STUDIES ON THE PHARMACOLOGY OF PHOLCODINE, CODEINE AND DEXTROMETHORPHAN IN MAN AND RAT

Thesis submitted for the degree of Doctor of Philosophy

by

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DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University and that to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Zhao Rong Chen

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CONTENTS

ABSTRACT

The aims of this thesis were to study the pharmacokinetics and pharmacodynamics of pholcodine, codeine and dextromethorphan in humans and in rats.

1. Original sensitive and specific HPLC assays were developed for the determination of (1) pholcodine; (2) codeine-6-glucuronide; (3) codeine, norcodeine and morphine and (4) dextromethorphan and three metabolites in plasma and in urine, which are suitable for the pharmacokinetic and metabolism studies in man and rat.

2. The pharmacokinetics and metabolism of pholcodine after single and chronic dosing were studied in six healthy human subjects. The pharmacokinetics and metabolism of pholcodine were substantially different from those of other chemically related compounds, such as codeine. Pholcodine had a very long plasma half-life which was about 15 times longer than that of codeine. The results indicate that the currently recommended dosage regimens for pholcodine may be inappropriate. Two new metabolites were isolated and one of them, a oxidative product of the morpholine ring, of pholcodine was successfully identified by HPLC, mass spectra and nuclear magnetic resonance spectra.

3. The pharmacokinetics of codeine were comparatively studied in 8 young subjects and 7 elderly patients. Codeine-6-glucuronide, the major metabolite of codeine, was directly determined in plasma and in urine for the first time. The plasma concentrations of codeine-6-glucuronide were 17 times higher than codeine but the plasma half-lives of the two compounds were similar in young subjects. The pharmacokinetics of codeine and codeine-6-

glucuronide in the elderly were altered compared with those in the young subjects. The absorption was delayed and the plasma half-lives for codeine and especially for codeine-6-glucuronide were increased. The plasma concentrations of codeine and codeine-6-glucuronide at steady state increased 2.4 and 3.8 times respectively and the renal clearances of codeine and codeine-6-glucuronide decreased 5.0 and 7.2 times respectively. The plasma concentrations of codeine and codeine-6-glucuronide at steady state were strongly correlated with the clearance of creatinine. All the pharmacokinetic changes were significantly correlated with age. β-glucuronidase, a widely used tool for the studies of glucuronides, is not suitable for the quantitative determination of codeine-6-glucuronide because of the incomplete hydrolysis.

4. The polymorphic metabolism of codeine was demonstrated in humans. The O-demethylation ratio of codeine was strongly correlated with that of dextromethorphan which is known to exhibit genetic polymorphism in the O-demethylation. This finding may have important clinical implications because codeine may not produce analgesia in the poor metabolisers who are unable to metabolise codeine to morphine. The preliminary results of the study on the genetic polymorphism of dextromethorphan suggested that the frequency of deficiency of this polymorphism in an Australian population was 3/52, which was similar to those in European countries (3-9%).

5. The μ-receptor binding affinities of codeine and its metabolites and several other opioids were studied in rat brain using the ligand ^{3}H-DAGO. The results showed that some of metabolites had similar or higher affinity to the μ-receptor than the parent compound and suggest they may be important in mediating analgesia. Pholcodine and dextromethorphan showed very low

binding affinities to the μ-opioid receptor which supports the previous findings that pholcodine and dextromethorphan have no analgesic effect.

6. Codeine O-demethylation to morphine in the brain was studied in the rat in vitro and in vivo. At 30 minutes after intraperitoneal administration of codeine, morphine was detected in the brain. However, after intraperitoneal administration of morphine, although a similar plasma morphine concentration was achieved, morphine was not detected in the brain. The results indicate a central (brain) conversion of codeine to morphine. The morphine concentrations in the rat brain after codeine administration peripherally were about 20 times that required to displace 50% of the μ-receptor ligand ^{3}H-DAGO. After incubation of codeine with NADPH system in vitro, more morphine was found in microvessel rich tissue than in the total homogenate. The results suggest that the O-demethylation of codeine to morphine occurs in microvessel tissue, possibly in endothelial cells. The biotrans-formation of codeine to morphine in the brain rather than in liver may explain the analgesic effect of codeine.

7. A cough recording system for measuring cough frequency was developed for a clinical study designed to compare the relative antitussive efficacy of codeine, pholcodine and dextromethorphan in patients. In a double-blind, placebo controlled pilot study, the antitussive effect of these agents in patients with chronic cough were studied.

PREFACE

The work described in this thesis was done in the Department of Clinical and Experimental Pharmacology, the University of Adelaide and the Department of Clinical Pharmacology of Royal Adelaide Hospital from April 1986 to August 1988. The initial aims of this thesis were to study the pharmacokinetics and cough suppressant effects of pholcodine, codeine and dextromethorphan in humans. As the studies were being carried out, some interesting findings, such as new metabolites of pholcodine, codeine polymorphic metabolism and the biotransformation of codeine to morphine in the brain, which were not suspected before, were observed. These findings attracted me so much, that a lot of time and energies have been used to investigate these other aspects. Thus, the focus has moved away from investigating the above drugs only as cough suppressants, and particular emphasis has been placed on the metabolism and analgesic activity of codeine.

Chapter 1.

GENERAL INTRODUCTION

1.1. Cough and cough suppressants

Cough is a protective physiological reflex serving to clear the respiratory passages of foreign materials and excess secretions. Cough appears in a wide variety of diseases (Skinner 1986), but it should not be suppressed indiscriminately. When cough is incapable of, or ineffective in, removing the provoking stimulus, secretions form constantly in response to this provocation. There are, however, many situations in which cough does not serve any useful purpose but may instead, cause serious medical problems (Bickerman & Itkin 1960) or prevent the patient from rest and sleep. In such situations, cough should be suppressed. Cough involves a complex reflex arc that begins with irritation of receptors. Cough receptors are situated mainly but not exclusively within the extrapulmonary airways (Sant'Ambrogio *et al* 1984; Banner 1986). The impulses from these receptors are conducted to a central area by way of afferent nerves and then passed down appropriate efferent nerves to expiratory musculature (Widdicombe 1980). A cough center has been identified in the medulla (Kase *et al* 1970; Chou and Wang 1975; Irwin *et al* 1977), but nothing is known of its central nervous organization. Drugs that can affect this complex mechanism directly or indirectly may possess antitussive activity.

A number of experimental models have been developed and used for evaluation of cough suppressants (Eddy *et al* 1969a). Cough may be induced experimentally in man by chemical stimuli, such as citric acid (Packman and London 1977; Empey *et al* 1979; Rees and Clark 1983; Pounsford and Saunders 1985; Lowry *et al* 1988), paraldehyde (Konar and Dasgupta 1959) and ammonia (Rosiere *et al* 1956). However, artificially induced cough in volunteer subjects is not necessarily the same as cough of pathological origins. Methods assessing pathological cough in humans have relied on subjective

evaluations or objective cough counting techniques. Subjective evaluation is highly variable, with an unacceptable margin for error (Bickerman and Rodgers 1980). Objective studies employing the actual recording of the cough are required to document the suppressant effect of antitussive agents (Svedmyr 1980). Many agents have been evaluated for their antitussive properties. Codeine, pholcodine and dextromethorphan are the most commonly used antitussives (Anonymous 1985). They are known to reduce cough as a result of their central actions (Jaffe and Martin 1985). Codeine and pholcodine are classified as narcotic antitussives while dextromethorphan as a non-narcotic antitussive agent (Jaffe and Martin 1985). Some opioids, such as codeine, have both analgesic and antitussive activities. Although they have been used clinically for 30-150 years, especially pholcodine and dextromethorphan, little is known about their fate in the human body and their relative antitussive effectiveness.

1.2. Opioid receptors and analgesia

Opioids play an important role in medical therapy because of their ability to relieve pain. Opioid-induced analgesia is the result of actions of the drug in the brain and involves several systems of neurotransmitters. The neurophysiological and pharmacological mechanism of analgesia remains unclear. However recent information has increased our understanding about some aspects of opioids and analgesia.

The opioid receptors have been extensively investigated and the present data indicate that several opioid receptors are present in the brain including μ, κ, δ and σ (Jaffe and Martin 1985). Analgesia has been associated with both μ and κ opioid receptors, while psychotomimetic effects have been ascribed to δ receptors and σ receptors are thought to be involved in alteration of affective behavior. The behavioral profiles of narcotics have indicated that μ-opioid receptor mediates supraspinal analgesia and κ receptor mediate spinal analgesia.

Morphine, a potent agonist of the μ-opioid receptor, has been widely used as an analgesic for many years and remains the standard against which new analgesics are measured. Structure-activity studies have shown that most drugs that act at the μ-opioid receptor contain a benzene ring and a basic tertiary amine with the spatial arrangement as found in morphine, and that a free phenolic hydroxyl group at position 3 is also very important for high potency. The replacement of the hydroxyl group by a methoxyl group decreases the binding affinity to the μ-opioid receptor markedly (Martin 1983; Jaffe and Martin 1985). Substitution at position 6 is not as important as at position 3.

The binding affinity of a compound to the μ-opioid receptor is the essential requirement for a centrally acting analgesic. However analgesic activity is influenced by many factors such as: agonistic versus antagonistic activity; resistance to metabolic breakdown and the affinity to the μ-opioid receptor of metabolites; binding to plasma proteins; and the ability to cross the blood brain barrier of the drug and its metabolites.

1.3. Codeine

1.3.1. Antitussive effect of codeine

Codeine is one of the numerous alkaloids contained in opium. The alkaloid is the methoxyl derivative of morphine and has been prescribed for its analgesic and antitussive properties for about 150 years (Jaffe and Martin 1985). The antitussive effectiveness of codeine has been demonstrated experimentally against cough induced by different stimuli in various animals (Eddy *et al* 1969b). A number of clinical trials of codeine as an antitussive drug in patients with cough of various origins have been conducted (Cass *et al* 1954; Mulinos *et al* 1962; Sevelius *et al* 1971). Most authors have found it to be superior to placebo, subjectively or objectively, in reducing cough frequency and intensity. These trials have also shown that a single dose of codeine of 30-60 mg or a daily dosage of 45-160 mg (divided dose) exerts an antitussive action (Eddy *et al* 1969b). Sevelius and coworkers (1971) reported that 15 mg of codeine orally reduced the frequency of pathological cough and progressively greater cough suppression was seen objectively as the dose was increased up to 60 mg. For many years, codeine has been used as a standard against which other antitussives have been tested (Bickerman and Rodgers 1980).

Codeine is known to reduce cough centrally (Kase *et al* 1970), although the exact mechanisms are still not clear. There is evidence that codeine binds in a saturable manner in the guinea-pig medulla, an area of the brain where the cough center is located (Chou and Wang 1975; Chau *et al* 1982).

1.3.2. Analgesic effect of codeine

Codeine is a commonly prescribed analgesic for mild to moderate pain, often combined with peripherally acting analgesics such as paracetamol or aspirin. The analgesic potency of codeine has been studied in various species of animals with different experimental methods. The results of these studies have generally shown that the analgesic potency of codeine was 1/10 to 1/3 of that of morphine after subcutaneous administration (Eddy *et al* 1968; Martin 1983). The analgesic effectiveness of codeine in man, in equianalgesic doses to morphine, was similar to that in animals (Eddy *et al* 1968; Jaffe and Martin 1985). Codeine itself has a much lower affinity to the μ-opioid receptor than morphine (Pert and Snyder 1973). Its analgesic effect was proposed to be exerted by the active metabolite morphine. However this hypothesis has been questioned based on results from both animal and human studies. It has been reported that parenteral administration of codeine, produced lower plasma concentrations of morphine (Rogers *et al* 1982), but resulted in stronger analgesic effects (Eddy *et al* 1968; Beaver *et al* 1978) compared with those after oral administration of codeine. The analgesic effects of other metabolites (Figure 1-3-1), especially the major metabolite codeine-6-glucuronide, have not been studied.

1.3.3. Assays for the determination of codeine and its metabolites in biological fluids

Numerous analytical methods for the determination of codeine in biological fluids have been reported. These methods include radio-labeled drug (Adler *et al* 1955; Yeh and Wood 1970b), radioimmunoassay (RIA) (Findlay *et al* 1977b; Butz *et al* 1983), gas chromatography (GC) (Schmerzler *et al* 1966; Sine *et al* 1973; Medzihradsky and Dahlstrom 1975; Brunson and Nash

1975; Cone 1976; Zweidinger *et al* 1976; Dahlström *et al* 1977; Bodd *et al* 1987), gas chromatography-mass spectrometry (GC-MS) (Clarke and Foltz 1974; Ebbighausen *et al* 1973b; Cole *et al* 1977; Chen *et al* 1982; Cone *et al* 1983; Quiding *et al* 1986) and high performance liquid chromatography (HPLC) (Nelson *et al* 1980; Tsina *et al* 1982; Visser *et al* 1983; Posey and Kimble 1983; 1984; Nitsche and Mascher 1984; Ginman *et al* 1985; Bedford and White 1985; Shah and Mason 1987). One feature of RIA is that the antibody is not specific for one compound, but cross-reacts with metabolites and other structurally similar compounds. The antibody to morphine, for example, often cross-reacts extensively with codeine (Gross *et al* 1974; Butler Jr 1978). The cross-reactivity of codeine-6-glucuronide, morphine and norcodeine with the anticodeine serum were 5%, 6.5% (Findlay *et al* 1977a,b) and 22% (Findlay *et al* 1978) respectively. This problem has been highlighted in the case of morphine, a metabolite of codeine. Hanks and Aherne (1985) compared the RIA with an HPLC method for morphine using the plasma samples from the same subjects after oral and intravenous administration of morphine sulfate. A good correlation was obtained between RIA and HPLC method for samples taken after intravenous administration, but after oral administration, the plasma morphine concentrations determined by RIA were 5-10 times higher than those determined by HPLC. Aherne and Littleton (1985) reported that morphine-6-glucuronide is an important factor interacting with morphine RIA. While the GC method is specific, the sensitivity, however, is often limited by the flame-ionization detector used and the absorption of compounds by column packings (Tsina *et al* 1982). Although GC-MS methods for codeine are sensitive and specific, the expensive and sophisticated instrumentation required is not available in many laboratories and the technique is time and cost-consuming for the analysis of a large number of samples. HPLC is specific and sensitive enough to study the pharmacokinetics of codeine at

therapeutic doses. Two of the assays mentioned above are for the simultaneous determination of codeine, norcodeine and morphine in urine (Posey and Kimble 1984) or in plasma (Shah and Mason 1987). However these methods are not satisfactory for pharmacokinetic study (see Chapter 4.1.). There is no assay reported on the direct determination of codeine-6-glucuronide, the major metabolite of codeine, in biological fluids. In addition, although many procedures have been described, only a few of them have been used in codeine pharmacokinetic studies (Vaughan and Beckett 1973; Findlay *et al* 1977a; Visser *et al* 1983; Quiding *et al* 1986; Bodd *et al* 1987).

1.3.4. Pharmacokinetics and metabolism of codeine

1.3.4.1. Absorption

Codeine is rapidly absorbed from the gastrointestinal tract, the peak plasma concentration occurring in about one hour (Findlay *et al* 1977a; Findlay *et al* 1978; Rogers *et al* 1982; Moolenaar *et al* 1983; Aylward *et al* 1984; Findlay *et al* 1986; Quiding *et al* 1986; Guay *et al* 1987) and the extent of absorption is virtually complete (Adler *et al* 1955; Vaughan and Beckett 1973; Bechtel and Sinterhanf 1978). Codeine is more rapidly absorbed and peak blood concentrations are higher following rectal administration than following oral administration (Moolenaar *et al* 1983). Absorption from alkaline solutions is more rapid than from acidic solutions (Moolenaar *et al* 1983) as might be expected.

1.3.4.2. Bioavailability

Vaughan and Beckett (1973) compared the 24 hour urinary excretion of unchanged codeine after oral administration of codeine phosphate with that after an intramuscular injection of the same dose and found the

bioavailability of codeine was 56-66%. Harris and coworkers (1982) reported in abstract form that the bioavailabilities of codeine in different formulations ("A.P.C.+ codeine phosphate tablet, codeine phosphate solution, codeine sulphate tablet") were similar. These were 62%, 60% and 57% respectively based on the area under the plasma concentration-time curve of codeine after oral administration compared with that after an intramuscular injection of the same dose. There was no difference in the bioavailability of codeine phosphate when it was administered as an oral solution, a rectal solution or a rectal suppository (Moolenaar *et al* 1983).

1.3.4.3. Distribution

Codeine is lipid soluble (3.98 octanol/pH 7.4, Moffat *et al* 1986) and penetrates cellular membrane barriers readily. It has been reported that codeine gets into the brain easily (Oldendorf *et al* 1972). Brain concentrations of codeine were much higher than those in plasma after peripheral administration (Gintzler *et al* 1976). Codeine has been reported to penetrate into human milk easily with an average milk/plasma area under the concentration-time curve ratio of 2 (Findlay *et al* 1983). A higher concentration of codeine in saliva has also been determined by Lee and coworkers (1986). In this study, the saliva/plasma concentration ratio was 4.2-5.5 after oral administration. Findlay and coworkers (1977a) determined the volume of distribution of codeine following an intramuscular injection to be 3.5±0.2 L/kg in six subjects. Quiding and coworkers (1986) reported the volume of distribution of codeine after seven oral doses to be 3.97±1.2 (2.27-5.70) L/kg in 12 subjects.

1.3.4.4. Protein binding

30% of codeine was bound to serum proteins in vitro (albumin 14.3%) determined by dynamic equilibrium dialysis, and the binding sites were the same as those for morphine and methadone (Judis 1977).

1.3.4.5. Metabolism

The biotransformation of codeine is catalysed by enzymes located in cells of various tissues, including the liver, small intestine, kidney, brain, placenta, adrenal cortex (Misra 1978). Of these the liver is the most important one. Codeine undergoes both phase I oxidative metabolism and phase II conjugative metabolism (Figure 1-3-1). "Bound" (glucuronide) codeine was first suggested to be an important biotransformation product of codeine in man in 1941 (Oberst *et al* 1941). It has been known that conjugation with glucuronic acid occurs at the 6-position of the codeine molecule (Way and Adler 1962; Yeh and Wood 1970a; Misra 1978). The O-demethylated and N-demethylated products, morphine and norcodeine respectively, were identified in urine after codeine administration in 1952 (Adler *et al* 1952). Both unconjugated and conjugated morphine and norcodeine can be found in urine after therapeutic dose of codeine (Mannering *et al* 1954; Adler *et al* 1955; Pærregaard 1958; Solomon 1974). Findlay and coworkers (1978; 1986) reported in volunteers taking 60 mg codeine phosphate the area under the plasma concentration-time curve of morphine, determined by RIA to be 10% of that of codeine. Quiding and coworkers (1986) found that the area under the plasma concentration-time curve of morphine, determined by GC-MS (a more specific method), as a percentage of that of codeine ranged from 0.9 to 4.4% with a mean of 2.6% after single and multiple 60 mg doses of codeine phosphate. Guay and coworkers (1987) reported that the percentage of

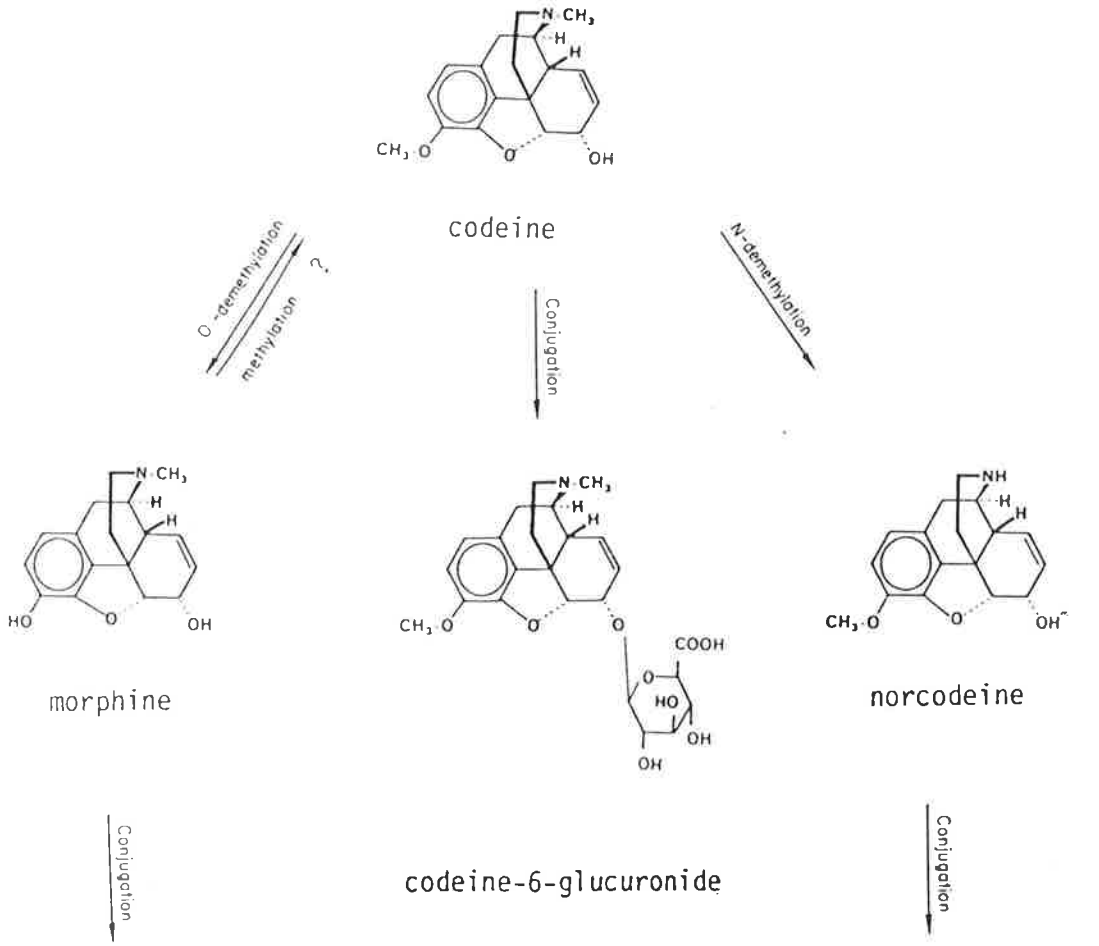


Figure 1-3-1 Metabolic pathways of codeine.

morphine/codeine area under the plasma concentration-time curve, based upon RIA, ranged from 8 to 32% (19±11%) after single codeine dosing and 10-40% (29±11%) after multiple codeine dosing. However, Nomof and coworkers (1977) did not detect any morphine or norcodeine in the blood samples, using GC, after intravenous infusion of codeine phosphate 60 mg/hour for 11 hours. Normorphine was detected (< 4% of the dose) in urine of man after taking 20 mg codeine phosphate (Ebbighausen *et al* 1973a). Other minor metabolites, in the guinea pig, reported by Cone and coworkers (1979, 1983) were hydrocodone, 6-α-hydrocodol, 6-β-hydrocodol, hydromorphone, 6-α-hydromorphol and 6-β-hydromorphol. It has been reported that codeine might be formed as the O-methylated metabolite of morphine in the rat, dog and in man (Böner and Abbott 1973; Yeh *et al* 1974). After oral or intravenous administration of morphine to man, 0.7-0.9% of the dose was recovered in urine as codeine (Böner and Abbott 1973).

Alcohol inhibited the O-demethylation of codeine but not N-demethylation in isolated rat hepatocytes (Bodd *et al* 1986). However there was no significant alteration in the O-demethylation in humans after oral administration of codeine phosphate (1 mg/kg) with and without ethanol (0.8 mg/kg) (Bodd *et al* 1987). Smoking does not influence the half-life or the area under the plasma concentration-time curve of codeine following oral administration (Rogers *et al* 1982).

1.3.4.6. Half-life

Findlay and coworkers, using RIA, determined the plasma half-life to be 3.3 hours following an intramuscular injection (1977a) and 2.9 hours following oral administration (1978). Similar values for the half-life of codeine were obtained by Rogers and coworkers (1982) and Quiding and

coworkers (1986), those being 2.8 hours and 2.5 hours respectively. The half-life of codeine, determined by RIA, was 5.9±2.0 hours in eight subjects who took 60 mg codeine phosphate syrup (Aylward *et al* 1984). Guay and coworkers (1987) reported the half-life of codeine, using RIA, to be 4.5±0.8 hours in six subjects. Bodd and coworkers (1987) studied the pharmacokinetics of codeine with a GC method, in the absence and presence of ethanol in humans and found the half lives to be 2.1±0.4 and 2.9±0.6 hours in the two groups respectively. These were not statistically significantly different.

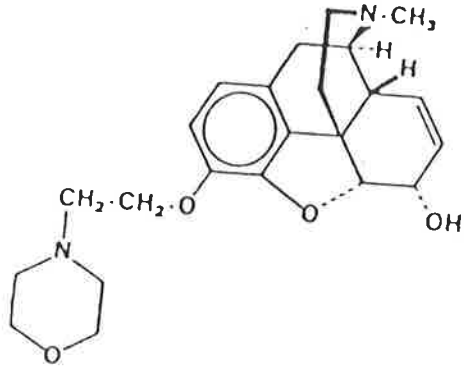
1.3.4.7. Excretion

Studies using ^{14}C-labelled codeine in man (Adler *et al* 1955) showed that codeine was excreted chiefly in urine, mainly as conjugates of codeine and its metabolites. Less than 1% of the dose was excreted in faeces after intramuscular injection. Urinary excretion was virtually complete in 24 hours, with 63-89% of the dose accounted for. An additional 2.2% of the dose was excreted in the period 24-72 hour after administration. Codeine and its metabolites were excreted in urine as percentage of dose after oral administration as follows: unchanged codeine, 5-17%; codeine conjugates, 32-46%; unconjugated and conjugated morphine, 5-13%; unconjugated and conjugated norcodeine, 10-21%. In a later study, Nomof and coworkers (1977) reported that the urinary excretion over 28 hours of total (free and conjugated) codeine, morphine and norcodeine were 56%, 6.4% and 5.6% of the dose respectively and that the maximum amount of codeine that can be metabolised is 30 mg/hour. These authors also suggested that codeine might be excreted by glomerular filtration and tubular secretion.

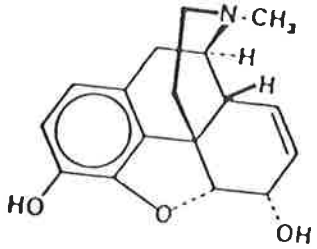
It should be noted that the determinations of codeine-6-glucuronide in the previous studies were based on enzymatic or acidic hydrolysis methods. Studies have shown that β-glucuronidase hydrolysed codeine-6-glucuronide

incompletely (Axelrod and Inscoe 1960, Yoshimura *et al* 1968; Guay *et al* 1988). Up till now there has been no report on the direct determination of codeine-6-glucuronide in plasma or in urine.

Toxicity in patients with renal failure after administration of therapeutic doses of codeine has been reported (Levine 1980, Matzke *et al* 1986). The pharmacokinetics of codeine in patients with renal failure were shown to be altered (Guay *et al* 1988). However, because of the serious cross-reactivity of the RIA employed in this study and the incomplete hydrolysis of codeine-6-glucuronide, the interpretation of the data is difficult. There are no data on the disposition and metabolism of codeine in the elderly.



pholcodine



morphine

Figure 1-4-1 Chemical structures of pholcodine and morphine.

1.4. Pholcodine

1.4.1. Antitussive effect of pholcodine

Pholcodine (3-O-morpholinoethylmorphine), a codeine-like antitussive agent (Figure 1-4-1), is chemically related to morphine, but unlike morphine and other opioids, it has little or no analgesic or euphorigenic activity and fewer withdrawal symptoms than codeine (Jaffe and Martin 1985, Eddy *et al* 1969). The antitussive potency of pholcodine as assessed in a variety of animal models has been reported to be equivalent to or superior to that of codeine (Cahen 1961; Eddy *et al* 1969b). Although the results of clinical trials were favorable to pholcodine (Snell and Armitage 1957; Oswald 1959; Bickerman and Itkin 1960; Heffron 1961; Mulinos *et al* 1960; 1962; Schofield 1963; Kelly 1963), these studies were deficient in several aspects: firstly, the cough frequency was estimated by non-objective methods; secondly the antitussive effect of pholcodine was not quantitatively compared with codeine in double-blind studies and thirdly in some of the studies, cough suppressant effect of pholcodine was assessed on artificial cough induced by chemical stimuli rather than pathological cough. Edwards and coworkers (1977) reported that pholcodine 15 mg with the antihistamine phenyltoxamine 10 mg orally produced a significant reduction in the frequency of cough, but there was no such effect for pholcodine 15 mg orally alone. Belcher and Rees (1986) found that pholcodine 10 mg orally increased the cough threshold induced by citric acid twofold compared with placebo.

Previous studies (Snell and Armitage 1957; Oswald 1959; Bickerman and Itkin 1960; Mulinos 1960; Heffron 1961; Mulinos *et al* 1962; Schofield 1963; Kelly 1963; Edwards *et al* 1977; Belcher and Rees 1986) on the antitussive effect of pholcodine used single oral doses. There is no report on the cough

suppressant effect of pholcodine after chronic administration. It seems likely that pholcodine may take a longer time to exert its antitussive effect after oral administration than the other antitussives such as codeine, because the the absorption of pholcodine was much slower than that of codeine (Neuvonen *et al* 1980; Findlay *et al* 1986). On the other hand, the cough suppressant effect of pholcodine after chronic administration, based on the currently recommended dosage regimen (10 mg 4-6 hourly, British National Formulary 1985), should be much stronger than that after single dose considering its long half-life (Neuvonen *et al* 1980; Findlay *et al* 1986).

1.4.2. Assays for the determination of pholcodine in biological fluids

Only a few reports on the pharmacokinetics of pholcodine have been published (Neuvonen *et al* 1980; Butz et al 1983; Findlay *et al* 1986). The methods used in these studies were based on RIA (Butz *et al* 1983; Findlay *et al* 1986) and ^{14}C-labelled drug administration (Neuvonen *et al* 1980). An ethylmorphine-6-hemisuccinate antiserum was used in the RIA for pholcodine. The cross-reactivity of pholcodine to the antiserum was 53%, but there was substantial cross-reaction with codeine (56%) and morphine (6.1%) (Butz *et al* 1983). Svenneby and coworkers (1983) reported that pholcodine interfered with the immunoassay for opiates in urine. The ^{14}C-pholcodine may be not suitable for the study of the disposition of the drug because the parent compound and its breakdown products can not be distinquished.

1.4.3. Pharmacokinetics and metabolism of pholcodine

1.4.3.1. Absorption

Pholcodine is readily absorbed from the gastrointestinal tract. The peak plasma concentration was achieved in about 5-6 hours (Neuvonen *et al* 1980; Findlay *et al* 1986).

1.4.3.2. Bioavailability

The bioavailability of pholcodine was reported to be 88% after the administration of 30 mg ^{14}C-pholcodine to six human subjects orally (Neuvonen *et al* 1980).

1.4.3.3. Distribution

Findlay and coworkers (1986), using RIA, reported the distribution volume (Vd/F) of pholcodine to be 43.3 ± 4.6 l/kg after oral administration of 60 mg of pholcodine. After oral and intravenous administration of ^{14}C-pholcodine in man, pholcodine was distributed into saliva. Serum and saliva concentrations of pholcodine were of the same magnitude (Neuvonen *et al* 1980).

1.4.3.4. Protein binding

Pholcodine was bound to serum proteins by 10% in vitro (Neuvonen *et al* 1980).

1.4.3.5. Metabolism

Butz and coworkers (1983) reported that 0.1-0.2% of the parent drug was transformed to morphine, determined by RIA, after administration of 10

mg/kg pholcodine to rats. Findlay and coworkers (1986) could not detect any morphine in the plasma samples from the human subjects who took 60 mg pholcodine. Little morphine (less than 1% of the dose) was found in urine after taking 30 mg ^{14}C-pholcodine in man (Neuvonen *et al* 1980). No conjugates, after β-glucuronidase hydrolysis, of pholcodine have been found in urine or plasma after oral or intravenous administration in man (Neuvonen *et al* 1980; Findlay *et al* 1986). Some metabolites of pholcodine in urine were demonstrated by autoradiograms after taking 30 mg ^{14}C-pholcodine in man (Neuvonen *et al* 1980). However none of them have been identified.

1.4.3.6. Half-life

The plasma half-life of pholcodine after intravenous administration of 30 mg ^{14}C-pholcodine in man was 230±35 hours based on radioactivity (Neuvonen *et al* 1980). Findlay and coworkers (1986), using RIA, determined the half-life to be 37.0±4.2 hours in volunteers taking single dose of 60 mg pholcodine orally. The large difference may be caused by the different analytical methods used.

1.4.3.7. Excretion

Pholcodine is mainly eliminated by the kidney. The cumulative excretion into urine averaged 20% of the dose within 24 hours and 50% over one week after intravenous administration of 30 mg ^{14}C-pholcodine to five human subjects. Unchanged pholcodine accounted for 30-50% of the total radioactivity in the urine. About 5% of the intravenous dose was excreted in faeces within one week and 10% of the dose was exhaled as $^{14}CO_2$ (Neuvonen *et al* 1980).

1.5. Dextromethorphan

1.5.1. Antitussive effect of dextromethorphan

Dextromethorphan, chemically related to codeine (Figure 1-3-1 and 1-5-1), is widely accepted as a safe and effective non-narcotic antitussive agent (Jaffe and Martin 1985). In spite of the absence of analgesic action (Eddy *et al* 1969b; Jaffe and Martin 1985), the antitussive effect of dextromethorphan has been confirmed in animal pharmacological models and in patients (Eddy *et al* 1969b). The earlier clinical trials in adult or paediatric patients with acute or chronic cough have shown that it can be as effective as codeine in therapeutic doses (Eddy *et al* 1969b). Calesnick and Christensen (1967) studied the antitussive effect of dextromethorphan and codeine on cough induced by citric acid after single oral doses and found that the dose-response curves of 10-40 mg of codeine and 15-30 mg of dextromethorphan were parallel but codeine was judged to have 2.2 times the potency of dextromethorphan on a weight basis. Matthys and coworkers (1983), however, investigated 16 patients with chronic cough in a double blind cross over design study. The authors found that both codeine and dextromethorphan at a dose of 20 mg were similarly effective in reducing cough frequency, while dextromethorphan lowered cough intensity to a greater degree than codeine and was considered the better antitussive by the majority of patients. Compared with codeine, dextromethorphan produces fewer subjective and gastrointestinal side-effects (Matthys *et al* 1983). Isbell and Fraser (1952, 1953) reported that dextromethorphan given orally or subcutaneously to nontolerant former addicts, in doses of 6-100 mg, exhibited no evidence of morphine-like behavior and that toxic symptoms (dizziness, diplopia, headache, nausea and vomiting) appeared following doses of 60 mg or more. The authors concluded

that dextromethorphan was devoid of addiction liability. However, abuse and toxic psychosis at very high doses have been described (Eddy *et al* 1969; Dodds and Revai 1967; Fleming 1986).

Dextromethorphan acts centrally to elevate the cough threshold for coughing (Chou and Wang 1975; Domino *et al* 1985). Selective sites for dextromethorphan have been identified in areas of the brain stem. These sites are distinct from opiate receptors and are also not associated with receptor binding sites for putative central neurotransmitters (Craviso and Musachio 1980; 1983; Snyder 1984).

1.5.2. Assays for the determination of dextromethorphan and its metabolites in biological fluids

Analytical methods for the determination of dextromethorphan and/or its metabolites in biological fluids have been reported, including paper chromatography (Willner 1963), fluorimetry (Ramachander *et al* 1977), RIA (Dixon *et al* 1978), GC (Furlanut *et al* 1977; Barnhart and Massad 1979; Pfaff *et al* 1983) and HPLC (Gillilan *et al* 1980; Achari *et al* 1984; Park *et al* 1984; East and Dye 1985; Mascher 1987). The RIA had a high cross-reactivity with the metabolites of dextromethorphan (40% with 3-methoxymorphinan, 4% with dextrorphan) with a limit of sensitivity of 4 ng/ml (Dixon *et al* 1978). A GC assay was sensitive for dextromethorphan (1 ng/ml) but was not sensitive enough for dextrorphan (20 ng/ml) (Pfaff *et al* 1983). East and Dye (1985) described four separate HPLC procedures for the analysis of dextromethorphan and its metabolites in plasma and urine. Although the assays are sensitive, they are not convenient. Mascher (1987) reported an HPLC assay with pre-column clean-up for the determination of dextrorphan and 3-hydroxymorphinan in plasma. This assay is very sensitive but no internal

standard was used. Park and coworkers (1984) presented a HPLC assay with cartridge column extraction for the simultaneous determination of dextromethorphan and three metabolites in urine. With this assay, all the four compounds were detected in nonhydrolysed urine after taking 60 mg dextromethorphan.

1.5.3. Pharmacokinetics and metabolism of dextromethorphan

1.5.3.1. Absorption

Because of the lack of a sensitive assay and the large interindividual variation in the plasma concentrations, only limited data on the pharmacokinetics of dextromethorphan in human are available. After oral administration, the peak plasma concentrations of dextromethorphan occur in about 1-4 hours (Barnhart and Massad 1979; Pfaff *et al* 1983; East and Dye 1985; Silvasti *et al* 1987), Dextrorphan, the 3-O-demethylated major metabolite, reached its peak plasma concentration in about 2 hours (Pfaff *et al* 1983; East and Dye 1985; Silvasti *et al* 1987). The total urinary recovery was 86.1% of the dose (Pfaff *et al* 1983) and these results indicate that dextromethorphan is well absorbed from the gastrointestinal tract. There are no significant differences in the absorption rate and extent between tablet and syrup preparations (Silvasti *et al* 1987). Fiese and Perrin (1968) reported that dextromethorphan was absorbed from the rat stomach at pH 2.0 and they suggested that the absorption is by passive diffusion and not by specialised transport process.

1.5.3.2. Bioavailability

There are no data available in humans because there is no intravenous form available for human use.

It has been suggested that dextromethorphan undergoes extensive first pass metabolism because the plasma concentrations of dextrorphan were up to 170 times higher than that of dextromethorphan (Pfaff *et al* 1983; East and Dye 1985; Silvasti *et al* 1987). This is supported by the observations that the mean area under the plasma concentration-time curve of dextromethorphan in the dog after oral administration was 3.8-18.0% of that after intravenous injection of the same dose (Dixon *et al* 1978; Barnhart and Massad 1979).

1.5.3.3. Distribution

The only information available is from a pharmacokinetic study in the dog (Dixon *et al* 1978). In this study, the volume of distribution was 5 l/kg after intravenous injection of 2 mg/kg of dextromethorphan.

1.5.3.4. Protein binding

This has not been studied.

1.5.3.5. Metabolism

Earlier studies have shown that dextromethorphan undergoes rapid and extensive hepatic metabolism and that the extent of metabolism is highly variable between subjects (Willner 1963; Dixon *et al* 1978; Barnhart and Massad 1979; Barnhart 1980; Pfaff *et al* 1983; Woodworth *et al* 1984; Schmid *et al* 1985; East and Dye 1985; Küpfer *et al* 1985; Küpfer *et al* 1986; Larrey *et al* 1987; Silvasti *et al* 1987). O- and N- demethylation followed by subsequent conjugation are the main metabolic pathways (Figure 1-5-1). Dextromethorphan is O-demethylated to dextrorphan, N-demethylated to 3-methoxymorphinan and N,O-demethylated to 3-hydroxymorphinan (Willner 1963; Pfaff *et al* 1983; East and Dye 1985). It has been shown that the metabolism of dextromethorphan resulting in 3-hydroxymorphinan is not only via N-

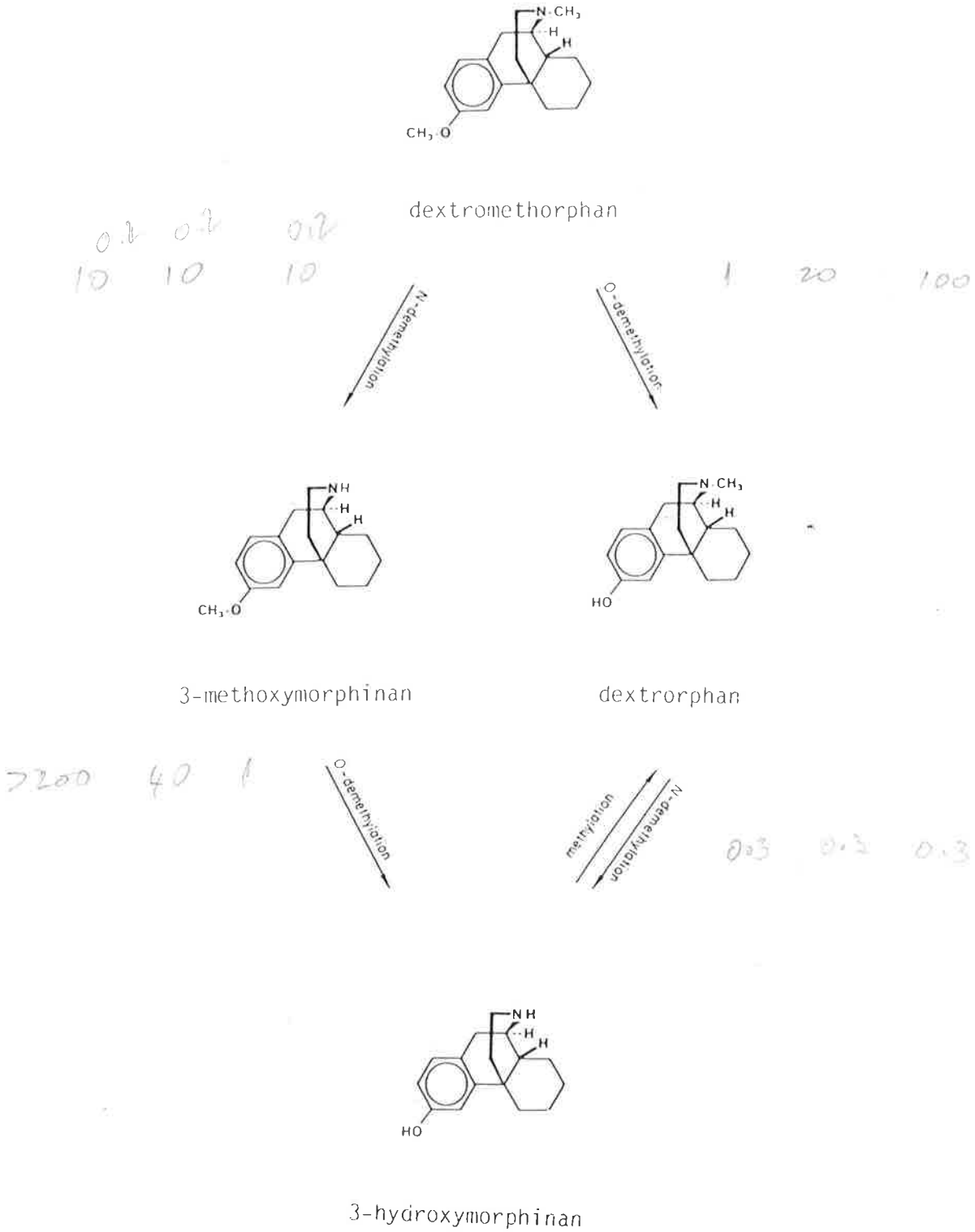


Figure 1-5-1 Metabolic pathways of dextromethorphan.

demethylation of dextrorphan but also via O-demethylation of 3-methoxymorphinan (Willner 1963). It has also been assumed that methylation of 3-hydroxymorphinan producing dextrorphan may occur simultaneously (Willner 1963) although the evidence is not strong. There is little information about the conjugates of the metabolites in plasma and urine. Ramachander and coworkers (1977) declared that almost all of the dextrorphan in plasma in one subject was present in the form of conjugates after determining, by fluorimetry, the plasma concentration of dextrorphan with and without enzymatic hydrolysis. Barnhart (1980) reported that 95% of dextrorphan and 3-hydroxymorphinan were present as conjugated forms in urine after a 30 mg oral dose. Conjugates of 3-methoxymorphinan were also detected in dog urine after administration of dextromethorphan (Barnhart 1980). Besides the metabolites mentioned above, 15 further metabolites and derivatives were characterised qualitatively by mass spectral techniques, suggesting a variety of additional metabolic degradation processes besides O- and N- demethylation (Köppel *et al* 1987).

Recently many studies have shown that dextromethorphan exhibits polymorphic metabolism (Pfaff *et al* 1983; Roy *et al* 1984; Woodworth *et al* 1984; Schmid *et al* 1985; Küpfer *et al* 1986; Larrey *et al* 1987). According to ratios of dextromethorphan/dextrorphan in urine, poor, intermediate or extensive metabolisers were phenotyped (Pfaff *et al* 1983; Woodworth *et al* 1984; Küpfer *et al* 1984; Küpfer *et al* 1985; Schmid *et al* 1985; Küpfer *et al* 1986; Larrey *et al* 1987). 3.9-9% of the population in European countries were found to have deficiency in the O-demethylation of dextromethorphan and phenotyped as poor metabolisers (Küpfer *et al* 1985; Schmid *et al* 1985; Larrey *et al* 1987). A significant relationship between dextromethorphan O-demethylation and debrisoquine 4-hydroxylation in humans has been

reported (Roy *et al* 1984; Küpfer *et al* 1984; Schmid *et al* 1985; Küpfer *et al* 1986). There is no such co-segregation with the mephenytoin polymorphism (Küpfer *et al* 1986). In human liver microsomes containing cytochrome P-450 db1/bufI, it has been demonstrated that dextromethorphan O-demethylation cosegregates with debrisoquine hydroxylation polymorphism (Dayer *et al* 1988). Dextromethorphan, together with bufuralol and debrisoquine, have been suggested to be the best studied substrates involved in the debrisoquine /sparteine-type genetic polymorphism of drug oxidation in man (Küpfer *et al* 1984; Roy *et al* 1984; Kronbach *et al* 1987; Dayer *et al* 1987).

1.5.3.6. Half-life

The plasma half-life of dextromethorphan is quite different between subjects because of the large interindividual variation of metabolism. Pfaff and coworkers (1983) reported that the plasma of extensive metabolisers contained only traces of dextromethorphan within the range of detection limit of the analytical procedure (1 ng/ml). The plasma concentration curves of the intermediate metabolisers and poor metabolisers were easily fitted to a two-compartment model with half-lives of the β-phase of 8.5-16.2 hours and 42-50 hours respectively. Silvasti and coworkers (1987) studied the pharmacokinetics of dextromethorphan in humans using a HPLC method with a lower sensitivity of 0.5 ng/ml. They found the maximum plasma concentration of dextromethorphan in extensive metabolisers to be 3-5 ng/ml with a half-life of 3.4 hours. The plasma half-life of dextrorphan reported by Pfaff and coworkers (1983) was 1.9 hours in extensive metabolisers and 6.2 hours in intermediate metabolisers. Plasma dextrorphan concentrations could not be detected in poor metabolisers by Pfaff and coworkers (1983) whose assay had a lower limit of sensitivity of 20 ng/ml. Silvasti and coworkers (1987) determined the plasma half-life of dextrorphan in extensive metabolisers to

be 2.7-4.0 hours. There is a lack on the information on the pharmacokinetics of dextrorphan in poor metabolisers. The half-life of 3-hydroxymorphinan in humans was 2.1 hours after they took dextromethorphan tablets and solutions (corresponding to 25 mg dextromethorphan base) (Mascher 1987). There is no information on the plasma half-life of 3-hydroxymorphinan in subjects with different metabolizing capacities.

1.5.3.7. Excretion

Dextromethorphan and its metabolites are excreted in urine in unconjugated and conjugated forms (Barnhart 1980; Pfaff *et al* 1983; Park *et al* 1984; East and Dye 1985). The excretion of the metabolites in urine in extensive metabolisers was almost complete after 24 hours (Pfaff *et al* 1983). The urinary recovery of drug 48 hours after drug administration, based on analysis of dextromethorphan, dextrorphan and 3-hydroxymorphinan was 0.18±0.12, 45.6±7.7 and 33.6±5.8% respectively representing a total of 79.4±3.9% in four subjects (Barnhart 1980). In this study 3-methoxymorphinan concentrations were too low to be quantitated under the conditions used. Pfaff and coworkers (1983) reported that 48 hours after administration of 25 mg dextromethorphan hydrobromide, an average amount of 62.5% of the dose was detected in the urine as dextrorphan, 23.5% as 3-hydroxymorphinan and 0.125% as dextromethorphan, giving a total of 86.1% in extensive metabolisers. With the intermediate metabolisers, 43.9% of the dose was excreted as dextrorphan, 26.5% as 3-hydroxymorphinan, 2.6% as dextromethorphan and 0.73% as 3-methoxymorphinan, giving a total of 73.6%, while in poor metabolisers, 10% of the dose was excreted as dextromethorphan, 3.9% as dextrorphan, 3.5% as 3-hydroxymorphinan and 1.7% as 3-methoxymorphinan with a total recovery of 19.3%. The urinary excretion was not dose or urine pH dependent (Küpfer *et al* 1986).

In summary, the pharmacology of pholcodine, codeine and dextromethorphan have been studied, but many problems and questions still exist:

(I) Pholcodine

1. The analytical methods for the determination of pholcodine in biological fluids are not specific.

2. The pharmacokinetics of pholcodine in humans have not been determined with specific analytical methods.

3. There are no data on the pharmacokinetics of pholcodine in humans after chronic administration.

4. Little information is available about the metabolism of pholcodine.

(II) Codeine

1. There is no assay reported on the simultaneous determination of codeine, norcodeine and morphine in plasma and urine, although many assays have been described for codeine or morphine.

2. There is no assay reported on the direct determination of codeine-6-glucuronide, the major metabolite of codeine, in biological fluids.

3. The pharmacokinetics of codeine-6-glucuronide have not been determined in man.

4. The metabolic pathways of codeine in humans have not been determined by specific analytical methods.

5. There are no data on the pharmacokinetics and metabolism of codeine in the elderly.

(III) Dextromethorphan

1. There is no assay reported on the simultaneous determination of dextromethorphan and metabolites in plasma and urine.

2. Whether the N-demethylation of dextromethorphan or the glucuronidation of the O-demethylated metabolites are under genetic control has not been reported.

3. There are no data on the genetic polymorphism of dextromethorphan metabolism in an Australian population.

(IV) Analgesia

1. The analgesic mechanism of codeine is not clear.

2. The comparative binding affinities of codeine and its metabolites to the μ-opioid receptor, which mediates analgesia, have not been studied concurrently with the highly specific ligand ^{3}H-DAGO.

3. The binding affinities of pholcodine, dextromethorphan and metabolites to the μ-opioid receptor have not been studied, although they were reported to have little or no analgesic effect.

(V) Antitussive effect

1. There is no accurate, practical and simple cough monitoring method available for the studies in patients with cough.

2. The antitussive effects of codeine, pholcodine and dextromethorphan in humans have not been quantitatively compared in well controlled clinical studies.

Thus the aims of this thesis were to develop specific and sensitive analytical methods, to try to answer some of the questions set out above regarding the pharmacokinetics and metabolism of pholcodine, codeine and dextromethorphan and to use modern technology to study the analgesic and antitussive effects of these drugs.

Chapter 2.

PHARMACOKINETICS AND METABOLISM OF PHOLCODINE IN HUMANS

2.1. Determination of pholcodine in biological fluids by HPLC

2.1.1. Introduction

As mentioned in 1.4.3., only two methods have been used for the determination of pholcodine in biological fluids (Neuvonen *et al* 1980; Butz *et al* 1983). The ^{14}C-labelled drug technique is not specific because the parent compound cannot be distinguished from its potential metabolites. The RIA also suffers from cross-reaction problems. Butz and coworkers (1983) were unable to use a specific antiserum to pholcodine, but used ethylmorphine-6-hemisuccinate to develop antiserum. This antiserum is nonspecific and has high cross reactivity with all alkyl substitutes at the 3-position. The cross-reactivity of this assay to pholcodine was 53% and for codeine and morphine was 53% and 6.1% respectively (Butz *et al* 1983). The extent of cross-reactivity to the potential metabolites of pholcodine is unknown. The need for a specific assay for the determination of pholcodine in biological fluids is clearly very important considering that the extent of urinary recovery of metabolites of pholcodine was at least as great as of the parent compound (Neuvonen *et al* 1980). The aim of this study was to develop a specific and sensitive HPLC assay for the determination of pholcodine in biological fluids in order to study the pharmacokinetics and possibly metabolism of pholcodine in humans.

2.1.2. Materials and methods

2.1.2.1. Reagents

All reagents were of analytical grade or of HPLC grade (see Appendix I). Pholcodine BP, codeine phosphate and morphine sulphate (F.H. Faulding

and Co. Limited, Adelaide, Australia) were all of British Pharmacopoeial grade quality.

2.1.2.2. Chromatography

The HPLC consisted of a SP 8770 isocratic pump (Spectra-Physics, San Jose, CA, U.S.A.), an LS-5 Luminescence Spectrometer (Perkin-Elmer Ltd, Beaconsfield, U.K.), a WISP 710 B automatic injector (Waters Assoc., Milford, MA, U.S.A.) and an Omniscribe B-5000 strip-chart recorder (Houston Instruments, Austin, TX, U.S.A.). The 15 cm x 4.6 mm I.D. stainless steel column was packed with Spherisorb 5 µm ODS-2 packing material (Phase Separations, Queensferry, U.K.). The final composition of the mobile phase was 16% acetonitrile, 0.035% triethylamine and distilled water to 100%, adjusted to pH 3.5 with orthophosphoric acid. The flow rate through the column at ambient temperature was 1 ml/minute which produced a back-pressure of 1.15 Kpsi (7.93 MPa). The excitation and emission wavelengths of the fluorescence detector were 230 and 350 nm respectively and the excitation and emission slits were set at 10 and 20 nm respectively.

2.1.2.3. Stock solutions

Pholcodine was made up as a 1 mg/ml stock solution in distilled water and was diluted to concentrations ranging from 2 to 200 ng/ml in drug-free plasma and saliva, and from 0.2 to 10 µg/ml in drug-free urine. Codeine, the internal standard (1 mg/ml in distilled water) was diluted in distilled water to 5 µg/ml for plasma and saliva analysis and 100 µg/ml for urine analysis.

2.1.2.4. Sample preparation

Plasma: 1 ml of plasma was pipetted into a 10 ml screw capped, tapered plastic tube to which was added 10 µl of the 5 µg/ml internal standard

solution and 0.5 ml of bicarbonate buffer (pH 9.6). The mixture was briefly vortexed, 4 ml of diethyl ether/chloroform/pentan-1-ol (2/1/1, by volume) were added and the tubes were placed on a rotary mixer for 10 minutes. The organic and the aqueous phases were separated by centrifugation at 2000 g for 10 minutes. The upper organic phase was transferred to a clean 10 ml screw capped, tapered plastic tube containing 100 µl of 0.1 N hydrochloric acid. The tubes were vortex-mixed briefly and than placed on a rotary mixer for 10 minutes. The two phases were separated by centrifugation at 2000 g for 5 minutes. The organic layer was discarded and the tubes were then placed in hot water for 10 minutes to remove all trace of organic solvents. An aliquot (10-30 µl) was injected onto the column via the automatic injector. Peak heights were measured manually and the peak height ratio of pholcodine to codeine was then calculated.

Saliva: Saliva samples were treated in exactly the same manner as the plasma samples.

Urine: 0.5 ml aliquot of urine was pipetted into a 10 ml screw capped plastic tube to which was added 25 µl of a 100 µg/ml internal standard solution. The samples were then handled in exactly the same manner as the plasma samples.

2.1.2.5. Statistical analysis

Standard curves were plotted as peak height ratio versus pholcodine concentration. Linear regression analysis was performed to determine the slope, intercept, their variability and the strength of the correlation. Precision was evaluated by spiking plasma and saliva with pholcodine to concentrations ranging from 5 to 200 ng/ml, and urine from 0.2 to 10 µg/ml,

performing replicate analysis intra- and inter-day and determining coefficients of variation.

2.1.3. Results and discussion

2.1.3.1. Chromatography

Figure 2-1-1 is a representative chromatogram from an injection of a solution containing a mixture of pholcodine, morphine and codeine each at a concentration of 2 µg/ml in distilled water. Morphine had a retention time of 3.5 minutes and a capacity factor of 1.75, pholcodine had a retention time of 5.5 minutes and a capacity factor of 3.25, whereas codeine had a retention time of 7.5 minutes and a capacity factor of 4.92. After evaluation of ultraviolet and electro-chemical detectors, the fluorescence detector was chosen. Ultraviolet detection, although providing sufficient sensitivity, was unacceptable because of interference from endogenous substances. Electro-chemical detection provided sufficient sensitivity for morphine and codeine but not for pholcodine. Unlike the others, fluorescence detection gave acceptable sensitivity and selectivity from interfering endogenous substances. The effects, on the retention time of the three compounds, of pH (Figure 2-1-2) and composition (Figure 2-1-3) of mobile phase were investigated. Firstly, increasing the pH from 2 to 5 resulted in an increase in retention time for all three compounds. Secondly, increasing the triethylamine concentration from 0.025 to 0.1% reduced the retention time for all three. Lastly, increasing the acetonitrile concentration from 12 to 30% resulted in a marked reduction in the retention time for codeine, a moderate reduction in retention time for morphine and a modest increase for pholcodine. As a result of the above, the final mobile phase of 16% acetonitrile and 0.035% triethylamine adjusted to pH 3.5 was chosen because it

permitted the baseline separation of the three compounds with reasonable analysis time and selectivity.

2.1.3.2. Linearity

Calibration curves showed good linearity between peak-height ratios and concentrations from 2 to 200 ng/ml for pholcodine in plasma and saliva ($r > 0.99$) and from 0.2 to 10 µg/ml for pholcodine in urine ($r > 0.99$). Representative standard curves for plasma , saliva and urine are shown in Figure 2-1-4 and 2-1-5. For plasma, saliva and urine standard curves, the 95% confidence intervals of the intercepts included the origin. The standard errors of the slopes were less than 5% for all three.

2.1.3.3. Precision and accuracy

The assay showed good precision at low and high pholcodine concentrations in plasma, saliva and urine. Table 2-1-1 shows the intra- and inter- day assay variability. Calculated concentrations were within 95% of added concentrations.

2.1.3.4. Recovery

The recovery of pholcodine at a plasma concentration of 100 ng/ml averaged (n=3) 80% and of codeine 95%. The 95% confidence intervals of the slopes of standard curves, prepared from the same samples (plasma, saliva and urine) stored at -20 °C, assayed 11 weeks apart, overlapped indicating no loss of pholcodine from the biological media.

2.1.3.5. Interference by other compounds

Interference by other drugs was studied by preparing and chromatographing aliquots of pure drugs and by analyzing samples from patients on

multiple drug therapy. None of the drugs in Table 2-1-2 interfered with the assay of pholcodine. Figure 2-1-6 shows a chromatogram from drug-free plasma and a plasma sample from a subject 0.5 hour after pholcodine ingestion (60 mg in linctus form). Figure 2-1-7 shows a chromatogram from drug-free saliva and a saliva sample 3 hours after ingestion in the same subject and Figure 2-1-8 shows a chromatogram from drug-free urine and a urine sample collected between 48-72 hours in this individual. In the chromatograms of urine and saliva samples, two additional peaks not found in any drug-free samples were observed at retention times of 4.5 and 6.5 minutes. These peaks are thought to be metabolites of pholcodine and their isolation and identification are discussed in Chapter 2.3.. This assay is sensitive and specific and appears suitable for studying the pharmaco-kinetics and disposition of pholcodine in man.

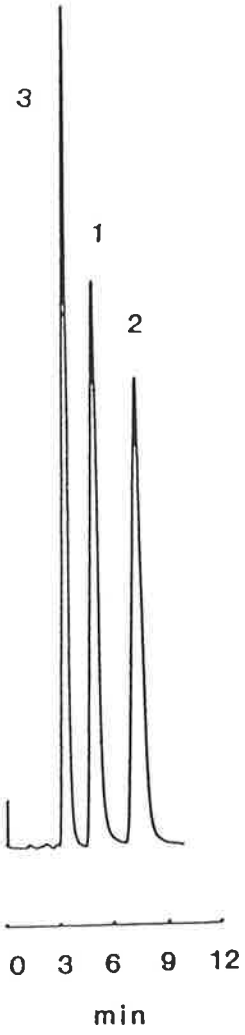


Figure 2-1-1 Chromatogram of a standard solution (in water) of pholcodine (1), codeine (2) and morphine (3).

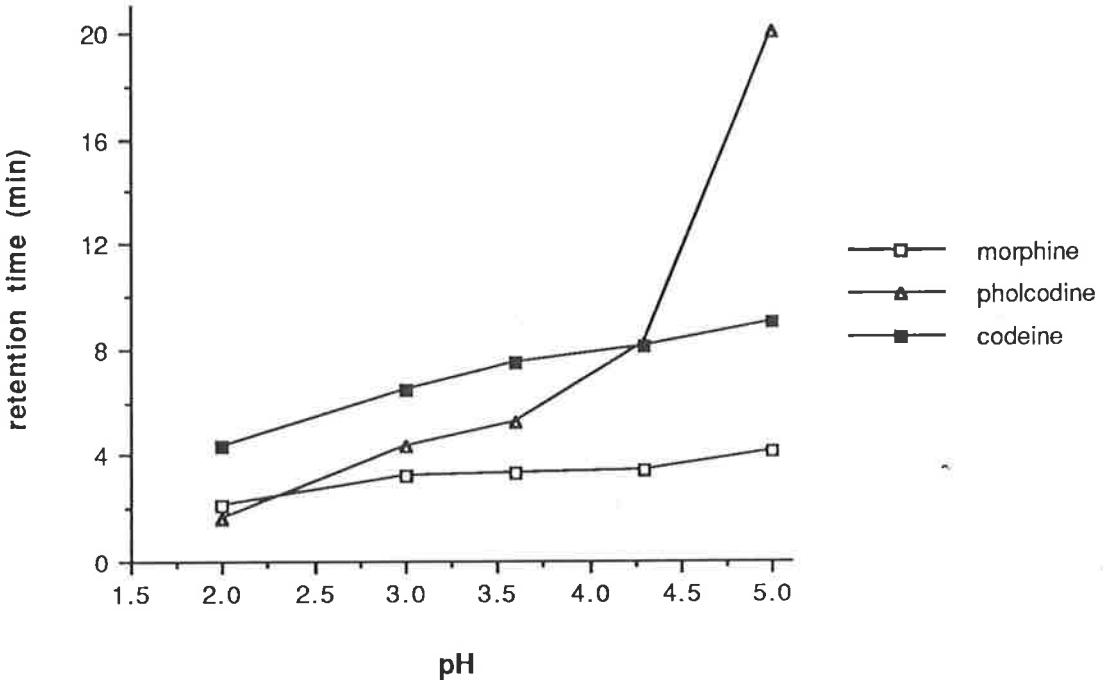


Figure 2-1-2 Effects of pH of the mobile phase (16% acetonitrile/0.035% triethylamine/distilled water to 100%) on the retention time of morphine, pholcodine and codeine.

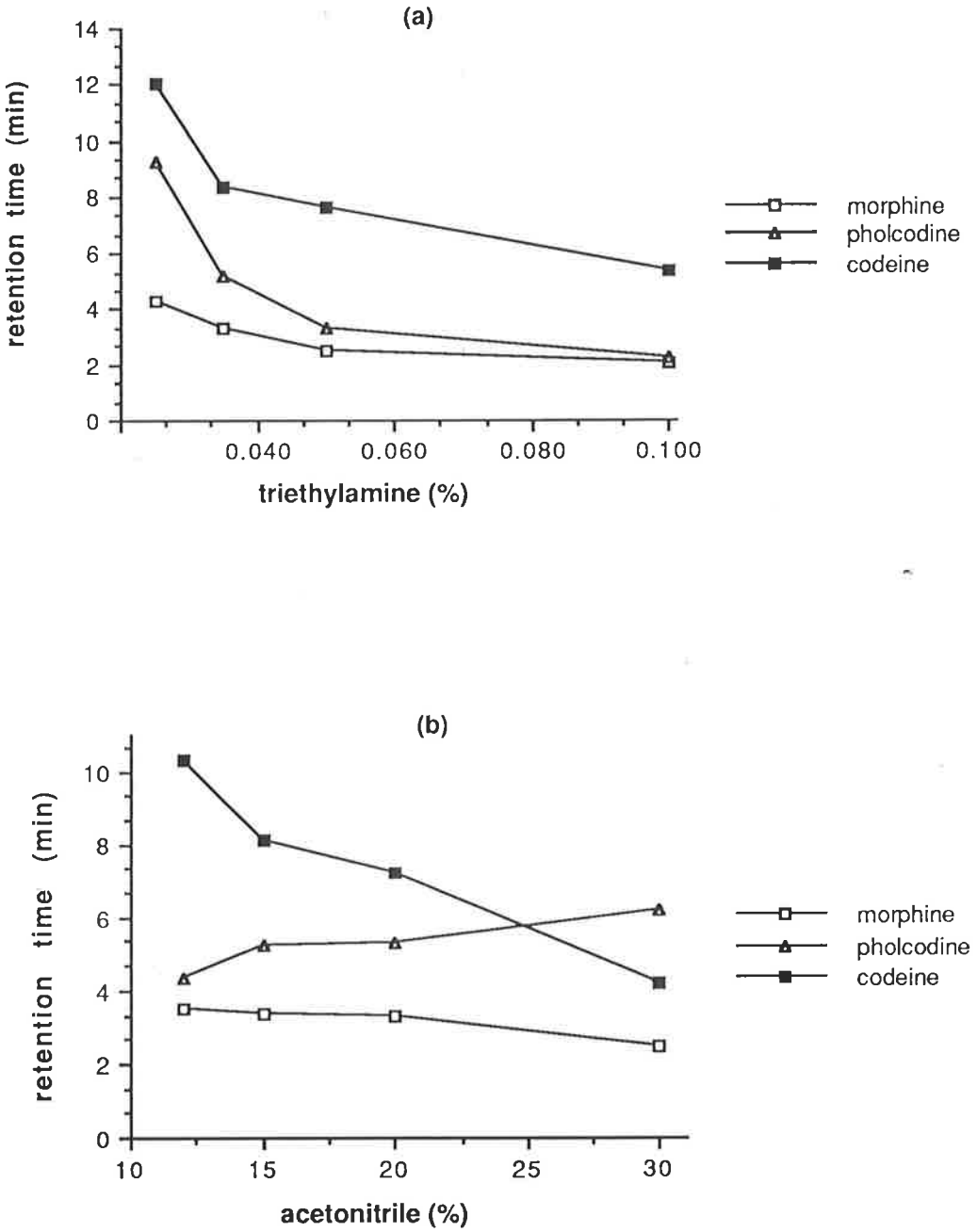


Figure 2-1-3 Effects of concentration of (a) triethylamine (in 16% acetonitrile/distilled water to 100%, pH 3.5) and of (b) acetonitrile (in 0.035% triethylamine/distilled water to 100%, pH 3.5) on the retention time of morphine, pholcodine and codeine.

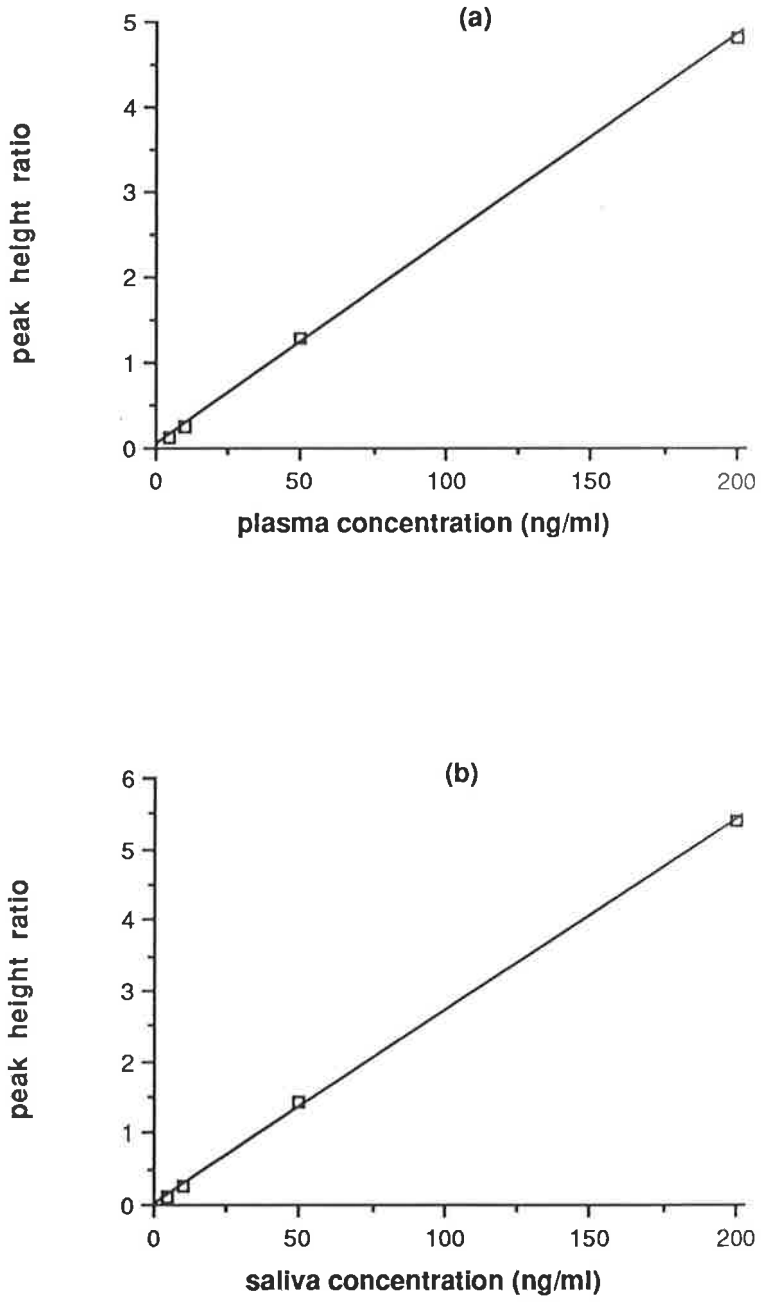


Figure 2-1-4 Representative standard curves for pholcodine in plasma (a), and saliva (b).

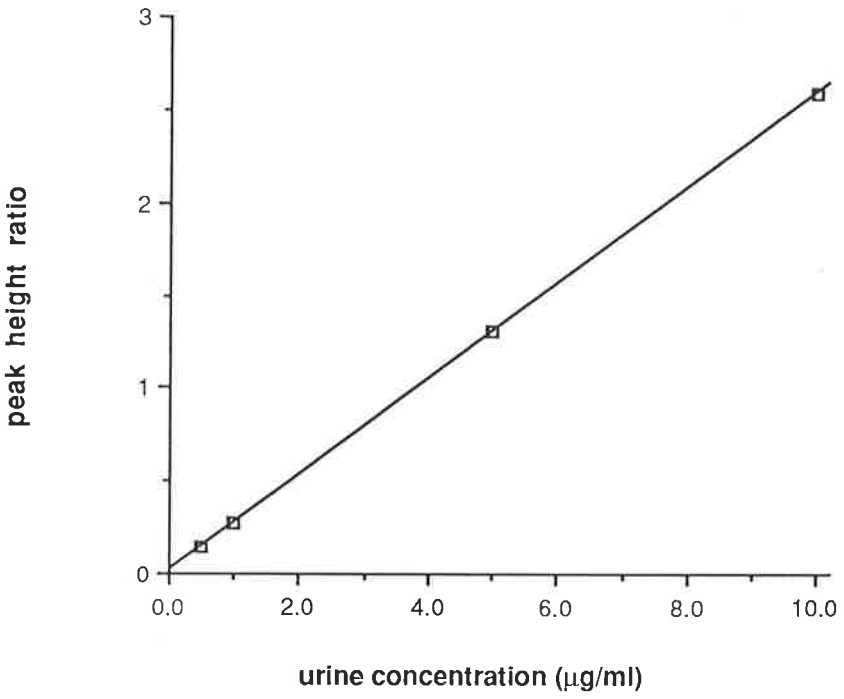


Figure 2-1-5 Representative standard curves for pholcodine in urine.

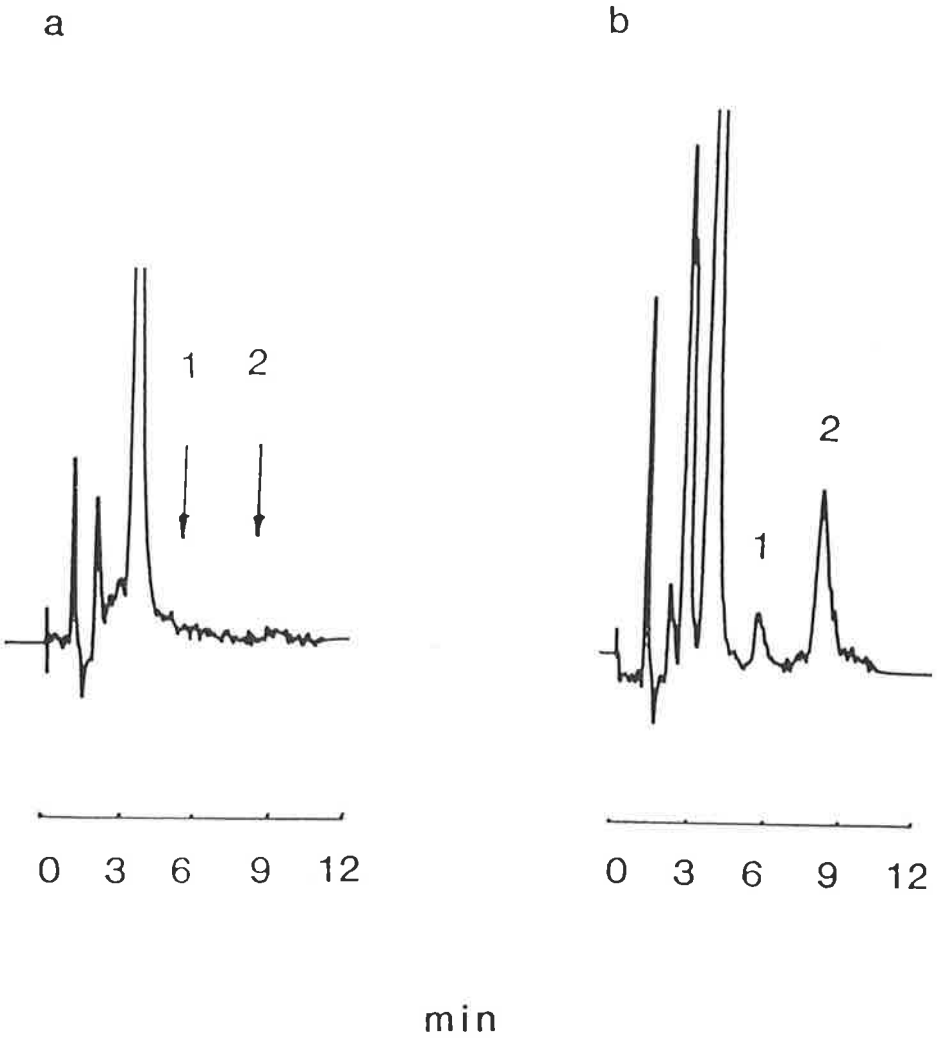


Figure 2-1-6 Chromatograms from an extract of (a) a drug-free plasma sample and (b) a plasma sample from the same subject 0.5 hour after ingestion of 60 mg pholcodine (pholcodine concentration = 15 ng/ml).

Peaks: 1 = pholcodine, 2 = codeine (internal standard).

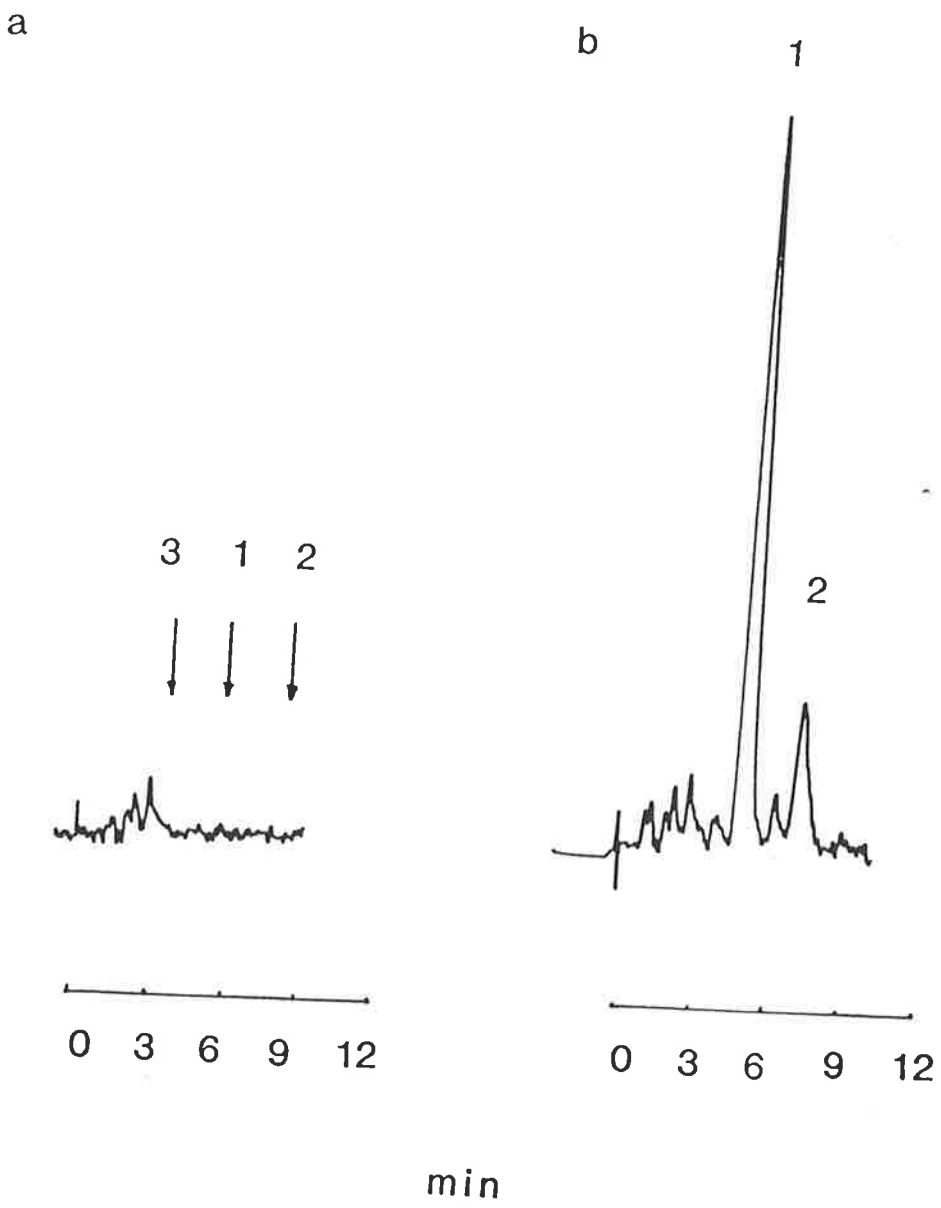


Figure 2-1-7 Chromatograms from an extract of (a) a drug-free saliva sample and (b) a saliva sample from the same subject 3 hours after ingestion of 60 mg pholcodine (pholcodine concentration = 196 ng/ml).

Peaks: 1 = pholcodine, 2 = codeine (internal standard) and 3 = morphine.

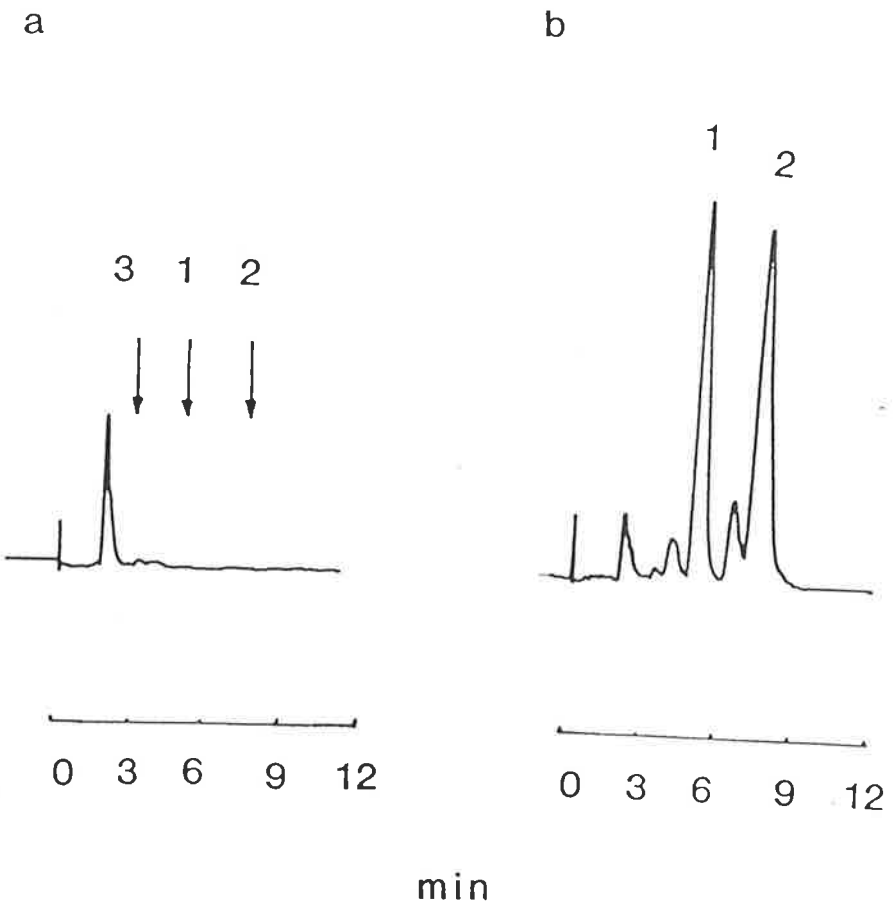


Figure 2-1-8 Chromatograms from an extract of (a) a drug-free urine sample and (b) a urine sample from the same subject during 48-72 hours after ingestion of 60 mg pholcodine (pholcodine concentration = 0.72 µg/ml).

Peaks: 1 = pholcodine, 2 = codeine (internal standard) and 3 = morphine.

Table 2-1-1 Assay reproducibility of pholcodine in plasma, saliva and urine

	concentration (ng/ml)	coefficient of variation (%)	number
intra-day			
plasma	10	3.92	10
	50	3.42	10
saliva	10	4.17	10
	50	3.62	10
urine	200	2.45	10
	1000	1.89	10
inter-day			
plasma	5	9.31	6
	200	7.78	6
saliva	5	9.47	6
	200	7.11	6
urine	500	4.89	6
	10000	2.27	6

Table 2-1-2 Drugs shown not to interfere with the pholcodine assay

tablet preparations and pure drugs tested	
amphetamine	dihydrocodeine
cocaine	dipyridamole
dextromethorphan	ethylmorphine
dextrorphan	naloxone
dextropropoxyphene	pentobarbital
plasma from patients on multiple drug therapy	
amiloride	lithium
amoxycillin	minoxidil
carbamazepine	paracetamol
digoxin	phenindione
flucloxacillin	salbutamol
frusemide	sorbide dinitrate
hydrochlorothiazide	theophylline
ibuprofen	verapamil

2.2. Pharmacokinetics of pholcodine in healthy volunteers

2.2.1. Introduction

Although pholcodine has been used as an antitussive drug for more than 30 years, little is known about its absorption and disposition in man. As has already been mentioned in Chapter 1., Neuvonen *et al* (1980) reported, in abstract form, that following intravenous administration of ^{14}C-pholcodine, the mean half-life of total radioactivity in plasma was 230 hours, and for "unchanged pholcodine" in urine 102 hours. Findlay *et al* (1986), using radio-immunoassay examined the pharmacokinetics of pholcodine given as a single 60 mg oral dose and reported a mean terminal half-life of 37 hours. The available data are thus in conflict and might have resulted from assay techniques whose specificities could be questioned. There are no data on the disposition of pholcodine following chronic administration.

The aims of this study were to determine:

(1) the pharmacokinetics and metabolism of pholcodine after 20 and 60 mg single doses;

(2) the pharmacokinetics and metabolism of pholcodine at steady state after chronic dosing with 20 mg 8 hourly;

(3) the extent of pholcodine binding to human plasma proteins

using the specific HPLC analytical method described in Chapter 2.1..

Table 2-2-1 Volunteer data

number	sex	age (year)	BW (kg)	BH (cm)	Cr (mM/L)	Cl_{cr} (ml/min)	GGT (U/L)	Albumin (g/L)
1	M	28	55	173	0.10	90	13	47
2	M	28	66	173	0.09	105	10	48
3	M	47	65	175	0.10	90	32	48
4	M	23	71	170	0.12	90	12	49
5	F	30	57	168	0.08	90	7	43
6	M	23	69	181	0.10	105	10	51

BW: body weight; BH: body height; Cr: serum creatinine concentration; Cl_{cr}: creatinine clearance; GGT: gamma glutamate transaminase; M: male; F: female.

2.2.2. Methods

2.2.2.1. Subjects

Six normal healthy volunteers who gave written informed consent participated in the study. The volunteers were aged 29.3±8.2 (mean ± SD) years and weighed 63.8±6.5 kg (Table 2-2-1). Prior to commencement of the study each subject underwent a physical examination and a venous blood sample was collected for biochemical (Multiple biochemical analysis, MBA 20) and haemotological (complete blood examination, CBE) examinations. These results were all normal. The creatinine clearance (Cl_{cr}) was estimated using the nomogram method (Siersbaek-Nielsen *et al* 1971) and results are shown in Table 2-2-1. All the volunteers were non-smokers. Other drugs and alcohol were prohibited for two days before pholcodine administration and for the duration of the study.

This study was approved by the Human Ethics Committee of Royal Adelaide Hospital and the Committee on the Ethics of Human Experimentation of the University of Adelaide.

2.2.2.2. Study design

Single dose study: The single dose study was conducted using a crossover design with each subject receiving, in a balanced randomised order, 20 and 60 mg pholcodine as single doses of 20 ml and 60 ml Linctus Pectolin® (F. H. Faulding and Co. Limited., Adelaide, Australia). Each 5 ml contained pholcodine base 5 mg, citric acid 50 mg, glycerol 1.5 ml, hydroxybenzoates 5 mg and water to 5 ml. There was a washout period of 3 to 4 weeks between the two doses. The doses were given at least one hour after a light breakfast. The linctus dispenser was rinsed with successive washings of

water (total volume 100 ml) which was ingested by the volunteer. Food was permitted after 4 hours and normal fluid intake was allowed. The volunteers were ambulant but confined to the laboratory for the first 8 hours of sampling. Ten ml venous blood samples were collected into heparinized plastic tubes at the following times: 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 8, 24, 32, 48, 72, 96, and 168 hours after dosing. Over the first 8 hours, the blood samples were collected via an indwelling catheter (Jelco^{TM}, 18 G i.v. catheter placement unit, Critikon, Tampa, USA), kept patent with a stylet (Jelco^{TM}), placed in a forearm vein. The subsequent samples were collected by single venepuncture. After chewing parafilm, mixed saliva samples (3 ml) were collected at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 8, 24, 32, 48, 72, 96, 120, 144 and 168 hours and saliva pH was recorded. All urine was collected at 0-8, 8-24, 24-32, 32-48, 48-72, 72-96, 96-120, 120-144 and 144-168 hours after dosing.

Chronic dose study: The second part of the study was conducted 3 to 8 weeks after completion of the single dose study. Subjects received 20 mg of pholcodine (as 20 ml Linctus Pectolin®) 8 hourly (0800, 1600, 2400 hour) for ten days. The final dose was given at 0800 hour on day 11 after a light breakfast. Prior to the dose (0800 hour) on days 1 to 11, and at 2, 4, 6, 8, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 hours after the last dose, saliva samples were collected. Plasma samples were collected at 0, 2, 4, 6 and 8 hours after the last dose on day 11. Urine was collected for a dosing interval (0-8 hour) on day 11 after the last dose.

Plasma was separated from blood cells by centrifugation. Urine pH was recorded and the volume was determined gravimetrically assuming a specific gravity of 1 g/ml. An aliquot of well mixed urine from each collection was retained for analysis. All samples were stored in stoppered vials at -20 °C until analysed for drug content.

2.2.2.3. Pholcodine analysis in biological fluids

Pholcodine concentrations in plasma, saliva and urine were determined by the HPLC method using fluorescence detection as described in Chapter 2.1..

2.2.2.4. Pholcodine and morphine conjugates analyses

The presence of conjugates of pholcodine and morphine in urine was inferred by the generation of pholcodine and/or morphine following enzymatic hydrolysis. The internal standard (codeine phosphate) was added to urine samples (0.3 ml) in 10 ml screw capped tubes. β-glucuronidase from Helix Pomatia type H-1 (Sigma Chemical Co., St. Louis, MO., USA) was added (0.3 ml of a 3500 U/ml in pH 5.0 acetate buffer), followed by incubation in a shaking water bath at 37 °C for 14 hours. Samples were analysed for pholcodine and morphine by the above method. The presence of the conjugates was also investigated by hydrochloric acid hydrolysis following a procedure described by Yoshimura and coworkers (1970) with modifications: To 2.5 ml of the urine from the chronic dosing study was added 0.05 ml of 40% $NaHSO_3$ solution and 3 ml of 37% HCL to bring the final acid concentration to 20%. The mixture was then heated in a boiling water bath for 60 minutes. To this hydrolysate was again added 0.05 ml of 40% $NaHSO_3$ and 3 ml 10 N NaOH solution, adjusted to pH 5.0, and then diluted with 0.2 M pH 5.0 acetate buffer to a final volume of 10 ml. An aliquot of the sample was analysed for the liberated pholcodine and morphine by the HPLC assay as above.

2.2.2.5. Plasma protein binding determination

The binding extent of pholcodine to plasma proteins was assessed in a microultrafiltration system (Model MMC Amicon Corp., Danvers, Mass, USA) with Diaflo YM 10 membranes (Amicon. Corp.). The system was housed in a

heated cabinet (thermostatically controlled) and binding was determined at 37 °C under a nitrogen atmosphere. The zero, two and four hourly plasma samples from the chronic dosing study in each subject were used. Preliminary studies were carried out to ensure that the compound did not sorb to the membranes or the glass walls of the ultrafiltration unit. The linearity of binding in vitro was assessed by spiking blank plasma with pholcodine at concentrations ranging from 5 to 200 ng/ml. The fraction unbound (f_u) was calculated as the concentration in the ultrafiltrate divided by the concentration in the original sample.

2.2.2.6. Pharmacokinetic and statistical analyses

The concentrations of pholcodine in saliva up to 2 hours after administration were not included in the calculations of the pharmacokinetic data, because of likely retention of part of the oral dose in the mouth during this time period.

Pharmacokinetic parameters for pholcodine were calculated by standard methods (Rowland and Tozer 1980). The maximum plasma concentration (C_{max}) and its time of occurence (t_{max}) and the plasma concentration at steady state (C^{ss}) after chronic dosing were determined directly from the observed data for each individual. The area under the plasma or saliva concentration-time curves to the last sampling time (AUC_{0-168}) following single dose administration and for the dosing interval (AUC_{0-8}) after chronic administration was calculated by the trapezoidal rule and the total AUC ($AUC_{0-\infty}$) after single doses was calculated as

$$AUC_{0-\infty} = AUC_{0-168} + C_p^{168}/\lambda_z^p \text{ or } C_s^{168}/\lambda_z^s$$

where C_p^{168} and C_s^{168} are the plasma and saliva concentrations at 168 hours respectively and λ_z^p and λ_z^s are the slopes of the terminal concentration-

time curves determined by linear regression analysis of the terminal concentration-time curves when plotted as their natural logarithm. Half-lives ($t_{1/2,z}$) in plasma and saliva were calculated as

$$t_{1/2,z} = 0.693/ \lambda_z^{p} \text{ or } \lambda_z^{s}$$

The half-life of pholcodine was also calculated using urine data in which the natural logarithm of the rate of urinary pholcodine excretion was plotted against the mid-points of the urine collection time periods. The slope of this line (λ_z^{u}) was used to calculate the half-life as

$$t_{1/2,z} = 0.693/\lambda_z^{u}$$

Renal clearance (Cl_R) was calculated as

$$Cl_R = A_e(t_1 - t_2) / AUC_{t1-t2}$$

where A_e is the amount excreted unchanged and t_1-t_2 represent time intervals. The fraction excreted unchanged (f_e) in urine was calculated as A_e divided by the dose administered. The volume of distribution (V) was calculated as

$$V = \text{dose} \cdot F/AUC \cdot \lambda_z^{p}.$$

Because the bioavailability (F) of pholcodine is not available, V/F was calculated.

The pharmacokinetic data from the 20 mg single dose study were used to predict the concentrations of pholcodine in plasma and in saliva during chronic administration. This prediction was made by using the equation:

$$C_{av} = AUC_{0-n\tau} / \tau$$

where τ is the dosing interval during chronic administration (8 hours) and $AUC_{0-n\tau}$ is the area under the single dose concentration-time profile from time zero to n (the number of doses given) times the dosing interval (Chiou, 1979).

Differences in pharmacokinetic data between treatments were analysed for statistical significance by the paired t-test. Correlations between plasma and saliva concentrations, between saliva concentration and saliva pH and between renal clearance and either urine pH or urine flow rate were determined by linear regression analysis and tested for differences by calculation of a 95% confidence interval for the slope. All data are reported as mean ± SD (standard deviation).

2.2.3. Results

2.2.3.1. Single dose studies

The mean plasma and saliva concentration-time profiles and urinary excretion rate versus mid-point time collections for pholcodine in six subjects following 20 and 60 mg oral doses are shown in Figure 2-2-1, 2-2-2 and 2-2-3 respectively. Table 2-2-2 and 2-2-3 contain the derived pharmacokinetic parameters. The time for pholcodine to reach the maximum plasma concentration was not different between the two doses ($P > 0.05$). The maximum plasma concentration for the 60 mg dose was between 1.75 and 3.05 times that of the 20 mg dose. Following attainment of the peak plasma concentration, plasma pholcodine concentrations declined in a mono-exponential manner. Tables 2-2-2 and 2-2-3 show that the elimination half-lives determined from plasma, saliva and urine excretion rate were not significantly different after the 20 mg dose and after the 60 mg dose ($P > 0.05$). There was no difference in half-life between the two doses when

determined using plasma, saliva or urine excretion rate data ($P > 0.05$). The combined mean half-lives in plasma were 48.6±10.4 hours, saliva 51.2±11.9 hours and urine 50.0± 12.2 hours. The mean area under the plasma concentration-time curve for the 60 mg dose was 2.3±0.3 (range 2.1-2.5) times that of the 20 mg dose. This was significantly less than the expected ratio of 3.0 ($P < 0.05$). The area under the saliva concentration-time curve for the 60 mg dose was 2.3±1.0 (range 1.2-3.3) times that of the 20 mg dose and was not significantly different from the expected ratio of 3.0 ($P > 0.05$). The mean area under the saliva concentration-time curve was 4.2±2.1 times higher than under the plasma concentration-time curve for the 20 mg dose, and 4.1±1.6 times for the 60 mg dose. There was a highly significant correlation ($r = 0.91$, $P < 0.01$) between plasma and saliva concentrations (Figure 2-2-4).

The cumulative excretion of unchanged pholcodine in urine is shown in Figure 2-2-5. The percentage of the dose excreted unchanged after 20 mg and 60 mg was 27 and 24% respectively ($P > 0.05$). There was no statistically significant difference ($P > 0.05$) in renal clearance of pholcodine between the two doses. There was a statistically significant ($r = 0.60$, $P < 0.01$) inverse relationship between urine pH and renal clearance of pholcodine (Figure 2-2-6a). There was however no correlation between urine flow rate and renal clearance of pholcodine (Figure 2-2-6b).

2.2.3.2. Chronic dose study

The pharmacokinetic parameters after chronic dosing are shown in Table 2-2-4. During the chronic dosing study, daily trough saliva concentrations reached a plateau by day 7 (Figure 2-2-7). The mean area under the saliva concentration-time curve during the dosing interval on day 11 was 1.97±0.44 mg·h/l which did not differ significantly ($P > 0.05$) from the single dose area under the saliva concentration-time curve (2.91±1.20 mg·h/l),

although in 5 of the 6 subjects, it was lower during the chronic study. By contrast, the mean area under the plasma concentration-time curve during the dosing interval on day 11 was 0.55±0.06 mg·h/l, being just significantly (P = 0.05) lower than the area obtained after the single dose (0.72±0.16 mg·h/l). The mean elimination half-life (53.5±2.3 hours) in saliva was not significantly different from the single dose half-lives (Tables 2-2-2, 2-2-3). Likewise the mean percentage of the dose excreted unchanged in urine (26.8±3.5) and the renal clearance (167±36 ml/min) were not significantly (P > 0.05) different from those following the two single doses (Tables 2-2-1, 2-2-2, 2-2-3). The mean ratio of saliva to plasma area under the concentration-time curves during the 8 hours dosing interval was 3.64±0.88 (range 2.40 to 5.06). The saliva pholcodine concentrations predicted from the single dose pharmacokinetic data are shown in Figure 2-2-7 and are not statistically different from the observed values (P > 0.05).

2.2.3.3. Plasma protein binding

The extent of plasma protein binding (Table 2-2-4) was 23.2±5.6% in the 6 subjects on chronic administration and was 21.5±3.1% when pholcodine was added to drug-free human plasma. The binding of pholcodine to plasma proteins was linear at plasma pholcodine concentrations from 5 to 200 ng/ml.

2.2.3.4. Metabolism

Morphine, and the conjugates of morphine after hydrolysis were not detected in urine (lower limit of assay sensitivity for morphine was 50 ng/ml in urine). Putative pholcodine conjugates were not detected in urine after both β-glucuronidase and hydrochloric acid hydrolysis. Two additional peaks were noted in the chromatograms of the plasma extracts (60 mg single dose and chronic dose), and in the extracts of urine and saliva samples after all

doses. These were subsequently shown to be metabolites of pholcodine and their identifications will be discussed in Chapter 2.3..

There were no adverse effects of pholcodine demonstrable in any of the volunteers during single or chronic studies.

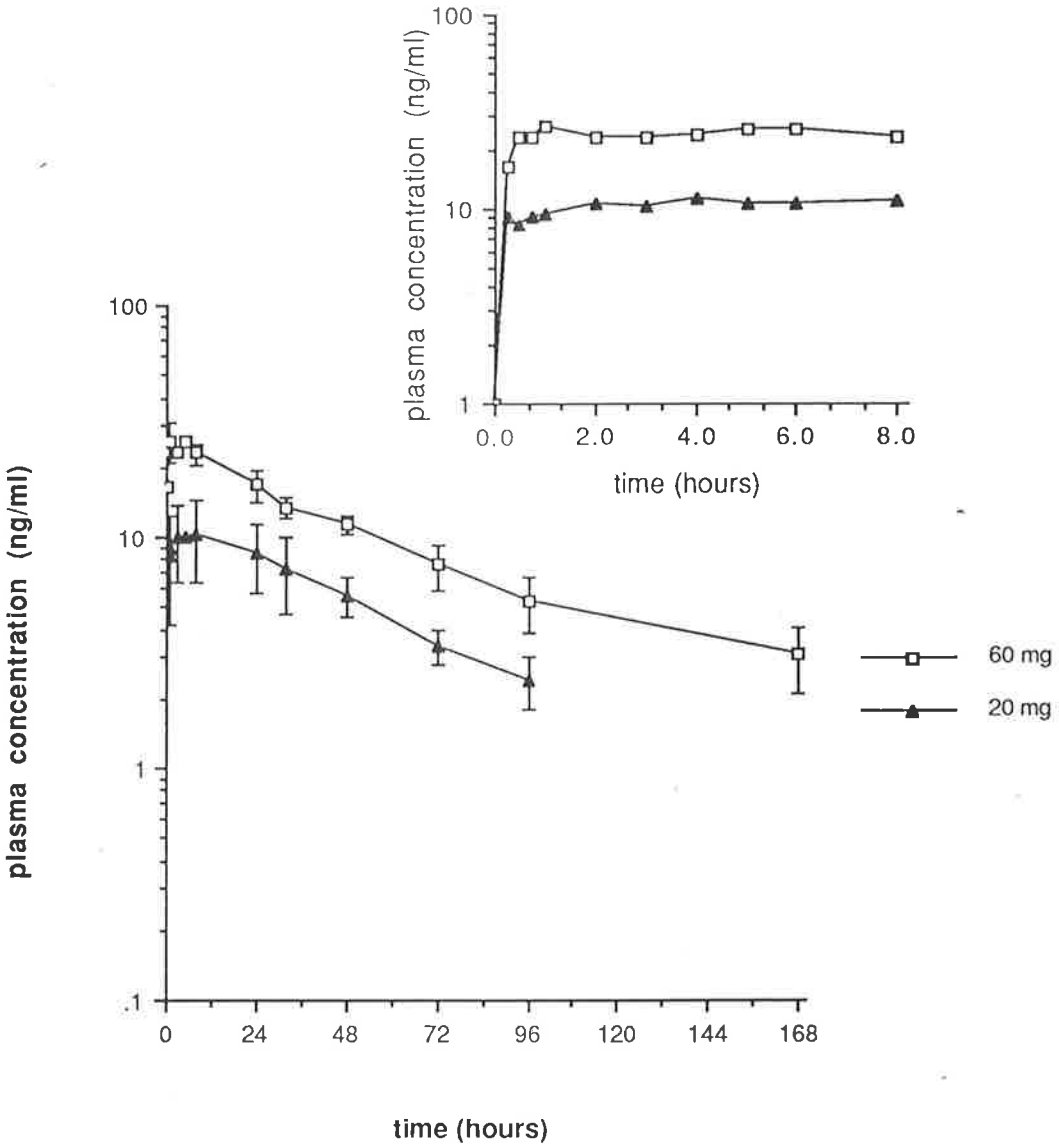


Figure 2-2-1 Plasma concentrations (mean±SD) of pholcodine after single 20 mg and 60 mg oral doses in six healthy volunteers. The inset shows the expanded plasma concentrations of pholcodine in the first 8 hours after dosing.

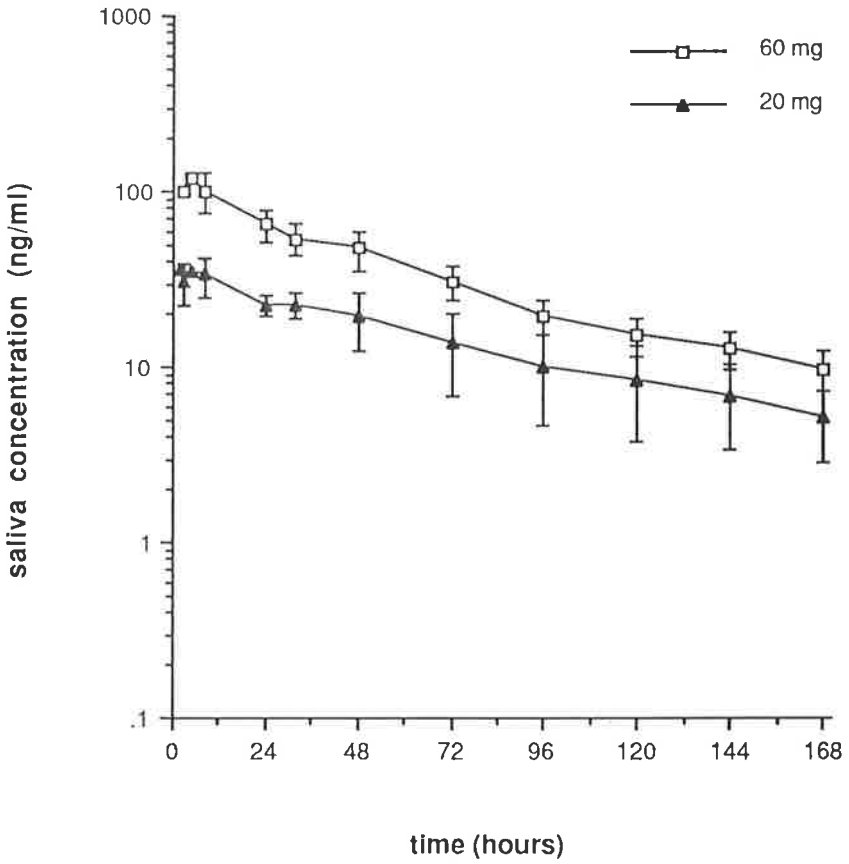


Figure 2-2-2 Saliva concentrations (mean±SD) of pholcodine after single 20 mg and 60 mg oral doses in six healthy volunteers.

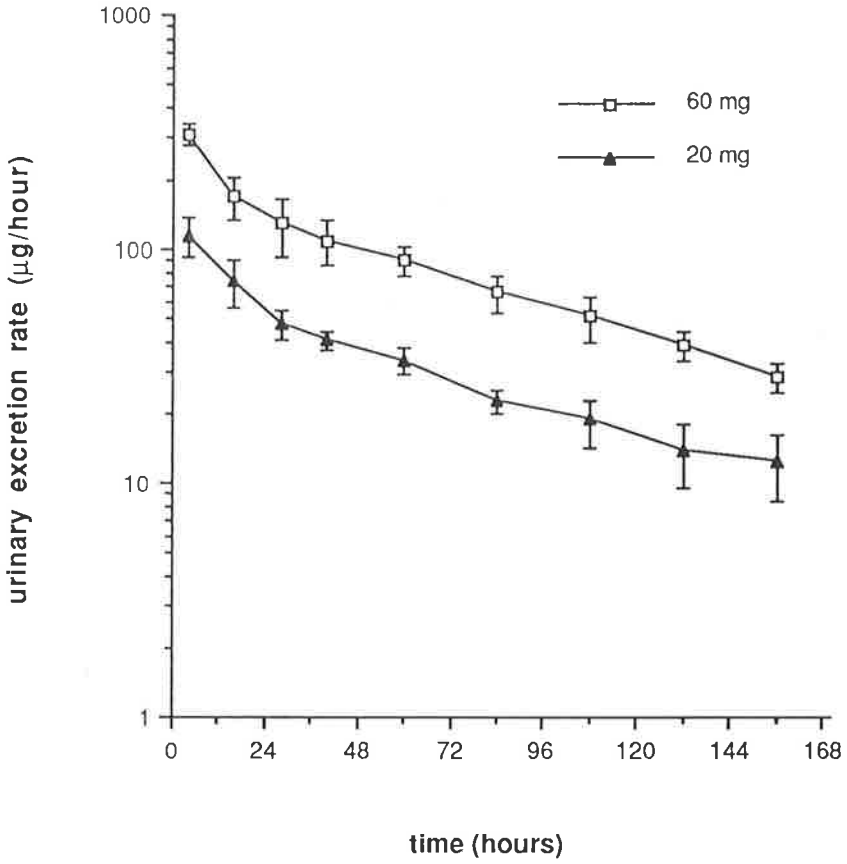


Figure 2-2-3 Urinary excretion rate versus mid-point collection time (mean±SD) of pholcodine after single 20 mg and 60 mg oral doses in six healthy volunteers.

Table 2-2-2 Pharmacokinetic parameters after a single 20 mg pholcodine in six healthy volunteers

parameters	subjects						mean	SD
	1	2	3	4	5	6		
t_{max} *(h)*	4.0	3.0	0.75	0.25	2.0	2.0	2.0	1.4
C_{max} *(ng/ml)*	12.1	9.6	11.1	25.4	9.7	8.9	12.8	6.3
$t_{1/2}$ *(h)*								
plasma	41.9	54.5	44.0	34.9	47.4	49.8	45.4	6.8
saliva	81.0	52.7	37.3	46.0	66.8	50.0	55.6	14.4
urine	53.6	57.6	42.0	32.8	51.7	49.8	45.5	10.3
$AUC_{0-\infty}$ *(mg·h/l)*								
plasma	0.70	0.66	0.62	1.05	0.67	0.62	0.72	0.16
saliva	4.17	2.62	1.63	1.95	4.91	2.19	2.92	1.21
V/F *(l/kg)*	31.4	36.2	31.4	13.5	35.9	33.6	30.3	8.5
Cl_R *(ml/min)*	123	129	141	63	136	162	126	33
f_e	0.30	0.26	0.26	0.24	0.32	0.27	0.27	0.03

Table 2-2-3 Pharmacokinetic parameters after a single 60 mg pholcodine in six healthy volunteers

parameters	subjects						mean	SD
	1	2	3	4	5	6		
t_{max} *(h)*	1.0	0.25	1.0	0.5	3.0	2.0	1.3	0.9
C_{max} *(ng/ml)*	36.7	29.3	28.6	43.5	26.2	26.3	26.3	6.4
$t_{1/2}$ *(h)*								
plasma	53.3	39.2	52.0	76.0	45.7	44.9	51.9	11.8
saliva	45.0	49.6	47.9	40.4	51.2	45.9	46.7	3.5
urine	36.0	39.2	55.5	76.4	45.7	60.7	52.1	15.0
$AUC_{0-\infty}$ *(mg·h/l)*								
plasma	1.52	1.44	1.51	2.34	1.65	1.55	1.67	0.31
saliva	4.97	7.53	5.19	6.09	8.72	7.18	6.61	1.33
V/F *(l/kg)*	53.8	35.6	46.3	36.3	42.0	36.4	41.7	7.2
Cl_R *(ml/min)*	133	171	181	95	171	146	150	30
f_e	0.20	0.25	0.27	0.22	0.28	0.23	0.24	0.03

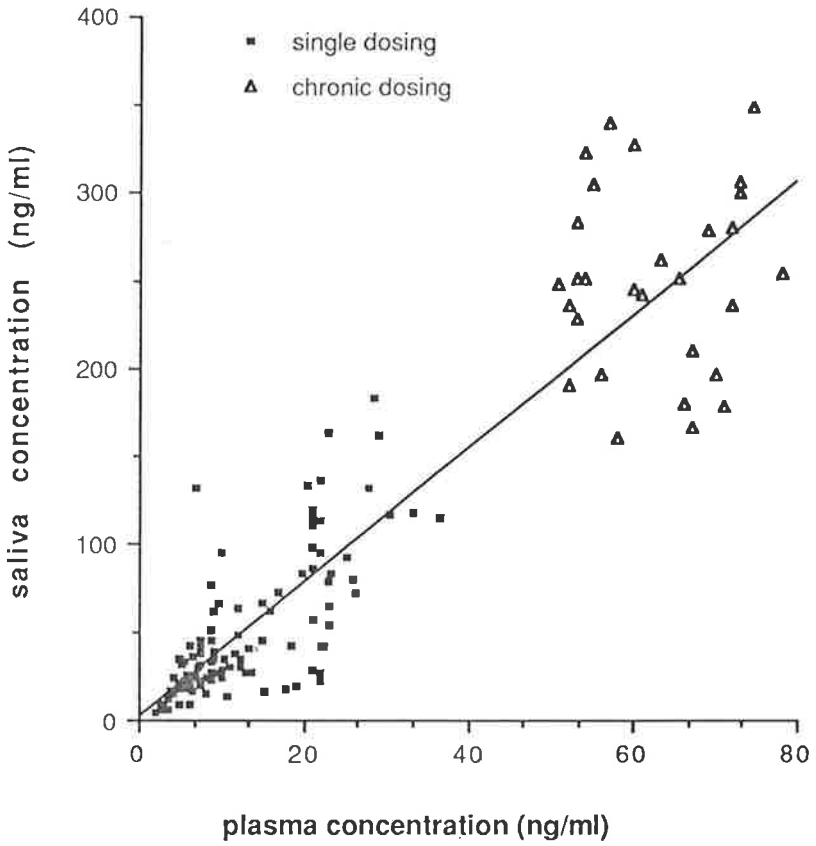


Figure 2-2-4 Correlation between the concentrations of pholcodine in saliva and plasma ($r = 0.91$, $P < 0.01$).

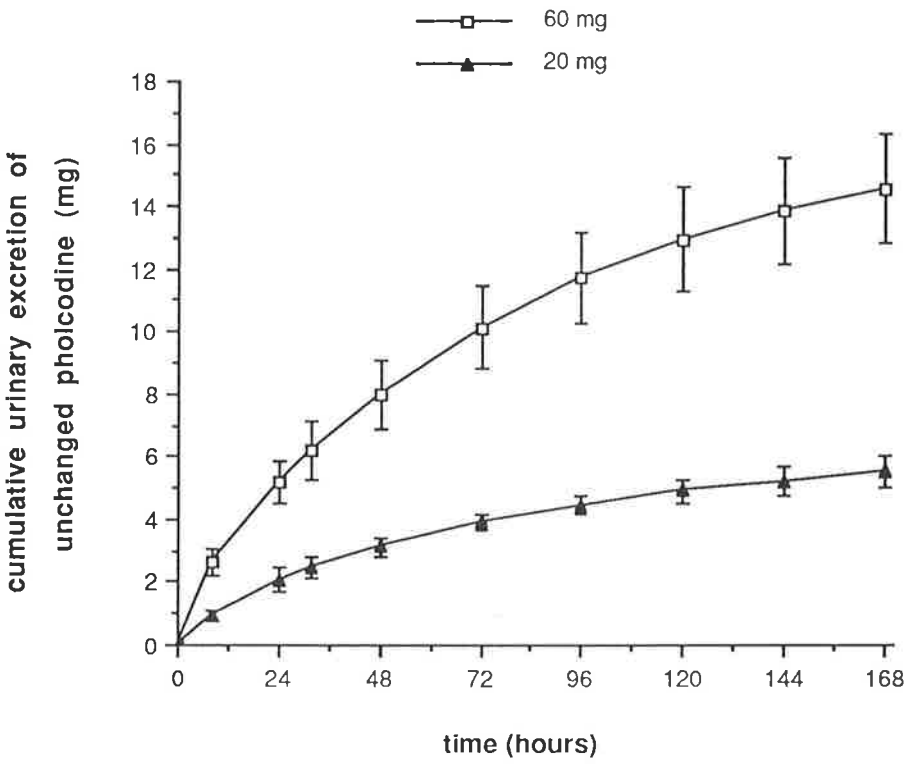


Figure 2-2-5 Cumulative urinary excretion (mean±SD) of unchanged pholcodine after single 20 mg and 60 mg oral doses in six healthy volunteers.

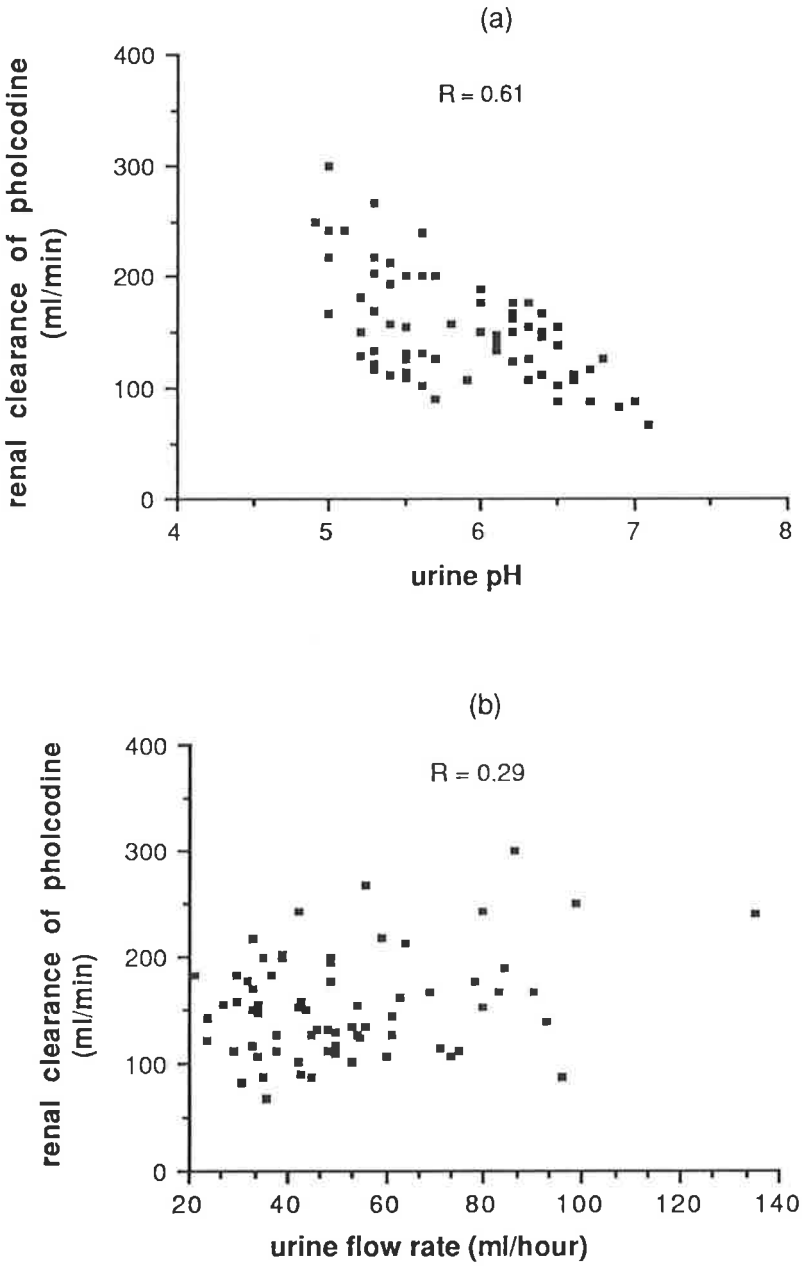


Figure 2-2-6 Correlation between the renal clearance of pholcodine and urine pH (a) and urine flow rate (b).

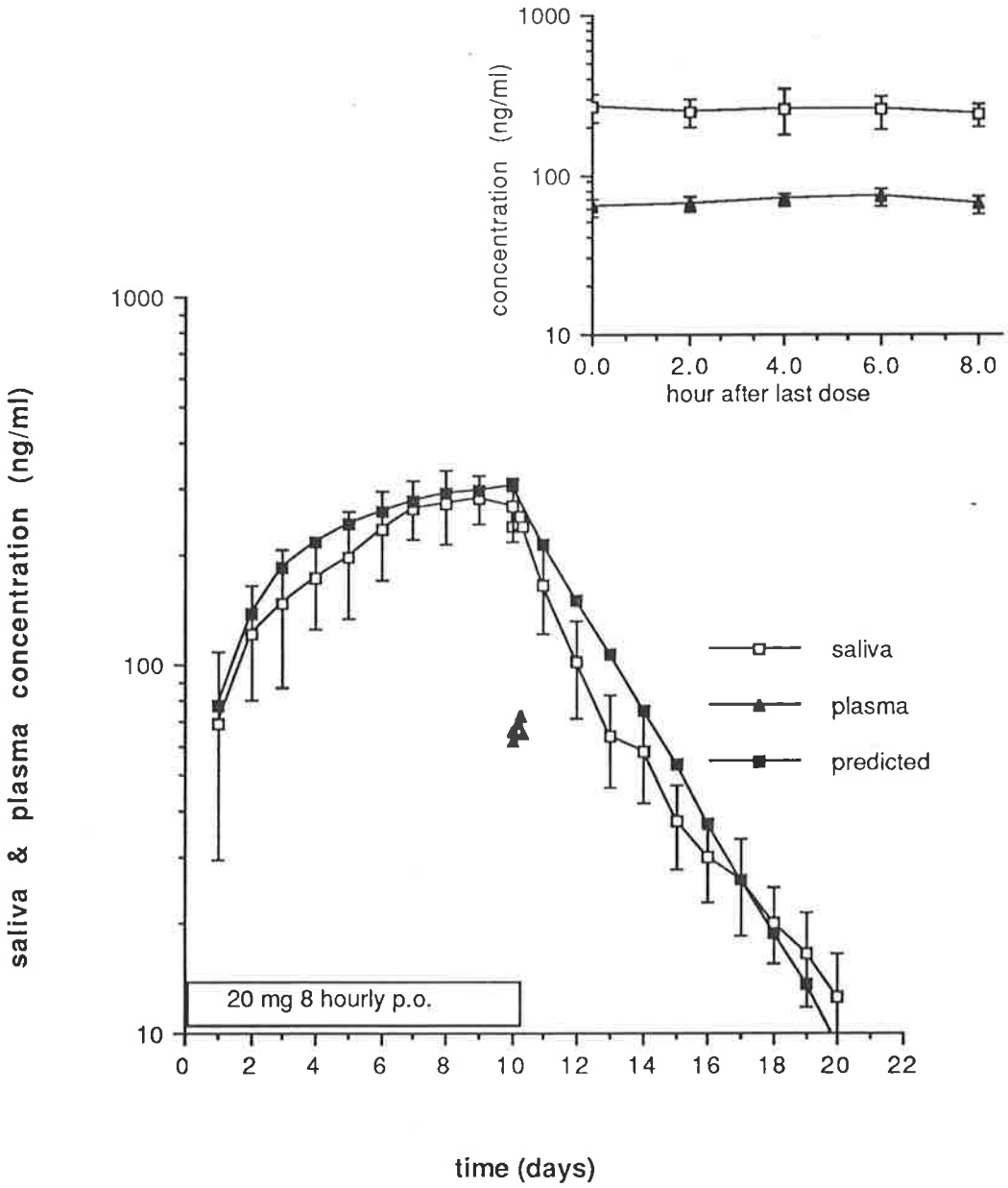


Figure 2-2-7 Plasma and saliva concentrations (mean±SD) of pholcodine after 20 mg oral pholcodine 8 hourly for 10 days in six healthy volunteers. The concentrations in saliva predicted from the single dose pharmacokinetics are shown. The inset contains the expanded data for the first 8 hours after the last dose on day 11.

Table 2-2-4 Pharmacokinetic parameters after chronic dosing of 20 mg pholcodine 8 hourly for 10 days in six healthy volunteers

parameters	subjects						mean	SD
	1	2	3	4	5	6		
saliva								
$t_{1/2}$ *(h)*	51.5	55.8	53.9	50.5	56.2	53.2	53.5	2.3
AUC_{0-8}*(mg·h/l)*	2.1	2.5	1.4	1.9	1.6	2.4	2.0	0.4
plasma								
C^{ss} *(ng/ml)*	59.6	68.2	60.5	72.0	58.0	74.5	65.5	7.0
AUC_{0-8}*(mg·h/l)*	0.53	0.49	0.57	0.58	0.48	0.62	0.55	0.06
fu (%)	76.2	69.7	71.6	81.3	77.0	84.7	76.8	5.7
urine								
Cl_R *(ml/min)*	180	166	162	137	230	128	167	36
fe	25.9	26.5	28.4	23.6	33.0	23.7	26.8	3.5

2.2.4. Discussion

There are limited data available concerning pholcodine pharmacokinetics in human. This is the first study to have used a specific chemical assay for the analysis of pholcodine in biological fluids in order to evaluate its disposition in man. The pharmacokinetics of pholcodine after single doses have been reported by Neuvonen and coworkers (1980) and Findlay and coworkers (1986). Differences exist in the results from those two studies compared with the present one. Firstly, the mean time to reach maximum plasma concentration of 2 hours reported here was much shorter than the 4-6 hours reported by Findlay and coworkers (1986). However they found the plasma concentration reached half of its maximum value within 30 minutes of drug administration. This was not noticed in this study. Secondly, after 60 mg of pholcodine in two separate studies, Findlay and coworkers (1986) reported a maximum plasma concentration of 27±6 ng/ml and 57±11 ng/ml and Neuvonen and coworkers (1980) attained a maximum plasma concentration of about 80 ng/ml after a 30 mg dose. The mean maximum plasma concentration of 26.3±6.4 ng/ml achieved in this study is similar to one of the values cited by Findlay and coworkers, but much lower than their second value. Thirdly, the mean plasma elimination half-life of 52±12 hours in this study is comparable to the value reported by Findlay and coworkers of 37±4 hours, but is shorter than the 102±10 hours from urine reported by Neuvonen and coworkers (1980). Finally, the area under plasma concentration-time curve of 1670±310 ng·h/ml is higher than that (1004±77 ng·h/ml) found by Findlay and coworkers (1986). The reasons for these discrepancies are not clear, but may relate to the differences in analytical methods employed by these authors.

The disposition of pholcodine after chronic administration to man has not been previously studied. The fact that the observed concentrations in both saliva and plasma were similar to those predicted from the single dose pharmacokinetic parameters indicates that the absorption and disposition of pholcodine is not dose or concentration dependent, at least within the dosage range employed in this study. The similar terminal half-lives after single doses and on cessation of chronic dosing support the above contention. There was a trend for the area under concentration-time curve during the dosing interval on day 11 for both saliva and plasma to be lower than the total area under concentration-time curve following the single dose. The reasons for this are not clear.

The fate of pholcodine in humans is still unclear. The urinary recovery of unchanged drug of about 25% indicates that a substantial proportion of the dose is not accounted for. The absorption of pholcodine was reported to be 88±26% (Neuvonen *et al* 1980). Thus most of the unaccounted portion must undergo metabolism, since only 5% of an intravenous dose was recovered as ^{14}C-pholcodine in faeces (Neuvonen *et al* 1980). There were no glucuronide or sulphate conjugates detected in urine. The two peaks which consistently appeared in all the urine and saliva chromatograms in every subject are metabolites of pholcodine. Their isolation and identification are described in Chapter 2.3..

There is evidence in the rat (Butz *et al* 1983) that morphine is a minor metabolite of pholcodine, accounting for less than 1% of the dose. In agreement with Findlay and coworkers (1986) and Neuvonen and coworkers (1980), I could not detect any morphine in urine even after enzymatic or acidic hydrolysis. Thus, pholcodine is unlikely to exert its pharmacological effect via morphine. Therefore the antitussive effect of pholcodine which

was shown to be comparable to codeine (Eddy *et al* 1969) can only be mediated by the parent compound and/or some metabolites other than morphine.

The saliva to plasma pholcodine concentration ratio was substantially greater than unity. This phenomenon results from a combination of factors: pholcodine is a basic compound, it has a high pK_a (9.3, Moffat *et al* 1986), is poorly plasma protein bound (23%) and is highly lipophilic. Codeine has similar physico-chemical properties to pholcodine, and its saliva to plasma concentration ratio is similar to that of pholcodine (Lee *et al* 1986; Chapter 4.3.). Based on the Henderson-Hasselbach equation, the predicted saliva to plasma ratio should be between 1.3 and 6.4 if saliva pH ranges from 6.5 to 7.2. The mean pH of saliva in these subjects was 6.8, which would result in a saliva to plasma ratio of 3.2. The mean ratio in my volunteers was 3.6. This somewhat higher than predicted value may reflect a contribution from ion trapping. The strong correlation between the saliva to plasma pholcodine concentration ratio and saliva pH ($r = 0.804$) is consistent with the Henderson-Hasselbalch equation and the known physico-chemical properties of pholcodine.

The renal clearance ranged from 63 to 181 ml/min and indicates that pholcodine must undergo tubular secretion in addition to glomerular filtration. In addition, the strong correlation between renal clearance and urine pH suggests that reabsorption must also occur.

The elimination half-life of pholcodine is substantially longer than that of the other commonly used antitussive agents such as codeine (2.8 hours, Quiding *et al* 1986) and dextromethorphan (3.0 hours, Aylward *et al* 1984). The possible reasons for this difference may reside in a larger distribution volume and/or slower clearance rate. The distribution volume of pholcodine could range between 30-40 l/kg, assuming complete bioavaila-

bility. By comparison, the distribution volume of codeine is 3.97 l/kg (Quiding *et al* 1986). Secondly, the total body clearance of pholcodine, again assuming complete gastro-intestinal absorption, could range from 400-600 ml/min, of which I have shown that renal clearance accounts for 25%. Thus, pholcodine is likely to be a drug with a intermediate hepatic extraction ratio. The long half-life of pholcodine indicates that there will be a large accumulation of pholcodine in the body after chronic dosing according to the currently recommended dosage regimen (10 mg 4-6 hourly, British National Formulary 1985) and that the maximal effects of pholcodine will be much later than codeine and dextromethorphan.

In conclusion, pholcodine has quite different pharmacokinetic characteristics from other antitussive drugs and this could be important in recommending appropriate dosage regimens in patients. The single and chronic dose pharmacokinetic data derived from this study suggest that the currently recommended dosage regimen for pholcodine, 10 mg up to 6 times daily, should be revised.

2.3. Isolation and identification of metabolites of pholcodine

2.3.1. Introduction

Almost nothing is known about the metabolism of pholcodine in humans. Study in rats showed that 0.1-0.2% of the parent drug was transformed to morphine after intravenous administration of 10 mg/kg of pholcodine (Butz *et al* 1983). However morphine in plasma was not detected after administration of a 60 mg oral dose of pholcodine in man (Findlay *et al* 1986). In the pholcodine pharmacokinetic studies (Chapter 2.2.), two additional peaks other than morphine were observed consistently in the chromatograms of the plasma (60 mg and chronic doses), saliva and urine samples. These peaks did not appear in any blank sample. The height of the peaks was related to the dose and the concentration of pholcodine. They were thought to be the metabolites of pholcodine.

The aims of this study were to isolate and identify these two metabolites.

2.3.2. Methods

2.3.2.1. Urine samples

All the urine samples collected after chronic doses in the pholcodine pharmacokinetic study (Chapter 2.2.) were used. In addition, one of the subjects took 60 mg pholcodine 8 hourly for 8 doses and urine was collected during the dosing period and for 72 hours after the last dose.

2.3.2.2. Extraction

Urine was made alkaline (pH 10) with sodium carbonate and mixed with equal volumes of distilled chloroform for 10 minutes. The organic phase was separated and the aqueous phase was again washed with an equal volume of distilled chloroform for 10 minutes. The chloroform extracts were combined and evaporated to dryness at 40 °C in a Rotavapor (Buchi CH-9230, Switzerland). The residue was redisolved in 0.05 N hydrochloric acid.

2.3.2.3. Separation

The two peaks were separated from pholcodine and from each other by HPLC (Chapter 2.1.). Aliquots of extracts were injected onto a C_{18} column via an automatic injector and the eluates were collected in glass tubes when the peaks appeared as monitored by the fluorescence detector. The collections of each of the two peaks were then adjusted to pH 10 with sodium carbonate and extracted with distilled chloroform three times. The combined chloroform extracts for each peak were then evaporated to dryness in the Rotavapor at 40 °C. The residues were redissolved in 0.05 N hydrochloric acid and transferred to a clean glass tube. The solutions were again made alkaline and extracted with distilled chloroform three times. Finally the combined chloroform extracts were evaporated to dryness and kept at - 20 °C until analysis.

2.3.2.4. Identification

HPLC: Aliquots of the extracts of the two peak collections were dissolved in the mobile phase and injected to the HPLC column to make sure that the extraction was successful and to estimate the amounts of the compounds assuming the same fluorescence response as pholcodine.

Mass spectra and NMR: The identification of the peaks by mass spectra (MS) and nuclear magnetic resonance (NMR) spectra was conducted by Dr. A. D. Ward of Department of Organic Chemistry, the University of Adelaide (see Appendix IV).

2.3.3. Results and discussion

There were two peaks which constantly appeared in the chromatograms of all the urine samples. One peak (II) came out before pholcodine and the other (I) after pholcodine (Figure 2-1-8b). The peak height of peak I was 5-10 times higher than peak II. These two peaks, in common with pholcodine, were well extracted by chloroform in alkaline pH. They were stable under the conditions described above. The chromatograms from the injection of peak collections were very clean and suggested that the the extraction and separation procedures for the two peaks were successful. It was important to use non-plastic containers for the collection and storage throughout the procedure of identification, because the phthalates released from plastic interfere with the analysis. The acid back extraction is also important to make the sample free from phthalates. In addition, chloroform should be distilled before use because the impurities in chloroform also influence the analysis.

Sufficient data for peak I were obtained from both mass spectra and NMR (see Appendix IV) and this metabolite could be well characterized. However, the information for peak II was insufficient to make any conclusion. Peak I had a molecular weight of 413 as determined by fast atom bombardment mass spectrometry, which is 14 mass units larger than that of pholcodine. This difference is caused by changes to the morpholinoethyl side chain, according to the data from the mass analysed kinetic energy spectrum (MIKES). The NMR spectra of pholcodine and peak I also showed that the

difference is related to the morpholine ring. All the data from mass spectra and NMR spectra strongly support that peak I has a >C=O group which has replaced the $-CH_2-$ unit in the morpholine ring (Figure 2-3-1).

Peak II was much smaller than peak I and its identification needs a lot more sample. This will be done at a later time.

The results from this pilot study have shown that the metabolism of pholcodine is different from that of other opioid compounds. The main metabolic pathways for opioid related compounds, such as codeine, are O-dealkylation and/or N-dealkylation and glucuronidation. These metabolic pathways appear to be unimportant for pholcodine. The biological activities of this metabolite (peak I) and the importance of this pathway in pholcodine metabolism needs further investigation after the compound is synthesised.

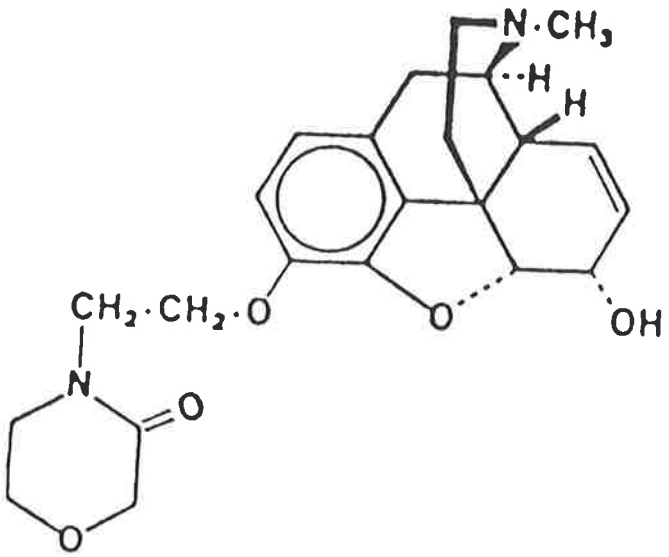


Figure 2-3-1. Chemical structure of the metabolite of pholcodine.

Chapter 3

POLYMORPHIC METABOLISM AND PHARMACOKINETICS OF DEXTROMETHORPHAN IN HUMANS

3.1. Simultaneous determination of dextromethorphan and three metabolites in plasma and urine by HPLC

3.1.1. Introduction

Although many analytical methods for the determination of dextromethorphan and/or its metabolites in biological fluids have been reported (Chapter 1.5.3.), there has been only one report of the simultaneous determination of dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan in urine by HPLC (Park *et al* 1984). This assay, however, used a relatively large sample volume (2 ml) and an extraction cartridge for sample clean-up. Because the metabolites undergo extensive glucuronide conjugation, whereas the parent compound does not, each sample has to be processed at least twice for the determination of unconjugated and conjugated compounds. Such a volume for plasma analysis may not be acceptable in pharmacokinetic studies, and the cartridge column extraction may add technical difficulties as well as cost to the method. There is no report on the simultaneous determination of the four compounds in plasma, although separate procedures for each compound have been described. Because of the polymorphic metabolism of dextromethorphan, plasma and urine concentrations of the parent compound and its demethylated metabolites vary markedly (Pfaff *et al* 1983, East and Dye 1985). In extensive metabolisers, compounds detectable in plasma after administration of dextromethorphan were mainly as the O-demethylated metabolites, dextrorphan and 3-hydroxymorphinan. In poor metabolisers, however, both O-demethylated metabolites and the parent compound were detectable (Pfaff *et al* 1983, East and

Dye 1985). To investigate the pharmacokinetics and metabolism of dextromethorphan in extensive and poor metabolisers, an assay for the simultaneous determination of the four compounds in plasma and urine is required. The aim of this study was to develop a sensitive and specific method for the simultaneous determination of dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan in plasma and urine using HPLC.

3.1.2. Materials and methods

3.1.2.1. Reagents

All reagents were of analytical grade or of HPLC grade (see Appendix I). Dextromethorphan hydrobromide, dextrorphan hydrobromide, 3-hydroxymorphinan hydrobromide, 3-methoxymorphinan hydrobromide (Roche products Pty. Ltd. NSW, Australia) and pholcodine (F. H. Faulding and Co. Ltd) were all of British Pharmacopoeial grade quality.

3.1.2.2. Chromatography

The HPLC consisted of a pump (M-6000A, Waters Associates Inc., Milford, Mass, U.S.A.), a LS-5 Luminescence Spectrometer (Perkin-Elmer Ltd, Beaconsfield, U.K.), a WISP 710 B automatic injector (Waters Associates) and an Omniscribe B-5000 strip-chart recorder (Houston Instruments, Austin, Tx, U.S.A.). The 15 cm x 4.6 mm I.D. stainless steel column was packed with Spherisorb 5 µm cyano packing material (Phase Separations, Queensferry, U.K.). The composition of the mobile phase was 17% acetonitrile, 0.06% triethylamine and 82.94% distilled water, adjusted to pH 3.0 with ortho-phosphoric acid. The flow rate through the column at ambient temperature was 1 ml/minute which produced a back-pressure of 1.5 Kpsi (10.3 MPa). The excitation and emission wavelengths of the fluorescence detector were 230

and 330 nm respectively and the excitation and emission slits were set at 10 and 20 nm respectively.

3.1.2.3. Stock solutions

Dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan were made up as a 1 mg/ml (base) stock solutions in distilled water and were diluted to concentrations ranging from 2 to 200 ng/ml in drug-free plasma and from 0.05 to 10 µg/ml in drug-free urine. Pholcodine, the internal standard (1 mg base/ml in distilled water) was diluted in distilled water to 1 µg/ml for plasma and 50 µg/ml for urine analysis.

3.1.2.4. Sample preparation

Plasma: 1 ml of plasma was pipetted into a 10 ml screw capped, tapered plastic tube to which was added 100 µl of the 1 µg/ml internal standard solution, and 0.5 ml of saturated sodium carbonate solution. The sample was briefly mixed, 4 ml of diethyl ether/chloroform/propan-2-ol (20/9/1, by volume) was added and the tubes were placed on a rotary mixer for 10 minutes. The organic and aqueous phases were separated by centrifugation at 2000 g for 10 minutes. The upper organic phase was transferred to a clean 10 ml screw-capped, tapered plastic tube containing 100 µl of 0.1 N hydrochloric acid. The tubes were placed on a rotary mixer for 10 minutes. The two phases were separated by centrifugation at 2000 g for 5 minutes. The organic layer was removed by aspiration. An aliquot (10-50 µl) was injected onto the column via the automatic injector.

Urine: 0.5 ml aliquot of urine was pipetted into a 10 ml screw capped plastic tube to which was added 50 µl of a 50 µg/ml internal standard solution.

The samples were then handled in exactly the same manner as the plasma samples.

β-glucuronidase hydrolysis: O.5 ml plasma or urine was mixed with 0.5 ml (8000 U/ml in pH 5 acetate buffer) of β-glucuronidase (Helix pomatia, type H-1, Sigma Chemical Co., St. Louis, MO, USA), followed by incubation in a water bath at 37 °C for 16 hours. Samples were assayed for drug concentrations in the same manner as those for unhydrolysed plasma and urine samples.

3.1.2.5. Statistical analysis

Peak heights were measured manually and the peak height ratio of dextromethorphan, dextrorphan, 3-hydroxymorphinan or 3-methoxymorphinan to pholcodine was then calculated. Standard curves were plotted as peak height ratio versus dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan concentrations. Linear regression analysis was performed to determine the slope, intercept, their variability and the strength of the correlation. Precision was evaluated by spiking drug-free plasma with dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan to concentrations of 10 and 200 ng/ml and urine at 0.5 and 10 µg/ml, performing replicate analyses with 10 samples intra-day and 4 samples inter-day and determining coefficients of variation. Accuracy was assessed by spiking plasma with dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan to concentrations of 10 and 200 ng/ml and urine at 0.5 and 10 µg/ml, assaying 10 times and calculating the estimated concentration from concurrently run standard curves.

3.1.3. Results and discussion

3.1.3.1. Chromatography

Figure 3-1-1 is a representative chromatogram from an injection of a solution in water containing a mixture of dextromethorphan, dextrorphan, 3-hydroxymorphinan, 3-methoxymorphinan and pholcodine, each at a concentration of 1 µg/ml. The limit of sensitivity defined as three times the baseline noise level for each compound was 1 ng/ml in plasma. 3-hydroxymorphinan had a retention time of 4.2 minutes and a capacity factor of 1.6; dextrorphan had a retention time of 5.1 minutes and a capacity factor of 2.2; 3-methoxymorphinan had a retention time of 7.6 minutes and a capacity factor of 3.8; dextromethorphan had a retention time of 10.5 minutes and a capacity factor of 5.6; pholcodine had a retention time of 6.3 minutes and a capacity factor of 2.9.

The cyano column was chosen after evaluation of C_{18} and cyano columns. It was very difficult to separate out the four compounds, especially for dextrorphan and 3-hydroxymorphinan, with the C_{18} column. Alterations in mobile phase composition and pH did not solve this problem. Substitution with a cyano column was effective. Variations in the composition and pH of the mobile phase were then explored (Figure 3-1-2, 3-1-3). Increasing the percentage of triethylamine resulted in a decrease of the retention time of all five compounds, while increasing the percentage of acetonitrile slightly increased the retention time of pholcodine but decreased the retention time for the other four compounds, and increasing the pH of the mobile phase increased the retention time of these five compounds. The mobile phase chosen resulted in optimal separation and chromatography. Several compounds were tested as candidates for the best internal standard. Morphine,

codeine, norcodeine and dihydrocodeine eluted too early and could not be separated from endogenous peaks. Cocaine was well separated from the analyte and endogenous peaks. However it was unstable and easily hydrolysed, especially in alkaline pH. Pholcodine was stable, and resulted in good separation from the analyte and endogenous peaks, and was finally chosen as the internal standard.

3.1.3.2. Linearity

Calibration curves showed good linearity between peak-height ratios and concentrations from 2 to 200 ng/ml for dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan in plasma ($r > 0.99$) and 0.05 to 10 μg/ml in urine ($r > 0.99$). Representative curves are shown in Figure 3-1-4. For plasma and urine standard curves, the 95% confidence intervals of the intercepts included the origin and the standard errors of the slopes were less than 5%.

3.1.3.3. Precision

The assay showed good precision at low and high concentrations in plasma and urine. Table 3-1-1 shows the intra- and inter- day assay precision in plasma and Table 3-1-2 shows the assay precision in urine. The precision for all compounds at different concentrations was less than 10%.

3.1.3.4. Accuracy

The accuracy of the assay for dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan in plasma and urine is shown in Table 3-1-3 and Table 3-1-4 respectively. For all the compounds accuracy was generally within 10%. In samples stored at -20 °C for 16 weeks, there was

no loss of dextromethorphan, dextrorphan, 3-hydroxymorphinan or 3-methoxymorphinan.

3.1.3.5. Recovery

The extraction recovery (Table 3-1-5) was determined by comparing the representative peak heights of extracted samples with the peak heights of standards of the same concentration. Although the extraction with chloroform or ether-chloroform provided good recovery for dextromethorphan, dextrorphan and 3-methoxymorphinan, the recovery for 3-hydroxymorphinan was very low. Adding propan-2-ol to the ether-chloroform mixture gave acceptable recoveries for all the four compounds and the internal standard pholcodine. After comparing the extraction efficiency with the organic solvent (ether-chloroform-propan-2-ol, 20/9/1 by volume) at volumes of 2, 4, 6 and 8 ml, 4 ml of the solvent was finally chosen for the extraction.

3.1.3.6. Interference by other compounds

Interference by other drugs was studied by analyzing samples from patients on multiple-drug therapy (Table 3-1-6.). None of the drugs in Table 3-1-6 interfered with the chromatograms of dextromethorphan, dextrorphan, 3-hydroxymorphinan, 3-methoxymorphinan or pholcodine. Figure 3-1-5 shows a chromatogram from drug-free plasma, a plasma sample from the subject 1.5 hours after ingestion of 30 mg dextromethorphan hydrobromide and the same plasma sample after β-glucuronidase hydrolysis. Figure 3-1-6 shows a chromatogram from drug-free urine, a sample of a 0 to 8 hour urine collection from an extensive metaboliser given 30 mg dextromethorphan hydrobromide and Figure 3-1-7 shows a chromatogram from a sample of a 0 to

8 hour urine collection from a poor metaboliser given 30 mg dextromethorphan hydrobromide.

The efficiency of the β-glucuronidase to hydrolyse conjugates of dextrorphan and 3-hydroxymorphinan has not been directly tested, because the pure conjugate compounds are not available at present. Barnhart (1980) reported that β-glucuronidase hydrolysis was equally effective as acidic hydrolysis. Gillilan and coworkers (1980) found that incubation of 1 hour at 37 °C with 1000 units β-glucuronidase per mililiter plasma adjusted to pH 5.0-5.3 gave complete hydrolysis. In this study, it was estimated by determining the total urinary recovery, based on analysis of dextromethorphan, dextrorphan, 3-hydroxymorphinan, 3-methoxymorphinan. The cumulative urinary recovery over 48 hours from a subject (subsequently classified as an extensive metaboliser) who took 30 mg dextromethorphan hydrobromide was about 96% of the dose (Figure 3-1-8) and these results suggest that the hydrolysis by β-glucuronidase on these particular conjugates under the conditions used was probably complete.

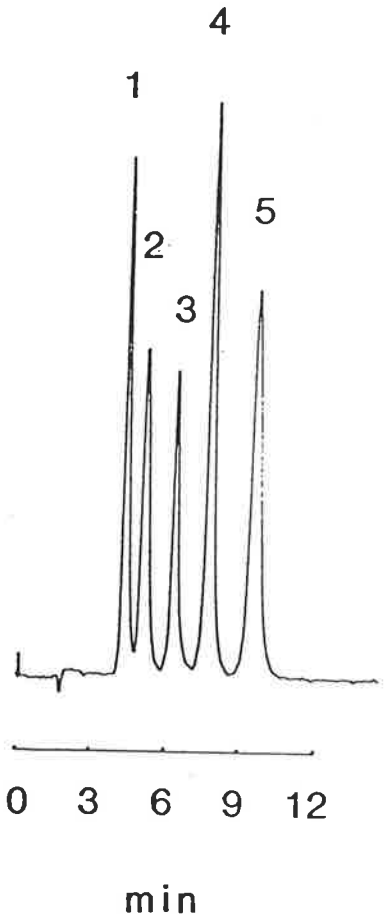


Figure 3-1-1 Chromatogram of a standard water solution of 3-hydroxymorphinan (1), dextrorphan (2), pholcodine (3), 3-methoxymorphinan (4) and dextromethorphan (5).

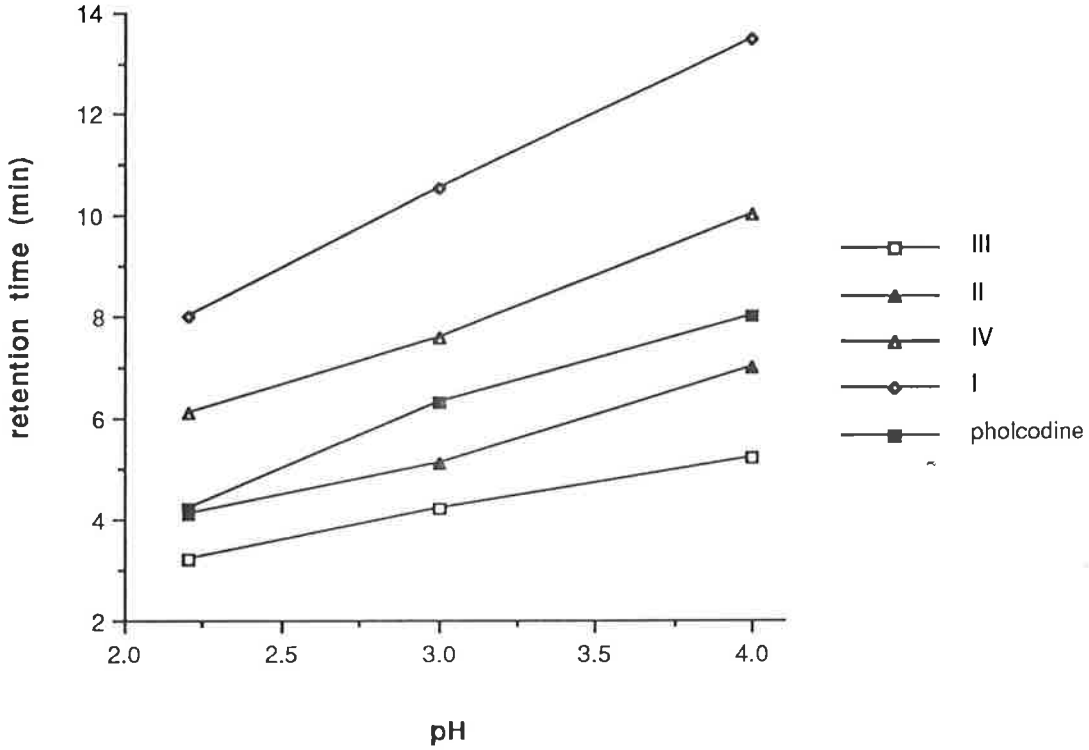


Figure 3-1-2 Effect of pH of the mobile phase (17% acetonitrile/ 0.06% triethylamine/distilled water to 100%) on the retention times of 3-hydroxy-morphinan (III), dextrorphan (II), 3-methoxymorphinan (IV), dextro-methorphan (I) and pholcodine.

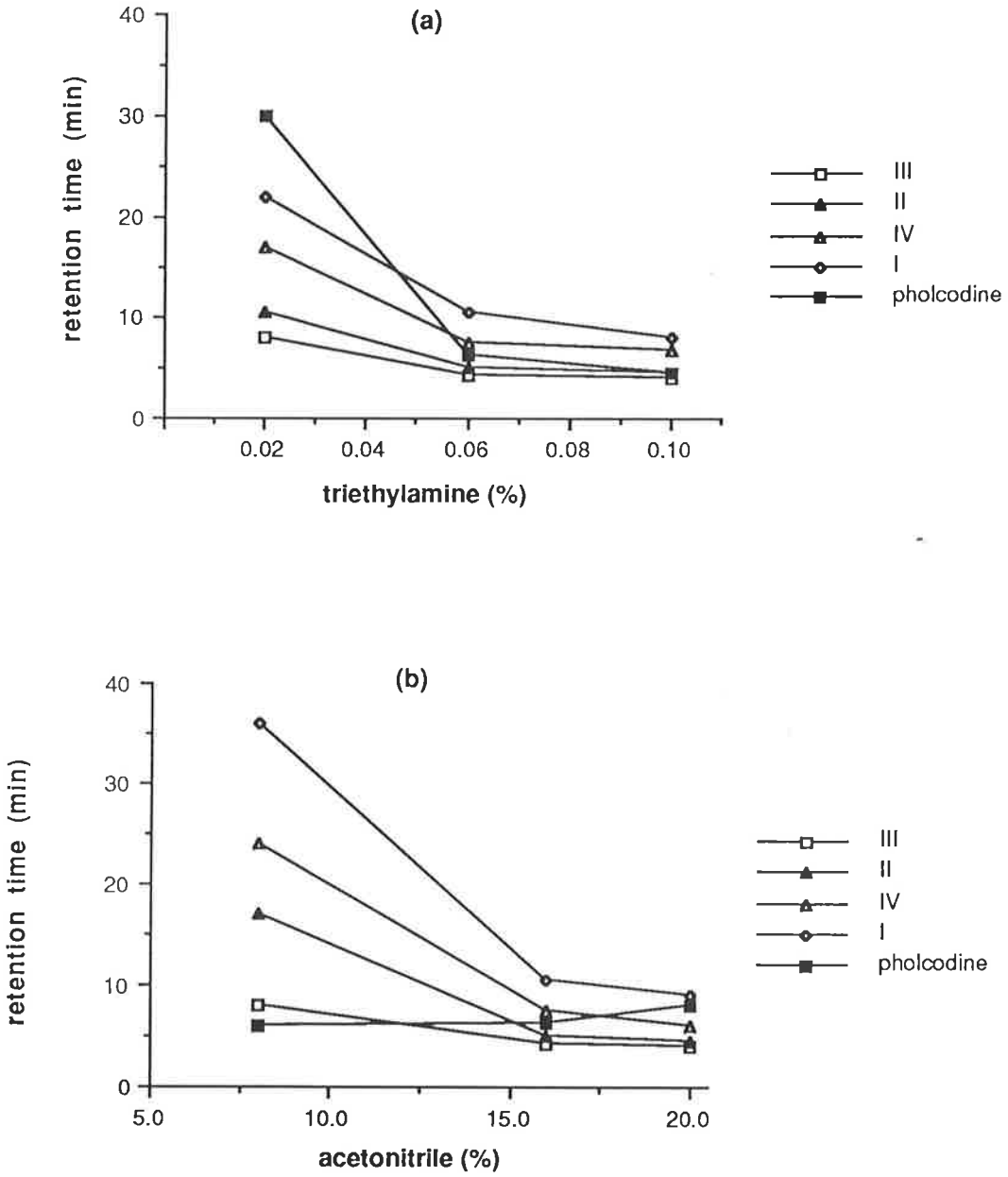


Figure 3-1-3 Effect of the concentrations of (a) triethylamine (in 17% acetonitrile/distilled water to 100%, pH 3.0) and (b) acetonitrile (in 0.06% triethylamine/distilled water to 100%, pH 3.0) on the retention times of 3-hydroxymorphinan (III), dextrorphan (II), 3-methoxymorphinan (IV), dextromethorphan (I) and pholcodine.

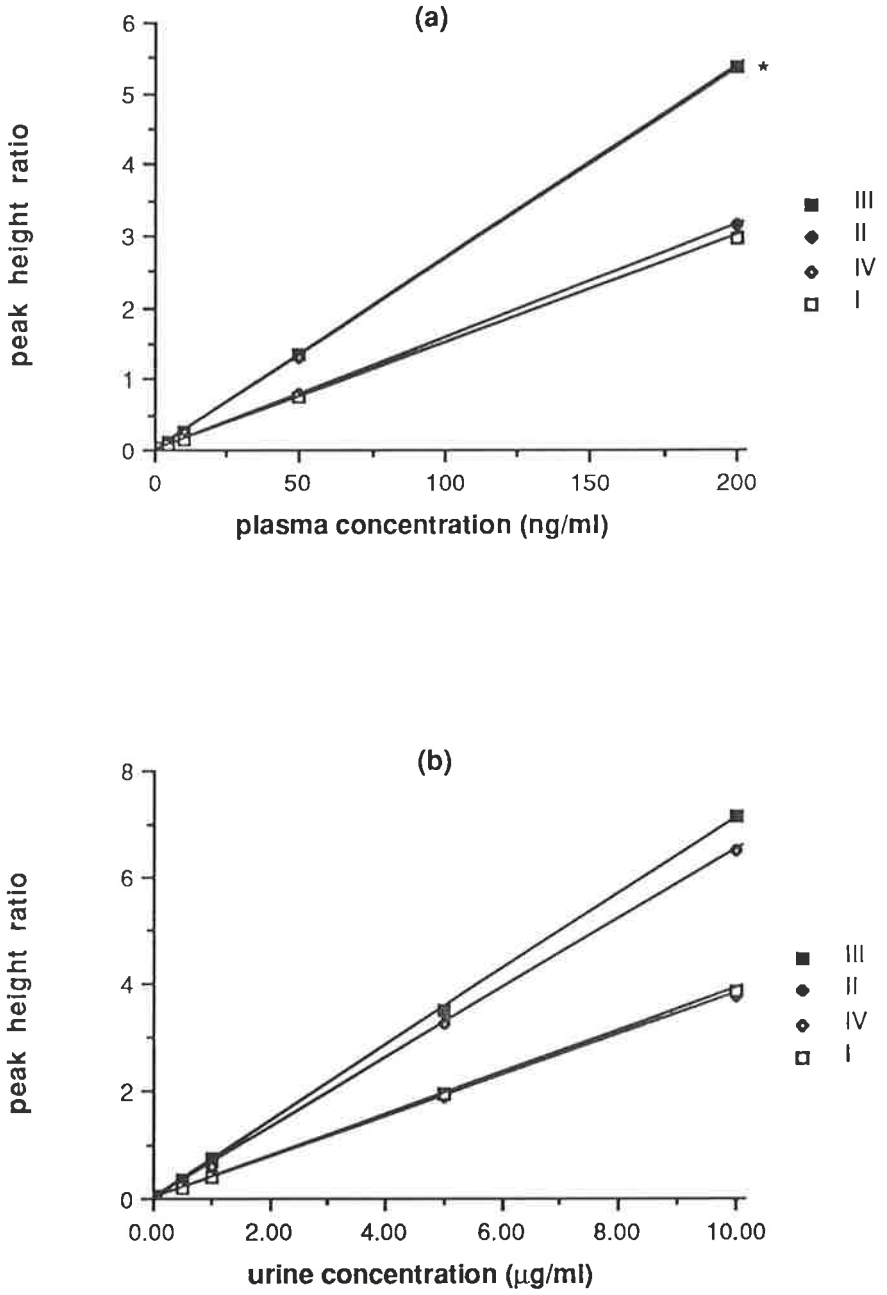


Figure 3-1-4 Representative standard curves for 3-hydroxymorphinan (III), dextrorphan (II), 3-methoxymorphinan (IV) and dextromethorphan (I) in plasma (a) and urine (b).

* The curves for III and IV overlapped.

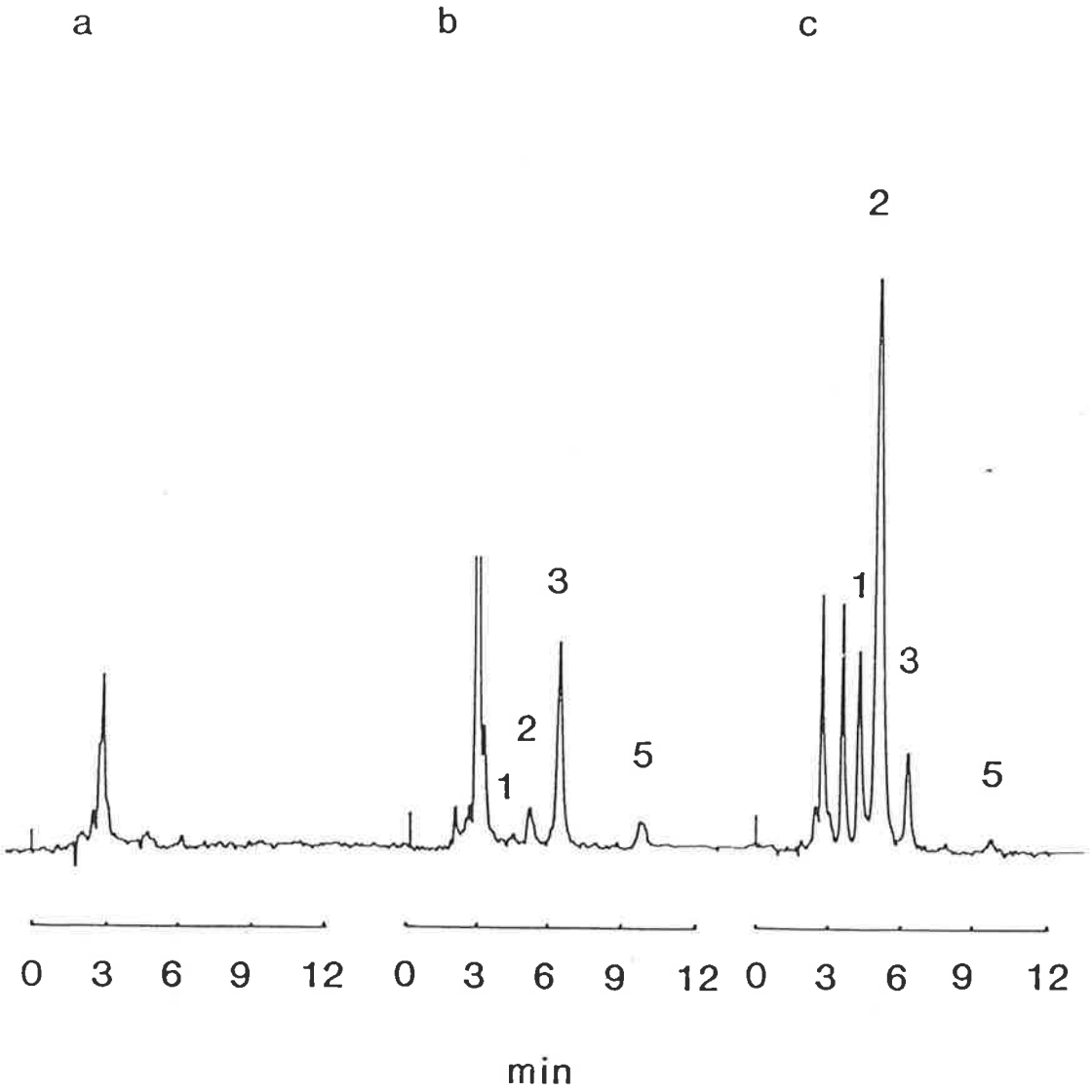


Figure 3-1-5 Chromatograms from an extract of (a) a drug-free plasma sample, (b) an unhydrolyzed plasma sample from a subject 1.5 hours after ingestion of 30 mg dextromethorphan hydrobromide (concentrations: 2 = 7.5 ng/ml; 5 = 3.7 ng/ml) and (c) the same plasma after β-glucuronidase treatment (concentration: 1 = 102 ng/ml; 2 = 484 ng/ml. sample was diluted 1 in 2.)

Peaks: 1 = 3-hydroxymorphinan; 2 = dextrorphan; 3 = pholcodine (internal standard); 5 = dextromethorphan.

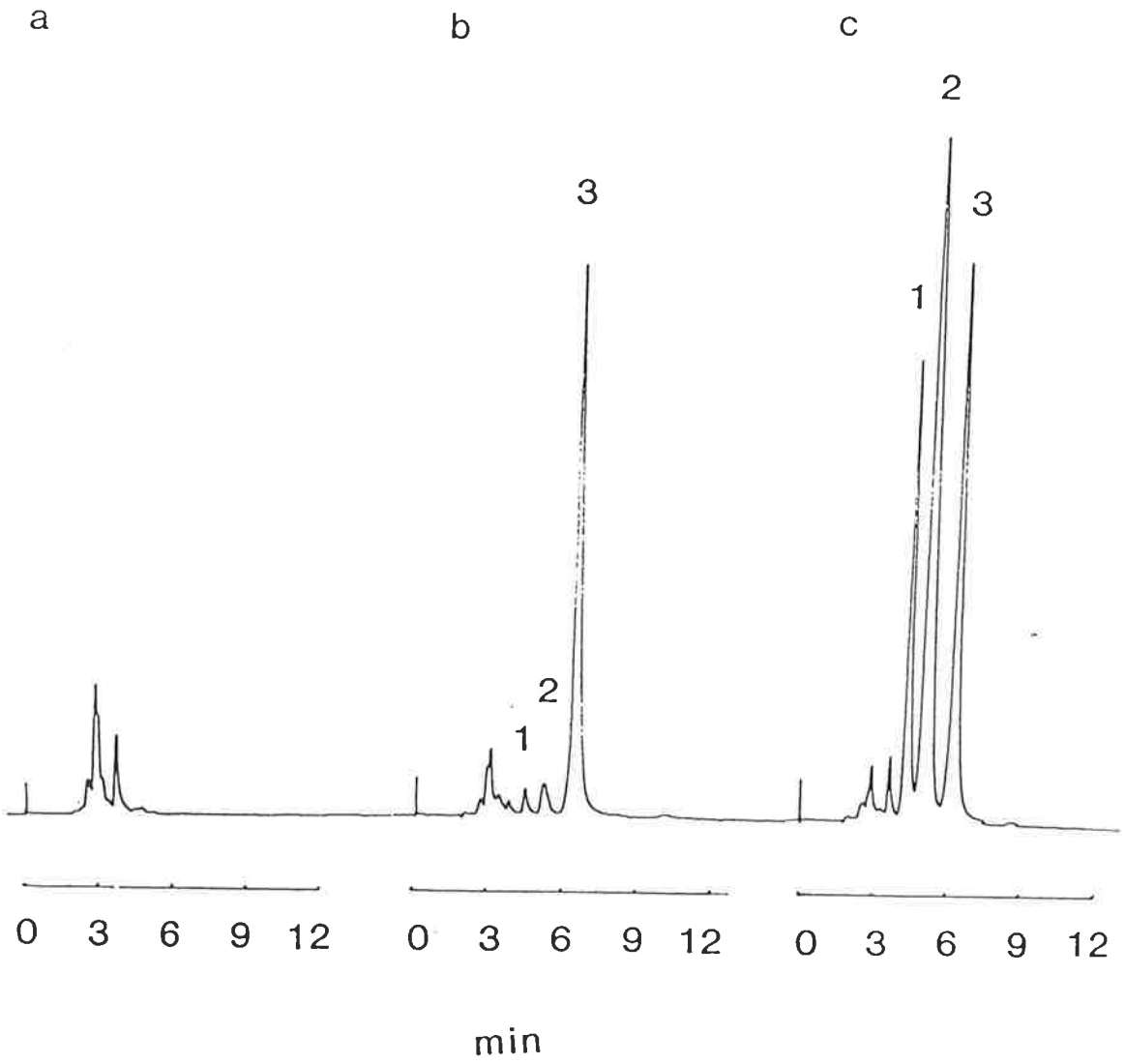


Figure 3-1-6 Chromatograms from an extract of (a) a drug-free urine sample, (b) an unhydrolyzed 0-8 hour urine sample from an extensive metaboliser after ingestion of 30 mg dextromethorphan hydrobromide (concentrations: 1 = 0.06 µg/ml; 2 = 0.23 µg/ml), (c) the same sample after β-glucuronidase treatment (1 = 1.1 µg/ml; 2 = 3.3 µg/ml).

Peaks: 1 = 3-hydroxymorphinan; 2 = dextrorphan; 3 = pholcodine (internal standard).

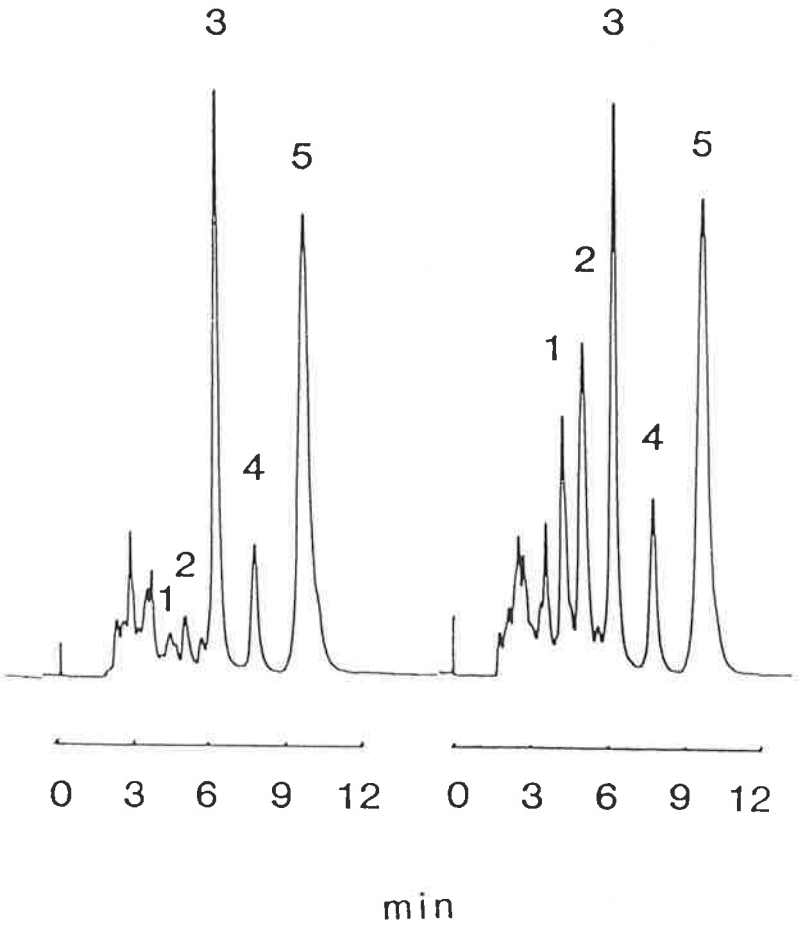


Figure 3-1-7 Chromatograms from an extract of (a) an unhydrolyzed urine sample from a poor metaboliser during 0-8 hours after ingestion of 30 mg dextromethorphan hydrobromide (concentrations: 1 = 0.2 μg/ml; 2 = 0.4 μg/ml; 4 = 0.35 μg/ml; 5 = 1.56 μg/ml) and (b) the same urine sample after β-glucuronidase treatment (concentrations: 1 = 0.9 μg/ml; 2 = 1.9 μg/ml; 4 = 0.39 μg/ml; 5 = 1.6 μg/ml).

Peaks: 1 = 3-hydroxymorphinan; 2 = dextrorphan; 3 = pholcodine (internal standard); 4 = 3-methoxymorphinan; 5 = dextromethorphan.

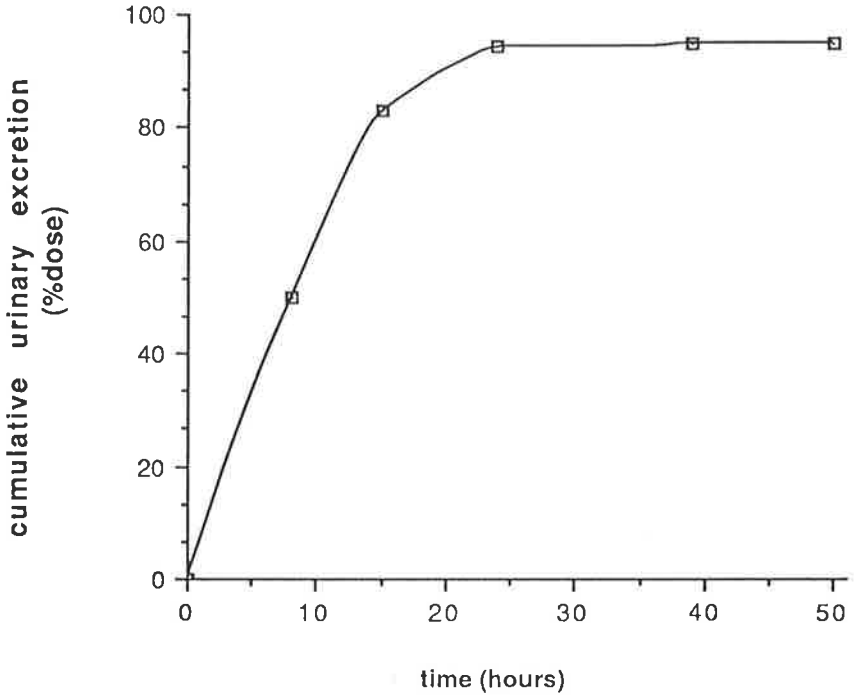


Figure 3-1-8 Cumulative urinary recovery as dextromethorphan and metabolites from a subject given 30 mg dextromethorphan hydrobromide orally.

Table 3-1-1 Assay variability for dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan in plasma

	concentration (ng/ml)	coefficient of variation (%)	number
intra-day			
I	10	4.9	10
	200	4.1	10
II	10	9.7	10
	200	1.9	10
III	10	7.0	10
	200	2.5	10
IV	10	5.7	10
	200	3.6	10
inter-day			
I	10	4.4	4
	200	5.1	4
II	10	6.8	4
	200	7.9	4
III	10	8.9	4
	200	2.1	4
IV	10	6.4	4
	200	1.8	4

I: dextromethorphan; II: dextrorphan;
III: 3-hydroxymorphinan; IV: 3-methoxymorphinan

Table 3-1-2 Assay variability for dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan in urine

	concentration (μg/ml)	coefficient of variation (%)	number
intra-day			
I	0.5	2.5	10
	10.0	1.8	10
II	0.5	3.4	10
	10.0	2.1	10
III	0.5	3.2	10
	10.0	2.4	10
IV	0.5	2.4	10
	10.0	2.1	10
inter-day			
I	0.5	7.9	4
	10.0	6.4	4
II	0.5	5.4	4
	10.0	4.4	4
III	0.5	1.6	4
	10.0	4.1	4
IV	0.5	1.9	4
	10.0	2.4	4

I: dextromethorphan; II: dextrorphan;

III: 3-hydroxymorphinan; IV: 3-methoxymorphinan

Table 3-1-3 Assay accuracy for dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan in plasma.

	plasma concentration (ng/ml)							
number	I		II		III		IV	
1	10.1	198.0	10.8	200.0	11.1	202.0	10.4	194.6
2	9.2	214.4	8.6	204.4	10.1	205.2	10.2	209.9
3	10.3	208.1	7.7	207.0	10.5	208.9	9.8	205.8
4	9.9	192.6	9.3	202.5	9.3	200.2	9.4	192.7
5	9.3	208.1	10.0	203.2	9.5	208.9	8.8	207.3
6	9.3	205.4	10.0	202.5	9.5	210.0	9.5	204.3
7	10.4	192.6	9.8	197.5	10.0	203.7	8.7	192.7
8	9.3	192.6	10.0	202.5	8.7	207.4	9.5	200.2
9	9.7	205.4	9.1	203.2	9.9	203.7	9.2	209.6
10	9.2	192.6	8.6	193.0	9.3	192.9	9.4	192.7
mean	9.7	201.0	9.4	201.6	9.8	204.3	9.5	201.0
SD	0.5	8.2	0.9	3.9	0.7	5.1	0.5	7.3
nominal	10.0	200.0	10.0	200.0	10.0	200.0	10.0	200.0

I: dextromethorphan; II: dextrorphan;
III: 3-hydroxymorphinan; IV: 3-methoxymorphinan

Table 3-1-4 Assay accuracy for dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan in urine

	urine concentration (μg/ml)							
number	I		II		III		IV	
1	0.51	10.0	0.52	10.0	0.52	10.0	0.53	10.6
2	0.49	10.2	0.50	9.8	0.49	10.0	0.50	10.9
3	0.48	10.1	0.49	9.9	0.48	9.8	0.49	10.6
4	0.48	9.8	0.49	10.0	0.48	9.9	0.49	10.5
5	0.51	10.1	0.49	10.0	0.48	10.2	0.51	10.8
6	0.48	9.7	0.46	9.6	0.47	9.6	0.50	10.2
7	0.49	9.7	0.47	9.7	0.48	9.6	0.50	10.3
8	0.48	9.8	0.48	9.7	0.48	9.8	0.50	10.4
9	0.48	9.9	0.49	10.3	0.48	10.1	0.51	10.7
10	0.48	10.0	0.48	10.0	0.51	10.3	0.49	10.7
mean	0.49	9.9	0.49	9.9	0.49	9.9	0.50	10.6
SD	0.01	0.18	0.02	0.21	0.02	0.24	0.01	0.22
nominal	0.50	10.0	0.50	10.0	0.50	10.0	0.50	10.0

I: dextromethorphan; II: dextrorphan;
III: 3-hydroxymorphinan; IV: 3-methoxymorphinan

Table 3-1-5 Extraction recovery for dextromethorphan, dextrorphan, 3-hydroxymorphinan, 3-methoxymorphinan and pholcodine from plasma at a concentration of 200 ng/ml.

	recovery (%)		
compounds	mean	SD	number
I	57	2.0	10
II	77	3.4	10
III	69	3.5	10
IV	60	1.9	10
pholcodine	63	3.6	10

I: dextromethorphan; II: dextrorphan;
III: 3-hydroxymorphinan; IV: 3-methoxymorphinan

Table 3-1-6 Drugs shown not to interfere with the assay of dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan

clofibrate	morphine
codeine	nifedipine
cyclophosphamide	norcodeine
diclofenac	norethisterone
digoxin	oestrogens
doxepin	oxazepam
doxorubicin	oxethazaine
flucloxacillin	paracetamol
folic acid	prednisolone
frusemide	pseudoephedrine
glyceryltrinitrate	quinine bisulfate
metformin	spironolactone
metoclopramide	temazepam
metoprolol	tolbutamide
miconazole	warfarin
minoxidil	

3.2. Polymorphic metabolism of dextromethorphan in humans

3.2.1. Introduction

In recent years there has been increasing interest in pharmacogenetic aspects of drug metabolism. Many studies were done following the discovery of polymorphic oxidative drug metabolism in man and several drugs have been found to exhibit distinct differences between extensive and poor metaboliser phenotypes. The most extensively studied drugs are debrisoquine and sparteine (Jacqz *et al* 1986). Metabolism of dextromethorphan in humans has been studied by several investigators (Willner 1963; Dixon *et al* 1978; Barnhart and Massad 1979; Barnhart 1980; Pfaff *et al* 1983; Woodworth *et al* 1984; Schmid *et al* 1985; East and Dye 1985; Küpfer *et al* 1985; Küpfer 1986; Larrey *et al* 1987; Silvasti *et al* 1987). Polymorphic metabolism in dextromethorphan O-demethylation was reported and a significant relationship between dextromethorphan O-demethyl-ation and debrisoquine 4-hydroxylation was established (Küpfer *et al* 1984, 1986). Recently, in human liver microsomes it has been demonstrated that dextromethorphan O-demethylation cosegregates with debrisoquine hydroxylation polymorphism (Dayer *et al* 1988). Dextromethorphan has been suggested to be one of the best substrates for studying the debrisoquine/sparteine-type genetic polymorphism of drug oxidation in man (Kronbach *et al* 1987).

In previous studies (Pfaff *et al* 1983; Schmid *et al* 1985; Küpfer 1986; Larrey *et al* 1987), the metabolic ratio was calculated as the ratio of O-demethylated metabolite dextrorphan to the parent drug. However, the O-demethylated metabolite dextrorphan is further N-demethylated to 3-

hydroxymorphinan (Figure 1-5-1). In addition, 3-methoxymorphinan, the N-demethylated metabolite of dextromethorphan, can also be O-demethylated to 3-hydroxymorphinan (Figure 1-5-1). Thus when the extent of O-demethylation is calculated, both of the O-demethylated metabolites (dextrorphan and 3-hydroxymorphinan) should be taken into consideration.

The aims of this study were to screen a population for different types of metabolisers of dextromethorphan using the assay described above (Chapter 3.1.) and to quantitate the urinary recovery of the parent compound and major metabolites. It was intended to identify poor, intermediate and extensive metabolisers of dextromethorphan so that those individuls can be used to study the genetic polymorphism of codeine (Chapter 4).

3.2.2. Methods

3.2.2.1. Subjects

Fifty two healthy volunteers, 36 male and 14 female (Table 3-2-1), who gave written informed consent participated in the study. The volunteers were aged 18-66 (27.3±5.2 mean ± SD) years and weighed 46-100 (67.9±11.9) kg. Prior to commencement of the study each subject underwent a medical history and physical examination. Subjects were excluded if had had any medical history of liver or renal disease and if they had ingested any drugs within one week of the study.

This study was approved by the Human Ethics Committee of Royal Adelaide Hospital and the Committee on the Ethics of Human Experimentation of the University of Adelaide.

3.3.2.2. Study design

Subjects received a single dose of 30 mg dextromethorphan hydrobromide orally as a capsule (prepared by the staff of the Pharmacy Department of the Royal Adelaide Hospital) before retiring at night and urine was collected overnight (8-10 hours). Urine volume was determined gravimetrically and recorded along with the urine pH. An aliquot of well mixed urine was retained in stoppered vials at -20 °C until analysed for drug content.

3.2.2.3. Drugs analysis in urine

Dextromethorphan and its three metabolites in urine were determined by the HPLC assay described in Chapter 3.1. and the conjugates of the compounds were determined following the procedure described in Chapter 3.1.2.4..

3.2.2.4. Calculation of the metabolic ratio

The metabolic ratio (MR) for O-demethylation was calculated as

$$MR = \frac{\text{Ae of total (dextrorphan+3-hydroxymorphinan)}}{\text{Ae of total (dextromethorphan+3-methoxymorphinan)}}$$

where Ae is the amount excreted in 8-10 hours urine, total refers to unconjugated and conjugated species, and the MR for N-demethylation was calculated as

$$MR = \frac{\text{Ae of total (3-hydroxymorphinan+3-methoxymorphinan)}}{\text{Ae of total (dextromethorphan+dextrorphan)}}$$

(re-calculation see appendix VII)

3.2.2.5. Statistical analysis

The antimodes between poor, intermediate and extensive metabolisers were determined by density function analysis with 99% confidence (Goldstein 1967). The following correlations were determined by linear regression analysis: the MR for O-demethylation and either the urine pH or urine volume; the MR for O-demethylation and N-demethylation.

3.2.3. Results

Large interindividual differences were observed in the extent of O-demethylation of dextromethorphan (Table 3-2-2). The frequency distribution histogram of the log O-demethylation ratio of dextromethorphan in 52 subjects is shown in Figure 3-2-1 and a probit plot is shown in Figure 3-2-2. Three distinct phenotypes were apparent. The antimode between extensive, intermediate and poor metabolisers, determined by density function analysis with 99% confidence limits, were 1.0 and 1.5. Three subjects had log O-demethylation ratios between - 0.52 and 0.31 and were classified as poor metabolisers. Four subjects had log metabolic ratios between 1.20 and 1.40 and were classified as intermediate. Forty five subjects with log metabolic ratios between 1.73 and 3.54 were classified as extensive metabolisers. The overnight (about 8-10 hours) urinary excretion of dextromethorphan and its metabolites for poor, intermediate and extensive metabolisers is shown in Table 3-2-3, 3-2-4 and 3-2-5 respectively. The urinary excretion of unchanged dextromethorphan in poor, intermediate and extensive metabolisers was 3.4 %, 1.6 % and 0.15 % of the dose respectively; the urinary excretions (Figure 3-2-3) of total (conjugated and unconjugated) dextrorphan were 2.0 %, 21.6 % and 30.2 % of the dose respectively and that of total 3-hydroxymorphinan were 0.8, 10.4 and 11.8 % of the dose respectively. 3-

methoxymorphinan, which was not detectable in most of the extensive metabolisers, only accounted for 0.4 % of the dose at most. The total overnight urinary recovery, as dextromethorphan and its three metabolites, in poor,intermediate and extensive metabolisers was 6.4, 33.7 and 42.1 % of the dose respectively. Dextrorphan and 3-hydroxymorphinan were mainly (> 95 %) in conjugated form. The percentage conjugated was not significantly different between the three types of metabolisers. Potential conjugates of dextromethorphan and 3-methoxymorphinan were not detected after β-glucuronidase hydrolysis. There was no significant correlation between the metabolic ratio and either urine pH (r = 0.002) (Figure 3-2-4) or the urine volume (r = 0.003) (Figure 3-2-5). The N-demethylation ratios in poor, intermediate and extensive metabolisers were not significantly different (P > 0.05). There was no correlation between O-demethylation and N-demethylation ratios (r = 0.08) (Figure 3-2-6). There were no adverse effects demonstratable in any of the subjects.

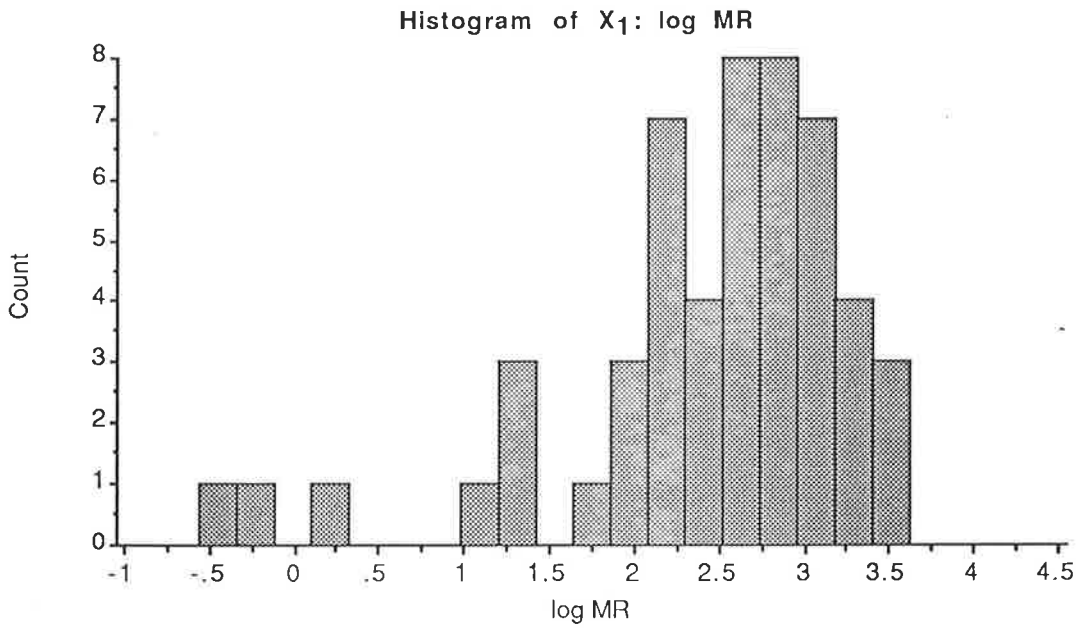


Figure 3-2-1 Frequency distribution histogram of the log metabolic ratios (MR) of O-demethylation of dextromethorphan in 52 subjects.

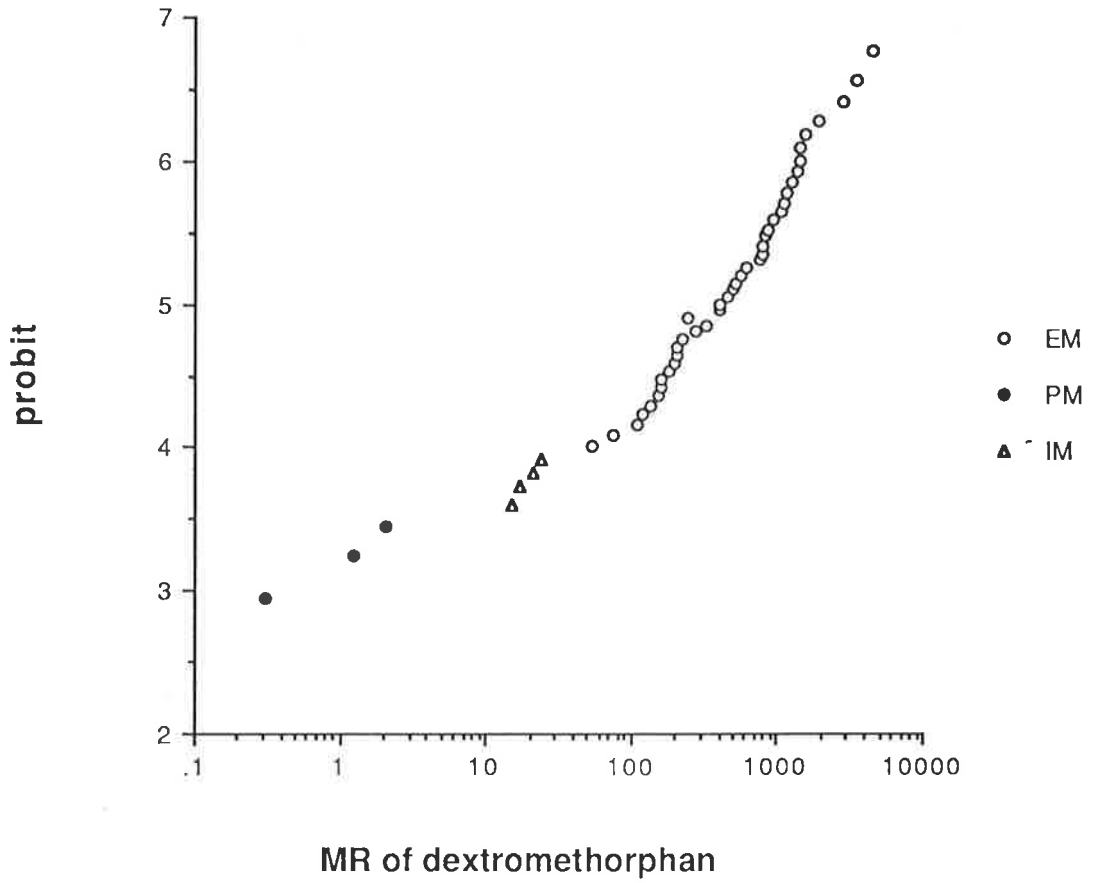


Figure 3-2-2 Probit plot of the frequency distribution of the metabolic ratios (MR) of O-demethylation of dextromethorphan. (PM = poor metabolisers; IM = intermediate metabolisers; EM = extensive metabolisers)

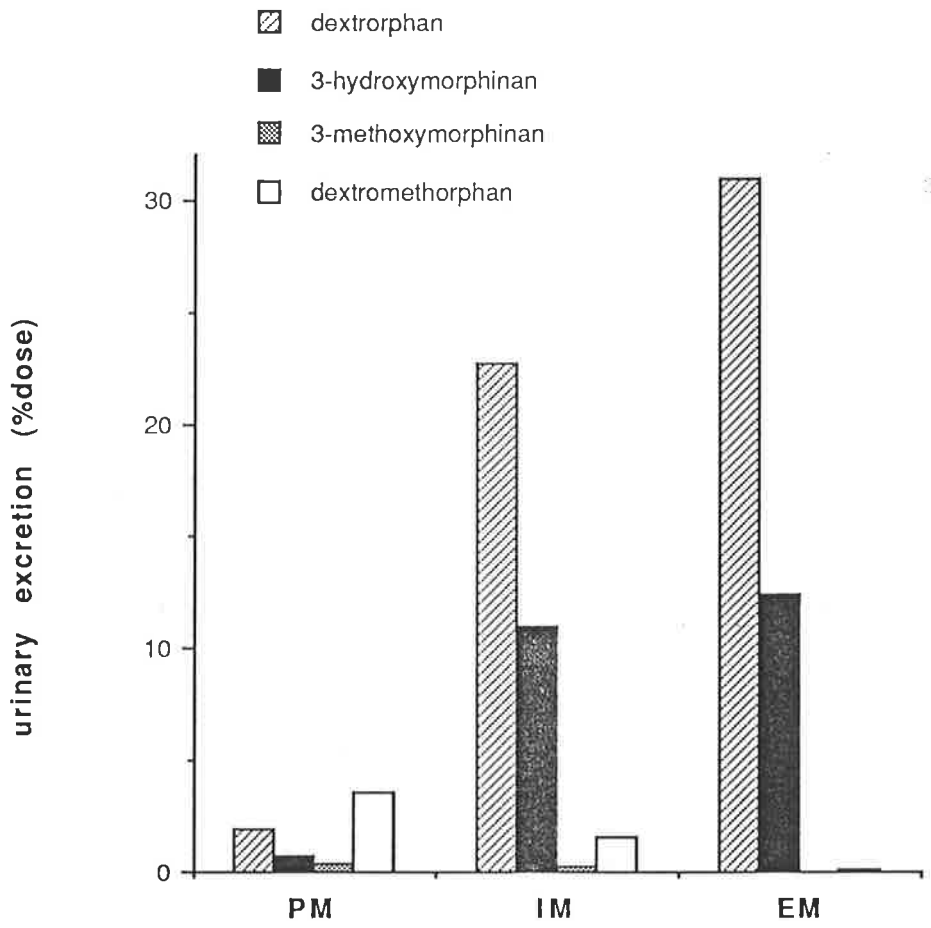


Figure 3-2-3 Mean urinary recovery of dextromethorphan and its metabolites in the three types of metabolisers. (PM = poor metabolisers; IM = intermediate metabolisers; EM = extensive metabolisers)

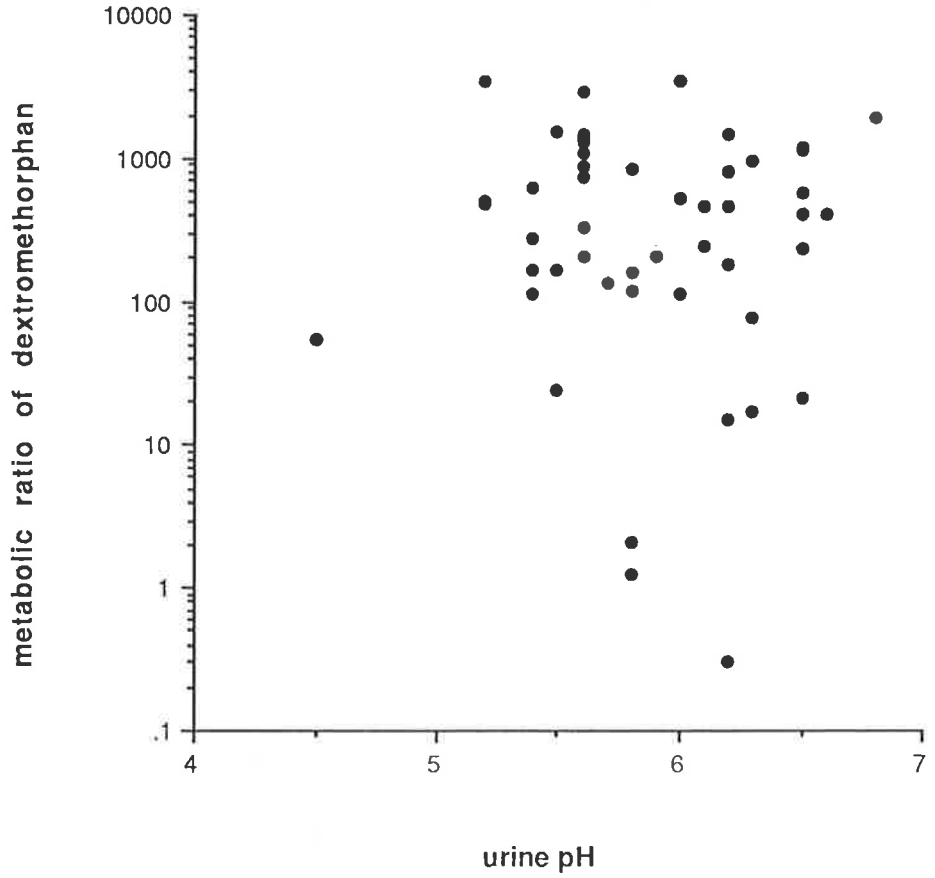


Figure 3-2-4 Correlation between the metabolic ratio and the urine pH.

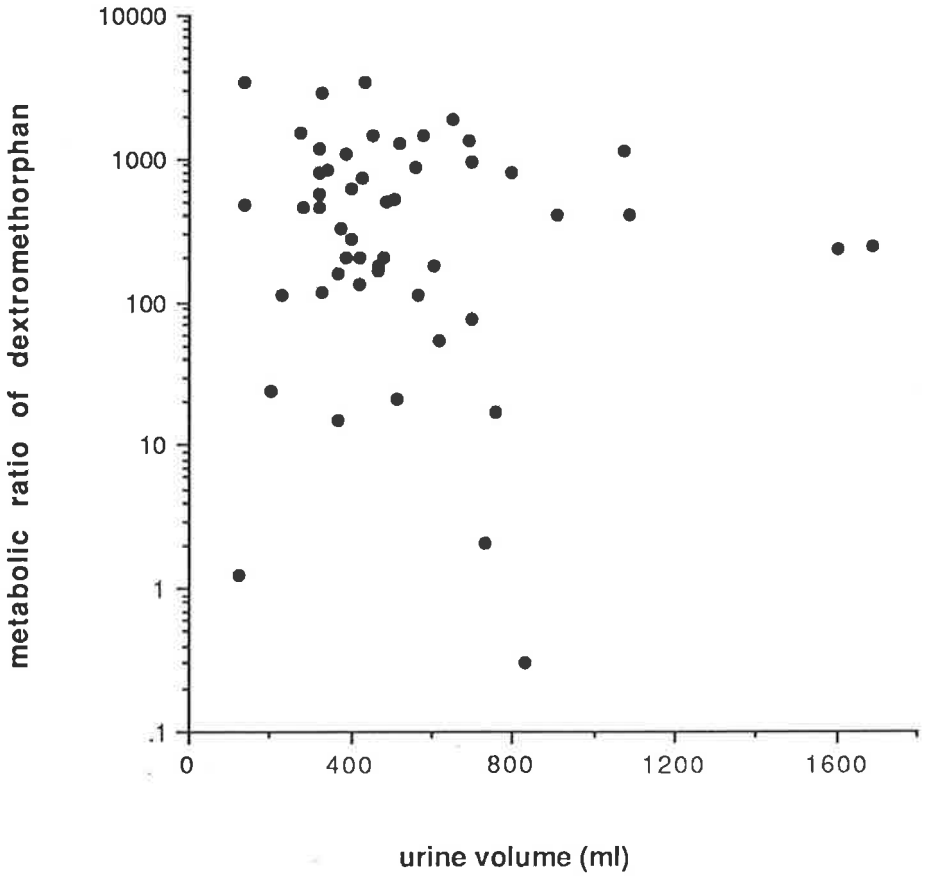


Figure 3-2-5 Correlation between the metabolic ratio and the urine volume.

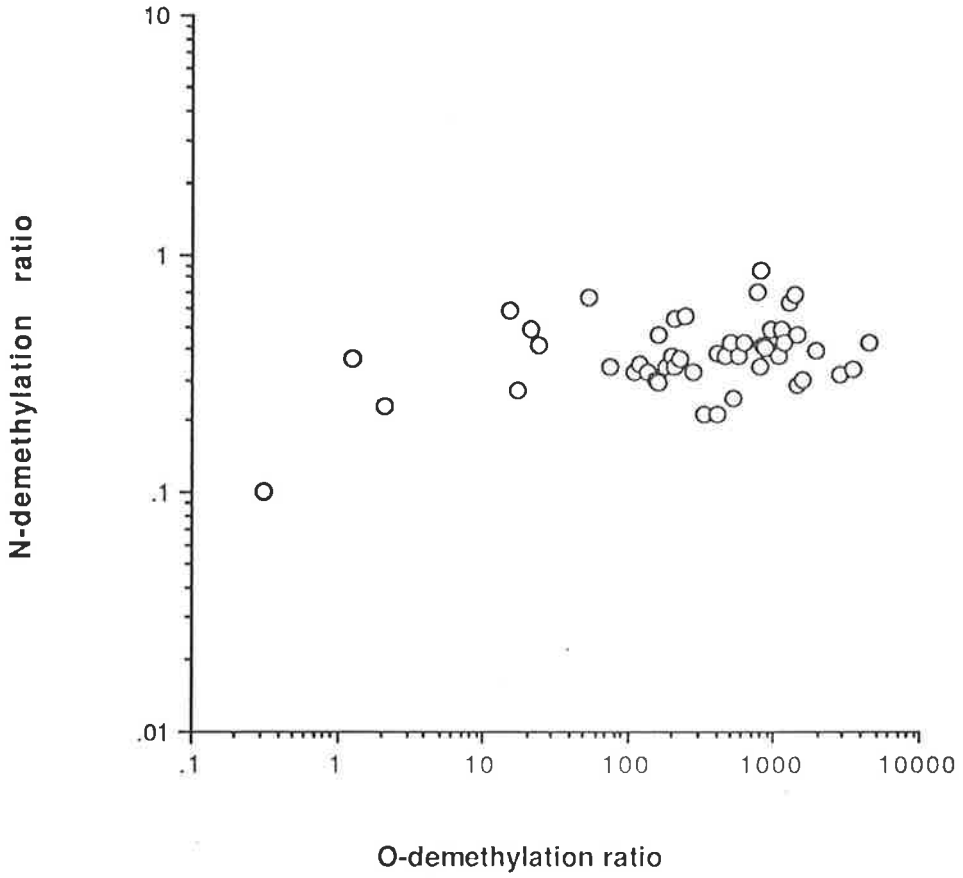


Figure 3-2-6 Correlation between the O-demethylation and N-demethylation metabolic ratios.

Table 3-2-1 Volunteer data

subject	sex	age (year)	body weight (kg)	body height (cm)
1	F	26	51	167
2	F	21	46	154
3	M	44	83	180
4	M	20	67	178
5	M	56	86	175
6	F	19	54	165
7	F	26	57	170
8	M	21	68	181
9	M	18	77	183
10	M	19	75	171
11	F	19	66	169
12	M	20	68	175
13	F	28	48	155
14	M	20	60	170
15	M	24	70	170
16	M	41	83	187
17	M	21	81	189
18	F	21	53	169
19	F	22	59	157
20	M	19	63	167
21	M	23	75	180
22	M	30	55	173
23	M	20	94	185
24	M	21	76	182
25	M	19	73	180
26	F	20	55	167
27	F	21	65	163
28	F	23	69	175
29	M	25	73	183

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Table 3-2-1 Volunteer data (continued)

subject	sex	age (year)	body weight (kg)	body height (cm)
30	M	66	70	172
31	M	25	78	159
32	M	25	76	170
33	F	33	92	175
34	M	37	87	173
35	M	24	76	182
36	F	53	62	166
37	F	18	59	175
38	M	22	60	163
39	M	38	71	182
40	F	22	66	164
41	F	19	57	170
42	M	36	68	170
43	F	25	60	163
44	M	37	72	184
45	M	22	80	188
46	M	22	74	176
47	F	43	75	160
48	M	49	65	175
49	M	21	100	176
50	F	20	80	175
51	M	30	70	165
52	M	30	65	176

Table 3-2-2 Metabolic ratios of dextromethorphan in 52 healthy volunteers

subject	urine pH	urine volume (ml)	O-MR	log O-MR	N-MR
1	6.2	830	0.3	-0.52	0.15
2	5.8	127	0.5	-0.31	0.24
3	5.8	733	2.2	0.35	0.24
4	6.2	367	15.7	1.20	0.58
5	6.3	761	17.6	1.25	0.27
6	6.5	515	21.8	1.34	0.48
7	5.5	203	24.9	1.40	0.42
8	4.5	620	56.7	1.75	0.65
9	6.3	700	79.6	1.90	0.34
10	5.4	232	115.3	2.06	0.32
11	6.2	570	117.4	2.07	0.48
12	5.8	330	124.4	2.10	0.35
13	5.7	425	142.1	2.15	0.32
14	5.8	367	163.3	2.21	0.30
15	5.5	470	169.5	2.23	0.29
16	5.4	470	172.4	2.24	0.46
17	6.2	605	190.9	2.28	0.34
18	6.2	470	191.7	2.28	0.32
19	5.6	420	212.0	2.33	0.37
20	5.9	480	215.8	2.34	0.34
21	5.6	390	239.7	2.38	0.36
22	6.5	1600	291.6	2.47	0.32
23	6.1	1690	346.3	2.54	0.21
24	5.4	403	364.0	2.56	0.55
25	5.6	375	427.2	2.63	0.38
26	6.5	910	432.6	2.64	0.21
27	6.6	1085	456.3	2.66	0.80
28	6.1	285	481.2	2.68	0.37
29	6.2	322	507.5	2.71	0.46

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Table 3-2-2 Metabolic ratios of dextromethorphan in 52 healthy volunteers (continued)

subject	urine pH	urine volume (ml)	O-MR	log O-MR	N-MR
30	5.2	140	520.4	2.72	0.42
31	5.2	490	552.7	2.74	0.25
32	6.0	510	589.5	2.77	0.37
33	6.5	320	656.1	2.82	0.43
34	5.4	400	658.8	2.82	0.53
35	5.6	430	789.4	2.90	0.70
36	6.2	800	831.6	2.92	0.84
37	6.2	325	851.6	2.93	0.34
38	5.8	340	861.1	2.94	0.41
39	5.6	560	919.3	2.96	0.40
40	6.3	700	1000.0	3.00	0.48
41	5.6	390	1122.8	3.05	0.37
42	6.5	1075	1198.6	3.08	0.48
43	6.5	320	1220.8	3.09	0.42
44	5.6	520	1357.9	3.13	0.63
45	5.6	690	1421.1	3.15	0.67
46	5.6	580	1530.5	3.19	0.46
47	6.2	455	1542.0	3.19	0.28
48	5.5	280	1615.8	3.21	0.30
49	6.8	650	2016.8	3.31	0.39
50	5.6	330	3000.0	3.48	0.31
51	6.0	435	3642.3	3.56	0.33
52	5.2	140	3659.6	3.56	0.42

O-MR: O-demethylation ratio;
N-MR: N-demethylation ratio.

Table 3-2-3 Urinary excretion of dextromethorphan and its metabolites in poor metabolisers

	dose in urine (%)				
subject	I	II	III	IV	total
1	7.2	1.7	0.67	0.70	10.4
2	1.5	0.6	0.25	0.24	2.5
3	2.0	3.5	1.20	0.11	6.8
mean	3.6	1.9	0.70	0.30	6.6
SD	3.2	1.5	0.50	0.30	3.9

I: dextromethorphan; II: dextrorphan;
III: 3-hydroxymorphinan; IV: 3-methoxymorphinan

Table 3-2-4 Urinary excretion of dextromethorphan and its metabolites in intermediate metabolisers

	dose in urine (%)				
subject	I	II	III	IV	total
4	2.6	27.2	17.2	0.23	47.3
5	1.2	19.2	5.3	0.22	25.9
6	1.4	26.1	13.0	0.37	40.9
7	1.0	18.3	8.0	0.04	27.3
mean	1.6	22.7	10.9	0.20	35.3
SD	0.7	4.6	5.3	0.12	10.4

I: dextromethorphan; II: dextrorphan;
III: 3-hydroxymorphinan; IV: 3-methoxymorphinan

Table 3-2-5 Urinary excretion of dextromethorphan and its metabolites in extensive metabolisers

	dose in urine (%)				
subject	I	II	III	IV	total
8	0.7	24.0	16.0	0.03	40.8
9	1.0	56.9	19.8	-	77.7
10	0.45	39.6	12.7	-	52.7
11	0.41	32.7	15.8	-	49.0
12	0.36	32.8	11.8	-	45.0
13	0.39	41.5	13.4	0.04	55.3
14	0.33	46.4	13.9	-	60.6
15	0.21	28.1	8.1	-	36.4
16	0.30	35.3	16.4	-	52.0
17	0.18	25.4	8.7	-	34.3
18	0.13	18.4	6.0	-	24.5
19	0.13	20.7	7.6	-	28.5
20	0.15	24.6	8.4	0.02	33.1
21	0.30	56.2	20.5	-	77.0
22	0.07	16.1	5.1	-	21.3
23	0.08	21.9	4.7	-	26.7
24	0.13	30.1	16.6	-	46.8
25	0.11	34.3	12.9	-	47.3
26	0.04	14.8	3.1	-	17.9
27	0.10	24.9	22.4	0.01	47.4
28	0.07	25.2	9.3	-	34.6
29	0.04	13.1	6.1	0.01	19.2
30	0.10	41.6	17.4	-	59.1
31	0.11	49.2	12.3	-	61.7
32	0.23	10.0	3.7	-	13.7
33	0.04	20.1	8.6	-	28.7
34	0.06	25.5	13.5	-	39.0
35	0.02	9.1	6.4	-	15.5
36	0.04	16.5	13.8	-	30.3

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Table 3-2-5 Urinary excretion of dextromethorphan and its metabolites in extensive metabolisers (continued)

	percentage of the dose				
subject	I	II	III	IV	total
37	0.06	37.5	12.8	-	50.4
38	0.05	28.3	11.6	-	40.0
39	0.08	50.1	20.1	-	70.3
40	0.03	21.4	10.4	-	31.8
41	0.05	43.7	16.0	-	59.8
42	0.05	39.7	19.1	0.02	58.9
43	0.04	50.7	21.3	-	72.0
43	0.02	19.7	12.4	-	32.1
45	0.03	26.7	17.8	-	44.6
46	0.03	27.6	12.8	-	40.4
47	0.02	25.1	7.1	-	32.3
48	0.03	31.8	9.4	-	41.2
49	0.03	42.9	16.7	-	59.6
50	0.02	34.3	10.7	-	45.0
51	0.02	54.9	17.9	-	72.9
52	0.01	21.1	8.8	-	30.0
mean	0.15	30.9	12.4	-	43.5
SD	0.19	12.6	5.1	-	16.8

I: dextromethorphan; II: dextrorphan;
III: 3-hydroxymorphinan; IV: 3-methoxymorphinan

3.2.4. Discussion

Studies on the polymorphism of dextromethorphan metabolism have been previously reported. In agreement with the findings of Pfaff and coworkers (1983), the phenotypes in the present subjects were divided into extensive, intermediate and poor metabolisers, based on the density function analysis. These phenotypes were also clearly distinguished on the distribution histogram and the probit plot of metabolic ratios. Although in some other previous studies (Schmid *et al* 1985; Küpfer 1986; Larrey *et al* 1987), the phenotypes were divided only into extensive and poor metabolisers, a similar distribution in metabolic ratios was observed. If the population is classified only as poor metabolisers and extensive metabolisers, those in the intermediate group would have to be assigned to either poor or extensive metabolisers groups. This would result in variations in the metabolic ratios for both groups. In addition, the classification of the population into 3 groups may provide a better basis for the study of the pharmacokinetics and pharmacodynamics of dextromethorphan in these different groups. In previous studies (Pfaff *et al* 1983; Schmid *et al* 1985; Küpfer 1986; Larrey *et al* 1987), the metabolic ratio was calculated from dextrorphan and the parent compound. However, 3-hydroxymorphinan, which is O- and N-demethylated, should also be considered when calculating the metabolic ratio. In this study, the urinary recovery of 3-hydroxymorphinan was about one third of that of dextrorphan. I have compared the two calculations of the metabolic ratio and found that although both calculations gave similar results in phenotyping, the calculation employed in this study resulted in a wider range of the metabolic ratio and a better separation between the three groups.

The conjugation of dextromethorphan and its metabolites has not been quantitatively studied. In this study, the O-demethylated metabolites were

extensively conjugated, whereas the parent compound and the N-demethylated metabolite did not appear to undergo conjugation. These results suggest that the conjugations occurs on the 3-O position. Although the total amount of dextrorphan and 3-hydroxymorphinan were very variable in the three types of metabolisers, the percentages of conjugation of the metabolites were not significantly different between extensive, intermediate and poor metabolisers. These results suggest that the conjugation reaction is not under genetic control.

Theoretically, urine pH might influence the urinary elimination of dextromethorphan and 3-methoxymorphinan, while the renal excretion of conjugates is less susceptible to changes in urine pH. Consequently, the O-demethylation ratio of dextromethorphan might be subject to changes in urine pH. However, as shown in Figure 3-2-4, there was no correlation between the urine pH and the O-demethylation ratio. A similar result was reported by Küpfer and coworkers (1986).

The frequency of the deficiency in the O-demethylation of dextromethorphan in this study was 3/52 which was similar to that (6%) in the oxidation of debrisoquine in an Australian population reported by Peart and coworkers (1986). Dextromethorphan has been demonstrated to cosegregate with the debrisoquine/sparteine oxidation pathway. It has been shown that a number of clinically useful drugs are affected by this genetic polymorphism and some of the drugs cause severe toxicities (Jacqz *et al* 1986). A single dose of dextromethorphan has few, if any, side effects and the differentiation of the 3 phenotypes is obvious. It may thus be a useful tool to predict abnormal responsiveness to other drugs which have severe side effects. It may also be useful in testing other drugs whose metabolism is potentially under genetic control.

3.3. A pilot study on the pharmacokinetics of dextromethorphan in humans

3.3.1. Introduction

Many studies have been done on the polymorphic metabolism of dextromethorphan (see Chapter 3.2.). However few data are available about its pharmacokinetics in humans. Large inter-individual variations have been noticed in a previous study (Pfaff *et al* 1983). After taking 25 mg dextromethorphan hydrobromide, the maximum plasma concentration of dextromethorphan was generally lower than 15 ng/ml, and the maximum plasma concentration of dextrorphan, the major metabolite of dextromethorphan, ranged from 0 to 690 ng/ml (limit of the assay sensitivity = 20 ng/ml). Unconjugated dextrorphan was not determined mainly because of lack of a sensitive and specific assay. In addition, another important metabolite, 3-hydroxymorphinan, was not determined.

The aims of this study were:

(i) to apply the high performance liquid chromatographic assay described in Chapter 3.1.;

(ii) to obtain some information on the pharmacokinetics of dextromethorphan and its metabolites to aid in the design of formal studies on the pharmacokinetics and pharmacodynamics of dextromethorphan (Chapter 5).

3.3.2. Methods

3.3.2.1. Subjects

Three volunteers, 2 male and 1 female, who gave written informed consent participated in the study. The volunteers were aged from 23 to 66 years and weighed from 62 to 76 kg. Prior to commencement of the study each subject was given a physical examination and a venous blood sample was collected for biochemical and hematological examinations. The results of the tests were within the normal ranges (Appendix II). These subjects were classified as extensive metabolisers.

This study was approved by the Human Ethics Committee of Royal Adelaide Hospital and the Committee on the Ethics of Human Experimentation of the University of Adelaide.

3.3.2.2. Study design

The subjects received 20 mg of dextromethorphan hydrobromide (as a capsule) 8 hourly (0800, 1600, 2400 hour) for seven doses. The final dose was given at 0800 hour on day 3. Ten ml venous blood samples were collected into heparinized plastic tubes via an indwelling catheter (Jelco^{TM}, 18 G i.v. catheter placement unit, Critikon, Tampa, USA), kept patent with a stylet (Jelco^{TM}), placed in a forearm vein. The blood sample collecting time were 0, 0.5, 1, 1.5, 2, 3, 4 and 8 hours after the last dose. All urine was collected over the interdosing interval (0-8 hours).

Blood samples were centrifugated immediately and plasma was separated. Urine volume was determined gravimetrically and recorded along with the urine pH. An aliquot of well mixed urine were retained for analysis.

All samples were stored in stoppered vials at -20 °C until analysed for drug content.

3.3.2.3. Drugs analysis in biological fluids

Plasma concentrations of dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan were determined by the high performance liquid chromatographic assay described in Chapter 3.1..

The presence of conjugates of dextromethorphan and its metabolites in plasma and urine was inferred by the generation of dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan following enzymatic hydrolysis as described in Chapter 3.1.2.4..

3.3.3. Results

The plasma concentration-time curves of dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan for the three subjects are shown in Figure 3-3-1, 3-3-2 and 3-3-3. The unconjugated dextrorphan in plasma was detected in all the three subjects but it was lower than 10 ng/ml. The unconjugated 3-hydroxymorphinan in plasma was not detected in the three subjects. Plasma dextromethorphan and 3-methoxymorphinan concentrations were detectable in subject 1 but not in the other two subjects. Dextromethorphan and its three metabolites in urine were detected both before and after enzyme hydrolysis and the urinary excretions are shown in Table 3-3-1. Dextrorphan and 3-hydroxymorphinan were extensively conjugated (> 95%) but dextromethorphan and 3-methoxymorphinan did not appear to undergo conjugation.

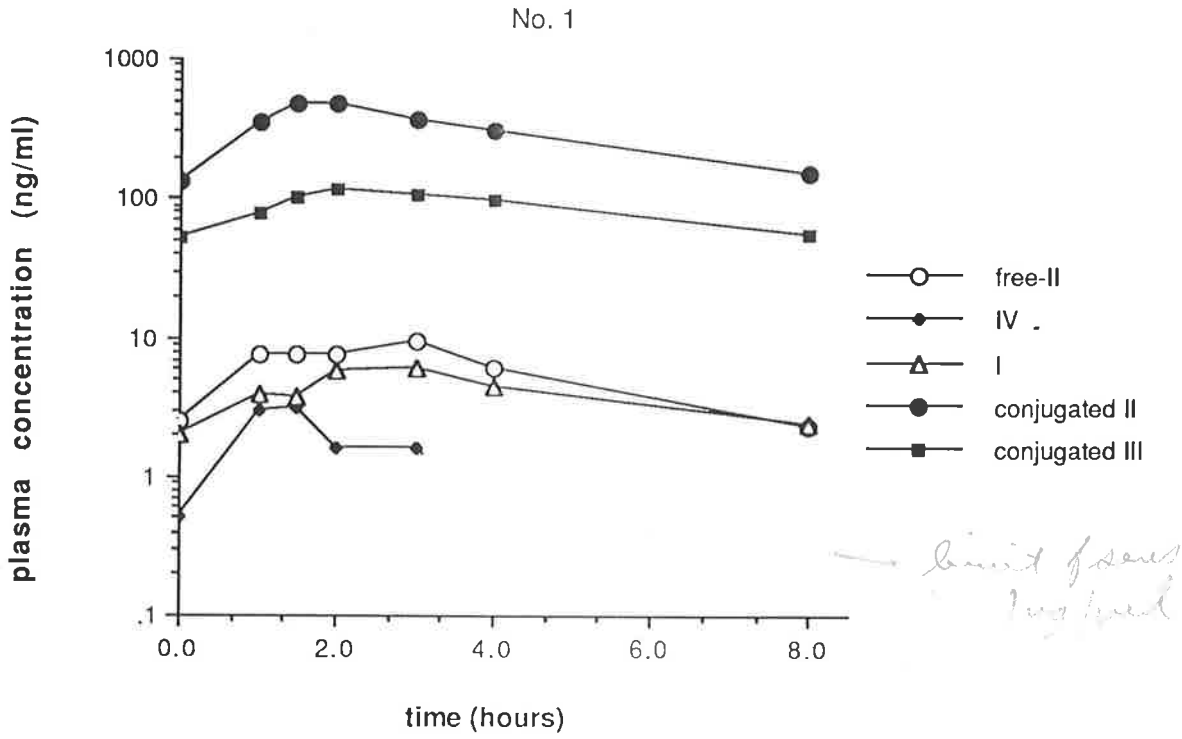


Figure 3-3-1. Plasma concentrations of dextromethorphan (I), dextrorphan (free-II), conjugated dextrorphan (conjugated II), conjugated 3-hydroxy-morphinan (conjugated III) and 3-methoxymorphinan (IV) in subject 1.

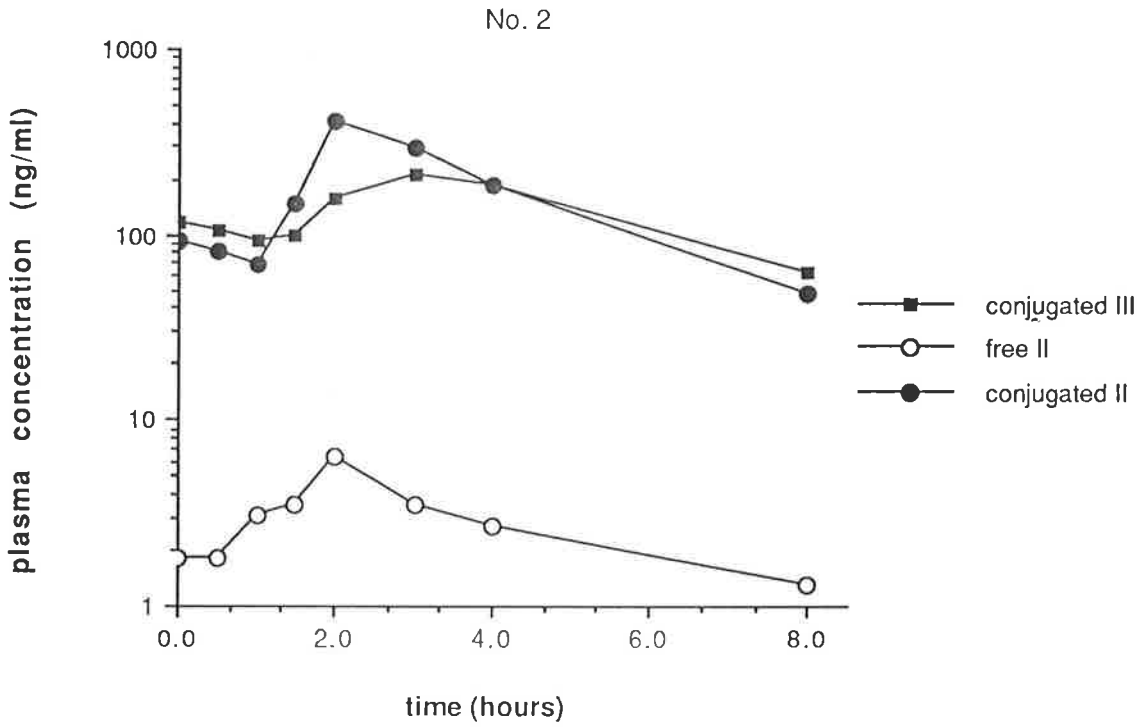


Figure 3-3-2. Plasma concentrations of dextrorphan (free-II), conjugated dextrorphan (conjugated II) and conjugated 3-hydroxymorphinan (conjugated III) in subject 2.

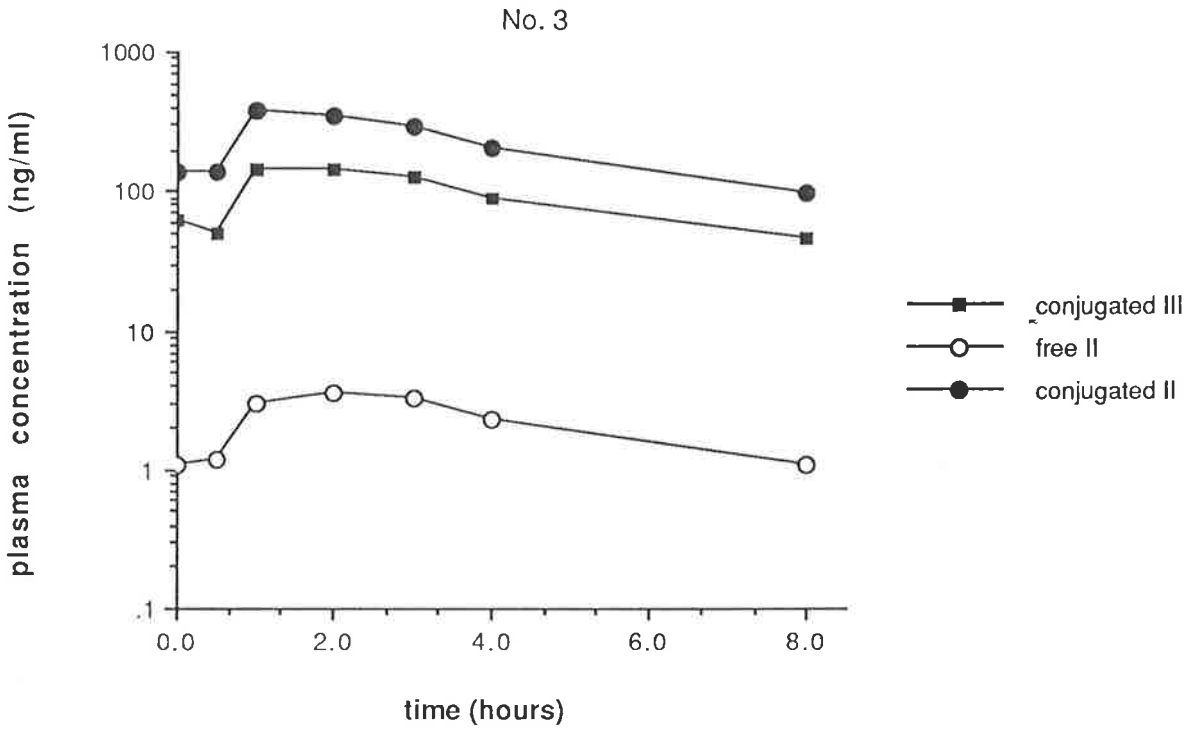


Figure 3-3-3. Plasma concentrations of dextrorphan (free-II), conjugated dextrorphan (conjugated II) and conjugated 3-hydroxymorphinan (conjugated III) in subject 3.

Table 3-3-1. Urinary recovery of dose (%) as dextromethorphan and its metabolites

compound	subject 1	2	3
dextromethorphan	0.1	0.05	0.05
dextrorphan	1.2	1.0	1.3
conjugated dextrorphan	26.9	24.8	31.8
3-hydroxymorphinan	0.4	0.3	0.3
conjugated 3-hydroxymorphinan	9.8	20.8	13.3
3-methoxymorphinan	0.01	-	0.01
total	38.4	47.0	46.8

3.3.4. Discussion

The results from this study were comparable to previous reports (Pfaff *et al* 1983; East and dye 1985). However in the previous studies, plasma concentrations of unconjugated dextrorphan were not determined mainly because of the limitation of the assay employed in these studies. To study the pharmacological effect of the drug and its metabolites, it is probably important to determine unconjugated compounds in plasma assuming that only the unconjugated drug has pharmacological activity. The unconjugated dextrorphan was detected in this study and its concentration was relatively higher than that of the parent drug. Although the plasma concentrations of conjugated dextrorphan and 3-hydroxymorphinan were extremely high, the plasma concentrations of the unconjugated compounds were very low. There are no data on their individual pharmacological activities. However, the antitussive effect of dextromethorphan has been well proved (Eddy *et al* 1969; Jaffe and Martin 1985). Thus, for any of them to be active, they must have a very high pharmacological potency. On the other hand, anything which may influence the plasma concentration of the unconjugated compounds may alter the pharmacological response dramatically. There are no data available on the pharmacological activities of dextromethorphan in different type of metabolisers. It is anticipated, however, that the pharmacological and toxicological responses of dextromethorphan in different metabolisers may be different considering the different plasma concentrations of the drug and metabolites.

The urinary recovery within 48 hours after a single dose was reported to be 20-86% of the dose (Pfaff *et al* 1983; East and dye 1985; Chapter 3.2.). There was about 45% of the dose recovered during a dosing interval from

urine in the three subjects. The lower recovery may have been due to incomplete collection of the urine sample.

Chapter 4.

STUDIES ON THE PHARMACOKINETICS AND PHARMACODYNAMICS OF CODEINE IN HUMANS AND IN RATS

4.1. Simultaneous determination of codeine, norcodeine and morphine in plasma and urine by HPLC

4.1.1. Introduction

To comprehensively study the pharmacokinetics and metabolism of codeine, it is necessary to determine its O- and N-demethylated metabolites, morphine and norcodeine respectively, and codeine-6-glucuronide in plasma and urine. Although many assays for individual compounds have been reported (Chapter 1.3.3.), only two procedures (HPLC) have been described for the simultaneous determination of codeine and its demethylated metabolites in plasma (Shah and Mason 1987) or in urine (Posey and Kimble 1984). The assay for urine samples was used as a indicator of codeine ingestion. There was no information about the accuracy and variability of the assay. Because of the relatively high concentrations of the substances in urine the assay for urine may not be suitable for plasma. The HPLC assay for plasma samples (Shah and Mason 1987), required a high voltage (1.2 V) for the electrochemical detection and had a large variability. Especially for codeine, the variability was 23% at a plasma concentration of 10 ng/ml. In addition the authors warn that "... the complexicity of the procedure does require attention to technical details such as sensitivity of the working electrode" (Shah and Mason 1987). This assay is unlikely to be suitable for analysis of large number of samples in pharmacokinetic studies. The aim of this study was to develop a specific and sensitive HPLC assay for the simultaneous determination of codeine, morphine and norcodeine in plasma and urine in humans. Because of the different physico-chemical character-

istics of glucuronide and base, codeine-6-glucuronide was not be able to be determined simultaneously in the above assay and will be determined by a separate assay (Chapter 4.2.).

4.1.2. Materials and methods

4.1.2.1. Reagents

All reagents and solvents were of analytical grade or of HPLC grade (see Appendix I). Codeine phosphate, morphine sulphate (F.H.Faulding and Co Limited, Adelaide, Australia), norcodeine (Eli Lilly and Co. Ind., U.S.A.) and dihydrocodeine bitartrate (Knoll AG Chemical Works, Ludwigshafen, FRG) were all of British Pharmacopoeial grade quality.

4.1.2.2. Chromatography

The HPLC consisted of a pump (M-6000A, Waters Associates Inc., Milford, Mass. U.S.A.), an LS-5 Luminescence Spectrometer (Perkin-Elmer Limited, Beaconsfield, U.K.), a WISP 710 B automatic injector (Waters Associates) and an Omniscribe B-5000 strip-chart recorder (Houston Instruments, Austin, Tx, U.S.A.). The 15 cm x 4.6 mm I.D. stainless steel column was packed with Spherisorb 5 µm cyano packing material (Phase Separations, Queensferry, U.K.). The composition of the mobile phase was 4% acetonitrile, 0.1% triethylamine and 95.9% distilled water, adjusted to pH 3.1 with orthophosphoric acid. The flow rate through the column at ambient temperature was 1 ml/minute which produced a back-pressure of 1.8 Kpsi (12.4 MPa). The excitation and emission wavelengths of the detector were 230 and 350 nm respectively and the excitation and emission slits were set at 15 and 20 nm respectively.

4.1.2.3. Stock solutions

Codeine, norcodeine and morphine were made up as 1 mg base per ml stock solutions in distilled water and were diluted to concentrations ranging from 5 to 200 ng/ml in drug free plasma and from 0.1 to 10 µg/ml in drug free urine. Dihydrocodeine, the internal standard, was diluted in distilled water to a concentration of 6.6 µg/ml for plasma analysis and 134 µg/ml for urine analysis.

4.1.2.4. Sample preparation

Plasma: 1 ml of plasma was pipetted into a 10 ml screw-capped, tapered plastic tube to which was added 10 µl of the 6.6 µg/ml internal standard solution, and 0.5 ml of 0.2 M bicarbonate buffer (pH 9.6). The sample was briefly mixed, 5 ml of chloroform was added and the tubes were placed on a rotary mixer for 10 minutes. The organic and aqueous phases were separated by centrifugation at 1500 g for 10 minutes. The upper aqueous phase was removed by aspiration and discarded and the organic phase was transferred to a clean 10 ml screw-capped, tapered plastic tube containing 100 µl of 0.1 N hydrochloric acid. The tubes were vortex-mixed briefly and then placed on a rotary mixer for 10 minutes. The two phases were separated by centrifugation at 1500 g for 5 minutes. An aliquot (10-50 µl) of the aqueous phase was injected onto the column via the automatic injector.

Urine: A 0.3 ml aliquot of urine was pipetted into a 10 ml screw capped plastic tube to which was added 25 µl of the 134 µg/ml internal standard solution. The samples were then handled in exactly the same manner as the plasma samples.

4.1.2.5. Statistical analysis

Peak heights were measured manually and the peak height ratios of the three compounds, codeine, norcodeine and morphine, to the internal standard, dihydrocodeine, were calculated. Standard curves were plotted as peak height ratio versus drug concentration and linear regression analysis was performed to determine the slope, intercept and their variability and the strength of the correlation. Precision was evaluated by spiking plasma with codeine, norcodeine and morphine to concentrations of 10 and 200 ng/ml, and urine at 0.1 and 10 μg/ml. Analysis was performed with 8 to 9 samples intra-day and 5 to 6 samples inter-day. Accuracy was assessed by spiking plasma with codeine, norcodeine and morphine to concentrations of 10 and 200 ng/ml and urine to 0.1 and 10 μg/ml, assaying 8 to 9 times and calculating the estimated concentration from concurrently run standard curves. Stability was evaluated by comparison of six standard curves prepared from the same samples over a 9 month period.

4.1.3. Results and discussion

4.1.3.1. Chromatography

Figure 4-1-1 is a representative chromatogram from an injection of a solution in water containing a mixture of codeine, norcodeine and morphine each at a concentration of 1 μg/ml and dihydrocodeine at a concentration of 3.3 μg/ml. Morphine had a retention time of 3.4 minutes and a capacity factor of 1.1, norcodeine had a retention time of 5.0 minutes and a capacity factor of 2.1, dihydrocodeine had a retention time of 6.1 minutes and a capacity factor of 2.8, whereas codeine had a retention time of 7.2 minutes and a capacity factor of 3.5. The cyano column was chosen after evaluation of C_{18} and cyano columns. Although a C_{18} column provided good separation of these four

compounds, morphine eluated too early and was interfered with by endogenous compounds in plasma samples. Alterations in mobile phase composition and pH did not solve this problem. Substitution with a cyano column was effective. Variations in the pH (Figure 4-1-2) and composition (Figure 4-1-3) of the mobile phase were then explored. Increasing the percentage of acetonitrile and/or triethylamine resulted in a decrease in the retention time of all four compounds, and increasing the pH of the mobile phase increased the retention time of these four compounds. The mobile phase chosen resulted in optimal separation and chromatography.

4.1.3.2. Linearity

Calibration curves showed good linearity between peak-height ratios and concentrations from 5 to 200 ng/ml for codeine, norcodeine and morphine in plasma ($r > 0.99$) and 0.1-10 μg/ml in urine ($r > 0.99$). Representative standard curves for codeine, norcodeine and morphine in plasma and urine are shown in Figure 4-1-4. For plasma and urine standard curves, the 95% confidence intervals of the intercepts included the origin and the standard errors of the slopes were less than 5%.

4.1.3.3. Precision

The assay showed good precision at low and high concentrations in plasma and urine and Table 4-1-1 shows the intra- and inter- day assay precision in plasma and that in urine is shown in Table 4-1-2. The precision, in most cases, was less than 10%.

4.1.3.4. Accuracy and stability

The accuracy of the assay for morphine, norcodeine and codeine in plasma and urine is shown in Table 4-1-3 and 4-1-4 respectively. Except for

morphine at 200 ng/ml in plasma, accuracy was greater than 95%. In samples stored at -20 °C for 9 months, there was no loss of codeine, norcodeine or morphine.

4.1.3.5. Recovery

Extraction recovery (Table 4-1-5) was determined by comparing the representative peak heights of extracted plasma samples (1 µg/ml) with the peak heights of standards of the same concentration. The pH of the initial extraction is critical for morphine. The reported extraction pH for morphine has ranged from 8.9 to 10.0 (Bedford and White 1985; Cone *et al* 1983; Posey and Kimble 1984). In this study, the optimal extraction of morphine occurred at pH 9.6 after comparing extractions at pH 8.9, 9.6 and 10.0. Adding diethyl ether to chloroform (2:1 by volume), in order that the organic phase is the upper phase, resulted in unacceptably poor recovery of norcodeine but not of codeine and morphine.

4.1.3.6. Interference by other compounds

Interference by other substances was tested by chromatographing chemically similar compounds and plasma samples from patients on multiple drug therapy. Agents which do not interfere with this assay are shown in Table 4-1-6. Figure 4-1-5 shows a chromatogram from blank plasma and a plasma sample from a subject 1.25 hours after ingestion of 30 mg codeine phosphate and Figure 4-1-6 shows a chromatogram from blank urine and a sample of the 0-12 hours urine from the same subject. There is a small peak in the plasma and urine samples which elutes near to but is resolved from the norcodeine peak in the chromatograms. In plasma, morphine and norcodeine could not be detected (limit of sensitivity 2 ng/ml). However in urine they could be quantitated. It should be noted that, especially in urine,

glucuronide conjugates of codeine, norcodeine and morphine are also present (Adler *et al* 1955), but not detected with this assay. A method for the quantitative determination of codeine-6-glucuronide in plasma and urine is described in the next section of this chapter.

In summary, a simple, sensitive and specific HPLC assay was developed for the simultaneous determination of codeine, morphine and norcodeine in plasma and in urine. This assay showed good accuracy and reproducibility and is suitable for the pharmacokinetic and metabolism study of codeine in humans (Chapter 4.3.).

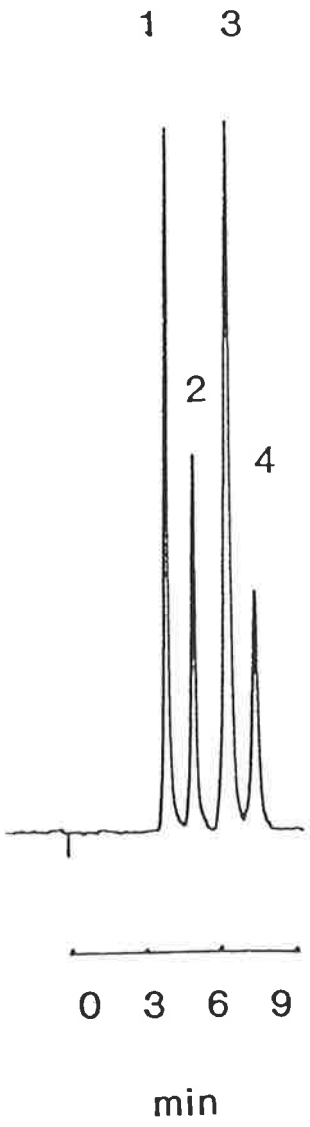


Figure 4-1-1 Chromatogram of a standard solution of morphine 1 μg/ml (1), norcodeine 1 μg/ml (2), dihydrocodeine 3.3 μg/ml (3) and codeine 1 μg/ml (4).

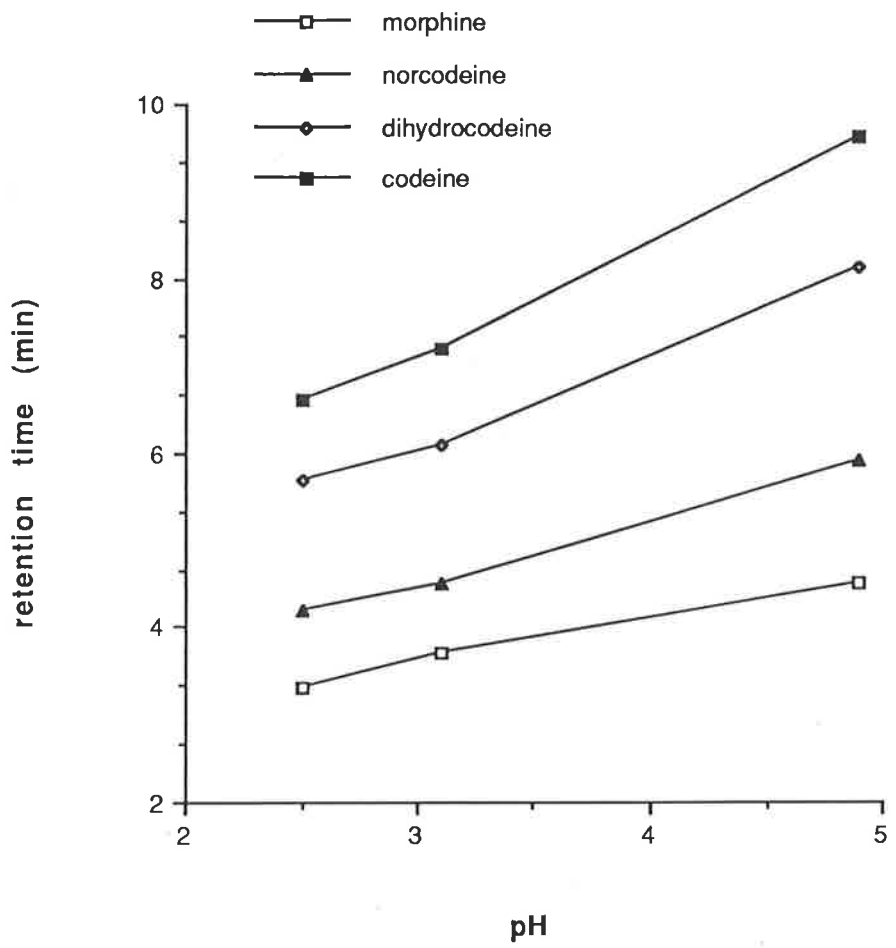


Figure 4-1-2 Effect of pH of the mobile phase (4% acetonitrile/0.1% triethylamine/distilled water to 100%) on the retention times of morphine, norcodeine, dihydrocodeine and codeine.

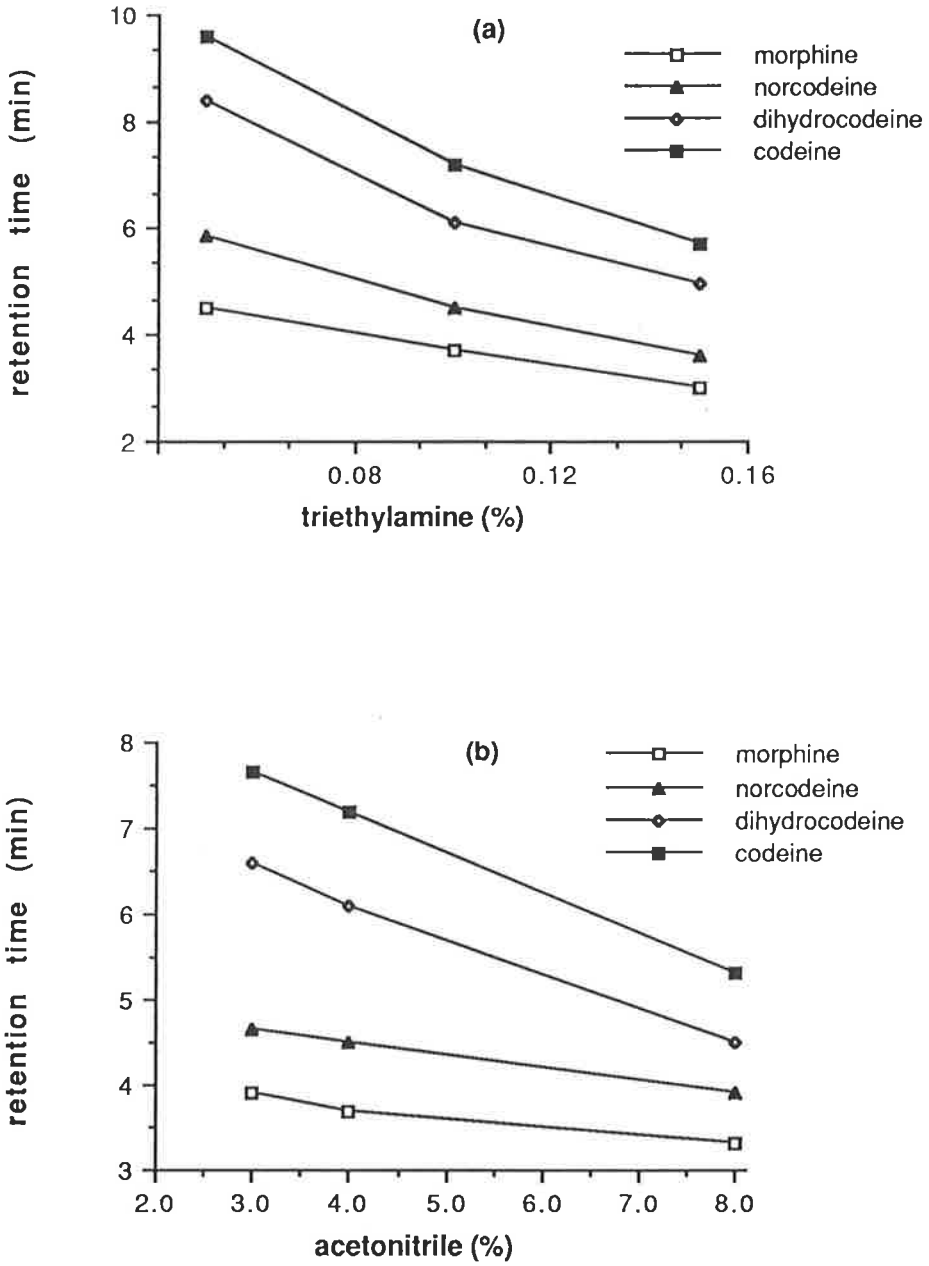


Figure 4-1-3 Effect of the concentrations of (a) triethylamine (in 4% acetonitrile/distilled water to 100%, pH 3.1) and (b) acetonitrile (in 0.1% triethylamine/distilled water to 100%, pH 3.1) on the retention times of morphine, norcodeine, dihydrocodeine and codeine.

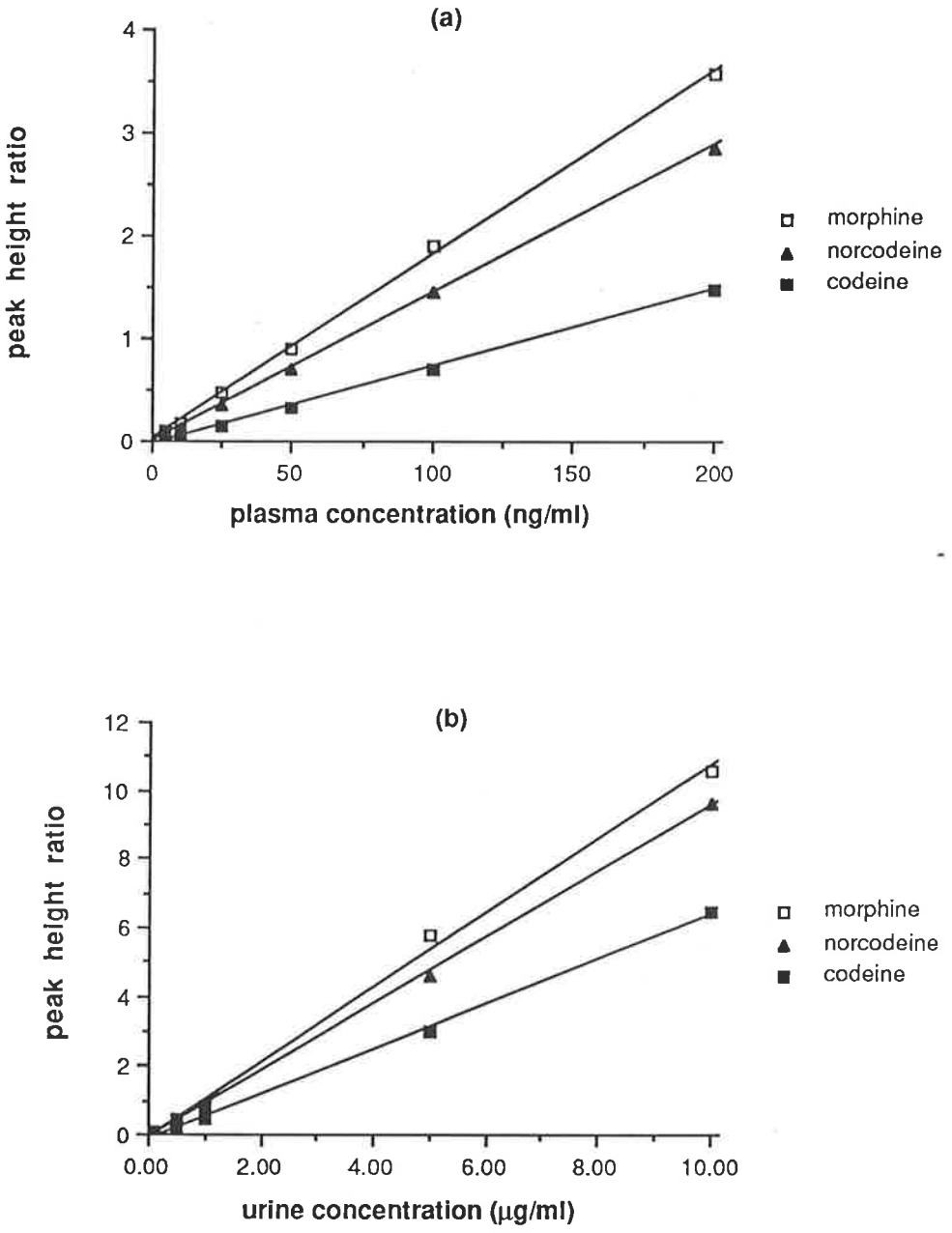


Figure 4-1-4 Representative standard curves for morphine, norcodeine and codeine in plasma (a) and urine (b).

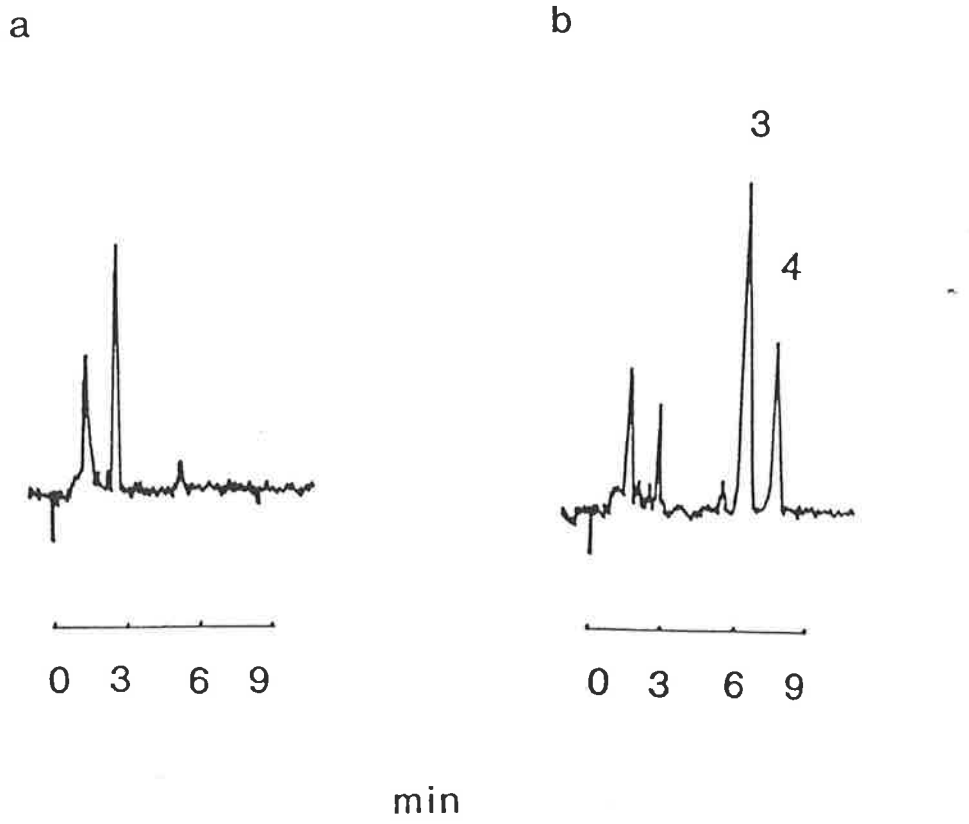


Figure 4-1-5 Chromatograms from an extract of (a) drug-free plasma sample and (b) a plasma sample from a subject 1.25 hours after ingestion of 30 mg codeine phosphate (codeine concentration = 39 ng/ml).
Peaks: 3 = dihydrocodeine (internal standard); 4 = codeine.

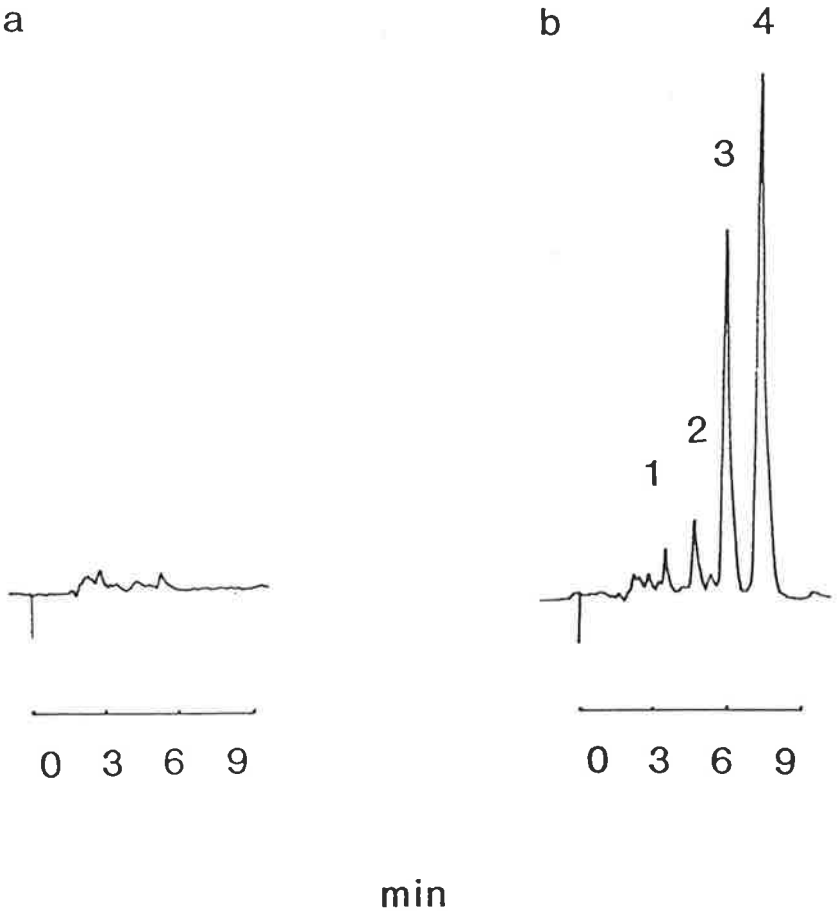


Figure 4-1-6 Chromatograms from an extract of (a) drug-free urine sample and (b) a 0-12 hours urine sample from the same subject (concentration: 1 = 0.8 µg/ml; 2 = 2.1 µg/ml; 4 = 14.5 µg/ml, sample diluted 1 in 2).

Peaks: 1 = morphine; 2 = norcodeine; 3 = dihydrocodeine (internal standard); 4 = codeine.

Table 4-1-1 Assay reproducibility of morphine, norcodeine and codeine in plasma

	concentration (ng/ml)	coefficient of variation (%)	number
intra-day			
morphine	10	7.71	9
	200	2.16	8
norcodeine	10	6.27	9
	200	1.31	8
codeine	10	6.27	9
	200	2.61	8
inter-day			
morphine	10	5.70	6
	200	10.21	6
norcodeine	10	7.67	6
	200	6.22	6
codeine	10	5.85	6
	200	1.42	6

Table 4-1-2 Assay reproducibility of morphine, norcodeine and codeine in urine

	concentration (μg/ml)	coefficient of variation (%)	number
intra-day			
morphine	0.10	8.42	9
	10.00	2.54	9
norcodeine	0.10	4.98	9
	10.00	2.52	9
codeine	0.10	6.38	9
	10.00	2.88	9
inter-day			
morphine	0.10	9.78	5
	10.00	5.98	5
norcodeine	0.10	7.83	5
	10.00	2.89	5
codeine	0.10	2.21	5
	10.00	3.41	5

Table 4-1-3 Assay accuracy of morphine, norcodeine and codeine in plasma

	plasma concentration (ng/ml)					
number	morphine		norcodeine		codeine	
1	9.2	195.4	10.8	198.9	10.0	196.0
2	9.5	184.0	9.8	195.8	9.3	200.5
3	10.6	185.0	9.6	198.6	11.2	210.6
4	11.3	189.0	10.4	200.0	9.9	202.5
5	9.1	194.3	10.4	203.2	9.9	205.1
6	10.1	192.6	10.2	203.6	9.7	200.9
7	9.9	189.4	9.6	198.6	10.7	200.0
8	9.1	188.9	8.9	198.2	9.9	193.5
9	10.4	—	10.8	—	11.0	—
mean	9.9	189.8	10.1	199.6	10.2	201.1
SD	0.8	4.1	0.6	2.6	0.6	5.3
nominal	10.0	200.0	10.0	200.0	10.0	200.0

Table 4-1-4 Assay accuracy of morphine, norcodeine and codeine in urine

	urine concentration (μg/ml)					
number	morphine		norcodeine		codeine	
1	0.11	9.24	0.09	9.37	0.10	9.86
2	0.11	9.74	0.10	9.88	0.10	9.94
3	0.10	9.91	0.10	9.95	0.11	10.47
4	0.10	9.85	0.09	10.1	0.10	10.71
5	0.12	9.68	0.10	9.97	0.11	10.47
6	0.11	10.09	0.11	10.28	0.10	10.03
7	0.09	10.00	0.10	10.02	0.09	10.47
8	0.09	9.63	0.10	9.80	0.11	10.22
9	0.10	9.73	0.09	10.03	0.10	10.52
mean	0.10	9.76	0.10	9.93	0.10	10.31
SD	0.01	0.25	0.01	0.25	0.01	0.30
nominal	0.10	10.00	0.10	10.00	0.10	10.00

Table 4-1-5 Recoveries (%) for codeine, norcodeine, morphine and dihydrocodeine from plasma.

number	morphine	norcodeine	dihydrocodeine	codeine
1	61	94	73	76
2	60	91	73	76
3	60	91	69	73
4	60	93	73	76
5	59	93	73	76
mean	60.0	92.4	72.2	75.4
SD	0.7	1.3	1.8	1.3

Table 4-1-6 Agents shown not to interfere with the assay

drugs in patients' plasma	pure drug preparations
digoxin	dextromethorphan
frusemide	dextrorphan
glyceryl trinitrate	dextropropoxyphene
metoclopramide	ethylmorphine
multivitamins	naloxone
paracetamol	oxycodone
polystyrene sulphonate	pholcodine
pseudoephedrine	codeine-6-glucuronide
quinine bisulphate	morphine-6-glucuronide
salbutamol	morphine-3-glucuronide
spironolactone	morphine-3-sulphate
temazepam	
theophylline	
tolbutamide	

4.2. Determination of codeine-6-glucuronide in biological fluids by HPLC using solid-phase extraction

4.2.1. Introduction

A major metabolite of codeine is codeine-6-glucuronide, the urinary recovery of which accounted for up to 50% of the dose after oral or intramuscular administration of codeine 0.3-0.4 mg/kg (Adler *et al* 1955). The plasma concentrations of codeine-6-glucuronide in man after taking 60 mg of codeine phosphate were over 10 times higher than that of codeine (Findlay *et al* 1977, Guay *et al* 1987). In these studies, however, the conjugate had not been identified as a chemical entity. The concentration of the conjugate was estimated from analysis of base released after acidic or enzymatic hydrolysis and this represents an indirect measurement of this metabolite. There is evidence that β-glucuronidase cannot release codeine-6-glucuronide completely (Axelrod and Inscoe 1960, Yoshimura *et al* 1968). Furthermore, it has also been reported that substances such as saccharo-1,4-lactone and sulfate ions in urine inhibit or interrupt the activities of β-glucuronidase (Levvy 1952, Boyland and Williams 1960, Combie *et al* 1982). Thus, the indirect assay using hydrolytic methods would probably underestimate or overestimate the codeine-6-glucuronide concentrations and lead to erroneous conclusions in studies on codeine disposition. Assays for the direct determination of codeine-6-glucuronide in biological fluids are not available at present. This may be due to an insufficient supply of codeine-6-glucuronide as the reference standard and the technical difficulties inherent in an assay for glucuronide.

The objective of this study was to develop a specific and sensitive HPLC assay for the determination of codeine-6-glucuronide in plasma and in urine.

4.2.2. Materials and methods

4.2.2.1. Reagents

All reagents and solvents were of analytical grade or of HPLC grade (see Appendix I). Dihydrocodeine bitartrate (Knoll AG Chemical Works, Ludwigshafen, FRG) was of British Pharmacopoeial grade quality. Codeine-6-glucuronide was synthesized by Dr. G. Reynolds of the School of Chemical Technology, South Australian Institute of Technology (Appendix III).

4.2.2.2. Chromatography

The HPLC system consisted of a pump (M-6000A, Waters Associates Inc., Milford, Mass. U.S.A.), a LS-5 Luminescence Spectrometer (Perkin-Elmer Limited, Beaconsfield, U.K.), a WISP 710 B automatic injector (Waters Associates) and an Omniscribe B-5000 strip-chart recorder (Houston Instruments, Austin, Tx, U.S.A.). The 15 cm x 4.6 mm I.D. stainless steel column was packed with Spherisorb 5-µm ODS-2 packing material (Phase Separations, Queensferry, U.K.). The composition of the mobile phase was 10% acetonitrile/0.03% triethylamine in 8 mM phosphate buffer, adjusted to pH 3.0 with orthophosphoric acid. The flow rate through the column at ambient temperature was 1 ml/minute which produced a back-pressure of 1.5 Kpsi (10.5 MPa). The excitation and emission wavelengths of the detector were 230 and 350 nm respectively and the excitation and emission slits were set at 15 and 20 nm respectively.

4.2.2.3. Stock solutions

Codeine-6-glucuronide was made up as 1 mg/ml (as codeine content) in distilled water and was diluted to concentrations ranging from 20 to 1000 ng/ml in drug-free plasma and from 0.2 to 100 µg/ml in drug-free urine. Dihydrocodeine, the internal standard, was diluted in distilled water to a concentration of 4 µg/ml for plasma analysis.

4.2.2.4. Sample preparation

Plasma: Extraction of codeine-6-glucuronide from plasma was based on the method of Svensson and coworkers (1982) for morphine-3- and 6-glucuronides, with modifications. A Sep-Pak C_{18} cartridge (Waters Assoc.) was pretreated with 10 ml of methanol, 5 ml of 25% acetonitrile in 10 mM phosphate buffer (pH 2.1) and 10 ml of distilled water. Plasma (0.5 ml) containing 50 µl of 4 µg/ml dihydrocodeine, was mixed with 3 ml of 0.5 M ammonium sulphate (pH 9.3) and was slowly passed through the pretreated cartridge. The cartridge was then washed with 20 ml of 5 mM ammonium sulphate (pH 9.3) and then 0.5 ml distilled water. Codeine-6-glucuronide and dihydrocodeine were eluted with 2 ml of 25% acetonitrile in 10 mM phosphate buffer (pH 2.1). The eluate was mixed with 3 ml of the 0.5 M ammonium sulphate buffer (pH 9.3) and injected into another pretreated cartridge. The cartridge was then washed with 20 ml of 5 mM ammonium sulphate (pH 9.3) and then 0.5 ml distilled water. The two compounds were eluted with 2 ml methanol. The eluate was evaporated to dryness in a Buchler vortex evaporator (Fort Lee, New Jersey, U.S.A.) and redissolved in 200 µl of 0.05 N hydrochloric acid. An aliquot (10-50 µl) of the solution was injected on the column via the automatic injector.

Urine: Urine was injected onto the HPLC column directly after dilution (1 in 5) with distilled water.

4.2.2.5. Statistical analysis

Peak heights were measured manually and the peak height ratio of codeine-6-glucuronide to the internal standard, dihydrocodeine, was calculated. For plasma analysis, standard curves were plotted as peak height ratio versus concentration, and for urine analysis, as peak height versus concentration. Standard linear regression analysis was used to determine the slope, intercept, their variability and the strength of the correlation for the above standard curves. Precision was evaluated by adding codeine-6-glucuronide to achieve plasma concentrations of 50 and 1000 ng/ml, and urine concentrations at 0.5 and 100 µg/ml. These samples were analysed 9 times within one day and once daily on six separate occasions and the coefficients of variation were calculated. Accuracy was assessed by adding codeine-6-glucuronide to achieve plasma concentrations of 50 and 1000 ng/ml and urine concentrations of 0.5 and 100 µg/ml. Each sample was assayed 9 times and the estimated concentrations were calculated from concurrently processed standard curves. Stability of codeine-6-glucuronide was assessed by adding codeine-6-glucuronide to achieve plasma concentrations of 50 and 1000 ng/ml and urine concentrations of 0.5 and 100 µg/ml. These were stored at -20 °C and assayed for both codeine-6-glucuronide and codeine on several occasions during a 12 week period.

4.2.2.6. Analysis of purity of synthesised codeine-6-glucuronide sample

The purity of the synthesized codeine-6-glucuronide sample was investigated by two independent methods as follows:

a. ***β-glucuronidase hydrolysis:*** 0.5 ml of codeine-6-glucuronide solutions (diluted in 0.2 M acetate buffer pH 5 to achieve concentrations of 2 µg/ml, 10 µg/ml and 100 µg/ml, n=5) were mixed with 0.5 ml β-glucuronidase (8000 U/ml in the 0.2 M acetate buffer) and incubated in a shaking water bath at 37 °C for 14 hours (overnight). The protein was removed by ultra-filtration using Centrifree^{TM} (Amicon Corporation, Danvers, MA, U.S.A.) ultrafiltration units at 2000 g for 15 minutes. Aliquots of the ultrafiltrate were analysed for codeine-6-glucuronide and codeine as described above for urine by direct injection onto the column. The purity was calculated as:

$$\text{purity} = \frac{\%\text{ of original sample recovered as codeine}}{\%\text{ of original sample released of codeine-6-glucuronide}}$$

The numerator of the above equation was calculated as the codeine concentration in the ultrafiltrate divided by the notional codeine concentration in the original codeine-6-glucuronide sample. The denominator was calculated as the codeine-6-glucuronide concentration in the original sample minus the codeine-6-glucuronide concentration in the ultrafiltrate divided by the codeine-6-glucuronide concentration in the original sample.

b. ***hydrochloric acid hydrolysis:*** The acid hydrolysis of codeine-6-glucuronide was performed following a procedure described by Yoshimura and coworkers (1970) with modifications: to 2.5 ml of codeine-6-glucuronide solution (concentrations of 2, 10 and 50 µg/ml, n=5) was added 0.05 ml of 40% $NaHSO_3$ solution and 3 ml of 37% HCL to bring the final acid concentration to 20%. The mixture was then heated in a boiling water bath for 60 minutes. To this hydrolysate, 0.05 ml of 40% $NaHSO_3$ and 3 ml 10 N NaOH solution were added to achieve a pH of 5.0. The mixture was diluted with 0.2 M pH 5.0 acetate buffer to a final volume of 10 ml. Aliquots of each sample were analysed for the liberated codeine as described above for urine. Codeine

solutions in the concentration range 2 to 50 µg/ml (n=2) were treated in the same manner.

4.2.2.7. Influence of urine on the hydrolysis of codeine-6-glucuronide by β-glucuronidase

The intrinsic influence of urine on the hydrolytic capacity of β-glucuronidase under the above conditions was investigated in two ways. Firstly, 10 µg codeine-6-glucuronide (n=2) in 0.5 ml drug-free urine or in 0.5 ml acetate buffer was added to tubes containing 0, 800, 2400, 4000 and 4800 U β-glucuronidase in 0.5 ml acetate buffer. Secondly, 10 µg of codeine-6-glucuronide in 10 µl distilled water was incubated with a constant amount of β-glucuronidase (4000 U in 0.5 ml 0.2 M acetate buffer) and varying volumes of urine (0.1, 0.2, 0.3, 0.4, 0.5,1.0 ml, n=2) were added. The final volume was adjusted to 1.0 ml with acetate buffer, except for the 1 ml urine sample in which 4000 U of β-glucuronidase was added as powder. The samples were processed and analysed as above.

4.2.3. Results and discussion

4.2.3.1. Chromatography

A representative chromatogram from an injection of a solution in water containing a mixture of codeine-6-glucuronide and the internal standard dihydrocodeine is shown in Figure 4-2-1. Codeine-6-glucuronide had a retention time of 5.9 minutes and a capacity factor of 2.9, and dihydrocodeine had a retention time of 11.5 minutes and a capacity factor of 6.7. Although a cyano column resulted in good chromatography, the internal standard peak was interfered with by endogenous substances from plasma and could not be separated by changing the composition and pH of the mobile phase. The C_{18} column gave good chromatographic separation of both

codeine-6-glucuronide and the internal standard, dihydrocodeine. Ultraviolet detection (210 nm) provided sufficient sensitivity for codeine-6-glucuronide. Fluorescence detection, however, gave both good sensitivity and better base line.

4.2.3.2. Linearity

The relationship between peak-height ratios and concentrations from 20 to 1000 ng/ml for codeine-6-glucuronide in plasma ($r > 0.99$) and for urine between peak-height and concentrations from 0.2 to 100 μg/ml ($r > 0.99$) were linear. Representative standard curves for codeine-6-glucuronide in plasma and urine are shown in Figure 4-2-2. For plasma and urine standard curves, the 95% confidence intervals of the intercepts included the origin; the standard errors of the slopes were less than 5%.

4.2.3.3. Precision

The assay showed good precision at low and high concentrations in plasma and urine. Table 4-2-1 shows the intra- and inter- day assay precision, whose coefficients of variation were less than 10%.

4.2.3.4. Accuracy

The accuracy of the assay for codeine-6-glucuronide in plasma and in urine is shown in Table 4-2-2. For plasma and urine samples with different concentrations of codeine-6-glucuronide, accuracy was greater than 95%. In samples stored at -20 °C for 12 weeks, the decomposition of codeine-6-glucuronide to codeine was less than 1%. The potential decomposition of codeine-6-glucuronide may influence codeine analysis very much in codeine pharmacokinetic study considering the very high concentrations of codeine-6-glucuronide.

4.2.3.5. Recovery

The Sep-Pak cartridge is very efficient for the separation of codeine-6-glucuronide from plasma, but some endogenous substances in plasma will be extracted at the same time. The second Sep-Pak purification step provides much cleaner chromatograms and its inclusion is especially important for the analysis of low concentrations. The recovery from plasma at a concentration of 1 µg/ml (mean± SD, n=4) was 74±2 % for codeine-6-glucuronide and 33±2 % for dihydrocodeine. The recovery for the internal standard was low but constant. The reasons for this are not clear. It is possible that because codeine-6-glucuronide and dihydrocodeine have different physico-chemical properties (such as pKa and polarities) and the conditions for cartridge column extraction for one compound may not necessarily be suitable for another. However the most important requirement for an internal standard is not recovery itself, but variability in recovery, which in the case of dihydrocodeine was low. The limit of detection for codeine-6-glucuronide was 10 ng/ml from plasma.

4.2.3.6. Interference by other compounds

Interference was studied by preparing and chromatographing aliquots of pure drugs and by analyzing samples from patients on multiple-drug therapy. None of the drugs in Table 4-2-3 interfered with the assay of codeine-6-glucuronide. A chromatogram from an injection of a solution of a mixture of codeine and its metabolites and congeners is shown in Figure 4-2-3.

Figure 4-2-4 shows a chromatogram from blank plasma and a plasma sample from a subject 1.25 hours after ingestion of 30 mg codeine phosphate

and Figure 4-2-5 shows a chromatogram from blank urine and a urine sample collected between 0 and 12 hours after dose from the same subject.

4.2.3.7. Purity of synthesized codeine-6-glucuronide

The purity of the synthesized codeine-6-glucuronide sample was estimated by enzymatic hydrolysis and acidic hydrolysis and the results are shown in Table 4-2-4. Both hydrolytic methods gave virtually identical results and indicated that the purity of the original sample was about 80%. After β-glucuronidase treatment, the codeine-6-glucuronide peak was still present on the chromatogram from samples containing both high and low concentrations. The percentages (Figure 4-2-6) of codeine-6-glucuronide released after β-glucuronidase hydrolysis at 2 and 10 μg/ml were the same, which indicated that β-glucuronidase can not release codeine-6-glucuronide completely even if sufficient amount of enzyme is present. This is in agreement with previous reports (Axelrod and Inscoe 1960; Yoshimura *et al* 1968). Acidic hydrolysis, resulted in complete codeine-6-glucuronide release, but concentrated acid and base are needed and the heating conditions made the procedure difficult and potentially dangerous. Many compounds including morphine may be broken down at these extreme conditions (Fish and Hayes 1974). Because the synthesised codeine-6-glucuronide was only used as a standard to determine the concentrations of codeine-6-glucuronide in plasma and in urine, the degree of purity of the standard will not influence the assay as long as the purity is known and is acceptably high.

4.2.3.8. Influence of urine on the activity of β-glucuronidase

The hydrolysis of codeine-6-glucuronide in acetate buffer and in urine with different concentrations of enzyme is shown in Figure 4-2-7.

Figure 4-2-8 shows the influence of urine volume on the degree of hydrolysis. Urine had a marked inhibitory effect on the hydrolytic capacity of β-glucuronidase. The characteristics of the curves in Figure 4-2-7 and 4-2-8 suggest that substances in urine inhibit probably competitively, the activity of β-glucuronidase. It has been reported that substances naturally occurring in urine such as sacchrro-1,4-lactone and sulfate ions are inhibitors of β-glucuronidase (Levvy 1952; Boyland and Williams 1960; Combie *et al* 1982). These results strongly support the need to assay codeine-6-glucuronide, and indeed all xenobiotics, in biological fluids by direct methods.

This is the first HPLC assay for the direct determination of codeine-6-glucuronide in biological fluids. This assay is sensitive (down to 10 ng/ml) and specific and is suitable for the pharmacokinetic and metabolism studies of codeine in man.

Figure 4-2-1 Chromatogram of a standard solution of codeine-6-glucuronide 1 μg/ml (1) and dihydrocodeine 3.3 μg/ml (2).

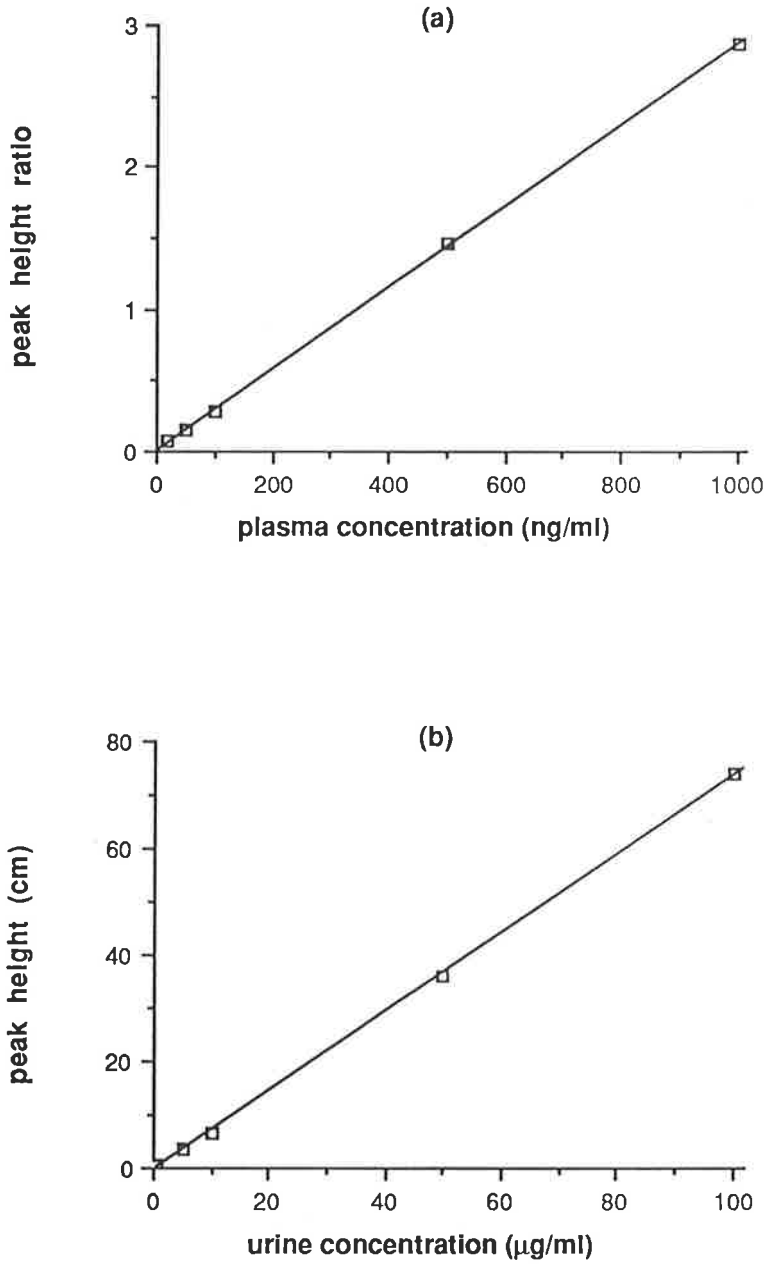


Figure 4-2-2 Representative standard curves for codeine-6-glucuronide in plasma (a) and urine (b).

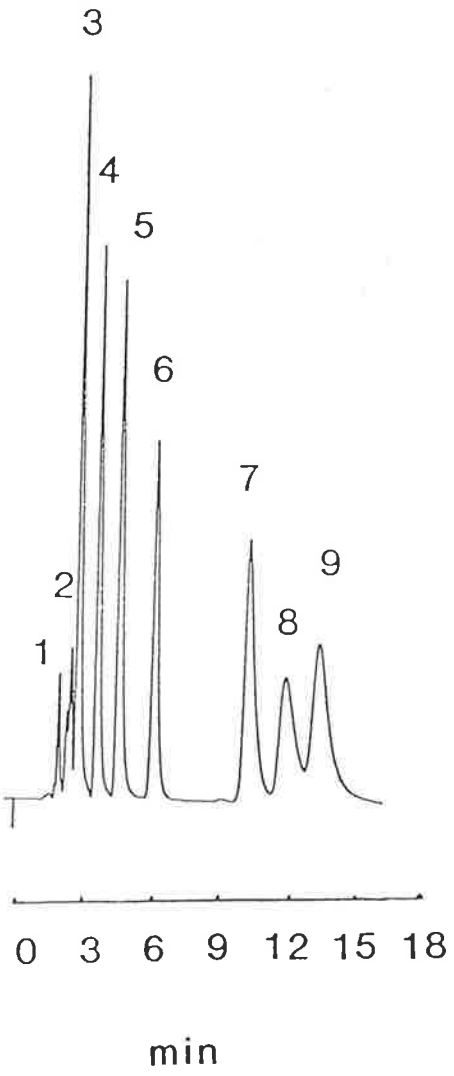


Figure 4-2-3 Chromatogram of a standard solution of (1) morphine-3-glucuronide, (2) morphine-3-sulphate, (3) morphine-6-glucuronide, (4) normorphine, (5) morphine, (6) codeine-6-glucuronide, (7) norcodeine, (8) dihydrocodeine and (9) codeine. Concentrations: 1-2 = 0.2 µg/ml; 3-9 = 1 µg/ml.

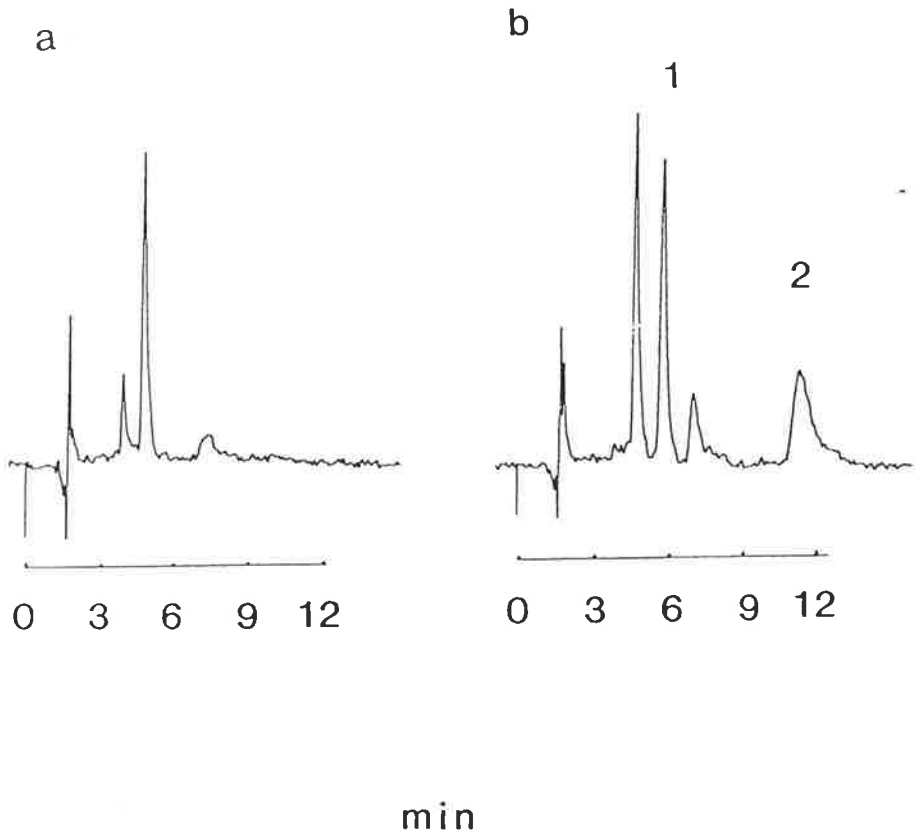


Figure 4-2-4 Chromatograms of (a) blank plasma sample and (b) a plasma sample from the same subject 1.25 hours after ingestion of 30 mg codeine phosphate (codeine-6-glucuronide concentration = 891 ng/ml)
Peaks: 1 = codeine-6-glucuronide; 2 = dihydrocodeine (internal standard).

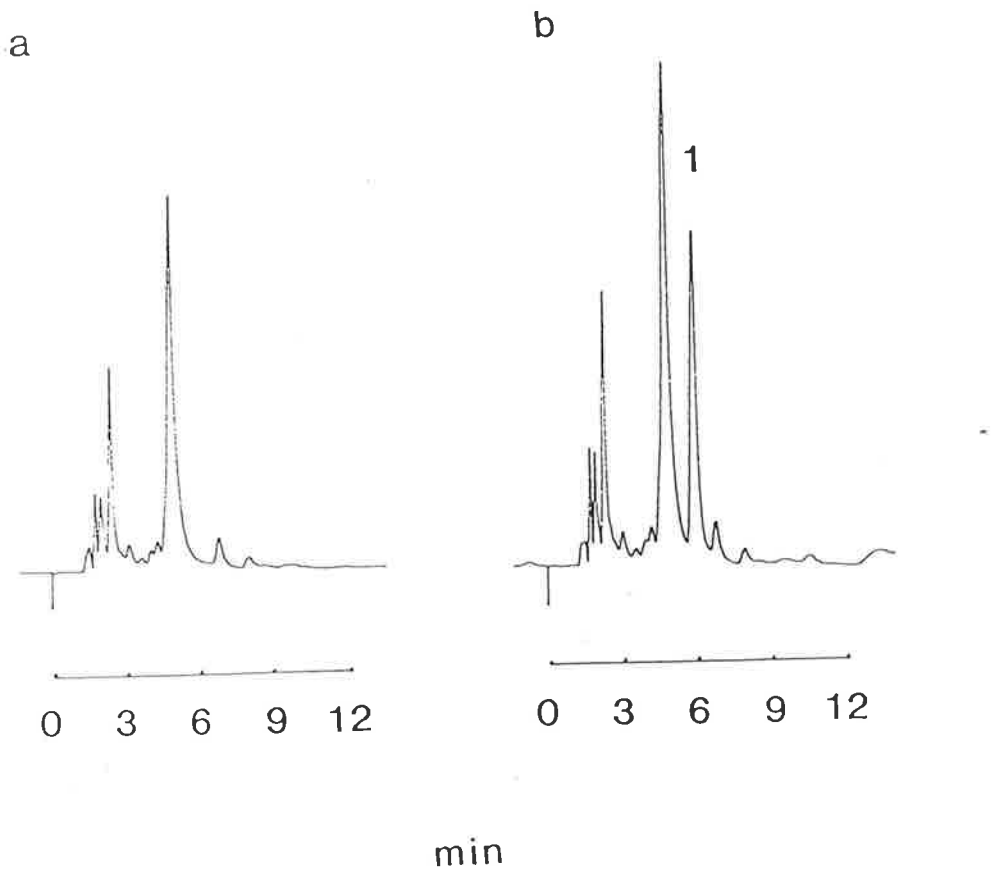


Figure 4-2-5 Chromatograms of (a) a blank urine sample and (b) a 0-12 hours urine sample from the same subject (codeine-6-glucuronide concentration = 58 µg/ml, sample diluted 1 in 5).

Peak: 1 = codeine-6-glucuronide.

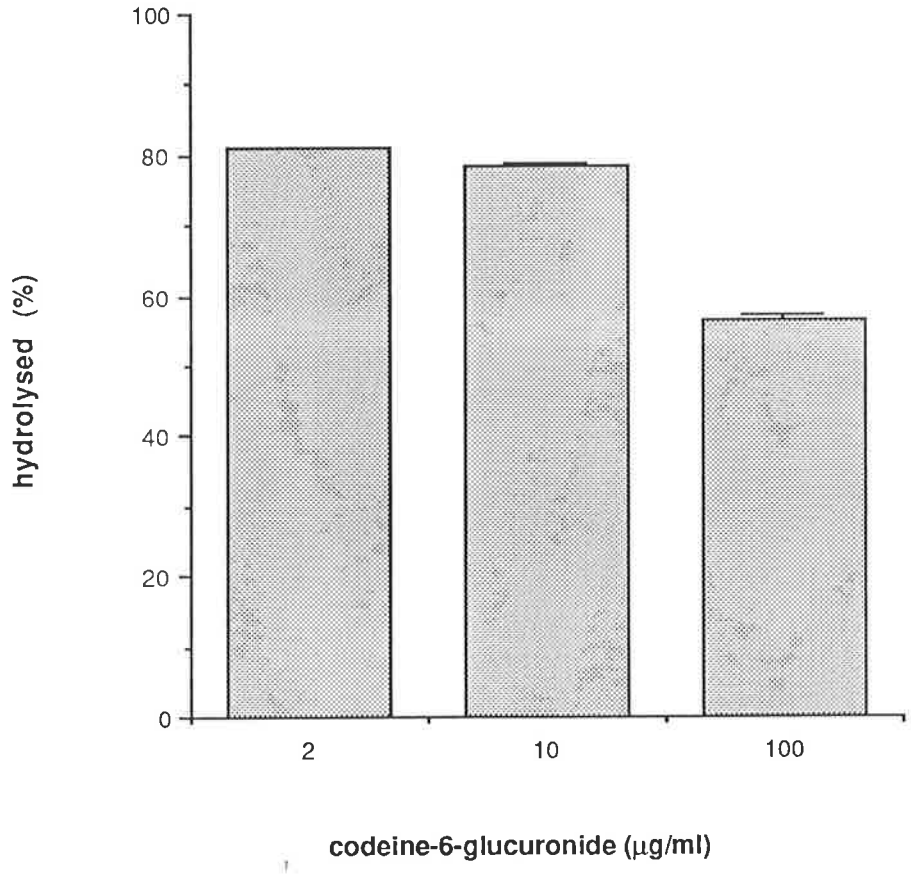


Figure 4-2-6 β-glucuronidase (4000 U/ml) hydrolysis (mean ± SD) of codeine-6-glucuronide at concentrations of 2, 10 and 100 µg/ml in acetate buffer (pH 5.0).

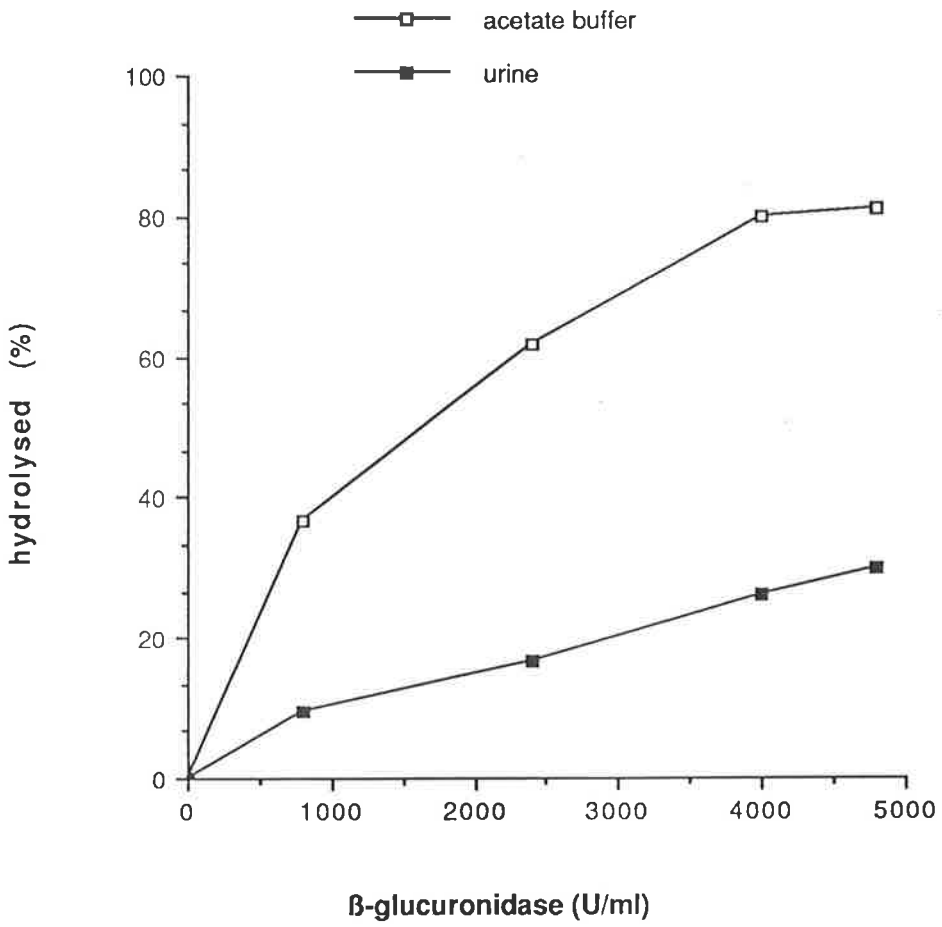


Figure 4-2-7 Effect of concentrations of β-glucuronidase on the hydrolysis of codeine-6-glucuronide (10 μg/ml) in acetate buffer (pH 5.0) and in urine.

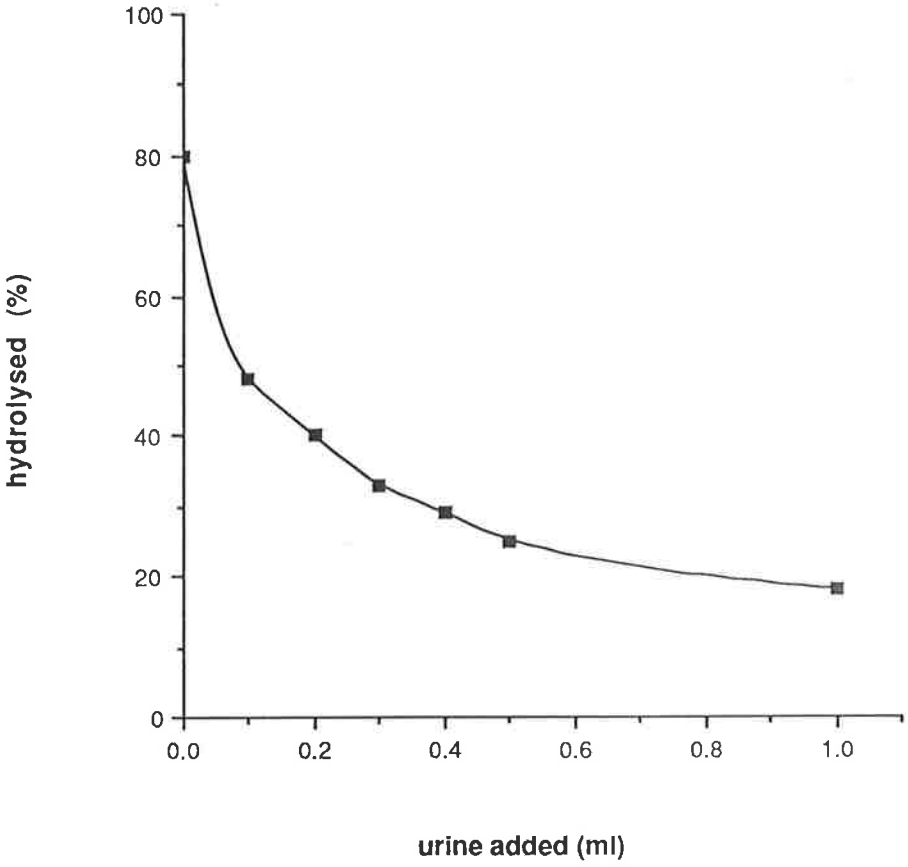


Figure 4-2-8 Influence of urine volume added to the incubation on the hydrolysis of codeine-6-glucuronide (10 µg/ml) by β-glucuronidase (4000 U/ml).

Table 4-2-1 Assay variability for codeine-6-glucuronide in plasma and urine

	concentration	coefficient of variation (%)	number
plasma (ng/ml)			
intra-day			
	50	4.2	9
	1000	3.4	9
inter-day			
	50	8.2	6
	1000	1.8	6
urine (μg/ml)			
intra-day			
	0.5	7.0	9
	100.0	2.0	9
inter-day			
	0.5	6.6	6
	100.0	1.8	6

Table 4-2-2 Assay accuracy for codeine-6-glucuronide in plasma and urine

number	plasma (ng/ml)		urine (μg/ml)	
1	49.5	1025	0.53	100.0
2	49.5	1059	0.46	100.0
3	48.9	1007	0.53	102.6
4	50.2	1025	0.46	102.6
5	52.9	991	0.46	97.4
6	51.2	957	0.53	97.4
7	49.5	1025	0.46	100.0
8	52.9	982	0.53	100.0
9	46.1	956	0.53	98.9
mean	50.1	1003	0.49	99.9
SD	2.1	35	0.03	1.9
nominal	50.0	1000	0.50	100.0

Table 4-2-3 Drugs shown not interfere with the codeine-6-glucuronide assay

drugs in patients' plasma	pure drug preparations
captopril	cocaine
digoxin	dextromethorphan
frusemide	dextrorphan
glyceryl trinitrate	dextropropoxyphen
metoclopramide	ethylmorphine
multivitamins	naloxone
paracetamol	oxycodone
polystyrene sulphonate	pholcodine
pseudoephedrine	
quinine bisulphate	
salbutamol	
spironolactone	
temazepam	
theophylline	
tolbutamide	

Table 4-2-4 Purity of synthesized codeine-6-glucuronide sample

hydrolysis method	concentration (μg/ml)	purity (%) mean	purity (%) SD	number
β-glucuronidase				
	2	80.2	0	5
	10	80.3	0.7	5
	100	81.7	1.6	5
hydrochloric acid				
	2	81.3	0	5
	10	80.9	2.3	5
	50	85.0	1.6	5

4.3. Pharmacokinetics and metabolism of codeine in healthy young volunteers

4.3.1. Introduction

The pharmacokinetics of codeine in man have been described in a few studies using radioimmunoassay (Findlay *et al* 1977a; 1978; 1986; Rogers *et al* 1982; Guay *et al* 1987) and gas chromatography-mass spectrometry (Quiding *et al* 1986). However, these studies have not provided satisfactory results because of several problems. Firstly, all the studies except one (Quiding *et al* 1986) were based on one radioimmunoassay method (Findlay *et al* 1977b). The results obtained using this method are open to criticism because the codeine antiserum has the potential to cross-react with some of the metabolites of codeine. For example, the codeine concentration required to inhibit the binding of tracer to antiserum by 50% was 3.4 ng/ml, and that for codeine-6-glucuronide was 72 ng/ml (Findlay *et al* 1977b). Thus, the binding affinity of codeine to the antiserum was about 20 times higher than that of codeine-6-glucuronide. However, plasma codeine-6-glucuronide concentrations have been shown to be at least 10 times higher than that of codeine (Findlay *et al* 1977b; 1986; Rogers *et al* 1982; Guay *et al* 1987; Bodd *et al* 1987). These data strongly suggest that codeine-6-glucuronide would interfere substantially with the radioimmunoassay for codeine. Secondly, in all studies to date, codeine-6-glucuronide, the major metabolite of codeine, was not determined directly. The concentrations of codeine-6-glucuronide in plasma or urine were estimated by analysing codeine after β-glucuronidase hydrolysis. Studies have shown that β-glucuronidase can not hydrolyse codeine-6-

glucuronide completely (Axelrod and Inscoe 1960; Yoshimura *et al* 1968; Guay *et al* 1988; Bodd *et al* 1987; Chapter 4.2.). In addition substances occurring naturally in urine inhibit the activity of β-glucuronidase (Levvy 1952; Boyland and Williams 1960; Combie *et al* 1982; Chapter 4.2.). For these reasons, Guay and coworkers (1988) stated "Assay values were corrected to reflect the efficiency of enzyme hydrolysis ...". The concentrations of codeine-6-glucuronide in these previous studies could therefore have been underestimated or overestimated. Thirdly, the pharmacokinetics after chronic dosing of codeine, have not been adequately compared with that after single dose administration. Quiding and coworkers (1986) studied the pharmacokinetics after chronic dosing of 60 mg of codeine phosphate, but little information was obtained about the single dose pharmacokinetics because of the short sampling period (2.8 hours). Guay and coworkers (1987) reported the plasma half-life of codeine, determined by radioimmunoassay, after single and chronic dosing to be between 4.5 and 5.6 hours compared with previous reports of 2.3-3.6 hours (Findlay *et al* 1977a; 1978; 1986; Rogers *et al* 1982; Quiding *et al* 1986). Fourthly, there is little information about the renal clearance of codeine and its metabolites because most of the previous investigators collected blood samples only.

Adler and coworkers (1955) were the first to have determined quantitatively the urinary excretion of codeine, morphine, norcodeine and their conjugates in humans after oral or intramuscular administration of ^{14}C-codeine 0.3-0.4 mg/kg. Nomof and coworkers (1977) studied the urinary excretion after intravenous infusion of variable doses of codeine, but the unconjugated and conjugated compounds were not distinguished. Since then the metabolism and disposition of codeine in humans have not been studied with modern analytical technology.

The aims of this study were to determine:

(i) the pharmacokinetics of codeine and codeine-6-glucuronide in healthy volunteers after single and chronic doses;

(ii) to determine the metabolism and disposition of codeine in healthy volunteers after single and chronic doses,

using sensitive and specific HPLC analytical methods described in Chapter 4.1. and Chapter 4.2..

4.3.2. Methods

4.3.2.1. Subjects

Eight healthy volunteers, 7 male and 1 female, who gave written informed consent, participated in the study. The volunteers were aged 27.3±5.2 (mean ± SD) years and weighed 67.9±11.9 kg (Table 4-3-1). Prior to commencement of the study each subject underwent a physical examination and a venous blood sample was collected for biochemical (MBA 20) and haematological (CBE) examinations. The plasma and urine creatinine concentrations were determined by a HPLC method (Huang and Chiou 1983). The creatinine clearance was calculated using the plasma and urine concentrations determined (see Chapter 4.3.2.4). Some of the results reflecting renal and hepatic functions are shown in Table 4-3-1. Drugs and alcohol were prohibited for two days before codeine administration and for the duration of the study.

This study was approved by the Human Ethics Committee of Royal Adelaide Hospital and the Committee on the Ethics of Human Experimentation of the University of Adelaide.

Table 4-3-1 Volunteer data

subject	sex	age (year)	BW (kg)	BH (cm)	Cr (mmol/L)	Cl_{cr} (ml/min)	GGT (U/L)	albumin (g/L)
1	M	25	68	170	0.10	102	17	48
2	F	27	48	166	0.06	105	19	46
3	M	23	83	190	0.09	132	16	50
4	M	37	83	177	0.09	125	45	52
5	M	20	58	170	0.08	120	12	54
6	M	30	63	173	0.10	90	15	50
7	M	26	70	185	0.11	97	5	52
8	M	30	70	160	0.10	102	23	51

BW: body weight; BH: body height; Cr: serum creatinine concentration; Cl_{cr}: creatinine clearance; GGT: gamma glutamate transaminase; M: male; F: female.

4.3.2.2. Study design

Single dose study: After an overnight fast, each subject swallowed a single dose of 30 mg codeine phosphate as a tablet (Fawns and McAllan) with 100 ml of water. Food was permitted after 3 hours and normal fluid intake was allowed over the duration of the sampling interval. The volunteers were ambulant but confined to the laboratory for the 12 hours of sampling. Ten ml venous blood samples were collected into heparinized plastic tubes via an indwelling catheter (Jelco^{TM}, 18 G i.v. catheter placement unit, Critikon, Tampa, USA), kept patent with a stylet (Jelco^{TM}), placed in a forearm vein. Sampling times were as follows: 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 3, 4, 6, 8 and 12 hours after codeine ingestion. At the same times mixed saliva samples (3 ml) were collected by chewing parafilm and the pH of saliva was recorded. All urine was collected over the intervals of 0-12, 12-24, 24-48 hours after dosing.

***Chronic dose study*:** The chronic dosing study was conducted in the same volunteers 1 to 3 weeks after completion of the single dose study. Subjects received 30 mg of codeine phosphate (as tablets) 8 hourly (at 0800,1600,2400 hour) for seven doses. The final dose was given at 0800 hour on day 3 after an overnight fast. Blood and saliva samples were collected at 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 4, 6 and 8 hours after the last dose and urine was collected over this interval.

Blood samples were centrifugated immediately and plasma was separated. Urine volume was determined gravimetrically and recorded along with the urine pH. An aliquot of well mixed urine was retained for analysis. All samples were stored in stoppered vials at -20 °C until analysed for drug content.

4.3.2.3. Drugs analysis in biological fluids

Unconjugated codeine, norcodeine and morphine analyses: Codeine, norcodeine and morphine concentrations in plasma, saliva and urine were measured by the HPLC method described in Chapter 4.1..

Codeine-6-glucuronide analysis: Codeine-6-glucuronide in plasma and urine was directly determined using the HPLC method described in Chapter 4.2..

Morphine and norcodeine conjugates analyses: The presence of conjugates of norcodeine and morphine in plasma and urine was inferred by the generation of norcodeine and morphine following enzymatic hydrolysis. The internal standard (dihydrocodeine) was added to plasma (1 ml) or urine samples (0.3 ml) in 10 ml screw capped tubes. β-glucuronidase from Helix Pomatia type H-1 (Sigma Chemical Co., St. Louis, MO., USA) was added (0.5 ml of a 5000 U/ml in pH 5.0 acetate buffer), followed by incubation in a shaking water bath at 37 °C for 16 hours. Samples were analysed for norcodeine and morphine by the method described in Chapter 4.1..

4.3.2.4. Analysis of creatinine concentrations

Creatinine concentrations in plasma and urine were determined by an HPLC method (Huang and Chiou 1983) with modifications. The HPLC consisted of a SP 8770 isocratic pump (Spectra-Physics), an ultraviolet detector (Model 440, Waters Assoc.), a WISP 710A automatic injector (Waters Assoc.) and an Omniscribe B-5000 strip-chart recorder (Houston Instruments). The column was a 33 cm x 4.6 mm I.D. stainless steel cation exchange column (Partisil-10, strong cation exchange, Whatman Chemical Separation Inc., Clifton, N.J.). The mobile phase comprised 10% acetonitrile

and 90% 0.02 M ammonium dihydrogen phosphate buffer (pH 4.8). The flow rate through the column was 1.5 ml/minute. The wavelength of the detector was 254 nm.

200 µl of acetonitrile were pipetted into a plastic tube containing 100 µl of plasma and the mixture was vigorously vortexed and then allowed to stand for 5 minutes. The tube was centrifugated at 2000 g for 5 minutes. Aliquots (10-30 µl) of the supernatant were injected to the column. Urine samples were diluted 40 times in 10% acetonitrile (in distilled water) and aliquots (10-30 µl) were injected onto the column. Standard curves of creatinine were performed in distilled water (0.1-2 mg/ml) since urine and plasma contain endogenous creatinine. The standard curve was linear over the concentration range employed. Neither codeine nor its metabolites interfered with the determination of creatinine in plasma or urine.

4.3.2.5. Pharmacokinetic and statistical analyses

The concentrations of codeine in saliva up to 1 hour after administration were not included in the calculations of the pharmacokinetic data, because of likely retention of part of the oral dose in the mouth during this time period. For easy comparison with codeine, the concentrations of codeine-6-glucuronide were calculated as the codeine content.

The maximum plasma concentration (C_{max}) and its time of occurence (t_{max}) were determined from the observed data. The elimination rate constant (λ_z) was calculated from the terminal portion of the semilogarithmic concentration-time curve using linear regression analysis. The area under the plasma or saliva concentration-time curves to the last sampling time following single dose (AUC_{0-12}) and chronic doses (AUC_{0-8}) administration

was calculated by the trapezoidal rule and the total AUC ($AUC_{0-\infty}$) for the single dose was calculated as

$$AUC_{0-\infty} = AUC_{0-12} + C_p12/\lambda_z^p \text{ or } C_s12/\lambda_z^s$$

where C_p12 and C_s12 are the plasma and saliva concentrations at 12 hours respectively. Half-lives ($t_{1/2,z}$) in plasma and saliva were calculated as

$$t_{1/2,z} = 0.693/\lambda_z^p \text{ or } \lambda_z^s$$

Renal clearance (Cl_R) was calculated as

$$Cl_R = A_e(t_1\text{-}t_2)\ /AUC_{t1\text{-}t2}$$

where $A_e(t_1\text{-}t_2)$ is the amount excreted unchanged over the urine collection interval t_1-t_2. Fraction excreted (f_e) in urine was calculated as A_e divided by the dose administered. Creatinine clearance (Cl_{cr}) was calculated as

Cl_{cr} = urinary excretion rate/plasma concentration

Differences in pharmacokinetic data between treatments were analysed for statistical significance by the t-test. Correlations between plasma and saliva concentration, saliva concentration and saliva pH and between renal clearance and either urine pH or urine flow rate were determined by linear regression analysis. Statistical significance was assumed when $P < 0.05$. All data are reported as mean ± SD.

4.3.3. Results

4.3.3.1. Codeine

The mean plasma and saliva codeine concentration-time profiles in eight subjects following single and chronic oral doses are shown in Figure 4-3-1 and 4-3-2. Tables 4-3-2 and 4-3-3 contain the derived pharmacokinetic

parameters of codeine after single and chronic dosing respectively. The maximum concentrations of codeine in plasma occurred in about one hour after dose, which were not different between the two treatments ($P > 0.05$). The maximum plasma concentration of codeine after chronic dosing was significantly higher than that after single dosing ($P < 0.05$). Plasma codeine concentrations declined in a monoexponential manner. The elimination half-lives determined from plasma and saliva after single and chronic dosing were not significantly different ($P > 0.05$). There were no differences between the half-lives of codeine after the two treatments ($P > 0.05$). The mean area under the plasma codeine concentration-time curve to infinity for the single dose was not significantly different from that during the dosing interval at steady state ($P > 0.05$). The area under the saliva codeine concentration-time curve for the single dose was significantly lower than that during the dosing interval following chronic dosing ($P < 0.05$). Codeine concentrations in saliva were on average 3.4 times higher than in plasma. There were significant correlations between saliva and plasma codeine concentrations ($r = 0.80$, $P < 0.01$) (Figure 4-3-3) and between the area under the saliva codeine concentration-time curve and saliva pH ($r = 0.83$, $P < 0.01$) (Figure 4-3-4).

4.3.3.2. Codeine-6-glucuronide

The mean plasma codeine-6-glucuronide concentration-time profiles after single and chronic dosing are shown in Figure 4-3-1 and 4-3-2. Table 4-3-4 and 4-3-5 contain the derived pharmacokinetic parameters of codeine-6-glucuronide after single and chronic dosing respectively. The maximum plasma concentrations of codeine-6-glucuronide occurred a mean of 1.2 hours after drug administration, which was slightly longer than that of codeine but was not significantly different ($P > 0.05$) and there were no

significant differences between the two treatments ($P > 0.05$). The mean maximum plasma concentration of codeine-6-glucuronide was 15 times higher than that of codeine, and there was no significant difference between single and chronic dosing ($P > 0.05$). The elimination half-life of codeine-6-glucuronide from plasma averaged 3.24±0.73 hours which was not significantly different between the two treatments ($P > 0.05$) and was not significantly different from that of codeine ($P > 0.05$). The mean area under the plasma concentration-time curve of codeine-6-glucuronide after the single dose was not significantly different from that during the dosing interval at steady state ($P > 0.05$). The mean area ($AUC_{0-\infty}$ single; AUC_{0-8} chronic) under the plasma concentration-time curve of codeine-6-glucuronide was 15.8±4.5 times greater than that of codeine. Codeine-6-glucuronide was not detected in saliva.

4.3.3.3. Renal clearance

The renal clearances of codeine and codeine-6-glucuronide were not significantly different between single and chronic dosing ($P > 0.05$). There was a statistically significant inverse relationship between urine pH and renal clearance for codeine ($r = 0.81$, $P < 0.01$) but no such relationship for codeine-6-glucuronide ($r = 0.08$) (Figure 4-3-5). There was no correlation between renal clearance and urine flow rate for either codeine ($r = 0.35$) or codeine-6-glucuronide ($r = 0.03$) (Figure 4-3-6). The urinary excretion of unchanged compound was not significantly different between single dose and chronic doses ($P > 0.05$).

4.3.3.4. Urinary excretion

The urinary excretion of codeine, norcodeine, morphine and their conjugates is shown in Table 4-3-6 and 4-3-7 and the combined proportions of

conjugated and unconjugated compounds are shown in Figure 4-3-7 and 4-3-8. There were no significant differences ($P > 0.05$) between the urinary excretion of codeine and its metabolites after the single dose and that during a dosing interval at steady state. The elimination of codeine and its metabolites was almost complete within 24 hours after a single dose (Figure 4-3-9; 4-3-10; 4-3-11). The O-demethylation ratio of codeine, calculated as total (conjugated + unconjugated) morphine divided by total codeine and norcodeine, and N-demethylation ratio of codeine, calculated as total norcodeine divided by total codeine and morphine, in the 8 subjects are shown in Figure 4-3-12.

Morphine and norcodeine in plasma after enzymatic hydrolysis were just detected in a few peak time samples (0.75-3.0 hours) after single and chronic dosages. The conjugates of morphine and norcodeine were not detected in saliva.

There were no adverse effects of codeine demonstrable in any of the volunteers during the single or chronic dosing studies.

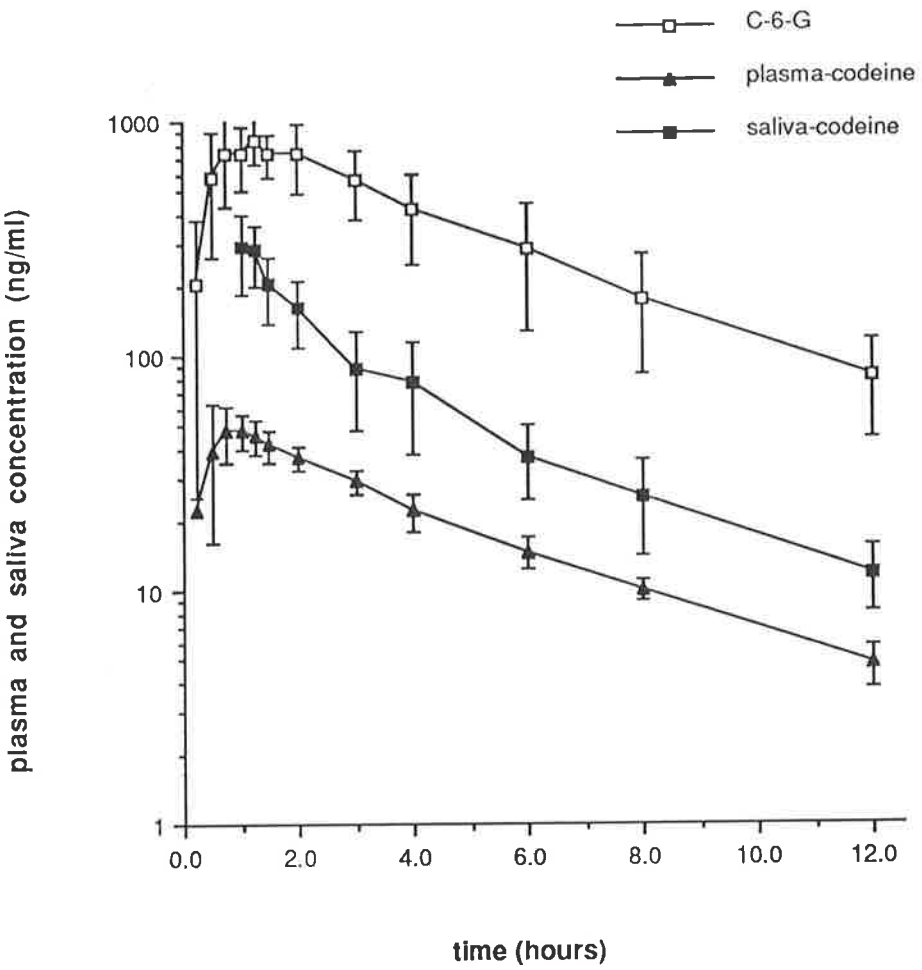


Figure 4-3-1 Plasma codeine, codeine-6-glucuronide (C-6-G) and saliva codeine concentrations (mean±SD) after a single 30 mg oral dose of codeine phosphate in 8 healthy volunteers.

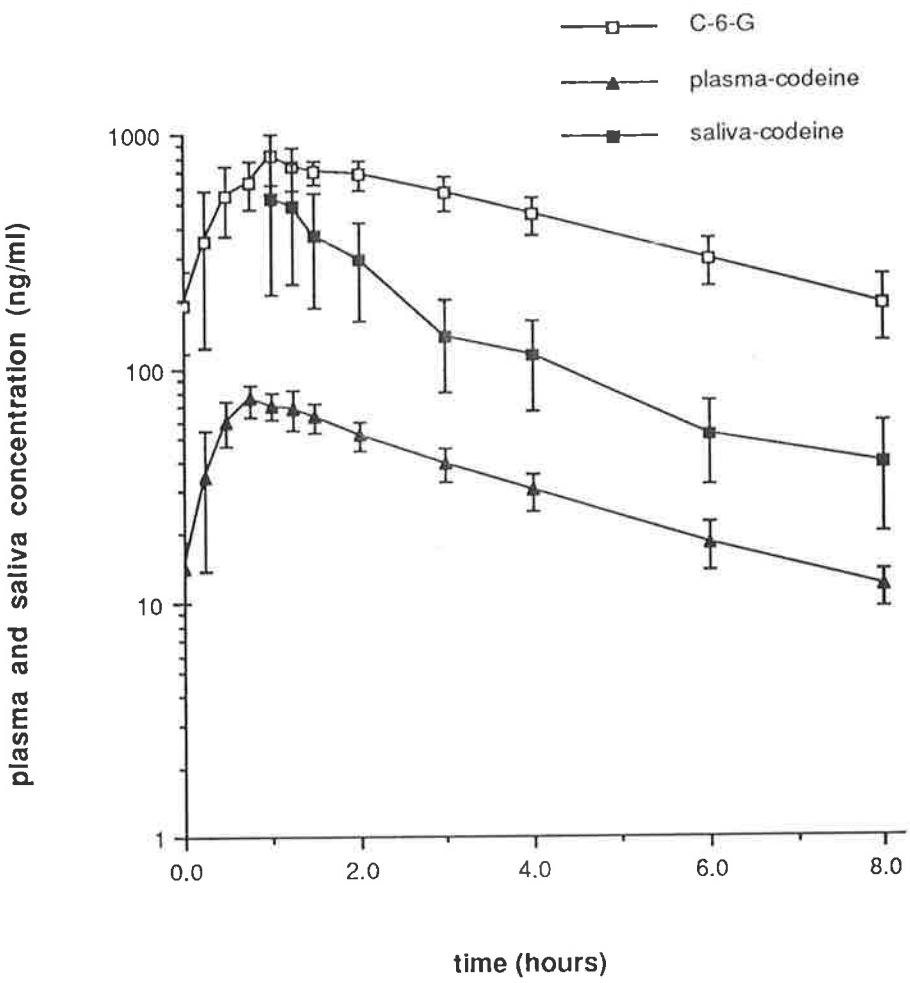


Figure 4-3-2 Plasma codeine, codeine-6-glucuronide (C-6-G) and saliva codeine concentrations (mean±SD) after 30 mg codeine phosphate 8 hourly for 7 doses in 8 healthy volunteers.

Table 4-3-2 Pharmacokinetic parameters of codeine after a single dose of 30 mg codeine phosphate orally

subject	$t_{1/2}$ (h)		C_{max}	t_{max}	$AUC_{0-\infty}$(mg·h/l)		Cl_R	f_e
	plasma	saliva	(ng/ml)	(h)	plasma	saliva	(ml/min)	(%)
1	3.22	2.82	67.6	0.75	0.24	0.56	178	11.1
2	2.77	2.74	81.8	0.50	0.26	1.06	157	10.8
3	3.63	3.11	41.4	1.00	0.24	0.59	67	4.3
4	3.17	3.19	47.6	1.00	0.22	0.76	167	9.5
5	2.81	3.18	55.7	1.25	0.26	0.74	208	14.0
6	3.15	2.98	51.3	0.75	0.24	0.89	260	16.6
7	3.60	2.72	42.3	1.50	0.27	0.70	189	13.2
8	3.59	4.13	47.8	1.00	0.24	0.50	238	15.2
mean	3.24	3.11	54.4	0.97	0.25	0.73	183	11.8
SD	0.34	0.45	13.8	0.31	0.02	0.18	59	3.9

Table 4-3-3 Pharmacokinetic parameters of codeine after chronic dosing of 30 mg codeine phosphate 8 hourly given orally

subject	$t_{1/2}$ (h)		C_{max}	t_{max}	AUC_{0-8}(mg·h/l)		Cl_R	f_e
	plasma	saliva	(ng/ml)	(h)	plasma	saliva	(ml/min)	(%)
1	3.13	3.81	71.4	0.75	0.24	0.53	191	11.9
2	3.37	3.29	87.0	1.25	0.32	1.77	201	16.7
3	3.25	2.84	72.7	0.75	0.24	0.67	250	15.4
4	2.78	2.20	66.8	0.75	0.28	1.42	219	16.0
5	2.63	2.35	68.3	1.00	0.24	1.07	100	6.2
6	2.44	2.39	87.7	1.25	0.25	1.17	246	16.3
7	2.91	2.48	79.2	0.75	0.34	0.98	265	23.4
8	2.68	2.17	79.2	0.75	0.27	0.57	110	7.9
mean	2.90	2.69	76.5	0.91	0.27	1.02	198	14.2
SD	0.33	0.59	8.0	0.23	0.04	0.43	63	5.5

Table 4-3-4 Pharmacokinetic parameters of codeine-6-glucuronide after a single dose of 30 mg codeine phosphate orally

subject	$t_{1/2}$ (h)	C_{max} (ng/ml)	t_{max} (h)	$AUC_{0-\infty}$ (mg·h/l)	Cl_R (ml/min)
1	2.95	967	1.25	3.88	57.1
2	2.31	1232	0.75	4.67	54.6
3	3.57	524	1.00	2.41	101.0
4	2.75	832	2.00	4.61	32.3
5	2.39	1089	2.00	4.54	52.9
6	4.71	637	1.25	4.22	59.6
7	4.32	937	1.25	7.41	36.2
8	2.76	1025	0.75	4.02	45.6
mean	3.22	905	1.28	4.47	54.9
SD	0.89	234	0.49	1.39	21.0

Table 4-3-5 Pharmacokinetic parameters of codeine-6-glucuronide after chronic doses of 30 mg codeine phosphate 8 hourly orally

subject	$t_{1/2}$ (h)	C_{max} (ng/ml)	t_{max} (h)	AUC_{0-8} (mg·h/l)	Cl_R (ml/min)
1	4.43	767	1.00	3.67	74.7
2	3.16	1009	1.00	4.05	64.0
3	2.31	660	1.00	2.38	111.2
4	3.12	759	2.00	3.63	46.5
5	3.06	891	0.75	3.51	51.7
6	3.39	832	1.25	3.51	54.6
7	3.43	828	1.00	3.77	53.8
8	3.23	1163	1.00	4.19	32.2
mean	3.27	864	1.13	3.59	61.1
SD	0.58	158	0.38	0.55	23.7

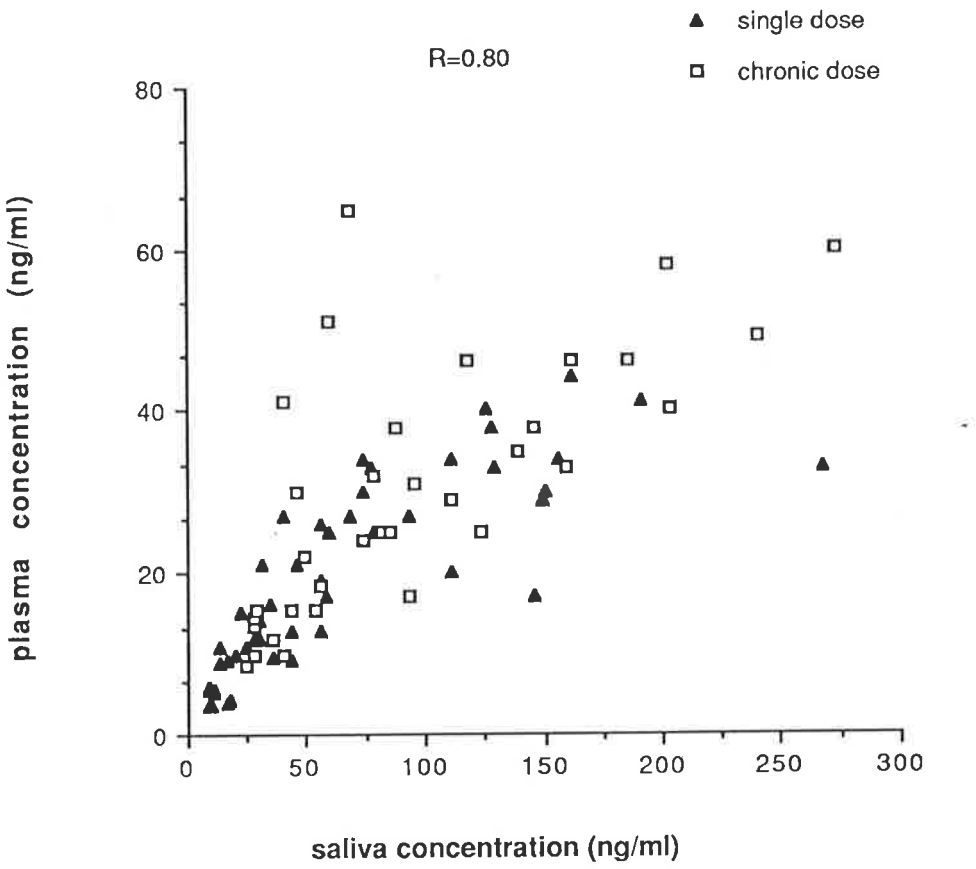


Figure 4-3-3 Correlation between the concentrations of codeine in plasma and saliva.

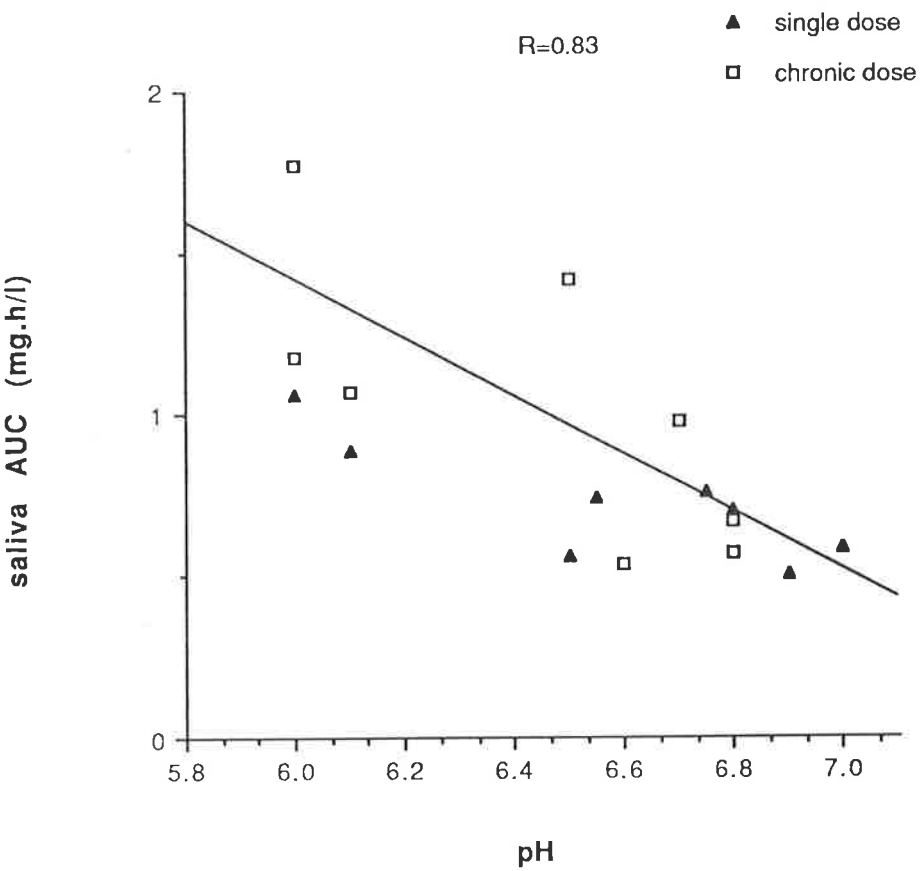


Figure 4-3-4 Correlation between the area under the saliva codeine concentration-time curve and the saliva pH.

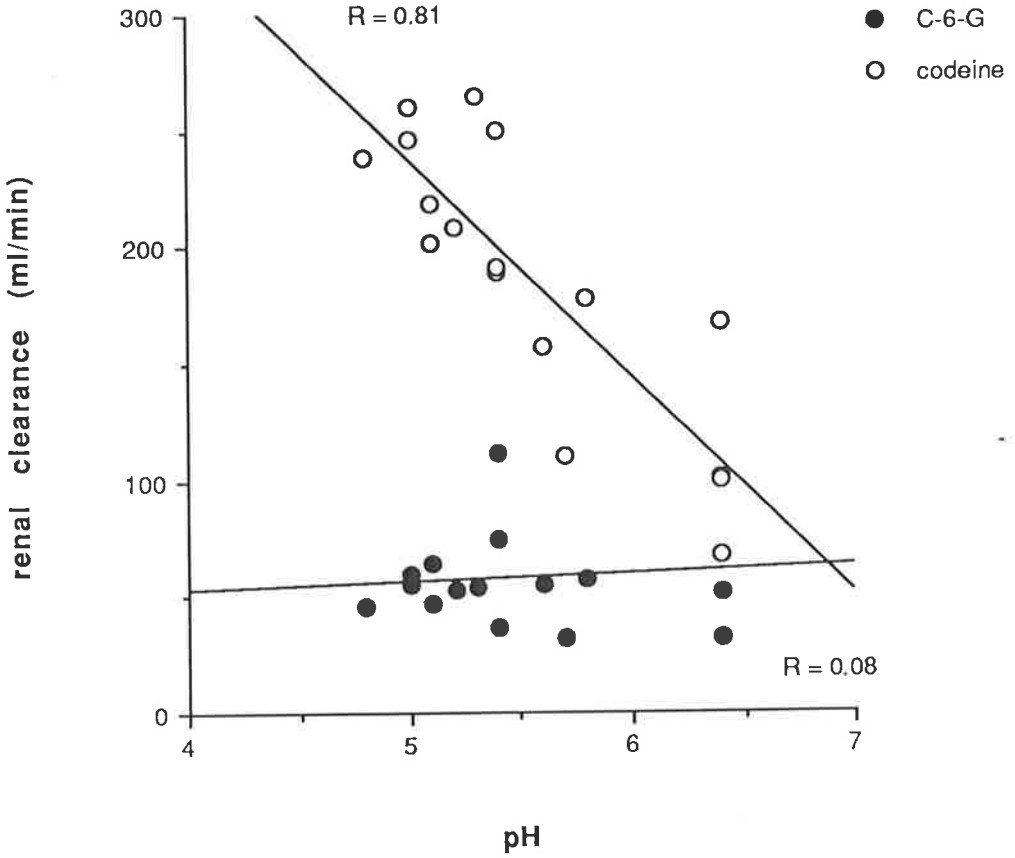


Figure 4-3-5 Correlation between the renal clearances of codeine and codeine-6-glucuronide (C-6-G) and urine pH.

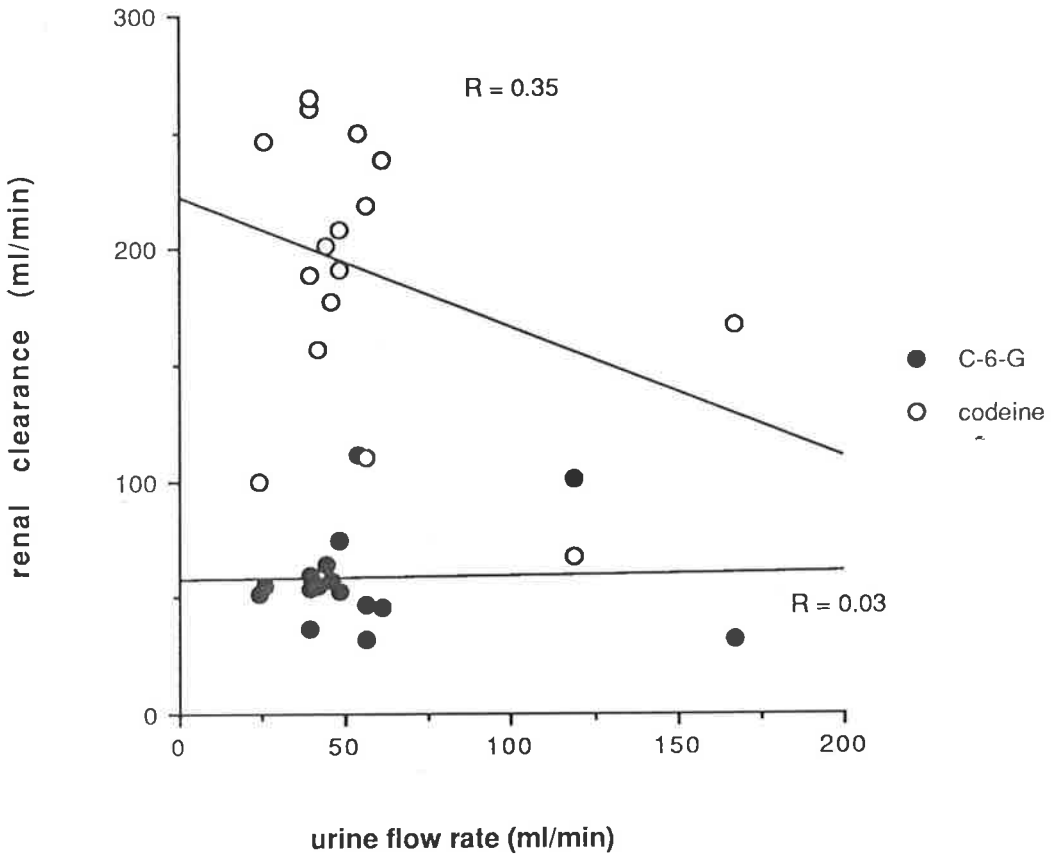


Figure 4-3-6 Correlations between the renal clearances of codeine and codeine-6-glucuronide (C-6-G) and the urine flow rate.

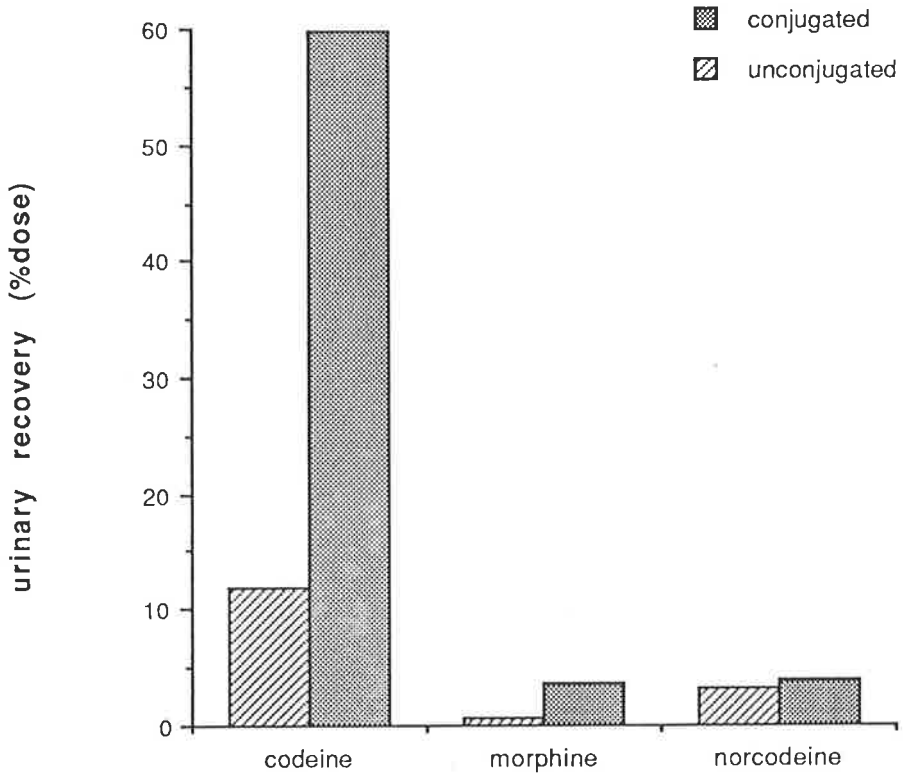


Figure 4-3-7 Mean percentage of unconjugated and conjugated codeine, norcodeine and morphine excreted in urine after a single oral dose of 30 mg codeine phosphate in 8 healthy volunteers.

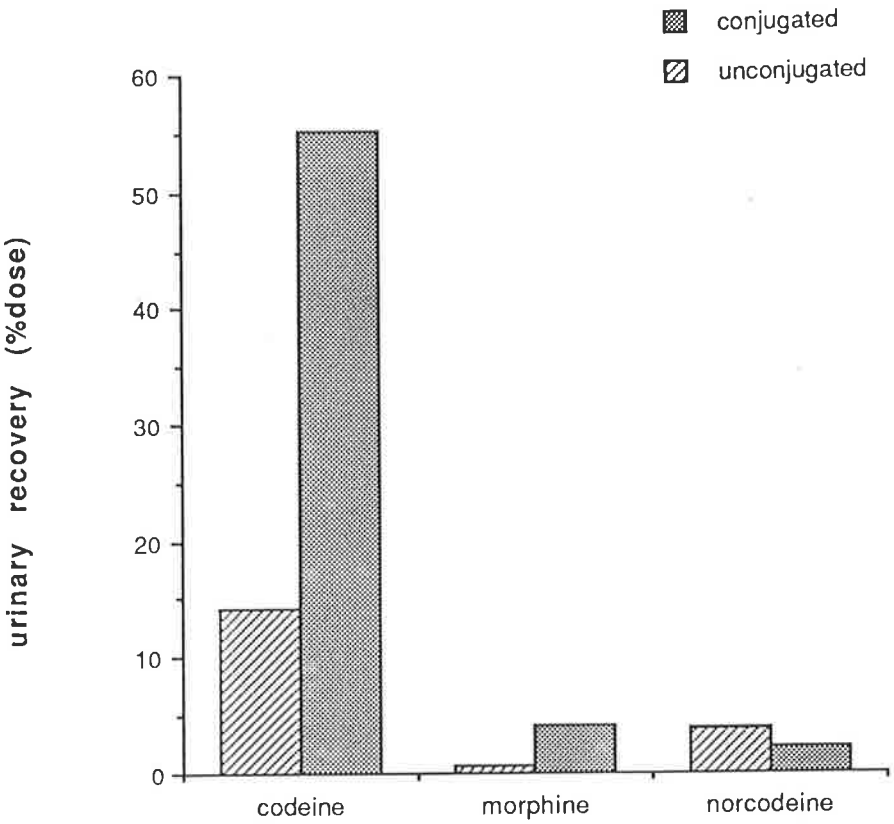


Figure 4-3-8 Mean percentage of unconjugated and conjugated codeine, norcodeine and morphine excreted in urine during a dosing interval after 30 mg codeine phosphate 8 hourly for 7 doses in 8 healthy volunteers.

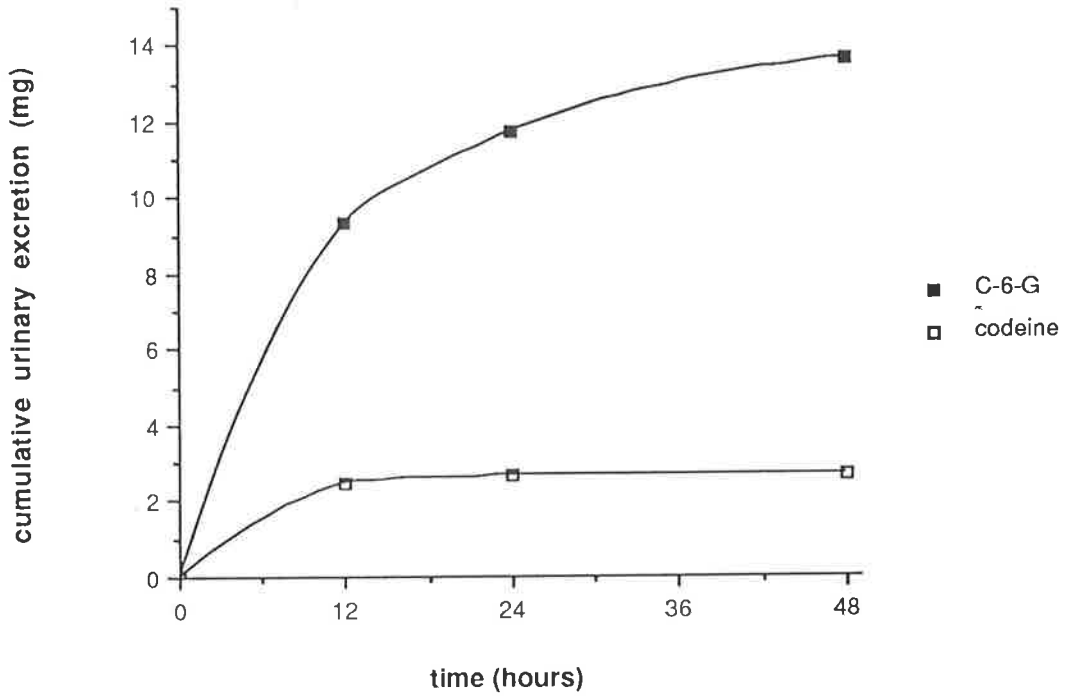


Figure 4-3-9 Mean cumulative excretion of codeine and codeine-6-glucuronide (C-6-G) in urine after a single oral dose of 30 mg codeine phosphate in 8 healthy volunteers.

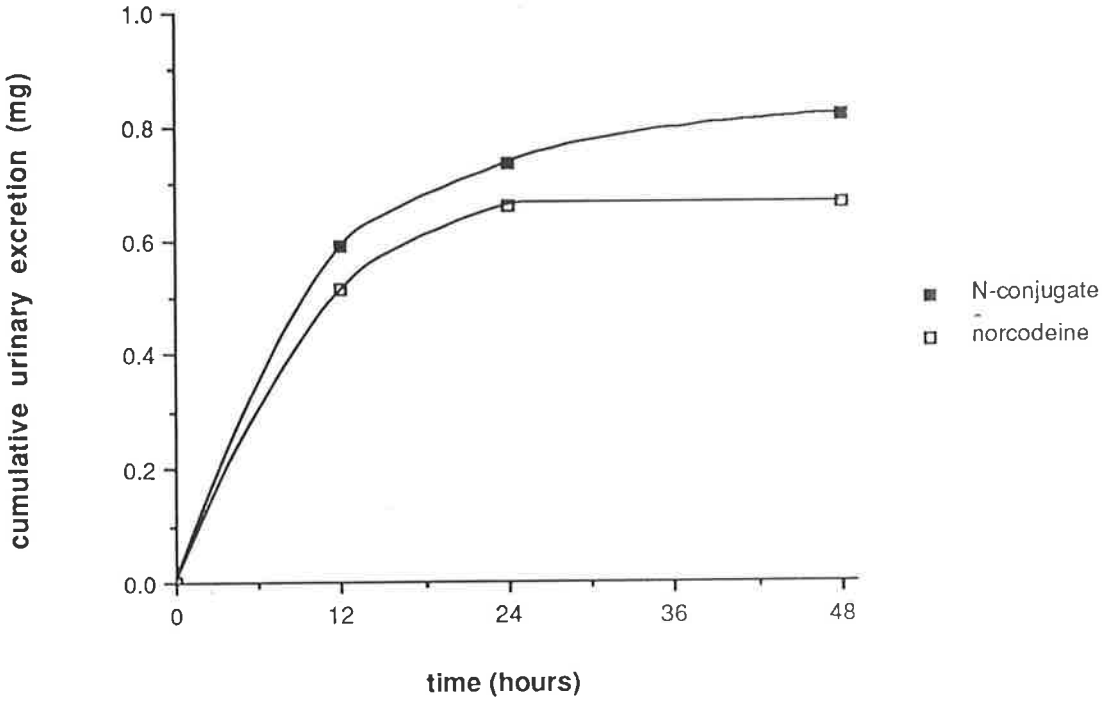


Figure 4-3-10 Mean cumulative excretion of norcodeine and its conjugates (N-conjugate) in urine after a single oral dose of 30 mg codeine phosphate in 8 healthy volunteers.

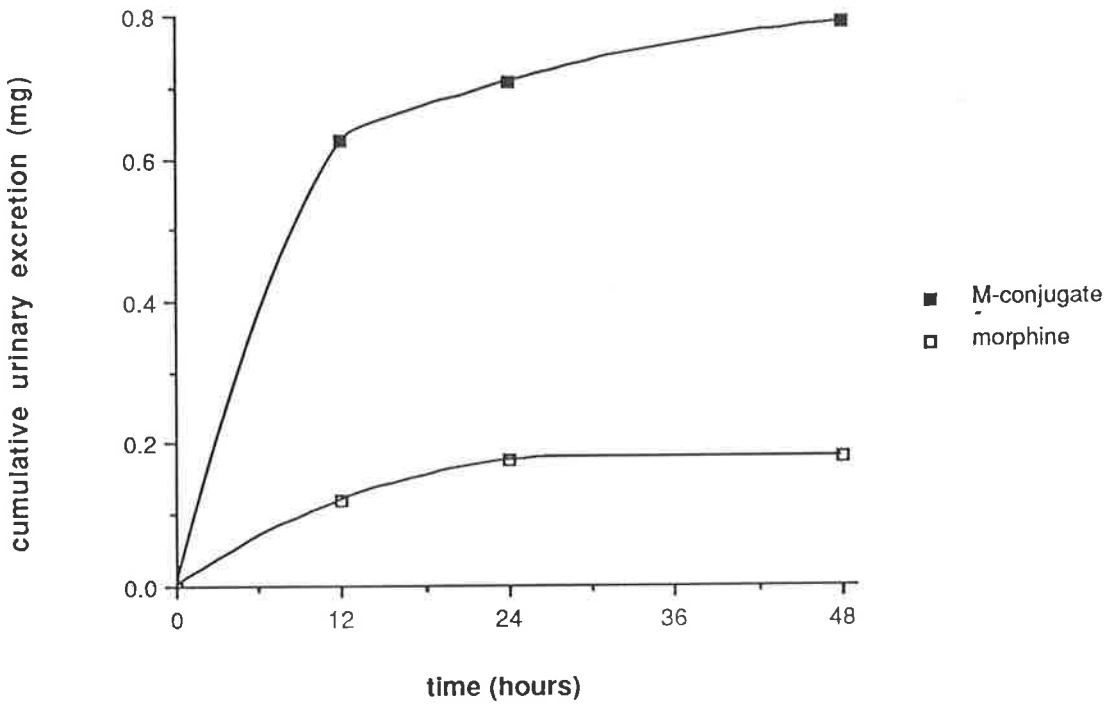


Figure 4-3-11 Mean cumulative excretion of morphine and its conjugates (M-conjugate) in urine after a single oral dose of 30 mg codeine phosphate in 8 healthy volunteers.

Table 4-3-6 urinary excretion (% of dose) of codeine and metabolites after a single oral dose of 30 mg codeine phosphate

subject	codeine	C-6-G	morphine	M-G	norcodeine	N-G	total
1	11.1	58.4	0.3	2.6	3.7	7.2	83.3
2	10.8	67.0	0.0	0.7	1.9	4.6	85.0
3	4.3	64.0	0.7	5.6	1.5	6.9	83.0
4	9.5	40.4	0.9	3.8	0.9	4.5	60.0
5	14.0	63.0	1.0	1.8	5.4	1.1	86.3
6	16.6	66.3	0.4	6.0	4.7	2.4	96.4
7	13.2	70.5	1.0	3.0	3.8	1.8	93.3
8	15.2	48.4	1.3	5.8	2.8	1.8	75.3
mean	11.8	59.8	0.7	3.7	3.1	3.8	82.8
SD	3.9	10.3	0.4	2.0	1.6	2.4	11.3

C-6-G: codeine-6-glucuronide; M-G: morphine-glucuronides;
N-G: norcodeine-glucuronides.

Table 4-3-7 Urinary excretion (% of dose) of codeine and metabolites after chronic oral doses of 30 mg codeine phosphate 8 hourly

subject	codeine	C-6-G	morphine	M-G	norcodeine	N-G	total
1	11.9	72.1	0.6	0.5	1.9	1.7	88.7
2	16.7	68.3	0.0	0.3	9.3	1.0	95.6
3	15.4	69.8	0.7	4.6	3.7	2.2	96.4
4	16.0	44.5	0.4	4.6	3.2	1.3	70.0
5	6.2	47.8	0.2	3.2	2.4	3.4	63.2
6	16.3	50.5	0.6	8.0	4.5	4.2	84.1
7	23.4	53.4	0.9	4.6	4.4	2.5	89.2
8	7.9	35.6	1.0	6.9	2.1	1.1	54.6
mean	14.2	55.2	0.6	4.1	3.9	2.2	80.2
SD	5.5	13.4	0.3	2.7	2.4	1.1	15.7

C-6-G: codeine-6-glucuronide; M-G: morphine-glucuronides;
N-G: norcodeine-glucuronides.

4.3.4. Discussion

The values obtained in this study for the maximum plasma concentration and its time of occurrence, the area under the plasma concentration-time curve and the plasma half-life of codeine after single dose administration are similar to some reported previously (Findlay *et al* 1977; 1978; 1986; Rogers *et al* 1982; Quiding *et al* 1986). There are differences, however, between my results and those of Guay and coworkers (1987). These investigators administered codeine sulphate orally to healthy volunteers on two occasions; once as a single 60 mg dose, and on the other occasion 60 mg every 6 hours for 9 doses. Firstly, the plasma elimination half-life of codeine in my subjects were 2.6-3.6 hours compared with 4.5-5.6 hours reported by Guay and coworkers. Secondly, in my study the mean areas under the plasma concentration curve were 250±20 ng/ml/h and 270±40 ng/ml/h after a single and chronic 30 mg doses respectively. If the kinetics of codeine between 30 and 60 mg are linear, the mean area under the plasma concentration curve after 60 mg codeine should be twice of that after 30 mg. However, the mean areas under the plasma concentration curve after a single and chronic 60 mg doses by Guay and coworkers were 2.6 times (649±308 ng/ml/h) and 3.2 times (855±504 ng/ml/h) higher respectively than those in the present study. In addition their data showed large variations in the kinetic parameters. For example, the mean value for area under the plasma concentration curve showed variations of up to 60%. These differences may have resulted from the nonspecific RIA used in Guay's study. The pharmacokinetics of codeine after multiple doses have been previously reported (Quiding *et al* 1986; Guay *et al* 1987). In contrast to the results of Guay and coworkers (1987), the maximum plasma concentration of codeine in

this study increased significantly after multiple dosing compared to the single dosing and this is in agreement with the findings of Quiding and coworkers (1986). The fact that the areas under the plasma concentration curve of codeine after single and chronic doses were not different indicated that the absorption and disposition were not changed after chronic dosing, at least at the doses used in this study although opioids may reduce gastro-intestinal motility (Jaffe and Martin 1985).

The pharmacokinetics of codeine conjugates have been studied by Guay and coworkers (1987) using β-glucuronidase hydrolysis and radio-immunoassay. In their study, assay values were corrected to reflect the efficiency of enzyme hydrolysis by the recovery of codeine after hydrolysis of standard concentrations of codeine-6-glucuronide in acetate buffer. However, the percentage of codeine-6-glucuronide hydrolysed by β-glucuronidase in buffer and in biological fluids are very different (see Chapter 4.2.) because substances in biological fluids inhibit the activity of the enzyme (Levvy 1952; Boyland and Williams 1960; Combie *et al* 1982). The maximum plasma concentrations of codeine-6-glucuronide in the present study were similar to those reported by Guay and coworkers, although there are some differences in the results between the two studies. Firstly, in contrast to the "apparent prolonged half-life of codeine conjugate" in healthy subjects (Guay *et al* 1987), the mean plasma half-life of codeine-6-glucuronide in my subjects was 3.2 hours which was not significantly different from that of codeine. Secondly, the renal clearance of codeine-6-glucuronide of 0.9 ml/min/kg in this study was larger than the 0.5 ml/min/kg reported by Guay and coworkers. These differences may have resulted from the incomplete hydrolysis and the back-calculation method in the study by Guay and coworkers (1987). The extent of hydrolysis of codeine-6-glucuronide by β-glucuronidase was dependent on the concentration of the

substrate (see Chapter 4.2.). In plasma samples from the first few hours after dosing, codeine-6-glucuronide concentrations were much higher than those in the last few hours' samples. The hydrolysis of codeine-6-glucuronide in the first few hours samples may be less complete than that in the samples from the last few hours under the same hydrolytic conditions. Thus the back-calculation method would probably lead to relatively lower estimated concentrations for the samples with higher codeine-6-glucuronide concentrations and vice versa. As a result, the half-life of codeine-6-glucuronide would be "prolonged". Furthermore, because the concentrations of the substances, which inhibit the activity of the β-glucuronidase, in plasma and urine are likely to be variable from subject to subject and from time to time in the same subject, it may not be possible to calculate the hydrolysis correctly.

The saliva to plasma codeine concentration ratio was substantially greater than unity. Similar results have been reported by Lee and coworkers (1986). The amount of codeine which distributes into saliva is a function of several factors. Some of these are: codeine is a basic compound; it has a high pK_a (8.2, Moffat *et al* 1986); it has low plasma protein binding (<30% Judis 1977); it is highly lipophilic (partition coefficient 3.98, octanol/pH 7.4, Moffat *et al* 1986); and saliva pH is lower than that of plasma. Pholcodine has similar physico-chemical properties to codeine and its saliva to plasma concentration ratio is similar to that of codeine (Chapter 2.2.). Based on the Henderson-Hasselbach equation, the predicted saliva to plasma ratio should be between 2.3 and 22 if saliva pH ranges from 6.0 to 7.0. The strong correlation between saliva to plasma codeine concentration ratio and saliva pH ($r = 0.72$) is consistent with the Henderson-Hasselbalch equation and the known physico-chemical properties of codeine. The finding that the area under the saliva codeine concentration-time curve during the dosing interval at steady state

was higher ($P < 0.05$) than that after single dose may be due to the difference ($p = 0.05$) of the saliva pH between the two treatments, which averaged 6.6 and 6.4 in single and chronic dose respectively. Because the drug concentrations in saliva are sensitive to saliva pH, a small change in the saliva pH will result in a large difference in the saliva drug concentrations. Codeine-6-glucuronide was not detected in saliva. This may be the result of the barrier effect of the cell membrane because codeine-6-glucuronide is very polar and highly water-soluble.

The renal clearance of codeine ranged from 67 to 265 ml/min and indicates that codeine must undergo tubular secretion in addition to glomerular filtration. In addition, the significant correlation between renal clearance and urine pH suggests that reabsorption must also occur. Codeine-6-glucuronide, a strongly acidic compound, fully ionized in plasma and urine should be readily eliminated from the body by the kidneys and its renal clearance should be at least as much as creatinine clearance. However, the renal clearance of codeine-6-glucuronide was much lower than creatinine clearance. Similar results for the morphine glucuronides have been reported in the dog (Garrett and Jackson 1979) and in man (Brunk and Delle 1974; Osborne *et al* 1988). The reasons for this are not clear. There are several possible mechanisms to explain this phenomenon. Firstly, although uncommon, the glucuronides may be reabsorbed after filtration. The reabsorption of some glucuronides, such as aldosterone-glucuronide and estrogen-glucuronide, have been reported (Frimpter *et al* 1963; Brown *et al* 1964; Moller and Sheikh 1983). Active transport of conjugated dihydromorphine, structurally similar to codeine, by the proximal portion of the renal tubules has been demonstrated in dogs and monkeys (Hug *et al* 1965; 1967). This process was inhibited by probenecid. Because of the high water solubility of glucuronides and because of the barrier effect of the cell

membrane, the reabsorption of glucuronides, if it exists, must be by an active or a carrier mediated mechanism. There may be a transport system for glucuronides or it may share the transport systems which exist for other compounds. Secondly, codeine-6-glucuronide may be deconjugated in the kidney and/or urinary tract. β-glucuronidase is present in many tissues and fluids in the body including the kidney and urine (Dutton 1980). Since deconjugation has been shown to occur in the systemic circulation, it may also occur in the kidney and/or urinary tract. Plasma protein binding is another factor which influences the renal clearance value. There are no data on the plasma protein binding of codeine-6-glucuronide. The plasma protein binding of morphine-3-glucuronide was very low (< 2%) in the dog (Garrett and Jackson 1979). Presumably, the plasma protein binding of codeine-6-glucuronide will be similar to that of the morphine glucuronides. Therefore plasma protein binding may not be important for codeine-6-glucuronide.

The urinary excretion of codeine and its metabolites in man has been investigated in several studies (Adler *et al* 1955; Vaughan & Beckett 1973; Nomof *et al* 1977). The total recovery from urine in this study (81±13% of the dose) is comparable to the findings of 63-86% by Adler and coworkers (1955) and 56-88% by Nomof and coworkers (1977). The recovery of unchanged codeine of 13±4.5% of the dose agrees well with previous results of 11% (Adler *et al* 1955) and 10% (Vaughan & Beckett 1973). The total (conjugated and unconjugated) morphine recovery of 5% is comparable to that of the previous observations, which were 5-13% (Adler *et al* 1955) and 4.5-7.4% (Nomof *et al* 1977). The total norcodeine recovery of 6.5%, however, was much lower than 16% reported by Adler and coworkers (1955) but similar to that of 5.6% by Nomof and coworkers (1977). My results which show that 36-70% of the dose is excreted in urine as codeine-6-glucuronide corroborate the previous findings that codeine glucuronide is the major quantitative

metabolite of codeine in man (Oberst 1941; Adler *et al* 1955). The present average value of 55%, however, is higher than that of 45% determined after acidic hydrolysis (Adler *et al* 1955). There is little information available about norcodeine conjugation in man. Adler and coworkers (1955) reported that in one subject, the ratio of the bound form (conjugated) to the free form (unconjugated) was 5.1. However the authors stated "... the values which in most cases were of doubtful significance", because of the inadequate assay sensitivity. The hydrolysis of morphine- and norcodeine- glucuronides by β-glucuronidase was not directly tested because these glucuronides were not available when the study was performed. It was reported that the percentage of hydrolysis of morphine-3-glucuronide in buffer by β-glucuronidase was 93% and for morphine-6-glucuronide was 65% (Yoshimura *et al* 1968). Svensson and coworkers (1982) reported that β-glucuronidase resulted in complete hydrolysis of morphine-3-glucuronide and 74% hydrolysis of morphine-6-glucuronide. The reasons for the difference are not clear, but may have resulted from the difference in their chemical structure. Morphine-3-glucuronide is a phenolic glucuronide whereas morphine-6-glucuronide is an alcoholic glucuronide. Because only 5-10% of the dose is metabolised to morphine and because 80% of morphine is conjugated to morphine-3-glucuronide (Säwe *et al* 1985), the hydrolysis for morphine glucuronide may be almost complete. There is little information about norcodeine conjugates. The hydrolysis of this glucuronide by β-glucuronidase may not be complete considering its structural similarity to that of codeine- and morphine- 6-glucuronide. However, the low concentrations of these glucuronides may have allowed maximal hydrolysis. Further investigation of these glucuronides by direct determination is necessary.

The extent of O-demethylation of codeine showed wide inter-individual variation after single and chronic doses. Subject 2 is particularly noteworthy since only a small amount of morphine was recovered in urine after single and chronic administration of codeine. The morphine/codeine ratio was much less than that of the average in the other subjects. This finding led me to investigate the possibility of polymorphism of codeine O-demethylation (see Chapter 4.5.).

4.4. Pharmacokinetics and disposition of codeine in elderly patients

4.4.1. Introduction

There are relatively few data available on the pharmacokinetics of codeine in humans considering its wide use as an analgesic and antitussive drug for many years. Specifically, the pharmacokinetics of codeine in the elderly have not been studied. The pharmacokinetics and pharmacodynamics of some other opioid analgesics such as morphine and pethidine are altered in the elderly. The serum concentrations of morphine were approximately 1.5 times higher in patients over 50 years of age than in young patients after intravenous injection (Berkowitz *et al* 1975). Plasma concentrations of pethidine after intramuscular or intravenous injection in patients over 70 years of age were about twice as high as those in patients under 30 years (Mather *et al* 1975). It has been reported that patients with impaired renal function suffered from prolonged respiratory depression when treated with morphine, because of the large accumulation of morphine-6-glucuronide (Osborne *et al* 1986). Matzke and coworkers (1986) observed morphine-like toxicity after therapeutic doses of codeine in three patients and these were reversed by injection of naloxone. Levine (1980) reported that severe narcosis occurred in a patient with chronic renal failure given therapeutic doses of codeine phosphate. The concentrations of codeine were within the "therapeutic range" and morphine was not detectable. However, they did not determine plasma concentrations of codeine-6-glucuronide or morphine-6-glucuronide. Recently Guay and coworkers (1988) reported that the codeine plasma half-life was increased 3-4 times after intravenous injection,

although the area under the plasma concentration-time curve was not significantly different, in patients with end-stage renal function compared with healthy volunteers. The pharmacokinetics of codeine in the elderly may also be altered considering the age-related reduction of renal function (Anderson and Brenner 1986).

The aims of this study were to study

(i) the pharmacokinetics and metabolism of codeine in elderly patients;

(ii) the pharmacokinetics of codeine-6-glucuronide in elderly patients, using sensitive and specific analytical methods.

4.4.2. Methods

4.4.2.1. Patients

Seven patients from The Royal Adelaide Hospital, 3 male and 4 female, who gave written informed consent participated in the study. These patients were receiving Panadeine Forte (containing codeine phosphate 30 mg and paracetamol 500 mg) for pain relief before enrolment in this study. The patients (Table 4-4-1) were aged 79.6±11.3 (mean ± SD) years and weighed 63.7±16.8 kg. Their diagnosis and medications are summarized in Table 4-4-2. Prior to commencement of the study each subject was given a physical examination and a venous blood sample was collected for biochemical (MBA 20) and haematological (CBE) examinations. The creatinine clearance was calculated using the plasma and urine creatinine concentrations determined by high performance liquid chromatography (see Chapter 4.3.2.4.). Some of the results reflecting the patients' renal and hepatic functions are shown in Table 4-4-1.

Table 4-4-1 Patients' data

	subject						
	1	2	3	4	5	6	7
sex	M	M	M	F	F	F	F
age (year)	88	61	84	94	82	69	79
BW (Kg)	67	80	82	55	76	45	41
Cr (mM/L)	0.18	0.08	0.15	0.07	0.15	0.15	0.07
Cl_{cr} (ml/minute)	9.5	120.0	50.1	39.2	20.1	26.3	14.4
urea (mM/L)	14.5	6.4	21.7	6.4	17.0	11.7	5.3
CB (μM/L)	1	1	5	1	1	37	1
TB (μM/L)	6	2	13	11	3	46	15
GGT (U/L)	29	20	80	7	11	130	6
ALP (U/L)	67	54	108	118	183	94	69
LD (U/L)	281	134	278	194	224	363	157
albumin (G/L)	37	46	37	43	38	26	35
Hb (G/dl)	11.2	15.6	10.3	12.9	10.7	15.1	10.7

BW: body weight; Cr: serum creatinine concentration; Cl_{cr}: creatinine clearance; CB: conjugated bilirubin; TB: total bilirubin; GGT: Gamma Glutamate Transaminase; ALP: alkaline phosphatase; LD: lactate dehydrogenase; Hb: hemoglobin.

Normal values are shown in Appendix II

Table 4-4-2 Diagnosis and medications

patient	diagnosis	medication
1	chronic heart failure	digoxin, frusemide, glyceryltrinitrate, captopril,temazepam, metoclopramide, paracetamol
2	arthritis of left knee	temazepam, paracetamol
3	fractured neck of femur	temazepam, cephalothin, frusemide, metoclopramide,prochlorperazine maleate, verapamil, clemastine fumarate, paracetamol
4	spinal fracture	paracetamol, heparin, miconazole
5	fractured neck of femur	digoxin, prochlorperazine maleate, pseudoephedrine, doxepin
6	fractured neck of femur	flucloxacillin, temazepam, tolbutamide, spironolactone, frusemide, folic acid, miconazole
7	fractured neck of femur	pethidine, temazepam, dextropropoxyphene, metoclopramide, paracetamol

This study was approved by the Human Ethics Committee of Royal Adelaide Hospital and the Committee on the Ethics of Human Experimentation of the University of Adelaide.

4.4.2.2. Study design

Each patient received a tablet of Panadeine Forte (containing codeine phosphate 30 mg and paracetamol 500 mg) 8 hourly (0800, 1600, 2400 hour) for at least seven doses. The final dose was given at 0800 hour on day 3. Ten ml venous blood samples were collected into heparinized plastic tubes via an indwelling catheter (Jelco^{TM}, 18 G i.v. catheter placement unit, Critikon, Tampa, USA), kept patent with a stylet (Jelco^{TM}), placed in a forearm vein. The blood sample collecting time were 0, 0.5, 0.75, 1, 1.25, 1.5, 2, 4, 6 and 8 hours after the last dose. All urine was collected over the 0-8 hour interdosing interval (Most patients had a urinary catheter.).

Blood samples were centrifugated immediately and harvested plasma stored in stoppered vials at - 20 °C until analysis. Urine volume was determined gravimetrically and recorded along with the urine pH. An aliquot of well mixed urine was retained in stoppered vials at -20 °C until analysed for drug content.

4.4.2.3. Drugs analysis in plasma and urine

Unconjugated codeine, norcodeine and morphine analyses: Codeine, norcodeine and morphine concentrations in plasma and urine were measured by the HPLC method using fluorescence detection as described in Chapter 4.1..

Codeine-6-glucuronide analysis: Codeine-6-glucuronide in plasma and urine was directly determined using the HPLC assay with fluorescence detection as described in chapter 4.2..

Morphine and norcodeine conjugates analyses:

Urine: The presence of conjugates of norcodeine and morphine in urine was inferred by the generation of norcodeine and morphine following enzymatic hydrolysis as described in 4.3.2.3..

Plasma: Plasma concentrations of morphine-3- and 6-glucuronide were determined by an HPLC method which was recently modified from the method by Svensson and coworkers (1982) and the method for codeine-6-glucuronide in Chapter 4.2.. The HPLC system consisted of a pump (Waters Assoc.), a LS-5 Luminescence Spectrometer (Perkin-Elmer Limited, Beaconsfield, U.K.), a WISP 710 B automatic injector (Waters Assoc., Milford, MA, U.S.A.) and an Omniscribe B-5000 strip-chart recorder (Houston Instruments, Austin, TX, U.S.A.). The 15 cm x 4.6 mm I.D. stainless steel column was packed with Spherisorb 5 µm ODS-2 packing material (Phase Separations, Queensferry, U.K.). The mobile phase comprised 26% acetonitrile in 10 mM phosphate buffer (pH 2.1) containing 1 mM dodecyl sulphate. The flow rate through the column was 1 ml/minute. The excitation and emission wave-lengths of the detector were 230 and 350 nm respectively. Plasma samples were treated in exactly the same manner as that for codeine-6-glucuronide except the internal standard was normorphine instead of dihydrocodeine. The 0, 1, 2, 4 and 8 hours' plasma samples from six elderly patients were analysed. For comparison, the 0, 1, 2, 4 and 8 hours' plasma samples after chronic dosing with 30 mg codeine phosphate 8 hourly for 7 doses from two young healthy subjects (Chapter 4.3.) were also analysed.

4.4.2.4. Creatinine analysis

Creatinine concentrations in plasma and urine were determined as described in Chapter 4.3.2.4..

4.4.2.5. Pharmacokinetic and statistical analyses

To facilitate comparison, the codeine-6-glucuronide concentrations were calculated as codeine content and morphine glucuronides as morphine content. The maximum plasma concentration (C_{max}) and its time of occurence (t_{max}) were determined from the observed data. The area under the plasma concentration-time curve to the last sampling time (AUC_{0-8}) following chronic dose administration was calculated by the trapezoidal rule. The plasma concentration at steady state (C_p^{ss}) was calculated as

$$C_p^{ss} = AUC_{0-8} / 8$$

The elimination rate constant (λ_z) was calculated from the terminal portion of the semilogarithmic concentration-time curve using linear regression analysis. Half-life ($t_{1/2,z}$) in plasma was calculated as

$$t_{1/2,z} = 0.693/ \lambda_z$$

Renal clearance (Cl_R) was calculated as

$$Cl_R = A_e(0-8) /AUC_{0-8}$$

where $A_e(0-8)$ is the amount excreted unchanged over the urine collection interval 0-8 hours. Renal creatinine clearance (Cl_{cr}) was calculated as

$$Cl_{cr} = \text{urinary excretion rate/plasma concentration}$$

Differences in pharmacokinetic data between the elderly patients and the healthy young subjects (Chapter 4.3.) were analysed for statistical

significance by the unpaired t-test. Correlations between renal clearance and either urine pH or urine flow rate and between renal creatinine clearance and renal codeine or codeine-6-glucuronide clearance were determined by linear regression analysis. Correlations between renal clearance and plasma concentrations at steady state were determined by linear regression analysis with logarithmic curve fit. Statistical significance was assumed when $P < 0.05$. All data are reported as mean ± SD.

4.4.3. Results

To compare with the elderly, data from the young subjects (Chapter 4.3.) after 30 mg codeine phosphate 8 hourly for seven doses were used. The average age of the elderly was 2.5 times that of young subjects. The body weight, however, was not significantly different ($P > 0.05$) between the two groups.

4.4.3.1. Codeine

The mean plasma concentration-time profile for codeine in the seven elderly patients is shown in Figure 4-4-1 (and for comparison, the young subjects) and Table 4-4-3 contains the derived pharmacokinetic parameters for codeine. The maximum plasma codeine concentration occurred 2.1 hours after the last dose, which was significantly later ($P < 0.05$) than in the young subjects (Table 4-3-3). The mean maximum plasma concentration of codeine was also significantly higher ($P < 0.05$) than that in young subjects. The plasma elimination half-life of codeine in the elderly was prolonged but was unable to be determined within the sampling period (0-8 hour). The area under the plasma codeine concentration-time curve over the dosing interval in the elderly was 2.4 times higher than in young subjects. There was a

significant correlation ($r = 0.70$, $P < 0.01$) between age and the steady state plasma concentrations of codeine (Figure 4-4-2).

4.4.3.2. Codeine-6-glucuronide

The mean plasma codeine-6-glucuronide concentration-time profile after chronic dosing for the elderly patients is shown in Figure 4-4-3 (and the healthy subjects) and derived pharmacokinetic parameters are shown in Table 4-4-4. The maximum plasma codeine-6-glucuronide concentration was significantly higher ($P < 0.05$) than in the young subjects (Table 4-3-4) and the occurrence of the maximum plasma concentration was significantly ($P < 0.05$) later than in the young subjects. The plasma half-life of codeine-6-glucuronide could not be determined because the plasma concentration-time curve during the sampling period was flat. The area under the plasma codeine-6-glucuronide concentration-time curve over the dosing interval was 3.8 times higher than in the young subjects and 21 times higher than that of codeine in the elderly. There was a significant correlation ($r = 0.86$, $P < 0.01$) between age and the steady state plasma concentrations of codeine-6-glucuronide (Figure 4-4-4).

4.4.3.3. Morphine and morphine glucuronides

The plasma concentrations of unconjugated morphine in six elderly patients were generally very low (< 5 ng/ml). In fact, it was just detectable in plasma in some of the patients. Because the concentration was close to the lower detection limit of the assay, plasma morphine concentrations could not be precisely quantitated. Both morphine-3-glucuronide and morphine-6-glucuronide were readily detected in the plasma samples from the six elderly patients (Figure 4-4-5). In the plasma from one of the two young subjects, however, only morphine-3-glucuronide was detected and it was about 10

times lower than in the elderly (Figure 4-4-5). Neither morphine-3-glucuronide nor morphine-6-glucuronide was detected in the plasma samples from the other young subject, from whose urine only a small amount of morphine (unconjugated and conjugated) was recovered.

4.4.3.4. Renal clearance

Creatinine clearance: The creatinine clearances calculated using the plasma and urine creatinine data in the elderly was substantially reduced and it was significantly ($r = 0.88$, $P < 0.01$) correlated with age (Figure 4-4-6).

Renal clearances of codeine: The renal clearances of codeine in the elderly were reduced by a factor of 5.0 by comparison with those in young subjects. The renal clearance of codeine was significantly correlated with the creatinine clearance (Figure 4-4-7, $r = 0.79$, $P < 0.01$). The plasma concentrations of codeine at steady state were significantly correlated with the renal codeine clearance (Figure 4-4-8, $r = 0.83$, $P < 0.01$) and more strongly correlated with the creatinine clearance (Figure 4-4-9, $r = 0.94$, $P < 0.01$). There was a significant correlation between the renal clearance of codeine and codeine-6-glucuronide (Figure 4-4-10, $r = 0.83$, $P < 0.01$). The renal codeine clearance was significant correlated with age (Figure 4-4-11, $r = 0.84$, $P < 0.01$).

Renal clearance of codeine-6-glucuronide: The renal clearances of codeine-6-glucuronide in the elderly were reduced by a factor of 7.2 by comparison with those in young subjects and the renal clearance of codeine-6-glucuronide was significantly correlated with the creatinine clearance (Figure 4-4-12, $r = 0.75$, $P < 0.01$). The plasma concentrations of codeine-6-glucuronide at steady state were significantly correlated with the creatinine clearance (Figure 4-4-13, $r = 0.84$, $P < 0.01$) and more strongly

correlated with the renal codeine-6-glucuronide clearance (Figure 4-4-14, r = 0.94, P < 0.01). There was a significant correlation between age and renal codeine-6-glucuronide clearance (Figure 4-4-15, r = 0.87, P < 0.01).

4.4.3.5. Urinary excretion

The percentage of the dose excreted in urine of codeine, norcodeine, morphine and their conjugates is shown in Table 4-4-5. The urinary excretion of all the compounds was lower than in the young subjects (P < 0.05). The amount excreted in urine for codeine (Figure 4-4-16, r = 0.73, P < 0.01) and codeine-6-glucuronide (Figure 4-4-17, r = 0.71, P < 0.01) was significantly correlated with the creatinine clearance. There were significant correlations between age and urinary excretion rate of codeine (Figure 4-4-18, r = 0.78, P < 0.01) or codeine-6-glucuronide (Figure 4-4-19, r = 0.87, P < 0.01).

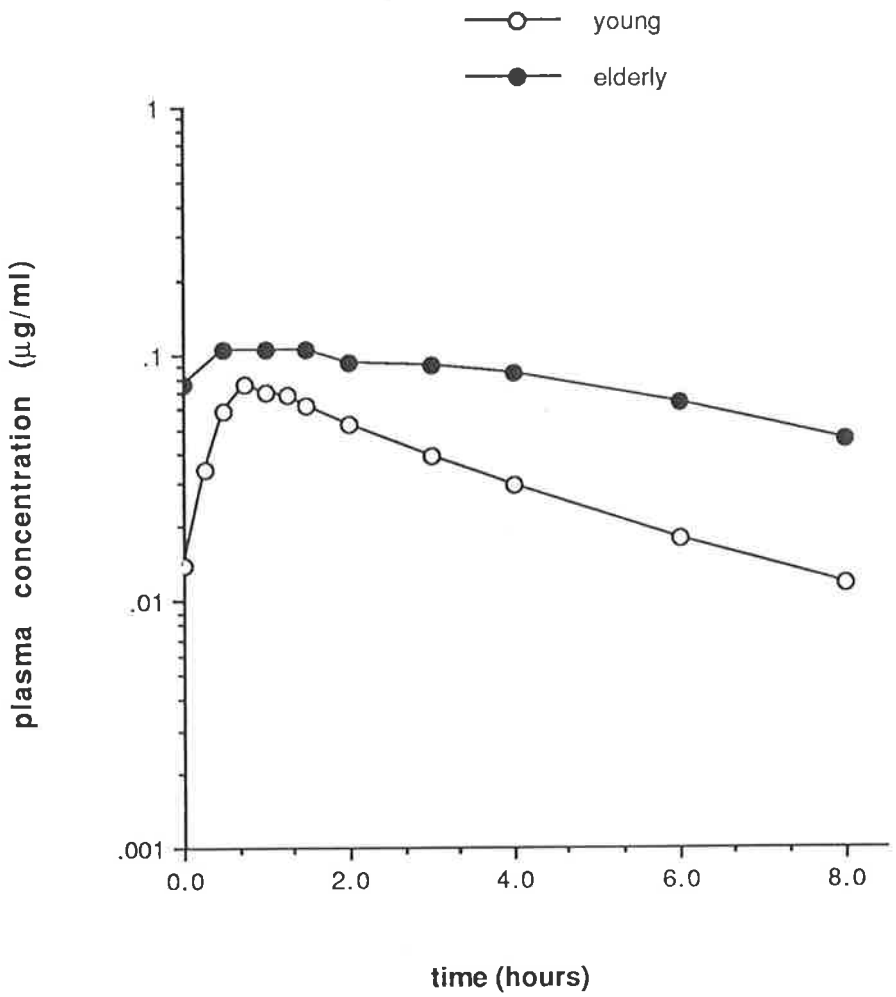


Figure 4-4-1. Mean plasma concentrations of codeine after 30 mg codeine phosphate orally 8 hourly for 7 doses in the elderly and young healthy subjects.

Table 4-4-3. Pharmacokinetic parameters for codeine in the elderly after codeine phosphate 30 mg 8 hourly for 7 doses

subject	C_{max} (ng/ml)	t_{max} (h)	AUC_{0-8} (mg·h/L)	C^{ss} (ng/ml)	Cl_R (ml/min)
1	195	3	1.15	144	11
2	68	1	0.29	36	154
3	91	1	0.26	33	30
4	79	3	0.47	59	20
5	206	3	1.00	125	5
6	278	1	0.63	79	8
7	88	3	1.08	135	13
mean	143	2.1	0.70	87	39
SD	82	1.2	0.38	47	57
young					
mean	76.5	0.9	0.27	34	198
SD	8.0	0.2	0.04	5	63
P	<0.05	<0.05	<0.01	<0.01	<0.001

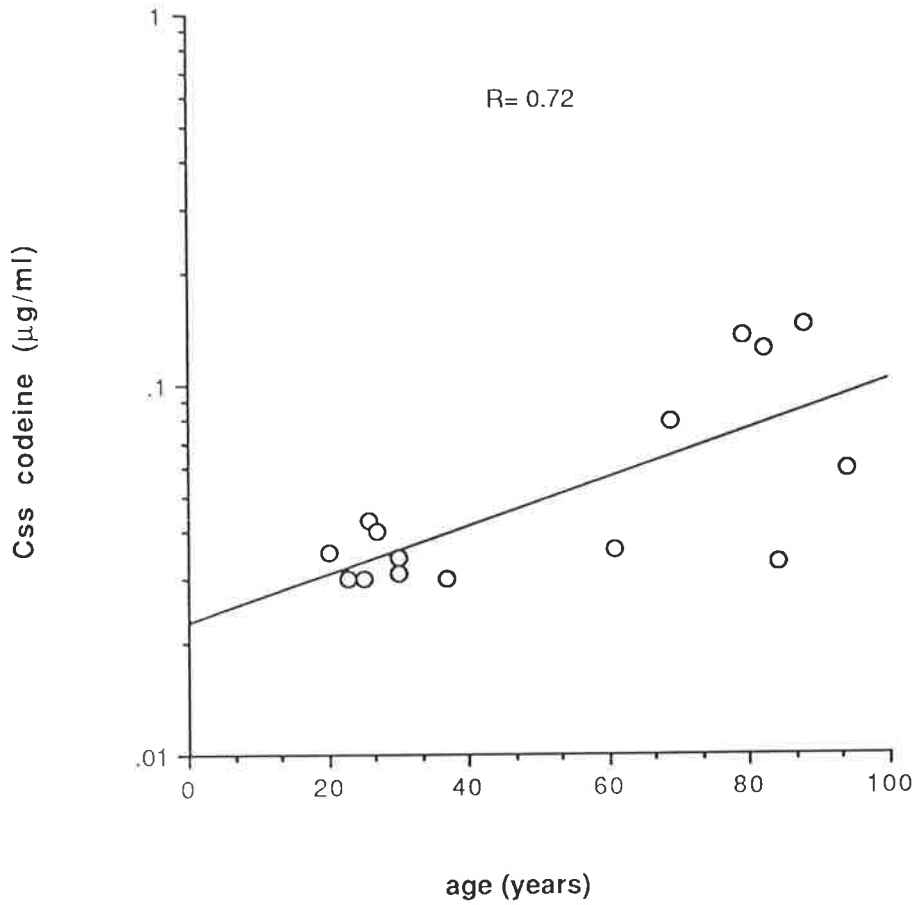


Figure 4-4-2. Correlation between age and the plasma codeine concentration at steady state.

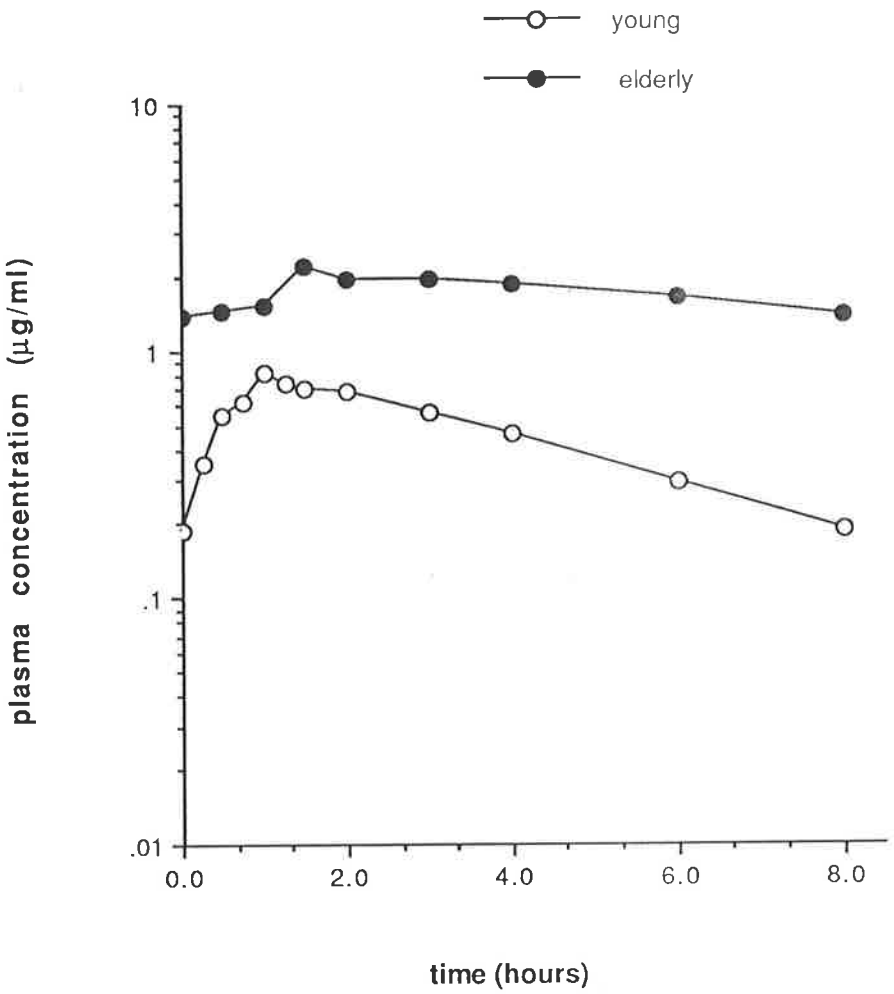


Figure 4-4-3. Mean plasma concentrations of codeine-6-glucuronide after 30 mg codeine phosphate orally 8 hourly for 7 doses in the elderly and young healthy subjects.

Table 4-4-4 Pharmacokinetic parameters for codeine-6-glucuronide in the elderly after codeine phosphate 30 mg 8 hourly for 7 doses

subject	C_{max} (ng/ml)	t_{max} (hour)	AUC_{0-8} (mg·h/L)	C^{ss} (ng/ml)	Cl_R (ml/min)
1	2500	4.0	19.0	2380	2.5
2	1407	2.0	7.1	890	15.5
3	3574	1.5	13.4	1680	4.8
4	1305	3.0	8.9	1110	7.8
5	4072	2.0	27.6	3450	0.9
6	2124	1.0	7.8	980	17.3
7	1149	2.0	12.1	1510	10.0
mean	2304	2.1	13.7	1720	8.5
SD	1150	1.1	7.4	920	6.3
young					
mean	864	1.1	3.6	559	61
SD	158	0.4	0.6	174	24
P	<0.01	<0.05	<0.01	<0.01	<0.001

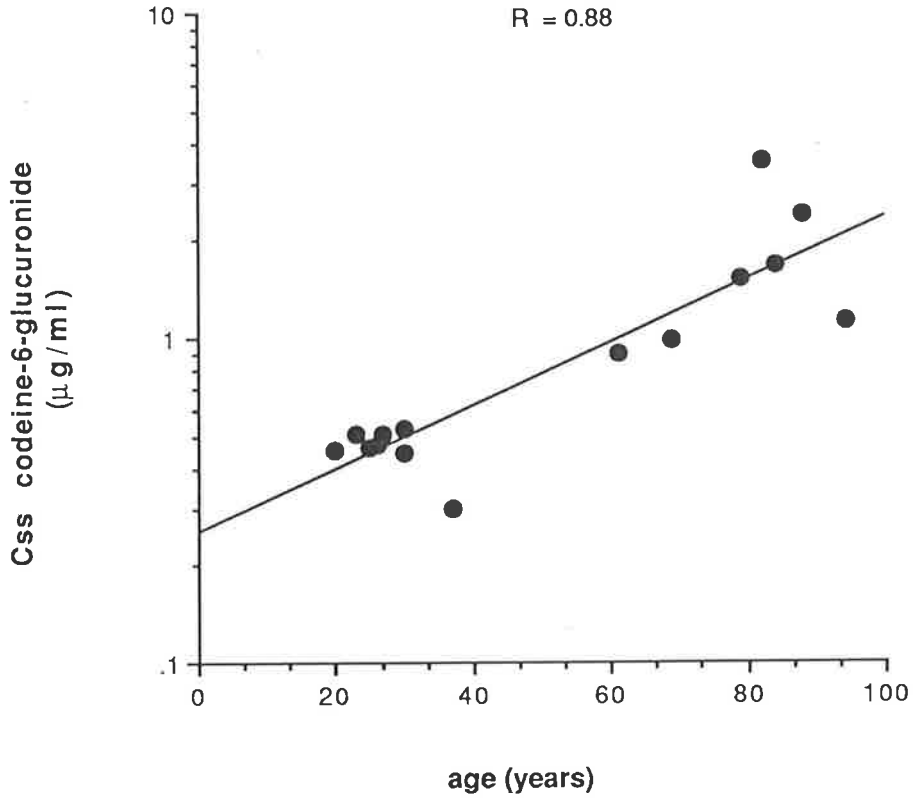


Figure 4-4-4. Correlation between age and the plasma codeine-6-glucuronide concentration at steady state.

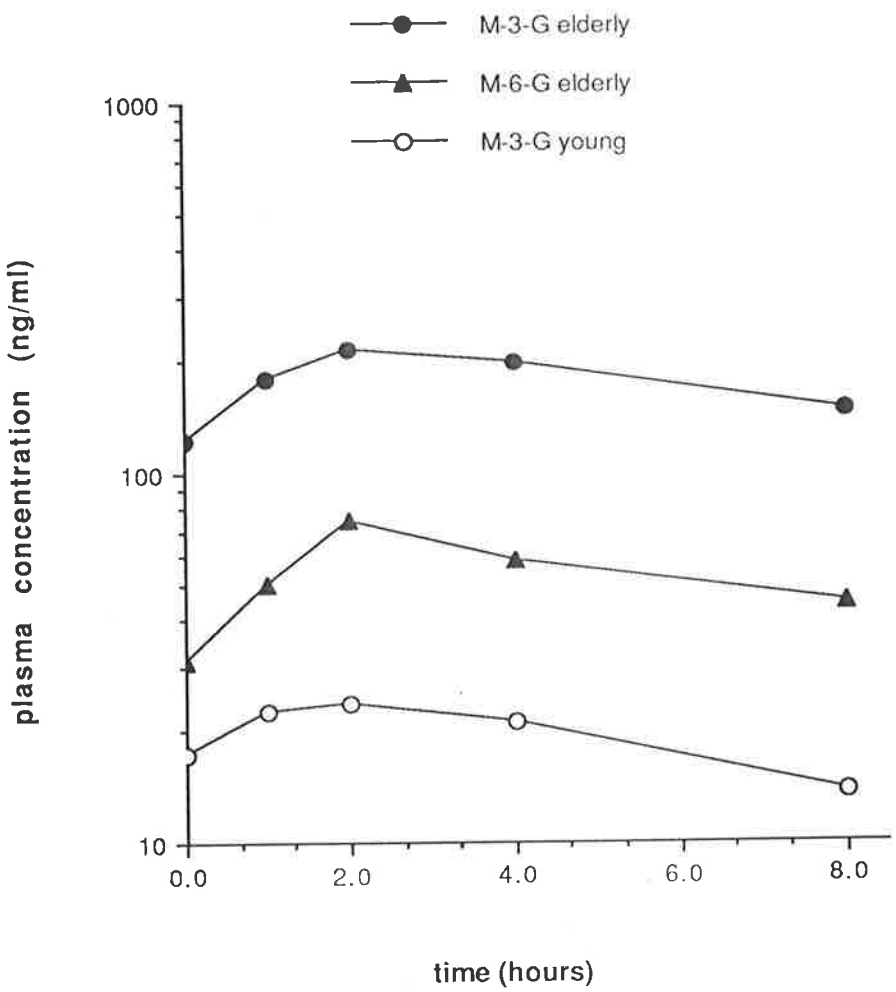


Figure 4-4-5. Mean plasma concentrations of morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G) after chronic dosing of 30 mg codeine phosphate 8 hourly in the elderly patients (n=6, mean±SD) and in one young subject.

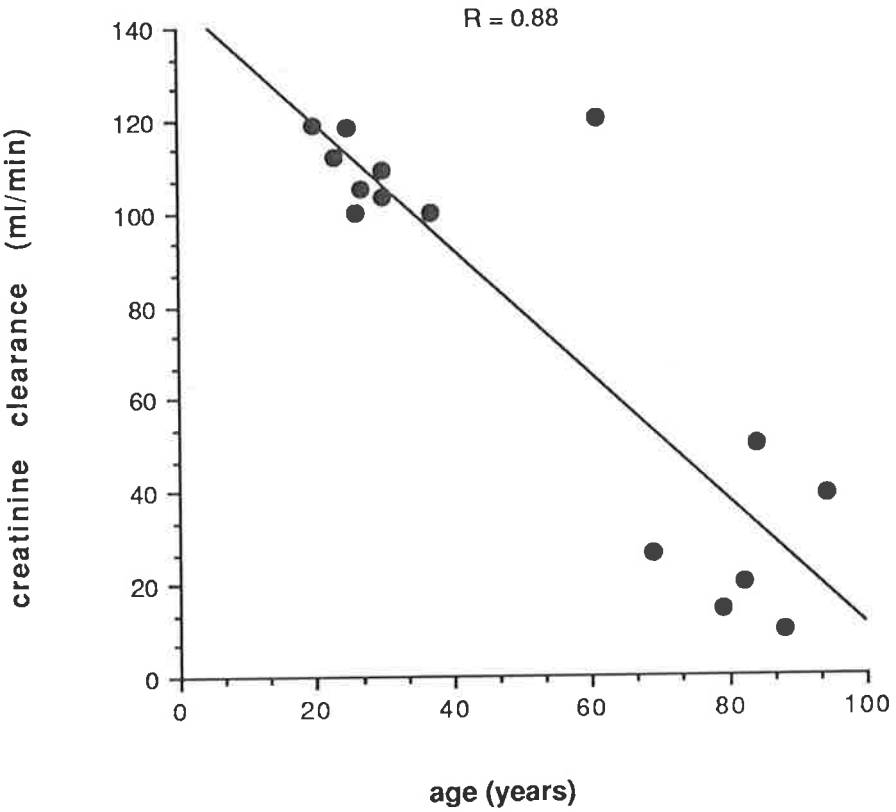


Figure 4-4-6. Correlation between age and creatinine clearance.

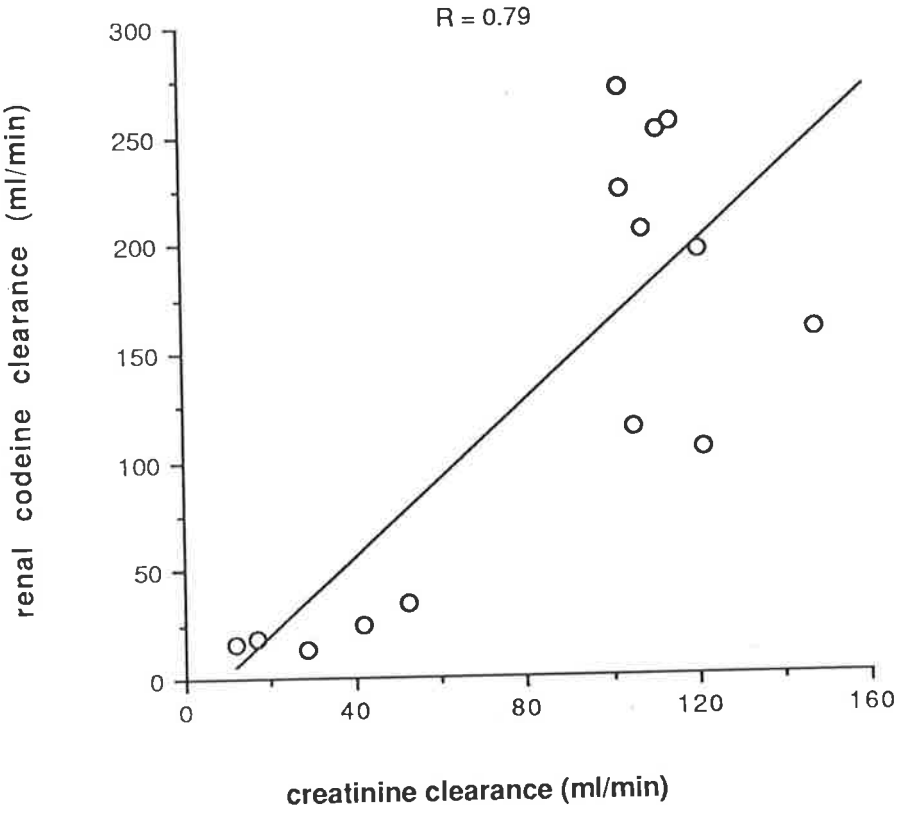


Figure 4-4-7. Correlation between creatinine clearance and the renal clearance of codeine.

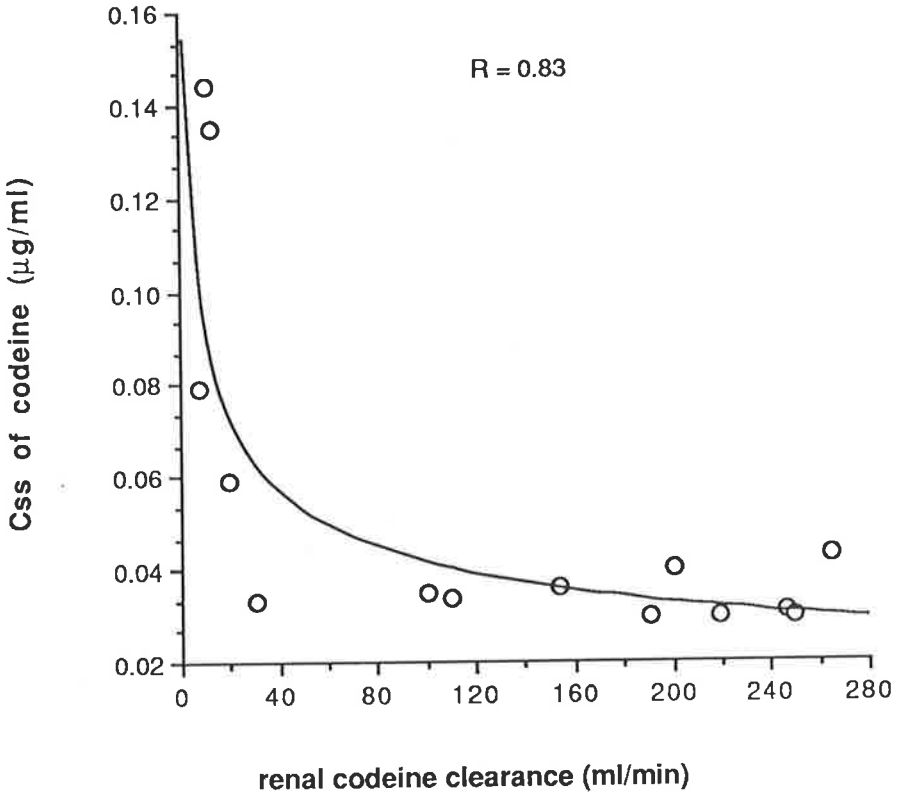


Figure 4-4-8. Correlation between renal codeine clearance and the plasma concentration of codeine at steady state.

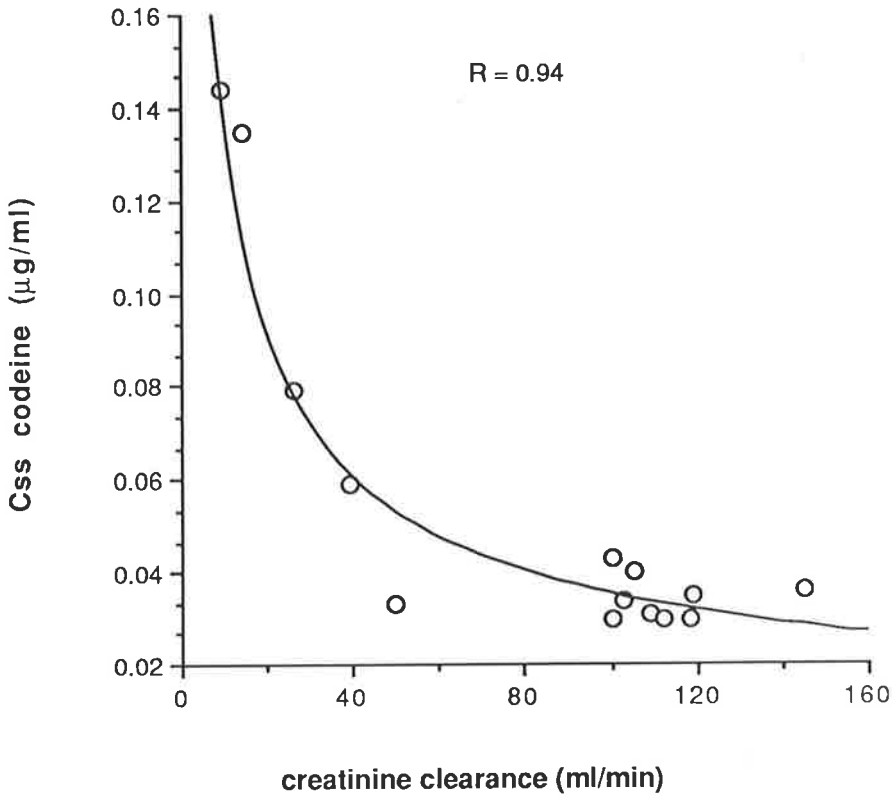


Figure 4-4-9. Correlation between creatinine clearance and the plasma concentration of codeine at steady state.

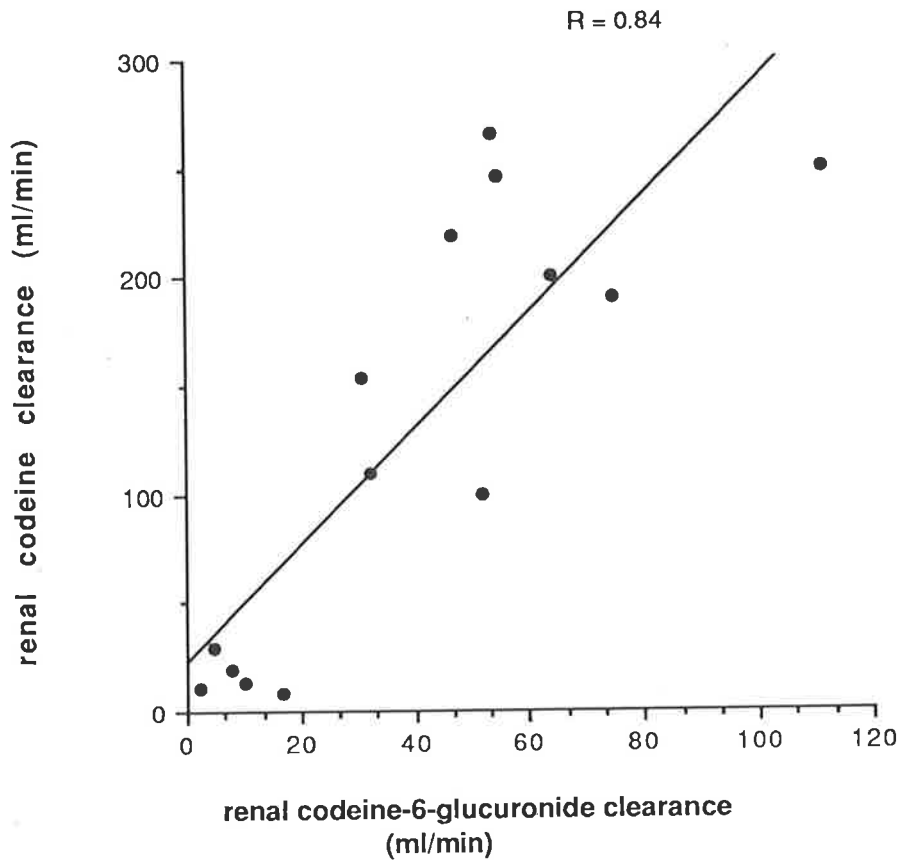


Figure 4-4-10. Correlation between renal clearances of codeine and codeine-6-glucuronide.

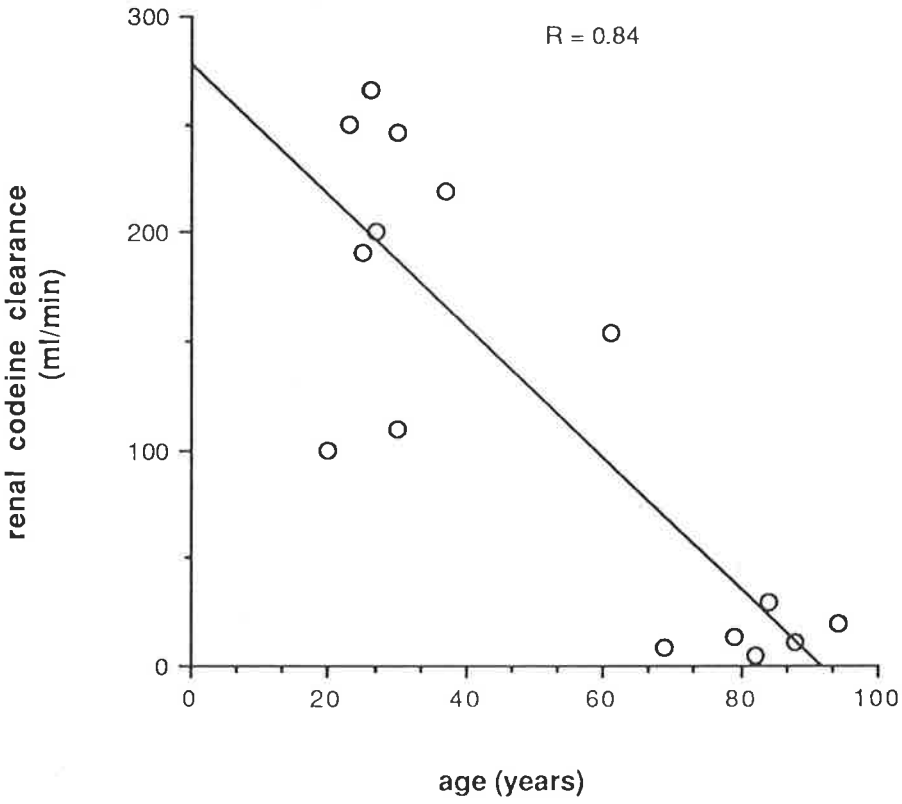


Figure 4-4-11. Correlation between age and renal clearance of codeine.

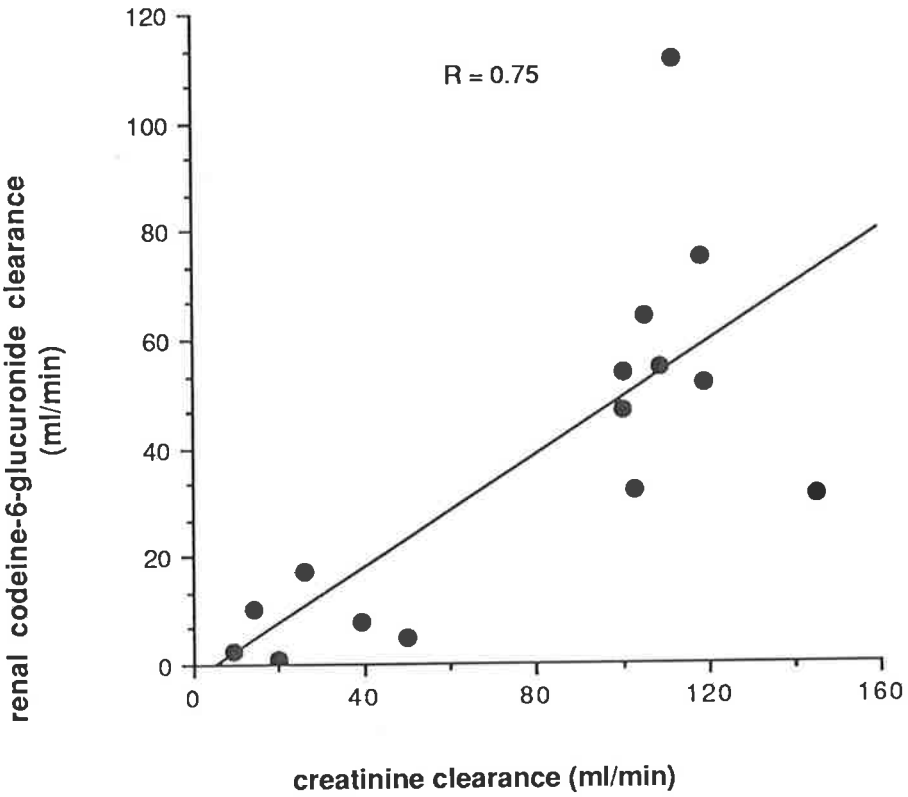


Figure 4-4-12. Correlation between creatinine clearance and the renal clearance of codeine-6-glucuronide.

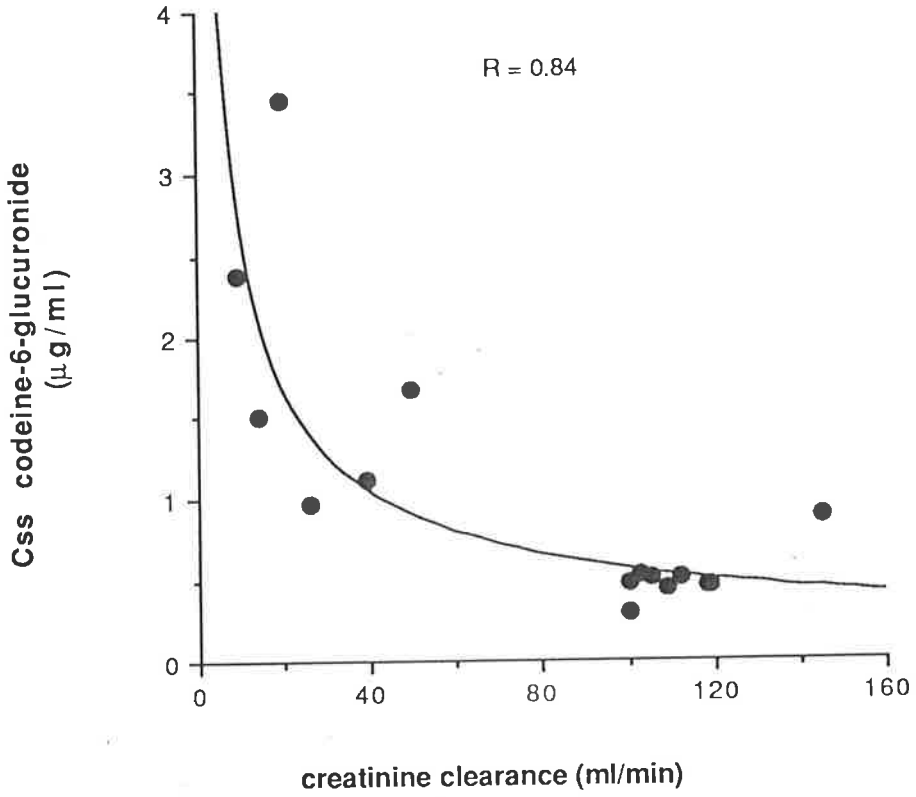


Figure 4-4-13. Correlation between creatinine clearance and the plasma concentration of codeine-6-glucuronide at steady state.

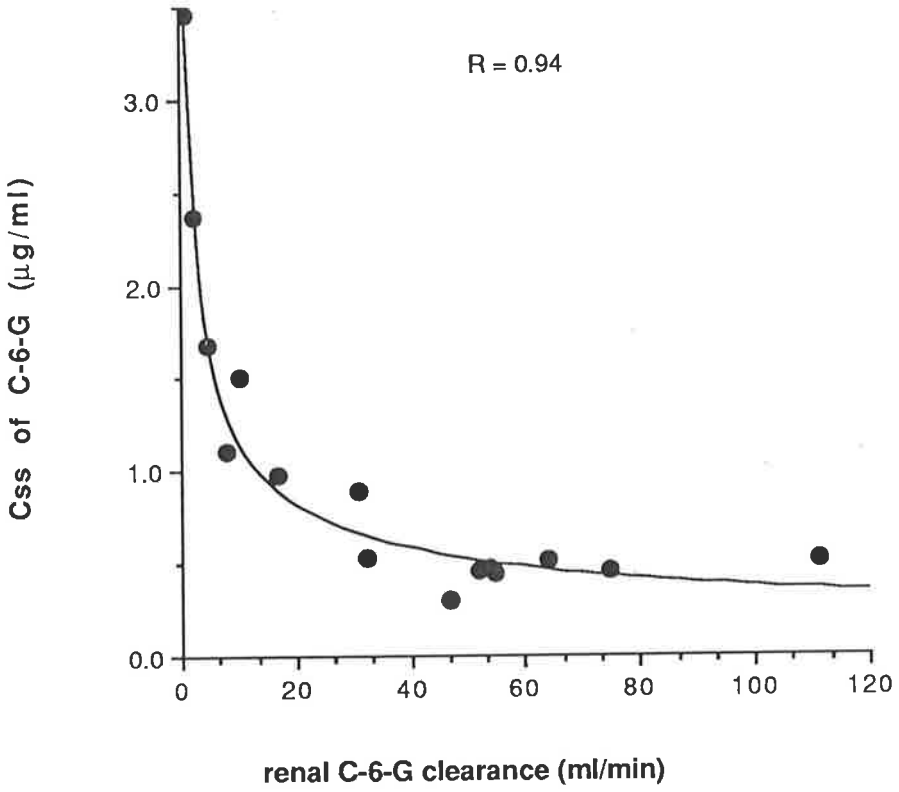


Figure 4-4-14. Correlation between renal codeine-6-glucuronide clearance and the plasma concentration of codeine-6-glucuronide at steady state.

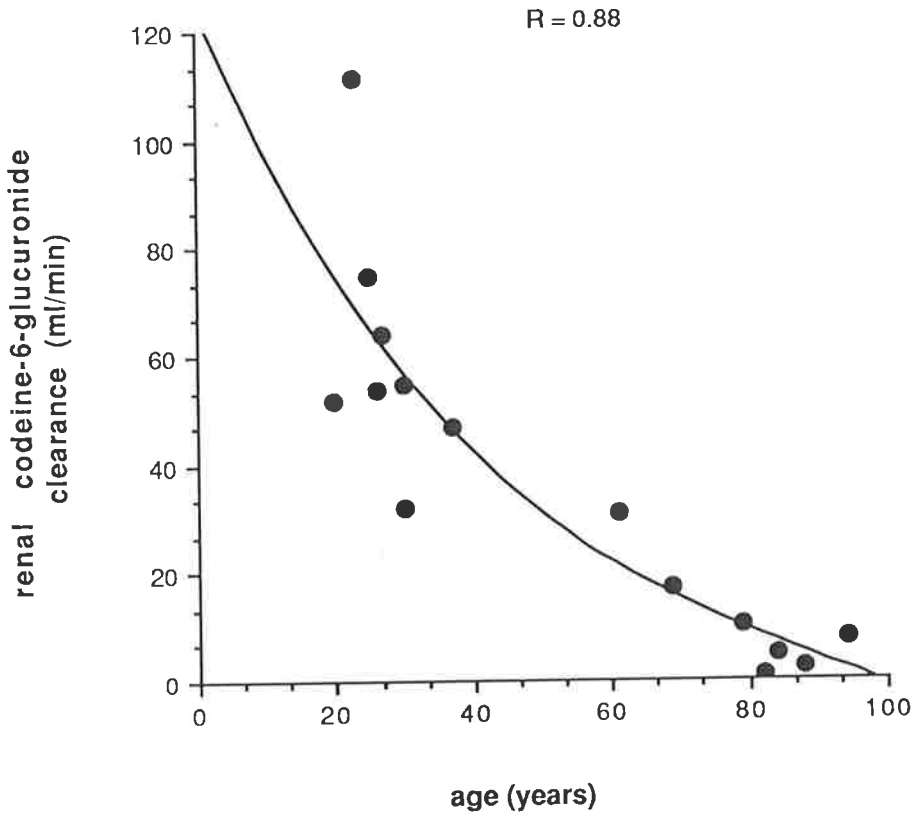


Figure 4-4-15. Correlation between age and renal clearance of codeine-6-glucuronide.

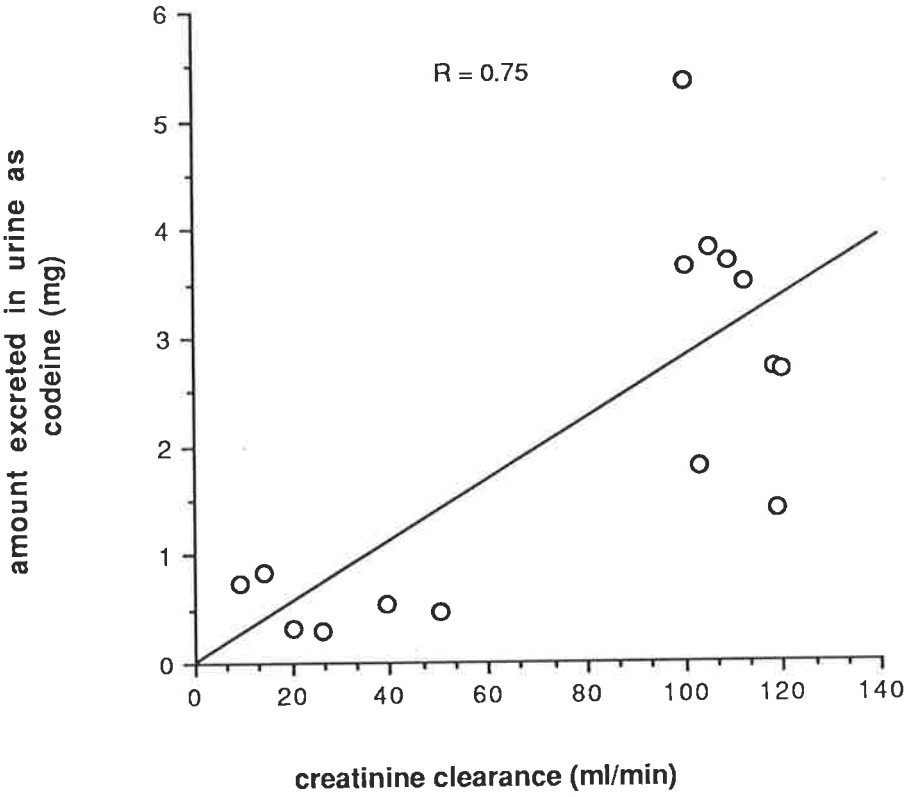


Figure 4-4-16. Correlation between creatinine clearance and the amount excreted in urine as codeine.

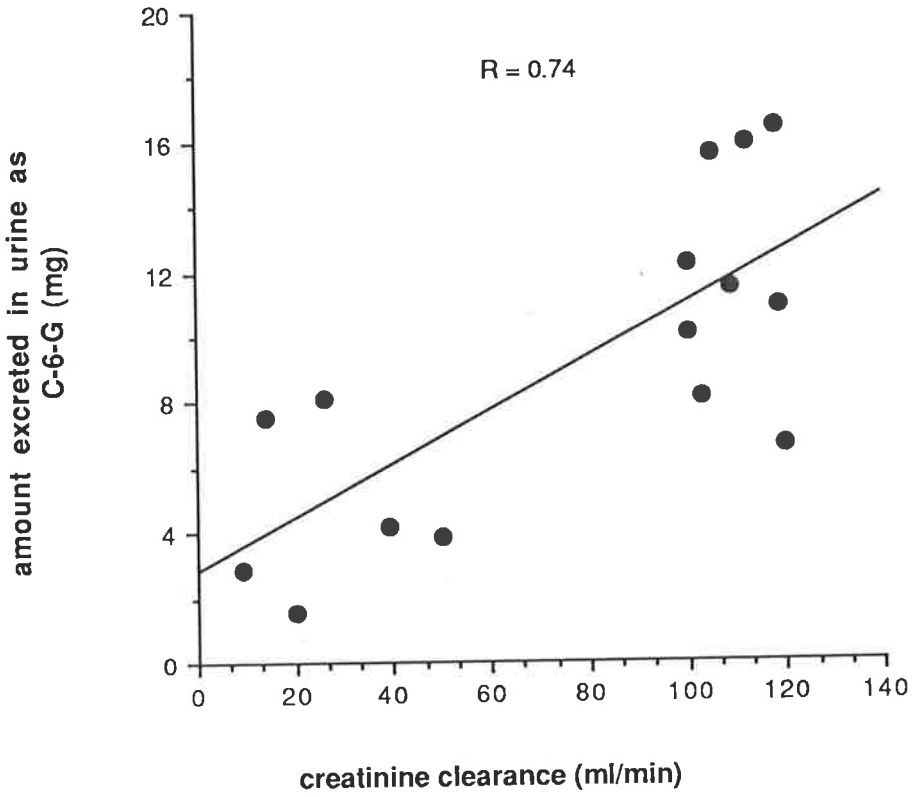


Figure 4-4-17. Correlation between creatinine clearance and the amount excreted in urine as codeine-6-glucuronide.

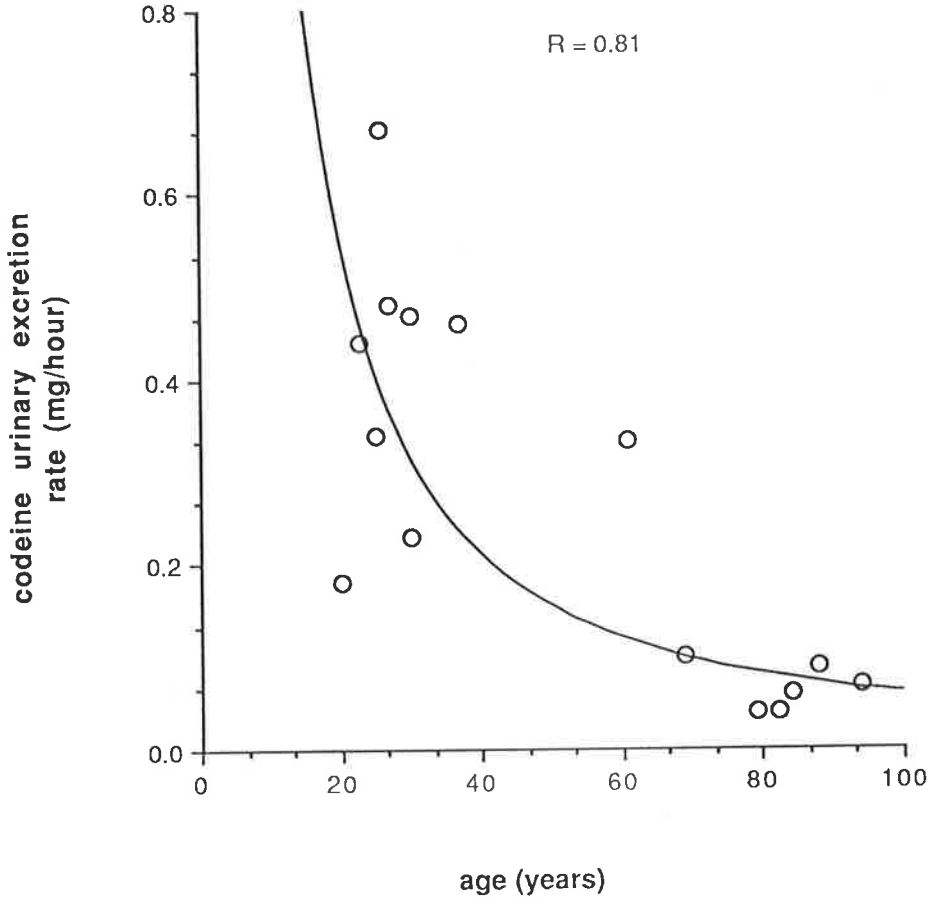


Figure 4-4-18. Correlation between age and the urinary excretion rate of codeine.

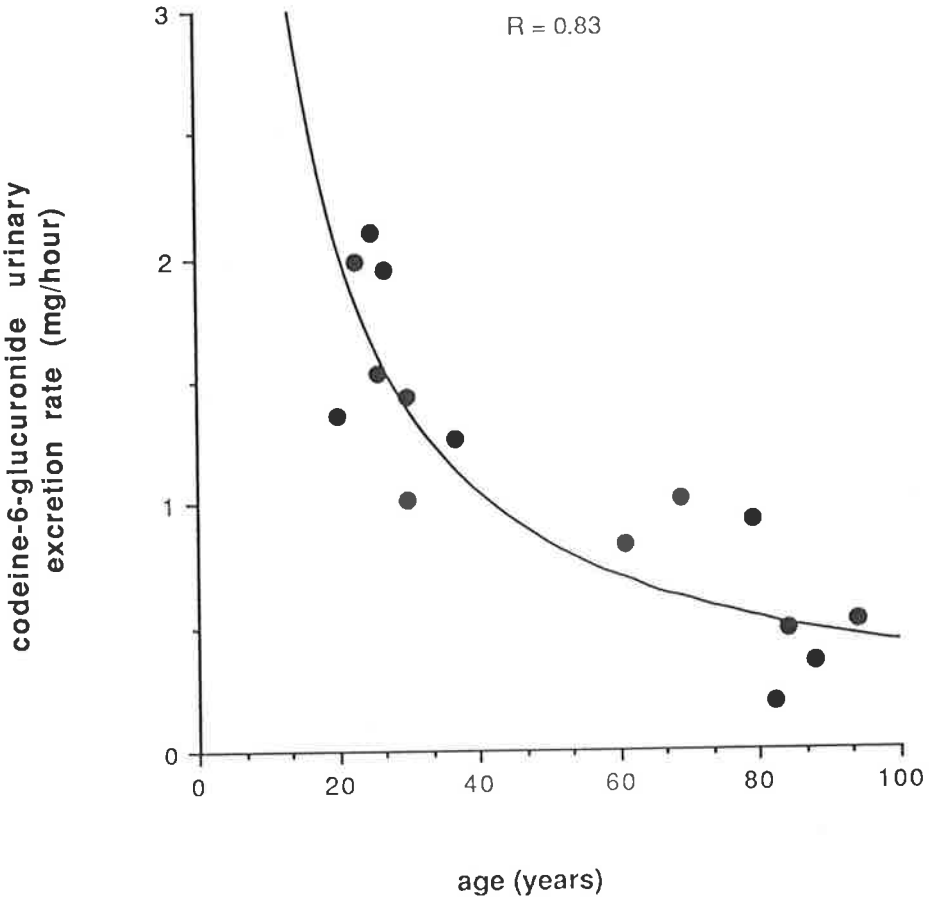


Figure 4-4-19. Correlation between age and the urinary excretion rate of codeine-6-glucuronide.

Table 4-4-5. Urinary excretion of codeine, codeine-6-glucuronide, total morphine and total norcodeine in the elderly after codeine phosphate 30 mg 8 hourly for 7 doses

	% dose excreted in urine				
subject	codeine	C-6-G	morphine*	norcodeine*	total
1	3.4	13.0	1.7	0.5	18.6
2	12.0	30.1	2.7	1.7	46.5
3	2.1	17.7	1.6	1.0	22.4
4	2.5	18.8	0.1	1.9	23.3
5	1.4	7.1	0.4	0.4	9.3
6	3.8	36.9	0.3	0.7	41.7
7	1.4	34	0.3	0.2	35.9
mean	3.8	22.5	1.0	0.9	28.2
SD	3.7	11.3	1.0	0.7	13.4

C-6-G: codeine-6-glucuronide;

*: unconjugated + conjugated

4.4.4. Discussion

To my knowledge there are no published data available on the pharmacokinetics of codeine in the elderly. Compared with the young subjects, the pharmacokinetics of codeine in the elderly were significantly altered. The occurence of the maximum plasma concentration was significantly later than that in the young subjects, and this suggests that the absorption rate of the drug might be reduced. There are several factors which can change the absorption from the gastrointestinal tract. The gastric emptying time, intestinal motility, number of functional absorbing cells and intestinal blood flow are all reduced in the elderly (Schmucker 1985). These changes might be expected to lead to decreased absorption. In addition, the tablets, Panadeine Forte, taken by the elderly were a mixture of codeine and paracetamol, whereas the young subjects took the tablets containing codeine phosphate only. Since the patients were all taking Panadeine Forte prior to enrolment in the study, this combination medicine was continued. There is no information whether paracetamol influences codeine absorption and/or disposition. This will be tested in this laboratory in a later time, as will the pharmacokinetics of codeine after Panadeine Forte in young subjects.

The renal clearances of codeine and codeine-6-glucuronide in the elderly were significantly reduced by comparison with the young subjects and significantly correlated with the creatinine clearance. The reduction in renal function with age has been well characterized (Schmucker 1985; Anderson and Brenner 1986). Glomerular filtration, tubular secretion and renal blood flow are all reduced in the aging kidney. In addition to the normal decline in the renal function, a variety of disease states, such as chronic heart failure or diabetes can further reduce renal drug elimination.

(The renal clearances of codeine and codeine-6-glucuronide have been discussed in Chapter 4.3.).

The plasma concentrations of codeine and codeine-6-glucuronide at steady state in the elderly were increased 2.4 and 3.8 times respectively by comparison with the young subjects. The strong correlation between the renal clearance and the plasma concentration at steady state of codeine and codeine-6-glucuronide indicated that the elevated plasma concentrations were mainly caused by the reduced renal elimination although some other factors might also contribute to it. Codeine is mainly metabolized by glucuronidation presumably mainly in the liver and only 10% of the dose is eliminated unchanged by the kidneys. One would anticipate that the impaired renal function will not cause large accumulation of codeine. There are two main factors, apart from renal function, which may be involved in causing the accumulation of codeine: (1) systemic deconjugation of codeine-6-glucuronide. β-glucuronidase has been demonstrated to be present in most tissues, particularly in liver, kidney, spleen, intestinal epithelium and endocrine organs and the levels of the enzyme were elevated in some disease states (Dutton 1980). Some investigators have shown that some drugs regenerate from their glucuronide conjugates and such a process occurs in renal failure (Fead and McQeen 1979, Guglar *et al* 1979, Fead 1980, Walle *et al* 1979); (2) reabsorption of codeine following biliary excretion of codeine-6-glucuronide and hydrolysis in the gut. Enterohepatic recycling of morphine conjugates has been shown to occur in the rat (Peterson and Fujimoto 1973; Walsh and Levine 1975), cat (Yeh *et al* 1971), dog (Garrett and Jackson 1979), rhesus monkey (Mellett and Woods 1956) and in man (Elliott *et al* 1954). It was reported that the reduction in the urinary excretion of oxazepam glucuronide in renal failure led to increase in biliary excretion of the

conjugate (Odar-Cederlöf *et al* 1977). Such a process may also operate with codeine. Studies will be performed in this laboratory to test these hypotheses.

Changes in the disposition of codeine in the elderly are secondary to the physiologic changes that alter organ function throughout the body. Renal function may be the most important one. Although the pharmacokinetics of codeine in patients with end-stage renal failure have been studied (Guay *et al* 1988), because of the non-specificity of the RIA method and because of the incomplete enzymatic hydrolysis of codeine glucuronide, the authors could not find any difference, except the prolonged half-life of codeine, in the pharmacokinetic parameters between healthy volunteers and the patients with renal failure. Thus Guay's study only provided little information on the pharmacokinetics of codeine in patients with renal failure. Studies on the pharmacokinetics of morphine in patients with renal failure, using specific HPLC methods and direct determination of the glucuronide, have shown that the pharmacokinetics are altered and the glucuronides of morphine accumulated to markedly high levels in plasma (Ball *et al* 1985; Osborne *et al* 1986; Säwe and Odar-Cederlöf 1987). Codeine is considered to be eliminated by hepatic metabolism, and no dosage adjustments are currently recommended in the elderly and in patients with renal failure (Cheigh 1977). My results have shown that the pharmacokinetics of codeine and especially codeine-6-glucuronide are markedly changed in the elderly, whose renal function was decreased. Codeine-induced narcotism have been reported recently (Levine 1980; Matzke *et al* 1986). Codeine-6-glucuronide may be an important factor in causing toxicity considering its enormously high concentrations in plasma and by analogy with the marked pharmacological activity of morphine-6-glucuronide (Yoshimura *et al* 1973; Joel *et al* 1985; Pasternak *et al* 1987; Osborne *et al* 1988). The binding affinity to the μ-opioid receptor of codeine-6-glucuronide is similar to that of codeine

(see Chapter 4.6.), but its plasma concentrations were more than 20 times higher than those of codeine in the elderly. Thus the large accumulation of codeine-6-glucuronide may be responsible for the toxicity of codeine in renal failure patients (Levine 1980; Matzke *et al* 1986). Codeine may have a higher risk of side-effects in the elderly than in young subjects because of the natural reduction of renal function with age. This may cause a considerable community problem since codeine is used frequently in the elderly. Therefore the dosage regimen for elderly may need to be modified.

Morphine glucuronides also accumulated in the plasma of the elderly patients receiving codeine, and this is in agreement with the previous findings in patients with renal failure (Barnes *et al* 1984; McQuay and Moore 1984; Ball *et al* 1985; Michie *et al* 1985; Sear *et al* 1985; Shelly and Park 1985; Osborne *et al* 1986; Säwe and Odar-Cederlöf 1985; 1987; Wolff *et al* 1988). The plasma concentrations of morphine glucuronides in the elderly were about 10 times higher than in young healthy subjects. This was not the original aim of this study, but the preliminary results have increased my interest to comparatively study the pharmacokinetics and disposition of morphine and its glucuronides after codeine administrations in the elderly and in young healthy subjects. All the plasma and urine samples from the young healthy subjects and the elderly are stored at - 20 °C for the future analysis.

The adverse effects of codeine were not specifically assessed in these patients because they were suffering from complex medical conditions, whose symptoms and signs may mimic some of the unwanted effects of codeine.

In summary, the pharmacokinetics of codeine and codeine-6-glucuronide in the elderly were significantly altered and correlated with the reduction of their renal function. Renal impairment results in large

accummulation of codeine and codeine-6-glucuronide which may account for toxicity of codeine. A dosage reduction may be necessary in the elderly and in patients with renal impairment.

4.5. Polymorphic metabolism of codeine in humans

4.5.1. Introduction

In the codeine pharmacokinetic study (Chapter 4.3.), there was only a small amount of morphine found in the urine of one of my volunteers after single and chronic administration of codeine. This finding could not be easily explained because the hepatic and renal functions of this subject were normal; there were no other drugs taken when this study was done; the urine pH and volume were similar to those of the others and the urinary excretion of the N-demethylated metabolite, norcodeine, and the total urinary recovery were not different from the other volunteers in my study.

Dextromethorphan is known to exhibit genetic polymorphism in its O-demethylation and it has been demonstrated that the metabolism is co-inherited with the hydroxylation of debrisoquine (Küpfer *et al* 1984, Schmid *et al* 1985; Kronbach *et al* 1987; Dayer *et al* 1988). Codeine has a similar chemical structure to and undergoes the same metabolic pathways as those of dextromethorphan (Figures 1-3-1 and 1-5-1). The similarities in the chemical structures and metabolic pathways in addition to the above findings led me to investigate the possibility of genetic polymorphism of codeine O-demethylation.

The aims of this study were to investigate:

(i) whether the O-demethylation of codeine is under genetic control;

(ii) whether the polymorphic O-demethylation of codeine is co-inherited with the polymorphic metabolism of dextromethorphan.

4.5.2. Methods

4.5.2.1. Subjects

Twelve subjects, 4 female and 8 male, selected from the dextromethorphan metabolism study (Chapter 3.2.) participated in this study. 3 poor metabolisers (PM), 2 intermediate metabolisers (IM) and 7 extensive metabolisers (EM) were recruited. The subjects were aged 31.8±11 (range 21-56) and weighed 68.6±13.3 (range 46-87). Drugs and alcohol were forbidden two days before and during the study.

This study was approved by the Human Ethics Committee of Royal Adelaide Hospital and the Committee on the Ethics of Human Experimentation of the University of Adelaide.

4.5.2.2. Study design

Each subject received a single oral dose of 30 mg codeine phosphate as a tablet (Fawns and McAllan) followed by 100 ml of water before going to bed. All urine produced overnight (8-10 hours) was collected. Urine volume was determined gravimetrically and recorded along with the urine pH. An aliquot of well mixed urine was stored in stoppered vials at -20 °C until analysed for drug content.

4.5.2.3. Drugs analyses in urine

Unconjugated codeine, norcodeine and morphine analyses: Codeine, norcodeine and morphine concentrations in urine were measured by the HPLC method described in Chapter 4.1..

Codeine-6-glucuronide analysis: Codeine-6-glucuronide in urine was directly determined using the HPLC method described in Chapter 4.2..

Morphine and norcodeine conjugates analyses: The presence of conjugates of norcodeine and morphine in plasma and urine was inferred by the generation of norcodeine and morphine following enzymatic hydrolysis as described in Chapter 4.3..

4.5.2.4. Metabolic ratio and statistical analyses

The metabolic ratio (MR) for the O-demethylation of codeine was calculated as

$$MR = \frac{\text{total morphine}}{\text{total norcodeine + total codeine}}$$

(re-calculation see appendix VII)

where total refers to unconjugated and conjugated. The associations between the metabolic ratios of codeine and dextromethorphan were determined by the Spearman rank correlation coefficient. The correlation between the metabolic ratio and urine pH or volume were determined by linear regression analysis. The differences between groups were determined by analysis of variance.

4.5.3. Results

The metabolic ratios of codeine and dextromethorphan in the 12 subjects are shown in Table 4-5-1 and the mean values for the poor, intermediate and extensive metabolisers are shown in Table 4-5-2. There was a significant correlation (r_s = 0.96; $P < 0.001$) between the metabolic ratios for the O-demethylation of dextromethorphan and codeine (Figure 4-5-1). The recoveries of total morphine, total norcodeine, codeine-6-glucuronide and

codeine following codeine administration are shown in Table 4-5-3. There were no significant differences in the N-demethylation and the glucuronidation of codeine between the three type of metabolisers. There were no significant correlations between the metabolic ratio of codeine and either the urine pH (Figure 4-5-2) or urine volume (Figure 4-5-3).

There were no side effects in any of the subjects during this study.

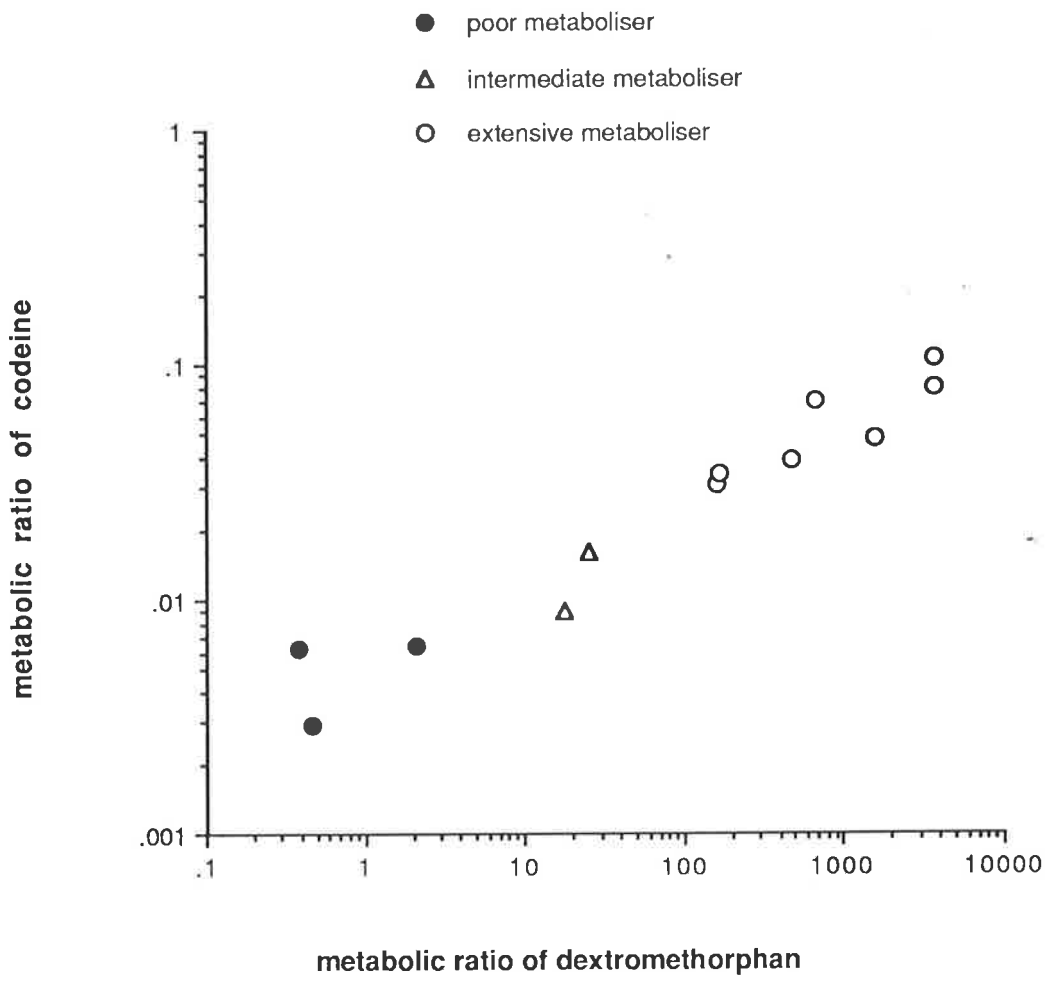


Figure 4-5-1. Correlation between the metabolic ratios of codeine and dextromethorphan (r_s = 0.96; P < 0.001).

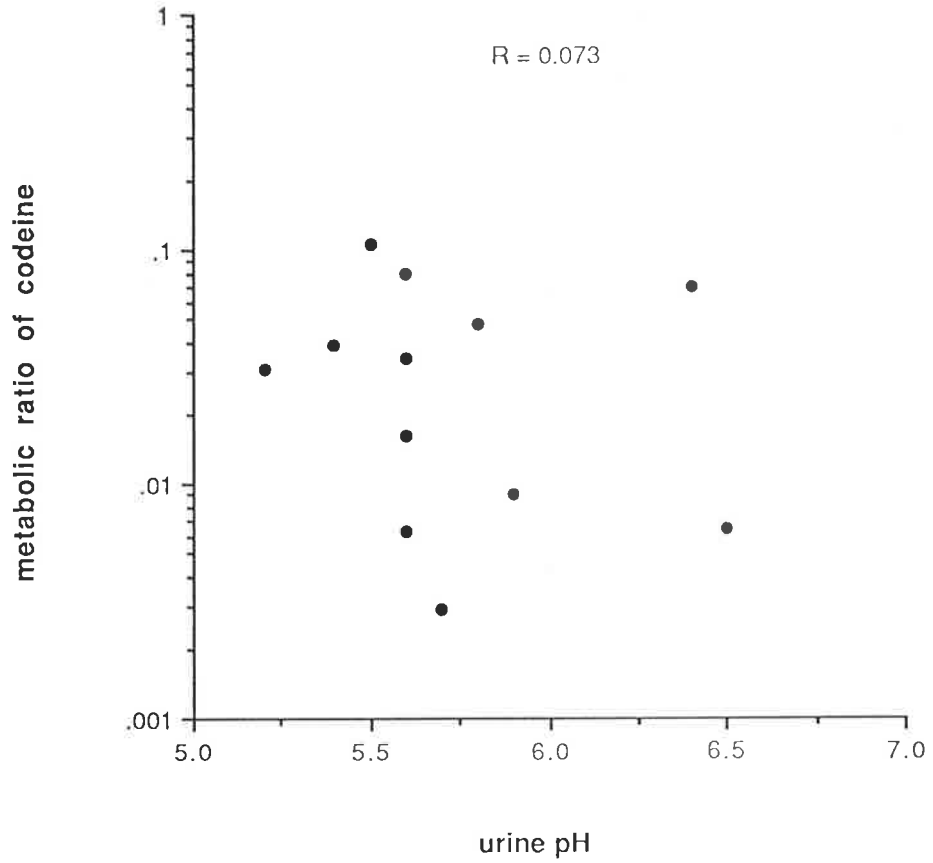


Figure 4-2-2. Correlation between the metabolic ratio of codeine and urine pH.

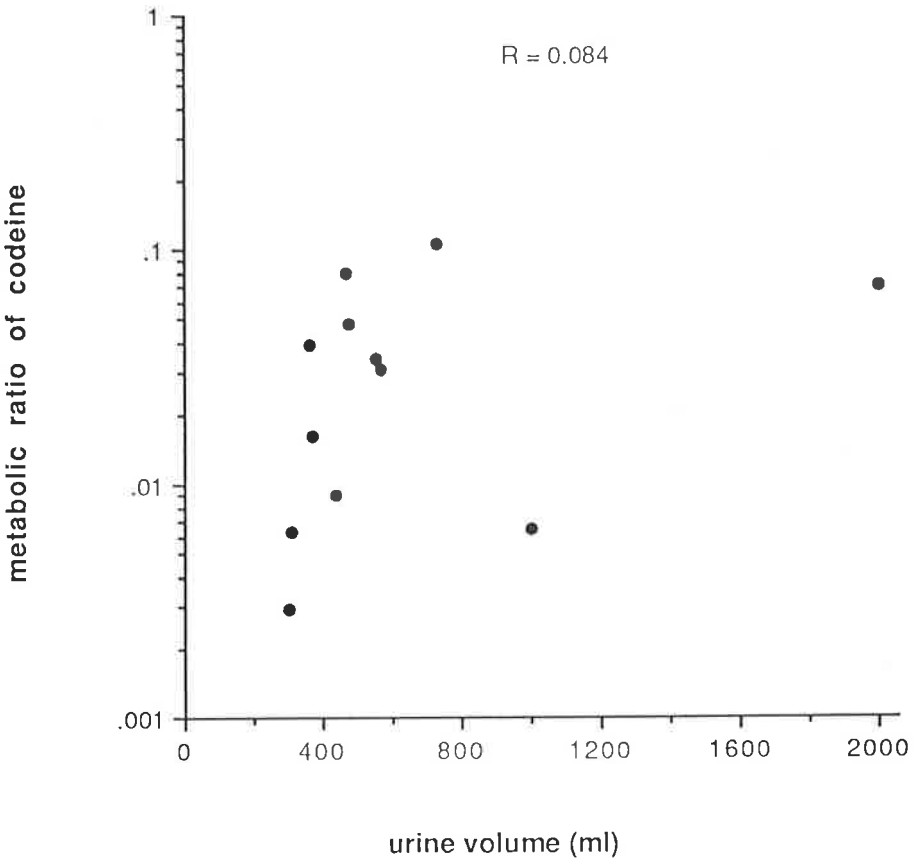


Figure 4-2-3. Correlation between the metabolic ratio of codeine and urine volume.

Table 4-5-1 Metabolic ratios of codeine and dextromethorphan in 12 healthy subjects

subject	urine pH	urine volume	metabolic ratio	
		(ml)	codeine	dextromethorphan
1	5.7	296	0.003	0.5
2	5.6	305	0.006	0.3
3	6.5	995	0.006	2.2
4	5.9	440	0.009	17.6
5	5.6	370	0.016	24.9
6	5.2	570	0.031	163.0
7	5.6	550	0.034	169.0
8	5.4	360	0.039	481.0
9	5.8	475	0.048	1542.0
10	6.4	2000	0.070	659.0
11	5.6	470	0.080	3659.0
12	5.5	730	0.106	3642.0

Table 4-5-2 Metabolic ratios of codeine and dextromethorphan in poor(PM), intermediate(IM) and extensive(EM) metabolisers (mean ± SD)

drug	PM n=3	IM n=2	EM n=7
codeine	0.005±0.002	0.012±0.005	0.058±0.028
dextromethorphan	0.98±0.97	21.25±5.16	1473±1557

Table 4-5-3 Urinary recovery (% of dose) of codeine and metabolites in poor(PM), intermediate(IM) and extensive(EM) metabolisers (mean ± SD)

drug	PM n=3	IM n=2	EM n=7	P
morphine	0.3±0.1	0.8±0.2	3.5±1.5	<0.05
norcodeine	3.5±2.2	2.3±0.7	4.6±2.1	>0.05
codeine	7.7±2.5	8.8±1.6	11.5±4.1	>0.05
codeine-6-glucuronide	49.0±4.4	51.6±7.7	46.7±7.7	>0.05
Total	60.2±7.9	62.6±5.5	62.8±6.9	>0.05

4.5.4. Discussion

The results from this study have shown that codeine O-demethylation in humans also exhibits genetic polymorphism. The strong correlation between the metabolic ratios for the O-demethylation of codeine and dextromethorphan suggests that the defect in the codeine O-demethylation is cosegregated with the debrisoquine/sparteine genetic oxidation polymorphism. These results are in agreement with a recently reported finding by Dayer and coworkers (1988). They found that morphine formation from codeine in human liver microsomes in vitro is dependent on the polymorphic monooxygenase known as cytochrome db1/bufI.

It has been reported that 5-10% (Clark 1985; Jacqz *et al* 1986) of the population have defects in the debrisoqine/sparteine oxidative metabolic pathway. Codeine has been widely used both alone and in combination with other analgesic and antitussive drugs. It is currently accepted that codeine exerts its analgesic effect via biotransformation to morphine (Jaffe and Martin 1985). The clinical consequences of the polymorphism of codeine O-demethylation are not clear. The findings in this study indicate that there may be a large number of people receiving an inactive compound (codeine) because of their inability to form morphine. Another possible clinical implication of this polymorphism is drug interactions. About 40 drugs have been reported to cosegregate with the debrisoquine genetic polymorphism (Jacqz *et al* 1986) and some of them are commonly used drugs such as β-blockers and antiarrhythmics. When codeine is given in combination with any of these drugs, alterations in metabolism and pharmacological effects of these drugs may occur (Kobayashi *et al* 1988), because of the competitive binding to the same drug metabolising enzyme, especially in the extensive metabolisers.

4.6. μ-opioid receptor binding affinity of codeine and its metabolites and some other opiates

4.6.1. Introduction

Several types of opioid receptors have been demonstrated to be present in the brain (Chapter 1.2.). The one most responsible for the analgesic activity of morphine, a potent and clinically important analgesic, has been designated the μ-opioid receptor. Before the advent of radioligands, receptor activity was mainly studied by measurement of physiological and biochemical response. The introduction of binding assays provided methods for the direct study of ligand-receptor interactions. Recently, a novel radioligand for μ-opioid receptor, ^{3}H-DAGO (Goldstein 1987), has become commercially available and this provides a useful tool for studying the potential for central analgesia of endogenous and exdogenous substances.

The binding affinity of morphine and its two glucuronide conjugates have been investigated and the results have shown that the affinity of morphine-6-glucuronide to the μ-opioid receptor is similar to that of morphine and morphine-3-glucuronide has very poor affinity (Pasternak *et al* 1987). Codeine is also an analgesic drug widely used clinically, but there is little information whether the parent drug and/or its metabolites mediate the analgesic effect of codeine.

The aim of this study was to test the binding affinities of morphine, codeine and their metabolites and some related opiates to the μ-opioid

receptor, to determine if any of these would be candidates as potential analgesics.

4.6.2. Methods

4.6.2.1. Animals

Male Wistar rats, weighing 250-350 g and aged 4 months, were used. This study was approved by the Human Ethics Committee of Royal Adelaide Hospital and the Committee on the Ethics of Animal Experimentation of the University of Adelaide.

4.6.2.2. Ligand

^{3}H-DAGO (Tyr*-D-Ala-Gly-N-Methyl-Phe-Gly-ol) was obtained from Du Pont NEN Research Products (St. Albany, Boston, MA, U.S.A.). The specific activity was 35.0 Ci/mmol and radiochemical purity was more than 99%. Unlabelled DAGO was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.).

4.6.2.3. Compounds

The following compounds were tested: morphine-3-glucuronide (Sigma Chemical Company, St. Louis, MO. U.S.A.), morphine-6-glucuronide, morphine-3-sulphate, normorphine (National Institute of Drug Abuse, Maryland, USA), morphine hydrochloric acid,codeine phosphate, oxycodone, pholcodine, (F.H. Faulding and Co. Ltd, Adelaide, Australia), norcodeine (Eli Lilly and Co. Ind., U.S.A.), codeine-6-glucuronide (synthesised by Dr. G. Reynolds, Appendix III), dextromethorphan, and dextrorphan (Roche Products Pty Ltd, Sydney, Australia).

4.6.2.4. Binding assay

The binding study was performed by modification of the method of Pert and Snyder (1973b). Rats were killed by decapitation and brain, after removal of the cerebellum, was homogenized in 10 ml of 0.1 M Tris buffer (pH 7.4, 0 °C). The homogenate was finally diluted to 110 volumes of tissue with the cold Tris buffer. 1.8 ml of this freshly prepared homogenate was incubated with 0.1 ml of 20 x 10^{-9} M ^{3}H-DAGO for 5 minutes at 20 °C and then 0.1 ml of the test compound solution (10^{-12} - 10^{-6} M) was added and incubated for 15 minute at 20 °C. Samples were cooled down to about 4 °C in an ice bath and then filtered under reduced pressure though Whatman glass-microfibres (2.5 cm GF/B Whatman International Ltd, Maidstone, England). The filters were washed twice with 8 ml of cold 0.1 M Tris buffer (pH 7.4). After addition of 10 ml liquid scintillation cocktail (Ready Value, Beckman Instruments, Inc. 2500 Harbor Boulevard Fullerton, CA, U.S.A.), radioactivity was determined by Liquid-Scintillation Counter (LS 3801 Beckman Instruments). Each sample was counted three times.

4.6.2.5. Statistical analysis

The equilibrium dissociation constant (Kd) of ^{3}H-DAGO-receptor interaction was determined from the Scatchard plot as a negative reciprocal of the slope (-1/β). IC_{50} was calculated from log dose-response analysis. The inhibition constant (Ki) was calculated as

$$Ki = \frac{IC_{50}}{1 + [L] / Kd}$$

where [L] is the concentration of the ligand ^{3}H-DAGO.

4.6.3. Results

^{3}H-DAGO had a very high affinity to the μ-opioid receptor with a Kd value of 0.04, which was similar to that reported previously (Wood 1986). The displacement curve of ^{3}H-DAGO by unlabelled DAGO is shown in Figure 4-6-1 and it had a similar slope to that of morphine. The binding curves of morphine and its metabolites are shown in Figure 4-6-2 and those of codeine and its metabolites are shown in Figure 4-6-3. The binding curves of oxycodone, pholcodine, dextromethorphan and dextrorphan are shown in Figure 4-6-4. All the binding curves had a similar slope. The IC_{50} and Ki values of these compounds are shown in Table 4-6-1.

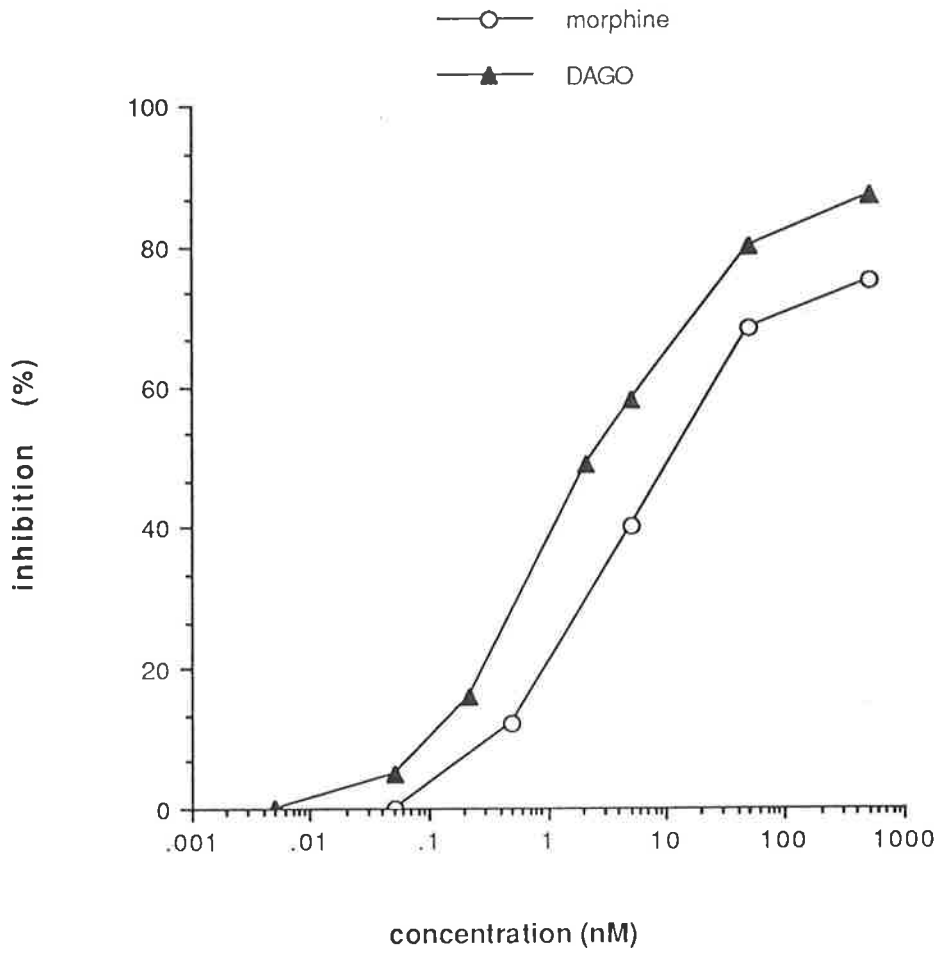


Figure 4-6-1. The displacement curves of ^{3}H-DAGO by unlabelled DAGO and morphine.

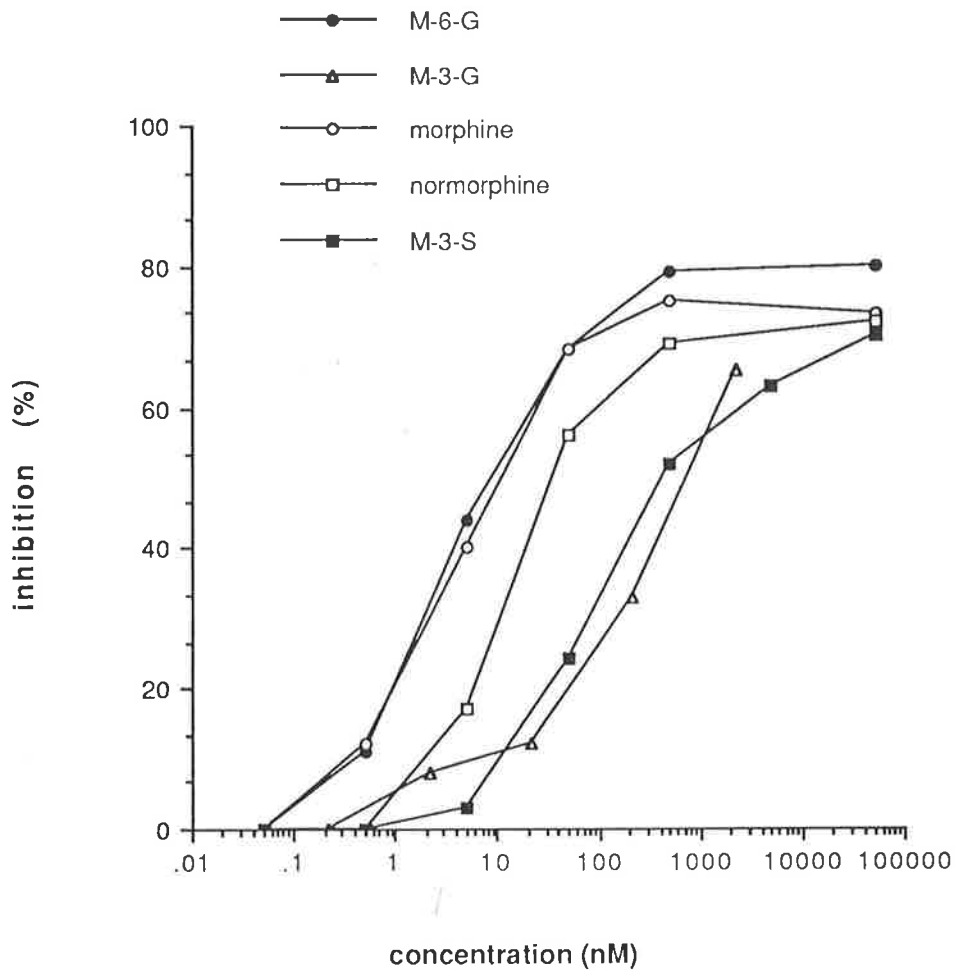


Figure 4-6-2. The displacement curves of ^{3}H-DAGO by morphine and its metabolites. (M-6-G: morphine-6-glucuronide; M-3-G: morphine-6-glucuronide; M-3-S: morphine-3-sulphate)

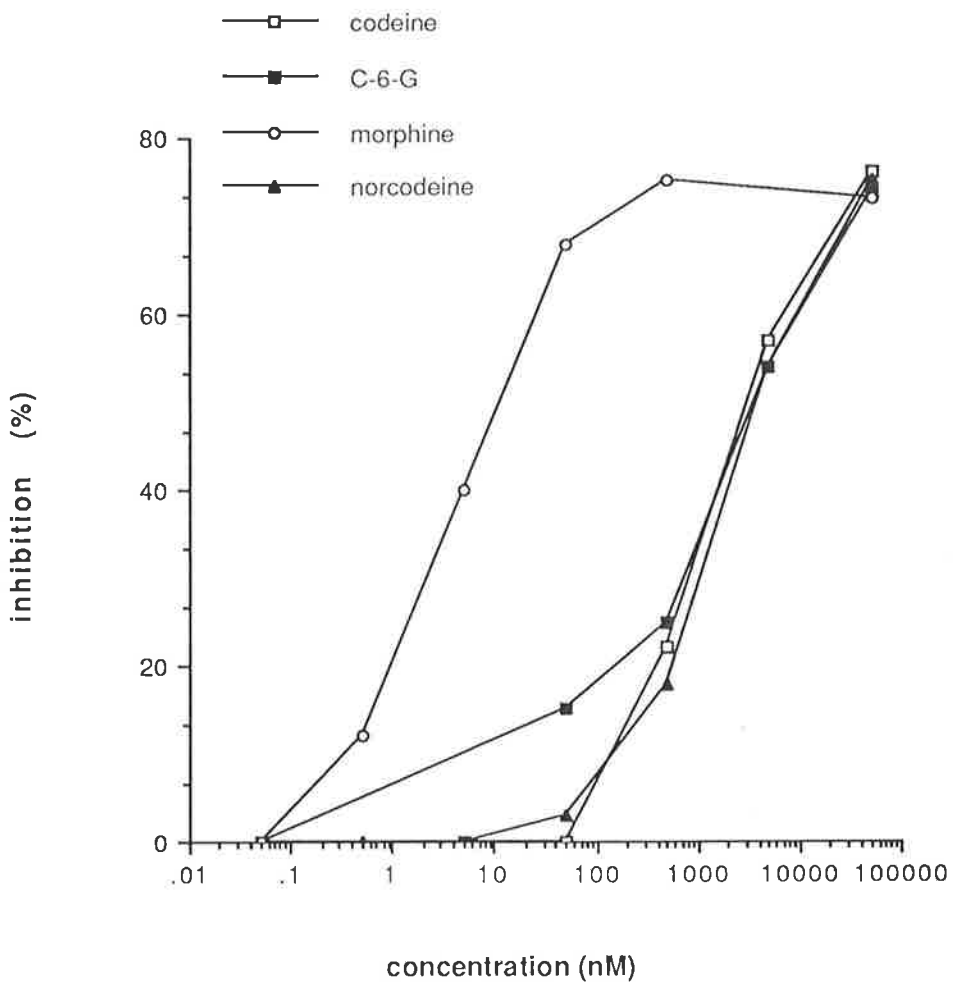


Figure 4-6-3. The displacement curves of ^{3}H-DAGO by codeine and its metabolites. (C-6-G: codeine-6-glucuronide)

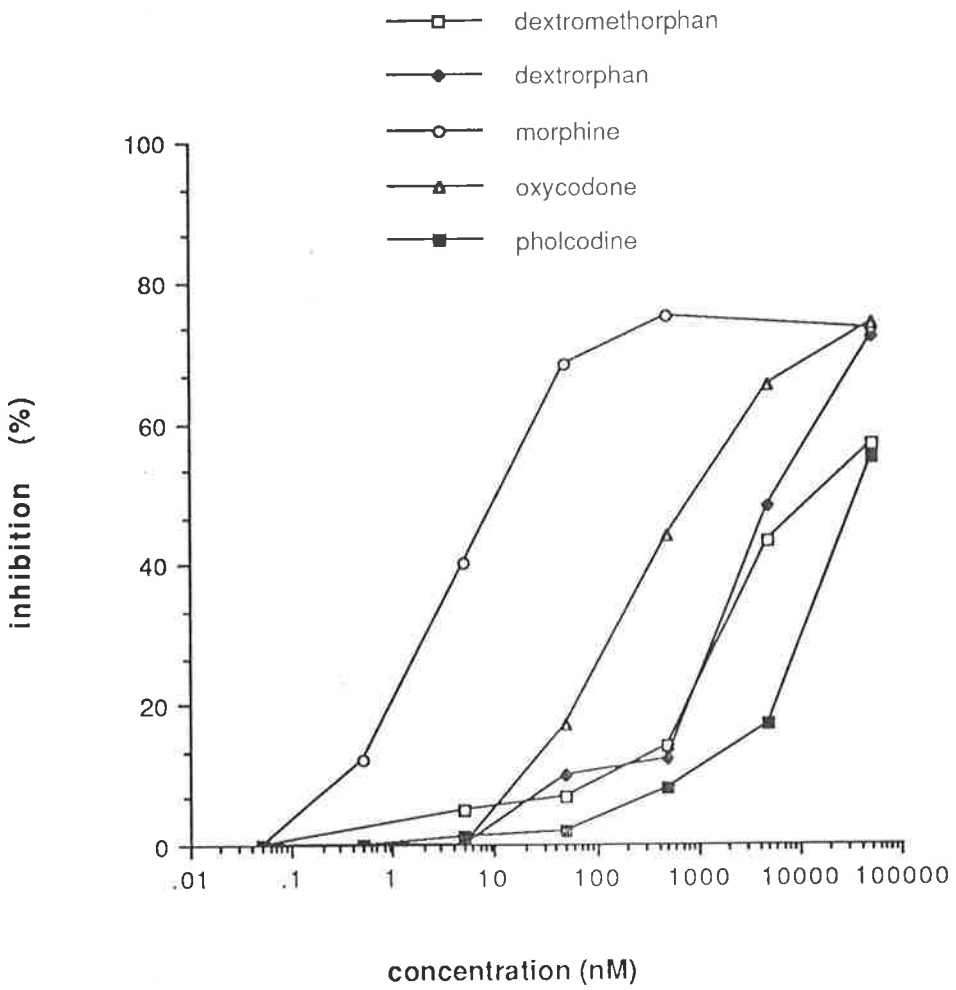


Figure 4-6-4. The displacement curves of ^{3}H-DAGO by oxycodone, pholcodine, dextromethorphan and dextrorphan.

Table 4-6-1 IC_{50} and Ki values

compound	IC_{50} (nM)		Ki
	mean	SD	
morphine-6-glucuronide	3.6	1	0.12
morphine	4.2	2	0.14
normorphine	34	6	1.16
morphine-3-sulphate	186	62	6.32
morphine-3-glucuronide	248	125	8.44
oxycodone	363	59	12.35
codeine-6-glucuronide	955	113	32.48
codeine	1380	52	46.94
norcodeine	1584	156	53.88
dextrorphan	1659	116	56.43
dextromethorphan	3981	1030	135.41
pholcodine	24547	9369	834.93

n = 4-6

4.6.4. Discussion

The plasma concentrations and urinary excretion of several opioid compounds and their metabolites have been determined in my studies (Chapters 2, 3, 4.). There is, however, little information on their relative pharmacological activities. μ-opioid receptor binding is a simple method for testing the affinity of xenobiotics to the receptor. Morphine is regarded as the reference against which other analgesic compounds are assessed and its binding affinity to the μ-opioid receptor has been studied using ^{3}H-DAGO as a specific ligand (Pasternak *et al* 1987). Morphine-6-glucuronide has been demonstrated to be an active metabolite for morphine analgesia and its binding affinity to the μ-opioid receptor was equal to or greater than that of morphine whereas that of morphine-3-glucuronide was much less than that of morphine (Pasternak *et al* 1987; Osborne *et al* 1988). The results from this study support these previous observations. Morphine-3-sulphate, conjugated at the same position as morphine-3-glucuronide in the morphine structure, had a similar binding affinity to the μ-opioid receptor as morphine-3-glucuronide. It has been demonstrated that the 3-hydroxyl group is very important in the analgesic effect of opioids and replacement of the 3-hydroxyl group diminishes the binding affinity to the μ-opioid receptor (Pert and Snyder 1973b).

Codeine is a commonly used analgesic. Its analgesic activity has been demonstrated both in animals and in man (Eddy *et al* 1969). It has been proposed that the biotransformation of codeine to morphine is responsible for the analgesic effect of codeine (Sanfilippo 1948). Although morphine has very high binding affinity to the μ-opioid receptor, the plasma concentrations of morphine after codeine administration were very low (Findlay *et al* 1978, 1986; Rogers *et al* 1982; Quiding *et al* 1986; Bodd *et al* 1987; Chapter

4.3.), and most of that was further metabolised to the less active form morphine-3-glucuronide (Adler *et al* 1955; Chapter 4.3.). In addition, morphine penetrates the blood brain barrier poorly (Oldendorf *et al* 1972; Hahn *et al* 1976; Mullis *et al* 1979). The analgesic potency of morphine given peripherally was 900 times less than when it was administered intra-cerebrally (von Cube *et al* 1970). Thus the hepatic morphine formation after codeine may not explain the analgesic effect of codeine. The binding affinity of codeine-6-glucuronide was 230 times less than morphine. However its plasma concentration was about 400 hundred times higher than morphine after codeine administration (Chapter 4.3.). Thus by analogy with morphine-6-glucuronide and because of its considerable plasma concentrations, it may contribute to the analgesic effect of codeine.

Oxycodone, chemically related to codeine, has an analgesic potency in between morphine and codeine (Jaffe and Martin 1985; Moffat *et al* 1986). It is metabolised by the same metabolic pathway as codeine and conjugated oxycodone is the major metabolite (Moffat *et al* 1986). The μ-opioid receptor binding affinity of oxycodone was relatively higher than codeine and lower than morphine in this study. Its analgesic effect may be mediated via the parent compound and/or it metabolites.

Pholcodine and dextromethorphan are commonly used antitussive drugs. Previous studies have shown that they are devoid of analgesic effect (Eddy *et al* 1969) and these findings were further supported by the results from this study. These results also indicate that their antitussive effect is mediated by other receptors rather than μ-opioid receptor.

This binding study has provided direct information on the compound-receptor interaction. However, relative concentrations of parent compound and metabolites need to be considered in addition to relative affinities. In

addition, the analgesic effect of a compound is affected by many factors. The most important determinant is the ability to pass across the blood brain barrier. This aspect is discussed in the next section of this chapter.

4.7. Morphine formation from codeine in rat brain: a possible mechanism of codeine analgesia

4.7.1. Introduction

Although codeine has been used as an effective analgesic drug for more than 100 years, its analgesic mechanism has been poorly understood. It has been proposed that codeine exerts its analgesic effect by hepatic biotransformation to morphine (Sanfilippo 1948). This hypothesis should be challenged because circulating morphine concentrations after therapeutic doses of codeine (60 mg) are very low (<10 ng/ml) (Findlay *et al* 1978, 1986; Rogers *et al* 1982; Quiding *et al* 1986; Bodd *et al* 1987; Chapter 4.3.), which are lower than the minimum plasma concentration (16 ng/ml) required for analgesia (Dahlstrom *et al* 1982), and because morphine has difficulty in penetrating the blood brain barrier (Oldendorf *et al* 1972; Hahn *et al* 1976; Mullis *et al* 1979). After administration of codeine about 5% of the dose was converted to morphine in rats (Johannesson and Wood 1964; Gintzler *et al* 1976) and humans (Adler *et al* 1955; Nomof *et al* 1977; Chapter 4.3.) and most of this was in the conjugated form (Adler *et al* 1955; Chapter 4.3.). Morphine concentrations in rat brain after systemic administration of morphine were only one-fifth of those in plasma (Hahn *et al* 1976). Interestingly, after oral administration of codeine in man, the analgesic potency was only 60% of the same intramuscular dose (Beaver *et al* 1978), although the oral administration of codeine resulted both in higher plasma concentrations and larger area under the plasma concentration-time curve of morphine (Rogers *et al* 1982). Codeine, although it has a lower affinity to the μ-opioid receptor than

morphine (Pert and Snyder 1973; Chapter 4.6.), has much greater lipid solubility and passes across the blood brain barrier much more readily (Oldendorf *et al* 1972; Gintzler *et al* 1976). Elison & Elliott (1963) demonstrated that codeine could be converted to morphine in rat brain slices. Recently it has been shown that salutaridine and thebaine can be converted to codeine and morphine in isolated rat brain microsomes (Kodaira and Spector 1988). Enzymes for drug metabolism have also been identified in the microvessels of rat brain (Ghersei-Egea *et al* 1988). After peripheral administration of codeine to rats, morphine was detected in the brain (Gintzler *et al* 1976; Johannesson and Schou 1963; Donnerer *et al* 1987). However the origins of the morphine are not clear. The aims of this study were:

(i) to investigate the O-demethylation of codeine to morphine in rat brain in vitro;

(ii) to determine the origin of the morphine in the brain after the peripheral administration of codeine.

4.7.2. Methods

4.7.2.1. Reagents

All reagents were of analytical degree (see appendix I). Codeine phosphate and morphine sulphate were British pharmacopoeial quality. NADPH, tris[hydroxymethyl]aminomethan (Sigma chemical company, St Louis, MO. USA) were of reagent grade. d-glucose and l-ascorbic acid were of analytical grade.

4.7.2.2. Codeine O-demethylation in rat brain in vitro

Male Wistar rats (weighing 250-350 g; aged 4 months) were killed by decapitation and the brains were removed and rinsed. After removing the

cerebellum and pial membrane, the brains were weighed and chilled on ice. The brains were homogenized in 10 volumes of cold artificial c.s.f. solution (NaCL 126 mM, KCL 2.68 mM, Na_2HPO_4 1 mM, $MgSO_4$ 0.88 mM, $NaHCO_3$ 22 mM, CaCL 1.45 mM, d-glucose 11 mM and ascorbic acid 0.57 mM) in a Ultra-Turrax® homogenizer (Janke & Kunbel KG, Ika Werk, Staufen i. Breisgau). The homogenate was preincubated at 37 °C for 5 minutes. 1.8 ml of the homogenate was pipetted into a incubation tube containing NADPH (final concentration 5 mM) and codeine at final concentrations ranging from 5 to 250 µM. The tubes were incubated with 5% CO_2 and 95% O_2 at 37 °C for 2, 5, 10, 20, or 30 minutes. The reaction was stopped by adding 1 ml of bicarbonate buffer of pH 9.6 and 5 ml of ether/chloroform (2/1, by volume). Morphine concentrations were determined by the HPLC assay described in Chapter 4.1..

Rat brain microvessels were isolated followed a procedure described by Head and coworkers (Head *et al* 1980). Both microvessels and the brain tissue were incubated as described above.

4.7.2.3. Codeine O-demethylation in rat brain in vivo

Codeine phosphate (20 mg/kg) or morphine sulphate (1 mg/kg) was administered to rats (Wistar, Male, 250-400 g) by intraperitoneal injection in a randomized order. The rats were killed at 0.5 or 1.0 hour after drug administration. Blood samples were collected and the brains removed. The cerebellum and the pial membrane were removed completely. The brains were weighed and homogenized in 0.1 M tris buffer pH 7.4 and diluted to a final volume of 4 ml. Aliquots of the brain and plasma samples were assayed for morphine and codeine concentrations by the HPLC assay described in Chapter 4.1..

This study was approved by the Committee on the Ethics of Animal Experimentation of the University of Adelaide.

4.7.2.4. Statistical analysis

Vmax and Km were determined from Michaelis-Menten equation and differences between treatments were determined by the t-test.

4.7.3. Results

4.7.3.1. Morphine formation in rat brain in vitro

The time course for morphine formation in rat brain is shown in Figure 4-7-1. The maximal formation of morphine occurred at 10 minutes. The concentration-formation rate curve is shown in Figure 4-7-2. The Vmax was 5.93 ± 0.16 nmol/g brain/h and the Km was 37.82 ± 4.99 μM. Morphine formation in microvessels and brain tissue is shown in Figure 4-7-3. The production of morphine in microvessels was 2 times higher than that in the brain tissue.

4.7.3.2. Morphine formation in rat brain in vivo

After intraperitoneal administration of morphine or codeine, morphine concentrations in rat brain are shown in Figure 4-7-4 and in plasma in Figure 4-7-5. The codeine concentrations in the brain and in plasma are shown in Figure 4-7-6. Morphine concentrations in plasma 30 minutes after intraperitoneal administration of 1 mg/kg morphine sulphate were not different ($P > 0.05$) from those after intraperitoneal administration of 20 mg/kg of codeine phosphate. However morphine concentrations in the brain 30 minutes after giving morphine could not be detected and at 60 minutes were significantly lower than after giving codeine ($P < 0.01$).

Codeine concentrations in the brain were about 3 times higher than that in plasma.

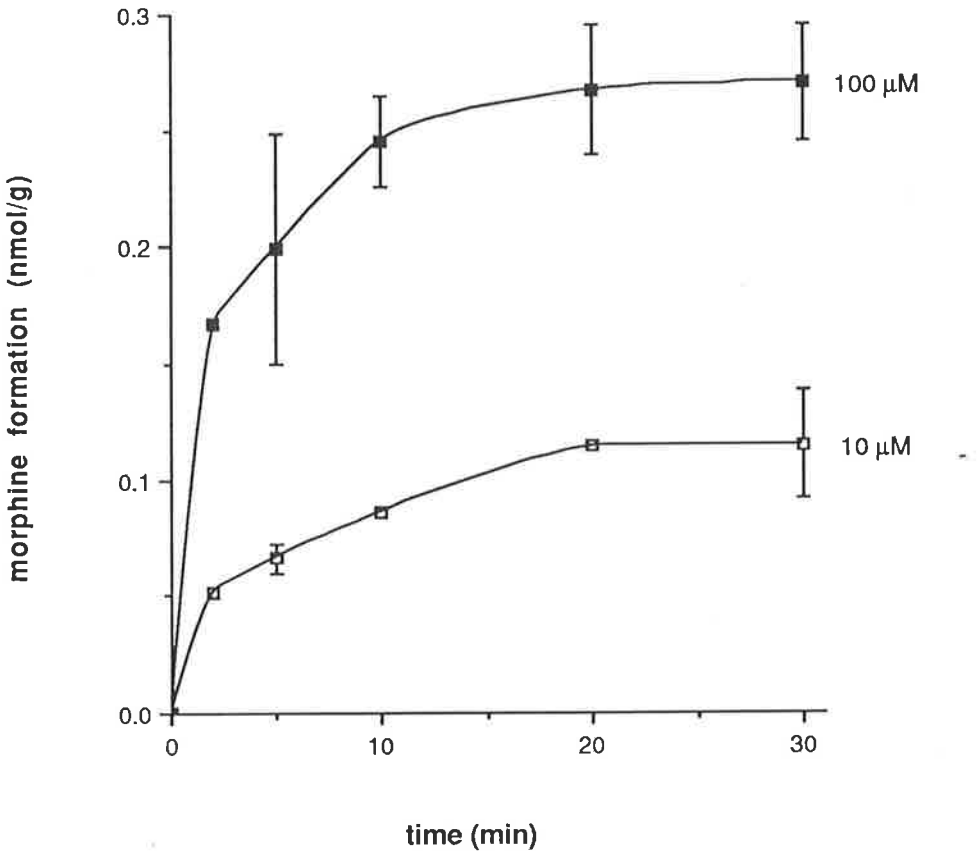


Figure 4-7-1 Time course of morphine production from codeine at concentrations of 10 and 100 µM in rat brain homogenate. Each point is the mean of 2 measures.

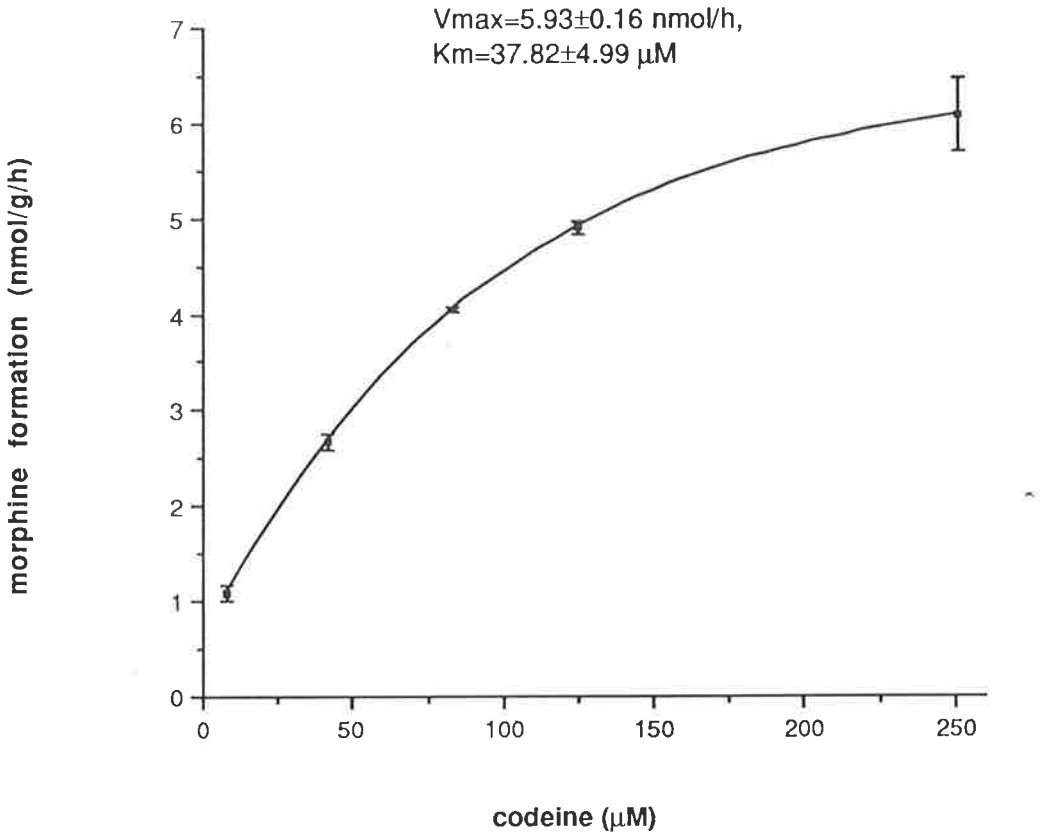


Figure 4-7-2 Kinetics of morphine production from codeine in rat brain homogenate. (mean±SD, n=3)

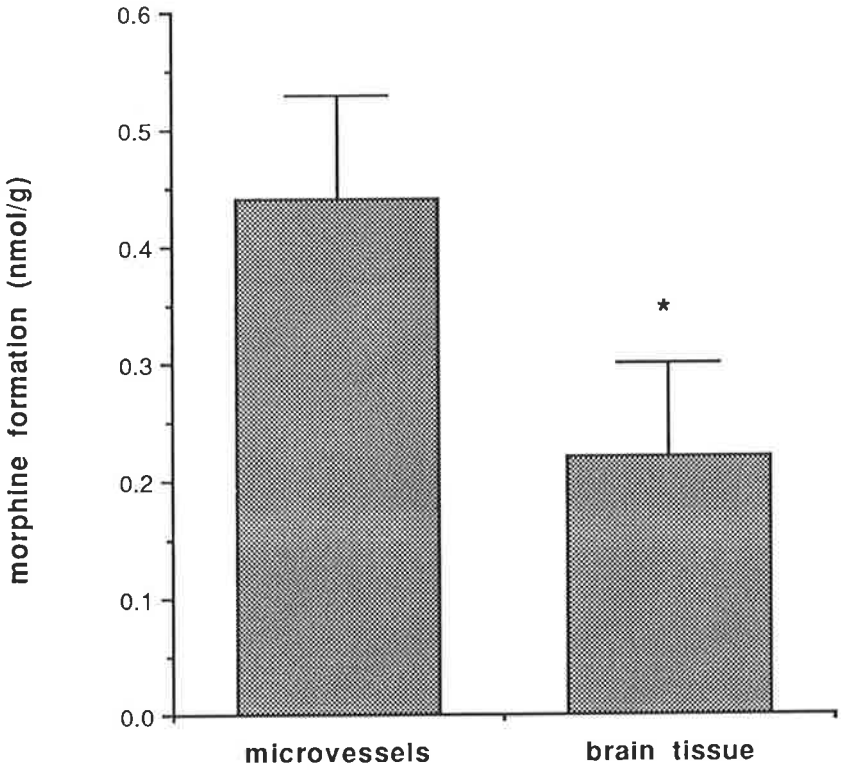


Figure 4-7-3 Morphine production from codeine in rat brain microvessels and brain homogenate (mean±SD, n=7).

* $P < 0.05$

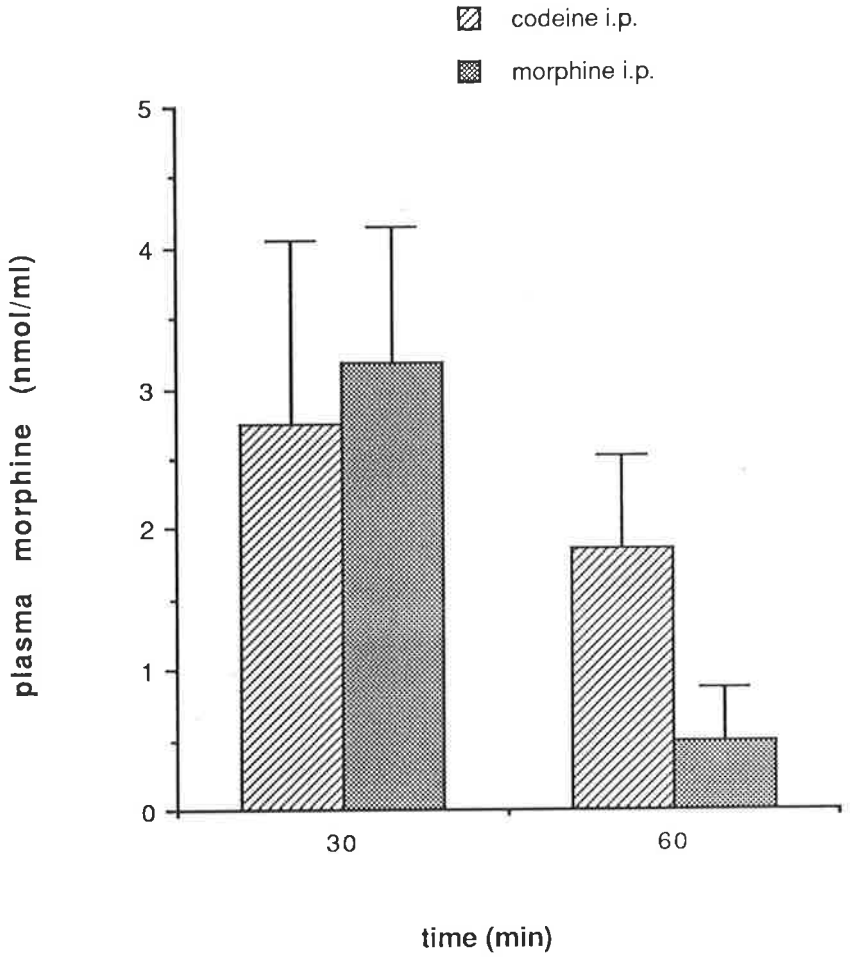


Figure 4-7-4 Morphine concentrations in plasma after intraperitoneal administration of 20 mg/kg of codeine phosphate or 1 mg/kg of morphine sulphate. (n=4)

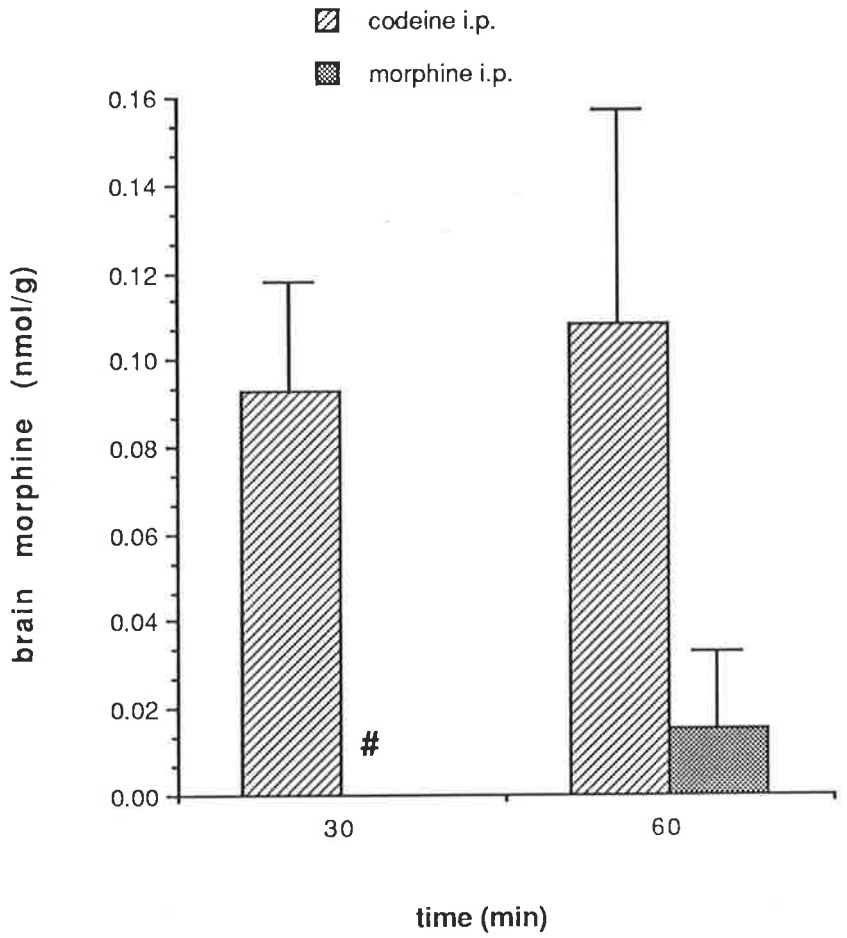


Figure 4-7-5 Morphine concentrations in the brain after intraperitoneal administration of 20 mg/kg of codeine phosphate or 1 mg/kg of morphine sulphate (n=4).

no morphine detected

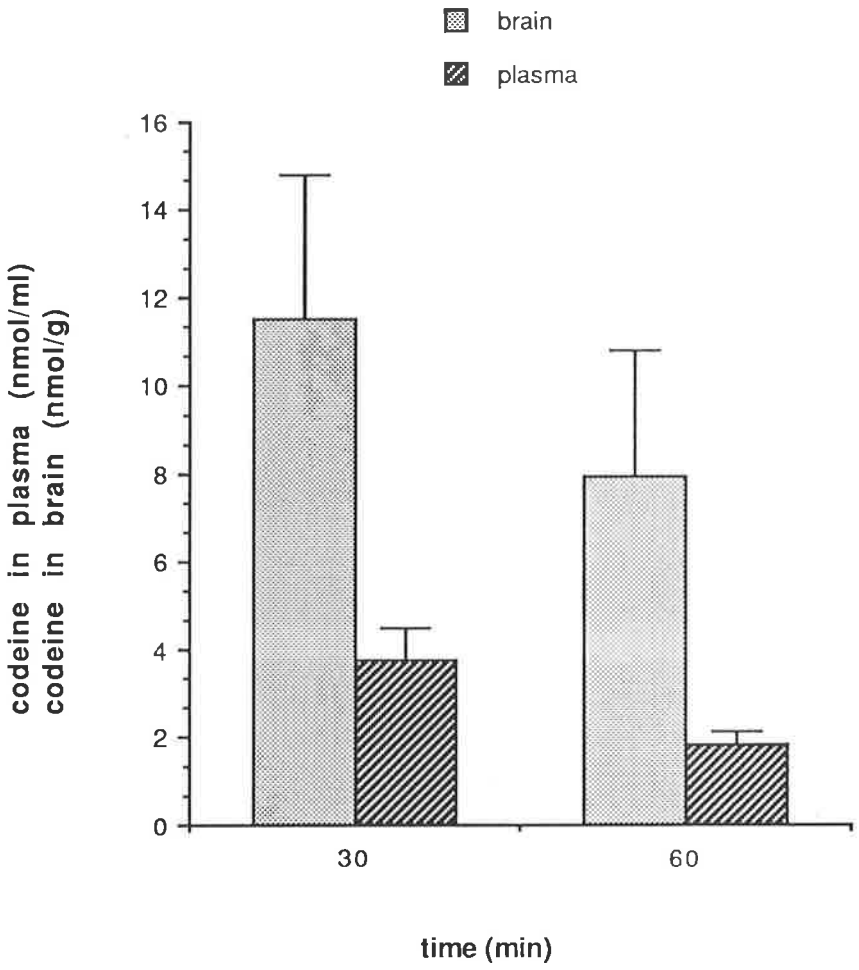


Figure 4-7-6 Codeine concentrations in plasma and in brain after intraperitoneal injection of 20 mg/kg of codeine phosphate. (n=4)

4.7.4. Discussion

Some studies have shown that morphine can be detected in the brain after systemic administration of codeine to the rat (Gintzler *et al* 1976; Johannesson and Schou 1963; Donnerer *et al* 1987). However it is unclear whether the morphine detected was formed in the brain or formed peripherally and then penetrated into the brain. In this study, at 30 minutes after intraperitoneal administration of morphine or codeine, the plasma morphine concentrations were similar. In the brain , however, morphine was detected only in the rats receiving codeine. At 60 minutes after drug administration a small amount of morphine was detected in the brain of the rats given morphine, but it was significantly lower than that in the brain of the rats given codeine. These findings suggest that morphine present in the brain after systemic administration of codeine was mainly formed from codeine in the brain, and only a little of the morphine formed systemically penetrated into the brain. Brain morphine concentrations at 60 minutes were not lower than at 30 minutes, although plasma morphine and brain codeine concentration started to decline. This indicates that morphine elimination from the brain was slower than codeine.

The ability of a drug to penetrate the blood brain barrier is attributable to the physico-chemical characteristics of a compound. One of the most important factors is lipid solubility. Codeine and morphine are quite similar in chemical structure. The only difference is that the codeine molecule has a methyl group attached at the 3-carbon position whilst the morphine molecule has a hydroxyl group at this position. The presence of the hydroxyl group causes morphine's very low lipid solubility (0.69 octanol/pH 7.4) and the methyl group endows codeine with considerable lipid solubility (3.98, octanol/pH 7.4). As a result of this chemical difference codeine enters

the brain much more quickly and extensively than morphine. For the same reason morphine in the brain comes out more slowly than codeine.

The in vitro formation of morphine from codeine in the brain homogenate was very rapid. In the first few minutes the production-time curve was linear and reached the V_{max} of 5.93 nmol/hour per gram brain in about 10 minutes. Kodaira and Spector (1988) incubated codeine with isolated microsomes from rat brain and the morphine formation was 14 pmol/hour per mg of protein. Although they did not report how much brain tissue was used to prepare the microsomes, the morphine production from codeine in this study was certainly more than that in microsomes reported by Kodaira and Spector. This is in agreement with previous findings (Law *et al* 1974) and probably indicates non-microsomal demethylation activity. Morphine formation in the microvessels was significantly greater than in the brain tissue separated from microvessels. This suggests that the O-demethylation of codeine occurs in the microvessels, most likely in the endothelial cells.

Endothelial cells in the brain have been shown to have the ability to metabolize drugs. The subcellular locations of the drug-metabolizing enzymes were in mitochondria and microsomes (Ghersei-Egea *et al* 1988). In pulmonary endothelial cells it has been shown that the enzymes are bound to the membrane of the endothelial cells both intracellularly and extracellularly (Bakhle 1982). The vast numbers of the endothelial cells, together with their position in the circulation and in the brain, confer a particular importance on their activities. Capillaries are extensively distributed in the brain and closely in contact with brain cells, nerve endings and receptors. The concentration of morphine required to displace 50% of the opioid receptor ligand ^{3}H-DAGO is about 4 nM in rat brain homogenate (Walther *et al* 1986; Chapter 4.6.). Morphine concentrations achieved in the rat brain after

intraperitoneal 20 mg/kg codeine were about 20 fold higher than this and would presumably have a significant analgesic effect.

There is little information available on the other metabolic pathways of codeine in the brain. Although N-demethylation of codeine was demonstrated in the rat brain (Elison and Elliott 1963; Fishman *et al* 1976), it is probably not as important as the O-demethylation, considering the affinity of norcodeine to the μ-opioid receptor is about the same as codeine which is 300 times less than morphine (Chapter 4.6.).

There is increasing interest in drug metabolism in the brain. There is no information, however, whether enzymes responsible for such metabolism in the brain are under genetic control as occurs in the liver. I have demonstrated that codeine O-demethylation is under genetic control and have assumed that this polymorphism affects at least the liver. If the O-demethylation of codeine in the brain exhibits the same defect in the poor metabolisers, codeine may be an inactive analgesic for these people.

The results from this study suggest that morphine can be biotransformed from codeine in the rat brain after systemic administration of codeine and the morphine formed centrally rather than peripherally may explain the analgesic effect of codeine.

Chapter 5

A PILOT STUDY ON THE ANTITUSSIVE EFFECT OF CODEINE, PHOLCODINE AND DEXTROMETHORPHAN IN PATIENTS WITH CHRONIC COUGH

5.1. Introduction

Codeine, pholcodine and dextromethorphan are the most commonly used antitussive drugs (Anonymous 1985). Their antitussive effects in man have been studied and have been reviewed by Eddy and coworkers (1969b). The antitussive properties of these drugs have been tested by determining their effect on provoked cough in healthy subjects or on spontaneously occurring cough in patients. Although the data from the provoked cough studies gave a valid indication of antitussive effect, experimental production of cough by irritants is subject to limitations and criticisms, and the results of such studies were not always predictive of how drugs affect spontaneous cough (Banner 1986). The previous antitussive studies in patients have suffered from criticisms because most of the studies were poorly controlled and based on non-objective evaluation (Eddy *et al* 1969b).

The measurement of cough has relied on subjective or objective evaluations. Because cough is such a common symptom and may persist for varying periods of time, adaption readily occurs, so that many patients may be unaware of the extent of their coughing and subjective evaluation is usually associated with an unacceptable margin for error. Several cough recording systems have been used in cough studies and have been proved to be successful in the measurement of cough frequency (Bickerman and Itkin 1960; Reece *et al* 1966; Calesnick and Christensen 1967; Sevelius *et al* 1971; Constant 1983; Matthy *et al* 1983; Cox *et al* 1984; Rühle *et al* 1984; Matthy *et al* 1985; Oldini and Vecchi 1987). Some investigators have also attempted to measure cough intensity (Constant *et al* 1983; Cox *et al* 1984; Matthy *et al* 1985; Oldini and Vecchi 1987), but this is still far from satisfactory. Of the recording systems used in the previous studies, the microphone-recorder system is simple and practical. However these systems were usually designed for use in

a private room and patients' activities were limited. These methods are not suitable for large clinical trials especially when cough is recorded for a long time.

The aims of this study were:

(i) to develop an accurate and practical cough recording system for clinical trials in both in-patients and out-patients;

(ii) to perform a pilot study to determine the antitussive effects of codeine, pholcodine and dextromethorphan using this cough recording system;

(iii) to determine if plasma drug concentrations could be related to cough suppression.

5.2. Methods

5.2.1. Equipments

A commercial Business Coder, microcassettes (Sony BI-500, Sony corporation, Japan) and a normal watch with chime (Kessel) were used. The alarm watch was installed on the recorder next to where the microphone is (Figure 5-1). This recorder is a voice-operated recorder. The tape moves automatically when there is sound and stops when there is no sound. The level of the sound to start the tape can be changed from "low" to "high", "low" for picking up relatively loud sounds and "high" for picking up not only loud but also low sounds. The alarm watch was set to chime hourly. When the recorder is on, a cough from the patient, the chime from the watch and other sufficiently loud sounds will start the tape immediately. The tape continues to run for 2 seconds after input sounds cease. The number of coughs in different units of time can be counted by playback of the cassettes. Before

using the device in the cough study, the sensitivity and the accuracy of the cough recording system were tested by comparing the cough number recorded from the tape recorder and that from hand counting by an observer sitting with the patients.

5.2.2. Drugs

Drugs used in this study were codeine phosphate, dextromethorphan hydrobromide, pholcodine and placebo. These drugs were made in 20 mg capsules which were of identical appearance. 10 capsules of each drug or placebo were packed into one bottle (for one patient). 40 bottles (10 for each drug) were numbered in a randomized order. These were prepared by the Pharmacy Department of the Royal Adelaide Hospital.

5.2.3. Patients

Royal Adelaide Hospital inpatients and out patients, who gave written informed consent participated in the study. Eleven patients have been studied so far. The patients (Table 5-1-1) were aged 47.5±15.2 (mean ± SD) years and weighed 67.1±9.0 kg. Their diagnosis and medications are summarized in Table 5-1-2. Prior to commencement of the study each subject was given a physical examination and a venous blood sample was collected for biochemical examination (MBA 20) and haematological examination (CBE). The creatinine clearance was estimated using a nomogram method (Siersbaek-Nielsen *et al* 1971). Some of the results reflecting the renal and hepatic functions are shown in Table 5-1-1.

This study was approved by the Human Ethics Committee of the Royal Adelaide Hospital and the Committee on the Ethics of Human Experimentation of the University of Adelaide.

5.2.4. Study design

This study was of a double-blind, placebo-controlled, randomized design. Before drug administration, cough frequency of the patient was assessed for eight hours (0800-1600 hour) by means of the portable tape recorder. At 1600 hours, the patient received a loading dose (because the half-life of pholcodine is very long) of two capsules of one of the drugs, and then the drug was taken as one capsule eight hourly for a further eight doses. On the second and third day of drug administration, the cough frequency was assessed from 0800 to 1200 hours. On day four, immediately before and at 0.5, 1, 1.5, 2, 4 and 8 hours after drug administration, 10 ml venous blood samples were collected into heparinized plastic tubes via an indwelling catheter, kept patent with a stylet (Jelco^{TM}, 18 G i.v. catheter placement unit, Critikon, Tampa, USA), placed in a forearm vein and all urine was collected over this eight hour period. Cough frequency assessment was performed throughout the duration of this period (0800-1600 hour).

Blood samples were centrifuged immediately and harvested plasma stored in stoppered vials at - 20 °C until analysis. Urine volume was determined gravimetrically and recorded along with the urine pH. An aliquot of well mixed urine was retained in stoppered vials at -20 °C until analysed for drug content.

5.2.5. Drugs analysis in plasma and urine

Unconjugated codeine and morphine analysis: Codeine and morphine concentrations in plasma and urine were measured by the high performance liquid chromatographic method using fluorescence detection as described in Chapter 4.1..

Codeine-6-glucuronide analysis: Codeine-6-glucuronide concentrations in plasma and urine were directly determined using the high performance liquid chromatographic assay with fluorescence detection as described in Chapter 4.2..

Pholcodine analysis: Pholcodine concentrations in plasma and in urine were determined by the high performance liquid chromatographic method as described in Chapter 2.1..

Dextromethorphan and its metabolites analysis: Dextromethorphan and its metabolites in plasma and in urine were determined by a high performance liquid chromatographic method as described in Chapter 3.1.

5.2.6. Pharmacokinetic and statistical analyses

The maximum plasma concentration (C_{max}) and its time of occurence (t_{max}) were determined from the observed data. The area under the plasma concentration-time curve to the last sampling time (AUC_{0-8}) following chronic doses administration was calculated by the trapezoidal rule.

The accuracy of the cough recording system was calculated as

$$\frac{\text{difference between hand and tape recorder counts}}{\text{number of hand counts}}$$

and the inhibition of cough was calculated as

$$\frac{\text{cough frequency before treatment - cough frequency after treatment}}{\text{cough frequency before treatment}}$$

Differences in cough frequencies between treatments were analysed for statistical significance by t-test. Correlations between plasma concentra-

tion at steady state and cough suppressant effect of the drug was determined by linear regression analysis. Statistical significance was assumed when $P < 0.05$. All data are reported as mean ± SD.

5.3. Results

The cough recording system was both sensitive and accurate. This system can pick up very weak cough and the sensitivity is adjustable according to the intensity of the cough and the noise of the environment. The accuracy of the system (Table 5-1-3) was < 1% from 15 hours assessment in two patients.

To allow this section of this thesis to be written, the code of this study from 1 to 11 was broken when the eleventh patient was completed. The study is still continuing. Of the 11 patients, 4 took codeine, 3 took pholcodine, 3 took dextromethorphan and 1 took placebo. The cough suppressant effects of these drugs in the 11 patients are shown in Figure 5-1-2. The cough suppressant effect and the plasma concentrations of codeine, pholcodine and dextromethorphan are shown in Figure 5-1-3-, 5-1-4 and 5-1-5 respectively.

Figure 5-1-1 Cough recording system

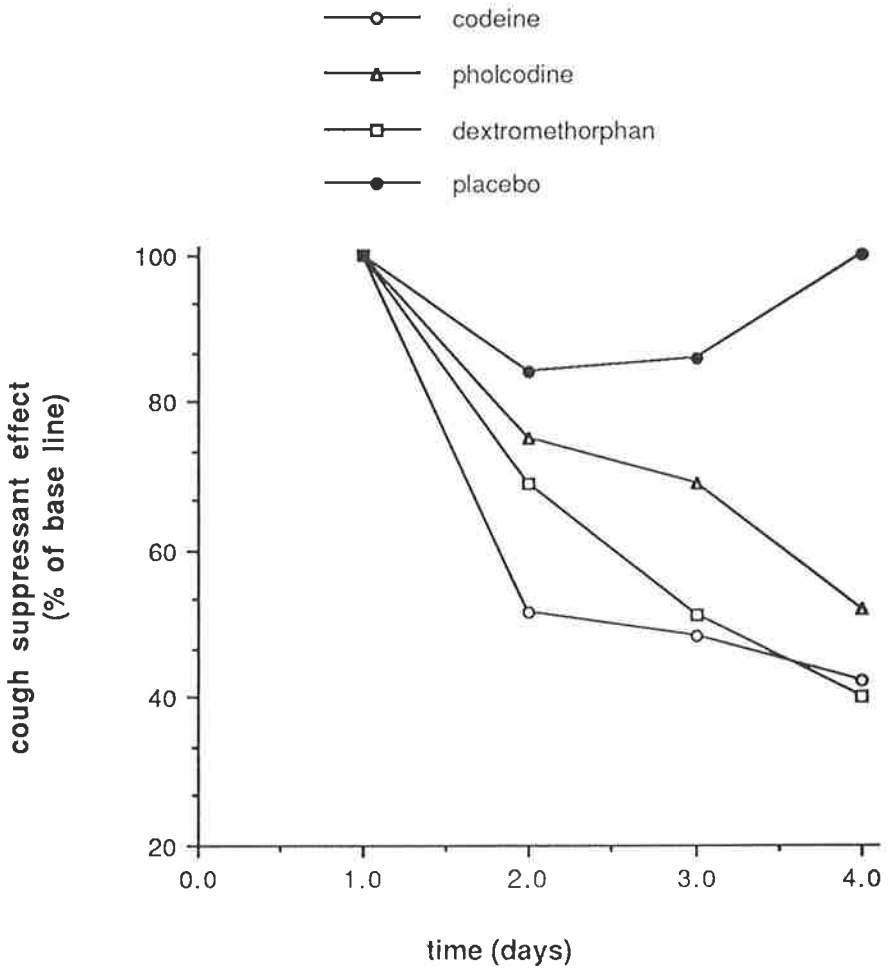


Figure 5-1-2 Mean antitussive effect-time profile of codeine (n=4), pholcodine (n=3) and dextromethorphan (n=3). Placebo n=1

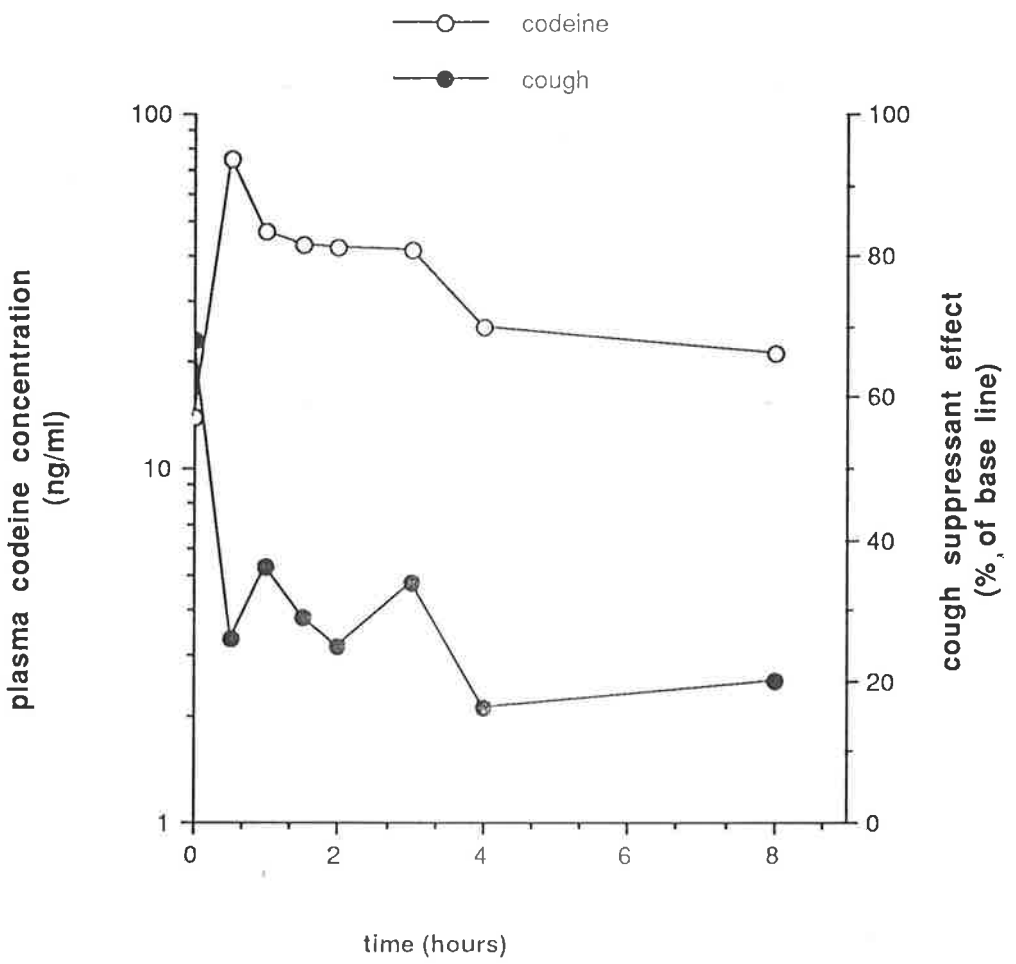


Figure 5-1-3 Antitussive effect and plasma concentrations of codeine (n=4).

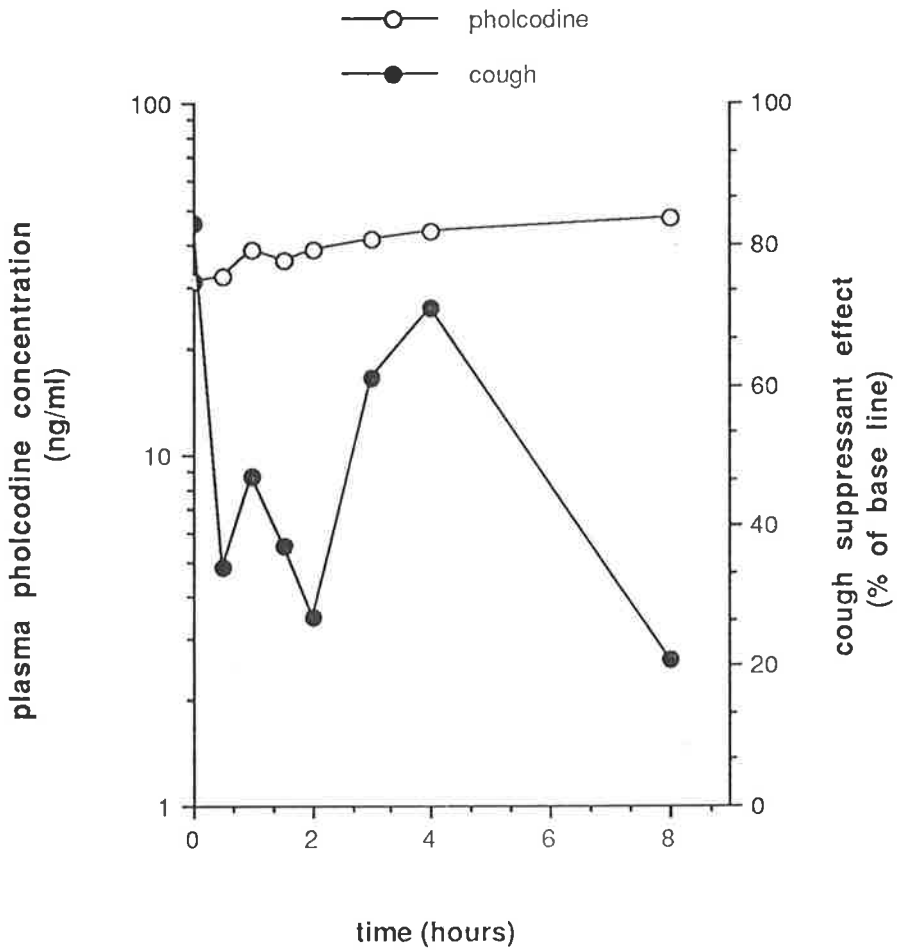


Figure 5-1-4 Antitussive effect and plasma concentrations of pholcodine (n=3).

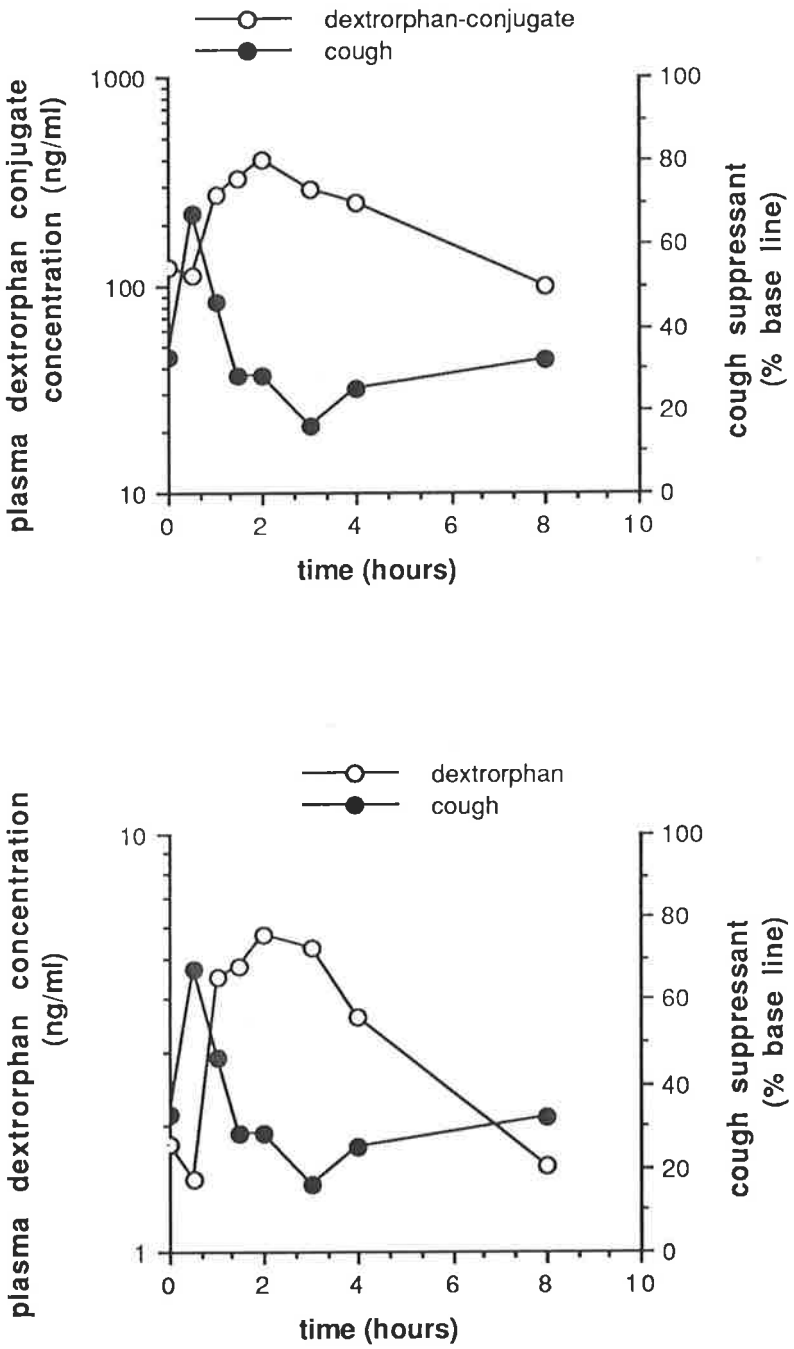


Figure 5-1-5 Antitussive effect and plasma concentrations of dextrorphan conjugate (top) and dextrorphan (bottom) (n=3).

Table 5-1-1 General information of the patients

patient	sex	age (year)	BW (kg)	smoker	Clcr (ml/min)	GGT (U/L)	ALP (U/L)	albumin (g/L)	Hb (g/dl)
1	M	48	62	N	105	95	91	36	12
2	F	64	68	N	75	21	91	42	15
3	F	53	62	N	73	11	41	44	15
4	F	28	82	Y	155	13	87	44	13
5	M	48	60	N	85	225	112	40	13
6	F	54	57	N	108	19	47	45	14
7	M	66	70	Y	90	14	78	44	13
8	F	26	54	N	95	20	91	43	13
9	M	54	78	Y	88	203	111	48	14
10	M	23	76	N	130	22	90	44	14
11	M	59	69	Y	90	17	12	36	13

BW: body weight; Clcr: creatinine clearance; GGT: Gamma Glutamate Transaminase; ALP: alkaline phosphatase; Hb: haemoglobin.

Normal values: see Appendix II

Table 5-1-2 Illness and medications

patient	diagnosis	medications
1	D.I.L.D.	cimetidine, glibenclamide, paracetamol, gentamicin, amoxycillin, temazepam.
2	asthma	paracetamol, terbutaline sulphate, beclomethasone dipropionate, theophylline, digoxin, amiloride, frusemide, temazepam.
3	D.I.L.D.	paracetamol, oxazpam, prednisolone, oestrogen, norethisterone.
4	bronchitis	none
5	D.I.L.D.	cimetidine, prednisolone, paracetamol, nitrazepam.
6	bronchitis, diabetes mellitus	metformin, chlorpheniramine, clofibrate, glibenclamide.
7	small cell carcinoma of lung	doxorubicin hydrochloride, nifedipine, cyclophosphamide, etoposide, prednisolone, temazepam, metoprolol, metoclopramide.
8	bronchitis	none
9	carcinoma of lung	chlorothiazide, 5-fluorouracil, dextropropoxyphene, paracetamol.
10	bronchitis	none
11	bronchitis	paracetamol, temazepam.

D.I.L.D. diffuse interstitial lung disease

Table 5-1-3 Accuracy of the cough recording system

time (hour)	cough/hour		difference
	hand count	recorder count	
patient 1			
1	102	101	1
2	38	38	0
3	75	75	0
4	83	85	-2
5	126	125	1
6	163	165	-2
7	127	127	0
patient 2			
8	64	64	0
9	184	186	-2
10	50	50	0
1	25	25	0
12	97	96	1
13	156	155	1
14	67	69	-2
15	54	54	0
total	1411	1415	

5.4. Discussion

This study was initially designed to be performed in 40 patients. However after one year, I had only successfully performed cough assessment and blood sampling in 11 patients. This study was temporarily stopped because of the limitation of time and the concern about the stability of the drugs and their metabolites in plasma and urine samples. There are several reasons why the recruiting of patients was so slow: (1) the patients had to have had chronic cough but to be well enough to participate in this study; (2) only about 50% of eligible patients agreed to participate; (3) several subjects were suitable, but because they underwent special treatments and examinations during the 4 day study period they could not be recruited; (4) errors in drug administration by the nursing stuff. The consideration of these problems may be helpful in future studies. Although the number of the patients is too small to allow any conclusions, each drug has shown a trend in decreasing cough frequency. In contrast, the one patient on placebo did not show such a trend. The maximum cough suppressant effects of the three drugs on day 3 after drug administration were similar. However, the time of the occurrence of maximal effect appeared different. Codeine reached its maximal effect on the first day after treatment, dextromethorphan on the second day and pholcodine on the third day. These differences may be the result of the different half-lives of the drugs. Pholcodine has a much longer half-life and takes a longer time to achieve the maximal plasma concentration. It may be necessary to take a plasma sample each day so that a plasma concentration-antitussive effect curve may be obtained. There was no obvious relation between the cough suppressant effects and the respective plasma concen-trations of these drugs because the cough numbers per hour varied greatly in all the patients before and after treatments. The variance

may have resulted from a number of factors such as eating, drinking, talking and changing position of the body. An assessment on a half day or one day bases provided much clearer results. Whether the metabolites of the drugs, especially pholcodine, have antitussive effect is not known. After administration of dextromethorphan, dextrorphan and its conjugate and 3-hydroxymorphinan conjugate were detected in plasma. Dextrorphan is possibly the more active compound.

These very preliminary results suggest that codeine, dextromethorphan and pholcodine have different antitussive properties. The occurrence of the maximum antitussive effect of pholcodine was much slower than dextromethorphan and codeine. These results suggest that pholcodine may not be a suitable drug for acute cough. The results of this pilot study are encouraging and more results and conclusions will be obtained when a larger study is finished.

Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

Codeine, pholcodine and dextromethorphan are commonly used cough suppressants, and in addition, codeine is widely prescribed as an analgesic. Their pharmacological activities in human have been studied but still many questions need to be answered. For example, although it has been used clinically for more than 100 years, the analgesic mechanism of codeine is still unclear. The pharmacokinetics and disposition of codeine, pholcodine and dextromethorphan have been incompletely studied because of lack of sensitive and specific analytic methods, especially for their metabolites which may be active. The work described in this thesis deals mainly with the pharmacokinetics and disposition of these drugs in humans. Some aspects of their pharmacodynamics in humans and in the rat have also investigated, including studies on the analgesic mechanism of codeine and study on the antitussive effects of codeine, pholcodine and dextromethorphan in patients.

To determine the concentrations of these compounds and their metabolites in biological fluids, four original sensitive and specific high performance liquid chromatographic assays have been developed. The assays for pholcodine and codeine-6-glucuronide are the first specific analytical methods for the determination of the compounds in biological fluids and the other two are also the first methods to simultaneously determine the drugs and metabolites in plasma and in urine, one of them for codeine, morphine and norcodeine and the other for dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan. These methods have shown good accuracy and provided sufficient specificity and sensitivity for the determination of pharmacokinetics and metabolism of these drugs after therapeutic doses in humans.

The pharmacokinetics and metabolism of pholcodine were studied in 6 healthy volunteers after single and chronic oral doses. The disposition and

metabolism of pholcodine in humans were substantially different from those of other chemically related compounds, such as codeine and dextromethorphan. The plasma half-life of pholcodine after single and chronic doses was 50 hours compared with those of 3 hours for codeine and dextromethorphan. The long half-life suggests that pholcodine should take a longer time to achieve its maximal pharmacological effects than codeine and dextromethorphan. Specifically, no antitussive effect after a couple of doses of pholcodine may not necessarily mean no antitussive effect after chronic administration. My results suggest that the currently recommended dosage regimens for pholcodine (10 mg 4 to 6 hourly) may be inappropriate and that pholcodine may not be the appropriate antitussive for short term (1-2 days) cough. It may however, be the drug of choice for suppression of chronic cough. More work is required to answer these questions. The metabolism of pholcodine is also different from that of codeine and dextromethorphan. In contrast to the great extent of glucuronide conjugation of codeine and dextromethorphan, pholcodine did not appear to undergo glucuronide conjugation. Morphine, a metabolite of codeine, was not detected in plasma and urine after single and chronic doses of pholcodine. This indicates that the putative antitussive effect of pholcodine is exerted via the parent compound and/or other metabolites rather than morphine. Two new metabolites of pholcodine were isolated and one of them was successfully identified by high performance liquid chromatography, mass spectrometry and nuclear magnetic resonance methods. This metabolite is the oxidative product of the morpholine ring of pholcodine. The differences in the metabolism between pholcodine and codeine may result from the difference in their molecular structures.

Studies were performed to determine the pharmacokinetics and metabolism of codeine after single and chronic oral doses in young healthy subjects and after chronic doses in the elderly. The pharmacokinetics of

codeine-6-glucuronide, the major metabolite of codeine, were also determined by a specific high performance liquid chromatographic method. The absorption and disposition of codeine in healthy young subjects after chronic doses were not changed compared with the single dose. The area under the plasma concentration-time curve of codeine-6-glucuronide was 17 times higher than the parent compound. The half-life of codeine-6-glucuronide was not significantly different from that of codeine. The high concentrations of codeine-6-glucuronide indicate that it may be a potentially important metabolite by analogy with the marked pharmacological activities of morphine-6-glucuronide. Morphine was just detectable in plasma after single and chronic administration of 30 mg codeine phosphate. The low plasma concentrations of morphine after therapeutic dose of codeine, in addition to its poor ability in crossing the blood brain barrier, do not support the hypothesis that codeine exerts its analgesic effect via hepatic formation of morphine. The urinary recovery of codeine-6-glucuronide was 55% of the dose administered, and those of morphine and norcodeine were 5% and 6% of the dose respectively. The renal clearance of codeine was 67 - 265 ml/minute and was pH dependant. The results indicate that codeine undergoes tubular secretion and reabsorption in addition to glomerular filtration. The renal clearance of codeine-6-glucuronide, which is usually thought to be readily excreted in urine, was 32 - 111 ml/minute and was lower than the glomerular filtration rate. The reasons for this are unknown, but could include re-absorption and/or deconjugation mechanisms for codeine-6-glucuronide in the kidney. The pharmacokinetics of codeine in the elderly were altered compared with those in the young subjects. The absorption was delayed and the plasma half-lives for codeine and especially for codeine-6-glucuronide were increased. The plasma concentrations of codeine and codeine-6-glucuronide at steady state increased 2.4 and 3.8 times respectively and the

renal clearances of codeine and codeine-6-glucuronide decreased 5.0 and 7.2 times respectively by comparison with the young subjects. The plasma concentrations of codeine and codeine-6-glucuronide at steady state were strongly correlated with creatinine clearance and also significantly correlated with the renal clearances of codeine and codeine-6-glucuronide. These results suggest that the elevated plasma concentrations of codeine and codeine-6-glucuronide are mainly caused by decreased renal function. All the changes in the pharmacokinetic parameters were significantly correlated with the age. Codeine is considered to be eliminated by hepatic biotransformation, and no dosage adjustments are currently recommended in aged patients and the patients with renal failure. My findings suggest that the dosing regimens of codeine for the elderly should take consideration of alterations in renal function.

β-glucuronidase has been widely used for both qualitative and quantitative determination of glucuronides in biological fluids. The hydrolysis of codeine-6-glucuronide by β-glucuronidase was investigated by direct determination of codeine-6-glucuronide and codeine, using specific high performance liquid chromatographic methods, after enzymatic hydrolysis. The hydrolysis of codeine-6-glucuronide by β-glucuronidase in acetate buffer solution was not complete with a maximum of about 80%. In urine, however, with the same concentration of β-glucuronidase, the hydrolysis was only 20-30% indicating substances naturally present in urine inhibited the activity of β-glucuronidase. My results suggest that β-glucuronidase hydrolysis may not be suitable for the quantitative determination of codeine-6-glucuronide.

The metabolism of dextromethorphan has been known to be under genetic control and dextromethorphan has been suggested to be the one of

the best drugs to study the debrisoquine oxidative polymorphism. In a screening study, the polymorphic metabolism of dextromethorphan was studied in 52 healthy volunteers. Based on the O-demethylation ratio (O-demethylated/non-O-demethylated fraction), three of them were identified as poor metabolisers, 4 as intermediate metabolisers and 45 as extensive metabolisers after density function analysis. The metabolic ratio for poor and extensive metabolisers averaged 1.2 and 883.0 respectively. The total urinary recovery in extensive metabolisers was 41% of the dose and 6% in poor metabolisers.

One of my volunteers in the codeine study was found to be unable to form morphine after ingestion of codeine. This finding, together with the similarities both in the structures and in the metabolic pathways of codeine and dextromethorphan, led me to investigate if the O-demethylation of codeine is under genetic control. Twelve volunteers, 3 poor metabolisers, 2 intermediate metabolisers and 7 extensive metabolisers of dextromethorphan from the above screening study, were studied. There was a very strong correlation between the metabolic ratios for the O-demethylation of dextromethorphan and codeine with a Spearman correlation coefficient of 0.96. The recovery of total morphine in the poor metabolisers following codeine administration was less than 0.3% of the dose and greater than 4% of the dose for the extensive metabolisers. The N-demethylation ratio, however, was similar in both groups. My results suggest that the O-demethylation of codeine is under genetic control and cosegregates with the dextromethorphan genetic polymorphism, which cosegregates with the debrisoquine oxidative polymorphism. The clinical consequences of this finding are not clear but it is possible that codeine may be potentially an inactive drug in those who are unable to metabolise codeine to morphine.

Morphine is a potent analgesic opioid and has a high binding affinity to the μ-opioid receptor. Recently, morphine-6-glucuronide has been shown to have similar analgesic effects to morphine. I have performed limited studies to test the binding affinity of codeine and its metabolites and several other opioids to the μ-opioid receptor in rat brain using the specific ligand ^{3}H-DAGO. Morphine and morphine-6-glucuronide showed similar binding affinities to the μ-opioid receptor, which was 70 times higher than that of morphine-3-glucuronide. The binding affinities of codeine, norcodeine and codeine-6-glucuronide to the μ-receptor in rat brain were similar, and about 230 times less than those of morphine and morphine-6-glucuronide. However the plasma codeine-6-glucuronide concentration after ingestion of codeine was about 500 times higher than that of morphine and may thus have some role in the analgesia of codeine. The binding affinities of dextromethorphan, dextrorphan and pholcodine to the μ-opioid receptor were at least 1000 times less than that of morphine. These results support the previous findings that dextromethorphan and pholcodine have no analgesic effects in animal and in man.

Although morphine has very high affinity to the μ-opioid receptor, less than 5% of the dose was converted to morphine after codeine administration, and most of this undergoes metabolism to the less active conjugate, morphine-3-glucuronide. The plasma concentrations of morphine after therapeutic doses of codeine were very low. In addition, morphine and its conjugates are very polar compounds and have difficulty passing across the blood brain barrier. The concentration of morphine in the receptor site would thus be even lower. Codeine is lipid soluble and crosses the blood brain barrier much more readily. Metabolism of xenobiotics in the brain has been reported. Codeine O-demethylation to morphine in the brain was studied in the rat in vitro and in vivo. After incubation of codeine with rat brain

homogenates, morphine was detected in the incubation solution and more morphine was found in microvessel rich tissue than in the total homogenate. These findings suggest that codeine is biotransformed to morphine in the brain, possibly in the endothelial cells of microvessels. In the in vivo study, rats were given codeine 20 mg/kg or morphine 1 mg/kg intraperitoneally. A similar plasma morphine concentration was achieved at 30 minutes after administration of codeine or morphine. Morphine in the brain, however, was only detected in the rats given codeine. The morphine concentrations in the rat brain after codeine administration peripherally were about 20 times higher than required to displace 50% of the μ-receptor ligand ^{3}H-DAGO. My results suggest that the morphine detected in the brain after peripheral administration of codeine, was mainly formed in the brain and the analgesic effect of codeine may be exerted by the morphine biotransformed from codeine in the brain rather than peripherally.

Although pholcodine, dextromethorphan and codeine have been widely used as antitussive drugs in patients for many years, their antitussive potency in man has not been well evaluated, mainly because of the lack of reliable and practical cough estimating methods. A cough recording system for measuring the cough frequency was developed for a clinical study designed to compare the relative antitussive efficacy of codeine, pholcodine and dextromethorphan in patients. In a double-blind, placebo controlled pilot study, the antitussive effect of these agents in patients with chronic cough were studied. The cough recording system was demonstrated to be working reliably. Preliminary results has been obtained and the study is still continuing.

The studies described in this thesis have provided new data on the pharmacokinetics, metabolism and some aspects of pharmacodynamics of

pholcodine, codeine and dextromethorphan, which will benefit the understanding and clinical applications of these drugs. Further work needs to be done to complete some of the areas I have explored, and to answer some of the questions which have arisen from the work presented in this thesis. Some of this effort should be devoted to the following:

(1) to complete the antitussive study on pholcodine, codeine and dextromethorphan in patients;

(2) to complete the determination of morphine glucuronides in plasma and in urine samples from the elderly patients and young healthy volunteers given codeine;

(3) to test the binding affinity pholcodine, codeine, dextromethorphan and their metabolites to the "cough-receptor";

(4) to study if any interactions occur between codeine and paracetamol after oral administration of the combination in humans;

(5) to further test my hypothesis on codeine analgesia by doing plasma and brain concentration-analgesic response curves; to test whether codeine-6-glucuronide is an active compound for analgesia, and whether it causes the toxicity of codeine in patients with renal failure;

(5) to test whether codeine is an inactive analgesic for the poor metabolisers who are not able to metabolise codeine to morphine;

(6) to test whether reabsorption of codeine-6-glucuronide occurs in the kidney; whether deconjugation of codeine-6-glucuronide occurs in the kidney and/or urinary tract; to test whether entero-hepatic re-circulation of codeine-6-glucuronide occurs in humans;

(7) to perform a study to determine the pharmacokinetics of dextromethorphan in poor metabolisers and in extensive metabolisers, and to assess plasma concentration-antitussive relationships in these groups;

(8) to try to synthese the metabolite of pholcodine and to determine it quantitatively and also to estimate its pharmacological activities.

APPENDICES

Appendix I.

Reagents involved in this thesis

reagent	grade	supplier
acetonitrile	HPLC	Ajax Chemicals
acetic acid	analytical	Ajax Chemicals
ammonium sulphate	analytical	Ajax Chemicals
anhydrous sodium carbonate	analytical	Ajax Chemicals
calcium chloride	analytical	Ajax Chemicals
chloroform	analytical	Ajax Chemicals
citric acid	analytical	Ajax Chemicals
diethyl ether	analytical	Ajax Chemicals
d-glucose	analytical	Ajax Chemicals
methanol	analytical	Ajax Chemicals
pentan-1-ol	analytical	Ajax Chemicals
potassium chloride	analytical	Ajax Chemicals
propan-2-ol	analytical	Ajax Chemicals
sodium acetate	analytical	Ajax Chemicals
sodium bicarbonate	analytical	Ajax Chemicals
sodium chloride	analytical	Ajax Chemicals
sodium dihydrogen phosphate	analytical	Ajax Chemicals
sodium hydrogen carbonate	analytical	Ajax Chemicals
sodium hydroxide	analytical	Ajax Chemicals
triethylamine	analytical	Ajax Chemicals
ascorbic acid	analytical	BDH Chemicals
hydrochloric acid	analytical	BDH Chemicals
orthophosphoric acid	analytical	BDH Chemicals
sodium bisulfite	analytical	BDH Chemicals
sodium hydrogen orthophosphate	analytical	BDH Chemicals

Ajax Chemicals, Port Fairy, Australia; BDH Chemicals, Sydney, Australia.

Appendix II.

Normal value ranges of clinical biochemical tests

(from the Institute of Medical and Veterinary Science, Adelaide.)

test	unit	range
creatinine	mmol/L	0.05 - 0.12
urea	mmol/L	0.25 - 0.45
albumin	g/L	39 - 48
haemoglobin	g/dl	13.5-18
glucose	mmol/L	3.8 - 5.8
conjugated bilirubin	µmol/L	1 - 4
total bilirubin	µmol/L	6 - 24
gamma glutamate transaminase	U/L	0 - 50
alkaline phosphatase	U/L	30 - 110
lactate dehydrogenase	U/L	110 - 230

Appendix III.

Synthesis of codeine-6-glucuronide

This was performed by Dr. G. Reynolds of the School of Chemical Technology, South Australian Institute of Technology, Adelaide.

The synthesis was performed following a procedure described by Yoshimura and coworkers (1968) with minor modifications: It was necessary to perform the Koenigs-Knorr reaction over 48 hours for complete disappearance of starting material.

The crude methyl[codeine-6-yl-2,3,4-tri-o-acetyl-β-D-glucopyranosid]-uronate was chromotographed on silica (Amicon 35-70μ) and the purified product eluted with 3% methanol in dichloromethane. The conversion to codeine-6-glucuronide was carried out as described by Yoshimura and coworkers (1968).

To verify the identity of the synthesised codeine-6-glucuronide (Figure iii-1), a ^{13}C NMR spectrum was measured on a solution of the product in D_2O and recorded on a Varian Gemini 200 RT NMR spectrometer (Figure iii-1). Assignments are based on resonances reported for codeine by Carroll and coworkers (1976). An attached proton test (APT) was also measured (Figure iii-1), using a procedure reported by Patt and Shoolery (1982).

The APT pulse sequence causes signals for quaternary carbons and CH_2's to appear above the base line of the spectrum whilst the signals for CH's and CH_3's point below. The results of the APT are in agreement with the assignments given for the ^{13}C resonances.

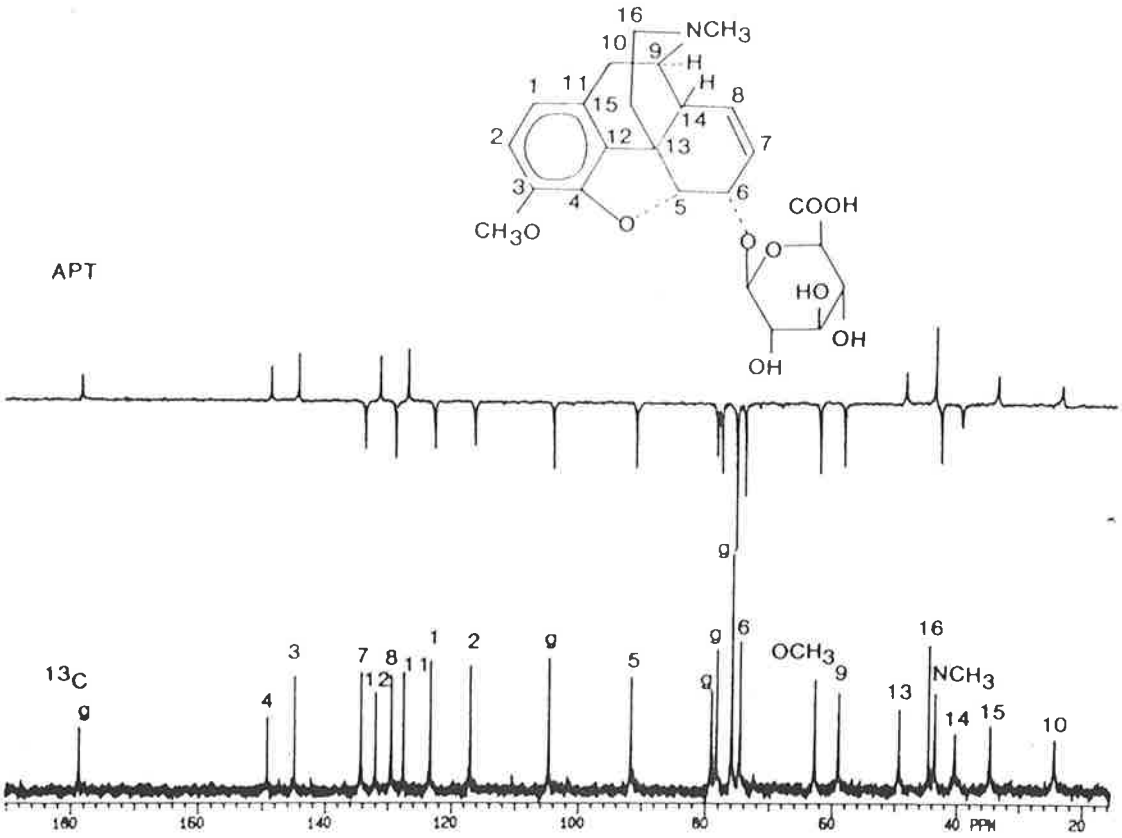


Figure iii-1 ^{13}C and APT N.M.R. spectra of codeine-6-glucuronide in D_2O. Numbers above signals in ^{13}C N.M.R. spectrum refer to Figure iii-1. Symbol g refers to carbons in sugar residue.

Appendix IV.

Identification of pholcodine metabolite by mass spectra and NMR

This was carried out by Dr. A.D. Ward Department of Organic Chemistry, the University of Adelaide.

Method:

Mass spectra: Mass spectra were measured on a VG ZAB 2F mass spectrometer using the fast atom bombardment technique and glycerol as the matrix. Mass analysed kinetic energy spectra (collision activated) were measured using helium as the collision gas.

Nuclear magnetic resonance (NMR) spectra: Nuclear magnetic resonance spectra were obtained on a Bruker CXP 300 instrument, using deuterochloroform as the solvent and tetramethylsilane as an internal reference. All chemical shifts are quoted as δ in parts per million.

Results and discussion:

The metabolite had a molecular weight of δ 413 as determined by fast atom bombardment (FAB) mass spectrometry (Figure iv-1b), compared to a molecular weight of δ 399 for pholcodine (Figure iv-1a). Using FAB conditions neither compound showed significant fragmentation ions in the higher mass region of the spectrum. When the mass analysed kinetic energy spectrum (MIKES) was determined on the molecular ion of pholcodine a prominent ion was observed at δ 114 mass units with a smaller peak at δ 100 (Figure iv-2). (This technique measures the fragmentations that occur directly from a particular ion, in this case the molecular ion). The formation of these peaks

corresponds to fragmentations involving the morpholine containing side chain as shown in the Scheme (Figure iv-3). Other prominent peaks in the MIKES spectrum of the molecular ion were observed at δ 395 (M-18), δ 355 (M-43), δ 341 (M-58), δ 312 (M-87) and δ 286 (M-113). The MIKES spectrum of the molecular ion of the metabolite (Figure iv-4) showed prominent peaks at δ 395 (M-18), δ 389 (M-24), δ 348 (M-65) and δ 286 (M-127). A weaker peak was observed at δ 128. Thus both systems cleave to give a peak at δ 286 and differ in the mass of the other fragment ion; δ 114 in pholcodine versus δ 128 in the metabolite. These data show that the extra δ 14 mass units in the metabolite are located in the morpholinoethyl side chain. This difference of δ 14 mass units between the two molecules can be most readily explained by the replacement of a $-CH_2-$ unit in this side chain with a >C=O group.

The ^1H nuclear magnetic resonance spectrum of pholcodine (Figure iv-5a) shows peaks which can be assigned, by comparison with the NMR spectra of codeine (Figure iv-6) and morphine, as follows (Table iv-1). A similar approach then allows some of the peaks in the NMR spectrum of the metabolite (Figure iv-5b) to be assigned.

The major difference in the two spectra relate to the signals from the morpholine ring which occur at δ 3.73 (OCH_2) and in the region δ 2.25-2.87 (NCH_2) in the pholcodine spectrum. [In morpholine (Varian NMR spectra catalog) itself the corresponding signals occur at δ 3.67 and at δ 2.87.] These peaks are considerably different in the spectrum of the metabolite, occurring at δ 4.17 (singlet, $-OCH_2C=O$), δ 3.86 (OCH_2CH_2) and δ 3.61 (NCH_2CH_2). The methylene attached to the aromatic oxygen shows signals in the δ 4.1-4.3 region in both spectra with the other methylene of the ethyl group apparently resonating in the δ 2.2-2.9 region. While further NMR studies will be necessary to clarify all the chemical shift details the available data rules

out the alternative lactone (Laurent and Bearn 1978) structure (as in Figure iv-7 X), (where the $NCH_2C{=}O$ singlet would be expected about δ 2.80) and agree with the chemical shift of a similar amide methylene (Figure iv-7 Y) which occurs at δ 4.20 (Perrone *et al* 1976).

The spectral data in total provides strong support for structure (Figure iv-7 Z) for the metabolite, although the synthesis of this structure and a direct comparison of the two materials will be necessary before the structure can be regarded as proved.

Table iv-1 Comparison of the NMR spectra of pholcodine and metabolite

pholcodine	**metabolite**	**assigment**
δ 6.65 (d)	δ 6.65 (d)	aromatic H
δ 6.53 (d)	δ 6.54 (d)	aromatic H
δ 5.68 (d)	δ 5.70 (d)	alkene H
δ 5.27 (d)	δ 5.29 (d)	alkene H
δ 4.87 (d)	δ 4.87 (d)	CH-O
δ 4.07=4.26 (m)	δ 4.10=4.31 (m)	
	δ 4.17 (s)	OCH_2=O
	δ 3.86 (t)	OCH_2CH_2 of ring
δ 3.74 (t)		morpholine O-CH_2
δ 3.70 (m)	δ 3,74 (m)	
	δ 3.61 (t)	NCH_2CH_2 of ring
δ 3.34 (bs)	δ 3.35 (bs)	
δ 3.07 (s)	δ 3.07 (bs)	
δ 3.01 (s)	δ 3.01 (s)	
δ 2.43 (s)	δ 2.44 (s)	N-CH_3
δ 2.25-2.87 (m)	δ 2.25-2.67 (m)	
δ 2.06 (dt)	δ 2.06 (dt)	
δ 1.87 (d)	δ 1.89 (d)	

d=doublet; m=multiplet; t=triplet; bs=broad singlet; s=singlet; dt=doublet of triplet.

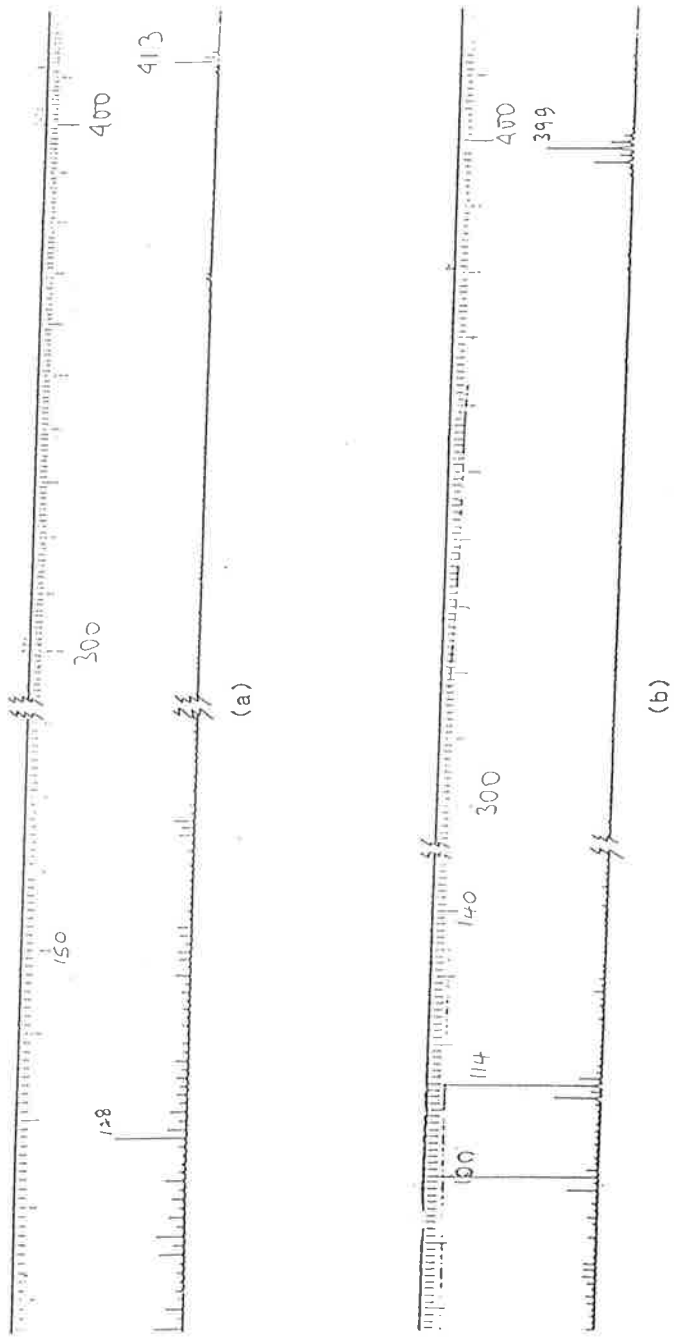


Figure iv-1. Fast atom bombardment mass spectrum of pholcodine (a) and metabolite (b).

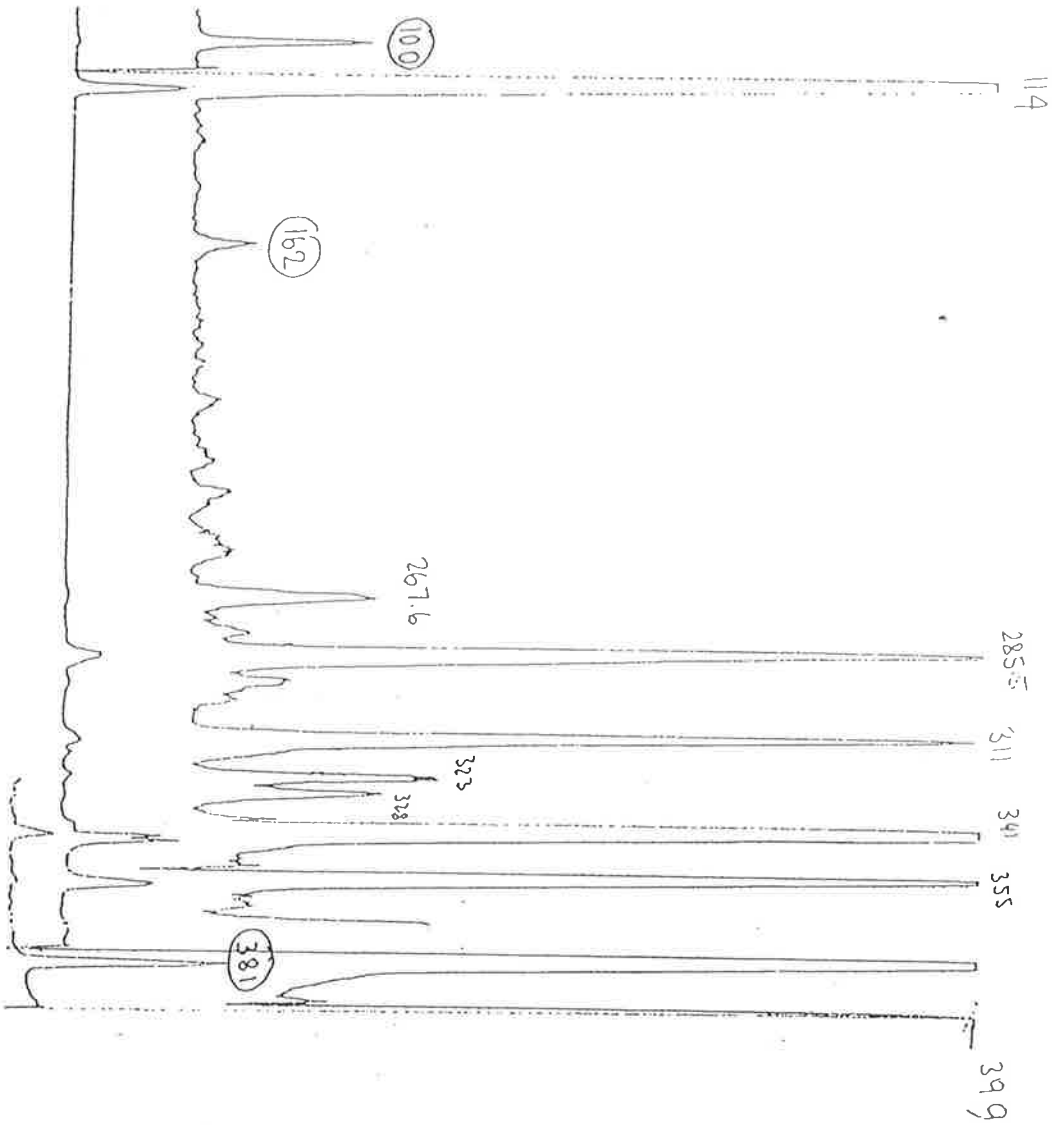


Figure iv-2. Mass analysed Kinetic energy spectrum of pholcodine.

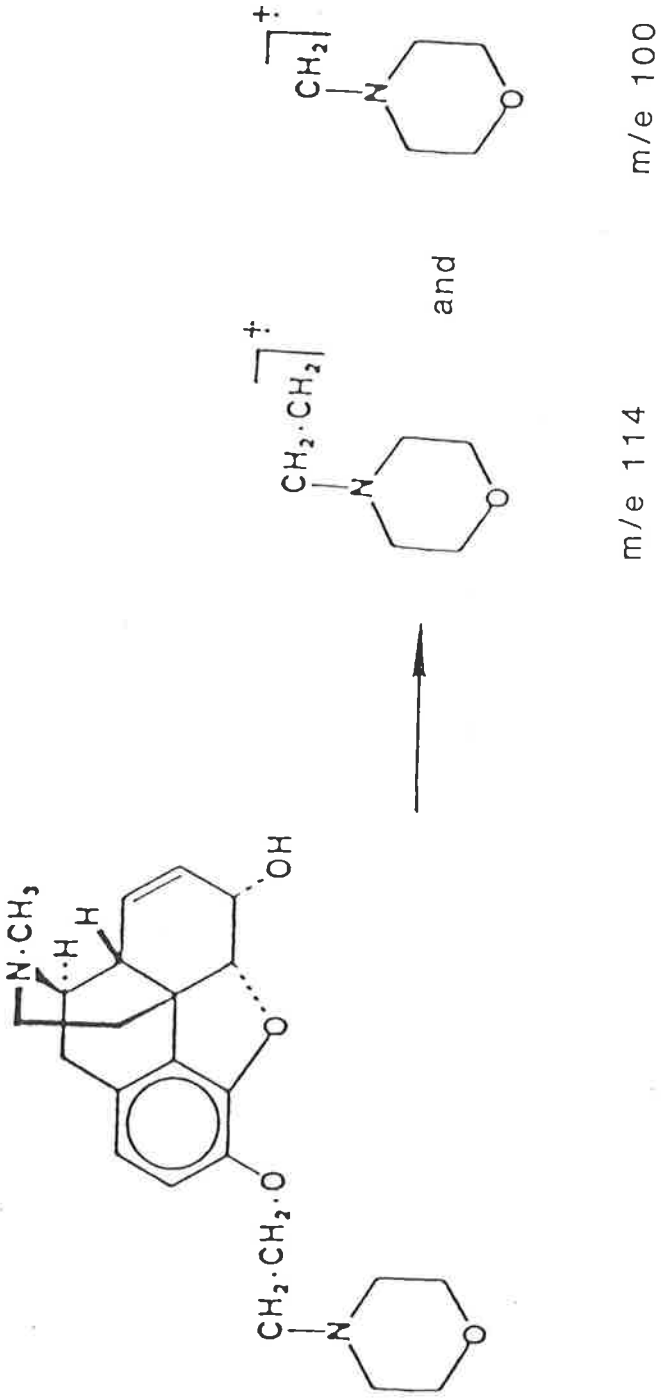


Figure iv-3. Scheme of fragmentations involving the morpholine containing side chain.

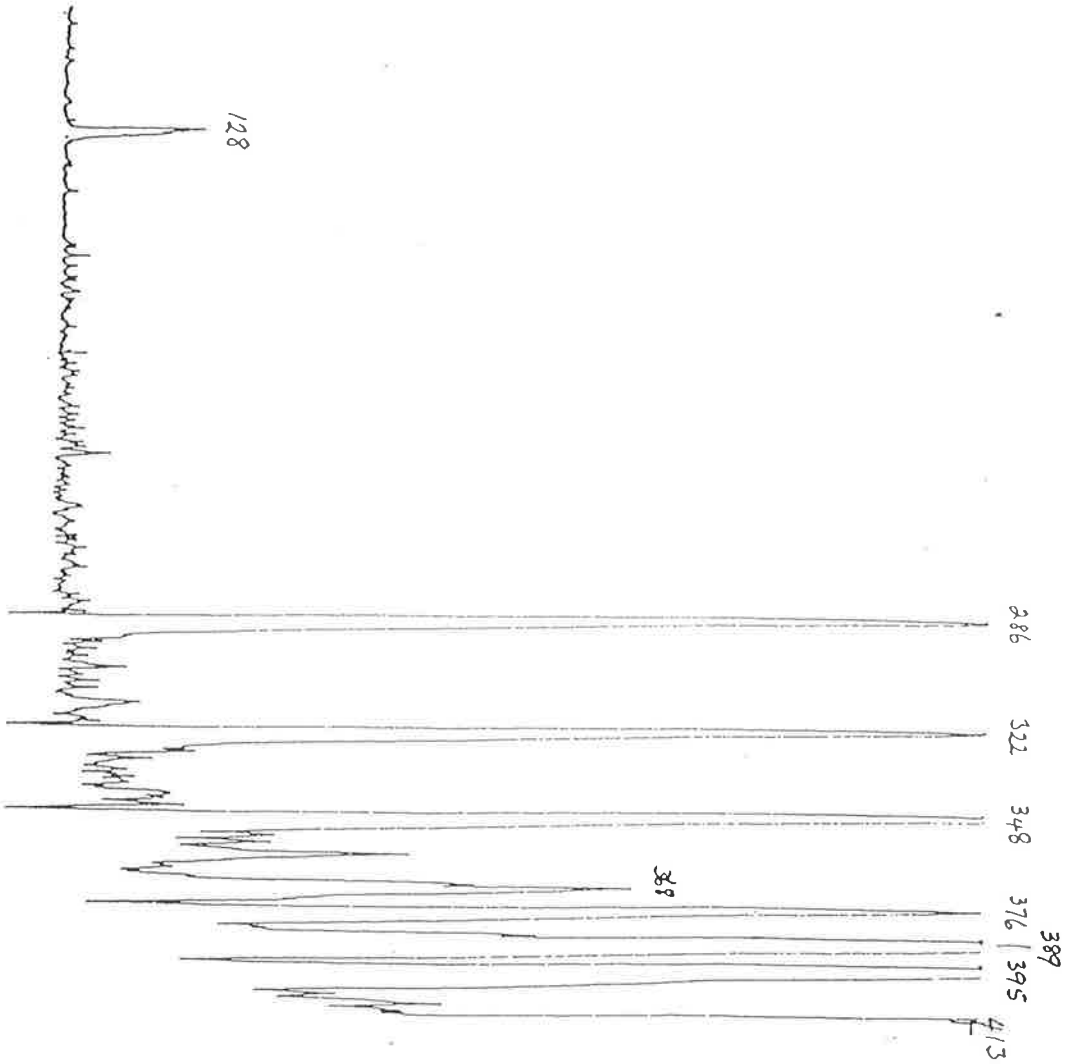


Figure iv-4. Mass analysed Kinetic energy spectrum of pholcodine metabolite.

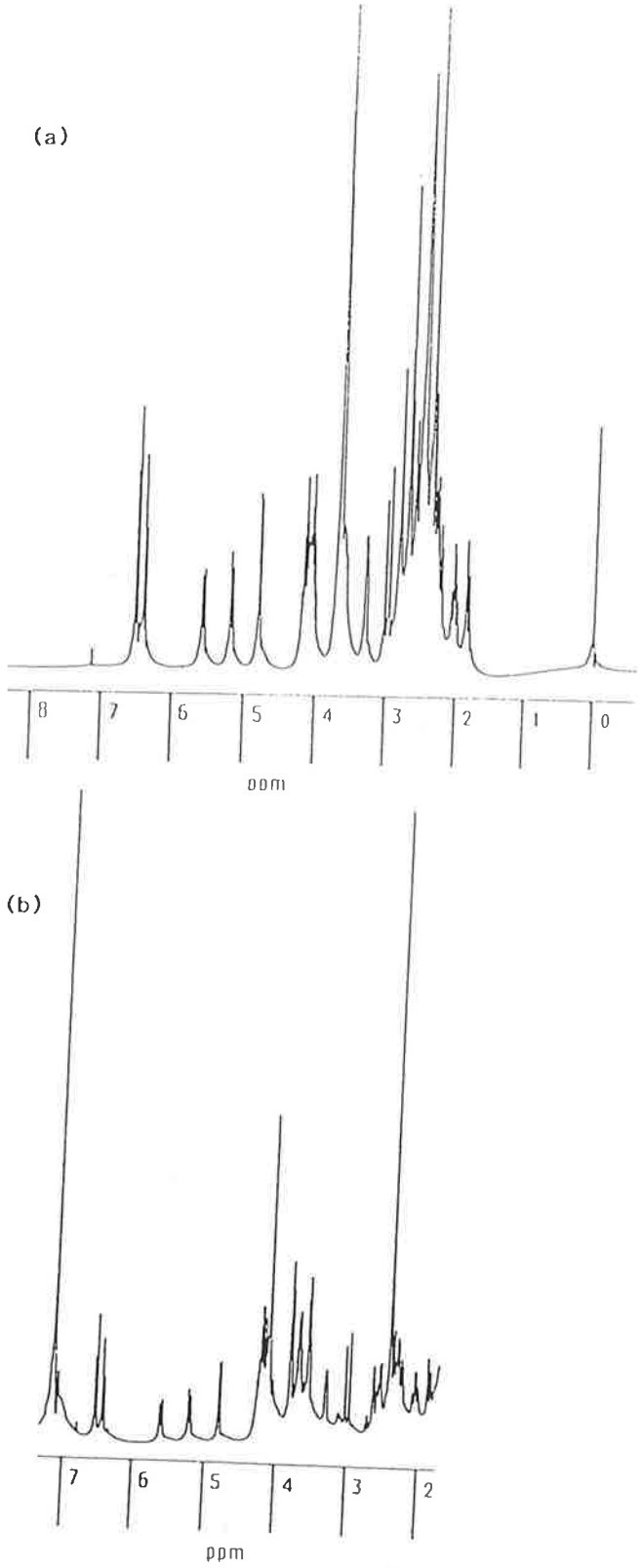


Figure iv-5. ^{1}H nuclear magnetic resonance spectrum of pholcodine (a) and metabolite (b).

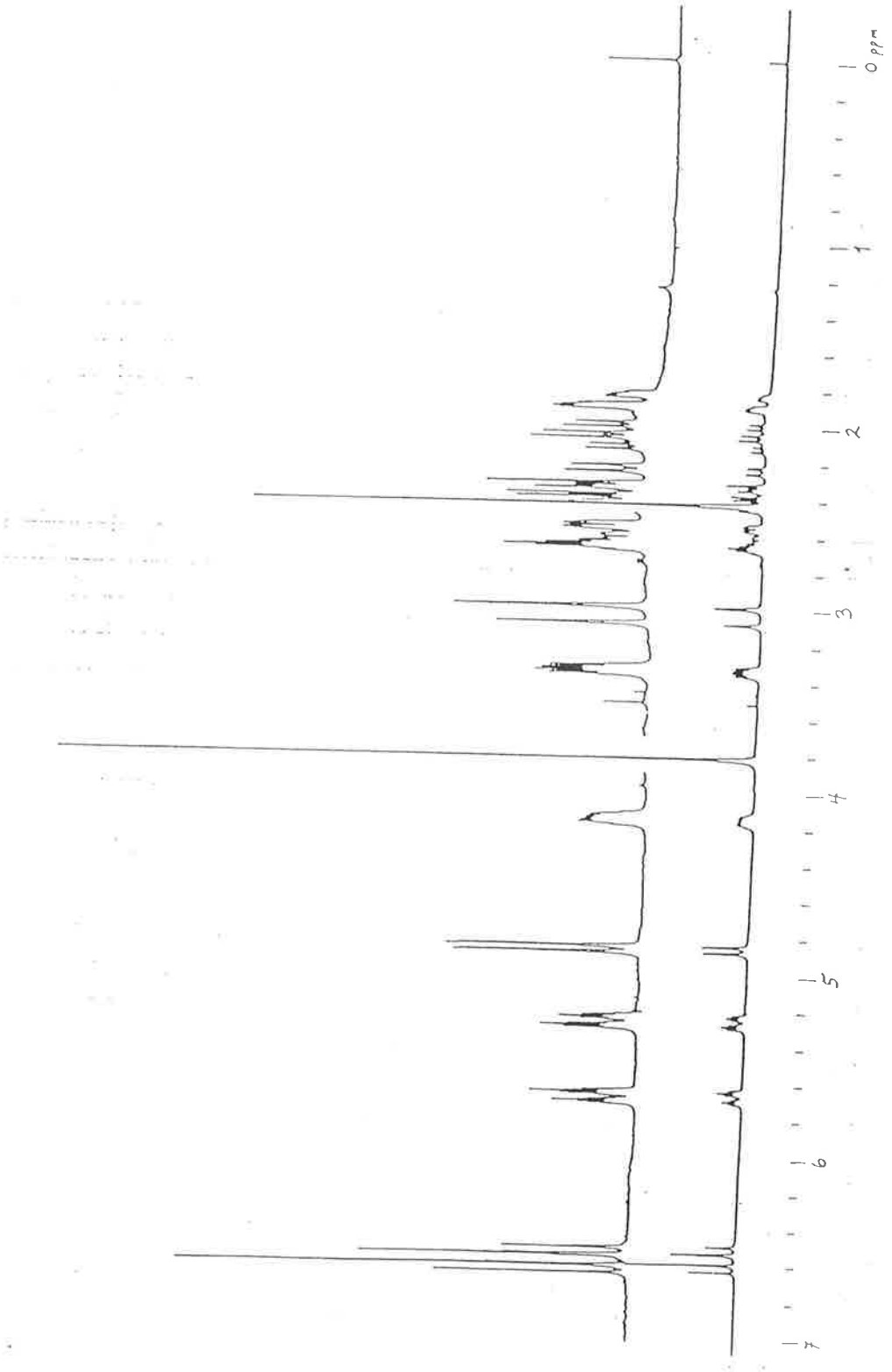
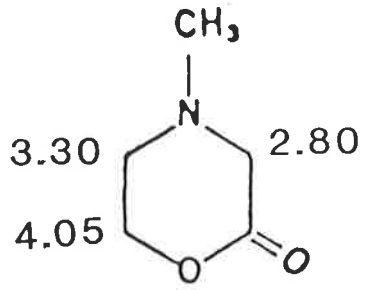
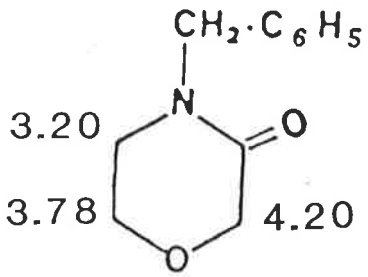


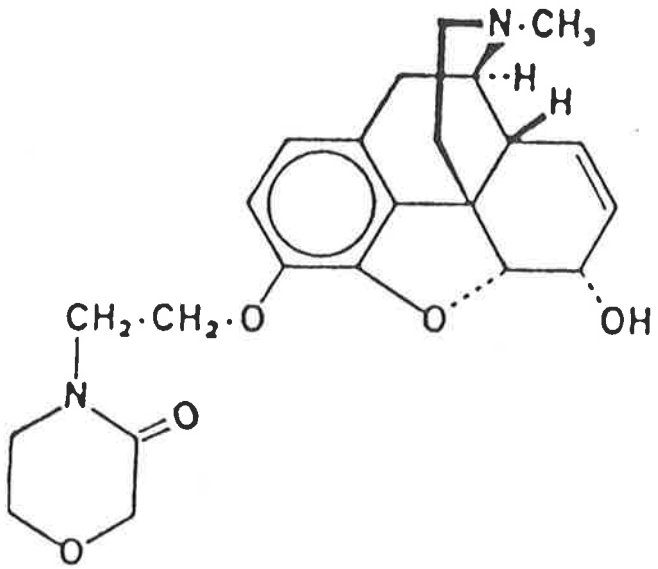
Figure iv-6. ^{1}H nuclear magnetic resonance spectrum of codeine.



(X)



(Y)



(Z)

Figure iv-7. Chemical structure of the metabolite

Appendix V.

List of publications

Work from this thesis has presented in the following publications, accepted publications and presentations at scientific meetings.

1. Chen ZR, Siebert DM, Somogyi AA, Bochner F. Determination of pholcodine in biological fluids by high performance liquid chromatography with fluorescence detection. *J Chromatogr Biomed Appl* 1988; 424: 170-6.

2. Chen ZR, Bochner F, Somogyi AA. Pharmacokinetics of pholcodine in healthy volunteers: single and chronic dosing studies. *Br J Clin Pharmacol* 1988; 26:445-453.

3. Chen ZR, Somogyi AA, Bochner F. Polymorphic O-demethylation of codeine. *Lancet* 1988;i;914-915.

4. Chen ZR, Bochner F, Somogyi AA. Simultaneous determination of codeine, norcodeine and morphine in biological fluids by high performance liquid chromatography with fluorescence detection. *J Chromatogr Biomed Appl* 1989 (in press).

5. Chen ZR, Bochner F, Somogyi AA, Siebert D. HPLC assay and pharmacokinetics of pholcodine in humans. *Xth International Congress of Pharmacology.* No. P82. August, 1987, Sydney, Australia.

6. Chen ZR, Bochner F, Somogyi AA. Single and chronic dose pharmacokinetics of pholcodine in humans. *21nd Annual Scientific Meeting of the Australian Society of Clinical and Experimental Pharmacologists and the*

Australian Pharmaceutical Science Association. December, 1987, Hobart, Australia.

7. Chen ZR, Irvine RJ, Somogyi AA, Bochner F. Morphine formation from codeine in rat brain: A possible mechanism of codeine analgesia. *22nd Annual Scientific Meeting of the Australian Society of Clinical and Experimental Pharmacologists.* December, 1988, Adelaide, Australia.

8. Chen ZR, Somogyi AA, Bochner F. Polymorphic O-demethylation of codeine to morphine in humans: Covarance with dextromethorphan O-demethylation. *22nd Annual Scientific Meeting of the Australian Society of clinical and Experimental Pharmacologists.* December, 1988, Adelaide, Australia.

9. Bochner F, Chen ZR, Young GD, Somogyi AA. Codeine disposition and metabolism in the elderly. *22nd Annual Scientific Meeting of the Australian Society of clinical and Experimental Pharmacologists.* December, 1988, Adelaide, Australia.

10. Irvine RJ, Chen ZR, Somogyi AA, Bochner F. Mu-opioid receptor binding affinity of some opiates and their metabolites. *22nd Annual Scientific Meeting of the Australian Society of clinical and Experimental Pharmacologists.* December, 1988, Adelaide, Australia.

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Appendix VII

One of the examiners of my thesis suggested that the metabolic ratios be re-calculated as follows:

$$\text{MR for O-demethylation} = \frac{\text{dextrorphan}}{\text{dextromethorphan}}$$

$$\text{MR for N-demethylation} = \frac{\text{3-hydroxymorphinan}}{\text{3-methoxymorphinan}}$$

The metabolic ratios for O- and N-demethylation recalculated by the above methods are shown in the following table and for comparison those calculated in this thesis are also shown. The O-demethylation ratios calculated by the suggested method were smaller than those from this thesis, and the range of the values of O-demethylation ratios were 0.24-2579 compared with 0.3-3660 from this thesis. Both methods have shown that the O-demethylation of dextromethorphan exhibits genetic polymorphism and the phenotypes were same, but that the intervals between the metabolic ratios for the phenotypes were smaller with the suggested method. The N-demethylation ratios were similar with both methods.

Metabolic ratios for O- and N- demethylation

subject	O-demethylation ratio		N-demethylation ratio	
	III + II / I =IV	II /I	III + IV /I + II	III /II
1	0.3	0.2	0.15	0.39
2	0.5	0.4	0.24	0.43
3	2.2	1.8	0.24	0.34
4	15.7	10.5	0.59	0.63
5	17.6	16.3	0.27	0.28
6	21.8	18.3	0.49	0.50
7	24.9	18.0	0.42	0.44
8	56.7	35.5	0.65	0.67
9	79.6	59.1	0.34	0.35
10	115.3	87.4	0.32	0.32
11	117.4	79.1	0.48	0.48
12	124.4	91.6	0.35	0.36
13	142.1	107.4	0.32	0.32
14	163.3	138.9	0.30	0.30
15	169.5	131.6	0.29	0.29
16	172.4	117.7	0.46	0.47
17	191.0	142.4	0.34	0.34
18	191.7	144.9	0.32	0.32
19	212.0	154.9	0.37	0.37
20	216.8	160.9	0.34	0.34
21	239.7	186.4	0.36	0.37
22	291.6	221.1	0.32	0.32
23	346.3	285.3	0.21	0.21
24	364.0	234.7	0.55	0.55
25	427.2	310.3	0.38	0.38
26	432.6	356.8	0.21	0.21
27	456.3	240.4	0.89	0.89
28	481.2	388.1	0.37	0.37
29	507.5	347.1	0.46	0.46
30	520.4	416.0	0.42	0.42
31	552.7	442.1	0.25	0.25
32	589.5	431.6	0.37	0.37
33	656.1	459.6	0.43	0.43
34	658.8	463.2	0.53	0.53
35	789.5	463.2	0.70	0.70
36	831.6	452.6	0.84	0.84
37	851.6	634.2	0.34	0.34
38	861.1	610.5	0.41	0.41
39	919.3	656.1	0.40	0.40
40	1000.0	673.7	0.48	0.48
41	1122.8	821.1	0.37	0.37
42	1198.6	808.9	0.49	0.49
43	1220.8	1161.4	0.42	0.42
44	1357.9	831.6	0.63	0.63
45	1421.1	852.6	0.67	0.67
46	1530.5	1046.3	0.46	0.46
47	1542.0	1201.5	0.28	0.28
48	1615.8	1247.4	0.30	0.30
49	2016.8	1452.6	0.39	0.39
50	3000.0	2284.2	0.31	0.31
51	3642.3	2747.4	0.33	0.33
52	3659.6	2578.9	0.42	0.42

I: dextromethorphan;
II: dextrorphan;
III: 3-hydroxymorphinan
IV: 3-methoxymorphinan.

As with dextromethorphan, the examiner suggested the metabolic ratio be re-calculated as follows:

$$\text{MR for O-demethylation} = \frac{\text{total morphine}}{\text{codeine}}$$

The metabolic ratios for O- demethylation re-calculated by the above method are shown in the following table and for comparison those calculated in this thesis are also shown. The O-demethylation ratios calculated by the suggested method were larger than those from this thesis, and the range of the values of O-demethylation ratios were 0.024-0.417 compared with 0.003-0.106 from this thesis. Both methods have shown a significant correlation between the metabolic ratios for the O-demethylation of codeine and dextromethorphan. The Spearman rank correlation coefficient is 0.89 with the suggested method compared with 0.96 from this thesis.

O-demethylation ratios

subject	O-demethylation ratio	
	suggested method	in this thesis
1	0.024	0.003
2	0.041	0.006
3	0.064	0.006
4	0.078	0.009
5	0.095	0.016
6	0.162	0.031
7	0.223	0.034
8	0.191	0.039
9	0.698	0.048
10	0.447	0.070
11	0.303	0.080
12	0.417	0.106