebrate n-acetylcholine (nAChR) and the γ-aminobutyric acid receptor complex (GABAR) are based on this general procedure (Lunt, 1980). However it should be emphasized that receptor purification is a relatively young science and there is no guarantee that these methods can be successfully applied to all neuroreceptors. In this chapter only the first step was adopted in order to obtain a crude but fairly reproducible opioid-membrane fraction for the naloxone-binding assay.

### 5.1.2 The Factors Which Affect the Final Results of Opioid Binding Activity

The opioid binding assay usually follows a pattern in which a receptor preparation is exposed to a radiolabelled ligand until an equilibrium is reached, after which time free ligand is separated from the receptor-bound ligand. Such an apparently simple procedure can unfortunately pose a number of practical problems. For example the number of opioid receptor sites with which the radioligand will interact in a specific, physiologically-relevant manner is likely to be a small proportion of the total number of potential binding sites in the tissue preparation (Lunt, 1980). The great majority of non-opiate receptors may well be physiologically irrelevant but may be defined as 'specific' binding sites within the terms of reference of many binding assays. The problem then is not simply to separate specific from non-specific binding but to distinguish between physiological receptors and 'other' binding sites. Therefore a lot of factors have their effects on the final results and so in our experiments these needed to be considered in the detailed design of the experiments.

#### 5.1.2.1 Specific And Non-specific Binding Sites

Firstly the number of receptor binding sites is usually small compared with the total number of sites with which the radiolabelled ligand can interact as shown above. This latter class of sites encompasses binding to the tissue preparation, to assay tubes, to filters and indeed to any part of the experimental system other than the receptor recognition site. The general procedure for distinguishing between the opiate-receptor and other binding sites is to use the 'cold ligand excess' method. In this procedure parallel measurements are made of the binding of radiolabelled ligand in the absence and presence of a large excess of unlabelled ligand (Lunt, 1980). Specific binding is then defined as total binding of radio-labelled ligand (in the absence of unlabelled ligand), minus the non-specific binding of radio-labelled ligand that remains in the presence of unlabelled ligands. The method is based on two assumptions; Firstly that the concentration of non-receptor-binding sites is greatly in excess of that of the receptor-binding sites and, secondly the receptor-bound radioactivity of the radiolabelled ligand is drastically reduced by addition of a large

excess of cold ligand, so becoming insignificant, but the level of non-receptor binding is essentially unchanged. The major shortcoming of the method is that 'specificity' is defined in terms of the relationship between the concentration of radiolabelled ligand and the $K_D$ (equilibrium dissociation constant) of the binding site (Briley *et al*, 1981). Thus any site whose $K_D$ is lower than or comparable with the concentration of radioligand used will emerge as a specific site. It is quite possible therefore that if differing concentration ranges of radiolabelled ligand are used then different 'specific' sites will appear. It frequently is the case that as ligands of higher specific radioactivity become available so workers use them at lower concentrations and find new specific binding sites. In our experiment nM concentration of radiolabelled $^3$H-naloxone were used as the radioactive ligand which is compatible with the concentration usually used in such experiments (Gillan *et al*, 1980).

Another aspect of the opiate-receptor-binding versus the non-opiate-receptor-binding problem is the saturability of the sites. It is often stated rather loosely that non-specific sites are unsaturable (Bielkiwicz, 1985). Clearly this can not be entirely true because no matter what the nature of the site there must be a finite number of them in the particular experimental system that is used. However, it is probable that there are many more non-specific sites than there are specific sites such that, when using concentrations of radiolabelled ligand in the generally used range of $10^{-9}$-$10^{-4}$ M, saturation of the non-specific sites is not approached. Therefore in our experiments we chose μM concentration of cold naloxone and nM concentration of radiolabelled naloxone as the experimental condition.

To some extent this can be achieved by a modification of the assay system so that specific binding of the radiolabelled ligand is assessed by displacement not with cold ligand but with a chemically-distinct agonist or antagonist. In this case the probability of competition between the radiolabelled ligand and the chemically-distinct displacing ligand for any common, non-physiological, high affinity site is greatly reduced. Indeed it was recommended some years ago (Bennett *et al*, 1978) that as a general rule a structurally-distinct ligand should be used for the displacement of specific binding. The extension of this approach is of course to displace the radioligand with a variety of compounds that are known from physiological

experiments to act at the appropriate neuroreceptor site. However in this thesis specific receptor binding was swamped by cold naloxone in experiments to give the non-specific binding of radiolabelled naloxone. The enhanced binding of radiolabelled naloxone in the absence of cold naloxone gave a difference disintegrations per minute count which was interpreted as specific binding of radiolabelled naloxone to opiate receptors. The effect of the Chinese Herb-Gou and other drugs on this receptor binding was used to see if any component of Gou showed specific binding to the opiate receptors.

### 5.1.2.2 Separation of Free From Bound Ligand

**Separation Time**

Whatever the method that is finally chosen, the single most important factor is the time taken to achieve a separation. In the great majority of cases binding measurements are made at equilibrium, therefore as soon as the free ligand is separated from the bound ligand that equilibrium is disturbed and bound ligand will dissociate until a new equilibrium is reached. The equilibrium association rate constant we defined as $k_{+1}$ and the dissociation rate constant as $k_{-1}$. In the majority of receptor binding assays, $k_{+1}$ is of the order of $10^6$ M per second. Most neuroreceptor binding assays are done with ligands with a $K_D$ in the range of $10^{-8}$ M (permitted separation time 10 sec) to $10^{-10}$ M (permitted separation time 15 min). The concentrations used in these experiments of this thesis were in the range of $10^{-9}$ M with permitted separations of the order of a minute or two.

**Vacuum Filtration**

Vacuum filtration offers possibly the fastest and most convenient method of achieving separation times of a few seconds. There are several commercial filtration chambers available; The millipore and Biorad versions are the most widely encountered. With such systems total filtration and washing times of 3-8 sec can be achieved routinely for sample volumes of up to 10 ml, using 2.5 cm diameter filters. Depending on the $K_D$ of the receptor-ligand pair, it may not be possible to wash the

retained material extensively and thus filter assays may suffer from high blank values. Non-specific absorption of free ligand to the filters may also be a problem. This can often be overcome by combinations of a variety of pre-treatments. For example the risk of dissociation of bound ligand during filtration and washing can be minimized by ensuring that all buffers are ice-cold. However even with all these precautions it is emphasized that satisfactory data will be obtained only if the $K_D$ is $10^{-8}$ M or less.

The opioid binding assays provide direct information about the recognition of a ligand by the receptor protein. In terms of physiological function this is only the first step in what may be a complex sequence of events that couples the recognition event to the cellular response. It is necessary therefore to take steps to ensure that the binding activity is indeed an integral part of a physiological receptor mechanism. There are very many examples in the literature of binding assays for almost all known receptor types. The methods follow a common pattern in which the receptor preparation is exposed to a radiolabelled ligand until an equilibration is reached, after which time free ligand is separated from the receptor-bound ligand. Such an apparently simple procedure can unfortunately pose a number of practical problems. Thus the number of neuroreceptor sites with which the radioligand will interact in a specific, physiologically-relevant manner is likely to be a small proportion of the total number of potential binding sites in the tissue preparation. The idea of the ligand-binding assay should be to use purified receptors directly, but in our laboratory conditions such work could not be done because we did not have access to such receptors. Thus the following assay experiments (see Section 5.2) were designed and utilised to investigate the effect of the Gou aqueous extract (see Chapter 2) on the naloxone to opioid receptor-binding activity. In these methods the influence of Gou extracts was assessed by its competition with radioactivity labelled naloxone for binding to the opioid receptors. In this sense the binding of the Gou extract was assessed indirectly since it was the radioactive disintegrations per minute DPM of the $^{3}$H-labelled naloxone which was directly measured.

### 5.1.3 The Kinetics of Opioid-receptor Binding Activity

The kinetics of binding of a ligand to its receptor are very similar to those of enzyme-substrate reactions. In typical ligand binding assays, radiolabelled ligand [$L^*$] is used and the receptor population [R] is provided by a membrane suspension. In such an experiment the following kinetics apply:

$$L^* + R \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} L^*R$$

The equilibrium dissociation constant, $K_D = \dfrac{K_{-1}}{K_{+1}} = \dfrac{[L^*][R]}{[L^*R]}$

If the total concentration of receptors in the tissue preparation is $[R_0]$,

$$[R_0] = [R] + [L^*R]$$

$$[L^*R] = \frac{[L^*][R_0]}{[L^*] + K_D} \qquad (I)$$

Bound ligand [$L^*R$] is usually separated from free ligand [$L^*$] by filtration and the analysis of the radioactivity disintegration counts per minute (DPM) on the filter paper enables the bound ligand to be determined. In practice such DPM values (total DPM) will be made up of two components; DPM due to specific ligand-receptor binding ([$L^*R$] above) and DPM due to non-specific interactions between ligand and other membrane components. These two components can be separated out by including a large excess of unlabelled ('cold') ligand in some incubations as explained in section 5.1. This will displace radiolabelled ligand from all receptor sites, thus giving an estimate of the remaining non-specific bound DPM. Such values can then

be subtracted from total DPM to give specific bound DPM values, as shown in **Figure 5.1** (Gilean *et al*, 1980; Cooper *et al*, 1986). The final curve obtained shows that ligand-receptor interactions are saturable, but the method assumes that there are vast numbers of non-specific binding sites so that, although there is a large excess of unlabelled ligand, there are still plenty of non-specific sites for binding radioactive ligand.

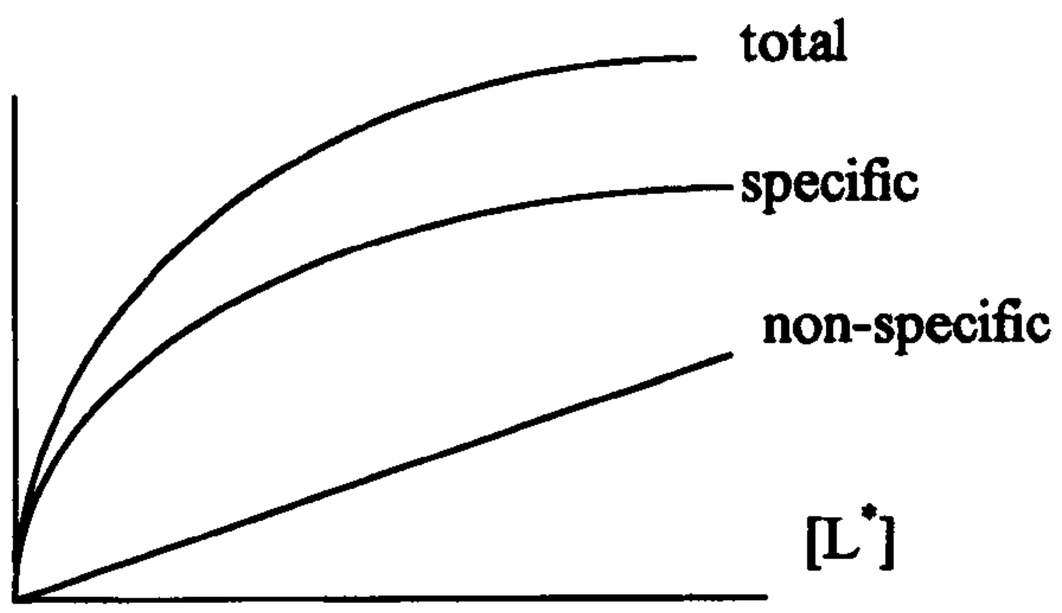



**Figure 5.1 Theoretical Ligand Binding Curve**

By re-arranging equation (I) the familiar Scatchard equation (II) is obtained.

$$\frac{[L^*R]}{[L^*]} = \frac{[R_0]}{K_D} - \frac{[L^*R]}{K_D} \qquad \text{(II)}$$

where, again, $[L^*R]$ refers to specific binding. Thus a graph of $[L^*R] / [L^*]$ (specific binding/free ligand) versus $[L^*R]$ (specific binding ) will (generally) produce a linear plot, enabling $K_D$ and $R_0$ to be determined (see **Figure 5.2**).

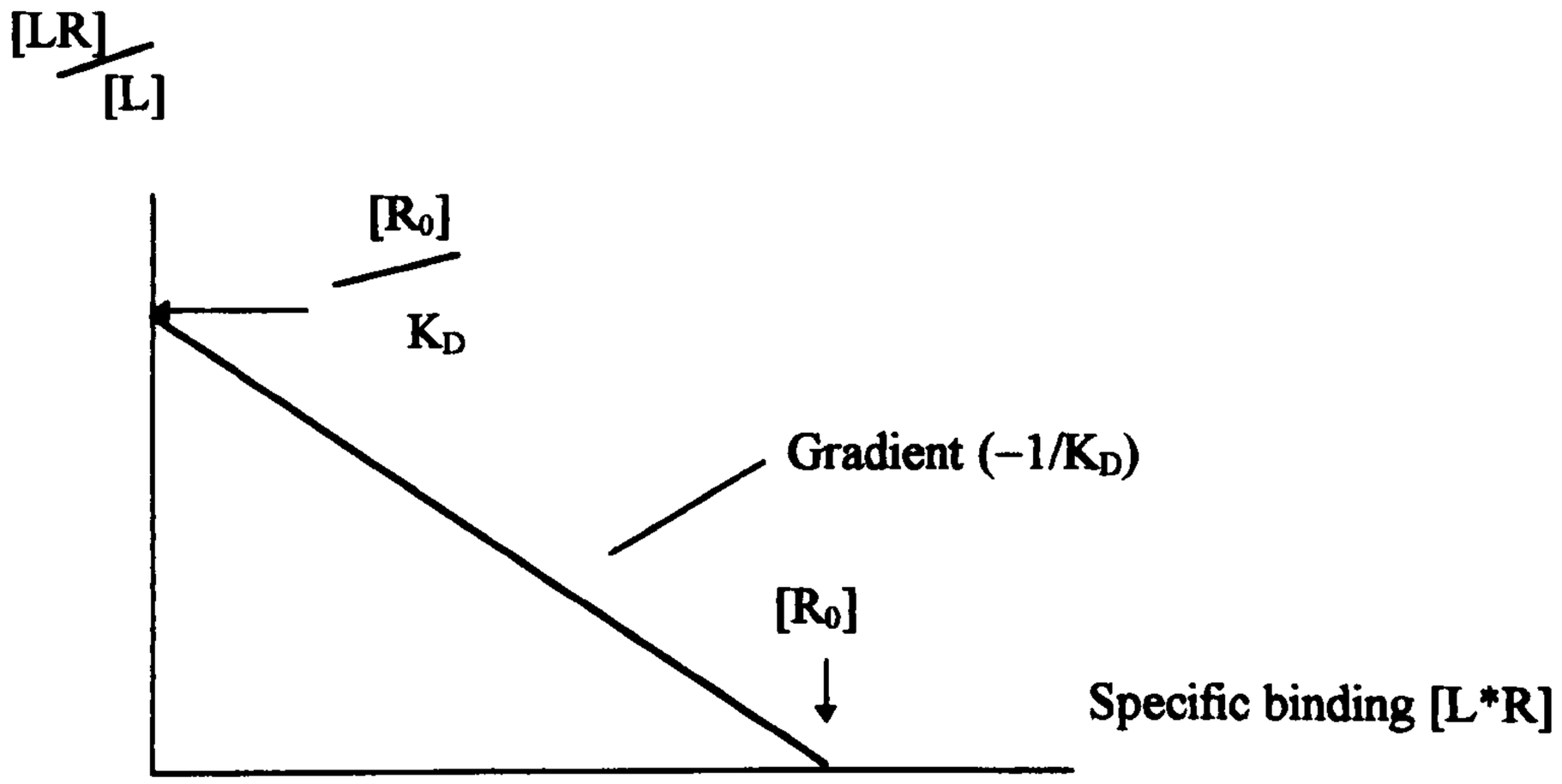



**Figure 5.2 Theoretical Scatchard Plot**

In this chapter the radiolabelled naloxone (N-allyl-2,3-$^3$H) which was purchased from NEN Ltd UK was used as the ligand for brain opiate receptors. This study was

designed to investigate the effect of Gou on opiate receptors. The detailed experimental procedures are discussed in following section (section 5.2).

## 5.2 Method

### 5.2.1 Preparation of Opiate Binding Receptors From Rat Brain Membranes

(Kosterlitz *et al*, 1980)

Rats (adult female wistar, 50 days old, 200g) were maintained in the University's Life-Science-Support unit under standard conditions. They were killed by carbon dioxide ($CO_2$) poisoning, decapitated and the brains rapidly removed. The tissue was then homogenised at low speed, using a citenco disrupter with teflon pestle, in 50 mM of ice-cold Tris-HCl, pH 7.4, 10 ml/g of wet weight of tissue. The homogenate was centrifuged at 49, 000 g for 20 mins at 4°C. The membrane pellet was then gently homogenised in 20 volumes of the Tris buffer and incubated for 45 minutes at 37°C in a shaking water bath. This suspension was then recentrifuged at 49, 000 g for 10 minutes and the pellet was then gently homogenised in 2 volumes of the Tris buffer. Aliquots of this suspension were assayed for protein using the Bradford method with Human Albumin as standard as described in chapter 3. The membrane suspension was then adjusted to a concentration of 10 mg/ml for use in the binding assay.

### 5.2.2 Experimental Methods of Opiate Binding

The ingredients were added to each tube in the order shown in the table overleaf. A typical incubation tube contained the following ingredients in a final volume of 1 ml,

| | |
|---|---|
| Receptor membrane suspension | 1.0 mg |
| [$^3$H]naloxone | 1-15 nM |
| Tris-HCl buffer | 50 mM sometimes with and sometimes without Chinese herbs (0.5 - 8 $mgml^{-1}$) |

Unlabelled naloxone (1-15 μM) was also included in some incubations. The reaction mixture was incubated in a water bath at 37°C for 30 minutes. This gave sufficient time for the binding to reach equilibrium at this temperature. After this time, each mixture was filtered through Whatman GF/B filters on the millipore filter manifold

under vacuum. Each filter was washed with 2×5 ml of ice cold Tris buffer, and placed upon a piece of tissue paper. When filtration was complete the filter papers were placed into scintillation-vial inserts, 5 ml of Ecoscint H was added and the vials were left for 2 days and then placed in the scintillation counter. A 'blank' vial containing only scintillation fluid was included as a control. From the results $K_D$ and $R_0$ were calculated and the changes resulting from the influence of the Chinese herb Gou on the binding activity of naloxone observed.

**Table 5.1 The Reaction Mixture of Naloxone-opioid Receptor Binding Assay**

| Tube No | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| [$^3$H]naloxone (0.1μM) (μl) | 10 1 nM | 101 1 nM | 10 1 nM | 40 4 nM | 40 4 nM | 40 4 nM | 80 8 nM | 80 8 nM | 80 8 nM | 150 15nM | 150 15nM | 150 15 nM |
| Cold naloxone (0.1mM) (μl) | - | - | - | - | - | - | - | - | - | - | - | - |
| Membrane(10mg/ml) suspension (μl) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 50 mM Tris-HCl buff (pH7.4) (μl) | 890 | 890 | 890 | 860 | 860 | 860 | 820 | 820 | 820 | 750 | 750 | 750 |
| Total (μl) | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 |
| Tube No | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
| [$^3$H]naloxone (0.1 μM) (μl) | 10 1 nM | 10 1 nM | 10 1nM | 40 4 nM | 40 4 nM | 40 4 nM | 80 8 nM | 80 8 nM | 80 8 nM | 150 15nM | 150 15nM | 150 15 nM |
| Cold naloxone (0.1 mM) (μl) | 10 1 μM | 10 1 μM | 10 1 μM | 40 4 μM | 40 4 μM | 40 4 μM | 80 8 μM | 80 8 μM | 80 8 μM | 150 15μM | 150 15μM | 150 15μM |
| Membrane (10mg/ml) suspension (μl) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 50 mM Tris-HCl buffer (pH7.4) | 880 | 880 | 880 | 820 | 820 | 820 | 740 | 740 | 740 | 600 | 600 | 600 |
| Total (μl) | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 |

# RESULTS AND DISCUSSION

## 5.3. The Determination of Protein Concentration in the Rat-Brain Membrane Preparation

The Bradford method was adopted to assay the protein concentration of rat brain membranes as described in chapter 3. The results were as shown in the following tables.

**Table 5.2 The Bradford Method to Determine The Protein Concentration of Rat Brain Membrane**

| The Concentration of Human Albumin (mg/ml) | | $A_{595}$ |
|---|---|---|
| 0.0 | | 0.000 |
| 0.2 | | 0.2603±0.0218 |
| 0.4 | | 0.3827±0.008 |
| 0.6 | | 0.5163±0.014 |
| 0.8 | | 0.6645±0.019 |
| 1.0 | | 0.7713±0.018 |
| **Rat brain membrane dilution :** | 1: 40 | 0.1293±0.025 |
| | 1: 20 | 0.2508±0.016 |
| | 1: 10 | 0.4879±0.023 |

**Based on these result, the standard curve was prepared as Figure 5.3.**

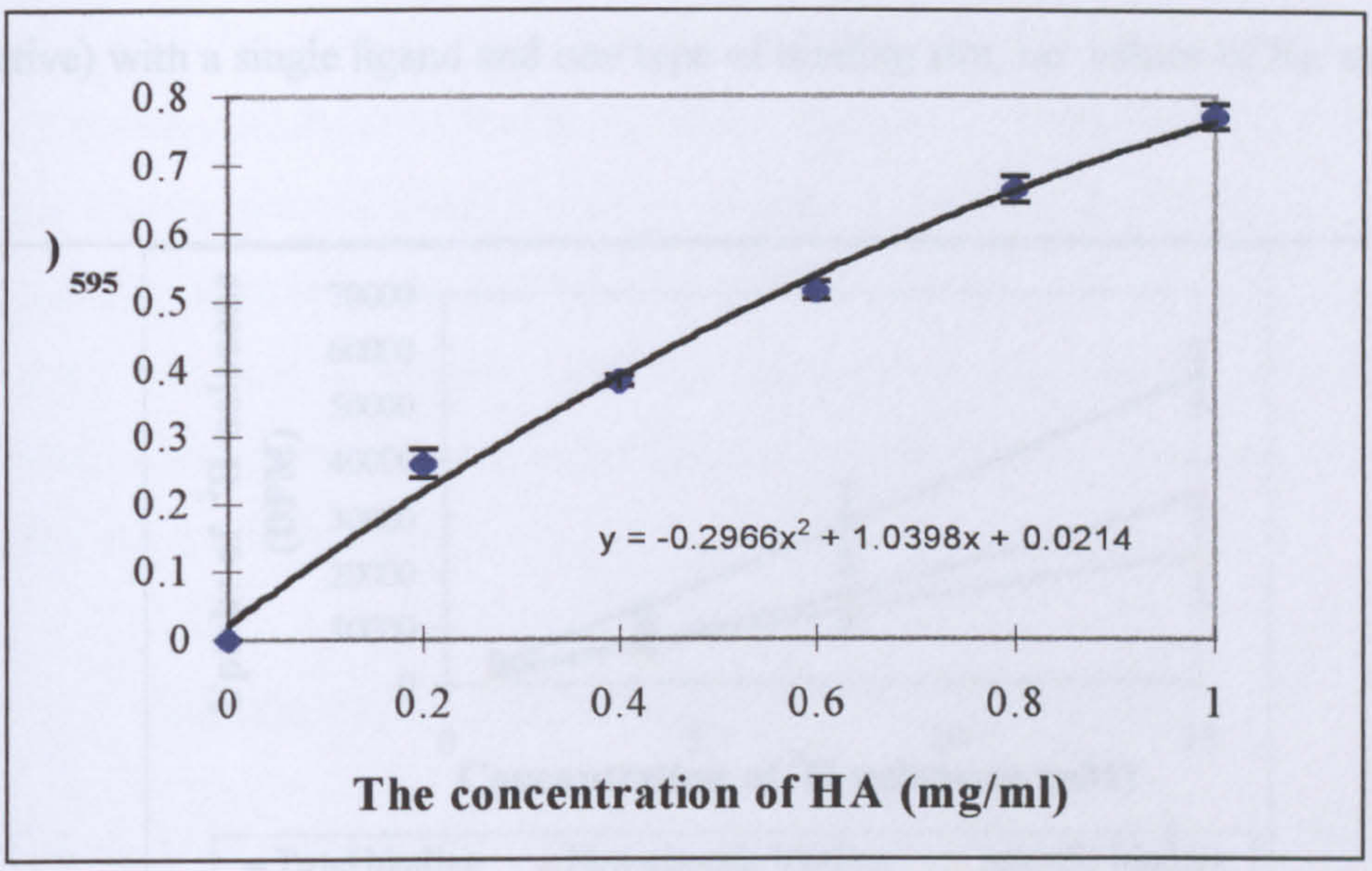



**Figure 5.3 The Determination of the Protein Concentration in the Rat Brain Membrane (Bradford Method)**

The rat-brain membrane was diluted at three dilutions. Human Albumin was used as a control to produce the standard curve.

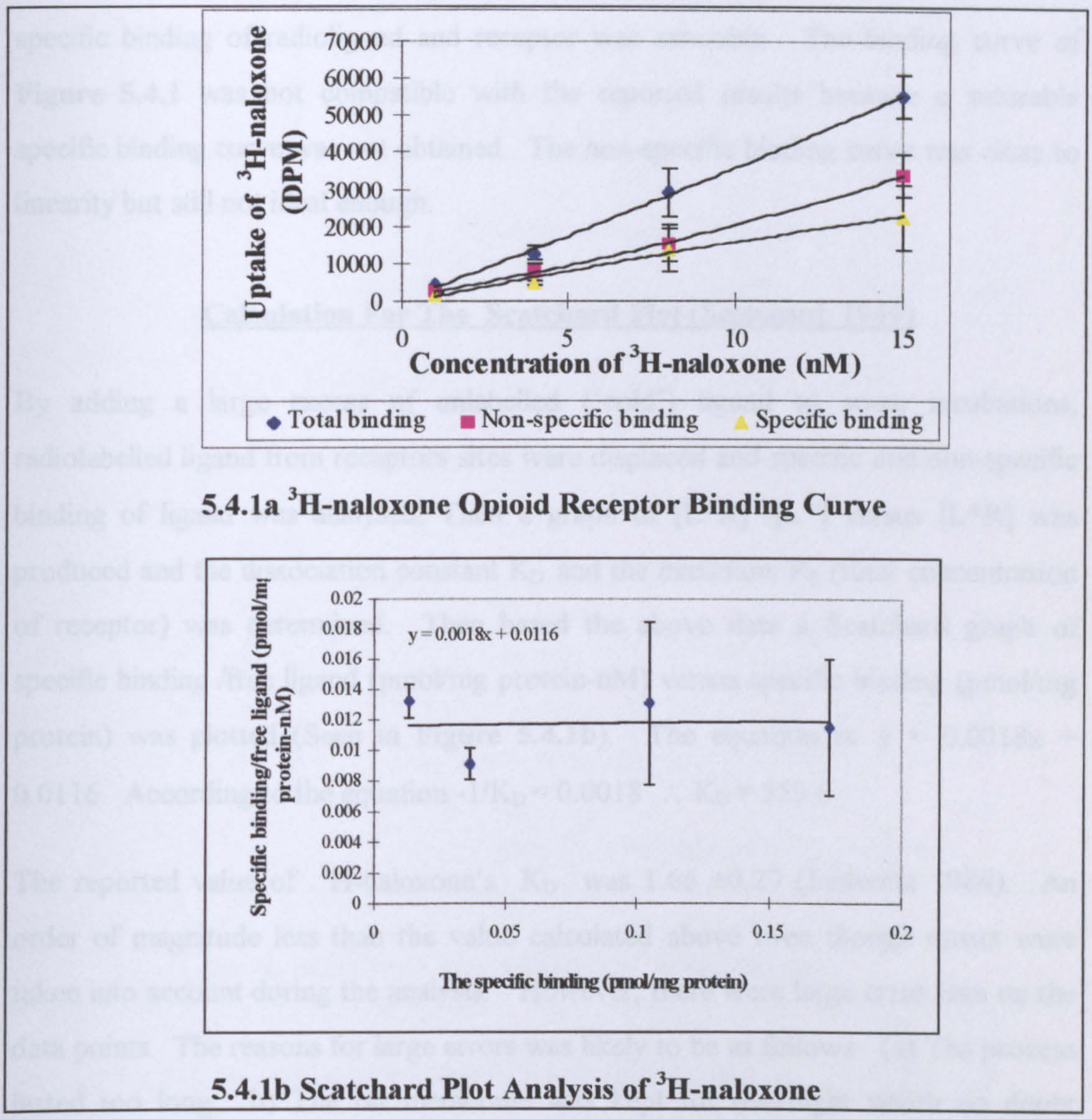
Using the rat brain membrane absorbance of **Table 5.2** and the equation for the curve of **Figure 5.3**, the protein concentrations could be calculated. They were : 0.528 mg/ml for the dilution of 1: 10, and 0.237 mg/ml for the dilution of 1:20, and 0.107 for the dilution of 1:40. Thus the average protein concentration in the original rat brain membrane was 4.77 ±0.50 mg/ml. The final volume of rat brain membrane was 40 mls, so the rat brain membrane contained 40×4.77 = 190.8 mg protein. The protein concentration of rat brain membrane was adjusted finally to 10 mg/ml.

## 5.4 The Design And Establishment of Naloxone-opioid Receptor Binding Assay

Before the activity of Gou was assayed, the naloxone-opioid receptor binding curve had to be established. In detail, 1 to 15 nM of $^{3}$H-naloxone and 1 to15 μM of cold naloxone were mixed separately as described in **Table 5.1**. In all, four batches of rat brain membranes were observed and all the results were as shown in **Figure 5.4.1** to **Figure 5.4.4**. The Scatchard Plots were also prepared for every binding curve so that the $K_D$ and $R_0$ could be obtained, assuming that independent binding (i.e. non-

cooperative) with a single ligand and one type of binding site, i.e. values of $K_D$ and $R_0$

.




**5.4.1a $^3$H-naloxone Opioid Receptor Binding Curve**




**5.4.1b Scatchard Plot Analysis of $^3$H-naloxone**

**Figure 5.4.1 First Binding Curves to The Rat-Brain-Receptor-Preparation of $^3$H-naloxone and Related Scatchard Plot (n=3)**

### (1) First Measurements of Naloxone Binding to the Rat-Brain-Receptor-Preparation

**Figure 5.4.1** shows results from first experiment and related Scatchard plot. From **Figure 5.4.1a** it can be found that the total binding gave the highest dpm count while receptor binding, i.e. specific binding, gave the lowest. Non-specific receptor binding was obtained from the difference between the total binding and the specific binding. The total binding of ligand to receptor (dpm) was very high as it was made up of two

components, dpm due to specific ligand receptor binding and dpm due to non-specific interaction between ligand and other membrane components. Non-specific binding of radioligand should increase linearly with increasing radioligand concentration whilst specific binding of radioligand and receptor was saturable. The binding curve of **Figure 5.4.1** was not compatible with the reported results because a saturable specific binding curve was not obtained. The non-specific binding curve was close to linearity but still not ideal enough.

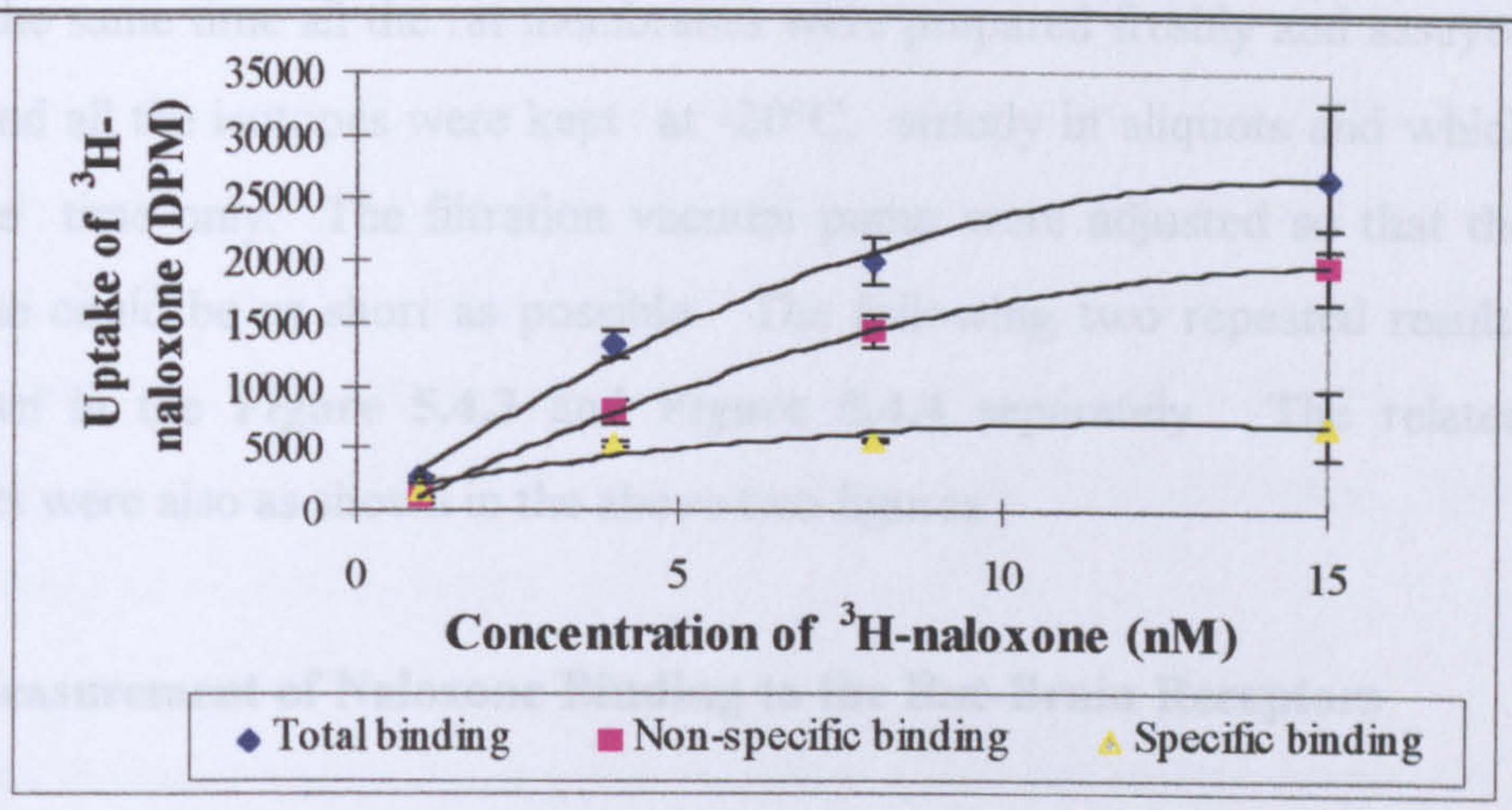
**Calculation For The Scatchard Plot (Scatchard, 1949)**

By adding a large excess of unlabelled ('cold') ligand to some incubations, radiolabelled ligand from receptors sites were displaced and specific and non-specific binding of ligand was analysed. Then a graph of [L*R] /[L*] versus [L*R] was produced and the dissociation constant $K_D$ and the maximum $R_0$ (total concentration of receptor) was determined. Then based the above data a Scatchard graph of specific binding /free ligand (pmol/mg protein-nM) versus specific binding (pmol/mg protein) was plotted (Seen in **Figure 5.4.1b**). The equation is: y = 0.0018x + 0.0116. According to the equation $-1/K_D = 0.0018$ $\therefore$ $K_D = 555.6$

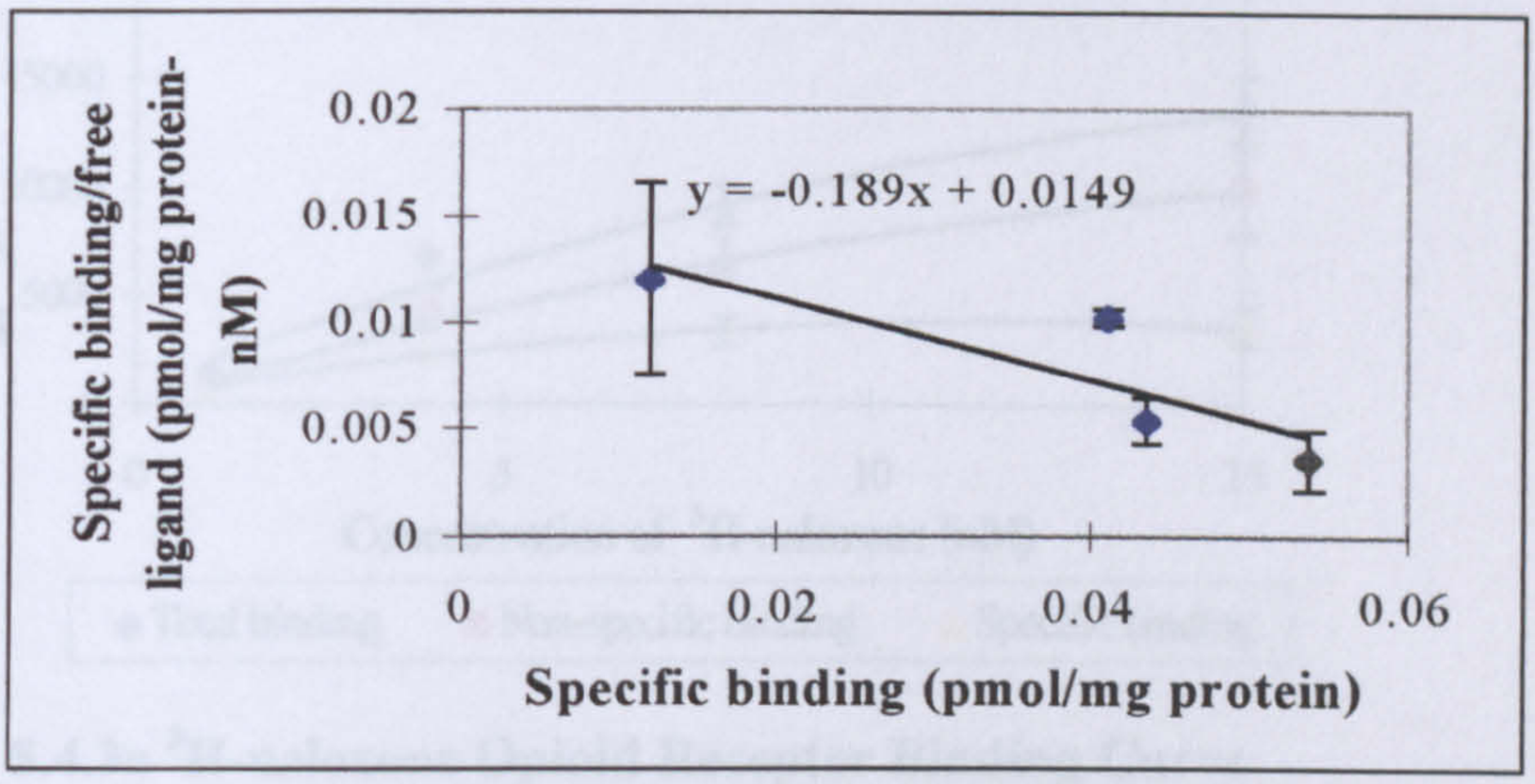
The reported value of $^3$H-naloxone's $K_D$ was 1.66 ±0.29 (Josterlitz 1984). An order of magnitude less than the value calculated above even though errors were taken into account during the analysis. However, there were large error bars on the data points. The reasons for large errors was likely to be as follows: (a) The process lasted too long; b) The rat membrane was kept for overnight which no doubt inactivated some receptor sites. Therefore the experimental conditions was needed to specified more clearly and experimental manipulations done more quickly and carefully.

**(2) Later Measurement of Naloxone Binding to the Rat-Brain Receptors**

In the second experiments the rat membranes were prepared freshly and assayed immediately. A more accurate filtration step was also adopted so that the separation time could be reduced. Otherwise the results were obtained as " first binding curve " and the results were as shown in **Figure 5.4.2**.




**5.4.2a $^3$H-naloxone Opioid Receptor Binding Curve**



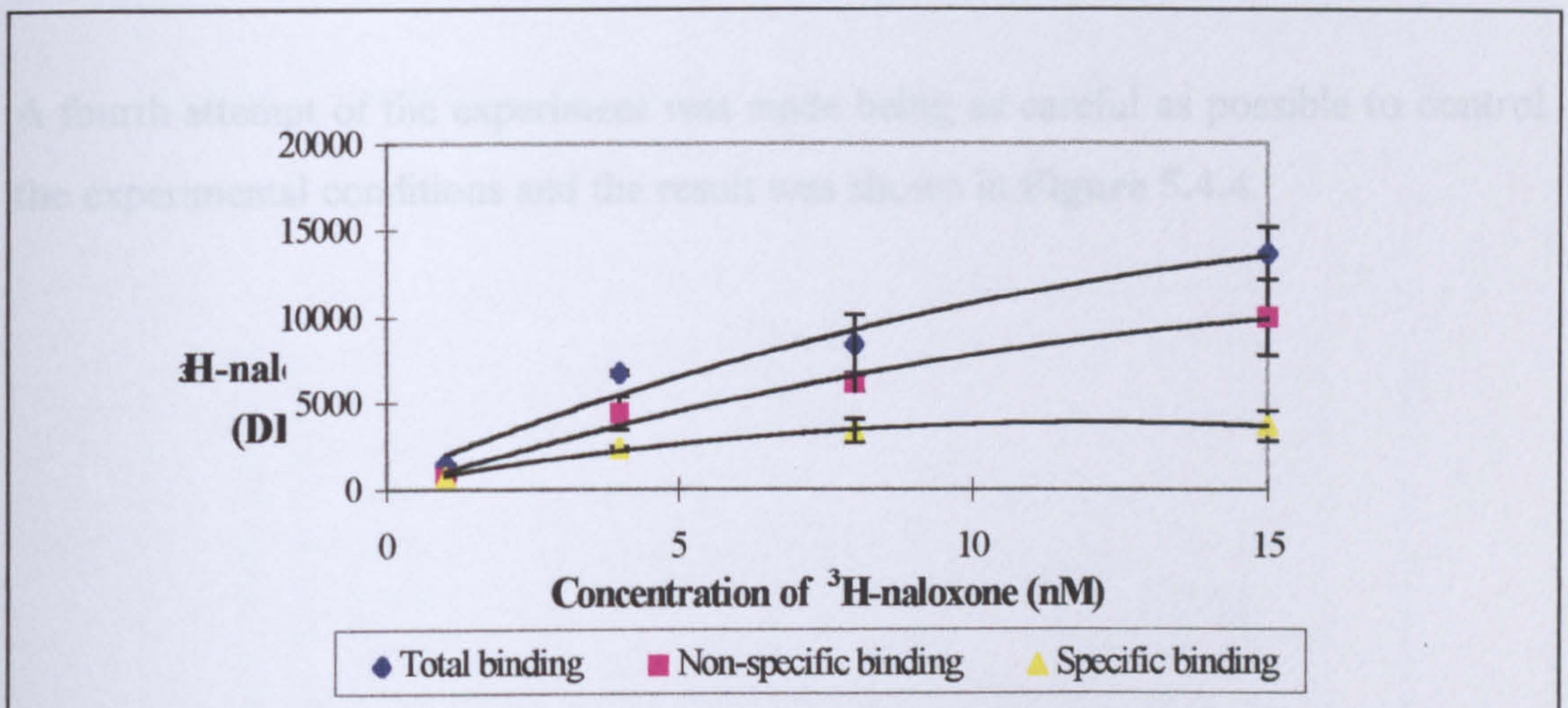

**5.4.2b Scatchard Plot Analysis of $^3$H-naloxone**

**Figure 5.4.2 Later Binding Curves to the Rat-Brain-Membrane-Receptor Preparation of $^3$H-naloxone and the Related Analysis (n=3)**

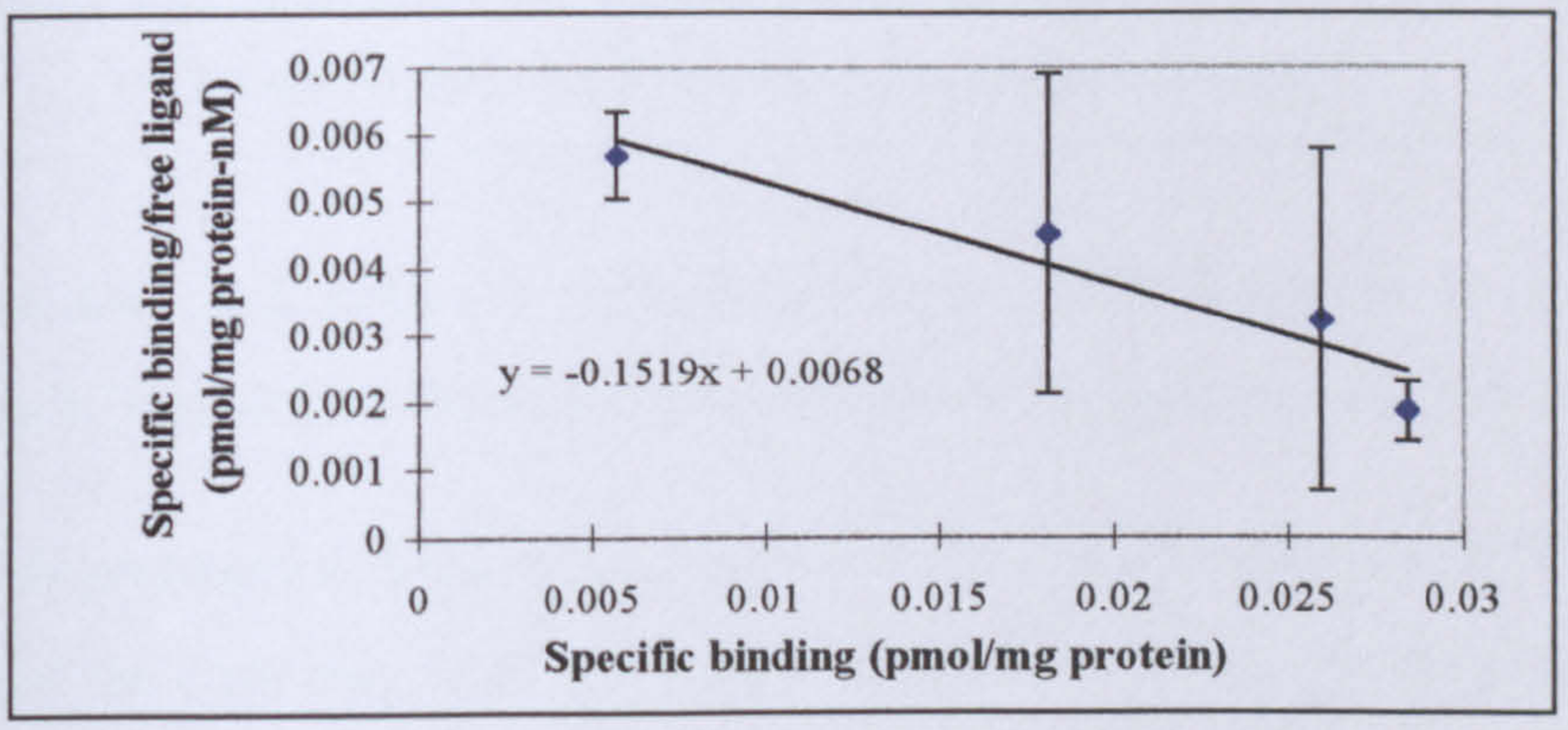
From **Figure 5.4.2** it can be found that the specific binding became saturable while non-specific binding was still not ideal enough. The Scatchard plot showed that y = -0.189x + 0.0149, giving $-1/K_D = -0.189$ $\therefore K_D = 5.29$ and $R_0 = 0.0149/0.189 = 0.0788$ pmol/mg protein. According to the reference (Kosterlitz, 1985) the $K_D$ of $^3$H-naloxone was 1.66±0.29 which is still statistically different from the above experimental value. This suggested that the experimental conditions were still not accurate enough and more improvements were needed. The experiment was repeated several times and every step of the procedure was done as carefully as

possible. At the same time all the rat membranes were prepared freshly and assayed immediately and all the isotopes were kept at -20°C, strictly in aliquots and which were used one time only. The filtration vacuum pump were adjusted so that the separation time could be as short as possible. The following two repeated results were as shown in the **Figure 5.4.3** and **Figure 5.4.4** separately. The related Scatchard plots were also as shown in the above two figures.

**(3) Third Measurement of Naloxone Binding to the Rat-Brain Receptors**




**5.4.3a $^{3}$H-naloxone Opioid Receptor Binding Curve**



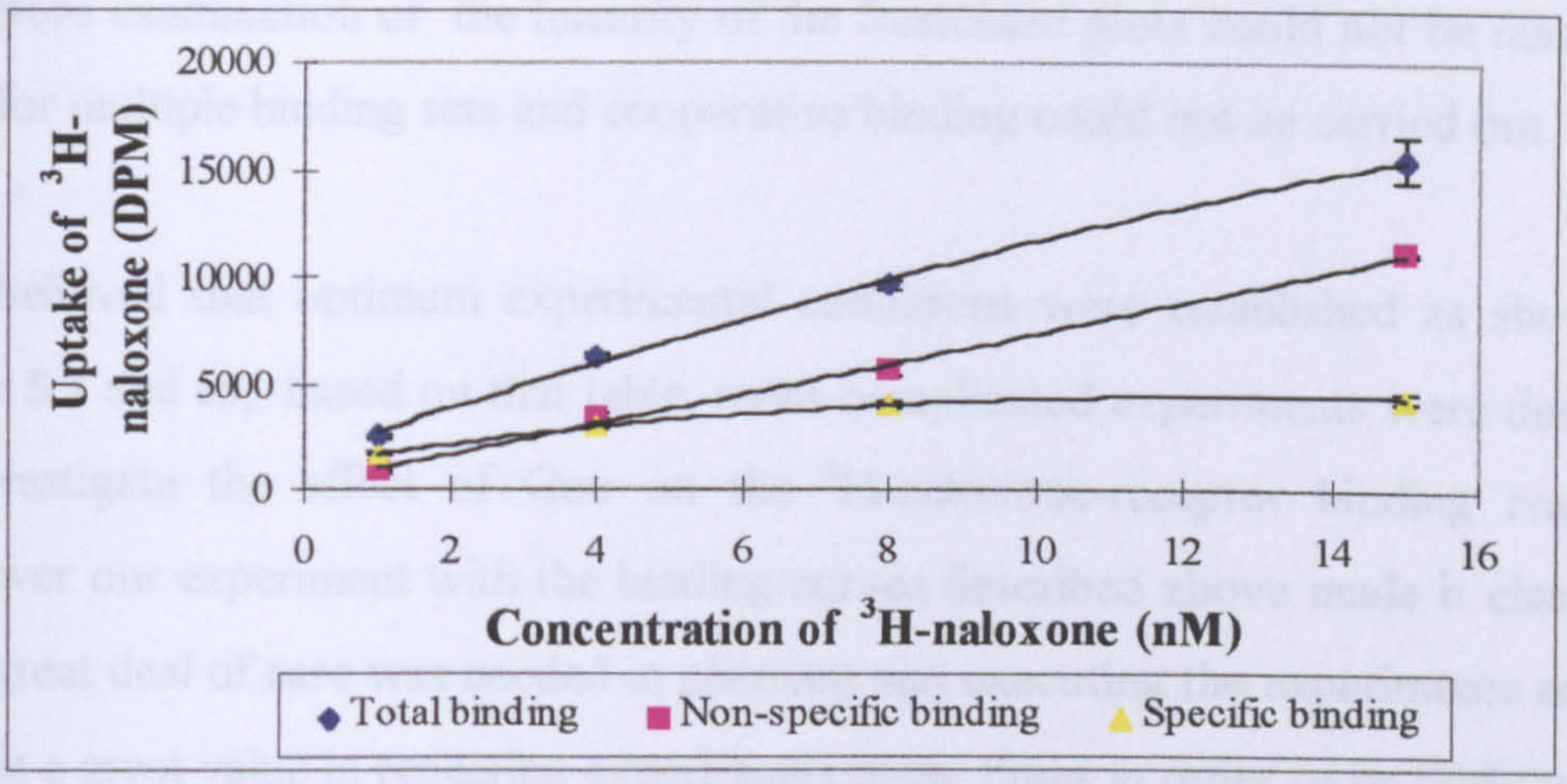

**5.4.3b Scatchard Plot Analysis of $^{3}$H-naloxone**

**Figure 5.4.3 A Third Set of Binding Curve to Rat-Brain-Membrane-Receptor-Preparation of $^{3}$H-naloxone and Related Analysis (n=3)**

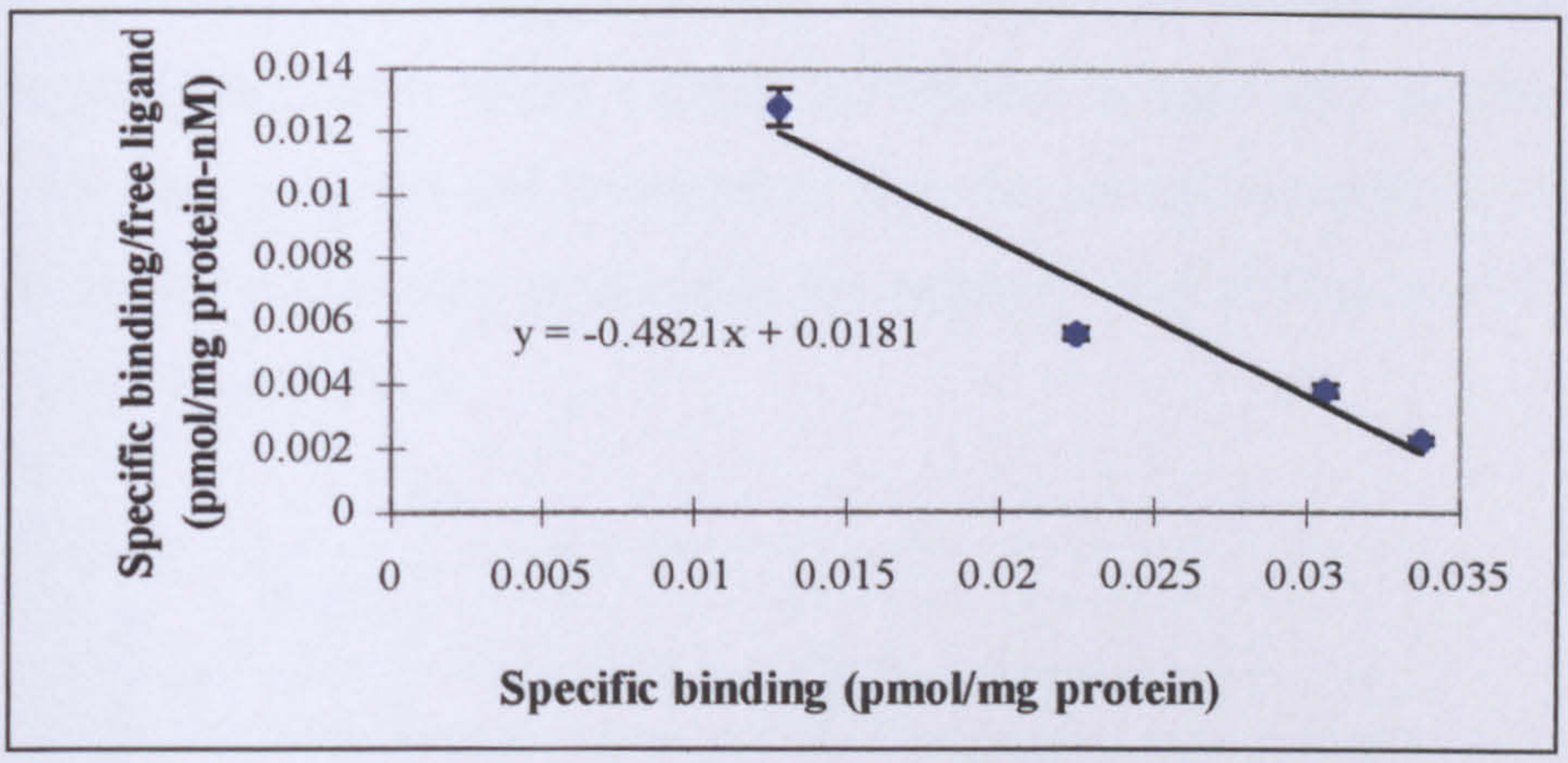
**Figure 5.4.3a** gave the quite good binding curve: the specific binding was saturable; The total binding was curved; The non-specific binding was unsaturable and approximately showed a straight line graph. The Scatchard plot also showed that $y = -0.1519x + 0.0068$; giving $-1/K_D = -0.1519$ or $K_D = 6.58$, with an $R_0$ value of $R_0 = 0.0068/0.1519 = 0.0448$ pmol/mg protein. Compared with work of Kosterlitz the $K_D$ was still too high. However the error bars were still large. This affects the accuracy of $K_D$ and $R_0$ values.

### (4) Fourth Measurement of Naloxone Binding to the Rat-Brain Receptors

A fourth attempt of the experiment was made being as careful as possible to control the experimental conditions and the result was shown in **Figure 5.4.4**.




**5.4.4a $^{3}$H-naloxone Opioid Receptor Binding Curve**




**5.4.4b Scatchard Plot Analysis of $^{3}$H-naloxone**

**Figure 5.4.4 A Fourth Set of Binding Curves of $^{3}$H-naloxone to the Rat-Brain-membrane-Receptor-preparation and Related Analysis (n=3)**

The result presented in **Figure 5.4.4** gave a much better binding curve (fourth Curve) finally and the error bars were also small. In this case the $K_D$ was equal to 1/0.4821= 2.07 nM which correlated well statistically with the work of Kosterlitz with a P value greater than 0.05 (Student's t test). The $R_0$ was calculated to be 0.0181/0.4821 = 0.0375 pmol/mg protein. It seems therefore that there is a high affinity of the ligand naloxone to the opiate receptor and this is why only a low dose of naloxone is required in the clinical treatment of morphine overdose. Only one $R_0$ value was obtained from the Scatchard graph, this assuming that the receptor had only one type

of binding site. However our experiments were restricted to only four data points. Therefore examination of the linearity of the Scatchard plots could not be made and tests for multiple binding sets and cooperative binding could not be carried out.

It is believed that optimum experimental conditions were established as shown in **Table 5.1** and so, based on that table, more complicated experiments were designed to investigate the effect of Gou on the $^{3}$H-naloxone-receptor binding reaction. However our experiment with the binding curves described above made it clear that (a) a great deal of care was needed in planning and executing the experiments and (b) there is a great value in repeating experiments many times in order to be fairly sure of the data and statistical accuracy of the results.

## 5.5 The Effect of Gou on Ligand-receptor Binding Activity

After the establishment of the opioid binding assay, the effect of Gou on this assay was investigated.

The following reaction mixture was prepared,

150 μl of 0.1 μM $^{3}$H-naloxone

5-80 μl of 100 mg/ml Gou aqueous extract

or 150 μl of 0.1 mM cold naloxone

100 μl of 10 mg/ml rat brain membranes

Then 50 mM Tris-HCl buffer (pH 7.4) was added until the final volume was 1000 μl.

The reaction mixture was incubated at 37°C for 30 minutes and then filtered using a Millipore Manifold Tower under vacuum conditions. In total four batches of rat membranes were prepared and observed so that the enough repeatibility could be obtained. All the results were as shown in the graphs plotted in **Figures 5.5.1**, **5.5.2** and **5.5.3 and Figure 5.5.4**.

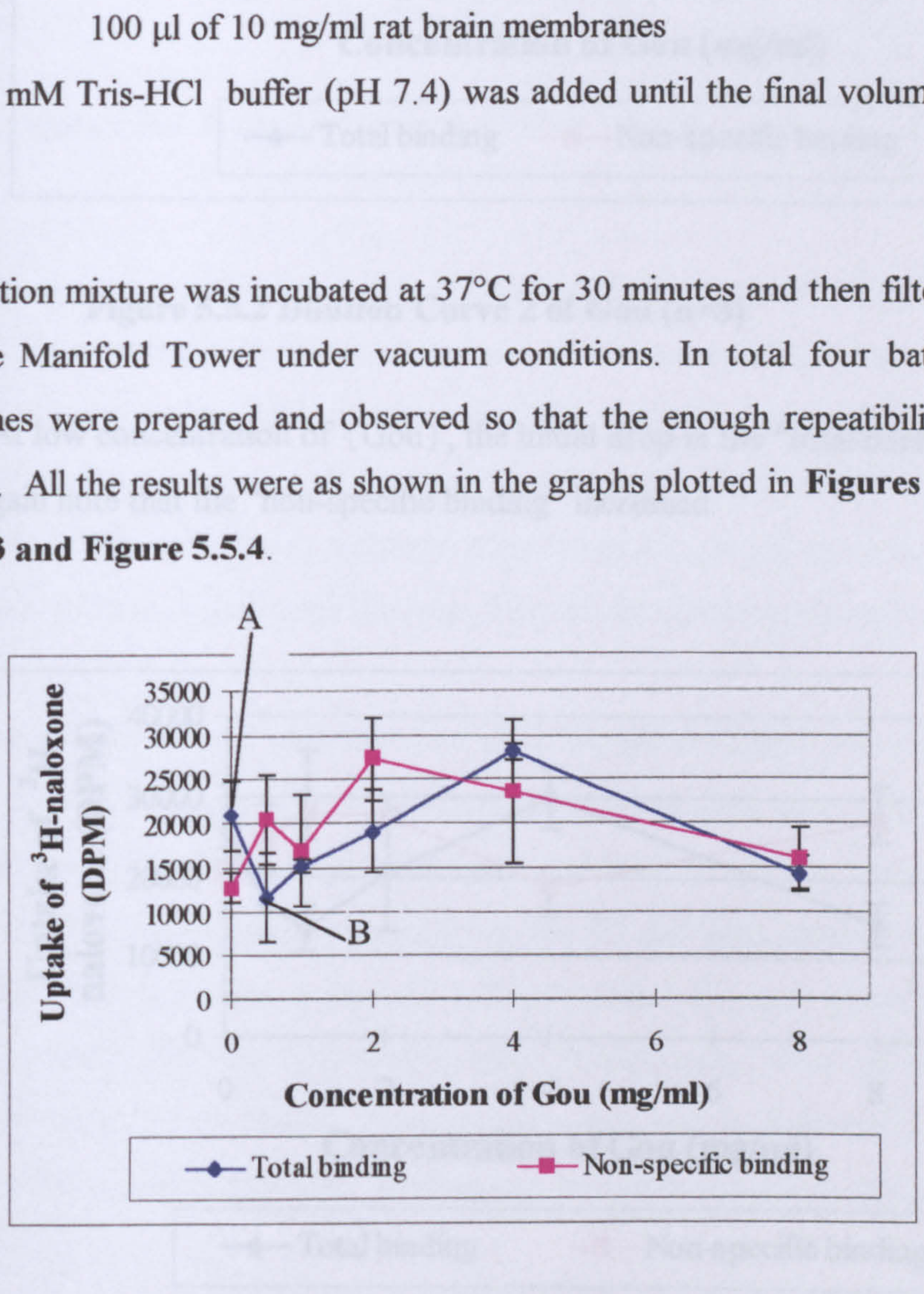



**Figure 5.5.1 Dilution Curve 1 of Gou (n=3)**

NB: The drop in the "total binding" curve from A to B showed low Gou concentrations in spite of an apparant trend where the "non-specific binding " increased.

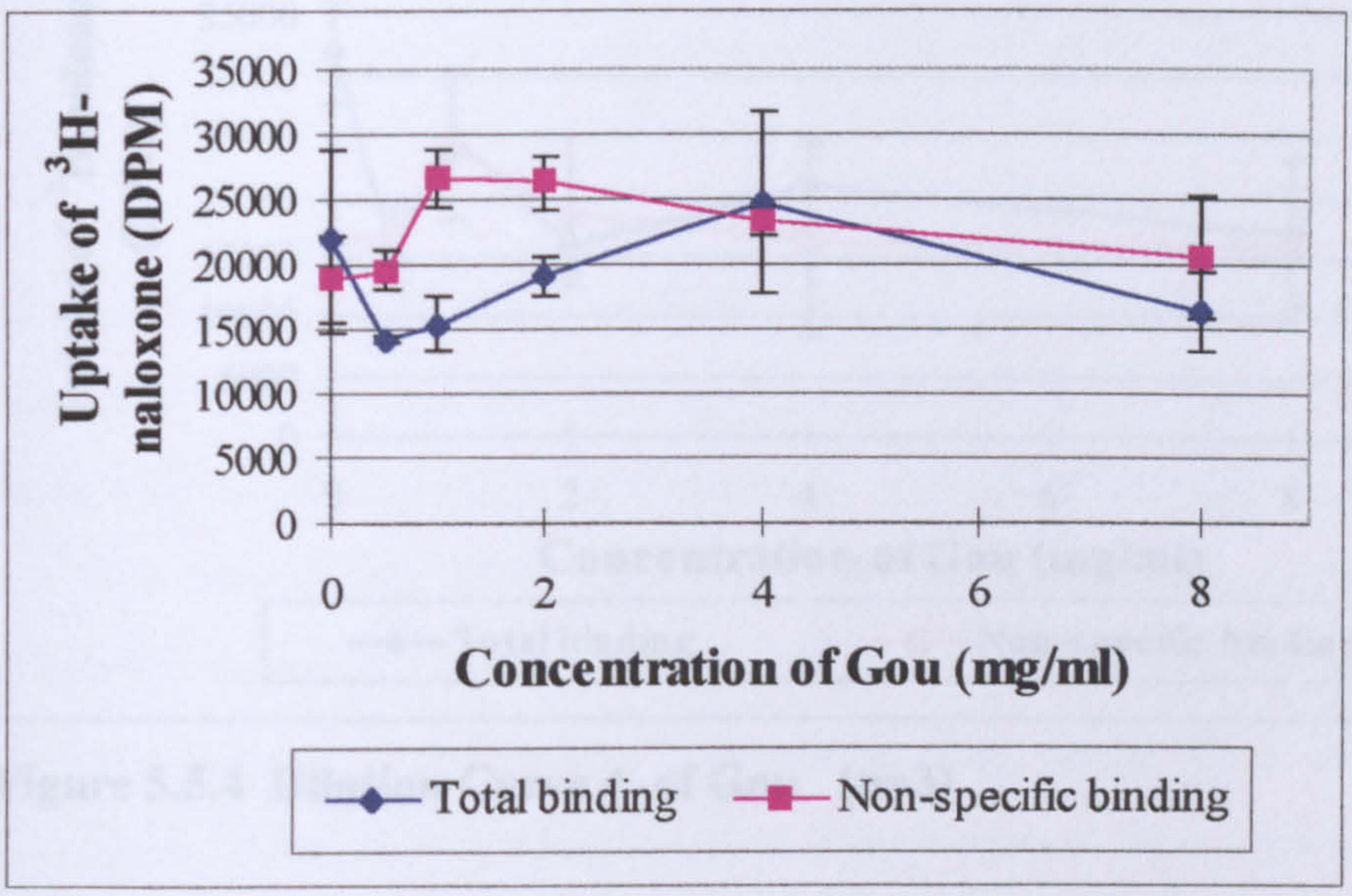



**Figure 5.5.2 Dilution Curve 2 of Gou (n=3)**

Note: At low concentration of [Gou], the initial drop in the "total binding ". Once again note that the "non-specific binding" increased.

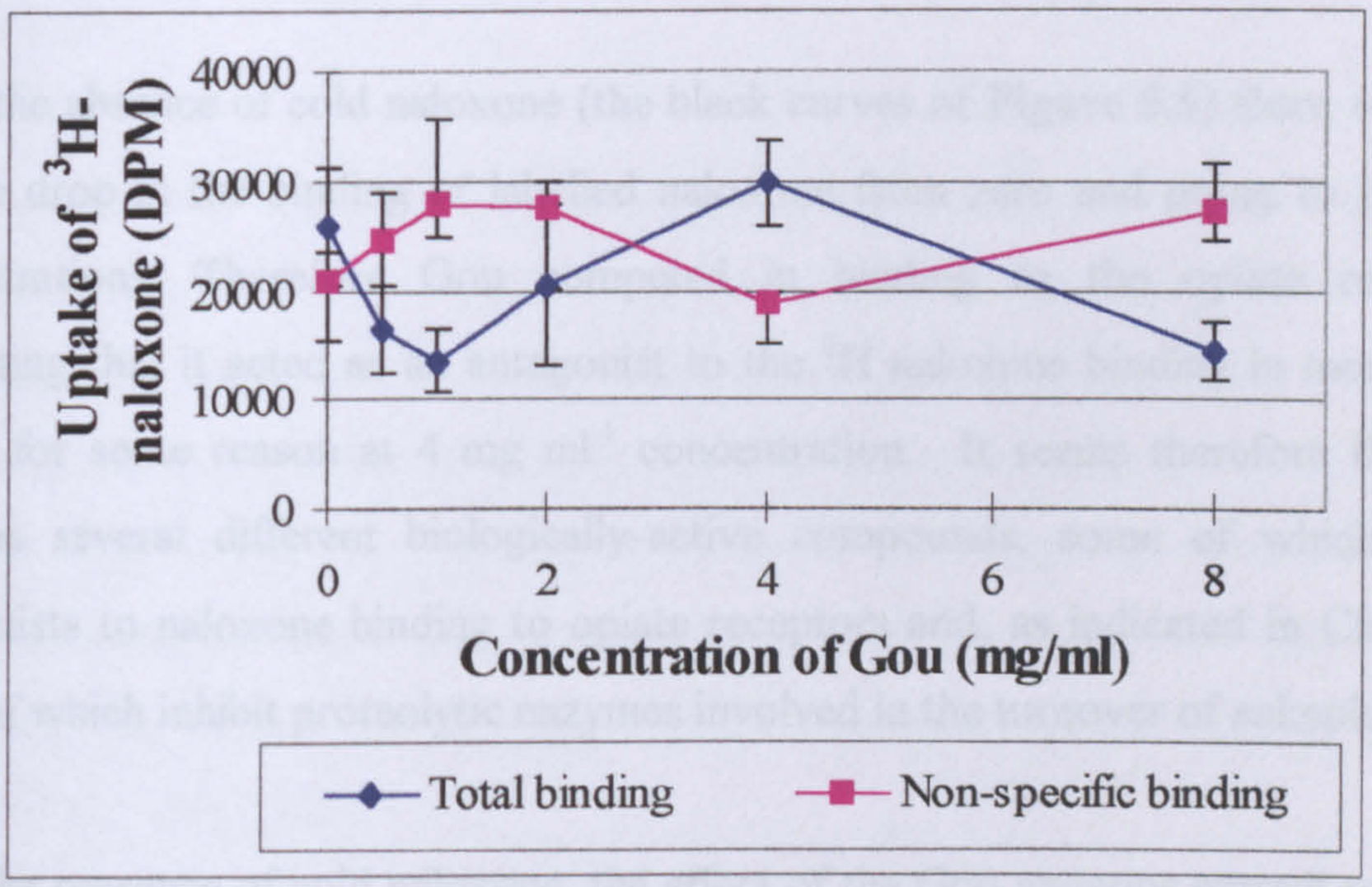



**Figure 5.5.3 Dilution Curve 3 of Gou (n=3)**

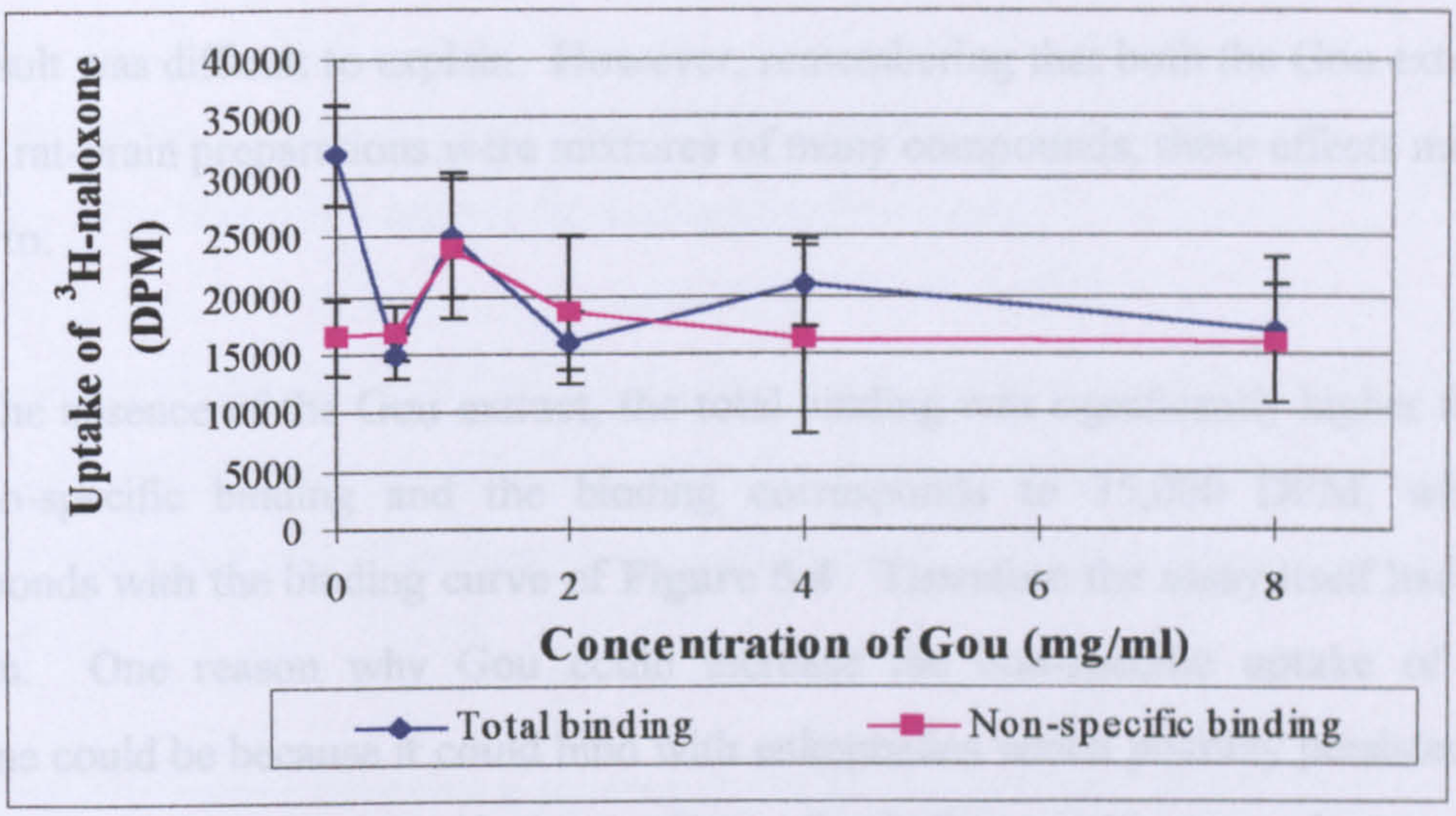



**Figure 5.5.4 Dilution Curve 4 of Gou (n=3)**

From **Figure 5.5.1 to Figure 5.5.4** it could be found that, even if all experimental conditions were exactly the same so far as it could be established, the result was still variable to some extent. This demonstrated that the naloxone-binding assay was not extremely repeatable. However the results presented in **Figure 5.5.1 to Figure 5.5.4** did show trends which are strongly suggestive of an antagonistic effect of a component of Gou on naloxone binding. The results may be summarised as follows:

1) In the absence of cold naloxone (the black curves of **Figure 5.5**) there was <u>in all cases</u> a drop in the binding of labelled naloxone from zero and going to low Gou concentrations. Therefore Gou competed in binding to the opiate receptors, suggesting that it acted as an antagonist to the $^{3}$H naloxone binding in most cases, except for some reason at 4 mg ml$^{-1}$ concentration. It seems therefore that Gou contains several different biologically-active compounds, some of which act as antagonists to naloxone binding to opiate receptors and, as indicated in Chapter 4, some of which inhibit proteolytic enzymes involved in the turnover of enkephalins.

2) In the presence of cold naloxone, the effect of the Gou aqueous extract on the $^{3}$H naloxone binding reaction became complicated: 1 to 2 mg. ml$^{-1}$ of Gou seemed to be able to <u>increase</u> the non-specific binding up to three times the DPM count compared with the control experiment at zero concentration of Gou. However at 4 mg. ml$^{-1}$ there was no significant difference with the control group at zero Gou concentration.

This result was difficult to explain. However, remembering that both the Gou extract and the rat-brain preparations were mixtures of many compounds, these effects might be due to:

a) In the absence of the Gou extract, the total binding was significantly higher than the non-specific binding and the binding corresponds to 35,000 DPM, which corresponds with the binding curve of **Figure 5.4**. Therefore the assay itself had no problem. One reason why Gou could increase the non-specific uptake of $^{3}H$ naloxone could be because it could bind with enkephalins which possibly persisted in the rat-brain-membrane preparations. The enkephalins could then change the mechanism of the $^{3}H$ naloxone-binding reaction in the mixture. How and why such a change could occur is still unclear and this explanation is thought to be not a good one since it is most unlikely that enkephalins were present in the rat-brain-membrane preparations.

b) It is possible that some components of Gou reacted with the $^{3}H$ naloxone itself to cause precipitation of the radio-labelled naloxone on the filter membranes, so enhancing the DPM counts in a way which would be extra to the expected non-specific binding.

There is no reason to expect that the drugs which inhibit the proteolytic degradation of enkephalins should bind to opiate receptors. However these drugs do have similar effects to some components of Gou, which clearly cannot be associated with opiate-receptor binding, but which may cause similar precipitation effects on cold naloxone to those discussed above. Therefore, to see if these apparent increases in non-specific binding, occurred also in the presence of the drugs, the opiate-binding procedure was carried out three times in the presence, respectively, of Bestatin, Captopril and Thiorphan.

## 5.6 Comparing the Effect of Standard NEP Inhibitors and Gou On Naloxone-opioid Receptor Binding Assay

In order to clarify the effect of Gou on the nonspecific binding measurements further, the comparison experiment of Gou and the standard inhibitors of peptidases (Bestatin, Captopril and Thiorphan) were investigated. The result were as shown in **Figure 5.6**.

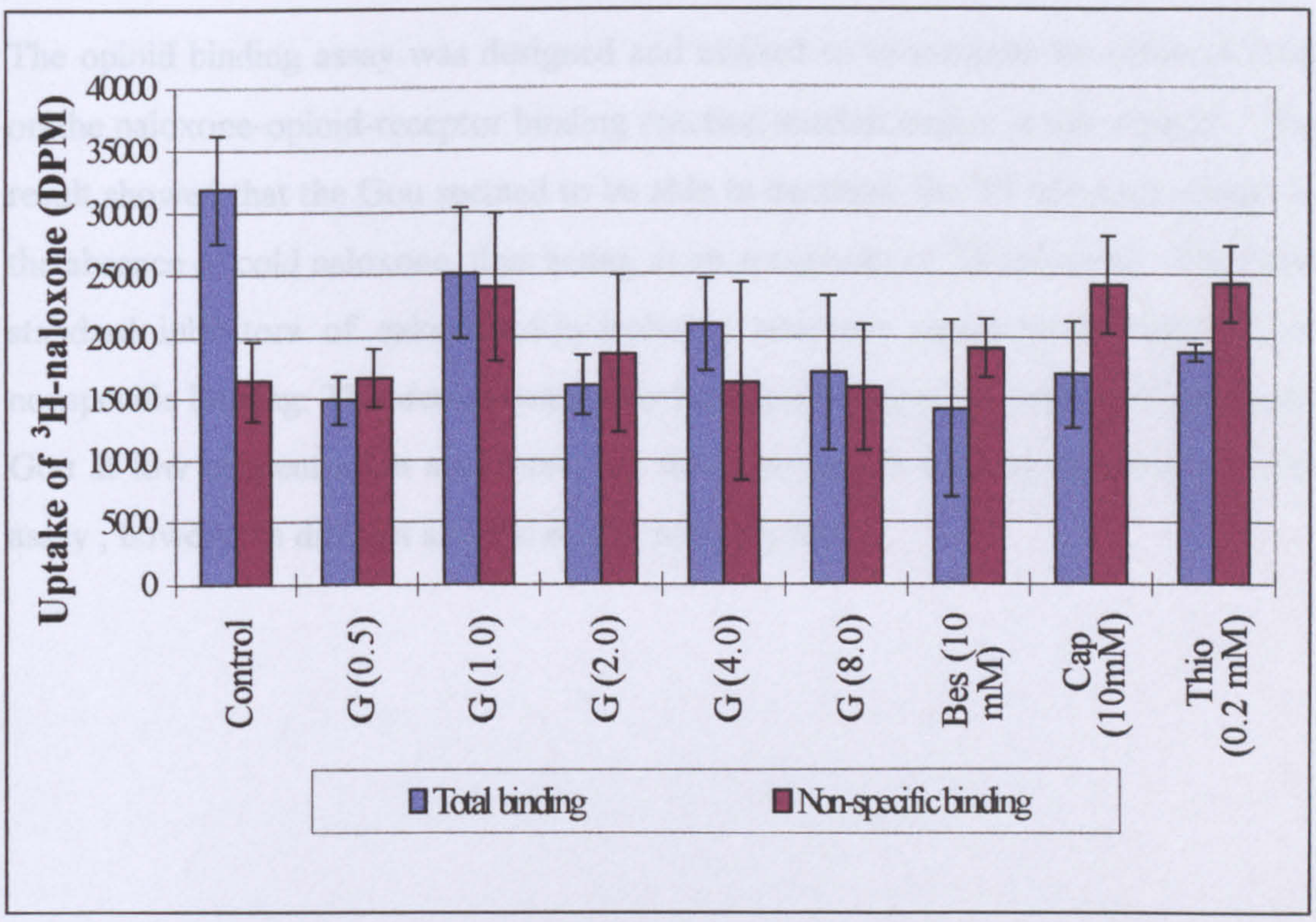



**Figure 5.6 The Comparison of the Effects of Standard NEP Inhibitors and Gou On Ligand-binding Assay (n=3)**

**Figure 5.6** shows that three standard inhibitors of enkephalin-hydrolysing enzymes increased the non-specific binding of naloxone-opioid receptor reaction systems. While in Gou's case it is more complicated, for the cases of Gou at 1.0 mg/ml to 8.0 mg/ml , the non-specific binding was lower than total binding. But in low concentration (2.0 mg /ml) the non-specific binding was higher than total binding. The above results suggested that the Gou preparation contains enkephalin-hydrolysing enzyme inhibitors which can increase the non-specific binding of

naloxone. But Gou might also contain other compounds which might antagonise the binding reaction of naloxone and opioid receptor. There seems to be a statistical reduction of the specific binding although so far as the author is aware there is no reason to expect the peptidase-inhibiting drugs to be competitive for binding to opiate receptors. The other effect is present also namely the increase in the $^{3}$H-naloxone binding to the membranes in the presence of cold naloxone.

## SUMMARY

The opioid binding assay was designed and utilised to investigate the effect of Gou on the naloxone-opioid-receptor binding reaction studied earlier in this chapter. The result showed that the Gou seemed to be able to decrease the $^{3}$H-naloxone uptake in the absence of cold naloxone, thus acting as an antagonist of $^{3}$H-naloxone. The three standard inhibitors of enkephalin-hydrolysing enzymes appeared to increase the nonspecific binding. This demonstrate they have not antagonism activity to naloxone. Gou at low concentration also increased the non-specific binding of naloxone. The assay , however is difficult and the results not very large.

# CHAPTER VI

# THE ISOLATION OF ACTIVE INGREDIENTS FROM GOU AND INVESTIGATION OF THEIR ACTIVITY

# INTRODUCTION

Although the literature has indicated that about 39 compounds have been obtained from Gou (Liu *et al*., 1991, 1993), it is not clear whether of these, of any, would show NEP inhibitory activity. The following procedures were adopted to fractionate the active ingredients from Gou crude extract:–

a) Sephadex G-10 gel filtration;
b) Reversed-phase preparative High Performance Liquid Chromatography (HPLC);
c) HPLC of the Gou aqueous extract and comparison of three single known components [rhynchophylline (Rhy), mitraphyline and pteropodine).

Fractions were characterised by absorption spectroscopy and activity against NEP. The HPLC separation pattern was used to investigate the stability of the Gou extract.

## 6.1 Reversed-phase Preparative High Performance Liquid Chromatography (HPLC)

### 6.1.1 Choice of Mode of Chromatography

The choice of chromatographic method is summarised in **Figure 6.1**.

**Figure 6.1 Flow Diagram Used for the Choice of Chromatographic Systems.** (Riley, 1997)

Based on the principle shown in **Figure 6.1** size-exclusion chromatography is traditionally recommended for the separation of compounds of molecular weight greater than 1000 and this is still the method of choice for the determination of the size distributions of biomolecules. Sephadex G-10 gel filtration was therefore chosen to determine the size distribution of compounds existed in Gou aqueous extract in this thesis. According to principle the main factors to consider in the choice of mode of chromatography for the separation of small molecules (RMM<1000) are ionic character and polarity. Ionic compounds are best separated by ion-exchange chromatography or reversed-phase chromatography. Therefore reversed-phase preparative HPLC was used to fractionate the active ingredients of Gou aqueous extract (Riley, 1997).

### 6.1.2 Reversed-phase Liquid Chromatography (RPLC)

Recently reversed-phase liquid chromatography has developed to be an effective and useful mode of chromatography for the analytical and preparative separations of compounds in the chemical, biological, pharmaceutical and biomedical sciences (Horvath *et al.*, 1976; Schomburg, 1988; Akiyama *et al.*, 1998). This partly derives from the fact that the technique requires an aqueous mobile phase and so it is generally compatible with most aqueous samples, samples can often be injected directly onto the column without pre-treatment. Even if pre-treatment of the sample is required, it is often relatively simple. For example the Gou crude extract was filtered before being injected onto the column. A wide range of polar- molecular analytes can be separated in this way.

The term reversed-phase liquid chromatography derives from the fact that the mobile phase is more polar than the stationary phase, which is the opposite of normal-phase chromatography. A typical reversed-phase system employs a hydrocarbonaceous phase covalently bonded to a silica gel and a hydro-organic mobile phase such as a mixture of water and acetonitrile.

The retention of macromolecules by the stationary phase arises from repulsion of non-polar (hydrophobic) regions of the solute molecules by the water molecules in

the mobile phase. The stationary-phase surface is saturated by molecules of polar solvent such as acetonitrile. Basically, all the possible solute-solvent-stationary phase interactions contribute to retention. For a given solute the logarithm of the capacity ratio is linearly related to the volume fraction of the polar solvent in the mobile phase, $\Phi$, their equations follows can be expressed this principle log $K' = \log k'_w - S\Phi$ where $k'_w$ is the capacity ratio of solute with a completely aqueous mobile phase (i.e. $\Phi=0$) and S is the slope coefficient. The above equation provides a covenient framework for characterisation of solvent strength and stationary phase polarity. The order of solvent strength are: methanol<acetonitrile<tetrahydrofuran. In this thesis Octadecsi-yllyl Silica (S5 ODS or $C_{18}$) was used as the stationary phase, and 0.075 TFA+100% water was used as mobile phase. Acetonitrile was the polar solvent (Riley, 1997), and TFA was used to keep the system always acid.

### 6.1.3 Reversed-phase Preparative HPLC Instrumentation

Briefly, the HPLC instrumental system can be described by the following diagram **(Figure 6.2)**.

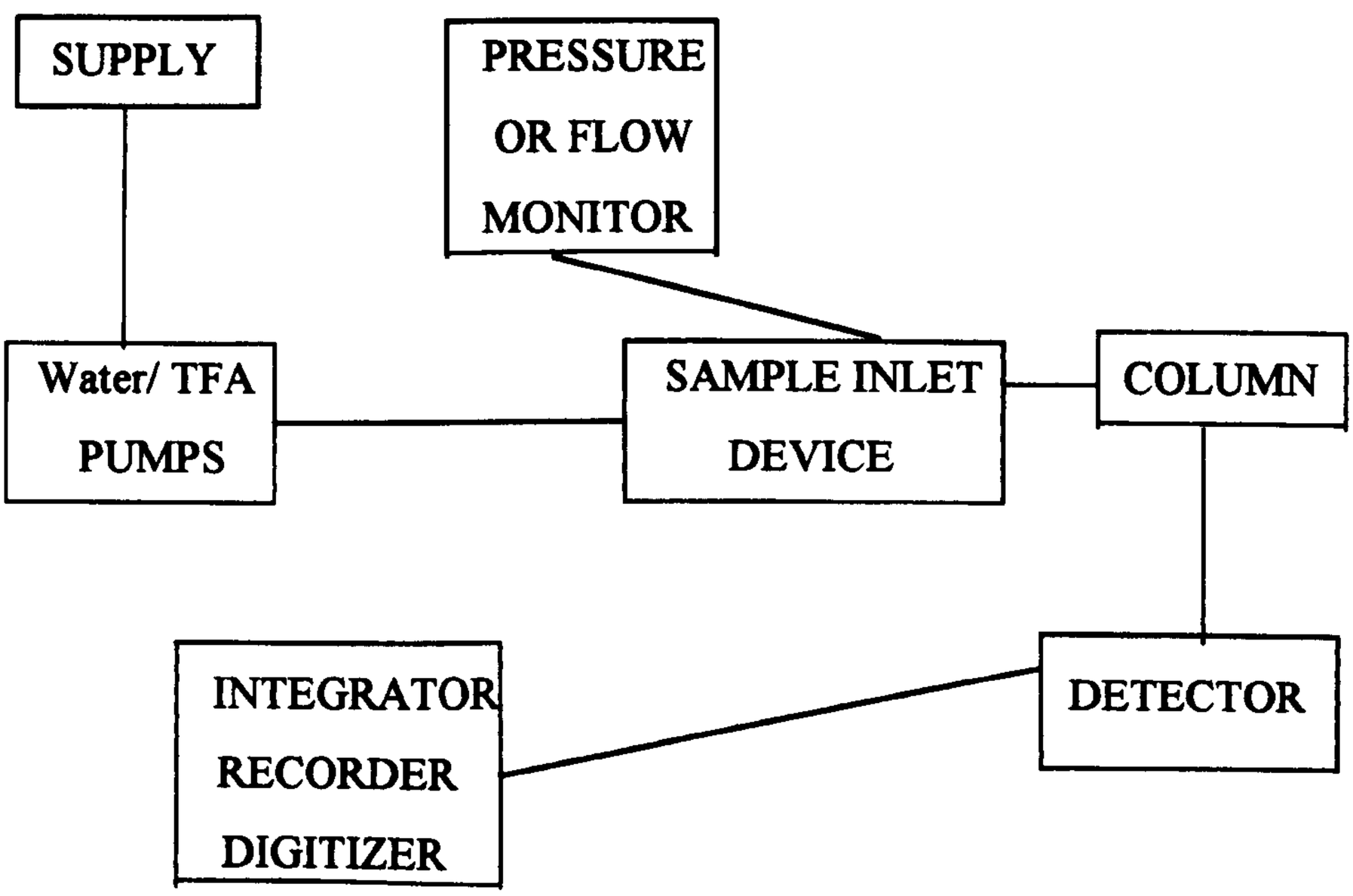



**Figure 6.2 Block Diagram of An HPLC Instrument**

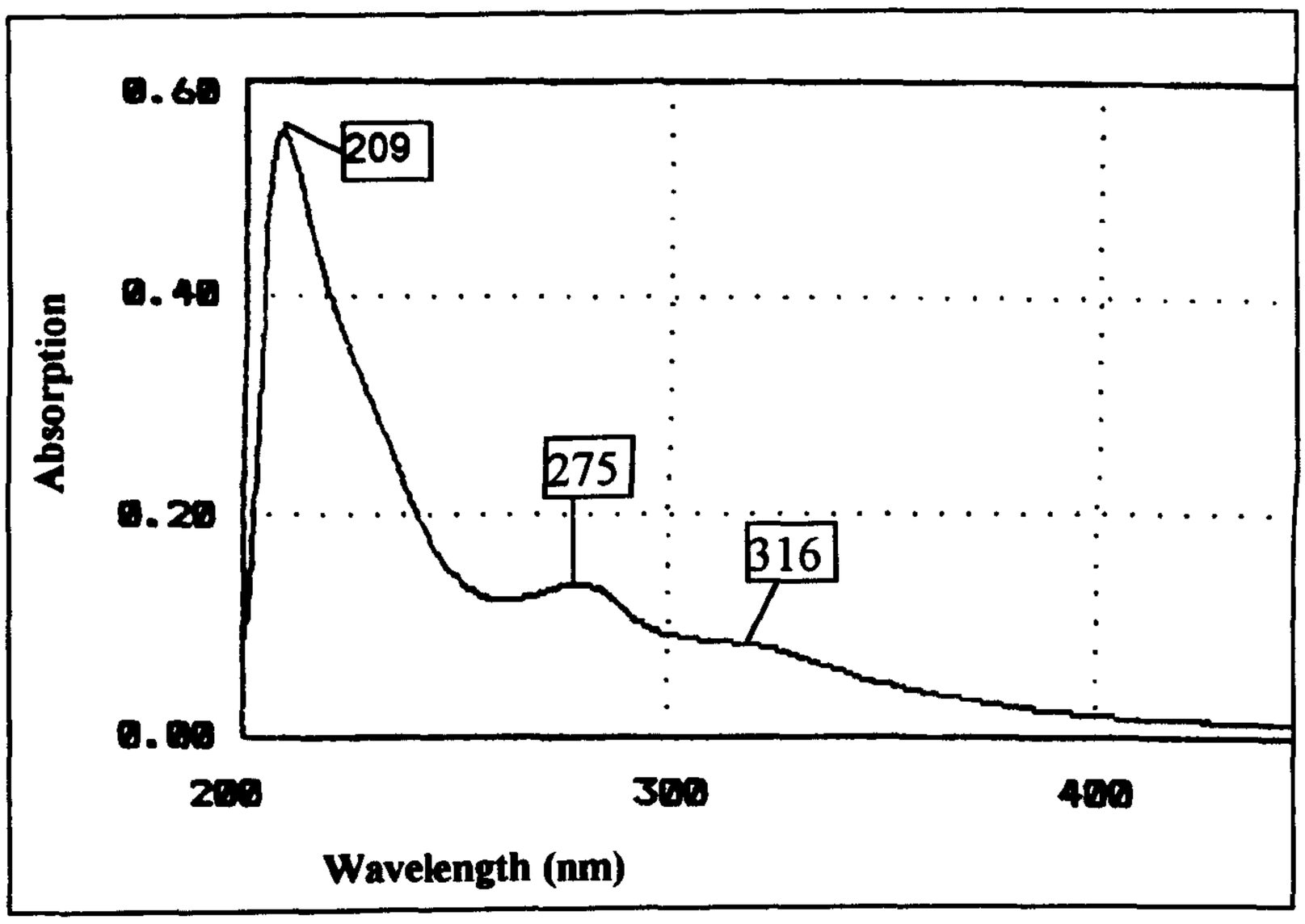
In this chapter reversed-phase HPLC was used to fractionate Gou aqueous extract so that the active ingredients could be obtained step by step. The detailed procedures of HPLC are described as in section 2.5 of chapter 2 and in section 6.6.1.

## RESULTS AND DISCUSSION

### 6.2 Spectral Analysis of Gou Crude Extracts

Both organic (methanol, Gmext) and aqueous (water, Gwext) extracts (prepared as described in section 2.1) of Gou were dissolved in 50 mM Tris-HCl (pH 7.4) at a final concentration of 100 mg/ml, then diluted with 50 mM Tris-HCl (pH7.4) until the final concentration was 0.5 mg/ml. In order to have an understanding for the spectral absorption of Gou crude extracts, the aqueous extract was measured for its absorption between 200 nm – 900 nm on a spectrophotometer, and the background absorption was designed to be subtracted on the machine. The organic extract was also measured for its absorption in the range of 200 nm to 900 nm. Both extracts showed absorption peaks only in the U.V. range (about 200 nm to 340 nm). The absorption curves were as presented in **Figure 6.3**. All the following absorption curves were obtained by subtracting the effect of absorption cells.

**(A) The U.V. Spectrum of the Gou Aqueous Extract (0.5mg/ml)**




**(B) The U.V. Spectrum of the Organic Extract of Gou (0.5mg/ml)**

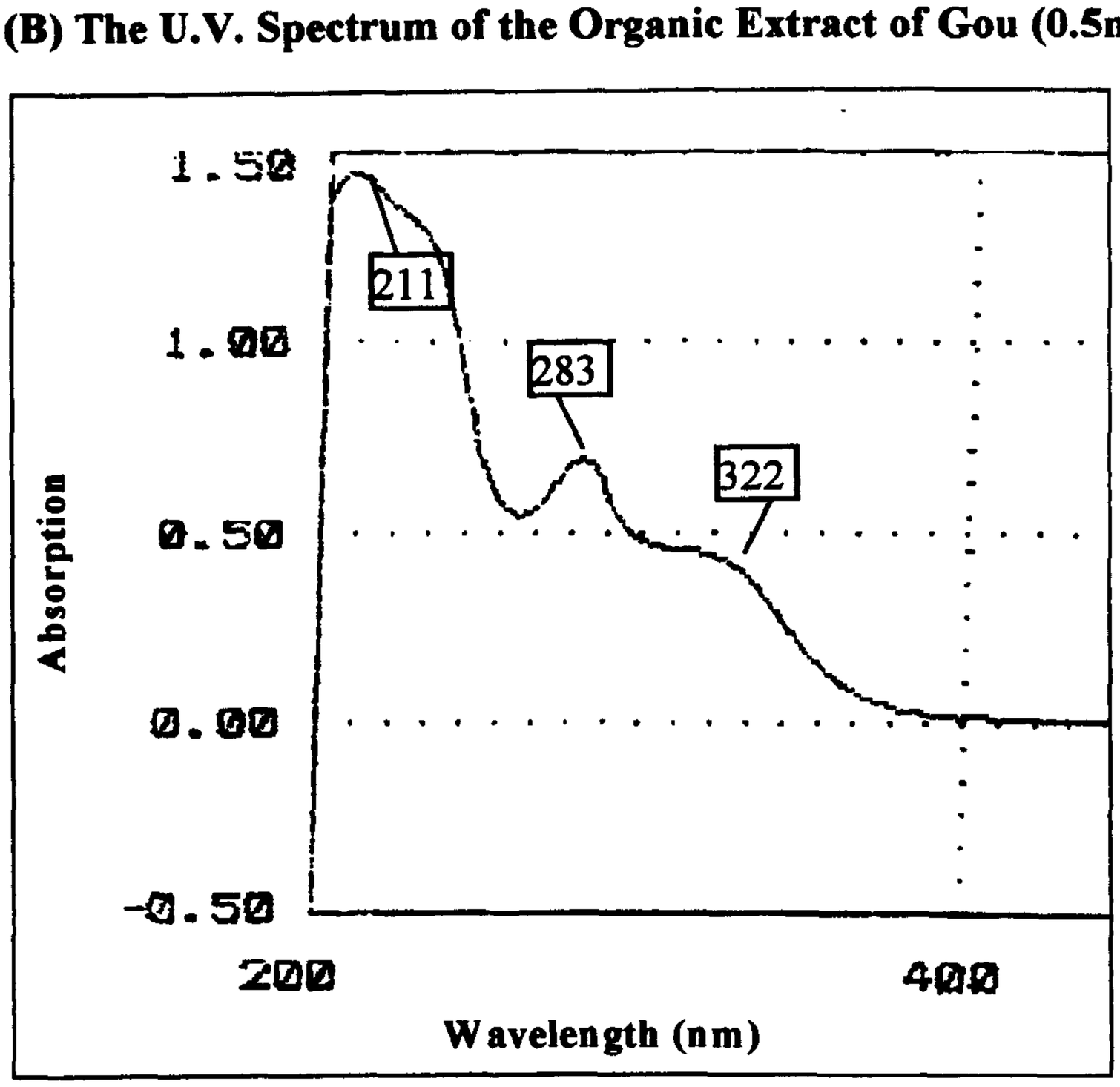



**Figure 6.3 U.V. Spectrum of the Gou Aqueous and Organic Extracts**

**( Concentration: 100 μg /ml)**

The U.V. spectrum as presented in **Figure 6.3** indicated that both Gou extracts showed absorption in the U.V. range. The aqueous extract was found to have

absorption peaks at 209 nm, 275 nm, 316 nm while the organic extract had peaks at 211 nm, 283 nm, 322 nm. Proteins are known to have absorption peaks in the region of 280 nm, and alkaloids have absorption peaks at about 220 nm to 240 nm. It can be assumed therefore that the crude extracts of Gou might contain protein or alkaloids. The above results were obtained by measuring the crude extracts and therefore experiments were essentially needed in order to carry out such measurements on single fractions of Gou.

## 6.3 The Fractionation of Gou By Sephadex G-10 and the Effects of the Fractions on NEP Activity

Spectrophotometry analysis showed that Gou aqueous extract might contain alkaloids or proteins. There are many molecules which have absorption peaks in the 280 nm range. In order to determine whether proteins were involved in NEP inhibitory activity, proteins were precipitated from the aqueous extract by addition of 50% ethanol. The pellet of precipitated proteins was separated from the supernatant by centrifugation at 13000 rpm for 5 minutes. Then the supernatant was added onto a Sephadex G-10 column (volume:5 ml) and eluted with 0.5 M sodium chloride. The eluted fractions were collected and their effects on NEP activity were measured. The hydrolysis percent of $^{3}$H(leu)-enkephalin for eluted fractions of Gou aqueous extract were as shown in **Figure 6.4**.

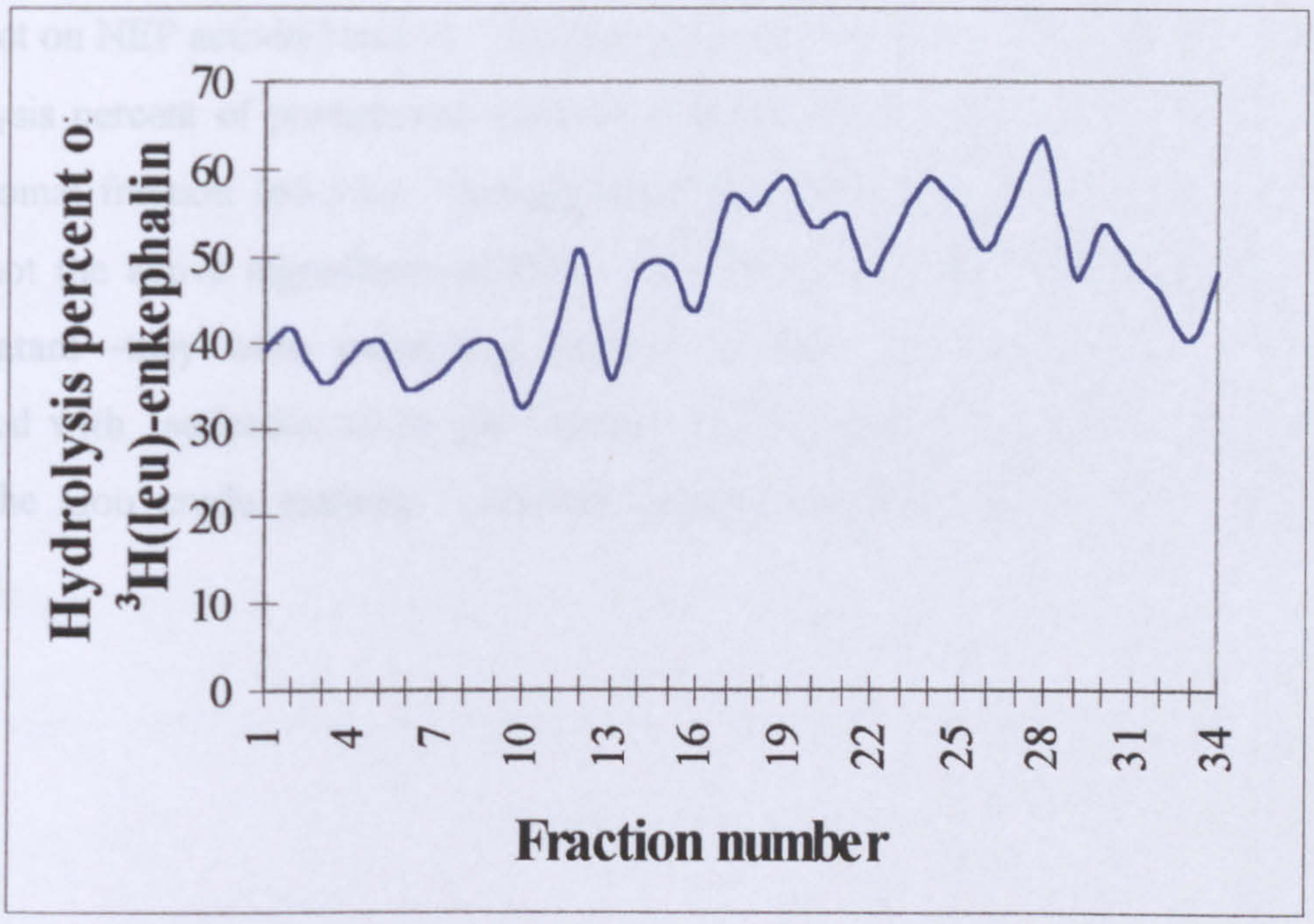



**Figure 6.4 The Hydrolysis Percent for the Fraction of Gou Aqueous Extract from A Sepadex G-10 Gel Filtration Column.**

0.5 M sodium chloride was used to wash the binding molecules, blue dextran was used as the marker . Loading volume 0.25 ml. Each fraction was assayed as in section 2.4 . The enzyme dilution was 1:20. The hydrolysis percent of crude enzyme (without adding fractions) was 65.5.

The results in **Figure 6.4** indicated that all the eluted fractions of Gou aqueous extract showed a lower hydrolysis percent of $^3$H(Leu)-enkephalin than the 65.5% hydrolysis produced by the crude rat kidney microsomal extract rich in NEP as described in section 4.1. But fraction 1 to 16 showed significantly lower hydrolysis percent than crude rat kidney microsomal extract ($P< 0.05$). Fraction17 to Fraction 34 did not show significantly lower hydrolysis percent than crude rat kidney microsomal extract. This suggested that the inhibitory ingredients of Gou were in the eluted fraction of 1 to 16. It also suggested Sepahdex G-10 gel filtration could not concentrate the active ingredients into one or two fractions.

The effect of the precipitated proteins (pellet) on NEP activity was also tested. The hydrolysis percent of $^3$H(leu)-enkephalin for the precipitated proteins (pellet) was: 63.6% (pellet was re-dissolved in 50 mM Tris-HCl buffer pH 7.4 and measured for

its effect on NEP activity) and 52.7 (in phosphate buffer). This demonstrated that the hydrolysis percent of precipitated proteins (pellet) was as high as crude rat kidney microsomal fraction (65.5%). It suggested that the precipitated proteins (pellet) were not the active ingredients of Gou. The active ingredients still existed in the supernatant---they were water and ethanol soluble. 50% ethanol pretreatment followed with sephadex G-10 gel filtration did not isolate the active compounds from the Gou crude extracts. Another specific isolation method was therefore needed.

## 6.4 HPLC Analysis of Gou Crude Extracts

### 6.4.1 High Performance Liquid Chromatography of the Gou Aqueous Extract

Aqueous extracts of Gou solutions at 10 mg/ml were applied to an HPLC column ($C_{18}$ / S5 $ODS_2$). The detailed procedure are given in section 2.4.2 of Chapter 2. The HPLC chromatograph of Gou is as shown in **Figure 6.5**.

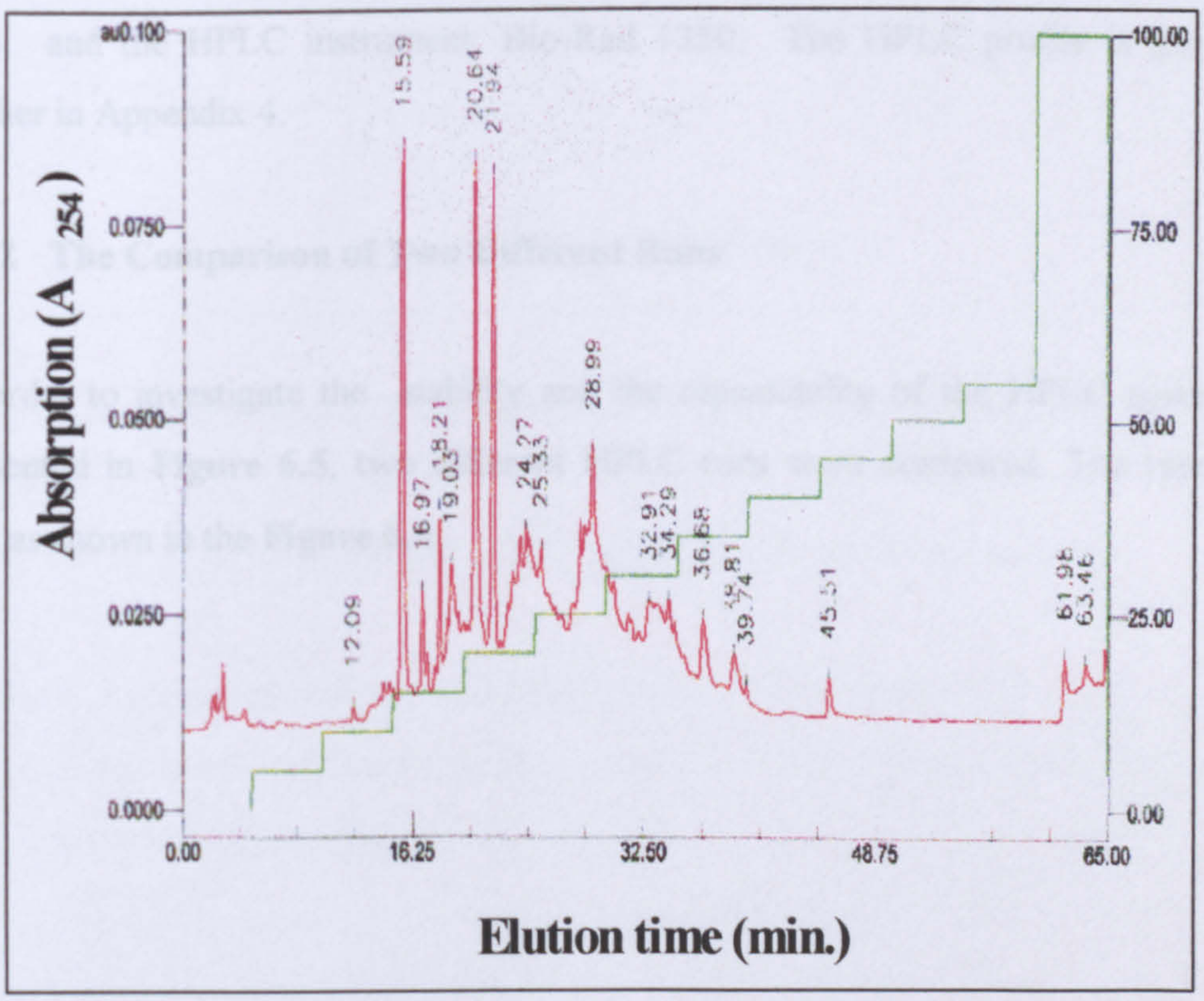



**Figure 6.5 A Typical HPLC Chromatograph of the Gou Aqueous Extract (10mg/ml)**. Loading volume: 50µl; Flow rate: 1ml/min; S5 ODS-2 $C_{18}$ column; Solvent system: 0.07% TFA (Trifluoroacetic acid) / 0.07% TFA+100% acetonitrile. (Typical of six runs)

The typical HPLC chromatograph presented in **Figure 6.5** showed that the aqueous extract of Gou contained at least 16 peaks in the solvent system of TFA/Acetonitrile. The elution times for above 16 peaks were: Peak1 at 12.09 minutes; peak2 at 15.59 minutes; peak 3 at 16.97 minutes; peak4 at 18.21

minutes; peak5 at 19.03 minutes; peak6 at 20.64 minutes; peak7 at 21.94 minutes; peak8 at 24.27 minutes; peak 9 at 25.33 minutes; peak10 at 28.99 minutes; Peak11 at 32.91 minutes; peak12 at 34.29 minutes; peak13 at 36.68 minutes; peak14 at 38.81 minutes; peak15 at 39.74 minutes and peak16 at 45.51 minutes. The S5 ODS2 column (made in Bio-Rad) and the TFA/acetonitrile solvent were therefore suitable for this separation system. The following HPLC experiments were therefore corrected out with the following parameters: Loading volume: 50μl; Column: S5 ODS2 (C18); Eluant A: 0.07%TFA; Eluant B: 0.07% TFA+100% Acetonitrile; Flow rate:1ml/min; Detector: 254 nm (0.0-0.1); and the HPLC instrument: Bio-Rad 1350. The HPLC profile is given further in Appendix 4.

### 6.4.2 The Comparison of Two Different Runs

In order to investigate the stability and the repeatability of the HPLC system presented in **Figure 6.5**, two different HPLC runs were compared. The result was as shown in the **Figure 6.6**.

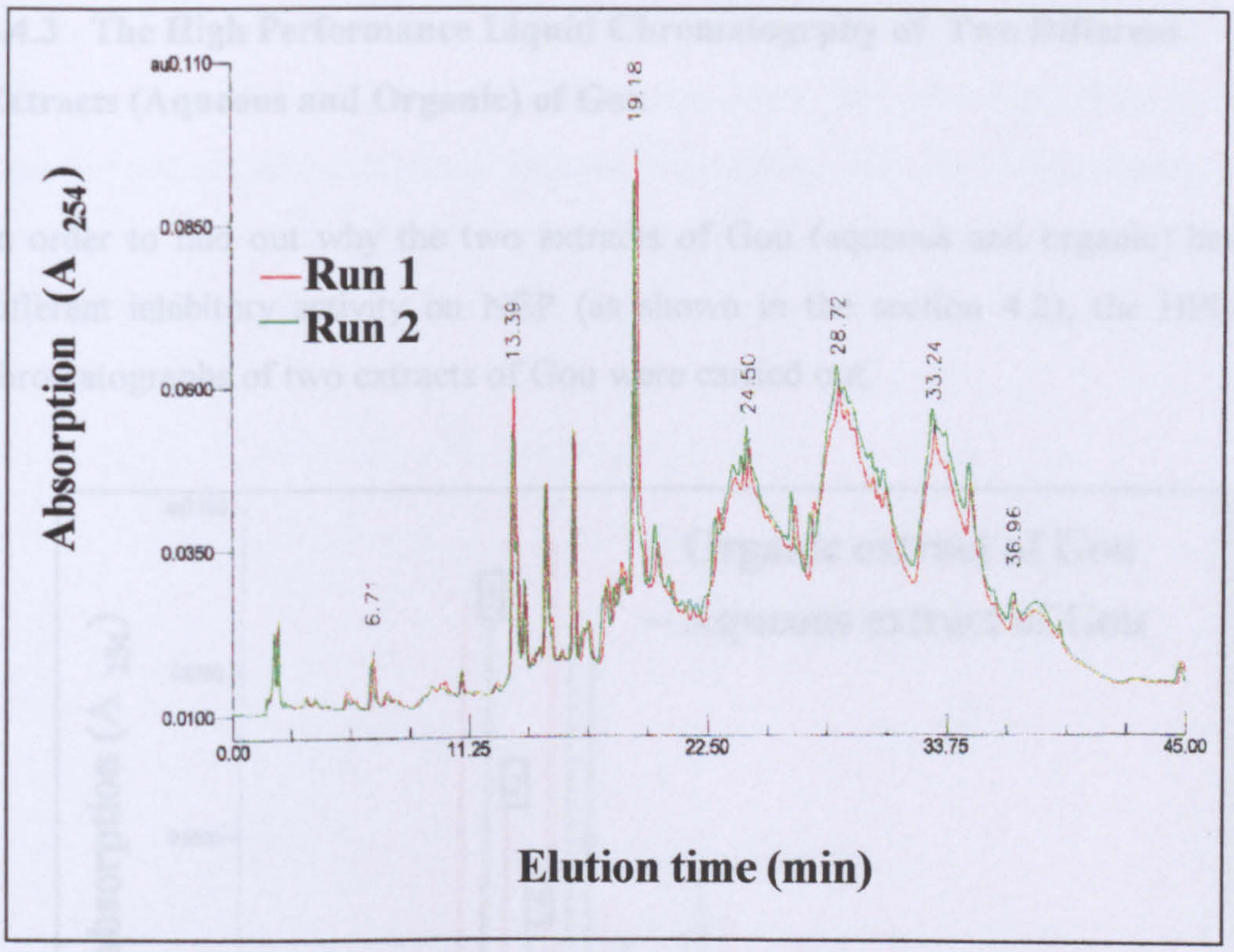



**Figure 6.6 Comparison of Two Different Runs of the HPLC of Gou Aqueous Extract.**

Run1: red plot; Run2: green plot . Both plots were run on the same day.

**Figure 6.6** suggested that the two different runs of Gou showed the same HPLC elute plots if they were run during same day. This demonstrated that the HPLC system itself is stable and repeatable.

### 6.4.3 The High Performance Liquid Chromatogrphy of Two Different Extracts (Aqueous and Organic) of Gou

In order to find out why the two extracts of Gou (aqueous and organic) have different inhibitory activity on NEP (as shown in the section 4.2), the HPLC chromatographs of two extracts of Gou were carried out.

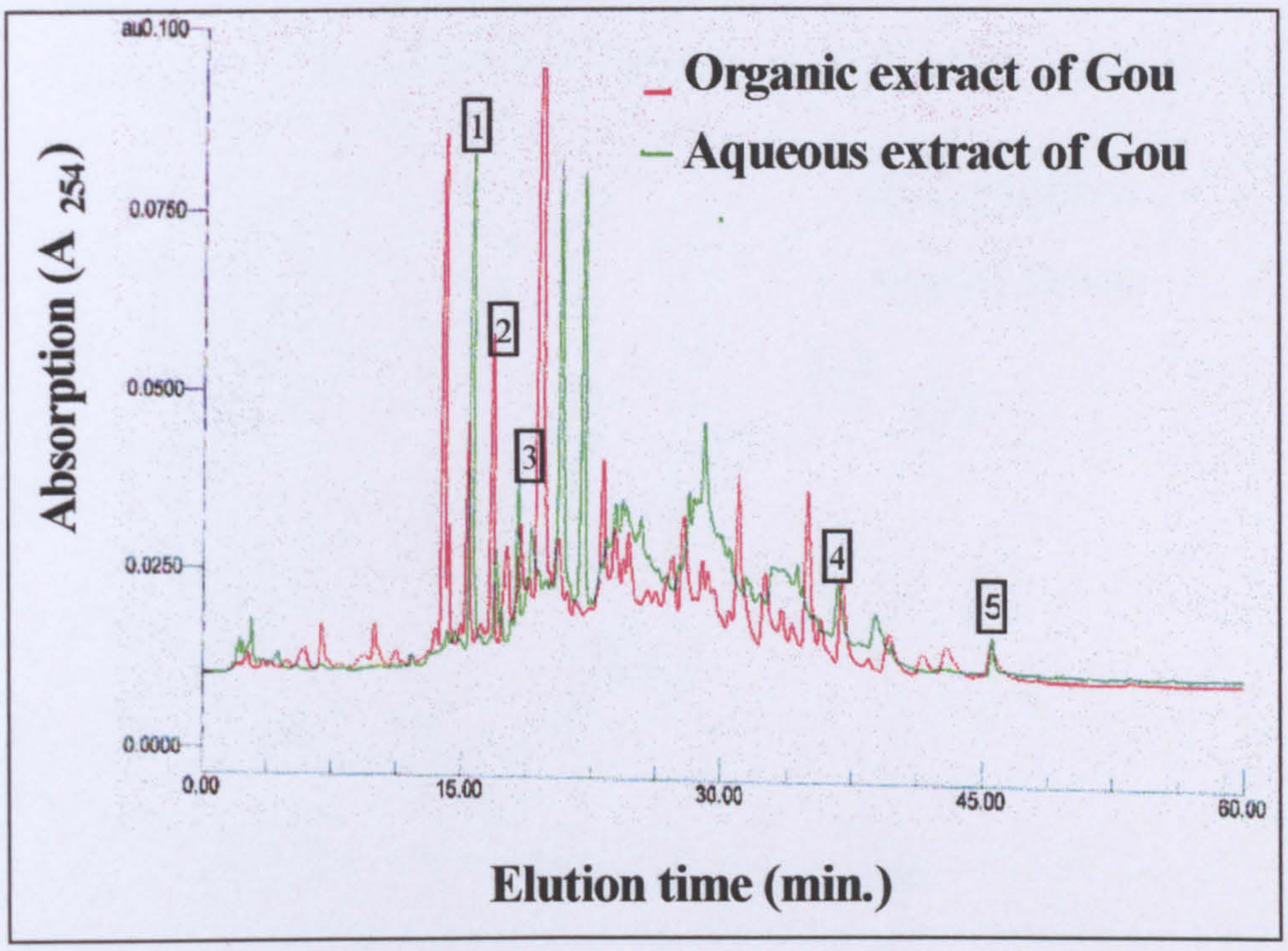



**Figure 6.7 The Comparison of Two Different Extracts of the Gou on HPLC Absorption**. Red run: Gwext (10mg/ml); Blue run: Gmext (10mg/ml). Other HPLC parameters were the same as shown in **Figure 6.5**.

The HPLC results presented in **Figure 6.7** showed that the two extracts had different HPLC chromatographs although five common peaks were observed. The elution time of the above five common peaks are as follows: peak1 eluted at 15.59 minutes, peak2 eluted at 17.25 minutes, peak3 eluted at 18.21 minutes, peak4 eluted at 36.68 minutes and peak5 eluted at 45.51 minutes. Other peaks were eluted at completely different times.

As the organic extract of Gou only showed limited inhibitory activity on NEP proteolysis (section 4.2), it is likely that the above five common peaks of the aqueous and organic extracts of Gou are not active. The active ingredients are thus likely to be contained in the other different peaks of the Gou aqueous extract or responsible for this limited activity.

### 6.4.4 The Time Stability Determination of Gou

In order to investigate the time stability of Gou the same batch of Gou aqueous extract was divided into equal aliqouts, and kept at –20°C. One aliquot was run HPLC at the first day (performed on 10/06/96) and another one was run HPLC about one year later (performed on 20/05/97). Then two different runs were compared in the following figure (**Figure 6.8**).

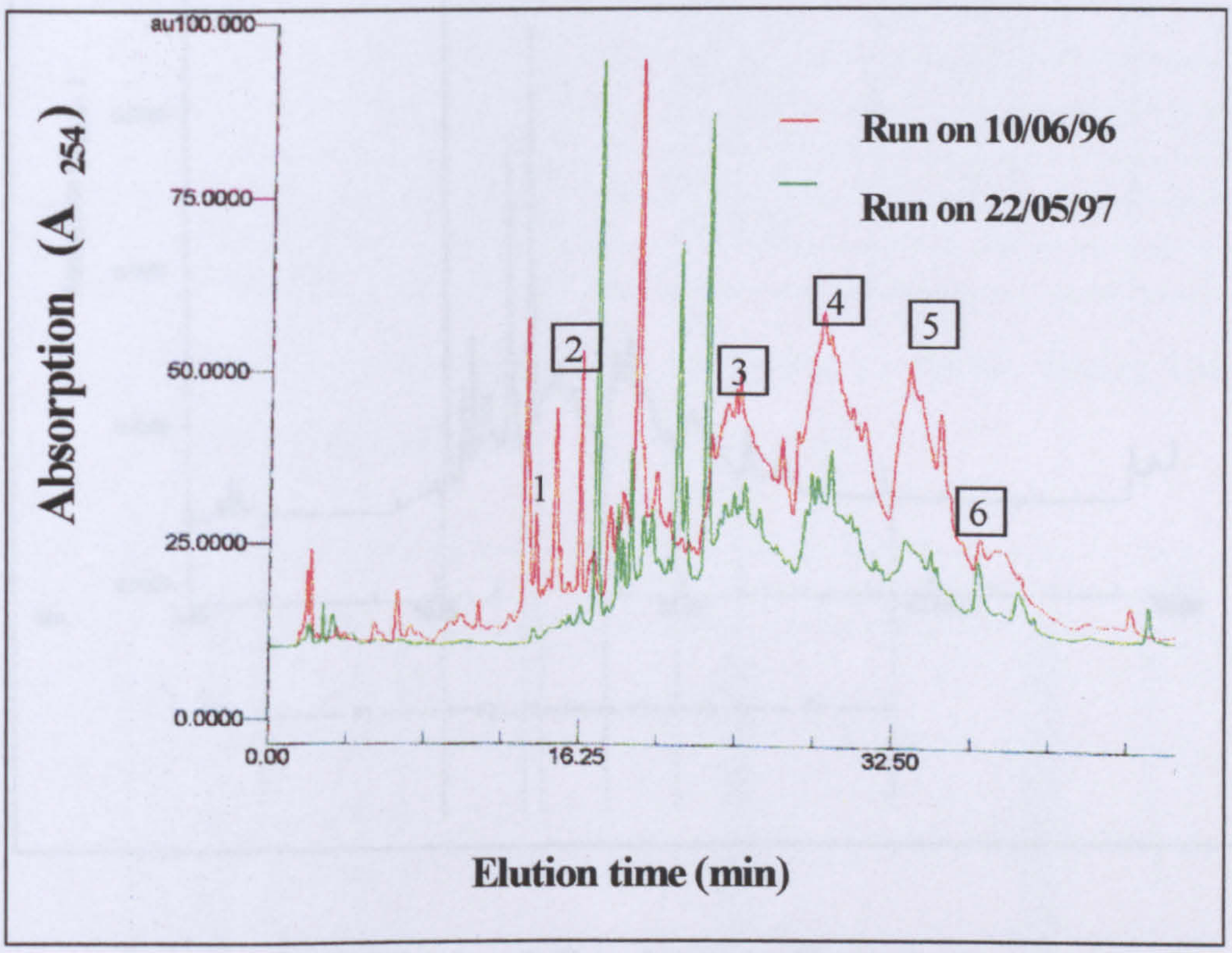



**Figure 6.8 The Determination of the Time Stability of Gou**

The result presented in the **Figure 6.8** showed that at least six peaks did not change with time. They are: peak 1 eluted 13.98 minutes, peak 2 eluted at 16.25 minutes, peak 3 eluted at 24.50 minutes, peak 4 eluted at 28.17 minutes, peak 5 eluted at 33.24 minutes, peak 6 eluted at 36.96 minutes and peak eluted at 39.41 minutes. Other peaks changed with time. All the following HPLC plots were obtained by means of the same batch of Gwext which was prepared on 10th June, 1996.

### 6.4.5 The Activity of Pooled HPLC Peaks on NEP

In order to develop the isolation of the active ingredients from Gou by the HPLC method, the HPLC fractions were pooled into seven parts, i.e. the eluant was collected as shown in figure (**Figure 6.9**).

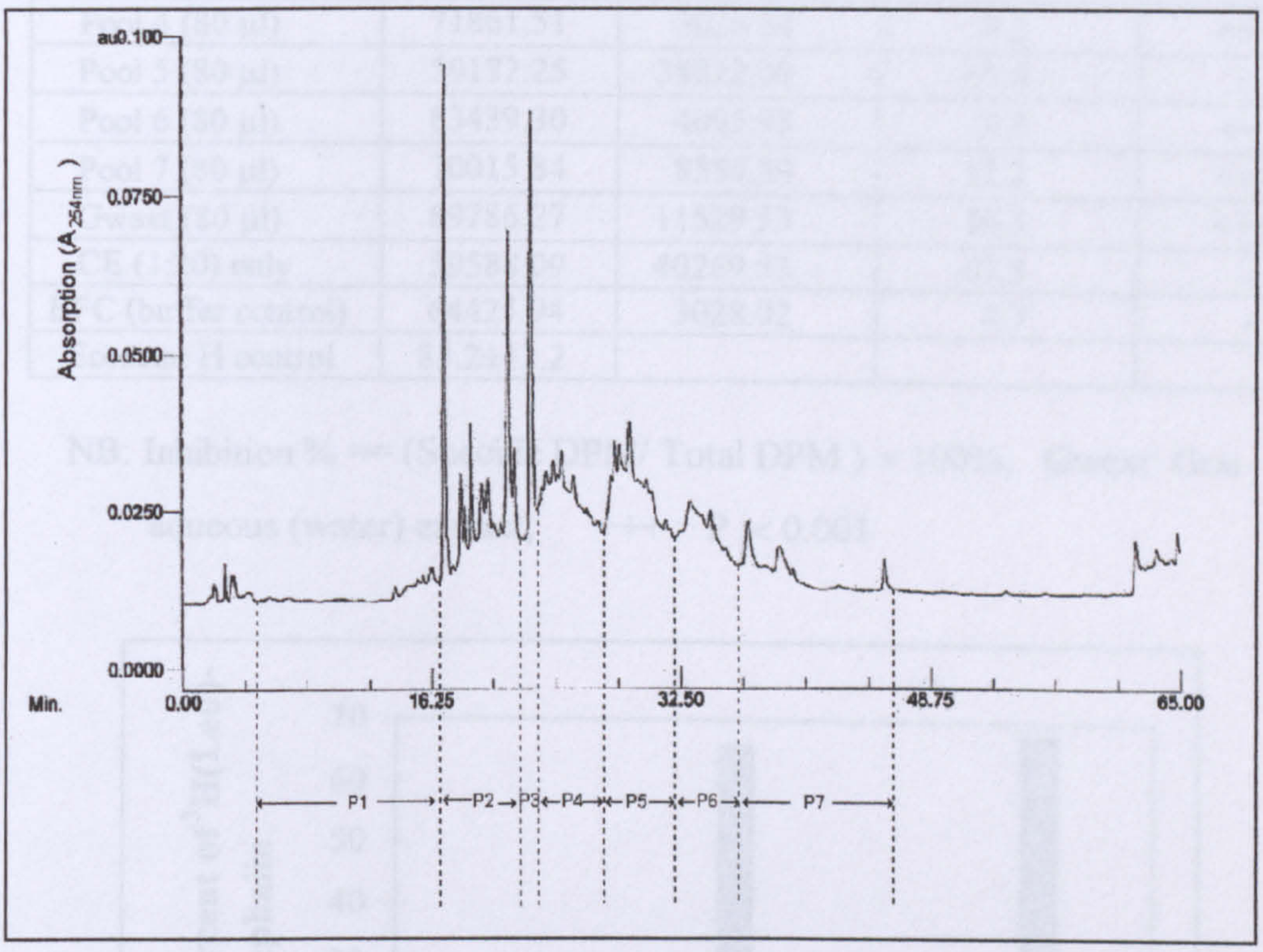



**Figure 6.9 The Pooled Peaks of Gou Aqueous Extract**.

As shown in the **Figure 6.9** every pool of fractions was evaporated and concentrated to dryness. Then the dried samples were washed with distilled water and dried again. Each pooled powder was dissolved in 80 μl of distilled water and then used for NEP assay. The assay of NEP actually described in the section of 2.3.5. The results were as shown in **Table 6.1** and charted in **Figure 6.10**.

**Table 6.1 The Effects of the different Pooled HPLC Peaks on NEP Activity (For the method used here was the same as section 2.4)**

| Specified Groups | Total DPM | Specific DPM | Hydrolysis % (HD%) | Inhibition % (IN%) |
|---|---|---|---|---|
| Pool 1 (80 μl) | 60554.34 | 2930.59 | 4.8 | +++ |
| Pool 2 (80 μl) | 63341.40 | 5039.92 | 8.0 | +++ |
| Pool 3 (80 μl) | 61893.73 | 3216.52 | 5.2 | +++ |
| Pool 4 (80 μl) | 71861.51 | 3026.34 | 4.2 | +++ |
| Pool 5 (80 μl) | 59172.25 | 38822.08 | 65.6 | - |
| Pool 6 (80 μl) | 83439.30 | 4095.98 | 4.9 | +++ |
| Pool 7 (80 μl) | 70015.84 | 8554.39 | 12.2 | +++ |
| Gwext (80 μl) | 69786.27 | 11529.53 | 16.5 | +++ |
| CE (1:20) only | 59588.09 | 40269.53 | 67.5 | - |
| BFC (buffer control) | 64425.94 | 3028.02 | 4.7 | - |
| Ecoscint H control | 83.2±12.2 | | | |

NB: Inhibition % = (Specific DPM/ Total DPM ) × 100%; Gwext: Gou aqueous (water) extract; +++ : $P < 0.001$

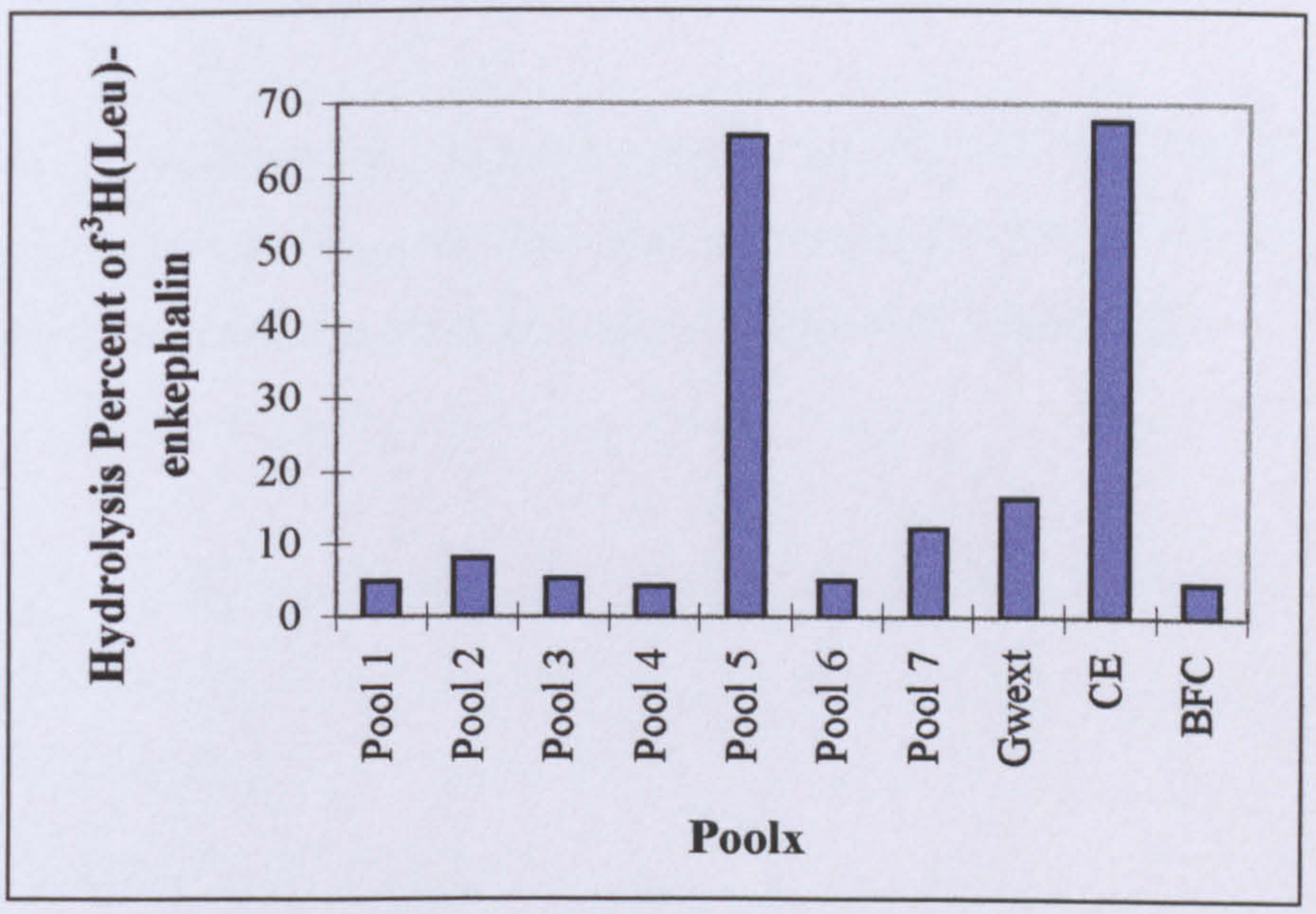



**Figure 6.10 The Effects of Pooled HPLC Peaks on NEP Activity**

The enzyme assay was the same as Chapter 3. The dilution of Crude enzyme (CE): 1: 20; BFC: Buffer control; Gwext: Gou aqueous (water) extract (80 μl of 100mg/ml);. CE: Crude rat kidney microsomal extract (NEP rich ).

The result presented in **Table 6.1** and **Figure 6.10** showed clearly that Pool 5 had the same level of hydrolysis percent of $^{3}$H(leu)-enkephalin as the crude rat kidney microsomal extract. It suggested that Pool 5 did not contain NEP inhibitor, while the other six Pools showed lower hydrolysis percent of $^{3}$H(leu)-enkephalin than the crude rat kidney microsomal extract. They showed positive inhibitory activity on NEP. Student's t-test showed their inhibitory activities are significant ($P< 0.001$). This suggested that the active ingredients of Gou aqueous extract were not a single compound but a mixture. They distributed in Pool 1, 2, 3, 4, 6 and 7, and might belong to a large group of compounds such as alkaloids, which have already been isolated from Gou by Liu *et al.* (1993). Therefore the positions of the peaks isolated from Gwext on HPLC plots were compared with such alkaloids.

## 6.5 The Comparison Experiments of Three Single Standard Compounds With Gou Crude (Aqueous) Extract (Gwext)

As mentioned in previous section, Liu *et al.* (1991, 1993) had successfully isolated about 39 compounds from Gou. All of them were alkaloids. And all the 39 compounds can be represented by the following three types of compounds:

(1) Tetracyclic-oxindole alkaloids: rhynchophylline(Rhy) etc.;

(2) Rauwolfia Alkaloids: hirsutine etc.;

(3) Pentacyclic-oxindole alkaloids: pteropodine, mitraphylline etc..

Three compounds (Rhy, mitraphylline and pteropodine) were kindly provided by Professor Feng of the Chinese Materia Medical Institute. The three compounds were run on HPLC under exactly the same experimental conditions as that of Gwext in **Figure 6.5**. The results were as shown in the following figures (**Figure 6.11a** to **Figure 6.11c**) and described in section 6.6.1, 6.6.2 and 6.6.3.

### 6.5.1 The Comparison of Rhy and Gwext

Rhy powder was prepared at 5 mg/ml in aqueous solution and diluted until the final concentration was 50 μg/ml, then 50 μl was analysed by HPLC plot to give the **Figure 6.11a**. Gwext at 10mg/ml was run HPLC as for **Figure 6.5**

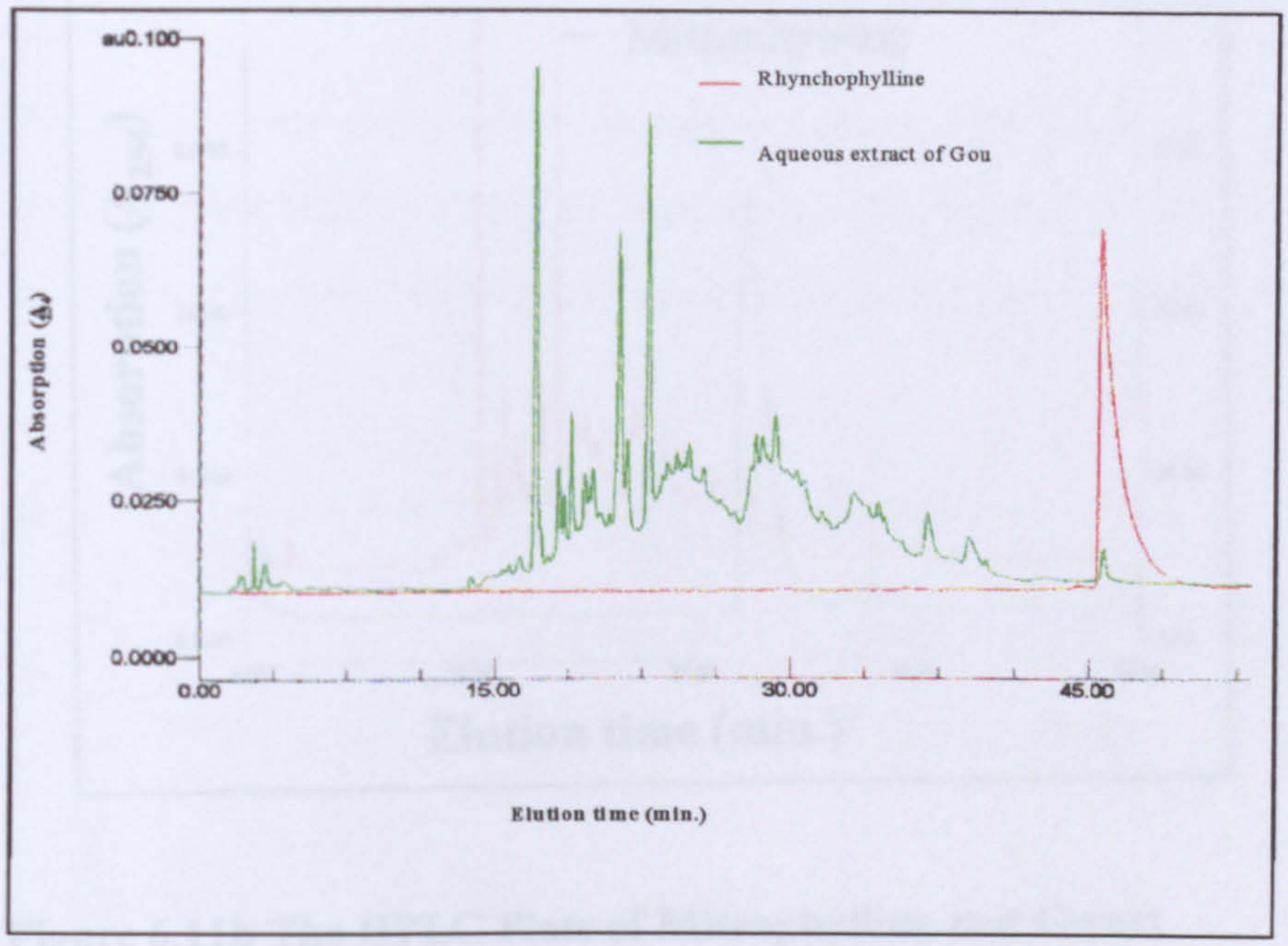



**Figure 6.11a The HPLC Plots of Rhy and Gwext**

As shown in the **Figure 6.11a** Rhy peak was eluted at the elution time of 46.5 minutes and at this elution time one peak was obtained on the Gwext plot. Rhy was in the range of pool 7.

### 6.5.2 The Comparison of Mitraphylline and Gwext

Mitraphylline was prepared at 500 μg/ml in aqueous solution and diluted until the final concentration was 50 μg/ml, then 50 μl was analysed by HPLC to give the plot in **Figure 6.11a**. Gwext was analysed as in **Figure 6.11a** . Both HPLC plots were as shown in **Figure 6.11b**.

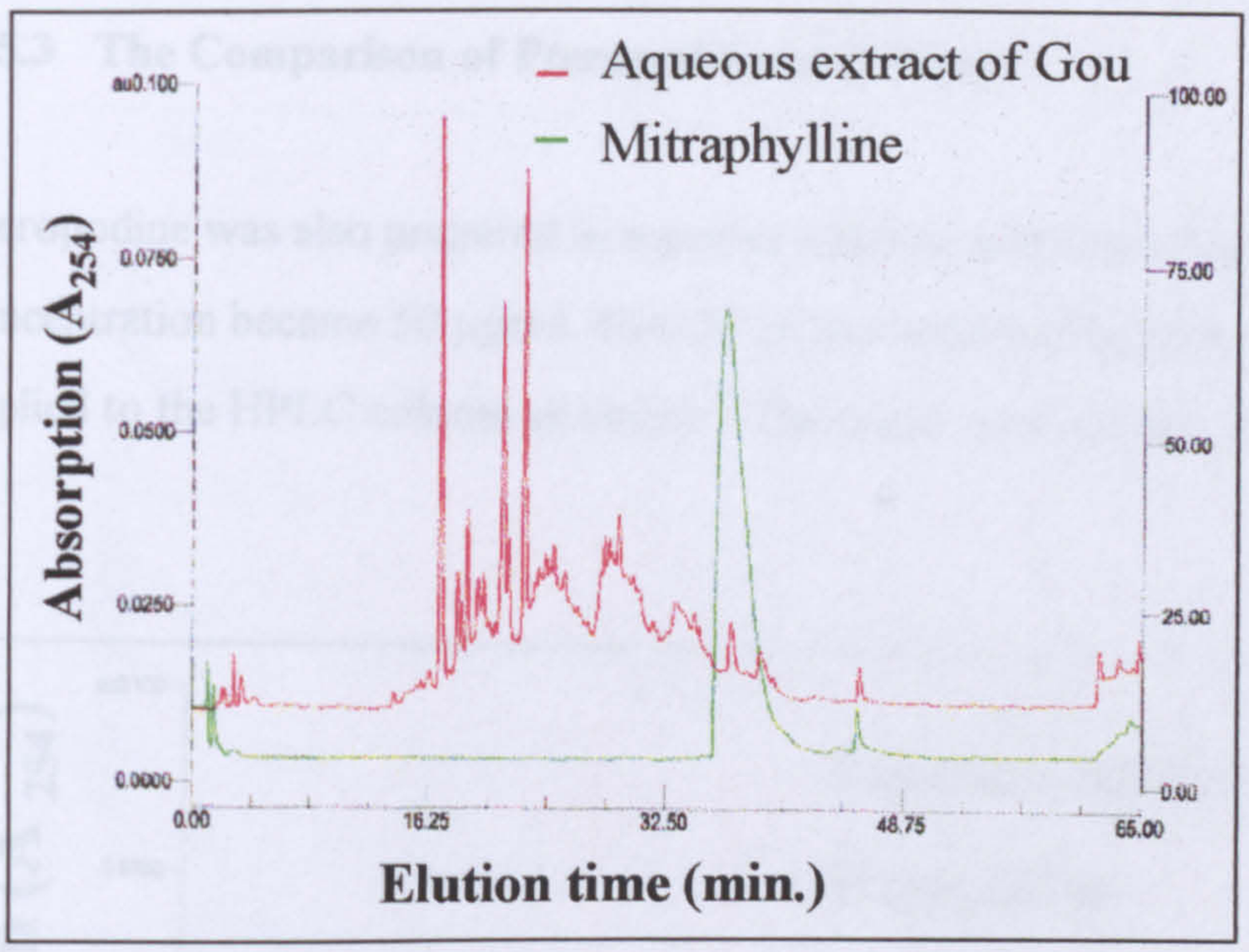



**Figure 6.11b The HPLC Plots of Mitraphylline and Gwext**

The plot of mitraphylline in **Figure 6.11b** showed that mitraphylline was eluted at the elution time of 37.5 minutes. As in **Figure 6.11a** there was a related peak observed in Gwext plot at the same elution time. Mitraphylline was also in the Pool 7 range.

### 6.5.3 The Comparison of Pteropodine and Gwext

Pteropodine was also prepared in aqueous solution and diluted until final concentration became 50 μg/ml, then 50 μl was analysed by HPLC. Gwext was applied to the HPLC column as before. The result were as seen in **Figure 6.11c**.

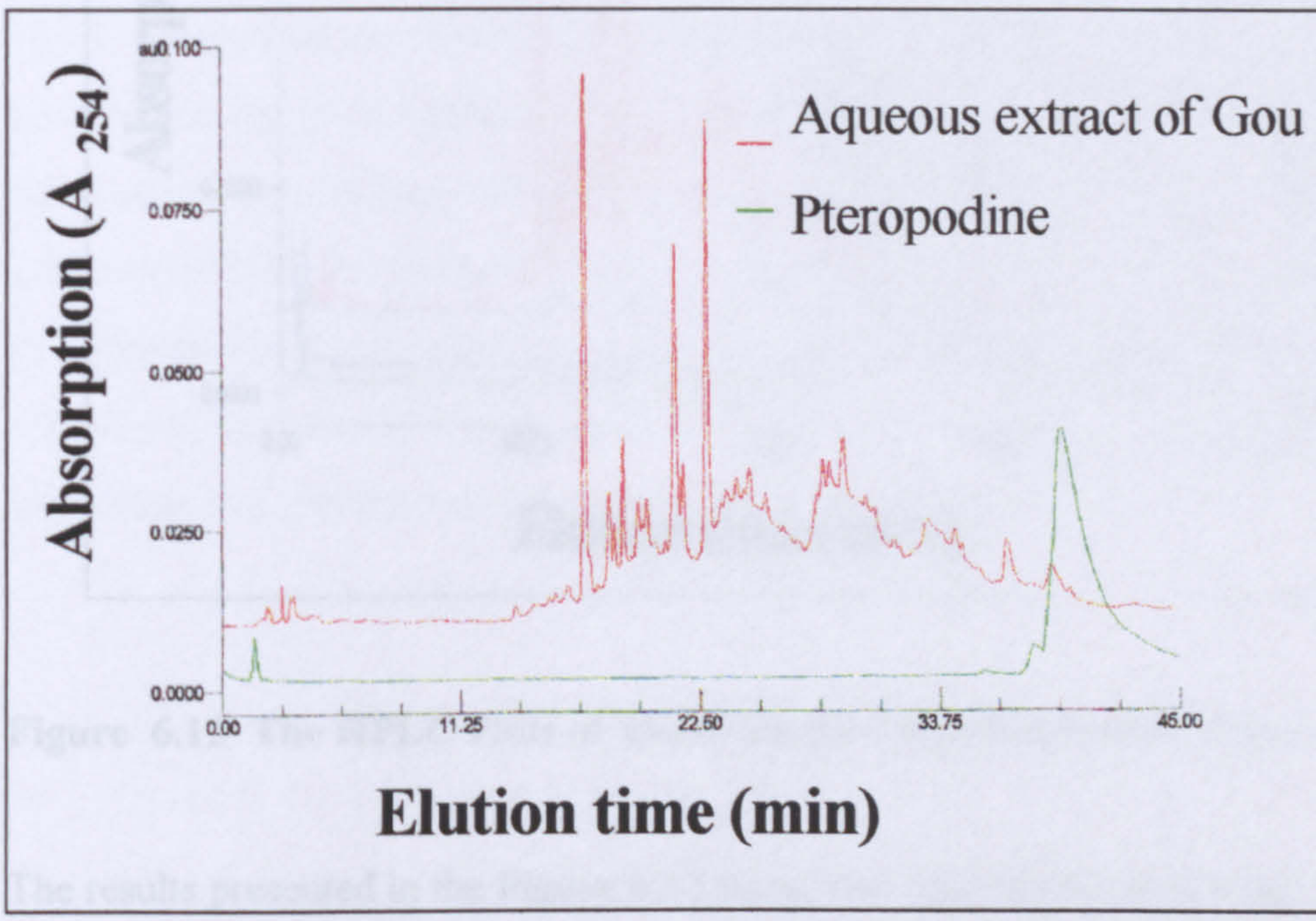



**Figure 6.11c The HPLC Plots of Pteropodine and Gwext**

As shown in **Figure 6.11c** the Pteropodine was eluted at the elution time of 39.66 minutes. The related peak was obtained at the elution time of about 38.86 minutes on Gwext plot. It was also in the range of Pool 7.

## CONCLUSION AND SUMMARY

### 6.6 Conclusion

The three single compounds were compared with Gwext in the same graph as shown as **Figure 6.12**.

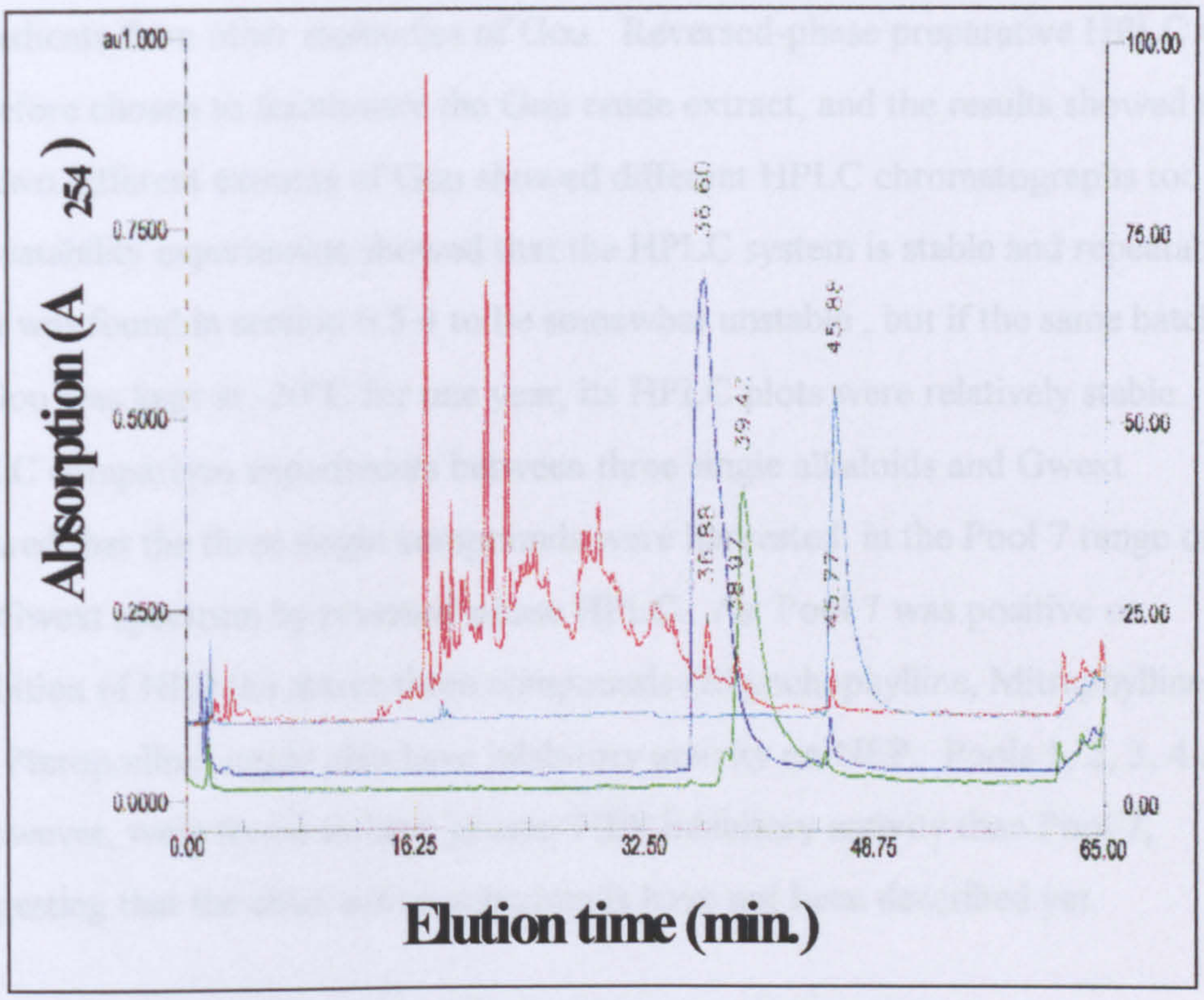



**Figure 6.12 The HPLC Plots of Three Single Compounds with Gwext**

The results presented in the **Figure 6.12** suggested that all the three single compounds presented in the area of Pool 7. As Pool 7 was already found to have inhibitory activity on NEP (section 6.6.5), above three compounds might be the active components of Pool 7. However, the inhibitory activity of Pools 1, 2, 3, 4, and 6 are greater than that of Pool 7. Clearly the aqueous extract of Gou contains many compounds with NEP inhibitory activity. This is unexpected and suggests that several different compounds may be active.

## 6.7 Summary

In this chapter the systematic chemical study of Gou crude extract was done so that the active ingredients could be fractionated step by step. The spectral analysis showed that both Gou aqueous (water) extract (Gwext) and organic (methanol) extract (Gmext) have absorption only in the U.V. range. And their absorption peaks are different (see section 6.4); 50% ethanol pre-treatment and

followed with sephadex G-10 column gel filtration can not separate the active ingredients from other molecules of Gou. Reversed-phase preparative HPLC was therefore chosen to fractionate the Gou crude extract, and the results showed that the two different extracts of Gou showed different HPLC chromatographs too. Repeatability experiments showed that the HPLC system is stable and repeatable; Gou was found in section 6.5.4 to be somewhat unstable , but if the same batch of Gou was kept at -20°C for one year, its HPLC plots were relatively stable. HPLC comparison experiments between three single alkaloids and Gwext showed that the three single compounds were harvested in the Pool 7 range of the Gwext spectrum by reversed-phase HPLC. As Pool 7 was positive on inhibition of NEP the above three compounds (Rhynchophylline, Mitraphylline and Pteropodine) might also have inhibitory activity on NEP. Pools 1, 2, 3, 4 and 6 however, were found to have greater NEP inhibitory activity than Pool 7, suggesting that the chief active compounds have not been described yet.

# CHAPTER 7

# CONCLUSIONS AND DISCUSSIONS

## 7.1 Known Analgesic Effects of Four Chinese Herbs

It has been known that the endogenous opioid peptides Met- and Leu-enkephalin are rapidly inactivated *in vivo*, by neutral endopeptidase (NEP E.C.3.4.24.11) (Malfroy *et al.*, 1978; Guyon *et al.*, 1979) and aminopeptidase N (APN E.C 3.4.11.2) (Hambrook *et al.*, 1976; Hersh, 1986); a further enkephalin inactivator is angiotensin-converting enzymes (ACE) (Waksman *et al.*, 1984; Giros *et al.*, 1986). In recent years the inhibition of enkephalin metabolism has been extensively investigated with the aim of testing the hypothesis that increasing the level of endogenous enkephalins in neuronal pathways implicated in pain transmission could lead to a "physiological" analgesia free of the major side effects of morphine (Roques and Fournie-Zaluski, 1986; Chipkin., 1986). Inhibitors of NEP and APN increase the level of endogenous enkephalin, and potent physiological analgesic responses should be produced without major side effects in all animals in pain for which morphine is normally prescribed (Fournie-zaliski *et al.*, 1984; Claude *et al.*, 1984; Nobel *et al.*, 1991, 1992, 1997; Chen *et al.*, 1998).

Based on the above background many scientists are interested in screening the inhibitors of the enkephalin-hydrolysing enzymes (including NEP, APN and ACE) and have obtained much knowledge in this field. French scientists are concentrating on biosynthesis of the enkephalin-like compounds which are sensitive to NEP, APN, DAP and ACE (Adjroud, 1995; Nobel *et al.*, 1991, 1992, 1997). So far they have developed a family of analgesics devoid of opioid side effects (Chen *et al.*, 1998; Nobel *et al.*, 1997), but no compound has actually been applied clinically. Compound RB 101 passed through clinical trial but was only slightly active after oral administration (Nobel *et al.*, 1991, 1992). Another compound RB 120 is currently on clinical trial ( Nobel *et al* , 1997). Japanese scientists are engaged in screening the natural inhibitors of these enzymes from micro-organisms (Kojima, 1990; Akiyama *et al.*, 1998; Otani, 1991, 1992; Tsurumi, 1995; Kimura, 1990; Tsuru *et al.*, 1992). However far from satisfactory results were obtained. As one kind of natural resource, plants are also worth investigating but so far no papers have been published. Traditional Chinese Herbs (TCH) have been used as analgesics for thousands of years (Yang *et al.*, 1985). Thus it

might be possible to find new analgesics by use of Chinese herbs clinically. This effect may be mediated by altering the metabolism of endogenous enkephalin by inhibiting NEP and APN, thus producing analgesia free of induced physical and psychic dependence.

So far Chinese scientists have done much work on screening new analgesics free of major side effects of morphine. Yang and his colleagues (1985) have successfully investigated the effects of three hundred cases of Chinese herbs on drug addiction *in vivo*. They used whole-animal tests to investigate the analgesic activity of twenty herbs, combined with data from treating opium and heroin addicts in Hong Kong. The results of three hundred cases were analysed and evaluated. The effects of Chinese herbs on withdrawal behaviour in morphine-addicted rats were also investigated. Four herbs (Qiang huo, Gou teng, Chuan xiong, Yan husuo) were found to reduce the three main withdrawal symptoms significantly. The effect of the above four herbs in combination on the morphine-withdrawal signs were also compared with that of endogenous opiate-like peptides (endorphin, enkephalin, dynorphin) and acupuncture. The analgesic effects of the above herbs were also studied in mice where the writhing test (a standard analgesic experiment—see section of *1.2)* caused a reduction of the writhing sign. Tests on the contraction of guinea pig ileum showed that the inhibition of the enzymes caused by the herbs was not blocked by naloxone. This suggested that none of the above herbs contained morphine-like substances showing that the analgesic activity was by a different mechanism. Based on the study of Yang *et al* (1985), it was decided in this thesis to investigate the effects of the above four listed herbs on enkephalin-hydrolysing enzymes (NEP, APN and ACE), so that the molecular mechanism for the analgesic activity of above four herbs might be clarified and the active ingredients identified.

The project involved screening for selective NEP inhibitors, mixed NEP/APN inhibitors and mixed NEP/ACE inhibitors. NEP was the key enzyme in this screening system.

## 7.2 NEP and Other Enkephalin-hydrolysing Enzyme Inhibitory Effects of Four Chinese Herbs

The crude microsomal fraction containing enkephalin-hydrolysing enzymes was therefore prepared from the rat kidney (section 2.3.1), and the enkephalin-hydrolysing enzymes assay was established (Chapter 3). By using selective NEP inhibitors or mixed inhibitors of NEP/APN, APN/ACE, and NEP/ACE, the inhibitory effects of four Chinese herbs Yan; Gou; Qiang and Chuan on NEP activity were tested and compared (Chapter 4). Aqueous extracts of Gou (Gwext) and Qiang (Qwext) showed significant inhibitory activity on NEP but results with organic extracts were not significant. Both aqueous and organic extracts of Yan (Ywext) and Chuan (Cwext) showed less inhibitory activity than Gou and Qiang. This suggested that the four herbs may have different bases for their analgesic activity (Yang *et al.*, 1985, 1986). Gwext showed the strongest inhibitory activity on NEP. The minimum concentration of Gwext needed to produce detectable NEP inhibition was 2.5 mg dried extract per ml and 50% inhibitory concentration ($IC_{50}$) was 2.7 mg dried extract per ml for a digestion time of 30 minutes. The experiments showed that **Gwext and Qwext were not only dual inhibitors but also triple inhibitors of NEP/APN/ACE**. So far there has been no report that triple inhibitors of the above three enzymes could be obtained, even RB120 is only a dual inhibitor of NEP/APN (Nobel *et al.*, 1997). The above results are therefore of great interest. The inhibitory effect of Gwext was compared with standard inhibitors and it was found that **Gwext in the experiment showed stronger inhibitory activity than that which was produced by the chemical inhibitor Thiorphan and it was even stronger than the combined effects of *Bestain and* Captopril**. Therefore the following work was concentrated on studying Gou.

## 7.3 Presence of Many Compounds with NEP Inhibitory Activity in Gou Aqueous Extract

To test whether one compound in Gou had inhibitory activity on the three enzymes, or several compounds acting together, a crude fractionation of Gou aqueous extract was done so that the active ingredients could be identified step by step (Chapter 6). The

spectral analysis showed that both Gou aqueous (water) extract (Gwext) and organic (methanol) extract (Gmext) have absorption peaks in the U.V. range. Reversed-phase preparative HPLC was chosen to fractionate the Gou crude extract, and the results showed that the two different extracts of Gou showed different HPLC chromatorams. These results partly explain why the two different extracts of Gou have different inhibitory activity on NEP. The Gwext was further fractionated by HPLC and **pooled into seven parts**, and the effect of each part on NEP activity was investigated. The results indicated that **Pool 5** had no inhibitory activity on NEP while the other six pools showed significant inhibitory activity on NEP proteolytic action. Three single compounds **(Rhynchophylline, Mitraphylline and Pteropodine)** isolated from Gou by Liu *et al* (1993) were compared with Gwext on HPLC plots and it was shown that the above three single compounds were eluted in the Pool 7 range of Gwext. This result suggested that above three compounds might be relevant to the inhibitory activity of Pool 7 on NEP proteolytic action. However, the HPLC result of Gwext showed that the *NEP inhibitory activity of Pools* 1, 2, 3, 4, and 6 are greater than that of Pool 7. Clearly *the aqueous extract of Gou contains many compounds with NEP inhibitory* activity. *This is unexpected and suggests that at least five active compounds have not yet been identified.*

### 7.4 Possible Activity on Opiate Receptor by Gwext

In order to analyse the pharmacological action of Gou's analgesic activity further, the opiate-receptor binding assay was used to investigate the effect of Gou aqueous extract on naloxone-opioid receptor binding reaction. The rat brain membrane was prepared as section 5.2.1 and the optimum ligand binding experiments were established. Then the effect of Gou on the naloxone-opioid-receptor binding reaction was investigated (Chapter 5). The result showed that the low concentration of Gou aqueous extract seemed to be able to decrease the $^{3}$H-naloxone uptake in the absence of cold naloxone. The Gou seemed to act as an antagonist of $^{3}$H-naloxone under this condition, while at 4 mg/ml the Gou aqueous extract increased the uptakes of $^{3}$H-naloxone and acted as an agonist. These results suggested Gou aqueous extract contained several compounds

which showed different activity on the naloxone-receptor binding assay. Gou had synergistic activity with naloxone which demonstrated that Gou's effect on opioid receptors was different from naloxone (morphine derivatives). The three standard inhibitors of enkephalin-hydrolysing enzymes (bestain, captopril and thiorphan) increase the nonspecific binding of naloxone showing that they have no antagonised activity to naloxone (**Figure 5.6**). At the same time Gou at low concentration also increased the non-specific binding of naloxone. Clearly Gou contains many different compounds which are active in the opioid system.

Combining with Gou's inhibitory effect on enkephalin-hydrolysing enzymes, it is assumed that one way for Gou's analgesic activity might be due to its inactivation of enkephalin-hydrolysing enzymes. This effect may be mediated by altering the metabolism of endogenous enkephalin by inhibiting NEP and APN.

## 7.5 The Composition of Gou

Chemical study of Gou extracts (*Uncaria rhynchophylline*) has been on going for about ten years. So far chemists have isolated and identified about 39 single compounds from Gou (Liu *et al.*, 1991, 1993). Most of them are alkaloids. The chemical structure of above 39 compounds were shown in following figure (**Figure 7.1**).

**Figure 7.1 The Chemical Structure of the Compounds Isolated from Gou (Liu *et al.*, 1991)** The structure of Rhynchophylline is 6, Mitraphylline and Pteropodine are 5.

**Figure 7.1** suggests that Gou has been well studied for its composition. However properties of the above 39 compounds are far less well studied so far.

The biological functions of some alkaloids among the above 39 compounds have been reported in recent years. Liu *et al* (1983) reported that they observed the hemodynamic effects of total alkaloids of *Uncaria macrophylla* in anaesthetized dogs. Cardiac output was determined by an *impedance* cardiograph. Intravenous (i.v.) infusion of the total alkaloids isolated from Gou produced a moderate hypotension accompanied by a marked bradycardia. The cardiac output increased temporarily and then decreased slightly, where as the stroke volume consistently elevated. The total peripheral resistance

diminished during initial hypotension. *The possible cause* of hypotension may be a reduction of both cardiac output and peripheral resistance. Considering *NEP can* degrade atrial natriuretic peptides (ANP) which mediate the cardiovascular system metabolism (Seymour, 1995; Robl *et al.*, 1996), it may be that Gou's inhibitory activity on NEP is related to these observations.

## 7.6 Concluding Remarks

Taken together the results presented in this study and Liu's report in 1983 , the pharmacological activity of Gou can be summarised as follows.

**(1) Inhibition of NEP/APN, so that the endogenous enkephalins could be kept at a high level and bind with opioid receptors thus producing analgesia;**

**(2) Inhibition of NEP/ACE, so the atrial natriuretic peptide (ANP) could be kept at a high level, thus resulting in an antihypertensive effect.** Above conclusion can also be expressed as in following chart **(Figure 7.2)**.

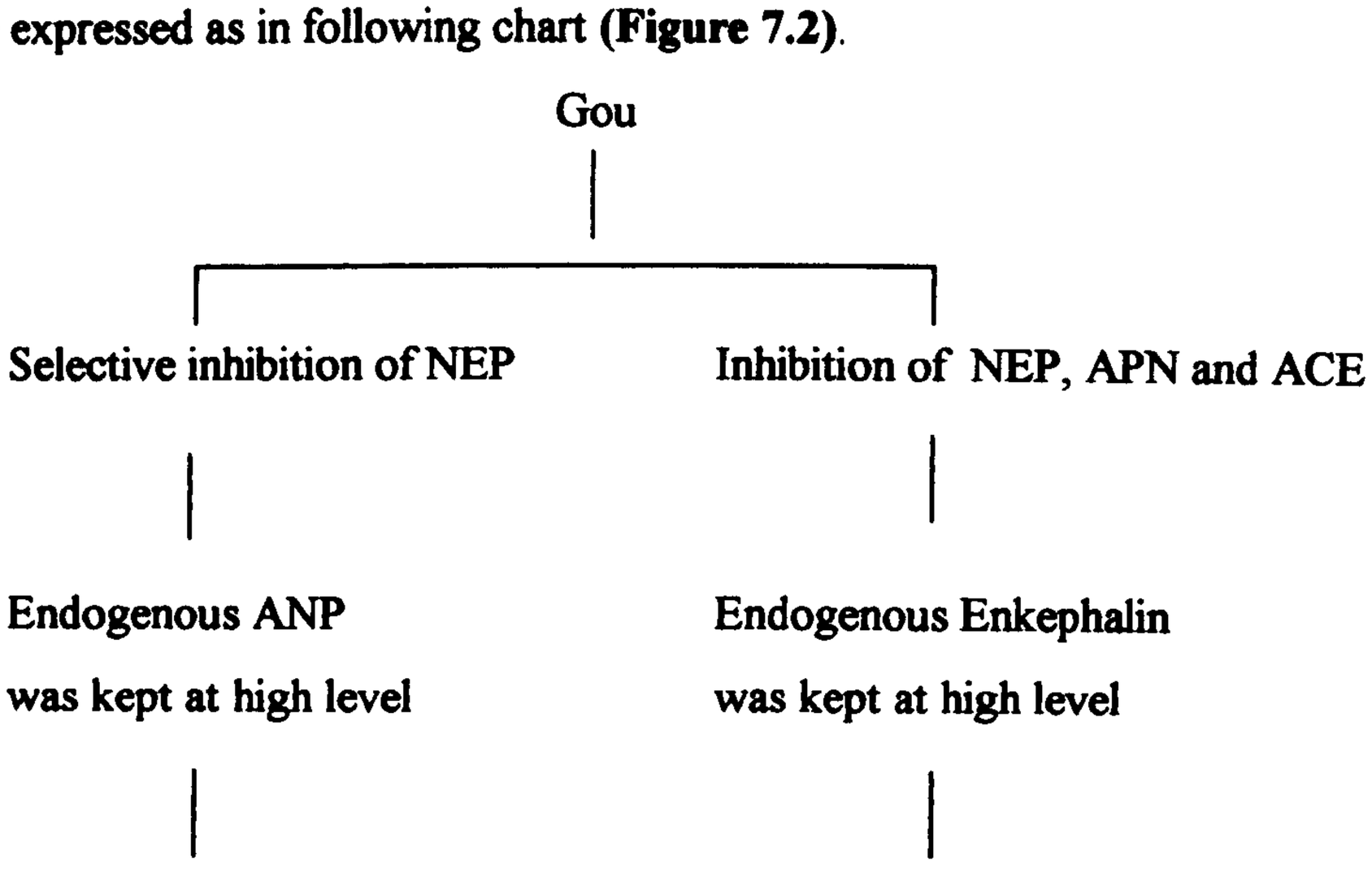



**Figure 7.2 A Possible Basis for Gou's Action**

## 7.7 Scope for Future Studies

Future studies will be concentrated on following :-

(1) Isolation of the 39 single compounds from Gou;

(2) Assay of the effect of the above 39 single compounds on enkephalin-hydrolysing enzyme (NEP and APN) activity;

(3) Design and utilization of a subtype opioid receptor binding assay to investigate the effect of the above 39 single compounds;

(4) Design and utilization of a standard analgesic assay in animals to observe the effect of active compounds obtained from Gou.

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# APPENDIX 1

## Amino acid abbreviations

| | | |
|---|---|---|
| A | ALA | Alanine |
| C | CYS | Cystine |
| D | ASP | Aspartic acid |
| E | GLU | Glutamic acid |
| F | PHE | Phenylalanine |
| G | GLY | Glycine |
| H | HIS | Histidine |
| I | ILU | Iso-leucine |
| K | LYS | Lysine |
| L | LEU | Leucine |
| M | MET | Methionine |
| N | ASN | Asparagine |
| P | PRO | Proline |
| Q | GLN | Glutamine |
| R | ARG | Arginine |
| S | SER | Serine |
| T | THR | Threonine |
| V | VAL | Valine |
| W | TRP | Tryptophan |
| Y | TYR | Tyrosine |

## APPENDIX 2

## SILVER STANING OF SDS-PAGE GELS

* Wear gloves at all times (Wash gloves before use, to remove talc.);
* Staining, developing, fixing and washing of gel are carried out on a flat bed rocker;
* Stain and developer need to be made up fresh.

1 Clean equipment/glassware with alcohol and $dH_2O$.

2 Wash gel in two changes of 50% **MeOH**. Each wash time is 15-30 minutes.

3 **STAIN**; Dissolve 1×NaOH pellet in 20 ml $dH_2O$, add 1.5 ml neat **ammonium hydroxide**;

Dissolve 0.8 g **silver nitrate** ($AgNO_3$) in 2.5 ml $dH_2O$;

With vigorous stirring of the ammonium solution, slowly add the silver solution dropwise. If a brown precipitation appears and will not clear, add drops of ammonium hydroxide. Dilute this solution to 200 ml (measuring cylinder).

4 Rinse gel in $dH_2O$ (few seconds).

5 Soak in silver stain for 20-40 minutes.

6 **DEVELOPER**; 2.5 ML 1% **citric acid**

250 µl **formaldehyde**

up to 500 ml (measuring cylinder).

7 Rinse gel in 2-3 changes of $dH_2O$.

8 Transfer gel to glass container, add developer. Brown bands should show in a few minutes. If precipitation appears, or developing is taking too long, pour off developer and add fresh.

9 Just before developing is complete, transfer gel to $dH_2O$. Developing will continue until developer is diffused out gel.

10 Rinse gel in 2-3 changes of $dH_2O$.

11 **FIX**; 45% **MeOH** (225 ml)

5% **acetic acid** (25 ml)

50% $dH_2O$ (250 ml)

12. Transfer gel to fix, leave for at least 30 minutes.

# APPENDIX 3

## The Student t Distribution

Early in this century it was shown by w.s. Gossett, writing under the name of 'Student', that the mean of a sample from normal distribution with unknown variance has a distribution that is similar to, but not quite the same as a normal distribution. He called it as the **t distribution**, and we still refer it as **Student's t distribution**. As the sample size increases the sampling distribution of the mean becomes closer to the normal distribution for large samples there is little point in doing so, since for large samples the methods give virtually identical answers and it is simpler to use the same method regardless of the sample size.

The t distribution has one parameter, a quantity called the **degrees of freedom**. The concept of degrees of freedom is one of the more elusive statistical ideas. In general the degrees of freedom are calculated as the sample size, inus the number of estimated

parameters. The degrees of freedom for the t distribution relate to the estimated standard deviation, which is calculated as variation around the estimated mean. Hence for a single sample of n observations we have n -1 degrees of freedom.

## One Sample t Test

Carry out a test of the null hypothesis that our data are a population with a specific 'hypothesized' mean. The test is called the **one sample t test**, and the value of t is calculated as

$$t = \frac{\text{sample mean - hypothesized mean}}{\text{standard error of sample mean}}$$

following the common for of hypothesis tests. If population mean is some value k, we can rewrite the formula as

$$t = \frac{x - k}{s/\sqrt{n}}$$

$$t = \frac{(x-k)\sqrt{n}}{s}$$

where x and s are the mean and standard deviation of the sample of size n. The magnitude of t is thus the average discrepqancy of the sample values from the hypothetical mean, divided by the standard error of the sample mean.

The **Table B4** was used to find the P value associated with an observed value of t. We can ignore the sign of t for a two-sided test, and look for the largest tabulated value of t below the observed value, the recommended level using the usual criterion of $P < 0.05$. Notice that statistical significance gives no information about the magnitude of energy deficit, nor the uncertainty of that estimate.

Note that we use t to indicate the observed value of the test statistic and also a particular value from the theoretical t distribution. For clarity I always use a subscript in the latter case. For many other statistical methods we use slightly different notation for these two purposes.

**Confidence Interval For The Median**

The methods using the t distribution to calculate a confidence interval or perform a t test require the data to be approximately normally distributed. We can calculate a confidence interval for a sample median without making any assumption about the distribution of the data. The data are ranked in ascending order, and the ranks of the

values defining the confidence interval are found from a table such as that given in **Table B11**. From that table the 95% confidence interval for the median is given by the data values.

For small samples the confidence interval for the median is rather wide, here being nearly twice as wide as the confidence interval for the mean given earlier. For larger samples of data that have a normal distribution the mean and median will be very similar and their confidence intervals will agree closely. It is preferable to use the median if the data are not near to normal.

## Table B4 the t distribution

The tabulated values of the t distribution correspondend to give two-tailed P values for different degrees of freedom. For the two-sided hypothesis test where the test statistic has a t distribution, the P value is less than a tabulated value of P if the test statistic is greater than the tabulated t value. The tabulated values are $t_{1-\alpha/2}$ where P $= \alpha$ .

Example: For an observed statistic test t = 3.21 on 20 degrees of freedom we have P< 0.01.

| x degrees of freedom | Two-tailed probability (P) | | | | | |
|---|---|---|---|---|---|---|
| | 0.2 | 0.1 | 0.05 | 0.02 | 0.01 | 0.001 |
| 1 | 3.078 | 6.314 | 12.706 | 31.821 | 63.657 | 636.619 |
| 2 | 1.886 | 2.920 | 4.303 | 6.965 | 9.925 | 31.599 |
| 3 | 1.638 | 2.353 | 3.182 | 4.541 | 5.841 | 12.924 |
| 4 | 1.533 | 2.132 | 2.776 | 3.747 | 4.604 | 8.610 |
| 5 | 1.476 | 2.015 | 2.571 | 3.365 | 4.032 | 6.869 |
| 6 | 1.440 | 1.943 | 2.447 | 3.143 | 3.707 | 5.959 |
| 7 | 1.415 | 1.895 | 2.365 | 2.998 | 3.499 | 5.408 |
| 8 | 1.397 | 1.860 | 2.306 | 2.896 | 3.355 | 5.041 |
| 9 | 1.383 | 1.833 | 2.262 | 2.821 | 3.250 | 4.781 |
| 10 | 1.372 | 1.812 | 2.228 | 2.764 | 3.169 | 4.587 |
| 11 | 1.363 | 1.796 | 2.201 | 2.718 | 3.106 | 4.437 |
| 12 | 1.356 | 1.782 | 2.179 | 2.681 | 3.055 | 4.318 |
| 13 | 1.350 | 1.771 | 2.160 | 2.650 | 3.012 | 4.221 |
| 14 | 1.345 | 1.761 | 2.145 | 2.624 | 2.977 | 4.140 |
| 15 | 1.341 | 1.753 | 2.131 | 2.602 | 2.947 | 4.073 |
| 16 | 1.337 | 1.746 | 2.120 | 2.583 | 2.921 | 4.015 |
| 17 | 1.333 | 1.740 | 2.110 | 2.567 | 2.898 | 3.965 |
| 18 | 1.330 | 1.734 | 2.101 | 2.552 | 2.878 | 3.922 |
| 19 | 1.328 | 1.729 | 2.093 | 2.539 | 2.861 | 3.883 |
| 20 | 1.325 | 1.725 | 2.086 | 2.528 | 2.845 | 3.850 |
| 21 | 1.323 | 1.721 | 2.080 | 2.518 | 2.831 | 3.819 |
| 22 | 1.321 | 1.717 | 2.074 | 2.508 | 2.819 | 3.792 |
| 23 | 1.319 | 1.714 | 2.069 | 2.500 | 2.807 | 3.768 |
| 24 | 1.318 | 1.711 | 2.064 | 2.492 | 2.797 | 3.745 |
| 25 | 1.316 | 1.708 | 2.060 | 2.485 | 2.787 | 3.725 |
| 26 | 1.315 | 1.706 | 2.056 | 2.479 | 2.779 | 3.707 |
| 27 | 1.314 | 1.703 | 2.052 | 2.473 | 2.771 | 3.690 |
| 28 | 1.313 | 1.701 | 2.048 | 2.467 | 2.763 | 3.674 |
| 29 | 1.311 | 1.699 | 2.045 | 2.462 | 2.756 | 3.659 |
| 30 | 1.310 | 1.697 | 2.042 | 2.457 | 2.750 | 3.646 |

## APPENDIX 4 Reversed-phase HPLC Elution Profile of Gou

The HPLC profile of Gou aqueous extract used in Chapter 6 was shown in following table.

**Table HPLC Elution Profile of Gou Aqueous Extract**

| TIME | % Pump_% | Pump_FLOW RATE |
|---|---|---|
| 0.00 | 100.00 | 0.00 | 1.00 |
| 0.10 | 100.00 | 0.00 | 1.00 |
| 5.00 | 100.00 | 0.00 | 1.00 |
| 5.01 | 95.00 | 5.00 | 1.00 |
| 10.00 | 95.00 | 5.00 | 1.00 |
| 10.01 | 90.00 | 10.00 | 1.00 |
| 15.00 | 89.90 | 10.10 | 1.00 |
| 15.01 | 85.00 | 15.00 | 1.00 |
| 20.00 | 85.00 | 15.00 | 1.00 |
| 20.01 | 80.00 | 20.00 | 1.00 |
| 25.00 | 80.00 | 20.00 | 1.00 |
| 25.01 | 75.00 | 25.00 | 1.00 |
| 30.00 | 75.00 | 25.00 | 1.00 |
| 30.01 | 70.00 | 30.00 | 1.00 |
| 35.00 | 70.00 | 30.00 | 1.00 |
| 35.01 | 65.00 | 35.00 | 1.00 |
| 40.00 | 65.00 | 35.00 | 1.00 |
| 40.01 | 60.00 | 40.00 | 1.00 |
| 45.00 | 60.00 | 40.00 | 1.00 |
| 45.01 | 55.00 | 45.00 | 1.00 |
| 50.00 | 55.00 | 45.00 | 1.00 |
| 50.01 | 50.00 | 50.00 | 1.00 |
| 55.00 | 50.00 | 50.00 | 1.00 |
| 55.01 | 45.00 | 55.00 | 1.00 |
| 60.00 | 45.00 | 55.00 | 1.00 |
| 60.01 | 0.00 | 100.00 | 1.00 |
| 65.00 | 0.00 | 100.00 | 1.00 |
| 65.10 | 100.00 | 0.00 | 1.00 |