A STUDY OF CAFFEINE AND ITS METABOLITES IN HUMAN BODY FLUIDS

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SUMMARY

This study describes the development and application of sensitive and specific, reverse-phase high performance liquid chromatography procedures for the measurement of caffeine and its metabolites in biological fluids. Satisfactory resolution of these compounds was achieved using Hypersil octadecylsilane columns with elution solvents consisting of buffered acetonitrile solutions containing tetrahydrofuran as an ion-pairing reagent. Eluted compounds were detected by monitoring their UV absorption at a wavelength of 280 nm. Conditions for processing body fluids for analysis were optimised using organic extraction techniques with proxyphylline incorporated as an internal standard. Maximum recovery of urine metabolites required the formation of ion-pair complex with tetrabutylammonium hydrogen sulphate. Serum caffeine levels determined by HPLC and a radioimmunoassay procedure showed good agreement. Furthermore, caffeine concentrations in saliva, following oral ingestion of caffeine, correlated well with corresponding values found in serum.

An assessment of the consumption of caffeinated beverages in a local population indicated that the dietary intake of caffeine in the U.K. is significantly higher than that previously reported for American and Canadian populations. Measurement of serum caffeine levels in non-pregnant subjects showed a mean value of 2.42 mg/l; 6% of the values were in excess of 5 mg/l which is comparable to the average peak level found following a 250 mg oral dose of caffeine.

Reference ranges are established for serum and salivary pharmacokinetic data and recoveries of metabolites from urine

of normal volunteers who received oral doses of caffeine. In addition the electrophysiological and psychopharmacological responses to oral doses of caffeine are reported following a collaborative study with the Institute of Psychiatry, London.

Evidence for alterations in the biotransformation of caffeine during pregnancy was obtained by comparing urine metabolite concentrations in pregnant women, receiving a known dietary intake of caffeine, with values obtained for a non-pregnant control group. It is suggested that hormonal influences on the hepatic drug metabolising enzymes may be implicated. Whilst pharmacologically significant concentrations of caffeine and its metabolites were detected in samples of maternal serum, cord blood and breast milk, no evidence was found to relate caffeine levels in the newborn infant with the onset of attacks of jitteriness or neonatal apnoea.

In a study of caffeine elimination in chronic liver disease, the clearance of caffeine in compensated cirrhotic patients was similar to that of normal controls. However for patients with decompensated liver disease, significant levels of impairement were found in both serum and salivary caffeine clearance. This change in the disposition of caffeine was also reflected in the profiles of it metabolites excreted in the urine. Since the biotransformation pattern of caffeine in these patients was shown to be normal, this impairement of caffeine clearance could be attributed to a reduction in intrinsic clearance resulting from a reduced mass of viable hepatocytes. Preliminary findings indicated that salivary caffeine clearance measurements might provide an accurate means of assessing the extent of hepato-cellular dysfunction.

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CONTENTS

ABBREVIATIONS USED IN TEXT

Methylxanthine metabolites

1MX	1-methylxanthine
1MU	1-methyluric acid
3MX	3-methylxanthine
3MU	3-methyluric acid
7MX	7-methylxanthine
7MU	7-methyluric acid
13MX	1,3-dimethylxanthine (Theophylline)
13MU	1,3-dimethyluric acid
17MX	1,7-dimethylxanthine (Paraxanthine)
17MU	1,7-dimethyluric acid
37MX	3,7-dimethylxanthine (Theobromine)
137MX	1,3,7-trimethylxanthine (Caffeine)
137MU	1,3,7-trimethyluric acid
PP	7β-hydroxypropyl-theophylline (Proxyphylline)
THF	Tetrahydrofuran
TBAS	Tetrabutylammonium hydrogen sulphate
T1/2	Half-life
k.el	Elimination constant
AUC	Area under elimination curve
Vd	Apparent volume of distribution

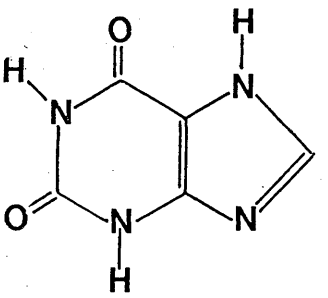
1. INTRODUCTION

Caffeine, theophylline and theobromine are three closely related alkaloids occuring in plants from which beverages, obtained from aqueous extracts, have been consumed for many years. Coffee, obtained from the seeds of Coffea arabica and related species contains caffeine. Tea, the leaves of Thea sinensis, contains caffeine and a small amount of theophylline, whilst cocoa, obtained from the seeds of Theobroma cacao, contains theobromine and caffeine. In recent years, additional dietary sources of caffeine have been introduced in the form of cola flavoured drinks made from nuts of the tree, Cola acuminata, and from many drugs including analgesics, antacids, and common cold remedies. Caffeine can be synthesised artificially from dimethyl urea and malonic acid.

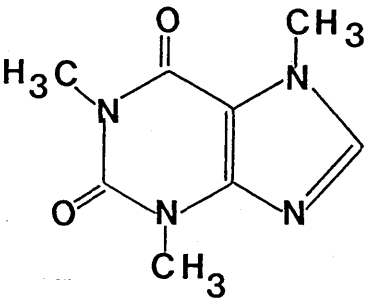
1.1. STRUCTURE, ABSORPTION AND DISTRIBUTION

The isolation of caffeine from green coffee beans was first reported in Germany by Runge (1820) and confirmed the same year by von Giese. In 1861 Strecker, using methyl-iodide, converted theobromine to caffeine and demonstrated that theobromine was a dimethylxanthine in structure and caffeine a trimethylxanthine. However, whilst the definitive stuctures of xanthine and caffeine were first described by Medicus in 1875, synthesis of caffeine was not achieved until a later study by Fischer and Ach in 1895.

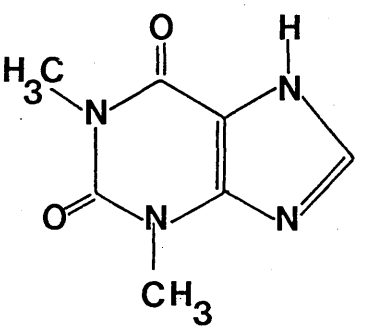
Caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine) are



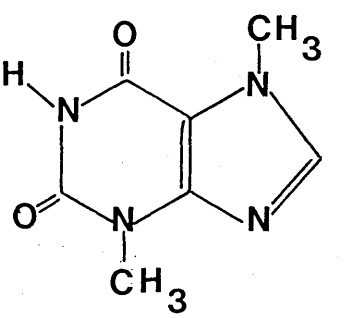
Xanthine



Caffeine



Theophylline



Theobromine

Fig 1.1 Structure of caffeine and related compounds.

methylated derivatives of the dioxypurine, xanthine, which is structurally related to uric acid and the nucleotide constituents of the nucleic acids, adenine and guanine (Fig 1.). The methylxanthines are readily absorbed after oral, rectal or parenteral administration and rapidly distributed throughout all tissues and organs. Oral administration of caffeine in man produces peak blood levels within 1-2 hours after ingestion, although this has been shown to vary according to the form in which it is administered (Marks and Kelly, 1973). Caffeine in coca-cola is absorbed much more slowly than that in tea or coffee, possibly as a result of a reduction in the rate of gastrointestinal blood flow caused by the lower temperature of coca-cola, or by the presence of sucrose.

The earliest reported study on the pharmacokinetics of caffeine was in 1953 when Axelrod and Reichenthal reported elimination half life values ranging from 2.5-4.5 hours in normal subjects following an oral dose of 7 mg/kg. However the present understanding of the pharmacokinetics of caffeine is mainly attributable to studies performed in the last decade. The combined data from several studies (Neims, 1979; Newton et al., 1981; Blanchard et al., 1983) have shown that the disappearance of caffeine from plasma or saliva follows first order kinetics after single oral doses of up to 10 mg/kg. Whilst investigating the effects of increasing doses (50-750 mg) on the plasma/ salivary pharmacokinetics of caffeine, Newton et al. (1981) showed that the apparent first-order elimination rate constant decreased linearly with dose whilst

total body clearance was unaffected (mean value 0.98 ± 0.38 $ml.kg.^{-1}\ min^{-1}$). There was also a trend towards increasing apparent volume of distribution with increasing dose. A linear relationship was observed between the area under plasma elimination curves and dose. In addition, dose and dose normalised plasma elimination curves were superimposable suggesting that caffeine obeys linear pharmacokinetics over the dose range investigated. The overall saliva/plasma concentration ratio was 0.74 ± 0.08.

The half life of caffeine for non-smoking, healthy adults is approximately 3-6 hours (Cook et al., 1976; Patwardhan et al., 1980; Newton et al., 1981; Zylber-Katz et al., 1984) although it has been reported that in habituallly heavy coffee drinkers, a period of up to 7 days may be required to completely decaffeinate their blood (Warren, 1969). In 1978 Parsons and Neims demonstrated that the mean value for elimination half-life in smokers (3.5 hr, n =13), was significantly less than than that for non-smokers (6.0 hr, n = 13) with corresponding clearance values of 155 ± 16 $ml.kg.^{-1}hr.^{-1}$ and 94 ± 18 $ml.kg.^{-1}hr$. Since no significant variations were found for the apparent volumes of distribution in smokers (720 ± 67 $ml.kg.^{-1}$) and non-smokers (610 ± 80 $ml.kg.^{-1}$) the differences were attributed to an induction of hepatic aryl hydrocarbon hydroxylase activity in smokers.

The plasma clearance of caffeine is significantly decreased in the third trimester of pregnancy (37 ml/kg/hr) producing an extended half. life of about 10 hours (Aldridge et al.,1981; Knutti et al., 1982). This,is probably due to changes in the

hormonal milieu since oral contraceptives produce a similar decrease in the rate of elimination of caffeine (Callahan et al, 1983). A delayed clearance is also observed in patients with severe liver disease (Statland et al., 1980; Desmond et al., 1980) and in newborns (Aranda et al., 1981), where elimination is slow due to immaturity of the enzymes involved in the biotransformation of caffeine. The low molecular weight of caffeine facilitates transport across the placenta (Goldstein and Warren, 1962) and accumulation may occur in foetal brain tissue (Galli et al., 1975). It is also secreted into maternal milk and has the ability to enter gonadal tissue (Goldstein and Warren, 1962).

1.2. METABOLISM

The metabolic fate of caffeine in man occurs primarily in the liver and, as in all other species (Bonati and Garratini, 1984), involves demethylation, oxidation and hydration reactions. The observed species differences in metabolite output are related to which particular pathways and reactions are dominant for a particular species, thereby favouring the formation of either demethylated xanthines, uric acids, or uracil derivatives of caffeine.

The earliest recorded study on caffeine metabolism in man was carried out in 1850 by Lehmann, who failed to detect traces of caffeine in urine samples collected following an oral dose. Evidence for the demethylation of ingested methyl-xanthines was first reported by Albanese (1895) who showed that dogs and rabbits fed large doses of caffeine or

theobromine excreted a monomethylxanthine. In 1901, Kruger and Schmidt concluded that the order of increasing stability of the methyl groups was 3,1 and 7 and that the methylated purines found in human urine were attributable to the 3-methyl demethylation of methylated purines present in food.

A significant contribution to the elucidation of the metabolism of methlxanthines was the introduction of paper chromatographic techniques capable of separating and detecting purines, pyrimidines and methylxanthines using only a few micrograms of material (Markham and Smith, 1949). In 1951, Weinfeld demonstrated that 1-methyluric acid and 1,3-dimethyl-uric acid were excreted in rabbit urine after feeding with theophylline or caffeine. A year later, Brodie et al. (1952) established that the major urinary metabolite of theophylline was 1,3-dimethyluric acid which accounted for 45% of the administered dose. Axelrod and Reichenthal (1953) showed, in three male subjects, that only 0.5%-1.5% of a 500 mg intra-venous dose of caffeine was excreted unchanged in the urine. However, whilst they reported plasma half-lives for both man and dog, caffeine metabolites were not analsyed in either plasma or urine. Although a report by Weinfeld and Christman (1953) confirmed that 1-methyl uric acid was an important metabolite of caffeine, and that 1,3-dimethyluric acid and 1-methyluric acid were the principal metabolites excreted following theophylline ingestion, the first metabolic pathways for each methylxanthine were proposed by Cornish and Christman in 1957.

By semi-quantitative analysis of the methylxanthines and

methyluric acids excreted by two volunteers following the ingestion of 1 g of theophylline, Cornish and Christman (1957) established that the main metabolites in urine after theophylline ingestion were 1,3-dimethyluric acid, 1-methyl uric acid, 3-methylxanthine and unchanged theophylline (10%). In a similar manner they also investigated the metabolism of caffeine and theobromine and found that for caffeine, the major urinary metabolites were 1-methylxanthine and 1-methyl uric acid. To a lesser degree 1,7-dimethylxanthine, 7-methyl-xanthine, 1,3-dimethyluric acid and unchanged caffeine were also excreted. Following the ingestion of theobromine, the major urinary metabolites were reported to be 7-methyl-xanthine, 3-methylxanthine, 7-methyluric acid and unchanged theobromine. However, the techniques used in these early studies were based on anion exchange and paper chromatography and the results were therefore mainly qualitative.

In recent years high performance liquid chromatography (HPLC), in combination with liquid scintillation counting and ultra-violet monitoring has been used to further elucidate the metabolic fate of labelled methylxanthines (Callahan et al. 1980,1982; Arnaud and Welsch 1980; Tang-Liu and Riegelman 1983; Tang et al. 1983). It is now well accepted that in man caffeine is metabolised by a series of sequential and parallel reactions (Fig. 1.2), the initial stages of which may be summarised as follows:

A. 3-N demethylation to yield 1,7-dimethylxanthine (paraxanthine).

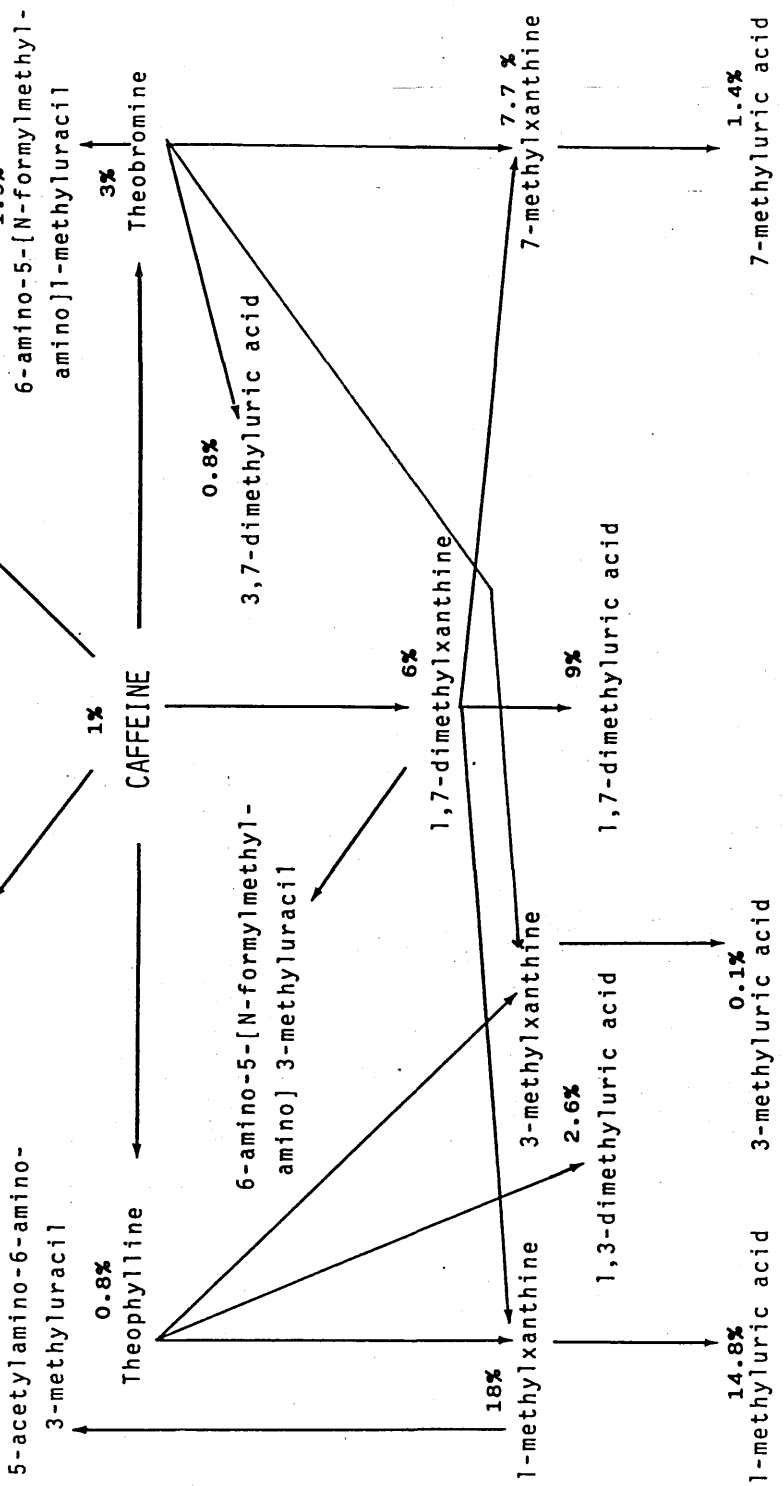


Fig 1.2 Quantitative metabolic pathway of caffeine in human (Arnaud and Welsch, 1981)
The number shown above the metabolite corresponds to the percentage of the dose found in urine.

B. 1-N demethylation to yield 3,7-dimethylxanthine (theobromine).

C. 7-N demethylation to yield 1,3-dimethylxanthine (theophylline).

These 3 reactions are probably catalysed by one or more of the cytochromes P-450 and yield formaldehyde as a secondary product. It has been estimated (Arnaud and Welsch, 1981) that approximately 95% of caffeine metabolism proceeds by an initial demethylation via these three reactions.

D. Hydroxylation of caffeine at the C-8 position to yield 1,3,7-trimethyluric acid which may also exist in its urate (salt) form. This reaction is again probably catalysed by one or more of the cytochromes P-450.

E. Hydration of caffeine at the 8,9 position to yield 1,3,7-trimethyldihydrouric acid and reversible ring opening to form 6-amino-5N-[formylmethylamino]-1,3-dimethyluracil. The enzyme(s) catalysing these reactions is not yet defined.

Although the reactions A-E all involve caffeine as a substrate, it is generally accepted that analogous reactions occur with the primary or secondary metabolites of caffeine.

In order to assess the importance of each of the three demethylation pathways, Arnaud and Welsch (1981) administered oral doses (4 mg/kg) of unlabeled paraxanthine, theobromine and theophylline to six non-smoking volunteers and quantitated the resulting urinary metabolites. Their report concluded that the quantitative importance of the initial demethylation reactions, in terms of metabolite recoveries were: 3-N 72%,

7-N 20% and 1-N 8%. A summary of the results of these findings is shown in Fig.1.2. In a cross over study involving the administration of oral doses of 1- methyl-^{14}C]-and [2-^{14}C] caffeine to 8 normal male subjects, Callahan et al (1982) were able to account for 81% of the total dose of caffeine administered. Although their chromatography system was unable to resolve 7-methylxanthine from 1-methyluric acid, theophylline from paraxanthine and 3,7-dimethyluric acid from 1-methylxanthine, their urinary profiles were similar to those reported by Arnaud and Welsch (1981). Faecal excretion of methylxanthines (where biliary metabolites could be included) accounted for less than 2% (± 1.7%) of the administered dose. One of the major urine metabolites found in this study corresponded to 5-acetylamino-6-amino-3- methyl uracil (AAMU), a urinary pyrimidine initially identified by Fink et al. in 1964 as an excretion product elevated after oral intake of caffeine. The excretion of this metabolite showed great interindividual variability, with recovery values ranging from between 7% to 35% of the administered dose (mean 14.7% ± 6.4%). Another radiolabelled polar metabolite called A2 was also detected (3% ± 1%) but this unidentified compound was found to be unstable in methanol solution left at room temperature, transforming to AAMU. The possibility that AAMU was also an artefact of isolation was later investigated by Tang et al. (1983) who, in confirming this hypothesis, isolated and identified 5-acetylamino-6-formylamino- 3-methyl uracil (AFMU) as a major metabolite of caffeine. Although this opened-ring metabolite of caffeine was initially isolated in

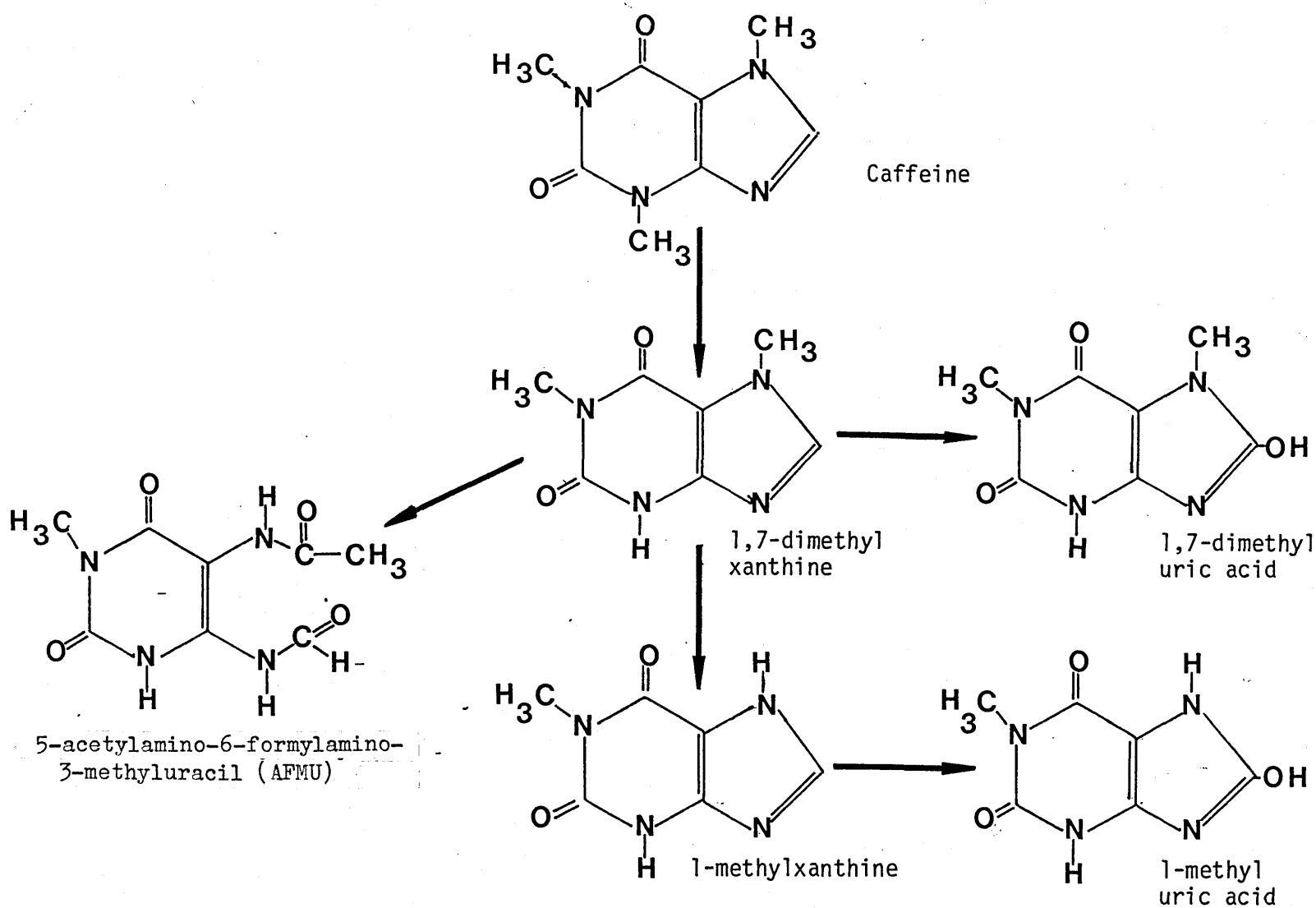


Fig 1.3. Metabolism of caffeine in man showing the formation of the five major metabolites.(Grant et al. 1984)

the urine of a volunteer fed 1-methylxanthine (Arnaud and Welsch, 1981), its urinary excretion has recently been found to be more rapid and quantitatively more important following paraxanthine administration. It is however possible that its first identification as a 1-methylxanthine metabolite could be a result of an incomplete clearance of caffeine due to an inadequate period of abstention from dietary xanthines prior to the study. (Fig 1.3)

The formation of AFMU has been shown to be mediated by the polymorphic N-acetyltransferase enzyme (Grant et al., 1983a) which is responsible for the acetylation of a number of endogenous substrates and drugs including isoniazid and sulfamethazine. Furthermore, it has been shown that the molar ratio of AFMU to 1-methylxanthine excreted in 24 hour pooled urine samples following the ingestion of 300 mg caffeine provides a simple index of acetylator status (Grant et al., 1983b).

In a comparative study of caffeine metabolism following an oral dose of [2-^{14}C]-caffeine in a 1:1 mixture of unlabelled caffeine and [1,3-^{15}N-8-^{13}C]-caffeine, Callahan et al. (1983) reported that the biotransformation of caffeine was similar in groups of normal males, ovulating females and females receiving oral contraceptives, despite a slower plasma clearance rate for caffeine in the latter group. Possible variations in the biotransformation of caffeine resulting from increasing age or its route of administration has recently been investigated by Blanchard et al., (1985), by comparing urinary metabolite profiles in a group of seven healthy young

men (aged 18-29 years) and in a group of five healthy elderly men (aged 66-71 years) following 5 mg/kg doses of caffeine, either as an aqueous oral solution or an intravenous infusion. The metabolite profiles in both groups showed no substantial variation with either the route of administration or age, with the exception that significantly greater amounts of 1-methyluric acid, 7-methyluric acid and 1,7-dimethyluric acid were excreted by elderly subjects.

1.3. PHARMACOLOGICAL PROPERTIES

The dietary xanthines have several pharmacological actions in common but differ markedly in the intensity of their actions. They stimulate the central nervous system, respiration, cardiac muscle and skeletal muscle; act on the renal tubules to produce a diuresis; relax smooth muscle, notably bronchial muscle and they cause coronary dilation. A structure/activity relationship exists between the three compounds. Methyl substitution on position 1 is associated with central nervous system (CNS) stimulation, diuretic effects are linked to the 3-methyl groups and cardiac stimulation with the methyl group at position 7. Caffeine therefore shows all three effects whilst theobromine possesses relatively weak CNS activity and theophylline has increased cardiac stimulant properties. All three compounds produce a diuresis from the presence of the 3-methyl group.

1.3.1. Central nervous system (CNS).

Caffeine and theophylline stimulate the CNS at all levels, the cortex being affected first folllowed by the medulla,

whilst the cord is stimulated only by very large amounts. Theobromine is virtually inactive in terms of CNS stimulation. Stimulation of the cortex occurs following the administration of 150-250 mg of caffeine, resulting in a more rapid and clearer flow of thought with less drowsiness and fatigue (Goldstein et al. 1965; Bruce et al. 1985). Caffeine also produces an increased capacity for sustained intellectual effort, decreased reaction time, and a more perfect association of ideas. Comparable effects of theophylline have not been carefully investigated. At higher doses, methyl-xanthines produce nervousness, restlessness, insomnia, tremors, hyperesthesia and other signs of CNS stimulation (Rall, 1980).

Caffeine and theophylline stimulate medullary respiratory, vasomotor and vagal centres but only in the presence of high blood levels. Following the administration of large amounts of caffeine or theophylline, the spinal cord may also be stimulated resulting in increased reflex excitability and lower motor centres may be directly excited. This may produce focal and generallised convulsions although in man, the toxic dose of caffeine is so large (over 10g) that human fatality from the consumption of caffeinated drinks is unlikely (McGee, 1980).

1.3.2. Cardiovascular system.

Caffeine and theophylline have prominent actions on the circulatory system although they are complex and sometimes antagonistic. The resulting effects depend largely on the conditions prevailing at the time of their administration and

the dose used.

Direct stimulation of the myocardium, particularly by theophylline, causes an increase in the force of contraction and cardiac output with an accompanying decrease in the venous filling pressure. Until recently, theophylline was used in the treatment of congestive heart failure but, the unpredictable absorption and distribution of theophylline in patients with compromised circulatory function frequently led to serious CNS toxicity (Piafsky et al. 1977).

At therapeutic plasma concentrations (10-20 mg/l) theophylline produces a modest increase in heart rate in normal individuals (Ogilvie et al. 1977). Similar effects for caffeine have been reported by Robertson et al. (1978) who also observed that noradrenaline, adrenaline and renin levels were significantly raised at 1 and 3 hours following the oral ingestion of caffeine (250 mg). However their release does not appear essential to the pressor effect of caffeine, since patients lacking a functioning autonomic nervous system have a normal or even exaggerated pressor response. After large doses, both methylxanthines produce definite tachycardia and sensitive individuals may experience other arrhythmias, such as premature ventricular contractions.

By direct action on the vascular musculature, the xanthines dilate the coronary, pulmonary and general systemic blood vessels although they may also contract blood vessels by stimulation of the medullary vasomotor centre. Dilation of the coronary arteries increaes coronary blood flow and the ablity of the heart for work, but the overall effect of the xanthines

on the systemic blood pressure is unpredictable. In the cerebral circulation, the xanthines increase vascular resistance thus decreasing cerebral blood flow and the oxygen tension of the brain. This vasoconstriction, rather than a decrease in cerebrospinal fluid pressure, may be responsible for the relief of hypertensive headaches by the xanthines (Rall, 1980).

1.3.3. Smooth muscle.

Xanthines, especially theophyline, relax the smooth muscles of the bronchi and are of value in the treatment of bronchial asthma. They may also overcome spasm of the biliary tract, produced in man by the injection of morphine or other opioids (Grubb and Burks, 1975), and theophylline containing compounds have been employed in the treatment of biliary colic.

1.3.4. Skeletal muscle.

Xanthines strengthen the contraction of isolated skeletal muscle, possibly by electrical stimulation or by an increased release of acetycholine. Caffeine increases the capacity for muscular work although it is argued that this may be due to stimulation of the central nervous system.

1.3.4. Gastric secretion

In man, moderate oral or parenteral doses of caffeine cause secretion of both acid and pepsin (Debas et al., 1971). In experimental animals pathological changes and ulcer formation have been shown to occur following high doses. Evidence suggesting that caffeine potentiates secretion of acid, induced by histamine or alcohol, is not convincing but careful attention should be made to the ubiquitous use of caffeinated

beverages in the pathogenesis and management of patients with peptic ulcers. Other ingredients of coffee have been shown to elevate plasma gastrin response in humans (Berkowitz et al. 1974) suggesting that coffee may be a more potent cause of stomach activity than caffeine alone. However, in a more recent study (McArthur et al., 1982), no single property of a variety of caffeinated beverages (including pH, caffeine content, osmolality, caloric content, buffering capacity and ionized calcium level) was found to be an adequate predictor of gastric acid secretion in humans.

1.3.5. Carbohydrate and lipid metabolism.

It is generally believed that caffeine raises blood sugar levels, possibly by the accumulation of cyclic AMP. Coffee has been shown to increase glucose tolerance in normal subjects (Feinberg et al., 1968) and to reduce it diabetics. Although no explanation for this has been proposed, caffeine inhibits glucose metabolism and antagonises the effects of insulin, which may lead to increased blood glucose levels when insulin production is faulty.

Caffeine elevates free fatty acid levels in plasma by stimulating cyclic AMP mediated lipolysis in adipose tissue (Bellet et al. 1968). This effect can be blocked by insulin and nicotininc acid and appears to be greater in young subjects perhaps indicating some degree of tolerance with increasing age. Tea drinking is negatively correlated with serum lipid levels in heart disease patients suggesting that it may contain a substance that counteracts the lipolytic effects of caffeine (Little et al. 1966). The prevalence of

atherosclerois appears to be higher among coffee-drinking than among tea-drinking populations (Young et al. 1967).

1.3.6. Other biochemical effects.

The xanthines, particularly theophylline have a well known diuretic effect which may be due to increased renal blood flow or a direct effect on the renal tubule. However, caffeine has also been shown to cause water retention (Ross, 1971) possibly from synergism between the action of antidiuretic hormone and cyclic AMP.

Caffeine readily permeates the liver and may alter the activity of drug metabolizing enzymes in the liver. In vitro, it has been shown to inhibit and reduce platelet aggregation induced by adenosine diphosphate but its effects on blood clotting are unknown. It depresses the formation of urea from ammonium salts and promotes creatinuria.

High doses of caffeine in rats produce an endocrine stress syndrome (Spindel and Wurtman, 1984) characterized by decreased serum TSH levels, suppressed pulsatile secretion of growth hormone, and elevated serum levels of corticosterone and β-endorphin. Similar responses have been shown in man following 500 mg doses but it is unlikely that these effects would be produced with normal patterns of coffee consumption. However, a non-coffee drinker who suddenly chose to drink large amounts of caffeine containing beverages or analgesics might well be expected to experience transient endocrine changes.

1.4. CELLULAR BASIS FOR THE ACTION OF THE METHYLXANTHINES

In spite of the widespread use of the methylxanthines as food constituents and as drugs, the molecular mechanisms mediating their pharmacological actions are not well understood. In order to explain their diverse effects, three basic cellular actions of methylxanthines have been studied, which in order of increasing sensitivity to methylxanthines are:

a) Translocation of intracellular calcium.

b) Inhibition of cyclic AMP phosphodiesterase.

c) Competitive antagonism of cell surface adenosine receptors.

Although caffeine has been shown to increase calcium levels in mammalian smooth muscle, heart muscle and skeletal muscle; the threshold for observations related to alterations in the permeablity to or binding of calcium in intracellular organelles, appears to be consistently greater than the maximal therapeutic concentrations for methylxanthines (Rall, 1980). Until recently, it was widely held that caffeine and theophylline acted in vivo by inhibiting phosphodiesterase and subsequently increasing levels of cyclic AMP. However, in intact animals, even relatively high doses of methylxanthines fail to increase tissue cAMP levels (Burg and Warner, 1975) and some inhibitors of phosphodiesterase that are 100-1000 times more potent than caffeine, fail to show similar behavioral effects (Snyder, 1984).

The past decade has produced considerable evidence to suggest that the observed behavioral and physiological effects

of caffeine may involve competitive antagonism at cell surface adenosine receptors (Fredholm, 1980; von Borstel and Wurtman, 1984; Snyder, 1984). In animals, adenosine produces sedation, bradycardia, hypotension, hypothermia, dilation of coronary and cerebral arteries, inhibition of platelet agregation, and suppression of locomotor activity. It also modifies the responses of the heart, vasculature, adipose tissue, and kidney to sympathetic stimulation. These effects, which are mediated through specific adenosine receptors located on the external surfaces of the plasma membranes of cell (Daly et al. 1981), are competitively antagonised by methylxanthines at concentrations similar to those found in plasma after the consumption of one to three cups of coffee (5-30 uM; Rall 1980).

The first biochemical analysis of adenosine receptor activity by Sattin and Rall (1970), showed that adenosine can increase the accumulation of cyclic AMP in brain slices by direct action on extracellular receptors which may be blocked by theophylline. The effects of adenosine on the enzyme adenylate cyclase, reveal two distinct sub-types of adenosine receptors which have been designated A1 and A2. These receptors show opposing effects in that adenosine, at micro-molar concentrations, inhances adenylate cyclase activity via A2 receptors ,whilst at nanomolar concentrations, it decreases adenylate cyclase activity through A1 receptors. There are also structure-activity differences between the receptors, the most striking being the marked stereospecificity in the effects of phenyl-isopropyladenosine (PIA) at A1 receptors,

with L-PIA being much more potent than D-PIA. At A2 receptors the two isomers have relatively similar effects.

Although most xanthines have similar potencies in blocking both A1 and A2 receptors, some exceptions exist between affinities for adenosine receptors and behavioral effects (Snyder, 1984). For example, 3-isobutyl-1-methylxanthine (IBMX) has about the same affinity as caffeine for adenosine receptors, but does not display locomotor enhancement at any dose examined. In addition, biphasic effects have been observed for some xanthines eg. at lower doses caffeine decreases locomotor activity whilst at higher doses it enhances it. IBMX decreases activity at all doses whilst 7-(β-hydroxyethyl)theophylline enhances locomotor activity at all doses evaluated.

The long term blockade of adenosine receptors by caffeine makes the cardiovascular system more sensitive to adenosine and also increases brain adenosine receptor density (von Borstel and Wurtman, 1984). It is possible that the onset of headaches and jitteriness associated with caffeine withdrawl (Goldstein et al., 1969) might reflect enhanced tissue sensitivity to endogenous adenosine which is a potent dilator of the cerebral vasculature. Although the effects of adenosine on cardiovasular tissues have generally been thought to be restricted to the particular tissue in which the adenosine was formed and released (Fredholm, 1980), it is possible that circulating adenosine might also function in a hormone-like role affecting vascular dynamics in tissues throughout the body (von Borstel and Wurtman, 1984).

1.5. MEASUREMENT OF CAFFEINE AND ITS METABOLITES IN BIOLOGICAL FLUIDS

Procedures for the measurement of caffeine in biological fluids have, in general, paralleled the development of analytical technology and have utilised ultraviolet spectrophotometrty, paper chromatography, thin layer chromatography (TLC), gas-liquid chromatography (GLC), high performance liquid chromatography (HPLC) and radioimmunoassay (RIA). The progress in methodology has not only increased the sensitivity, specificity and ease of determinations but has also permitted the measurement of metabolites.

1.5.1. Ultraviolet spectrophotometry

Ultra-violet spectrophotometry was first utlised by Ishler et al.(1948) for the measurement of caffeine in coffee, a procedure later adapted by Fisher et al.(1949) for monitoring caffeine levels in the urine of greyhounds and horses. Prior to this, the determination of caffeine involved extraction with chloroform followed by a nitrogen estimation or the application of a non-specific colorimetric method. The first definitive method for the determination of caffeine in biological fluids was described by Axelrod and Riechenthal (1953). Caffeine was extracted with benzene from a biological matrix saturated with sodium chloride at pH 7-8 and then back extracted with 5N HCl before direct measurement of its absorbance at 273 nm. Problems related to the effects of benzene in altering the spectral absorbance curve of caffeine were later overcome by Routh et al.(1969) using a differential

absorption method at 285 nm, in which the same extract at two different pH values was used for the sample and reference solutions. However, U.V. spectrophotometric procedures are time consuming, require large sample volumes and are prone to interference from caffeine metabolites and co-administered drugs.

1.5.2. Immunoassay techniques

A radioimmunoassay for the measurement of caffeine in serum, described by Cook et al.(1976) offered many advantages in terms of sensitivity and specificity. An antiserum, prepared by immunizing rabbits with 7-[5-carboxypentyl]-1,3-dimethylxanthine-bovine serum albumin conjugate, was incubated with serum samples for 2 hours in the presence of a [8-^{3}H] caffeine radioligand, before separation of bound and unbound ligand with dextran-coated charcoal. The requirement for a sample volume of only 20 µl and a detection limit of 20 ng/ml, led to the procedure being used in several kinetic studies.

Non-isotopic immunoassays for the measurement of caffeine have received limited attention. An enzyme multiplied immunoassay technique (EMIT) has been developed for theophylline (Syva Inc.), using an antiserum produced in a manner similar to that described for RIA. The enzyme-labelled drug is made by coupling theophylline at the 8-position with glucose-6-phosphate (Gunshaw et al.,1977), the activity of which is monitored spectrophotometrically by the reaction between NAD and glucose-6-phosphate. Although a similar system could be adopted for the measurement of caffeine, the

manufacturers have declined to develop the assay in view of its limited application. A substrate labelled fluorescent immunoassay (SLFIA) has been described by Biggs et al.(1985) although the antiserum used in the procedure showed extensive cross reactivity with theophylline.

1.5.3. Thin layer chromatography

Thin layer chromatography techniques have been employed in both therapeutic monitoring and, in combination with the use of radiolabelled caffeine, for studies of the biotransformation of caffeine. A procedure for the measurement of caffeine and theophylline in serum, saliva or urine was described by Reichert (1978) for monitoring the treatment of neonatal apnoea with methylxanthines. Using a Keiselgel 60 F254 chromatography plate, samples were applied directly onto a wet ethanol spot and developed using a solvent system of ethylacetate: methanol: 25% ammonia. A detection limit of about 1 µg/ml was achieved by measuring the UV absorbance with a dual wavelength scanner, in a reflection mode using a sample wavelength of 273 nm and a reference wavelength of 315 nm.

Two dimensional chromatography of radiolabelled caffeine, using a silica gel plate (Merck F254, 0.25 mm thickness) was used by Arnaud (1976) to resolve the various metabolites of caffeine in urine. The sample spots were developed first with chloroform: methanol (4:1, v/v) and then with chloroform: acetone: n-butanol: concentrated ammonium hydroxide (3:3:4:1, v/v). Using this procedure, the chromatographed compounds may be identified using a radioscanner and/or autoradiography, or

by scintillation counting following removal of the spots by scraping or cutting of the plate. A one dimensional procedure with chloroform: acetone: n-butanol: concentrated ammonium sulphate (3:3:2:0.5, v/v) described by Ferrero and Neims (1983) separated theophylline, theobromine, 1,7-dimethyl-xanthine, 1,3,7-trimethyluric acid and 6-amino-5-[N-formyl-methylamino]-1,3-diaminouracil.

1.5.4. Gas-liquid chromatography

The application of gas chromatography to the measurement of caffeine in plasma was first described by Grab and Reinstein in 1968. Samples were prepared by an alkaline extraction with chloroform, using hexobarbital as an internal standard, before injection onto a 3% OV17,Chromosorb W 100-120 mesh AW/DMCS column at an operating temperature of 200°C. The effluent was monitored using a flame ionization detector at 260°C.

In recent years, the introduction of alkali flame ionization detectors, specific for nitrogen/phosphorus containing compounds, has resulted in the development of methods showing significant improvements in both sensitivity and specificity (Cohen et al., 1978; Bonati et al, 1979;). These have included a procedure for the measurement of caffeine in urine utilizing solid phase extraction columns (Delbeche and Debackere, 1983). However, these methods have received only a limited application to studies on the kinetics of caffeine, due mainly to their inability to measure metabolites. In addition, a comparison between the use of GLC using nitrogen-phosphorus detection and high performance

liquid chromatography techniques (Bonati et al., 1979) showed that HPLC gave linear responses over a wider concentration range with improved precision at lower concentrations and subsequently lower limits of detection.

The combination of GLC with mass spectrometry has provided a valuable system for the identification of metabolites of caffeine (Merriman et al., 1978).

1.5.5. High performance liquid chromatography

The present understanding of the disposition of caffeine is largely attributable to the application of high performance liquid chromatographic techniques which have permitted the simultaneous measurement of both caffeine and its methylxanthine metabolites. Procedures have been described for the measurement of caffeine in plasma/serum, urine, saliva, milk, cerebrospinal fluid, semen, amniotic fluid, aqueous humour, and a variety of tissue affusates, perfusates and extracts.

Measurement of caffeine and its dimethylxanthine metabolites in serum and saliva.

The various methods described for the measurement of caffeine in serum are summarized in Table 1.1. They differ mainly with regard to their chromatographic conditions and mode of sample preparation. Although normal phase systems have shown adequate resolution and specificity for both caffeine (Van der Meer and Hass, 1980) and its metabolites (Arnaud and Welsch, 1982, Wahllander et al., 1985), most procedures have favoured the improved reliability and simplicity of reverse phase systems (Desiraju et al., 1977; Foeander et al., 1980;

REFERENCE	COLUMN	MOBILE PHASE	SAMPLE PREPARATION
a) Normal phase chromatography			
Van Der Meer and Hass (1980)	5 µm silica	Tetrahydrofuran: dichloromethane (20:80, v/v)	Dichloromethane extraction
Arnaud and Welsch (1982)	Lichrosorb Si-60	Chloroform:isopropanol: acetic acid (92:7:1, v/v)	Chloroform:isopropanol extraction (95:5, v/v)
Wahlander et al., (1985)	Lichrosorb Si-60	Dichloromethane containing formate buffer in methanol	Chloroform:isopropanol extraction (1:1, v/v)
b) Reverse phase chromatography			
Foenander et al., (1980)	5 µm octadecylsilane	12% acetonitrile in 10mM acetate buffer, pH 4.0	Dichloromethane extraction
Desiraju (1977)	10 µm octadecylsilane	12% methanol in 0.05M KH PO4, pH 4.7	Direct injection following ultrafiltration
Blanchard et al., (1980)	10 µm octadecylsilane	15% acetonitrile in 10mM acetate buffer, pH 4.0	Acetonitrile precipitation
Haughey et al., (1982)	5 µm octadecylsilane	Acetonitrile:phopsphoric acid (85%):water (13:0.5:86.5, v/v)	Chloroform:isopropanol extraction (80:20, v/v)
Scott et al., (1984)	5 µm octadecylsilane	Acetonitrile:tetrahydrofuran: 50 mM acetate buffer, pH 4.0 (4:1:95, v/v)	Chloroform:isopropanol extraction (85:15)
Pickard et al., (1986)	8 µm Waters radial -pak octadecylsilane	1% acetic acid:methanol (83:17, v/v)	Bond-Elut C18 extraction columns (Analytichem Inc.)

Table 1.1 HPLC procedures for the measurement of caffeine in serum and saliva

Blanchard et al., 1982; Pickard et al., 1986). However, the inability of reverse phase systems to adequately resolve theophylline and 1,7-dimethylxanthine has impeded their application to metabolite studies. Recent procedures for the measurement of theophylline, have shown that these problems of specificity may be improved by the incorporation of ion-pairing reagents, such as tetrahydrofuran (Miksic and Hodes, 1979) or tetrabutyl ammonium chloride (Farrish and Wargin, 1980), into the mobile phase.

Sample preparation has been achieved either by the use of organic extraction or deproteinization techniques, involving precipitation with acetonitrile solutions (Blanchard et al., 1980; Christensen and Neims, 1985) or ultrafiltration (Desiraju et al., 1977). Organic extraction with dichloromethane or chloroform/isopropanol mixtures has been mainly adopted in preference to deproteinization techniques in order to overcome problems encountered with peak distortion (Jowett, 1981) and interference from cephalosporin antibiotics (Kelly et al., 1978). More recently solid phase extraction columns, containing reversed-phase octadecylsilane bonded silica (Bond-Elut C18), have been used for both the measurement of caffeine and theophylline in serum (Pickard et al., 1986; Hartley et al., 1986). Whilst their use reduces analysis time by eliminating the need for solvent evaporation and reconstitution in the mobile phase, these advantages are offset by their high cost.

b) Measurement of methylxanthine metabolites in urine

The techniques used in early studies of the metabolism of caffeine and theophylline (Brodie et al., 1952; Cornish and Christman, 1957) were very cumbersome, involving anion-exchange chromatography followed by paper chromatography; then, spectrophotemetry and colorimetry. Quantitative analysis of these metabolites was not achived until 1974 when, Thompson et al. (1974) separated theophylline metabolites using an Aminex A-5 cation exchange resin. However, the sample preparation was extremely time consuming and laborious since it involved an initial prefractionation of the urine into methylxanthine and uric acid fractions using a Dowex 2-X8 anion exchange resin (Cl- form, 50-100 mesh). These fractions were then injected separately onto the ion-exchange column.

Aldridge et al. (1979) determined caffeine metabolites using a simple gradient elution system (1.5%-7.5% aceto-nitrile in 0.5% glacial acetic acid) on a reversed phase column (μ Bondapak C-18) following organic extraction of urine samples by means of chloroform/isopropanol (85:15). However, although the procedure was used by Callahan et al. (1981) and Arnaud and Welsch (1981) for studies on the metabolism of caffeine, it failed to resolve 7-methylxanthine from 1-methyluric acid, theophylline from 1,7-dimethylxanthine or 3,7-dimethyluric acid from 1-methyl xanthine. In addition, extraction recoveries were consistently low for 1-methyluric acid (36.3%), 7-methyluric acid (55.6%) and 3,7-dimethyluric acid (58.5%).

Recently, it has been shown that the recovery of methyl

xanthines from urine may be significantly improved by the formation of an ion-pair complex prior to extraction (Tang-Liu and Riegelman (1982). The sample, internal standard (β-hydroxy - ethyltheophylline), 0.1M tetrabutylammonium hydrogen sulphate, and 0.01M sodium carbonate buffer, pH 11.0, were mixed for 30 seconds before and after the addition of about 1 gram ammonium sulphate. This mixture was then extracted with ethylacetate: chloroform: isopropanol (45:45:10) and, following centrifugation, the organic layer was evaporated to dryness under nitrogen. The residue was then reconstituted in an aqueous solution of 50mM tetrabutylammonium hydrogen sulphate and 10mM sodium acetate, pH 4.9. Chromatoraphy was then performed using a 5 μm Ultrasphere ODS column and a gradient elution sytem consisting of Solvent A, the aforementioned reconstitution solution, and Solvent B which consisted of solvent A mixed with an equal volume of 50% methanol, pH adjusted to 4.8 with glacial acetic acid. A gradient increase of 0-45% increase in solvent B resolved 15 methylated xanthines and uric acids within 30 minutes, the column effluent being monitored at 280 nm. However, the retention of 7-methylxanthine was found to be sensitive to trace amounts of methanol retained in the stationary phase of the column, and a re-equilibration period of 30 minutes was necessary to resolve 7-methylxanthine from 3-methyluric acid.

MEASUREMENT OF CAFFEINE AND ITS DIMETHYLXANTHINE METABOLITES IN SERUM AND SALIVA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

2. MEASUREMENT OF CAFFEINE AND ITS DIMETHYLXANTHINE METABOLITES IN SERUM AND SALIVA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In recent years, high performance liquid chromatography (HPLC) has been extensively applied to the specific analysis of theophylline or caffeine, but the simultaneous measurement of caffeine and its dimethylxanthine metaboltes has received little attention. Foenander et al.(1980) described a procedure wherein dichloromethane extracts of serum were chromatographed on a octadecylsilane column using 12% acetonitrile in acetate buffer, pH 4, as a mobile phase. However, dichloromethane extraction produced a low recovery and the chromatography system was unable to resolve theophylline from 1,7-dimethyl-xanthine (paraxanthine), a metabolite of caffeine often present in significant concentrations in biological fluids. Improved specificity was shown in a normal phase procedure adopted by Arnaud and Welsch (1981) for the measurement of theophylline, theobromine, paraxanthine and caffeine in serum and saliva. Samples were extracted using a chloroform/ isopropanol mixture but their results showed considerable variation in recovery between xanthines. Moreover, normal phase systems are prone to rapid column deterioration.

The specificity problems encountered with reverse phase systems for the measurement of methylxanthines may be improved by the incorporation of tetrahydrofuran (Miksic and Hodes, 1979) or tetrabutylammonium chloride (Farrish and Wargin, 1980) into the mobile phase. This report describes the

development of a procedure, designed initially for the simultaneous measurement of caffeine, theophylline and theobromine in serum and saliva but later modified to permit the estimation of 1,7-dimethylxanthine, the primary metabolite of caffeine found in serum and saliva. The correlation between caffeine levels in serum and saliva is investigated and the results obtained by HPLC analysis are compared with those obtained using a radioimmunoassay procedure.

2.1. Materials and Equipment

Caffeine was purchased from BDH Chemicals Ltd. Poole, England; theophylline, 7β-hydroxypropyltheophylline (proxyphylline), 1-methyluric acid, and 1,7-dimethylxanthine from Sigma Chemical Company Ltd., Poole, Dorset, England; 7-methyluric acid, 7-methylxanthine, 3-methylxanthine, 1-methylxanthine, 1,3-dimethyluric acid, 1,7-dimethyluric acid and 1,3,7-trimethyluric acid from Adams Chemical Company, Round Lake, Illinois, USA.

Stock solutions (200 mg/l) of the xanthines were prepared in distilled water. The working standards of caffeine, theophylline, and theobromine were made by further diluting the stock solution in horse serum (Wellcome Reagents Ltd. Beckenham, England) to give concentrations of 5-30 mg/l.

Analar grade chloroform, isopropanol, tetrahydrofuran (BDH Chemicals Ltd., Dorset, England) and HPLC grade acetonitrile (Rathburn Chemicals Ltd., Walkerburn, Scotland) were used throughout the study.

HPLC Equipment

Initial studies were performed using an Applied Chromatography Systems (ACS) twin headed reciprocating pump (Model 750/03). This was later replaced by an ACS model 300/02 single headed, dual channel, reciprocating pump. Both pumps were adjustable to provide a constant flow rate of 0.1 to 9.9 ml/min.

Eluted peaks were detected using an ACS 750/11E UV monitor fitted, with an 8 µL flow cell, and integrated using a Spectra Physics Model 4270 computing integrator. Samples were injected via a Rheodyne loop injector fitted with a 50 µL loop.

Chromatography was performed using stainless steel columns (100 x 4.5 mm id.) packed, by a slurry technique, with 5 µm Hypersil octadecylsilane (Shandon, London, England). Methanol (AR) was used as the packing solvent. The packing material (1.8g) was added to a stainless steel column packing reservoir which was then filled to capacity (50 ml) with methanol and the contents mixed continuously on a magnetic stirrer for approximately 5 minutes. After securing the reservoir lid in place, the solvent inlet line was attached to a constant pressure air pump and the reservoir outlet line attached to an empty column containing a 2 µm frit on the outlet end reducing union. The packing pump was then started and the air regulator adjusted to produce a pressure of 4,000 psi which was maintained until the solvent flow from the outlet end of the HPLC column had ceased. After allowing the pressure to return to zero, the column was disconected fron the packing system, installed into the analytical HPLC system and primed with the

appropriate solvent system for at least 30 minutes before injecting a standard mixture. The suitability of the column for use in analytical studies was assessed by inspection of peak symmetry and calculation of the number of theoretical plates. For the latter, a minimum value of 40,000 per metre was required.

2.2. Separation and identification of caffeine and its dimethylxanthine metabolites by reverse phase HPLC.

The optimal chromatography conditions for the separation of an aqueous standard (10 mg/l) containing caffeine, theobromine, theophylline and 1,7-dimethylxanthine (paraxanthine) was investigated using a series of solvent systems consisting of acetonitrile or methanol solutions, prepared in distilled water or acetate buffer, as described in Table 2.1. The eluted peaks were detected using a UV monitor set at a previously established optimum wavelength of 280 nm. In order to improve the resolution between 1,7-dimethyl-xanthine (paraxanthine) and theophylline, and thus eliminate previously described problems of specificity (Miksic and Hodes, 1979; Farrish and Wargin, 1980), the use of mobile phases incorporating tetrahydrofuran and tetrabutylammonium chloride as ion-pairing reagents were investigated.

Results

The separation of methylxanthines, using reverse phase high performance liquid chromatography, was found to be highly sensitive to changes in both the pH and polarity of the mobile

MOBILE PHASE	FLOW RATE (ml/min)	THEOBROMINE mins	THEOBROMINE k´	PARAXANTHINE mins	PARAXANTHINE k´	THEOPHYLLINE mins	THEOPHYLLINE k´	CAFFEINE mins	CAFFEINE k´
a) Hypersil 5 um octadecylsine column (250 x 4.5 mm i.d.)									
12% acetonitrile in 40mM acetate buffer, pH 4.0	1	5.5	0.93			7.0	1.54	11.5	3.36
20% acetonitrile in 40mM acetate buffer, pH 4.0	1	3.6	0.42			4.0	0.76	5.2	1.00
12% acetonitrile in 40mM acetate buffer, pH 5.0	1	5.6	1.00			7.2	1.67	12.0	3.44
12% acetonitrile in dist. water	1	6.1	1.10			8.1	2.1	14.2	3.86
20% acetonitrile in dist. water	1	3.9	0.39			4.5	0.64	6.2	1.28
20% methanol in 40mM acetate buffer, pH 4.0	1	7.2	1.61			10.4	2.78	17.0	5.11
b) Hypersil 5 um octadecysilane column (100 x 4.5 mm i.d.)									
8% acetonitrile in 40mM acetate buffer, pH 4.0	1.5	2.5	1.5			3.7	2.7	7.5	6.5
4% acetonitrile: 5mM tetra-butylammonium chloride in 40mM acetate buffer, pH 4.0	1.5	2.4	2.0	3.8	3.87	4.3	4.37	8.1	9.12
4% acetonitrile: 1% tetrahydrofuran in 40mM acetate buffer, pH 4.0	1.5	2.0	2.3	2.9	3.8	3.1	4.2	5.3	7.8

Table 2.1 Chromatography retention data for the separation of methylxanthines

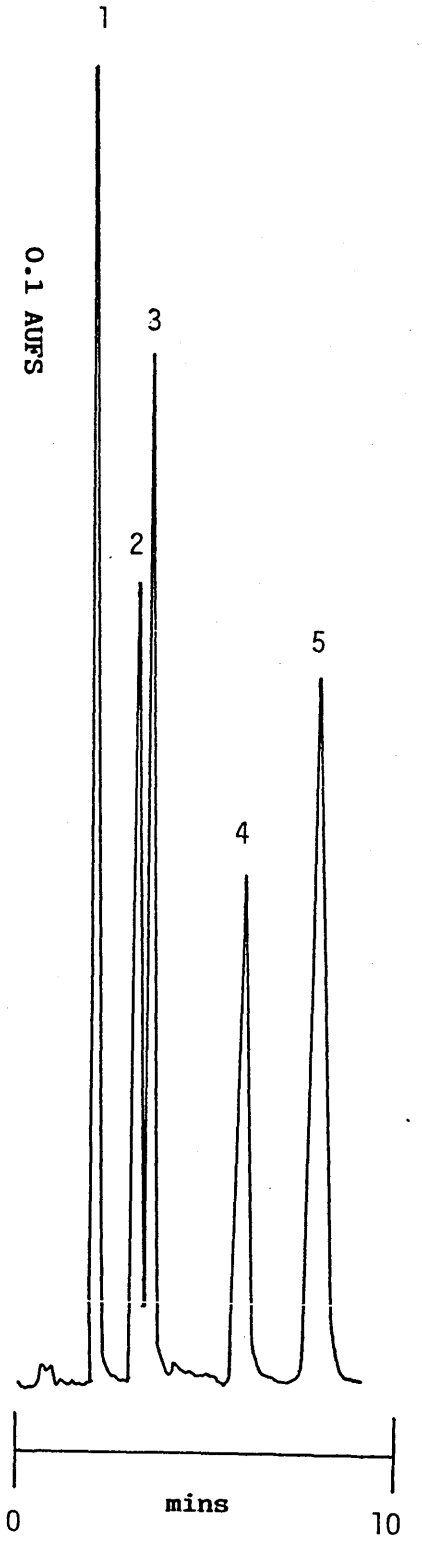


Fig 2.1 Chromatography of an aqueous solution of methylxanthines (10mg/l)

Chromatographic conditions as described in text.

Peaks; 1 = Theobromine 2 = 1,7-dimethylxanthine

3 = Theophylline 4 = Caffeine 5 = Proxyphylline

Compound	Capacity factor (k´)	Retention time (min)
Theobromine	2.3	2.0
Theophylline	4.2	3.1
Caffeine	7.8	5.3
Proxyphylline	9.8	6.3
7-methyluric acid	0.5	0.9
7-methylxanthine	1.0	1.2
1-methyluric acid	1.3	1.4
3-methylxanthine	1.7	1.6
1-methylxanthine	1.8	1.7
1,3-dimethyluric acid	2.0	1.8
1,7-dimethyluric acid	3.2	2.5
1,7-dimethylxanthine	3.8	2.9
1,3,7-trimethyluric acid	5.0	3.6
Paracetamol	-	-
Phenobarbitone	-	-
Primidone	-	-
Phenytoin	-	-
Trimipramine	1.0	0.6

Table 2.2 Chromatography retention data for the separation of methylxanthines using a solvent system of 4% acetonitrile: 1% tetrahydrofuran in 40mM acetate buffer, pH 4.0.

-: Indicates that the compound did not chromatograph under the conditions used.

phases (Table 2.1.). The addition of tetrahydrofuran or tetrabutylammoniumhydrogen hydrogen chloride produced similar and significant improvements in the resolution of theophylline and 1,7-dimethylxanthine. Optimum chromatographic conditions, in terms of resolution and retention time, were achieved using a moblie phase of 1% tetrahydrofuran/4% acetonitrile (v/v) in acetate buffer, pH 4. The use of this eluent at a flow rate of 1.5 ml/min produced a pressure drop of 60 bars. The separation of an aqueous standard solution using this system is shown in Fig. 2.1.

The specificity of the chromatography system was examined by injecting aqueous preparations of methylxanthine metabolites and a number of potentially interfering compounds, the retention times of which are shown in Table 2.2.

2.2.1. Electrochemical detection of methylxanthines

The suitability of direct current (dc) amperometric detection for the measurement of caffeine, theobromine and theophylline was assessed using an EDT Model LCA 10 detector fitted with a glassy carbon electrode and operated at an oxidizing potential of +1.3 volts. The electrochemical responses of the methylxanthines were monitored following the chromatographic separation of an aqueous standard solution using a solvent system of 12% acetonitrile in acetate buffer, pH 4.0, at a flow rate of 1 ml min.

Results

Chromatograms comparing the amperometric detection of a 100 ng injection of aqueous standard solutions with detection by

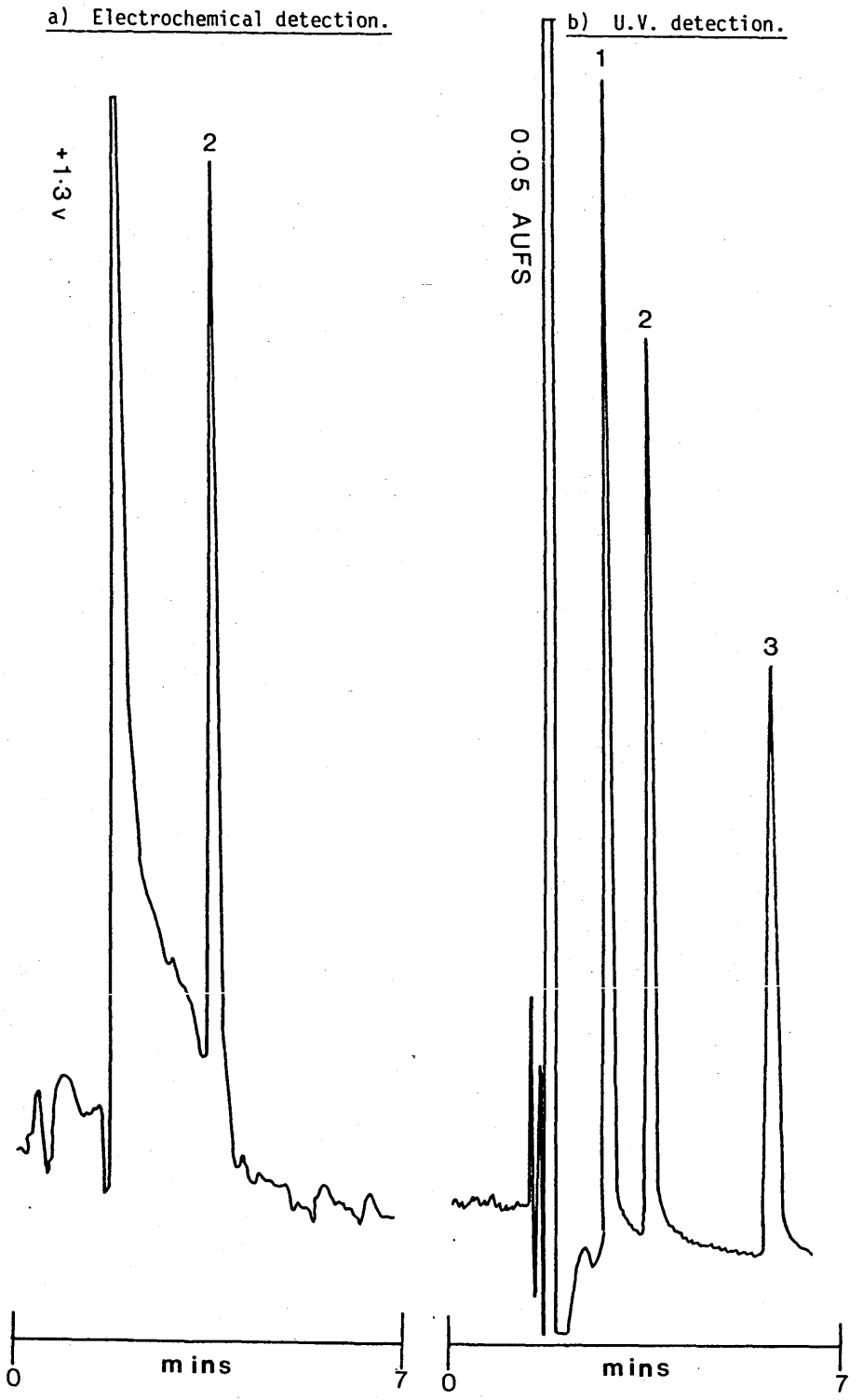


Fig 2.2 Monitoring of an eluted methylxanthine solution (100ng) using a) electrochemical detection and b) UV monitoring at 280 nm.
Chromatographic conditions as described in text.
1 - theobromine, 2 - theophylline, 3 - caffeine

UV at 280nm are shown in Fig.2.2. At an operating potential of +1.3 volts only theophylline was detected using dc amperometric detection. Although it has been shown that caffeine and theobromine are detectable using differential pulse amperometry (Lewis and Johnson, 1978), these facilities were not available and subsequently further use of amperometric detection was considered unsuitable.

2.3. The extraction of methylxanthines from serum.

In order to eliminate the previously reported specificity problems associated with deproteinization techniques (Jowett, 1981; Kelly et al., 1978), an organic extraction procedure was investigated for sample preparation. The extraction efficiency of a series of organic solvent systems were examined by estimating the recovery of caffeine, theophylline, theobromine and 1,7-dimethylxanthine from two pooled serum samples, labelled A and B, which had been spiked with 4ug/ml and 8ug/ml amounts of aqueous standard. The extraction solvent sytems investigated were a) dichloromethane, b) chloroform, c) chloroform/isopropanol (85:15 v/v), d) ethylacetate and e) ether/dichloromethane (60:40 v/v)

100 µl serum, 200 µl 0.2M HCl and 5 ml of extraction solvent were vortexed mixed for 30 seconds in a stoppered tube and then centrifuged at 2,000 rpm (approx. 1500 g) for 5 minutes. 4 ml of the organic phase was then transferred to a clean tube and evaporated to dryness under a stream of air in a water bath at 40° C. The residue was dissolved in 100 µl of the

mobile phase and 50 ul injected onto the HPLC column. The recovery of the methylxanthines from serum was calculated by comparing the peak heights with those obtained from the injection of an unextracted aqueous standard solution (200ng/50ul).

Results

The recovery data for each of the solvent systems studied is shown in Table 2.3. Optimum recovery was achieved using a chloroform/isopropanol mixture (85:15 v/v) which produced recovery values in excess of 90% for each methylxanthine. Chromatograms of serum samples extracted using this system are shown in Fig.2.3. Although dichloromethane extraction was used in a procedure described by Foenander et al. (1980), these results suggest that recovery using this solvent is low, and in the case of caffeine, extremely variable.

2.3.1.The recovery of methylxanthines from saliva and aqueous solutions.

Using the procedure previously described for serum, the suitability of chloroform/isopropanol (85:15) extraction for the measurement of methylxanthines in saliva was investigated by determining the recovery from two saliva samples, spiked with 4 mg/l and 8 mg/l amounts of an aqueous standard. For comparison, aqueous solutions of similar concentrations were extracted in the same manner.

Results.

The recovery of methylxanthines from saliva was similar to that found for serum (Table 2.3.), thus confirming the suitability of the procedure for the extraction of methyl-

SOLVENT	MATRIX	PERCENTAGE RECOVERY			
		Theobromine	Paraxanthine	Theophylline	Caffeine
Chloroform/isopropanol (85:15, v/v)	Water	93.7	98.5	96.7	98.7
	Serum	90.8	95.8	94.2	93.3
	Saliva	89.1	93.8	90.3	92.3
Dichloromethane	Serum	66.2	*	66.1	58.8
Ethylacetate	Serum	64.6	*	81.8	77.5
Ether/dichloromethane (60:40, v/v)	Serum	46.5	*	69.5	76.7

Table 2.3. Solvent extraction of methylxanthines from serum and saliva

Methylxanthines were measured as described in text. Each value is the mean of five determinations carried out at concentration levels of 4 and 8 mg/l of the matrix.
* denotes not measured

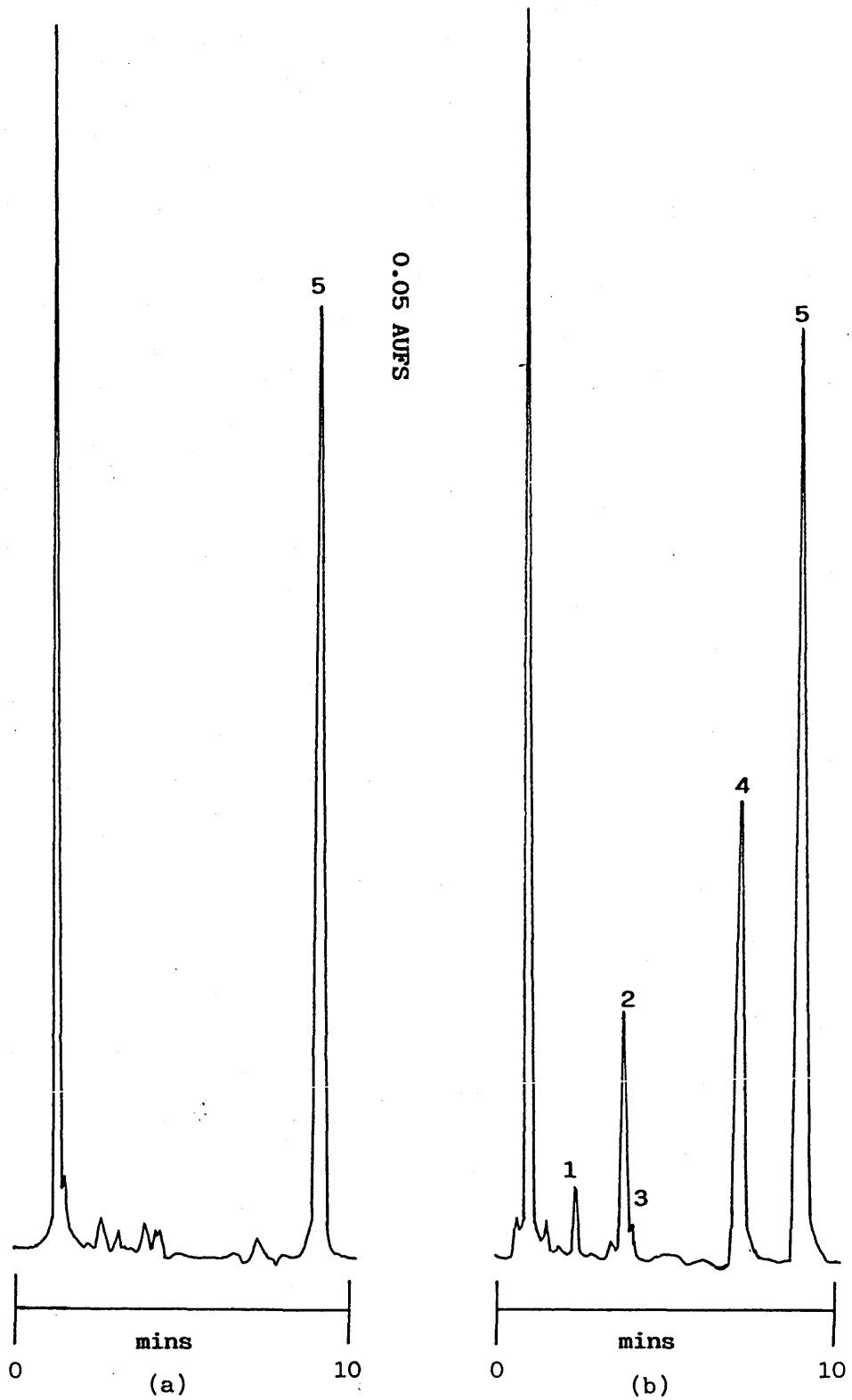


Fig 2.3 Chromatography of extracted serum samples collected a) before and b) 4 hours following an oral dose of caffeine (400 mg).

Chromatography conditions as described in text. Peak identification as in Fig 2.1

xanthines from saliva. In addition, the close agreement between aqueous recovery values and those obtained using serum/saliva matrices suggests that standardization of the procedure may be achieved using aqueous solutions.

2.3.2. Selection of an internal standard

In order to minimize any loss of precision, through either variations in extraction recovery or volume of injection, the use of an internal standard was adopted. Selection was based on examining the retention data of internal standard solutions (200 mg/l) used in previously described HPLC procedures for caffeine and theophylline. These included, 8-chlorotheophylline (Adams et al., 1976; Foenander et al., 1980), 7-hydroxypropyltheophylline (Evenson and Warren, 1976; Naish et al., 1979; Blanchard et al., 1980) and carbamazepine (Van der Meer and Hass, 1980).

Using a solvent system of 12% acetonitrile in acetate buffer, pH 4.0, at a flow rate of 1 ml/min 8-chlorotheophylline was unresolved from theophylline and carbamazepine was not detected. However, 7-hydroxypropyltheophylline was suitably resolved from all the standard methylxanthines. Following extraction by the previously described procedure, the recovery of 7-hydroxypropyl theophylline was found to be 95.4%, confirming its suitability for use as an internal standard.

2.3.3. Standardization and detection limits.

A series of standard solutions (range 1-30 mg/l) containing theobromine, theophylline, paraxanthine and caffeine, were

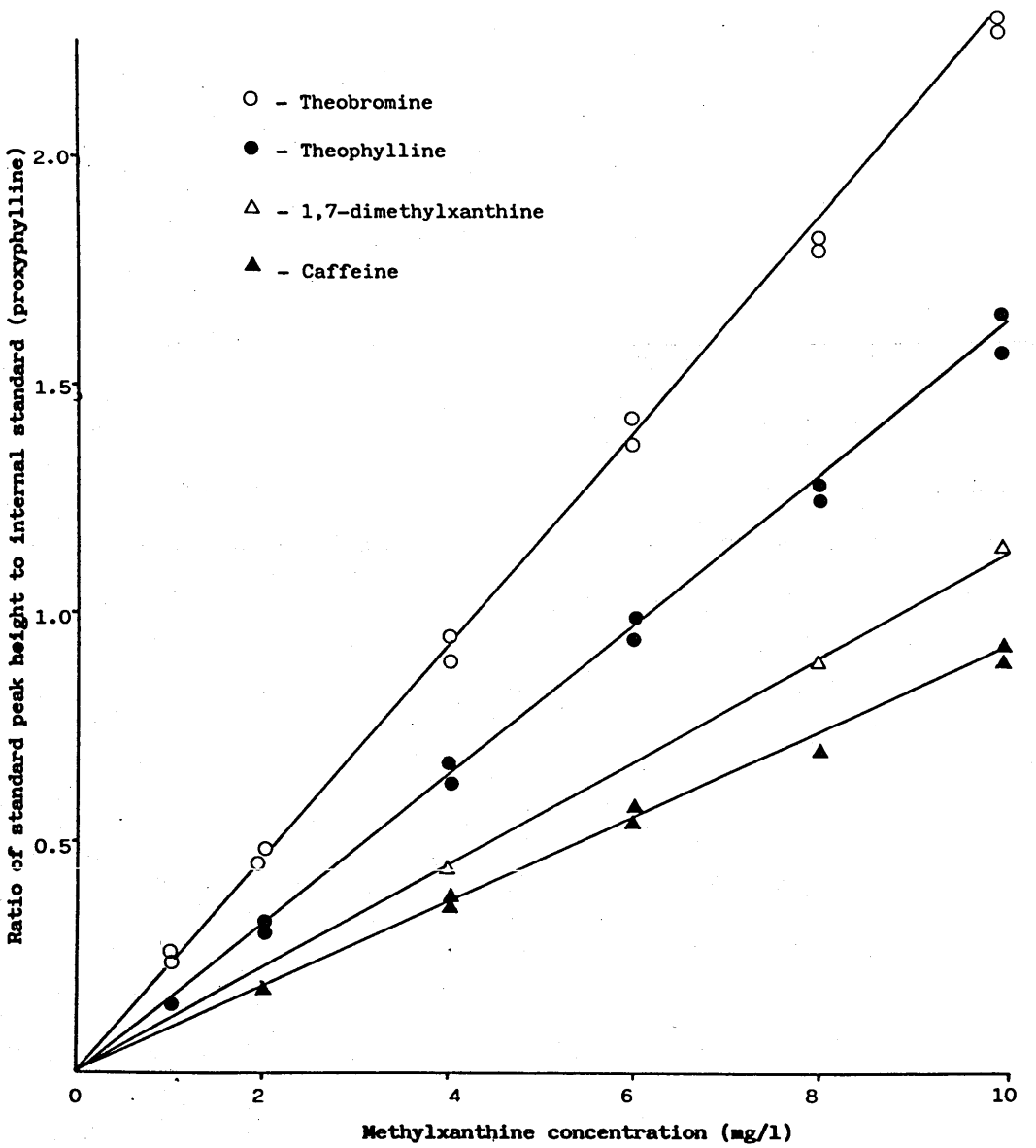


Fig 2.4 Standard curves for theobromine, theophylline, 1,7-dimethyl xanthine and caffeine following extraction of spiked serum samples.

prepared using horse serum as a diluent. Duplicate extractions of these solutions, using proxyphylline (800 ng/tube) as an internal standard, were chromatographed and standard curves prepared by plotting the methylxanthine/proxyphylline peak height ratios against concentration. The detection limit for each methyl- xanthine was established by determining the minimum concentration of a standard solution, which, when extracted, produced a signal to noise ratio of 4:1 at 0.01 absorbance units full scale (AUFS). A blank extraction of the horse serum was also chromatographed in order to detect the presence of any interfering peaks.

Results

For each methylxanthine, the standard curves showed a linear response over the range investigated. Linear regression analysis showed corresponding coefficient of variation values (r) for theobromine, theophylline, paraxanthine and caffeine of 1.000, 1.000, 0.998 and 0.999 respectively. Standard curves covering the normal expected range (1-10 mg/l) of these methylxanthines are shown in Table 2.4. Limits of detection were established as 100 ng/ml for theobromine, theophylline and paraxanthine and 200 ng/ml for caffeine. The extract of horse serum itself showed no significant interference.

2.3.4. Reproducibility of method

The within batch precision of the method was assessed by chromatographing replicate extractions of serum and saliva samples spiked with 4 mg/l and 8 mg/l amounts of an aqueous standard (200 mg/l). Between batch precision was studied by

Sample	Theobromine	Theophylline	Paraxanthine	Caffeine
Serum A	4.41	4.37	4.01	6.73
	4.78	4.74	4.00	7.67
	4.84	4.78	3.97	7.13
	4.98	4.86	4.09	7.73
	5.04	4.86	4.28	7.66
Mean	4.81	4.72	4.07	7.38
S.D.	0.25	0.2	0.13	0.44
CV%	5.13	4.3	3.08	5.9
Saliva A	4.35	4.25	4.21	5.20
	4.49	4.38	4.58	5.17
	4.38	4.25	4.00	4.76
	4.49	4.29	4.13	5.37
	4.38	4.3	4.20	4.96
Mean	4.42	4.29	4.22	5.09
S.D.	0.07	0.05	0.22	0.24
CV%	1.49	1.24	5.11	4.72
Serum B	7.6	7.59	7.31	7.84
	8.08	8.16	8.00	7.97
	7.99	8.0	8.26	8.0
	8.06	7.91	8.30	8.13
	8.06	7.94	8.18	8.3
Mean	7.96	7.92	8.01	8.05
S.D.	0.20	0.21	0.41	0.17
CV%	2.55	2.62	5.09	2.2
Saliva B	7.68	7.79	8.14	11.16
	8.34	8.4	8.00	11.47
	8.0	8.09	8.27	11.45
	7.9	8.0	8.23	11.32
	7.9	8.0	7.66	10.71
Mean	7.96	8.06	8.06	11.22
S.D.	0.24	0.22	0.25	0.31
CV%	3.02	2.75	3.06	2.78

Table 2.4 Within batch precision for measurement of methylxanthines in serum and saliva.

Sample	Day	Theobromine	Theophylline	Paraxanthine	Caffeine
Serum A	1	4.83	4.38	4.48	4.49
		4.74	4.26	-	4.58
	2	4.88	4.52	4.30	4.83
		4.79	4.53	-	4.81
	3	5.14	4.74	4.76	4.13
		5.00	4.51	-	4.42
	4	4.94	4.55	4.33	4.80
		4.75	4.74	-	4.93
Mean		4.89	4.53	4.47	4.65
S.D.		0.12	0.15	0.21	0.27
CV %		2.5	3.3	4.7	5.8
Serum B	1	8.29	7.89	8.24	11.88
		8.17	7.78	-	11.11
	2	8.31	8.22	7.72	11.72
		8.21	8.22	-	11.88
	3	8.43	8.17	8.41	12.24
		8.50	8.22	-	12.39
	4	8.18	8.18	8.16	12.10
		8.69	8.77	-	12.44
Mean		8.35	8.19	8.13	11.97
S.D.		0.12	0.26	0.29	0.39
CV %		1.5	3.2	3.6	3.3

Table 2.5 Between batch precision for the measurement of methylxanthines in serum

the repeated analysis of two serum samples "A" and "B" which had been spiked in a similar manner. For quantitation, the ratio of peak heights of the xanthines to those of the internal standard were compared with those obtained for appropriate working standards processed in a similar manner.

Results

The results summarized in Table 2.4 and Table 2.5 showed good precision for both within and between batch analysis. Within batch analysis of serum samples produced overall coefficient of variation values for theobromine, theophylline, 1,7-dimethylxanthine and caffeine of 3.8%, 3.5%, 4.1% and 4.2% respectively. Similar results were shown for saliva samples with corresponding values of 4.7%, 2.0%, 4.1% and 3.8%. The mean coefficients of variation for serum samples A and B showed respective values of 2.0%, 3.3% and 4.6%, confirming that the precision of the method was maintained for between batch analysis.

2.4. .Correlation between caffeine concentrations in serum and saliva.

A series of serum and saliva samples, collected simultaneously from 52 hospital staff and outpatient volunteers were assayed for caffeine by the procedure previously described. For the collection of saliva samples, volunteers were requested to rinse their mouths out with tap water, and about 5 minutes later, collect approximately 2 ml of saliva into a plastic container. In order to overcome sample contamination with sputum, volunteers were requested not to "clear their

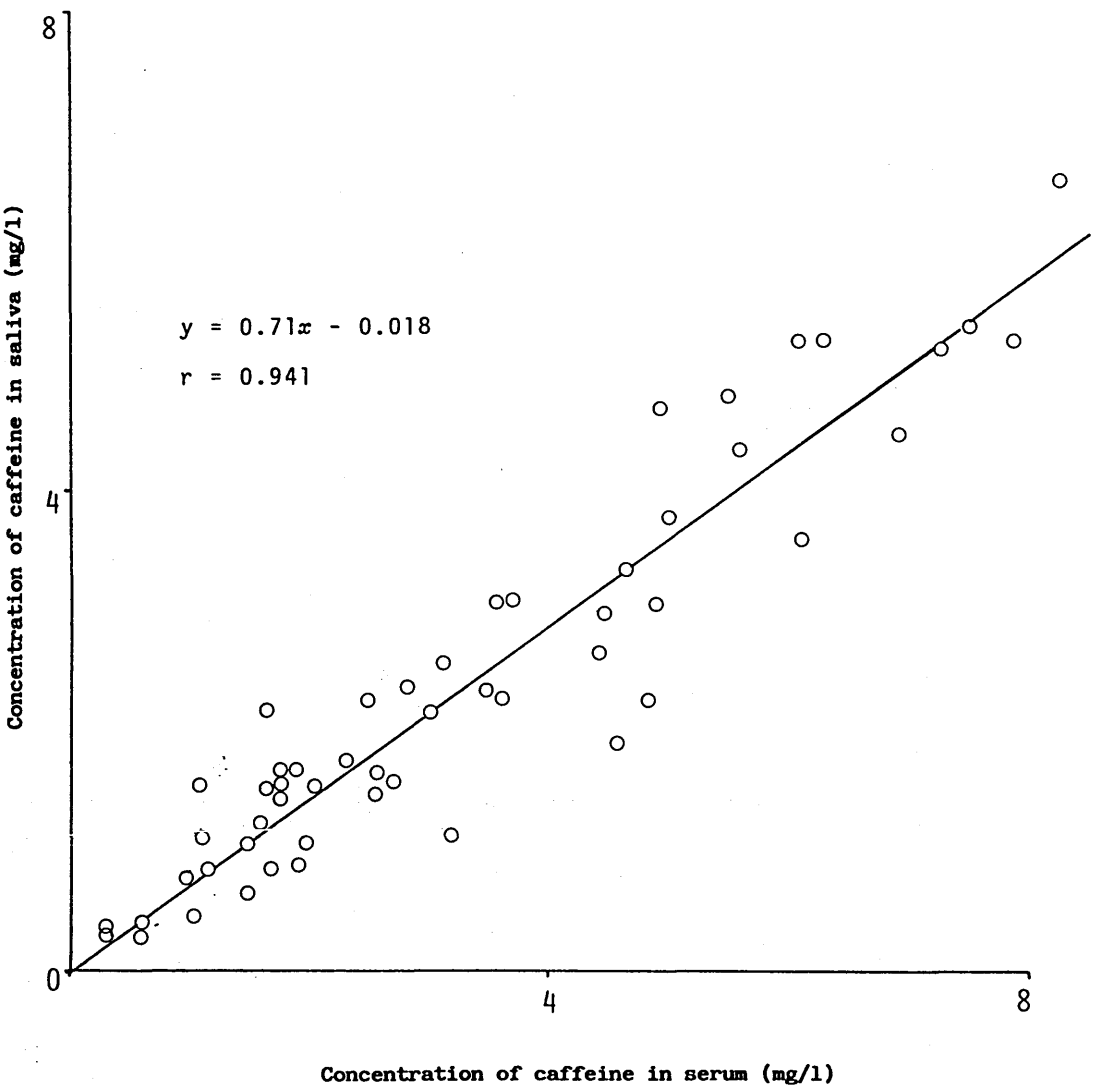


Fig 2.5. Correlation between caffeine levels in serum and saliva.

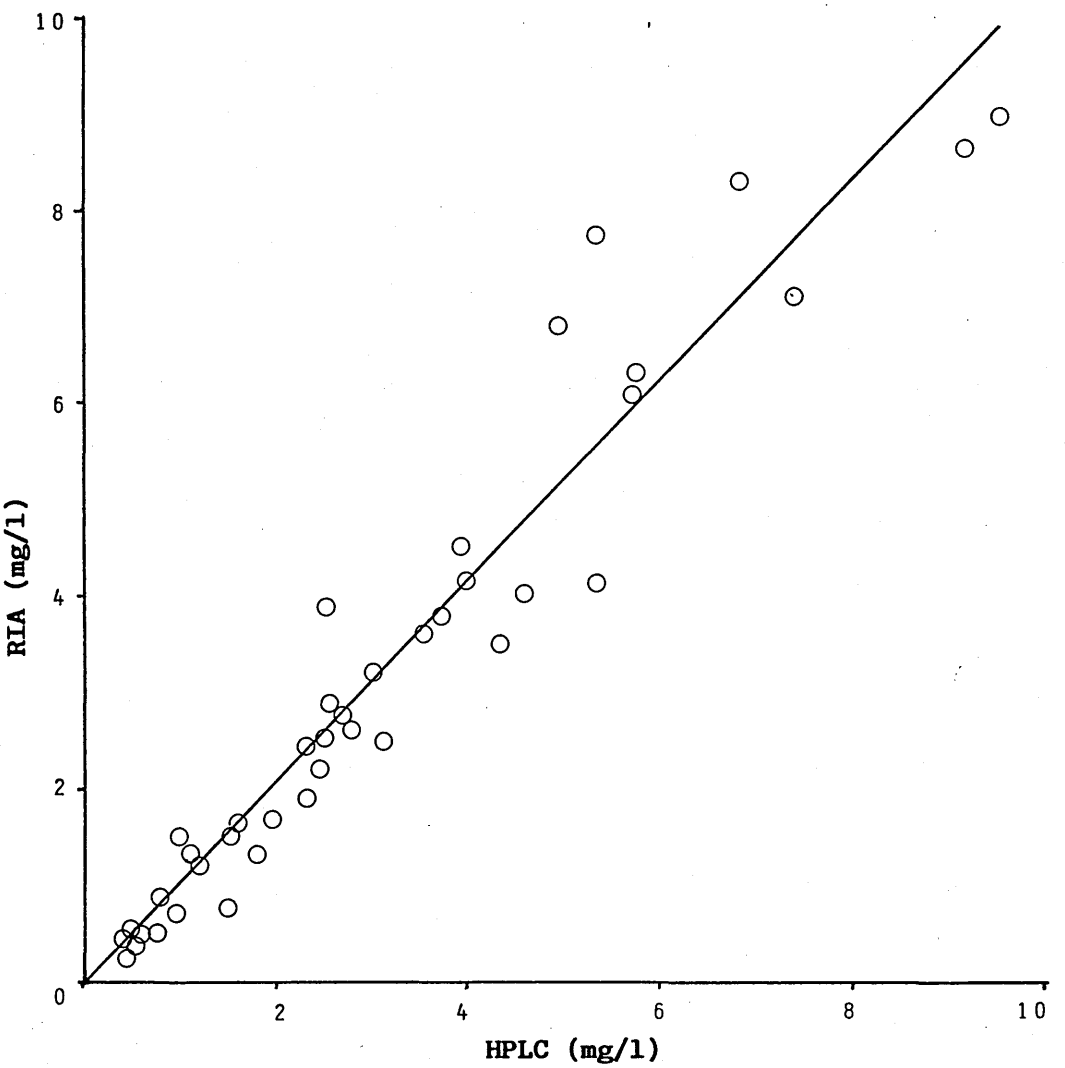


Fig 2.6. Correlation between serum caffeine estimations by HPLC and RIA

(y =1.04 x - 0.063 ; r = 0.962)

throats" during collection.

Results

The relationship between results obtained for caffeine concentrations in serum and saliva is shown in Fig. 2.5. Linear regression analysis showed a coefficient of correlation (r) of 0.941 with a regression line, $y = 0.710x - 0.018$; where y is the value for saliva and x is the value for serum. These results indicate that the analysis of caffeine in saliva can be used to reflect concentrations in serum.

2.5. Correlation between serum caffeine measurement by HPLC and radioimmunoassay.

In a collaborative study with St Luke's Hospital, Guildford, a comparison was made between the results of caffeine determinations made on 42 serum samples using a radioimmunoassay procedure (Cook et al., 1976) and values obtained using the described HPLC procedure.

A scattergraph of the results obtained by each procedure is shown in Fig. 2.6. The correlation coefficient (r), calculated by linear regression analysis, was 0.962 with a regression line, $y = 1.04x - 0.063$ where y is the value obtained by RIA and x the value by HPLC. These values confirm a good correlation between the two procedures.

DISCUSSION

Spectrophotometric methods for the measurement of caffeine, theophylline and theobromine in biological fluids (Schack and Waxler, 1949; Axelrod and Reichenthal, 1953; Fellenberg and

Pollard, 1979) require large sample volumes and are non-specific whilst gas chromatography procedures (Cohen et al., 1978; Schwertner, 1979; Chambers, 1979) have long analysis times and low sensitivity. The use of radioimmunoassy (Cook et al., 1976) and enzyme immunoassay techniques may be used to overcome some of these limitations but they are unable to provide simultaneous determinations of the methylxanthine metabolites of caffeine.

The method described in this study utilises the simplicity and reliability of reverse-phase HPLC which overcomes the problems of column deterioration often encountered with normal phase procedures. The incorporation of tetrahydrofuran into the mobile phase resulted in improved specificity by resolving theophylline from 1,7-dimethylxanthine thus permitting the simultaneous determination of caffeine and its dimethyl-xanthine metabolites. Sample preparation using organic extraction was adopted in preference to the more rapid deproteinization techniques in order to overcome associated problems encountered with peak distortion (Jowett, 1981) and interference from cephalosporin antibiotics (Kelly et al., 1978). Optimum conditions for extraction were obtained using a chloroform/ isopropanol mixture (85:15 v/v) at pH 4. The recovery values and precision using this extraction procedure was shown to be very satisfactory and results suggest that aqueous standards may be suitably employed as an alternative to those prepared in drug free serum. Direct injection of saliva samples, diluted 1:4 with the internal standard, showed good reproduciblity and results of caffeine analysis

correlated well with those obtained using organic extraction ($r = 0.995$). However this technique resulted in a rapid deterioration of column performance and subsequently organic extraction is recommended for routine use.

The good correlation obseved between caffeine levels in serum and saliva indicates that analysis of saliva can be used to reflect concentrations in serum. The non-invasive nature of saliva sampling is of considerable advantage in therapeutic drug monitoring and pharmacokinetic studies, and the ability to perform at least 25 analyses in one working day makes this HPLC procedure highly suitable for these applications. In addition the procedure may also be used to determine methyl-xanthine concentrations in beverages by direct injection of diluted samples into the HPLC system.

Experience has shown that the procedure described is robust and economical with no significant problems of late eluting peaks, column deterioration or interference from other drugs.

MEASUREMENT OF THE URINARY METABOLITES OF CAFFEINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

3. MEASUREMENT OF THE URINARY METABOLITES OF CAFFEINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

The methods currently available for the measurement of caffeine metabolites in urine have been reviewed in section 1.5.5. However most studies involving the analysis of methylxanthine metabolites have utilised the procedure described by Aldridge et al. (1979) in which urine is extracted with a chloroform/iso-propanol mixture (85:15 v/v) before chromatographing the metabolites on a C-18 μ-bondapak column (Waters Associates) using a concave gradient of 1.5%-7.5% acetonitrile in 0.5% acetic acid. However, a significant limitation of this method is its inability to resolve 7-methylxanthine from 1-methyluric acid, theophylline from 1,7-dimethylxanthine or 3,7-dimethyluric acid from 1-methylxanthine. In addition, extraction recoveries are low for 1-methyluric acid (36.3%), 7-methyluric acid (55.6%) and 3,7-dimethyluric acid (58.5%).

In order to overcome these limitations, a procedure has been developed which offers a significant improvement in both recovery and specificity and facilitates the separation and quantitation of thirteen metabolites within a 20 minute period. In an attempt to optimize sample preparation a critical comparison is made between results obtained from the direct injection of diluted urine samples with those produced following ion-pair extraction using a procedure similar to that previously described by Tang-Liu and Riegelman (1982).

CHROMATOGRAPHIC CONDITIONS

3.1. Materials and equipment

The HPLC equipment, reagents and caffeine metabolites were similar to those described in section 2.2. Additional reagents included tetrabutylammonium hydrogen sulphate (Sigma Chemicals Ltd. Poole, Dorset); disodium tetraborate, 1M phosphoric acid, ethylacetate, sodium carbonate, sodium bicarbonate and ammonium sulphate (Analar grade, BDH chemicals Ltd. Poole, Dorset).

For the provision of elution gradients an Applied Chromatography Systems (ACS) 750/36 decilinear programmer was used. Chromatographic columns of 5 μm Hypersil octadecyl-silane 250 x 4.5 mm i.d. (Shandon, London), slurry packed by the procedure previously described, were used throughout.

A stock metabolite standard solution was prepared according to the procedure described by Thompson et al. (1974). Each xanthine metabolite (10 mg) was dissolved in approximately 35 ml of 0.08M disodium tetraborate, $Na_2B_4O_7$, 10 H2O; the pH was then adjusted to 7 with 1M phosphoric acid before diluting to 100 ml with distilled water.

3.2.Separation and identification of caffeine and its metabolites by reverse phase HLPC.

a) Isocratic separation of methylxanthines

The suitability of isocratic reverse phase chromatography systems (incorporating acidic acetonitrile/water mixtures as mobile phases), for the separation of caffeine and its dimethylxanthine metabolites, has been clearly established in

Section 2. However the use of a similar mobile phase (4% acetonitrile/1% tetrahydrofuran in acetate buffer pH 4.0) for the separation of a complex mixture of methylxanthines resulted in poor resolution of the early eluted, high polarity metabolites. The subsequent application of a less polar isocratic system, consisting of 2.5% aqueous acetonitrile, improved the resolution of the more polar metabolites at the expense of a prolonged retention time for caffeine and an inability to resolve 1,7-dimethylxanthine from theophylline (Table 3.1). These findings confirmed the limitations of isocratic chromatography systems in separating metabolite mixtures of varying polarity, clearly indicating the requirement for a technique involving gradient elution.

b) Gradient elution chromatography

The optimisation of chromatography conditions for the separation of an aqueous solution (50 mg/l) containing caffeine and eleven of its metabolites, was investigated using a variety of elution gradients consisting of acetonitrile mixtures in acetate buffer. In addition, the resolution of 7β-hydroxypropyl theophylline (proxyphylline) was examined to assess its suitability for use as an internal standard. The effects of variations in pH and ionic strength of the mobile phase were investigated and also the addition of the ion-pairing agent, tetrahydrofuran.

Results

The initial optimistion of chromatography conditions and many of the early metabolite studies were performed using a pre-packed Hypersil ODS column (Column A), commercially

SOLVENT SYSTEM	RETENTION TIME (mins) 7MU	7MX	1MU	3MX	1MX	13MU	37MX	17MU	17MX	13MU	137MU	137MX	PP
COLUMN A													
2.5% Acetonitrile in distilled water	-	-	4.3	6.0			11.8		20.5	20.5		54.2	
Gradient 1 in 50 mM Acetate buffer pH 4.0	8.1	9.9	9.9	11.0	12.2	15.2	16.8	18.8	20.1	20.1	22.2	24.0	
Gradient 2 in 50 mM Acetate buffer pf 4.0	5.8	6.7	6.8	7.5	8.3	10.2	11.6	13.3	15.0	15.0	16.7	20.1	
Gradient 3 in 10 mM Acetate buffer pH 4.0	6.1	6.8	7.3	7.8	8.8	9.8	10.5	12.4	13.1	13.5	14.5	16.9	17.3
Gradient 3 in 50 mM Acetate buffer pH 4.0	6.2	6.8	7.2	7.7	8.8	10.3	11.0	13.0	13.8	14.4	15.5	18.0	18.4
Gradient 3 in 10 mM Acetate buffer pH 4.2	6.2	7.0	7.4	8.0	9.0	10.0	10.7	12.6	13.3	13.7	14.7	17.0	17.5
Gradient 3 in 50 mM Acetate buffer pH 4.2	6.0	6.7	7.1	7.6	8.7	9.7	10.4	12.0	12.8	13.3	14.2	16.7	17.2
Gradient 3 in 10 mM Acetate buffer pH 4.4	6.0	7.0	7.2	8.0	9.0	9.9	10.7	12.3	13.2	13.6	14.6	17.0	17.4
Gradient 3 in 50 mM Acetate buffer pH 4.4	5.9	6.7	7.0	7.7	8.8	9.7	10.5	12.2	13.2	13.6	14.6	17.0	17.4
Gradient 3 in 50 mM Acetate buffer pH 4.8	6.2	7.5	7.5	8.5	9.4	10.5	11.4	13.0	14.1	14.3	15.4	18.0	18.5

Table 3.1. Chromatography retention data of methylxanthine metabolites

For key to abbreviations refer to page 3. Details of gradient systems are shown in Fig. 3.1b

SOLVENT SYSTEM	RETENTION TIME (mins) 7MU	7MX	1MU	3MX	1MX	13MU	37MX	17MU	17MX	13MU	137MU	137MX	PP
COLUMN B													
Gradient 3 in 10 mM Acetate buffer pH 4.0	4.6	4.8	5.7	5.8	7.3	8.7	9.1	11.8	11.8	12.7	13.9	15.7	16.9
Gradient 3 in 10 mM Acetate buffer pH 4.4	4.7	5.0	5.9	6.2	7.7	9.2	9.4	11.9	12.3	13.2	14.3	16.2	17.2
Gradient 3 in 10 mM Acetate buffer pH 4.6	4.5	5.0	5.7	6.1	7.5	9.0	9.2	11.5	12.0	12.7	13.8	15.7	16.9
Gradient 3 in 10 mm Acetate buffer pH 4.8	4.2	4.9	5.2	6.1	7.5	9.1	9.1	11.0	12.0	12.8	13.7	15.8	17.0

Table 3.1b. Chromatography retention data of methylxanthine metabolites

For key to abbreviations refer to page 3.

Key to gradient systems

Gradient 1 - 4.8 - 44.8% Acetonitrile in acetate buffer (v/v)
Gradient 2 - 3.8 - 73.8% Acetonitrile in acetate buffer (v/v)
Gradient 3 - 0 - 12.8% Acetonitrile + 1% Tetrahydrofuran in acetate buffer (v/v)

obtained from Shandon U.K. Ltd. However, as result of a gradual deterioration in performance, it was eventually replaced by a "in-house" slurry packed column (Column B) which showed characteristic changes in optimal chromatogrphic conditions. These were mainly reflected by variations in pH; the optimum value for column A being pH 4.0 compared to levels varying between pH 4.6-4.8 for the columns prepared "in-house". In order to demonstate these effects, data is reported in Table 3.1 and Table 3.1b. for the use of columns A and B respectively.

For column A, optimum separation of the metabolite standard solution was achieved using a gradient elution system of 0-12.5% acetonitrile in 1% tetrahydrofuran, pH 4.0. The eluent for pump A consisted of 1% tetrahydrofuran in 10 mM acetate buffer, pH 4.0, and for pump B, 15% acetonitrile and 1% tetrahydrofuran in 10 mM acetate buffer, pH 4.0. Solvent A was pumped for 5 minutes before introducing a stepwise gradient of 5%, 10%, 2% increase in B per minute, using a segment interval of 5 minutes. A flow rate of 1.5 ml per minute was maintained throughout. Similar conditions were found to be optimal for column B with the exception that both solvents were prepared in 10mM acetate buffer pH 4.6. Chromatograms showing the separation of an aqueous solution of methylxanthines using columns A and B are shown in Fig. 3.1 and 3.2 respectively.

Whilst variations in ionic strength (10mM-50 mM) and slight differences in the acetonitrile concentration resulted in minimal effects on the resolution of methylxanthines, the pH of the mobile phase was found to be critical in obtaining

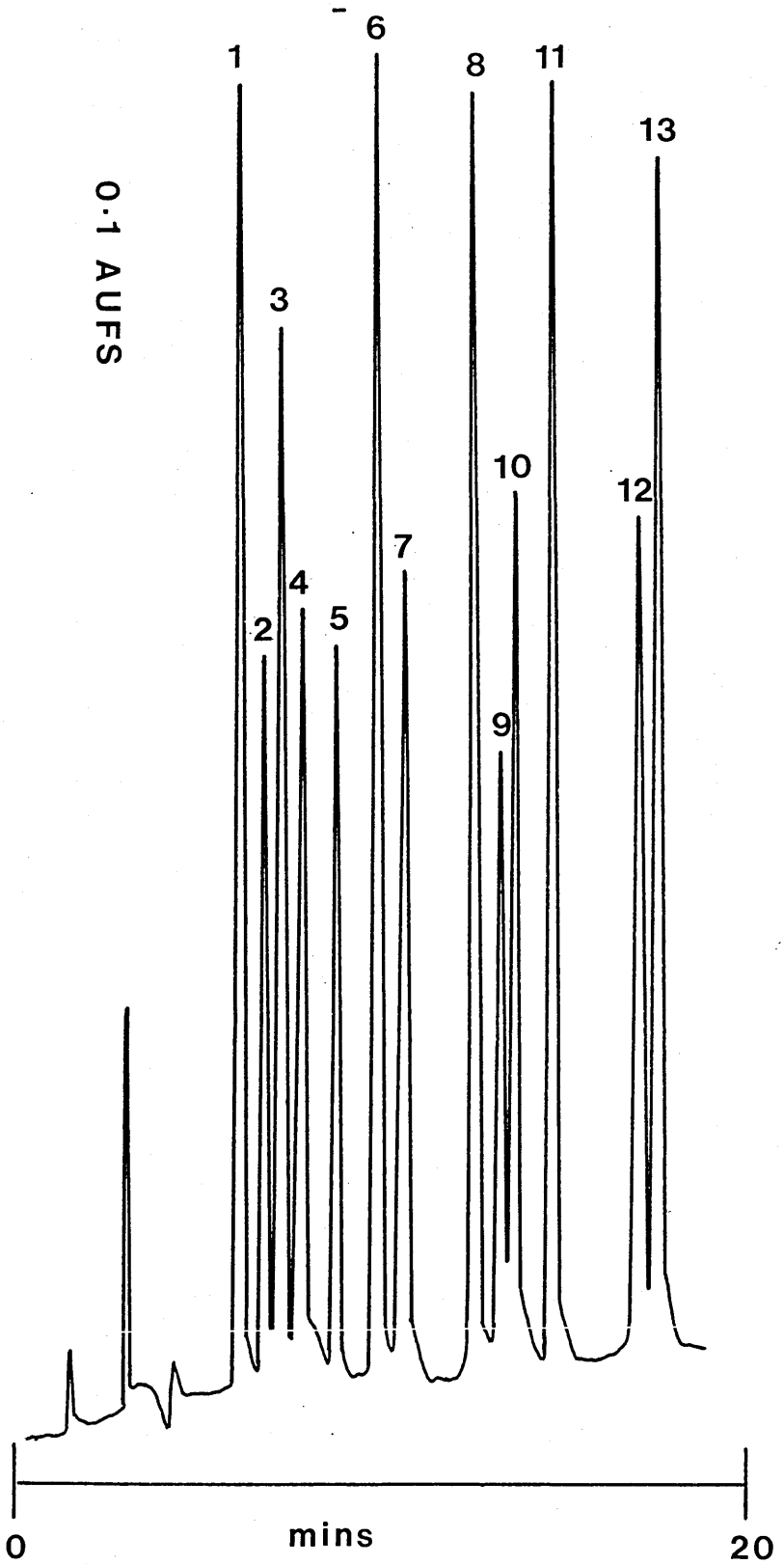


Fig 3.1 Chromatography of an aqueous methylxanthine standard solution (50mg/l) using Hypersil ODS Column A. (Chromatography conditions as described in text)

PEAKS: 1= 7-methyluric acid, 2= 7-methylxanthine, 3= 1-methyluric acid,
4= 3-methylxanthine, 5= 1-methylxanthine, 6= 1,3-dimethyluric acid,
7= theobromine, 8= 1,7-dimethyluric acid, 9= 1,7-dimethylxanthine,
10= theophylline 11= 1,3,7-trimethyluric acid, 12= caffeine,
13= proxyphylline (internal standard)

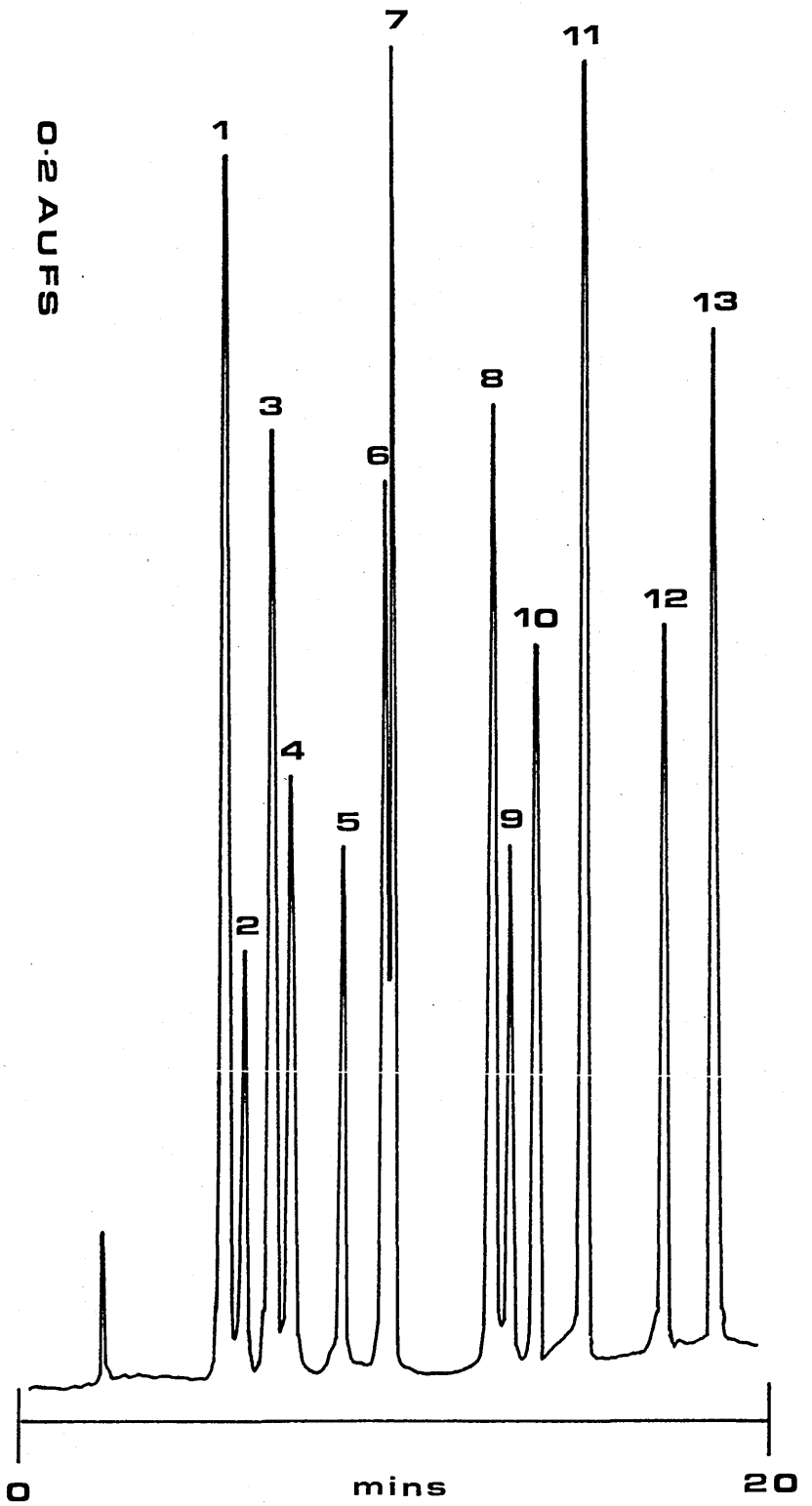


Fig 3.2 Chromatography of an aqueous methylxanthine standard solution (50mg/l) using Hypersil ODS, Column B.

Chromatographic conditions are described in text.

Peak identification as in Fig 3.1.

maximum separation of the early eluted peaks. For example, an increase from pH 4.0 to pH 4.4 when using column A, resulted in an almost total loss of resolution between 7-methylxanthine and 1-methyluric acid, whilst for column B, a slight reduction in pH from 4.6 to 4.4 led to an inability to resolve 1-methyluric acid from 3-methylxanthine and 1,7-dimethyluric acid from 1,7-dimethylxanthine. In agreement with the findings of Section 2., the addition of tetrahydrofuran to the mobile phase was found to be essential in achieving optimal separation of theophylline and 1,7-dimethylxanthine. A period of 5 minutes, pumping solvent A only, was found to be an acceptable period of re-equilibration beween gradient runs.

SAMPLE PREPARATION

3.3. Direct Injection

Urine samples were centrifuged and then diluted 1 in 10 with an aqueous solution of the internal standard, proxyphylline (10 mg/l). An aliquot (50 µl) of the diluted sample was then injected directly onto the column without further preparation. For quantitation, the peak height ratios of eluted metabolites were compared with those obtained from the injection of an aqueous standard solution (50 mg/l) processed in a similar manner.

An assessment of detector response linearity was made by measuring the peak heights of eluted peaks following the injection of standard solutions ranging from 25-200 mg/l. Within batch precision was studied by replicate analysis (n=4) of a 24 hour urine sample collected from a volunteer receiving

a controlled dietary intake of 600 mg (Urine A) and a urine sample collected from a Mormon volunteer spiked with methylxanthine metabolites to a concentration of 25 mg/l. Between batch precision was investigated by analysis of a similar spiked urine sample on 3 consecutive days.

The specificity of the procedure was investigated by chromatographing a series of random urine samples, collected from a Mormon population on a strict caffeine free diet. The results obtained were expressed in terms of mg/g creatinine; urine creatinine levels being determined using the alkaline picrate reaction described by Jaffé.

The suitability of urine "clean-up" procedures was studied using C-18 Sep-pak cartridges (Walters Associates), micro-columns containing kieselguhr (Extrelut, Merck Ltd), and ultrafiltration using the Amicon Micropartition System. The procedures adopted for use with these systems are descibed in Appendix 1. They were evaluated by estimating the post treatment recovery of a methylxanthine standard and their ability to eliminate non-specific substances present in a urine sample collected from a Mormon volunteer.

Results

Detector linearity was found to be acceptable over a concentration range of 25-200 mg/l (mean coefficient of correlation, r = 1.0) confirming the suitability of a one point calibration. Within batch precision data is summarized in Table 3.2. The mean coefficient of variation for the within batch precision of diluted urine samples was 8.64% (SD. 5.11)

COMPOUND	DIRECT INJECTION				ION-PAIR EXTRACTION			
	Urine A		Urine B		Urine A		Urine B	
	Mean	C.V.	Mean	C.V.	Mean	C.V.	Mean	C.V.
7MU	7.8	22.5	29.2	16.8	4.6	19.6	24.0	6.3
7MX	19.4	10.5	35.2	7.1	17.7	6.2	29.3	4.3
1MU	99.9	0.8	25.4	8.0	80.9	3.6	27.0	1.9
3MX	10.2	9.7	30.8	6.1	12.1	10.3	29.8	3.1
1MX	56.5	7.9	28.2	4.4	54.7	2.1	33.3	3.7
13MU	8.3	5.2	30.6	8.9	7.5	24.2	26.0	7.4
37MX	9.0	8.2	30.7	8.3	4.4	21.2	31.9	5.4
17MU	36.3	1.3	29.4	7.4	36.7	8.3	29.1	4.0
17MX	19.9	8.1	31.5	9.4	14.7	9.8	29.0	1.9
13MX	4.7	5.6	32.3	5.9	*	*	31.3	5.2
137MU	4.4	19.6	30.7	5.5	5.5	15.9	27.3	2.9
137MX	7.3	13.8	31.3	6.4	5.0	16.0	29.2	3.6
MEAN		9.43		7.85		12.47		4.15
S.D.		6.56		3.18		7.37		1.67

Table 3.2. Within-batch precision for analysis of methylxanthines in urine
(* = not detected) For key to abbreviations refer to page 3

Urine A: 24 hr sample obtained from a volunteer after a caffeine intake of 600 mg per 24 hr. Urines B and C: separate urine specimens collected from Mormon subjects and spiked with methylxanthines to give in each case a final concentration of 27.78 mg/g creatinine. Results are given as mean of four determinations and expressed as mg/g of creatinine. CV = coefficient of variation (%).

with values ranging from 4.4% (1-methyluric acid) to 19.7% (7-methyluric acid). Between batch precision, studied by analysis of a spiked urine sample on 3 consecutive days, showed an overall mean coefficient of variation of 8.58 (S.D. 4.39) with corresponding ranges of 1.5% (caffeine) to 15.5% (7-methyluric acid).

The methylxanthine levels determined in urine samples from Mormon volunteers on a strict caffeine free diet are shown in Table 3.4. Significant blank values (>3 mg/l) were obtained for 7-methylxanthine, 1-methyluric acid, 1,3- dimethyluric acid 3-methyl xanthine, 1-methylxanthine, and theobromine, although the latter three were affected by detection of an interfering peak on a single occasion.

Whilst the use of Sep-Pak cartridges as a sample "clean-up" procedure showed complete recovery of all the methylxanthines, interfering peaks were still present on post extraction chromatograms of urine. Similar results were obtained following the use of the ultrafiltration technique. Although the recovery values following "Extrelut" extraction were acceptable for caffeine and the dimethylxanthines (93-105%), low recoveries were found for the mono-methylxanthines (25-75%) and the uric acid metabolites (both mono- and di-) were almost totally retained on the column.

3.4. Organic extraction of methylxanthines

In view of the limitations of the direct injection procedure, imposed by the finding of high blank values, attempts to improve specificity were made by investigating the

use of organic extraction techniques. The following solvent systems were assessed for their suitability by calculating the recovery of an aqueous methylxanthine standard (100 mg/l) following extraction.

a) Chloroform/isopropanol with acetate buffer pH 4.0.

b) Ethylacetate/chloroform/isopropanol with acetate buffer pH 4.0, 7.0, 11.0.

c) Ion-pair extraction using tetrabutylammonium hydrogen sulphate.

Metabolite recovery values were calculated by comparison of peak height values with those obtained by direct injection of unextracted aqueous standard.

3.4.1.Organic extraction using a) chloroform/isopropanol (85:15 v/v) and b) ethylacetate/chloroform/isopropanol (45:45:10 v/v).

Aqueous standard solution (100 µl), 50 µl of internal standard (proxyphylline, 200 mg/l), 100 µl of buffer solution were placed in a stoppered tube and vortex mixed for 1 minute following the addition of 5 ml extraction solvent. After centrifugation at 2000 rpm (1500 x g) for 5 minutes, a 4ml aliquot of the organic layer was transferred to a clean tube and evaporated to dryness at 45 C under a stream of air. The residue was reconstituted with 500 ul of 1% tetrahydrofuran in acetate buffer, pH 4.0, and then injected onto the HPLC column.

3.4.2. Ion-pair extraction

Aqueous standard solutions (200 µl), 50 µl of internal standard solution (proxyphylline, 200 mg/l), 200 µl 0.1M tetrabutylammonium hydrogen sulphate and 100 µl of pH 11 buffer solution (0.1M sodium carbonate/0.1M sodium bicarbonate 90:10 v/v), to produce a resulting pH of approximately 6.5, were placed in a stoppered tube. The contents were vortex mixed, before and after the addition of approximately 0.5g of ammonium sulphate, and then extracted by vortex mixing with 5 ml of a solution containing ethylacetate/chloroform/ isopropanol (45:45:10 v/v) for at least 1 minute. After centrifugation for 5 minutes at 2,000 rpm (1500 x g), a 4 ml aliquot of the organic layer was transferred to a clean tube and evaporated to dryness at 45°C under a stream of air. The residue was dissolved in 500 µl of 1% tetrahydrofuran, pH 4.8, 50 µl of which was injected onto the column.

Results

The recovery values for each of the solvent systems investigated are summarized in Table 3.3. Significant differences in recovery between these systems, were present for only 7-methyluric acid and 1-methyluric acid. In comparison with chloroform/isopropanol extraction, the use of ethylacetate/chloroform/isopropanol, without the presence of an ion-pairing agent, gave an overall lower recovery, especially at an alkaline pH. However, a significant advantage of the latter solvent system was that the organic layer formed the upper phase after centrifugation, thus eliminating the requirement for removal of the aqueous phase prior to

EXTRACTION SYSTEM	PERCENTAGE RECOVERY											
	7MU	7MX	1MU	3MX	1MX	13MU	37MX	17MU	17MX	13MU	137MU	137MX
a) Aqueous												
Chloroform/isopropanol (85:15, v/v) pH 4.0	47.4	93.2	61.0	94.7	98.4	96.8	101.7	95.0	98.9	96.8	104.2	94.4
Ethylacetate/chloroform /isopropanol (45:45:10, v/v) pH 4.0.	21.6	75.2	35.9	84.0	89.7	92.6	96.9	91.6	95.5	97.5	105.4	106.0
Ethylacetate/chloroform /isopropanol (45:45:10, v/v) pH 7.0	20.7	75.2	33.2	80.9	88.1	91.8	95.7	87.3	93.8	93.8	103.6	104.2
Ethylacetate/chloroform /isopropanol (45:45:10, v/v) pH 11.0	5.6	77.5	14.7	84.0	91.3	84.9	95.7	73.6	99.0	98.8	103.6	98.5
Ion-pair 0.05M TBAS pH 6	74.9	89.2	74.2	85.0	88.2	92.4	85.1	87.0	96.4	96.2	86.3	92.4
Ion-pair 0.2M TBAS pH 6	73.2	90.8	80.8	91.6	92.6	96.4	74.4	87.0	101.9	92.6	84.9	95.3
Ion-pair 0.2M TBAS pH 6	71.5	84.0	76.8	88.1	90.1	87.4	95.1	85.9	96.4	90.1	83.2	92.0
b) Urine												
Ion-pair 0.1M TBAS pH6	74.5	81.3	65.9	89.6	85.4	73.7	96.3	83.6	79.3	85.4	91.9	97.8

Table 3.3 Extraction recoveries for methylxanthine metabolites

For key to abbreviations refer to page 3

sampling. The recovery of 7-methyluric and 1-methyluric acid was significantly improved by the addition of tetrabutyl ammonium hydrogen sulphate (TBAS) and subsequently this system was adopted for further investigation. Slight variations in the concentration of TBAS (0.05M-0.2M) produced only minimal differences in recovery values.

3.4.3. Standardization

As a result of the close correlation in recovery values for aqueous solutions and spiked urine samples using the ion pair extraction procedure, standardization using aqueous solutions was adopted. A series of standard solutions, covering a range of 0-100 mg/l, were prepared in distilled water and extracted in duplicate using proxyphylline (1 ug/tube) as an internal standard. The reconstituted extracts were then chromatographed and standard curves prepared by plotting the metabolite/ proxyphylline peak height ratios against concentration.

Results

The relationship between concentration and the peak height ratios were linear for each metabolite. The mean coefficient of correlation (r), calculated using least squares regression analysis, was 0.988 (range = 0.992-0.999).

3.4.4. Reproducibility and specificity

The within batch precision of the procedure was studied by replicate analysis (n=4) of a 24 hour urine sample collected from a volunteer receiving a controlled daily intake of 600 mg

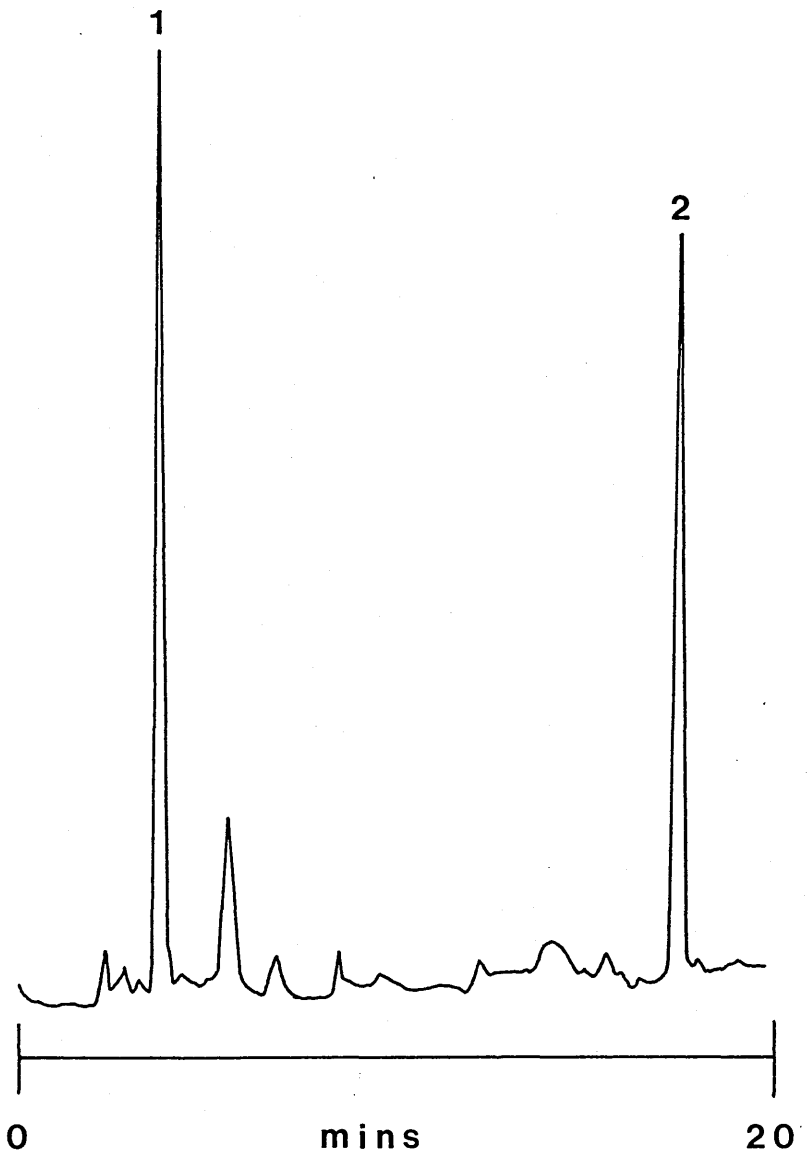


Fig 3.3 Chromatography of an extracted urine sample from a Mormon volunteer.

Chromatography conditions as described in text for column A.

Peaks: 1 = Uric acid 2 = Proxyphylline

(Urine A) and a urine sample collected form a Mormon volunteer spiked with methylxanthine metabolites to a concentration of 25 mg/l. Quantition of the metabolites (Table 3.2.) showed mean coefficient of variation values of 8.12% (SD = 6.65) with corresponding ranges of 2.75% (1-methyluric acid) to 15.81% (1,3-dimethyluric acid). Between batch precision, studied by analysis of a spiked urine sample on three consecutive days, showed an overall mean coefficient of variation of 8.04% with corresponding ranges of 3% (1-methylxanthine) to 12.2% (1,7-dimethylxanthine).

Specificity was examined by measurement of methylxanthine levels in urine samples obtained from Mormon volunteers on a strict caffeine free diet. Values of less than 1.5 mg/g creatinine were found for each metabolite (Table 3.4), demonstrating a considerable improvement in specificity over the direct injection procedure. A chromatogram of a Mormon urine extract is shown in Fig. 3.3

3.5.COMPARISON OF URINARY METHYLXANTHINE METABOLITE PROFILES BY DIRECT INJECTION AND ION-PAIR EXTRACTION PROCEDURES.

A comparative study of the two procedures was made by analysing urine samples, collected from normal volunteers receiving a controlled dietary intake of caffeine (300, 450 or 600 mg) in the form of pre-weighed aliquots of instant coffee. Following a 24 hour period of equilibration to the restricted caffeine intake, the volunteers collected two consecutive 24 hour urine samples of which the volumes were recorded and aliquots stored at - 20^{o}C until analysis. The controlled

caffeine intake was maintained throughout the study and all other forms of caffeine were excluded from the diet.

In order to assess the suitability of the procedures for monitoring metabolite excretion following administration of other methylxanthines, urine samples were also collected and analysed from hospital inpatients receiving theophylline therapy.

Results

The analysis of 24 hour urine samples from patients receiving oral doses of theophylline (16 mg/kg/day) produced peaks corresponding to theophylline, uric acid, 1-methyluric acid, 3-methylxanthine and 1,3-dimethyluric acid (Fig 3.4). Urinary excretion products identified from individuals receiving a controlled intake of caffeine included the compounds named above plus caffeine, 7-methyluric acid, 7-methylxanthine, 1-methylxanthine, theobromine, 1,7-dimethyluric acid, 1,7-dimethylxanthine and 1,3,7-trimethyluric acid (Fig 3.5). The methylxanthine levels obtained using both procedures are summarized in Table 3.4.

Statistical analysis of the results obtained for each procedure was made using a Student t-test and linear regression analysis. A favourable correlation was found for the major metabolites, 1-methyluric acid ($r = 0.867$, $P < 0.1$), 1-methylxanthine ($r = 0.930$, $P < 0.05$), and 1,7-dimethyluric acid ($r = 0.783$, $P < 0.1$), although the direct injection procedure results showed a slight positive bias. For the remaining metabolites, the correlation between the two procedures decreased in proportion to concentration although a

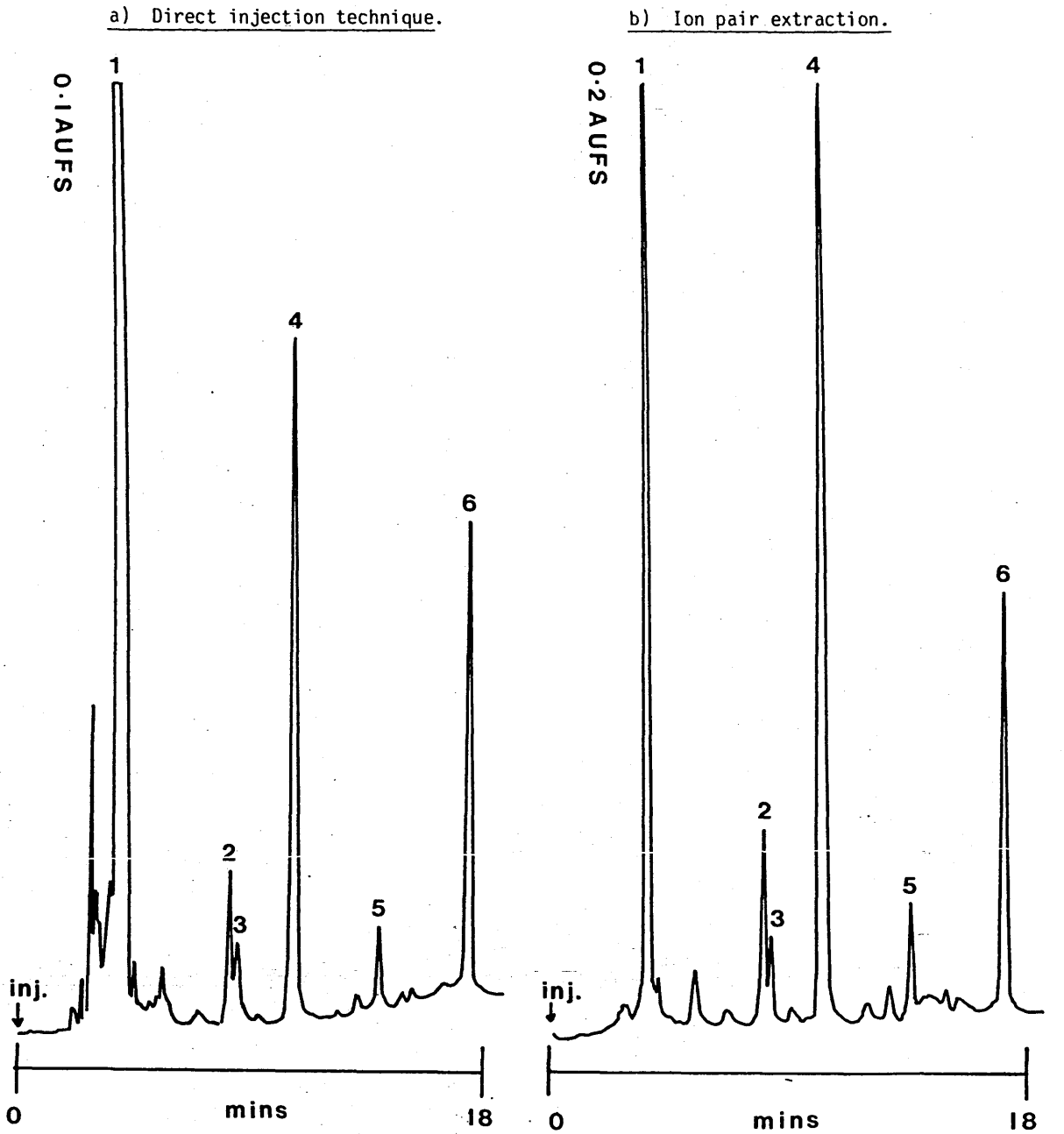


Fig 3.4 Chromatograms of urine sample received from a subject receiving theophylline therapy assayed by a) direct injection technique, b) ion pair extraction.
Chromatographic conditions as described in text for Column A.

PEAKS: 1= uric acid, 2= 1-methyluric acid, 3= 3-methylxanthine, 4= 1,3-dimethyluric acid, 5= theophylline, 6= proxyphylline (internal standard).

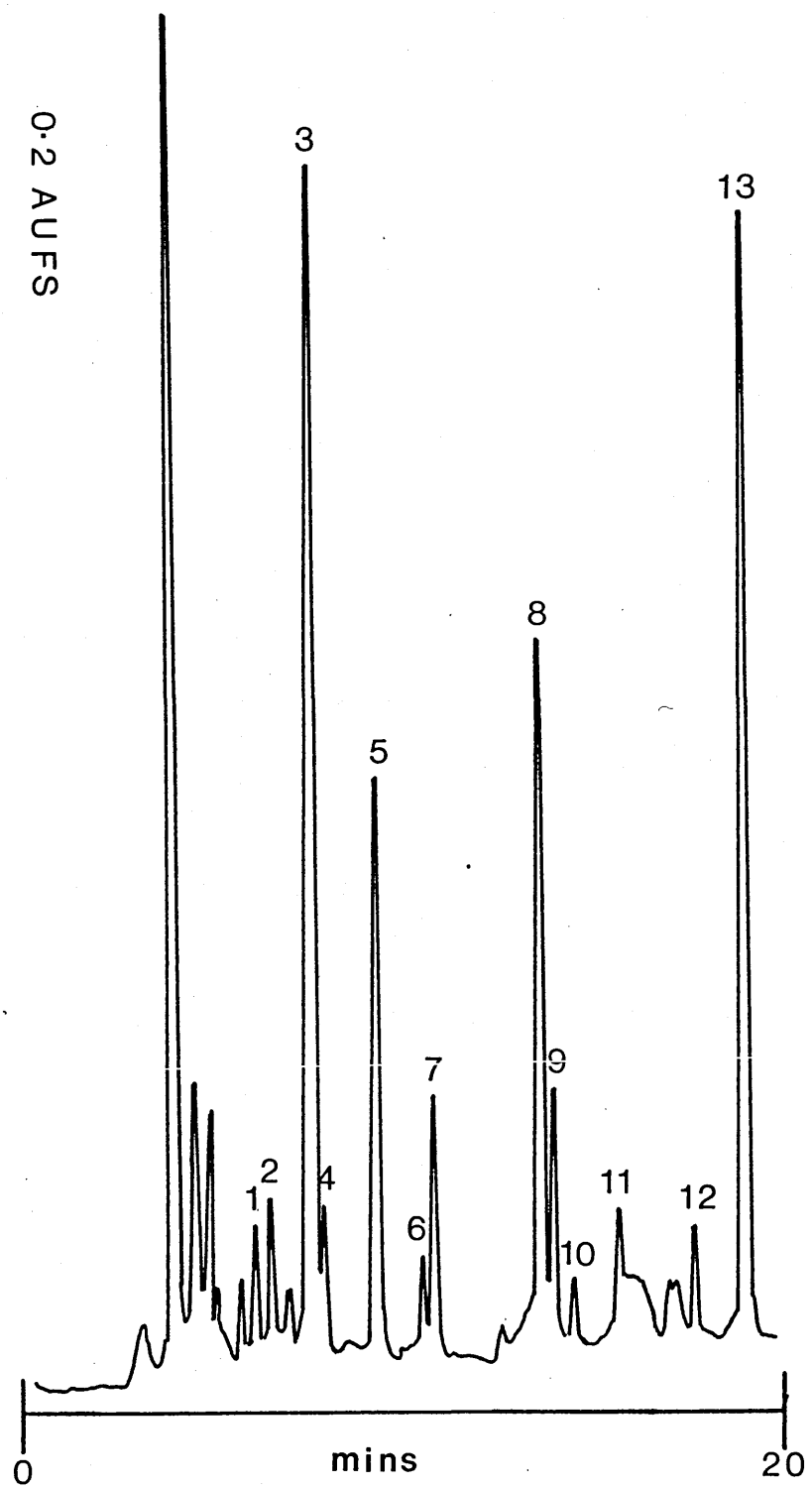


Fig 3.5 Chromatogram of a urine sample collected 0-24h following oral ingestion of 500mg caffeine (ion pair extraction technique). Chromatographic conditions as described in text for Column B. Peak identification as in Fig 3.1.

CAFFEINE INTAKE AND ASSAY PROCEDURE		CONCENTRATION IN URINE (mg/g creatinine) 7MU	7MX	1MU	3MX	1MX	13MU	37MX	17MU	17MX	13MU	137MU	137MX
A. Direct Injection													
300 mg/24 hrs (n=4)	mean	4.73	10.90	51.90	3.13	30.20	2.98	2.13	27.10	14.63	+	+	17.93
	S.D.	1.30	1.27	6.85	4.93	7.01	3.61	2.66	9.12	1.03	+	+	6.45
450 mg/24 hrs (n=5)	mean	4.40	14.64	80.24	13.70	48.66	7.14	8.96	24.98	16.08	1.66	3.48	12.16
	S.D.	2.09	2.49	27.65	5.04	9.50	1.49	2.82	6.49	5.22	1.16	2.91	7.34
600 mg/24 hrs (n=2)	mean	11.65	18.80	120.60	10.47	62.87	9.10	13.83	35.20	20.65	4.90	3.33	4.75
	S.D.	1.53	6.00	26.60	5.33	4.76	2.31	6.48	1.48	1.65	0.51	0.81	1.01
Mormon group (n=13)	mean	0.66	3.15	2.68	3.61	5.16	3.06	3.39	0.59	0.22	1.48	0.47	0.32
	S.D.	0.72	3.35	1.89	2.82	4.64	2.36	5.87	0.75	0.78	1.66	0.70	0.80
B. Ion-pair extraction													
300 mg/24 hrs (n=4)	mean	5.43	10.21	50.32	7.93	28.68	5.13	5.35	24.38	15.75	2.43	2.05	7.40
	S.D.	1.29	2.52	14.75	1.56	7.63	2.53	1.21	7.97	2.08	1.72	0.74	3.56
450 mg/24 hrs (n=5)	mean	4.28	17.34	75.14	12.60	47.52	5.82	6.06	24.76	15.94	3.16	1.92	5.73
	S.D.	1.31	2.61	39.8	2.07	9.49	0.92	3.90	7.88	1.88	2.23	0.64	2.36
600 mg/24 hrs (n=2)	mean	5.10	17.58	98.20	11.48	54.90	6.83	6.70	30.93	19.95	2.63	2.70	7.03
	S.D.	1.17	1.86	11.30	1.40	5.06	0.74	1.53	6.67	1.01	1.18	0.48	1.15
Mormon group (n=13)	mean	0.75	0.17	0.79	0.62	1.08	0.20	0.37	0.35	0.78	1.42	0.35	0.30
	S.D.	0.92	0.43	0.66	0.72	1.09	0.33	0.73	0.45	1.58	1.60	0.36	0.58

Table 3.4 Urinary excretion of methylxanthine metabolites in healthy subjects
(+ = present but not measurable) For key to abbreviations refer to page 3.

For the mormon group, one random specimen was collected for each subject. With the rest of the subjects, two 24 hr urine samples were collected and analysed separately and the average value used for evaluation of the mean excretion for the respective subject group (n = 2,4 or 5)

significant difference was only observed for unchanged caffeine.

DISCUSSION

Whilst reverse phase chromatography techniques have made a significant contribution to the present understanding of the biotransformation of caffeine they have been generally non-specific. This has been mainly reflected by their inability to resolve 7-methylxanthine from 1-methyluric acid, theophylline from 1,7-dimethylxanthine or 3,7-dimethyluric acid from 1-methylxanthine. In contrast the procedure described herein eliminates most of these problems of non-selectivity.

In order to optimise sample preparation in terms of selectivity, precision and preparation time, an assessment has been made of the use of direct injection of diluted urine and a procedure involving the formation and extraction of an ion-pair complex. The extraction procedure described shows excellent selectivity and much improved recovery from previous techniques based on chloroform/isopropanol extraction. Variations in recovery are relatively insignificant using the direct injection technique and, with a much reduced preparation time, it has obvious advantages over extraction techniques. Also the requirement of peak height correction using the internal standard was found to be minimal. However, it is apparent that the technique has limitations due to high blank values and the occasional presence of an interfering peak. These problems were not resolved by the use of ultrafiltration or purification using C-18 Sep-Pak (Water's

Associates) cartridges prior to injection. The use of "Extralut" columns for sample purification was rejected due to low recovery values for certain metabolites.

The chromatography procedure described achieves separation of the xanthine metabolites within 20 minutes, and a re-equilibration period of only 5 minutes permits the analysis of approximately twenty samples within one working day or more if automatic sampling is used. The short period of equilibration can be largely attributed to the use of acetonitrile, in preference to methanol as a solvent component, since reports have shown (Muir et al., 1980) that, in order to ensure adequate resolution of the early eluted peaks when using methanol based solvents, all traces of methanol must be removed from the stationary phase of the column between injections. This necessitates re-equilibration periods of at least 30 minutes using a flow of buffer solutions only. In the absence of an ion-pairing agent, the retention of some of the methyluric acids was inadequate for acceptable resolution. The addition of tetrahydrofuran as a counterion to the solvent system resulted in a considerable increase in retention of these metabolites despite the fact that the pH of the mobile phase was lower than their pKa values (about 5.5). The effects of pH were most noticeable with the retention times of 1-methyluric acid and 1,3-dimethyluric acid. For example, in using column B, the lowering of the pH to 4.4 resulted in a marked reduction in the retention and resolution of these compounds, presumably by reducing the fraction of these molecules in the ionic state which are capable of ion-pairing.

Increasing the pH to 5.0 resulted in the opposite effect where the retention times of 1-methyluric acid and 1,3-dimethyluric acid were increased by virtue of the higher equilibrium concentration of the paired, neutral species. Whilst the resolution of 1,3-dimethyluric acid and theobromine was relatively poor using Column B, these represent only minor metabolites of caffeine metabolism and for theophylline metabolites studies, theobromine interference in the measurement of 1,3-dimethyluric acid levels is negligible.

Slight variations in the resolving properties of different batches of HPLC packing materials are not uncommon (unpublished observations). Whilst the differences observed in this report could be the result of variations in packing techniques, ie commercial vs "in-house", this is considered unlikely. Following consultation with Shandon U.K. and subsequent examination of a series of Hypersil ODS batches, it was clearly established that such marked effects were infrequent; optimum resolution being generally obtained at pH 4.6. It was concluded that the altered selectivity in Column A may have been due to previous applications using octyl -sulphonic acid, clearly indicating the disadvantages of using HPLC columns for multifunctional analysis.

The use of an ion-pair extraction procedure considerably improved the recovery of the methyluric acid metabolites. At pH 6.5, uric acids are in an ionized form which permits them to form an ion-pair with the tetrabutylammonium counter ion. This can then be effeciently extracted using the solvent system described. Extraction was enhanced by the addition of

ammonium sulphate to the system to produce a salting out effect. The effects of pH and concentration of ion-pairing agent were in agreement with those previously described by Tang-Liu and Riegelman (1981). Using this extraction procedure a batch of twenty samples may be processed for chromatography in appproximately 1 hour.

Both the direct injection and ion-pair extraction procedures have proved robust in use with no problems of late eluting peaks and minimal deterioration of column performance. When required, peak resolution may be rapidly restored by repacking the top few millimetres of the column and the use of a guard column is considered unnecessary. The use of direct injection offers a slight improvement in the within-batch precision when compared with the extraction procedure, but for the major metabolites, both procedures show satisfactory reproducibility.

In summary both the procedures described in this study have some value in investigating the pharmacokinetics and metabolism of methylxanthines. In studies where a precise determination of values is required, the extraction procedure described is the method of choice. However, experience has shown that, providing suitable cut-off values are applied to compensate for the potentially high blank values, the convenience of direct injection of diluted urine is of value where quantitation is only required for the major metabolites. Applications include screening for toxicity or the detection of alterations in the pattern of metabolite excretion resulting from diseased states such as chronic liver disease.

4. HUMAN CONSUMPTION OF CAFFEINE

4. HUMAN CONSUMPTION OF CAFFEINE

4.1. Introduction

The consumption of caffeine from natural sources has been enjoyed throughout the world for centuries. Historical records show that tea has been popular in the Orient since about 350 A.D. where initially its raw leaves were infused with water to produce a medicinal potion. However, tea was first brought to Europe in 1559 by the Venetian traveller Gian Battista Ramusio, and found its way to England during the early 17th century. The East India Company brought its first consignment from China in 1664. Although coffee was similarly introduced to Europe in the seventeenth century, the earliest mention of coffee beans being consumed as a hot beverage was in the tenth century. However coffee for use as a food may have been cultivated in Ethiopia in early 575 A.D. Cocoa and other caffeine containing chocolate products also have a long history. Chocolate drinks became popular first in Spain where, in 1519, the spanish conquerers were served a sweetened chocolate drink by the Aztec Emperor Montezuma. A way of making milk chocolate bars was invented in Switzerland in 1876.

The major ingredient use of caffeine is as flavouring component in cola-type soft drinks which originated around the beginning of this century (Dr Pepper in 1885, Coca-cola in 1886, Pepsi-cola in 1896 and Royal Crown cola in 1912). To a lesser extent, caffeine is also used as a flavouring in baked foods, dairy deserts, puddings and fillings, and in some sweets. Caffeine was first chemically isolated in 1820 and has

since been used therapeutically in neonatal apnoea, acne and other skin disorders, migraine headaches and as a bronchial and cardiac stimulant. Caffeine also occurs in various "over the counter" products used as analgesics, diuretics, slimming aids, allergy relief preparations and mild stimulants.

4.2. Sources, levels and consumption estimates of caffeine

Estimates of the caffeine content of various food products have shown considerable variations (Burg, 1975), especially with reference to tea and coffee (Table 4.1). The differences may be partially explained by non-standardization of analytical procedures and reference volumes (ie."cup" size), although they may also be influenced by the plant variety and growing conditions of the natural product. The largest variation occurs with brewed coffee where levels depend on the form (eg. ground roasted vs instant), the method of brewing (eg. percolator vs filter), the amount of coffee used and the brewing time.

Representative values for each of the major sources were standardized by Barone and Roberts (1981) for consumption estimates in American populations. The selected values were:

Coffee

a) Ground roasted	85 mg per 5 oz cup
b) Instant	60 mg per 5 oz cup
c) Decaffeinated	3 mg per 5 oz cup

Tea

a) Leaf or bag	40 mg per 5 oz cup
b) Instant	30 mg per 5 oz cup

Product	Volume or weight	Caffeine content (mg) Range	Average	Reference
Roasted and ground coffee	5 oz	60-124	83	Burg (1975)
(percolated)	5 oz	-	74	Gilbert (1981)
Instant coffee	5 oz	40-108	59	Burg (1975)
	5 oz	-	66	Gilbert (1981)
	5 oz	-	66	FDA (1980)
Roasted and ground coffee	5 oz	2- 5	3	Burg (1975)
(decaffeinated)	5 oz	-	2	Gilbert (1981)
Instant coffee (decaffeinated)	5 oz	2- 8	3	Burg (1975)
Roasted and ground coffee (drip)	5 oz	-	112	Gilbert (1981)
Instant coffee (percolated and drip)	5 oz	29-176	-	Gilbert (1981)
Tea	5 oz	8- 91	27	Gilbert (1981)
Bagged tea	5 oz	-	42	Burg (1975)
	5 oz	28- 44	-	FDA (1980)
Leaf tea	5 oz	30- 48	41	Burg (1975)
Instant tea	5 oz	24- 31	28	Burg (1975)
Cocoa - African	5 oz	-	6	Burg (1975)
- South American	5 oz	-	42	Burg (1975)
Cocoa	5 oz	-	5	FDA (1980)
	5 oz	< 40	-	Gilbert (1981)
	5 oz	2- 7	4	Zoumas et al. (1980)
Chocolate bar	30 g	-	20	Gilbert (1981)
Milk chocolate	1 oz	-	6	FDA (1980)
	1 oz	1- 15	6	Zoumas et al. (1980)
Chocolate milk	8 oz	2- 7	5	Zoumas et al. (1980)
Baking chocolate	1 oz	-	35	FDA (1980)
	1 oz	18-118	60	Zoumas et al. (1980)
Regular colas	6 oz	15- 23	-	NSDA (1982)
Decaffeinated colas	6 oz	trace		NSDA (1982)
Diet colas	6 oz	1- 29	-	NSDA (1982)
Decaffeinated diet colas	6 oz	trace	-	NSDA (1982)

Table 4.1 Caffeine content of food products (Barone and Roberts, 1984)

Cola (except caffeine free)	18 mg per 6 oz glass
Cocoa, hot chocolate	4 mg per 5 oz cup
Chocolate milk	5 mg per 8 oz glass

A survey by the Market Research Corporation of America reported a mean daily caffeine intake for all adults as being 2.6 mg/kg. This compared favourably with previous surveys by Graham (1978) and Gilbert (1981) which reported respective daily consumption estimates of 206 mg and 200 mg per adult. Data from a nationwide food consumption survey (NFCS), which included only consumers of caffeine containing products, reported a total mean consumption of 345 mg for all groups. Respective average daily caffeine intake values from coffee, tea and cola flavoured drinks were 233 mg, 76 mg and 25 mg. Caffeine intake from various chocolate products was less than 6 mg per day. Whilst consumption estimates for children (under 18 years of age) are more variable than those for adults, the mean daily intake of caffeine for all American children is estimated to be 1 mg/kg.

Bruce and Lader (1986) have reported that of 51 prescribable analgesic preparations available in the UK, 12 contain caffeine; the mean content being 26 mg (SD = 15 mg, range 7.5-50 mg). For "over the counter" (OTC) preparations of systemic analgesics, 32 out of 89 forms listed in the OTC Index, 1985, were reported to contain caffeine. The mean content was 35 mg (SD = 21 mg, range 7.5- 95 mg).

4.3. A study of caffeine consumption in the United Kingdom

In view of the absence of reports on the dietary intake of caffeine in the U.K., a limited study (n= 174) was made to assess the nature and quantity of caffeine consumption in a random population. Subjects were requested to complete a questionnaire (Appendix 2) giving details of their age, sex, daily consumption of caffeinated beverages, smoking habits, current drug therapy and information on any adverse effects experienced through caffeine consumption or withdrawl. In addition, the caffeine content of various commercial brands of coffee, tea, cola and drinking chocolate was determined using the HPLC procedures previously described. Aqueous dilutions of the beverages were directly injected onto the analytical column and the peak heights compared with values obtained from injection of aqueous standard solutions.

Results

A summary of the caffeine concentrations of the various beverages analysed in this study is shown in Table 4.2. In agreement with previous estimates (Table 4.1), the values showed considerable variation, especially for the caffeine content of percolated and filter coffee extracts. With the exception of chocolate drinks, where significant amounts of theobromine were present (43-209 mg/200 ml), the levels of other methylxanthines were minimal.

In order to provide an accurate comparison with previously reported studies, consumption estimates were calculated using the standardized values selected by Barone and Roberts (1982). The results are summarized in Table 4.3 and shown as a histo-

PRODUCT	CONCENTRATION (mg per 200 ml cup) CAFFEINE	THEOPHYLLINE	THEOBROMINE
Coffee			
a) Instant coffee			
Nescafe´	75	-	-
Gold Blend	62	-	-
b) Percolated coffee			
Various blends	74 - 263	-	-
Safeways Kenya	120	-	-
c) Filter coffee			
Kenco continental	52	-	-
Safeways original	77	-	-
d) Decaffeinated			
Cafe Hag	1	-	-
Gold Blend Coffee Beans	6	-	-
Tea			
a) Bagged tea			
Tetley	40	-	-
Quick Brew	39	-	-
Co-operative 99	48	1.2	4.8
Marks and Spencers extra strong	49	-	-
b) Leaf tea			
Typhoo	51	-	-
Co-operative 99	85	1.2	6.4
PG tips	193	-	-
China	38	-	-
c) Instant			
Nestea	60	-	-
d) Decaffeinated			
St James´	3	-	-
Luaka	4	-	-
Cola flavoured drinks			
Coca-cola	18	-	-
Pepsi-cola	13	-	-
Safeways	10	-	-
Chocolate drinks			
Cadbury´s drinking choc.		-	58
Sainsbury´s hot choc.	2	-	43
Sainsbury´s chocolate	8	-	209

Table 4.2 Methylxanthine levels of common beverages

Beverages were prepared using manufacturers recommended amounts ie. one teaspoonful of coffee per 200ml cup, one teabag per 200ml cup, and one teaspoonful of leaf tea per 200ml. Tea preparations were allowed to brew for 5 minutes before sampling

AGE GROUP	No. SUBJECTS	AVERAGE DAILY CONSUMPTION OF CAFFEINE (mg) COFFEE	TEA	CHOCOLATE	COLA	TOTAL
10 - 20	15	173.1 (233.3)	120.0 (141.2)	1.07 (2.90)	2.36 (6.46)	296.6 (267.7)
21 - 40	80	209.5 (152.0)	138.0 (144.6)	0.31 (1.22)	0.45 (1.41)	348.2 (176.7)
41 - 60	60	204.0 (188,7)	166.7 (143.2)	1.17 (2.82)	0.10 (0.77)	371.9 (192.8)
61 - 80	19	149.5 (150.0)	254.7 (179.0)	0.79 (1.87)	0	405.0 (165.1)
Total	174	198.4 (171.4)	159.1 (150.3)	0.72 (2.13)	0.47 (2.19)	358.7 (189.3)
Smokers	53	229.9 (212.8)	190.2 (183.5)			420.9 (248.3)
Non-smokers	121	184.0 (149.9)	145.7 (133.1)			329.3 (152.4)

Table 4.3 Average daily consumption of caffeine in the United Kingdom. Values are expressed as the mean caffeine intake per group. Figures in parenthesis represent one standard deviation

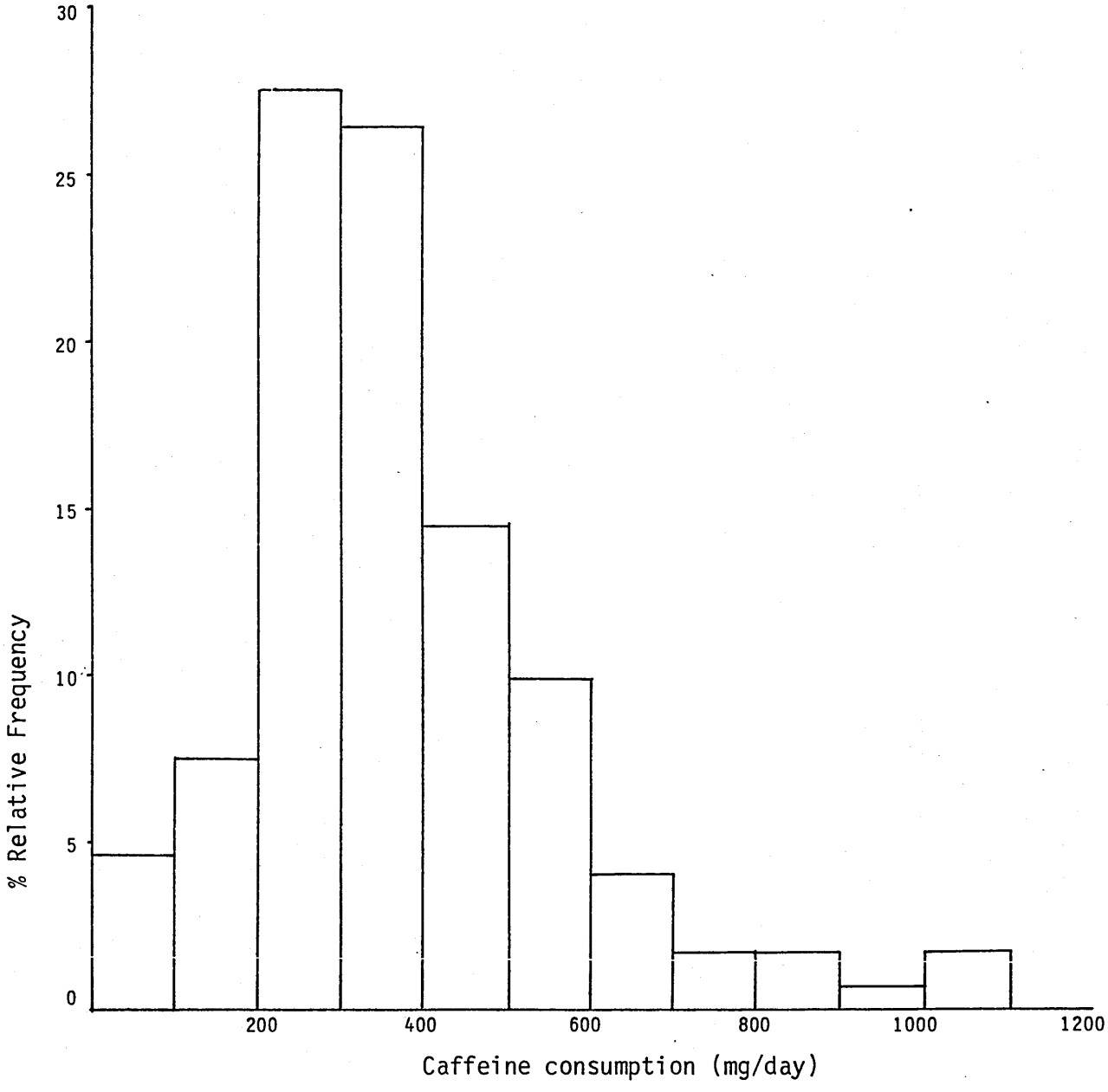


Fig 4.1 Dietary intake of caffeine in a U.K. population.

gram in Fig 4.1. Although there appeared to be a trend for increased dietary intake with increasing age, the difference in total caffeine intake between the highest (61-80 years) and lowest (10-20 years) consumption groups was not statistically significant ($P < 0.2$). However the increased consumption in smokers (421 ± SD. 248), compared to non-smokers (329 + SD. 152) was significant ($P < 0.01$).

In terms of mean estimates for the total population studied, coffee drinking was found to be the major source of dietary caffeine, accounting for 55% of per capita intake. The consumption of tea accounted for 44% whilst caffeine intake from chocolate and and cola flavoured drinks was minimal (less than 0.4%). The contributions of the various sources to the total caffeine intake were constant for all age groups, with the exception of subjects aged 61-80 years, where, caffeine intake from the consumption of tea (63%) was significantly higher than that from coffee (37%). The increased caffeine intake in smokers was not related to an increased consumption of a particular beverage since the ratio of coffee versus tea consumption was similar for both groups.

DISCUSSION

Previous surveys involving American and Canadian adult populations have reported mean daily caffeine intakes of 2.6 mg/kg (Market Research Corporation of America, 1977), 206 mg (Graham, 1978), 200 mg (Gilbert, 1981) and 345 mg (Nationwide Food Consumption Survey-NFCS, 1982). The mean caffeine intake for children of all ages from 5-18 years, on a body weight basis, has been estimated to be approximately 1 mg/kg (Morgan et al., 1982). However with the exception of reports by Galliano (1982) and Bruce et al. (1986), which reported respective mean values of 621 mg (SD = 210) in 20 adults and 426 mg (SD = 146) in nine adults, estimates of caffeine consumption in the U.K. based on large samples are not available.

Since the caffeine content of food products may vary considerably according to the type, source, "cup-size", method of brewing and plant variety, consumption estimates based solely on retrospective questionnaires have many limitations. Whilst a more accurate estimate may be made by concurrent analysis of the actual beverages consumed, this approach is technically difficult when dealing with a large population. However from the data obtained in this survey certain generalised observations can be made. The consumption estimates suggest that the caffeine intake in the U.K. is significantly higher than that reported for American or Canadian populations. Although the NFCS of America reported a similar mean total caffeine intake, the study only included

habitual consumers of caffeinated beverages. The indications that caffeine intake is higher in smokers than non-smokers is in agreement with the conclusions made by Istvan (1984) following a review of previous studies, which primarliy involved male subjects, of the inter-relationships between tobacco, alcohol and caffeine. The report also found a positive relationship between the consumption of alcohol and coffee although it was mainly limited to those reporting heavy use of either substance.

In agreement with reports from the USA and Canada, the data from this survey suggests that the combined consumption of coffee and tea account for the vast majority of per capita caffeine intake in the U.K. However whilst the proportion attributed to the consumption of coffee appears to be similar (appprox 60%), the consumption of tea is significantly higher (44% versus 15-30%) whilst caffeine intake from chocolate and cola flavoured drinks is lower. This was particular evident in the age group, 61-80 years, where the consumption of tea was the major dietary source of caffeine. Although the total caffeine intake, calculated on the basis of number of cups consumed, was marginally higher for this group, this may represent an overestimation since elderly people often prefer weaker brews of tea and coffee (unpublished observation). Since it has been suggested that coffee consumption may increase as the caffeine content decreases (Koslowski, 1976), it is possible that a similar relationship may exist for this age group with respect to tea.

5. PHARMACOKINETICS AND METABOLISM OF CAFFEINE IN NORMAL SUBJECTS

5. PHARMACOKINETICS AND METABOLISM OF CAFFEINE IN NORMAL SUBJECTS

5.1.Pharmacokinetics of caffeine in serum and saliva following oral administration.

The first reported study on the pharmacokinetics of caffeine was in 1953 when Axelrod and Reichenthal reported elimination half life values ranging from 2.5-4.5 hours in normal subjects following an oral dose of 7 mg/kg. However the present understanding of the pharmacokinetics of caffeine is mainly attributable to studies performed in the last decade. In 1978 Parsons and Neims compared the salivary elimination curves of caffeine in groups of healthy smokers (n=13) and non-smokers (n=13). The mean half-life in smokers (3.5 hr) was significantly less than that in smokers (6.0 hr) with corresponding clearance values of 155 ± 16 $ml.kg^{-1}hr^{-1}$ and 94 ± 18 $ml.kg^{-1}hr^{-1}$. Since no significant differences were found between the apparent volumes of distribution for the smoking (720 ± 67 $ml.kg^{-1}$) and non-smoking (610 ± 80 $ml.kg^{-1}$) groups, the increased clearance of caffeine in smokers was attributed to an induction of hepatic aryl hydrocarbon hydroxylase activity Additional studies on the half-life of caffeine have reported mean values of 4 hours in seven subjects (Cook et al., 1976) and 5.5 ± 2.6 hours in thirteen males following a 250 mg oral dose of caffeine (Patwardhan et al., 1980).

The effects of increasing doses (50-750 mg) on the plasma/ salivary pharmacokinetics of caffeine was investigated by Newton et al. (1981). The apparent first-order elimination

rate constant decreased linearly with dose whilst total body clearance was unaffected (mean value 0.98 ± 0.38 ml.kg^{-1} min^{-1}). There was a trend towards increasing apparent volume of distribution with increasing dose. A linear relationship was observed between the area under plasma concentration vs time curves and dose; dose and dose normalised plasma concentration vs time plots were superimposable suggesting that caffeine obeys linear pharmacokinetics over the dose range investigated. In 5 subjects kinetic data obtained following an oral dose of 300 mg on 3 occasions showed good reproducibility. The overall saliva/plasma concentration ratio was 0.74 ± 0.08.

The noninvasive nature of saliva sampling and the ability to obtain multiple samples without loss of blood or exposure of paediatric or geriatric patients to discomfort and potential skin irritation and infection makes it ideal for pharmacological investigations. In addition, for many drugs, concentrations in saliva reflect the free fraction of the drug, whereas data obtained from plasma concentrations represents both the free and protein-bound forms of the drug. In plasma, The ratio of free to protein-bound drug varies with age and disease state and may also be affected by alterations in albumin concentrations and the presence of compounds that can displace the drug from its binding site on albumin. As pharmacological effects are generally attributed to the free form of a drug, measurements of drug concentrations in saliva should therefore be, therapeutically more meaningful.

An earlier study in this thesis (Section 2.3.5) showed a favourable correlation between caffeine levels in serum and

saliva (R = 0.941). However, before saliva samples can be used with confidence in pharmacokinetic studies it is essential to establish that data obtained from saliva is a true reflection of serum kinetics. The reports by Parsons and Neims (1978) and Newton et al. (1981) provide only limited information to substantiate this relationship. In the following studies, the suitability of salivary caffeine determinations is further investigated by comparing pharmacokinetic data, derived from caffeine levels measured in samples of serum and saliva, collected simultaneously following oral doses of caffeine. In addition, an assessment is made of caffeine levels in a random non-pregnant population. Approval for this study was obtained from the Bromley Health District Ethics Committee.

5.1.1. Study 1.

Following an overnight abstention from all forms of caffeinated beverage, samples of serum and saliva were collected from 4 normal volunteers for estimation of basal caffeine levels. The subjects then received either 2 cups of strong coffee (equivalent to 180 mg of caffeine) or a gelatine capsule containing 200 mg anhydrous caffeine. Additional samples of serum and saliva were collected at 20 mins, 40 mins, 1 hr, 2 hr, 4 hr and 6 hr post caffeine dose. Blood samples were centifuged and samples of serum and saliva were stored at - 20° C until required for analysis. Prior to analysis, the thawed saliva samples were centrifuged at 2,000 rpm for approximately 5 minutes to remove mucoid particles. Following analysis by the previously described techniques,

serum and saliva caffeine concentrations were plotted semilogarithmically as a function of time and the slope of the descending curve (elimination constant,k) was calculated by least squares linear regression analysis. The concentration of caffeine at zero time (Co) was calculated by extrapolation of the regression line. It was assumed that the kinetic data conformed with first order kinetics of a one compartment model (Newton et al., 1982). The area under the plasma (AUC-p) and saliva (AUC-s) concentration/time curves from 0 to infinity were estimated by the trapezoidal method. Pharmacokinetic data was determined using the following equations: T1/2 = 0.693/k, apparent volume of distribution = dose/Co, and total plasma and saliva clearance (Cl) = dose/AUC. Student's t test was used for analysis of data.

Results

A summary of the pharmacokinetic data obtained from this study is shown in Table 5.1 (N1-N4). Additional data is also included from a volunteer (N13) who received an oral dose of 400 mg as described in study 2 of this section. The estimated values for caffeine in serum and saliva showed a linear agreement throughout. Least squares regression analysis of the levels for each subject showed a mean coefficient of correlation of 0.982 (SD = 0.005); the overall mean for the combined values for the 5 subjects was 0.965. The mean saliva:serum caffeine concentration ratio was 0.82 (SD = 0.10).

A comparison of pharmacokinetic data derived from serum caffeine determinations versus data obtained from salivary

Subject	Sex/Age	Wt. (kg)	Dose mg	Tl/2 (h)		k.el ($h.^{-1}$)		Vd ($l.kg^{-1}$)		AUC ($mg.l^{-1}h^{-1}$)		Cl ($ml.kg^{-1}min^{-1}$)	
				Ser	Sal	Ser	Sal	Ser	Sal	Ser	Sal	Ser	Sal
N1	F/26	60	180	5.7	4.9	0.12	0.14	0.44	0.48	43.5	44.1	1.15	1.14
N2	F/26	65	180	3.4	3.1	0.20	0.22	0.42	0.49	33.9	19.9	1.42	1.82
N3	M/32	70	200	3.7	4.5	0.19	0.15	0.37	0.43	48.5	44.1	0.98	1.08
N4	M/56	80	200	3.1	2.8	0.22	0.25	0.47	0.54	29.2	22.9	1.42	1.82
N13	M/42	82	400	4.1	4.8	0.17	0.14	0.54	0.63	53.7	58.3	1.51	1.39
Mean				4.00	4.02	0.175	0.170	0.448	0.514	41.8	37.8	1.296	1.450
S.D.				1.02	0.99	0.042	0.054	0.063	0.076	10.1	16.1	0.222	0.357
Significance (P)				<0.9		<0.9		<0.9		<0.25		<0.8	
Correlation (r)				0.760		0.755		0.982		0.974		0.759	

Table 5.1 Pharmacokinetics of caffeine in serum and saliva following oral administration to normal healthy volunteers.

Pharmacokinetic data was calculated using least squares regression analysis. Levels of significance (P) for values in serum vs saliva were calculated using a paired Student's t-test. Linear regression analysis was used to obtain coefficient of correlation (r).

Abbreviations: Tl/2 - half-life, k.el. - elimination constant, Vd - apparent volume of distribution, AUC - area under elimination time curve, Cl - clearance

measurements showed good correlation. Respective coefficient of correlation values for elimination half life, elimination constant, volume of distribution, area under curve and clearance were 0.760, 0.755, 0.982, 0.974 and 0.759. The mean values for these parameters were similar for both serum and saliva. Whilst values for apparent volume of distribution were consistently higher in saliva than in serum, an analysis of the results using a paired Student's t test showed that the difference was not statistically significant ($P < 0.9$).

5.1.2. Study 2.

Whilst the number of subjects involved in Study 1 was too small for an accurate statistical evaluation, the correlation between kinetic data in serum and saliva was considered sufficiently acceptable to permit further kinetic studies to be performed using saliva samples only. In order to provide reference ranges of pharmacokinetic data for future studies, additional groups of volunteers were investigated. The study was performed using the same conditions as for Study 1 with the exception that the administered oral dose consisted of either 200 mg or 400 mg of anhydrous caffeine contained in a gelatine capsule. For subjects N5-N12, saliva samples were collected at times 0, 0.5, 1, 1.5, 2, 2.5, 4, 6, 8, 10 and 12 hours following oral caffeine whilst for the subjects N13-N21 samples were collected at times 0, 0.5, 1, 2, 3, 4, 6, 16, 16 and 24 hours. Samples were stored at -20° C until required for analysis. Salivary caffeine determinations were made using the previously described techniques and, for subjects N13-N21,

measurements were also made of the dimethylxanthine metabolites of caffeine (theobromine, theophylline and paraxanthine). For subjects N5-N12, a comparison was made of pharmacokinetic data obtained by linear regression analysis with data obtained from a computer based non-linear kinetics programme. The computer system consisted of an intelligent user interface linked to the "NONLIN" programme supplied by Upjohn Ltd. Using this system, a polyexponential equation is used to fit concentration-time data by a weighted iterative non-linear least-squares regression algorithm.

Results

A summary of the subject characteristics, caffeine doses and kinetic parameters, calculated from salivary caffeine determinations, is shown in Table 5.2. Only one subject (N12) was a smoker and the volunteers were all free from any form of medication, including oral contraceptives.

The mean values for elimination half life (4.35 ± SD 1.95), elimination constant (0.185 ± SD 0.07) and total salivary clearance (1.605 ± SD 0.555) were in agreement with those previously reported by Parsons and Neims (1978) for a non-smoking population. A wide variation was found for elimination half life values (2.6-9.1 hours). For one subject (N12) the enhanced clearance could be attributed to the inducing effects of smoking on the microsomal enzyme, aryl hydrocarbon hydroxylase, whilst in contrast, the impaired clearance observed for subject N7 may possibly have been related to an habitual abstinence in the consumption of caffeinated beverages.

A statistical comparison, using Student's t-test, of the

Subject	Sex/Age	Wt. (kg)	Dose mg	T1/2 (h)	k.el (h^{-1})	Vd ($l.kg^{-1}$)	AUC ($mg.l^{-1}h^{-1}$)	Cl ($ml.kg^{-1}min^{-1}$)
N5	M/29	67	200	2.7 (4.1)	0.26 (0.17)	0.35 (0.30)	40.1	1.26
N6	M/56	76	200	3.2 (3.1)	0.22 (0.22)	0.57 (0.56)	19.2	2.48
N7	F/22	58	200	9.1 (12.0)	0.08 (0.06)	0.62 (0.73)	65.7	0.85
N8	F/24	65	200	3.8 (3.8)	0.18 (0.18)	0.55 (0.55)	31.2	1.72
N9	F/34	60	400	2.9 (3.7)	0.24 (0.19)	0.50 (0.55)	58.0	1.92
N10	M/32	73	400	3.5 (3.3)	0.20 (0.21)	0.55 (0.50)	45.8	1.99
N11	M/24	80	400	5.0 (5.1)	0.14 (0.14)	0.74 (0.74)	47.7	1.75
N12	M/20	73	400	2.6 (2.6)	0.27 (0.27)	0.49 (0.55)	35.1	2.60
N13	M/42	82	400	4.8	0.14	0.63	58.3	1.39
N14	M/35	75	400	8.1	0.09	0.55	90.0	0.99
N15	F/30	46	400	2.1	0.33	0.63	89.3	1.62
N16	F/39	60	400	5.8	0.12	0.42	81.5	1.35
N17	M/41	70	400	4.1	0.14	0.38	59.1	1.61
N18	M/42	80	400	3.8	0.18	0.38	73.1	1.54
N19	M/35	69	400	3.9	0.18	0.41	112.6	0.87
N20	M/22	71	400	5.9	0.12	0.23	103.4	0.91
N21	F/38	67	400	2.6	0.27	0.38	40.9	2.43
Mean				4.35	0.185	0.502		1.605
S.D.				1.95	0.070	0.129		0.555

Table 5.2 Salivary pharmacokinetics of caffeine in healthy normal subjects calculated using non-linear kinetics.

For comparison, corresponding values calculated using least squares regression analysis are shown in parenthesis. Refer to Fig 5.1 for key to abbreviations.

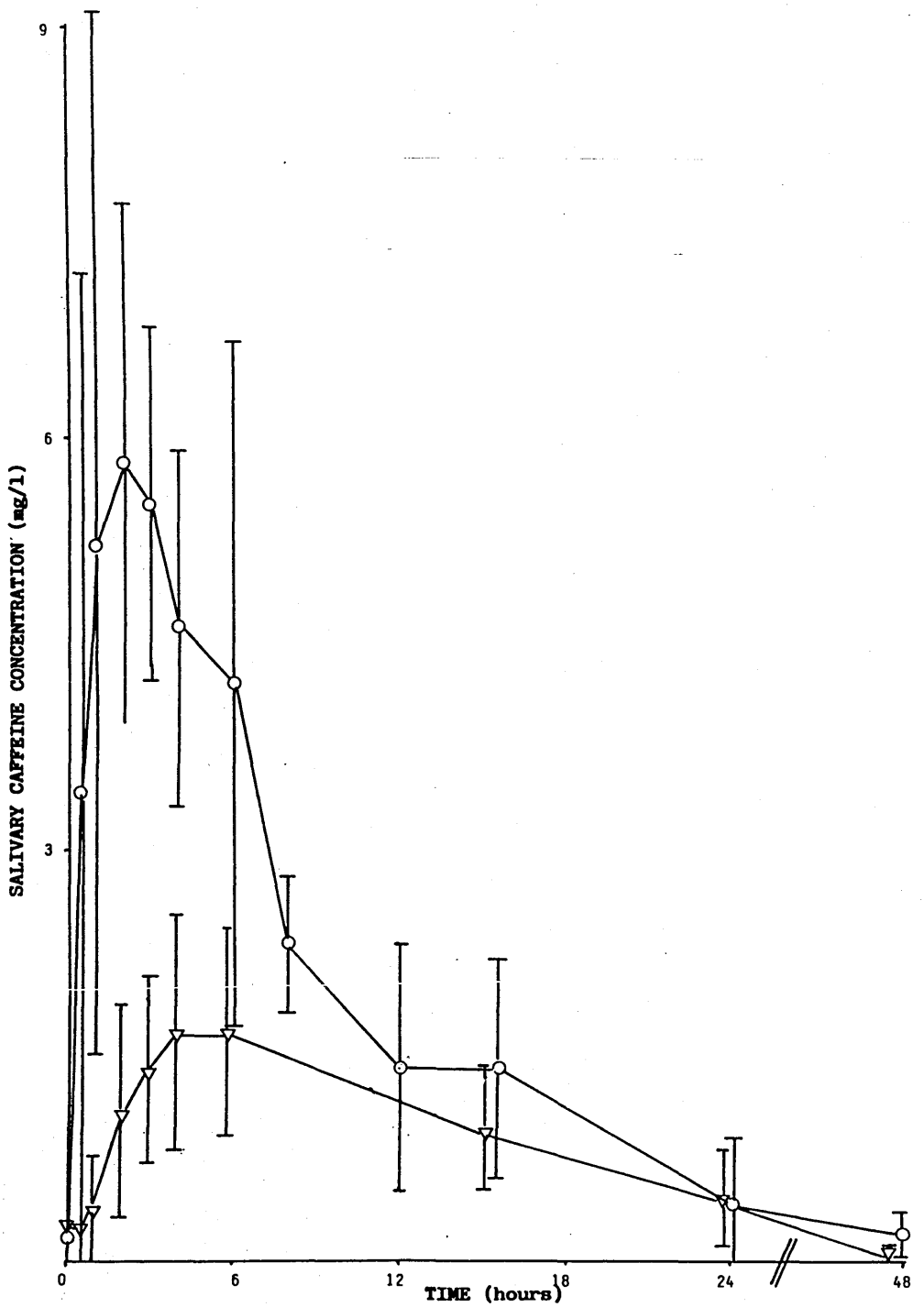


Fig 5.1 Mean caffeine (○) and 1,7-dimethylxanthine (▽) levels in saliva following oral dose of caffeine (400mg).
Vertical line represents one standard deviation.

kinetic data calculated using linear vs non-linear kinetics showed no significant differences between values for elimination constant ($P < 0.9$) and volume of distribution ($P < 0.9$). Whilst the values for elimination half life were statistically different ($P < 0.001$) this was mainly attributable to the wide variation observed in values for subject N7.

A plot of the mean salivary levels of caffeine and paraxanthine for subjects N13-N21 is shown in Fig 5.1. Peak levels of paraxanthine (mean = 1.9 mg/l ± SD. 0.7) were obtained 5.4 hours (SD = 2.87) following oral caffeine administration (400 mg). The levels of theobromine and theophylline were similar throughout to those found in basal samples (less than 1 mg/l).

5.2.Measurement of caffeine levels in a non-pregnant population

In order to provide a "reference range" for caffeine levels in a normal non-pregnant population, serum caffeine determinations were made on a series of samples obtained at random from both normal volunteers and patients attending hospital out-patient clinics (n = 202). Since some of the symptoms of chronic anxiety states and thyrotoxicosis mimic those associated with caffeine toxicity eg. tachycardia, anxiety, nervousness, tachypnoea and gastrointestinal disturbances, the relationship between circulating caffeine levels and the occurence of these symptoms was investigated in a group of congruent patients.

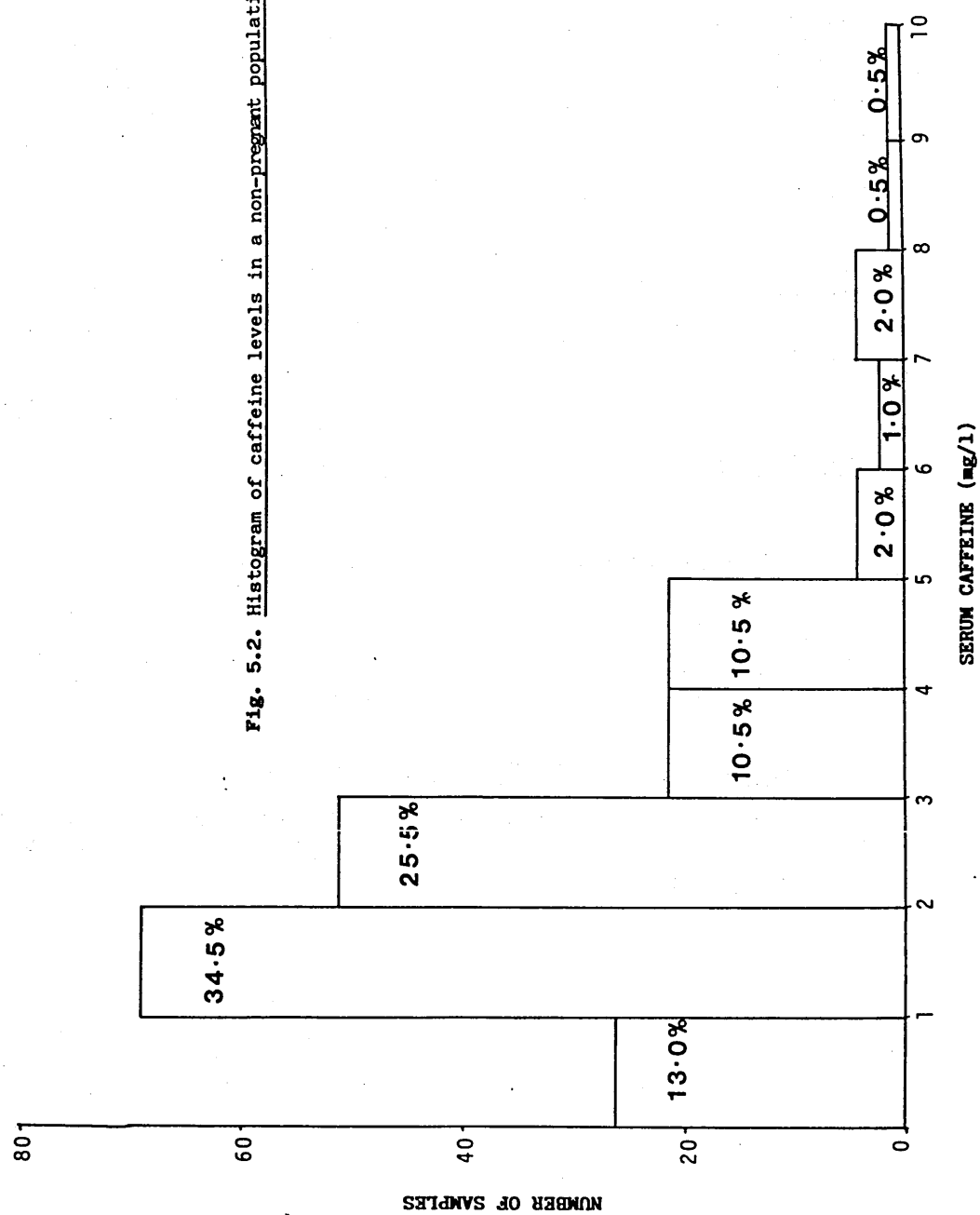


Fig. 5.2. Histogram of caffeine levels in a non-pregnant population

Results

A summary of the caffeine levels, determined in samples of serum obtained from the participants of this study, is shown in Fig. 5.2. The overall mean caffeine level was 2.42 (SD = 1.59) with values ranging from 0 to 9.2 mg/l. In agreement with the findings of previous report by Smith et al., (1982), 6% of the population studied showed caffeine levels in excess of 5 mg/l.

The mean serum caffeine level for 72 patients with symptoms related to caffeine toxicity was 2.44 mg/l (SD = 1.72); a result similar to that found for the total population studied. Significantly elevated levels of 9.2 and 8.2 mg/l were found in two subjects. Although requests were made to obtain repeat samples, and information on their normal dietary caffeine intake, a return was only received for the former subject. The "follow-up" serum caffeine level on this patient was 3.9 mg/l with an estimated daily caffeine intake of 320 mg.

5.3.Urine metabolite profiles of caffeine in normal subjects

On commencing this study in 1981, literature reports on the metabolism of caffeine were limited to those described by Brodie et al. (1956), Arnaud and Welsch (1980) and Callahan et al. (1980) which have been summarised in Section 1.2. By administering radiolabelled caffeine to healthy normal sublects, the latter two studies clearly established that the metabolic fate of caffeine primarily involves demethylation and oxidation in the liver before excretion by the kidneys; unchanged caffeine accounted for only 1-3% of the administered dose. However analytical limitations prevented the resolution

of 7-methylxanthine from 1-methyluric acid, 1,7-dimethylxanthine from theophylline, and 3,7-dimethyluric acid from 1-methylxanthine.

In this study, the more specific methodology described in Section 3 was used to evaluate the rate of appearance and recovery of caffeine metabolites in urine samples collected from normal healthy subjects following oral administration of unlabelled caffeine. Cummulative data was then used to establish a reference range for metabolite excretion in subjects receiving a single bolus ingestion.

5.3.1. Study 1

Four healthy male subjects, aged 20-55 years, were enrolled for this study after a careful explanation of its requirements. The subjects were all non-smokers and receiving no prescibed or non-prescribed forms of medication. Following a 24 hour period of abstention from all forms of caffeinated beverages and chocolate, urine samples were collected to establish basal levels of caffeine and its metabolites. An oral dose of anhydrous caffeine (200 or 400 mg) contained in a gelatine capsule was then administered to each subject with approximately 200 ml of water. Further urine samples were then collected for periods of 0-2 hr, 2-4 hr, 4-6 hr, 6-8 hr, 8-10 hr, 10-12 hr, and 12-24 hr. The volumes of the urine samples were recorded and aliquots stored at -20° C until assayed using HPLC. Prior to analysis the samples were diluted 1:10 with the internal standard (Proxyphylline, 10 mg/l) and then injected directly onto the column without further preparation.

	METHYLXANTHINE EXCRETION (% administered dose)												
	7MU	7MX	1MU	3MX	1MX	13DU	37MX	17DU	17DX	13MU	137MU	137MX	Total
0-2 hr													
Mean	0.24	0.51	0.67	0.25	0.27	0.14	0.28	0.22	0.20	0.04	0.14	0.19	3.15
S.D.	0.09	0.21	0.29	0.10	0.36	0.13	0.18	0.11	0.11	0.07	0.11	0.20	0.96
2-4 hr													
Mean	0.20	0.38	1.05	0.21	0.78	0.14	0.19	0.75	0.29	0.05	0.12	0.22	4.36
S.D.	0.09	0.13	0.38	0.07	0.16	0.06	0.06	0.47	0.14	0.07	0.11	0.19	0.71
4-6 hr													
Mean	0.22	0.44	1.69	0.23	1.17	0.19	0.24	1.08	0.42	0.03	0.12	0.17	6.0
S.D.	0.12	0.21	0.99	0.09	0.53	0.13	0.19	0.77	0.16	0.05	0.11	0.11	2.71
6-8 hr													
Mean	0.26	0.56	1.91	0.31	1.58	0.30	0.29	1.40	0.47	0.04	0.12	0.13	7.36
S.D.	0.14	0.15	0.26	0.09	0.31	0.15	0.15	0.66	0.31	0.06	0.06	0.06	1.40
8-10 hr													
Mean	0.28	0.56	2.45	0.31	1.49	0.31	0.30	1.16	0.40	0.06	0.06	0.06	7.41
S.D.	0.16	0.18	1.13	0.09	0.31	0.16	0.22	0.49	0.15	0.04	0.03	0.04	1.99
10-12 hr													
Mean	0.26	0.52	2.13	0.29	1.28	0.23	0.21	1.09	0.40	0	0.04	0.04	6.46
S.D.	0.13	0.16	0.75	0.10	0.17	0.07	0.09	0.58	0.29	0	0.06	0.05	0.84
12-24 hr													
Mean	0.97	1.99	9.04	1.13	4.50	0.84	1.06	3.50	1.23	0	0.20	0.16	24.31
S.D.	0.43	0.87	1.87	0.57	1.95	0.40	0.59	2.33	1.11	0	0.26	0.20	5.06
0-24 hr													
Mean	2.43	4.94	18.98	2.72	11.09	1.98	2.24	6.88	3.37	0.20	0.79	0.89	56.50
S.D.	0.54	1.00	4.92	0.41	1.24	0.68	0.95	0.51	1.87	0.21	0.62	0.41	4.19

Table 5.3 Mean metabolite excretion following an oral dose of caffeine (200 or 400 mg)

For key to abbreviations refer to page 3

Results

The urinary recoveries of caffeine metabolites for the specified time intervals are summarised in Table 5.3. Despite a variation in the administered dose, the total recovery for the 24 hr period was similar for each subject (mean value 56.5% ± SD 4.2). These values are compared in Table 5.4 with data obtained from the previous studies of Arnaud and Welsch (1981) and Callahan et al. (1982).

The major metabolites excreted in the urine during the 24 hr period were 1-methyluric acid (18.98% ± SD 4.92), 1-methyl xanthine (11.09% ± SD 1.24) and 1,7-dimethylxanthine (3.37% ± SD 1.87). Unchanged caffeine accounted for only 0.89% of the administered dose. The overall mean value of metabolites present in the basal urine was 5.49 mg/l with values ranging from 0.93 mg/l for theophylline to 13.7 mg/l for 1-methyluric acid. Whilst significant amounts of metabolite were recovered during the time interval 12-24 hr, the periods of maximal output were 2-4 hr for caffeine; 6-8 hr for 1-methylxanthine, 7-methylxanthine and 1,7-dimethyluric acid; and 8-10 hr for 1-methyluric acid.

5.3.2. Study 2

A group of normal volunteers consisting of 4 male subjects (aged 26-42 yrs) and 5 female volunteers (aged 24-28 yrs) were enrolled for this study after explanation of the protocol. All subjects were non-smokers and receiving no medication with the exception of 2 females who were receiving oral contraceptives. Since the subjects were all habitual consumers of caffeine (mean daily intake = 280 mg) they were requested to abstain

	CAFFEINE METABOLITE EXCRETION (% OF ORAL DOSE ADMINISTERED)												
	7MU	7MX	1MU	3MX	1MX	13U	37X	17U	17X	13X	137U	137X	Total
N.S.													
0-24 hr	1.0	3.2	16.7	1.3	13.0	0.8	0.5	7.8	6.2	0.7	1.5	1.1	53.8
24-48 hr	1.0	2.2	10.6	1.8	5.2	1.3	1.5	3.6	1.9	0.6	0.2	0.4	30.3
A.A.													
0-24 hr	2.0	2.4	8.6	1.4	5.5	1.8	1.5	5.2	3.8	1.3	2.2	4.4	40.1
24-48 hr	0	0	1.7	0.6	1.0	0.6	0.5	0.4	0	0	0	0	4.7
G.M.													
0-24 hr	0.4	1.9	17.2	0	9.3	1.3	2.3	7.0	3.3	0.3	0.6	1.3	45.1
24-48 hr	0.4	1.2	3.0	0.3	1.5	0.6	0.6	0.8	0.3	0	0	0.3	8.9
M.H.													
0-24 hr	0.3	3.1	19.0	1.1	10.8	0.7	1.4	5.7	4.5	0.4	1.2	3.9	52.0
24-48 hr	0.3	1.6	5.9	0.7	1.1	0.3	0.7	1.0	0	0	0	0	11.5
Me.H.													
0-24 hr	0.6	2.4	14.8	1.2	3.8	0.8	2.0	5.4	4.7	0	0	1.7	37.3
24-48 hr	0	1.2	4.1	1.3	2.5	1.1	0.6	1.7	0.4	0.4	0	0	13.3
F.L.													
0-24 hr	0.9	1.8	12.0	0.8	11.6	0.8	1.7	6.9	1.2	0.3	0.7	1.8	40.3
24-48 hr	0.9	0	4.2	1.0	1.8	1.0	1.3	1.9	0.8	0.5	0	0	12.5
M.P.													
0-24 hr	1.0	1.1	30.3	1.1	15.8	0.7	2.9	6.7	2.4	0.2	0.4	0.5	62.7
24-48 hr	0.3	1.2	3.0	2.1	1.4	0	0.2	0	0	0	0.4	0	8.5
B.P.													
0-24 hr	1.4	2.5	13.2	1.1	3.2	0.6	1.5	4.5	2.5	0.7	0.3	1.0	32.2
W.B.													
0-24 hr	0.5	2.0	6.3	0.6	4.0	0.6	1.1	3.9	1.7	0.3	0.3	0.5	21.8
24-48 hr	0.2	1.8	1.9	0.6	1.7	0.3	0.4	0.9	0.6	0	0.1	0.1	8.6
Mean metabolite excretion													
0-24 hr	0.9	2.3	15.3	1.0	8.6	0.9	1.7	5.9	3.4	0.5	0.8	1.8	42.8
S.D.	0.5	0.7	6.9	0.4	4.6	0.4	0.7	1.3	1.6	0.4	0.7	1.4	12.3
0-48 hr	1.2	3.4	19.9	2.0	11.3	1.6	2.4	7.4	4.0	0.6	1.0	2.0	56.5
S.D.	0.6	1.2	8.5	1.0	4.9	0.6	0.6	2.0	2.0	0.5	0.7	1.4	16.4

Table 5.4 Methylxanthine metabolite excretion of normal volunteers following an oral dose of caffeine (400 mg)

For key to abbreviations refer to page 3

METABOLITE	METABOLITE EXCRETION (% DOSE ADMINISTERED)			
	Arnaud + Welsch[1] (1981)	Callahan[2] (1982)	Study A[3] (Re: page 113)	Study B[4] (Re: page 265)
1-methyluric acid	11.7	14.8	19.9	19.7
1-methylxanthine	11.7	18.1	11.3	11.6
1,7-dimethyluric acid	9.0	5.4	7.4	7.3
7-methylxanthine	6.4	9.9	3.4	3.8
1,7-dimethylxanthine	6.0	5.9	4.0	4.9
3,7-dimethylxanthine	2.6	1.9	2.4	1.8
3-methylxanthine	2.6	4.1	2.0	2.7
1,3-dimethyluric acid	2.6	1.8	1.6	1.6
1,3-dimethylxanthine	0.7	0.9	1.6	0.7
7-methyluric acid	*	*	1.2	1.5
1,3,7-trimethyluric acid	2.0	1.3	1.0	0.6
1,3,7-trimethylxanthine	1.0	1.2	0.8	2.1

Table 5.5 Summary of studies on caffeine metabolism in man

1. Total metabolite excretion for 48 hour period following an oral ingestion of unlabelled caffeine (8 mg/kg).

2. Total metabolite excretion for 48 hour period following an oral dose of [2- ^{14}C]-caffeine (5 mg/kg).

3. Total metabolite excretion for 48 hour period following an oral dose of unlabelled caffeine (400 mg).

4. Mean metabolite excretion of two consecutive 24 hour periods for subjects receiving a controlled dietary intake of caffeine (300-750 mg/24h).

from all forms of dietary caffeine for a 24 period before the study and for its duration. On day 1 of the investigation, a 400 mg bolus dose of anhydrous caffeine, contained in a gelatine capsule, was administered orally to each subject. Total urine outputs were then collected for the time intervals 0-24 hr and 24-48 hr; urine volumes were recorded and aliquots stored at -20° C until required for analysis. Methylxanthine metabolite levels were determined by HPLC using the previously described ion-pair extraction procedure.

Results

The urinary methylxanthine metabolite profiles for each subject invstigated are summarized in Table 5.4 and Fig 5.5. The overall mean recovery of the administered dose for the 48 hour period of urine collection was 56.5% (SD = 16.4). In agreement with the results of study 1, the principal urinary products of caffeine were shown to be 1-methyluric acid (19.9 ± SD 8.5), 1-methylxanthine (11.3 ± SD 4.9) and 1,7-dimethyl-uric acid (7.4 ± SD 2.0); unchanged caffeine accounted for only 2% (± SD 1.4) of the administered dose. Although the mean total metabolite recovery for the first 24 hour period was lower than the mean value found for a similar period in study 1 (56.5 ± SD 4.19 vs 42.8 ± 12.3), the difference was not statistically significant. Since the total recovery values for study 1 were obtained using combined data from serial urine collections, assayed using the less specific direct injection technique, the difference probably reflects a cummulative blank effect.

The metabolite profiles showed no variations related to the

individuals sex or the concurrent use of oral contraceptives. For one subject (U8), the total metabolite recoveries were consistently low, although the pattern of distribution was unaltered. Whilst this low recovery may be explained in terms of incomplete urine collection, this was considered unlikely since the subject was familiar to research techniques.

The results of this study are in agreement with two previous reports (Arnaud and Welcsh, 1981; Callahan et al., 1982) of metabolite recoveries obtained following administration of radiolabelled caffeine. The lower recovery of 7-methylxanthine in the present study, with a corresponding increase in the recovery of 1-methyluric acid, is probably explained by the improved specificity of the HPLC procedure in terms of its ability to resolve 7-methylxanthine from 1-methyl uric acid. A similar explanation may be made for the variation in recovery values for 1,7-dimethylxanthine and theophylline, which were also poorly resolved in the HPLC procedures used for the earlier studies.

DISCUSSION

The primary objective of the studies described in this section has been to establish suitable reference ranges, using the previously developed analytical procedures, for the pharmacokinetic and metabolic aspects of caffeine metabolism in normal healthy volunteers. The data obtained agrees favourably with results of previous studies thus confirming its reliability for use in future studies on the disposition of caffeine.

The non-invasive nature of saliva sampling and the ability to obtain multiple samples prompted further investigation into the suitability of saliva measurements for obtaining pharmacokinetic data. The close correlation between caffeine levels in serum and saliva confirms that salivary determinations may be used to accurately reflect the concentration of caffeine in serum. Thus the same pharamacokinetic principles applied to blood can be used when salivary drug concentrations are measured. This concept is supported by a recent study by Zylber-Katz et al. (1984) which reports a similar ratio (0.79 ± SD 0.02) and degree of correlation for caffeine levels in saliva versus plasma. For comparison, the mean saliva: plasma caffeine ratios in seven neonates (Khanna et al., 1980) and in six healthy nursing mothers (Hildenbrandt et al., 1983) have been reported as 0.71 and 0.73 respectively.

The transfer of drugs from plasma to saliva is mainly a passive, pH dependent process; drug concentrations in saliva generally representing the free fraction of the drug. Since pharmacological effects are produced only by the free form of the drug, salivary concentrations are more meaningful. For acidic drugs with pKa values greater than 8.5 and basic drugs with pKa values of 5.5 or less no correction is required for the salivary pH. However, since caffeine is a weak base (pKa 14), there is a tendancy for it to accumulate in saliva in accordance with the pH partition theory. Evidence for this has been shown by Zylber-Katz et al. (1984) who demonstrated that the actual ratio for salivary caffeine concentration versus the free fraction in serum was 1.47 (SD = 0.05).

Determination of the fraction of drug bound to plasma proteins (Fb) may be obtained using the following equation described by Dvorchik and Vesell (1976).

$$Fb = 1-K\frac{[Saliva]}{[Plasma]}$$

where K is a proportionality factor which takes into account plasma and salivary pH as well the pKa of the drug (Martin et al. 1974).

$$\text{For bases, } K = 1+10^{-(pHp-pKa)} \; / 1+10^{-(pHs-pKa)}$$

For caffeine the calculated values for K at salivary pH 6.5 and 7.0 are 0.126 and 0.399 respectively.

The urine metabolite data derived from this study shows that the majority of the metabolites of caffeine are recovered within the first 24 hours after dosing. The distribution of metabolites is in agreement with the previous reports by Arnaud and Welsch (1981) and Callahan et al. (1982) which indicates that the metabolic fate of caffeine is primarily demethylation and oxidation in the liver with unchanged caffeine accounting for only 1-3% of an administered dose. In man, the increasing stability of the methyl groups is 3-N, 1-N, and 7-N and although the initial demethylation may occur at any of these three positions, yielding formaldehyde as a secondary product, the reported quantitative importance of the reactions are 3-N demethylation, 72%; 1-N demethylation, 20%; and 7-N demethyl- ation, 8% (Arnaud and Welsch, 1982). Subsequently the majority of administered caffeine undergoes 3-N

demethylation to produce 1,7-dimethylxanthine, the primary metabolite found in serum. The major metabolites excreted in urine are 1-methyl- uric acid, 1-methylxanthine and 1,7-dimethylxanthine.

Since the completion of these studies, further reports on the biotransformation of caffeine have been made by Callahan et al. (1983), Branfman et al. (1983), Tang et al. 1983, and Blanchard et al. (1985). Data form these studies agree favourably with the metabolite recovery values found in this report. Whilst it has not been possible in this study to measure the acetylated uracil metabolites of caffeine, such as 5-acetylamino-6-amino- 3-methyluracil (AAMU) and 5-acetylamino -6-formylamino-3-methyl uracil (AFMU), it is probable that the unaccounted metabolites for the normal volunteers (ca. 44%) represents the formation of such compounds.

Symptoms associated with the withdrawl of caffeine were experienced by many of the volunteers participating in these studies. The reported effects included headaches, drowsiness, lethargy and severe muscle aching in the thighs and legs. In agreement with previous reports by Driesbach and Pfieffer (1943) and Greden et al. (1980), the most commonly reported effect to the abstention of caffeine was a withdrawl headache, mainly occuring after a period of approximately 12 hours. In all cases the withdrawl effects were eliminated by the ingestion of caffeine. Adverse effects associated with the intake of caffeine included mild tachycardia, light headiness and insomnia.

Although it has been reported that anxiety states may in

some cases be attributed to "caffeinism" (Greden, 1974; Greden et al., 1978; Uhde et al., 1984), no relationship could be found in this study between serum caffeine levels and the presence of symptoms associated with caffeine toxicity. However recent reports by Boulenger et al. (1984) and Lee et al. (1985) have suggested that anxiety disorder patients may have an increased sensitivity to caffeine which leads to a decrease in consumption. Since this in turn would lead to a reduction in circulating caffeine levels, it can be concluded that the random monitoring of caffeine levels in serum or saliva is of minimal clinical value in detecting caffeine related anxiety states.

6. THE ELECTROPHYSIOLOGICAL AND PSYCHOPHARMACOLOGICAL EFFECTS OF CAFFEINE IN NORMAL HEALTHY HUMANS

6. THE ELECTROPHYSIOLOGICAL AND PSYCHOPHARMACOLOGICAL EFFECTS OF CAFFEINE IN NORMAL HEALTHY HUMANS

INTRODUCTION

The pharmacological and physiological effects of caffeine in normal subjects have been reviewed in Section 1. Reports on the acute effects of caffeine (Robertson et al., 1978, 1981) have shown that following an oral dose of caffeine (250 mg) systolic blood pressure is significantly increased, plasma adrenaline levels are doubled and nor-adrenaline levels may be increased by 75%. A small, but significant decrease in heart rate has also been observed. Levels of dopamine and unconjugated 3-methoxy-4-hydroxy-phenyl-ethyleneglycol (MHPG) are reported to be unaltered (Robertson et al., 1981; Charney et al., 1984). The ability to develop tolerance to the humoral and haemodynamic effects of caffeine is well established (Robertson et al., 1984; Ammon et al., 1983). Whilst significant increases in plasma cortisol levels have been recorded in subjects receiving large doses (750 mg) of caffeine (Ühde et al., 1983), such effects are probably behavioral since the neuroendocrine reponse to caffeine is reported to be minimal (Spindel et al., 1984).

The ability of caffeine to improve both motor and mental efficiency was first demonstrated in 1912 by Hollingworth. The administration of 150-250 mg of caffeine increases alertness, stimulates attention and restores performances degraded by factors such as fatigue and boredom (Weiss and Laties, 1962; Goldstein, 1965). At higher doses caffeine produces nervous-

ness, restlessness, insomnia, tremors, hyperesthesia, tachypnoea, tachycardia and gastrointestinal disturbances (Greden, 1974; Gilbert, 1976). Withdrawl of caffeine in habitual users may produce severe headaches, nausea, vomiting, fatique, mental depression and rhinorrhoea (Dreisbach and Pfeiffer, 1943).

Clinically, the symptoms associated with excessive caffeine consumption ("caffeinism") are difficult to distinquish from those found in anxiety disorders (Greden, 1974). However, whilst there is a strong positive correlation between caffeine consumption and scores of trait-anxiety, as a group, patients with anxiety disorders consume no more caffeine than controls (Boulenger and Uhde, 1982). This suggests that an increased sensitivity to the effects of caffeine may exist in anxious patients through altered pharmacokinetic (Levy and Zylber-Katz, 1983) and/or pharmacodynamic responses.

The aim of this study was to establish baseline pharmacokinetic and psychopharmacological values for responses to single doses of caffeine in healthy human subjects. In collaboration with Professor M. Lader and Dr M. Bruce of the Institute of Psychiatry, Denmark Hill, London, the effects of caffeine (250 mg or 500 mg) and placebo on physiological, psychological measures and subjective feelings were studied in a double blind, cross over study in normal healthy volunteers. Each subject was tested on three different occasions, at least one week apart. The psychophysiological test sequences were performed by Dr M. Bruce using where possible an on line PDP-12A computer for the rapid and accurate aquisition,

storage and analysis of data.

METHODS

Approval for this investigation was obtained from the Ethics Committee (Research) of the Institute of Psychiatry. Nine normal healthy adult volunteers, 4 males and 5 females, were enrolled for this study after a careful explanation of the protocol. All were habitual caffeine users; the mean daily intake as assessed by retrospective questionnaire was 428 mg (SD = 146), range 230 - 670 mg. Instructions were given to abstain from caffeine and alcohol for a 24 hour period prior to each test day and to have a light breakfast.

On arrival in the laboratory at about 12 noon, a cannula was inserted into a forearm vein and kept patent with heparin. Electroencephalogram (EEG) scalp electrodes were applied and electrodes were also attached to the forearm and thumb to measure skin conductance. Following the collection of a basal blood sample, the subjects were asked to provide a sample of urine and then collect all subsequent urine passed for the 5 hour duration of the investigation. A pre-drug test sequence was then performed before administering a capsule containing either 250 mg or 500 mg of caffeine or placebo in matching capsules, using a randomised design. Test sequences were repeated at 1,3 and 5 hours later, details of which are summarized in Appendix 3. At hourly intervals throughout the test period, 5 ml samples of venous blood were collected and centrifuged with minimum delay. Total urine volumes were recorded and aliquots (30 ml) were stored along with the samples of plasma at -20° C for later analysis of caffeine and

its metabolites by HPLC.

An appraisal of the psychophysiological data was made by calculating a four-way analysis of variance, the main sources of variance being subjects, drugs, occasions and times. Differences between drugs were obtained from the drugs x times interaction. Differences between means were assessed using the 0.05 critical difference.

RESULTS

a) Plasma levels of caffeine and its dimethylxanthine metabolites

The absence of methylxanthines in pre-drug blood samples confirmed that each subject was adequately decaffeinated. Following the oral administration of caffeine, plasma levels of the drug rose rapidly producing peak values at 1-2 hours (Fig 6.1). The levels of theobromine and theophylline showed only a minimal increase over basal levels whilst plasma concentrations of paraxanthine rose steadily to the 5 hour point. Pharmacokinetic data, calculated using a computer based non-linear kinetics programme is summarized in Table 6.1. Whilst precise calculation of pharmacokinetic parameters was impeded by the limited number of sample points, the overall mean values for half-life (4.62 + SD = 2.17), elimination constant (0.193 + SD = 0.08) and volume of distribution (29.3 + SD = 5.55) were similar to those reported in Section 5. for saliva samples obtained from normal volunteers. The close correlation between values obtained following 250 mg versus

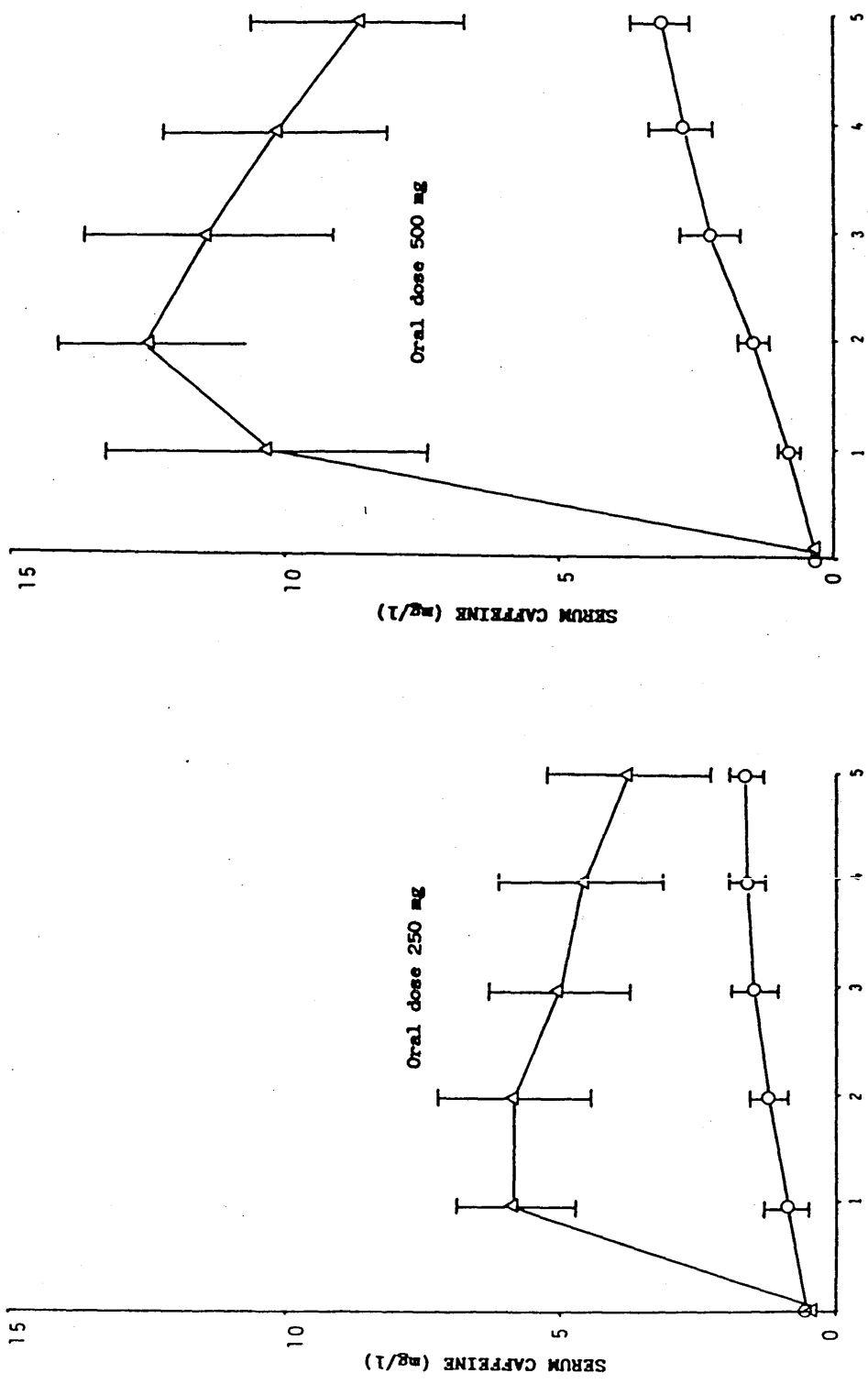


Fig 6.1 Mean serum caffeine (▽) and 1,7-dimethylxanthine (○) levels following the oral ingestion of caffeine in 9 subjects.

Subject	C max mg/l		C max Time (h)		T1/2 (h)		k.el ($h.^{-1}$)		aVd (l)		AUC (0-∞) ($mg.l^{-1}h^{-1}$)	
	250	500	250	500	250	500	250	500	250	500	250	500
S1	7.6	14.1	2	3	*	8.0	*	0.09	*	29.8	*	200
S2	6.6	13.6	2	2	8.2	*	0.09	*	31.3	*	90.5	*
S3	7.0	13.7	2	2	2.2	*	0.32	*	23.2	*	39.9	*
S4	4.9	10.4	1	2	3.1	3.6	0.22	0.19	31.5	38.5	30.2	149.4
S5	4.7	12.4	1	2	2.4	3.2	0.28	0.22	31.2	20.0	23.2	86.9
S6	6.8	13.5	1	2	4.8	6.3	0.14	0.11	31.8	28.2	52.5	149.9
S7	5.8	9.7	1	2	3.8	4.0	0.18	0.17	38.5	34.4	34.5	124.7
S8	4.9	10.8	1	2	4.5	2.9	0.15	0.24	38.1	26.4	40.0	64.3
S9	7.2	13.9	1	1	5.6	2.3	0.12	0.30	24.9	14.4	77.7	84.1
Mean	6.16	12.45	1.33	2.00	4.33	4.33	0.187	0.188	31.3	27.4	48.5	122.8
S.D.	1.11	1.70	0.50	0.50	1.97	2.06	0.081	0.074	5.4	8.2	23.8	47.6

Table 6.1 Plasma pharmacokinetics of caffeine in normal healthy volunteers following oral doses of 250 and 500 mg.

Abbreviations: Cmax - Peak caffeine concentration, T1/2 - half-life, k.el - elimination constant, Vd - apparent volume of distribution, AUC - area under elimination curve, (* Insufficient points for accurate curve fitting)

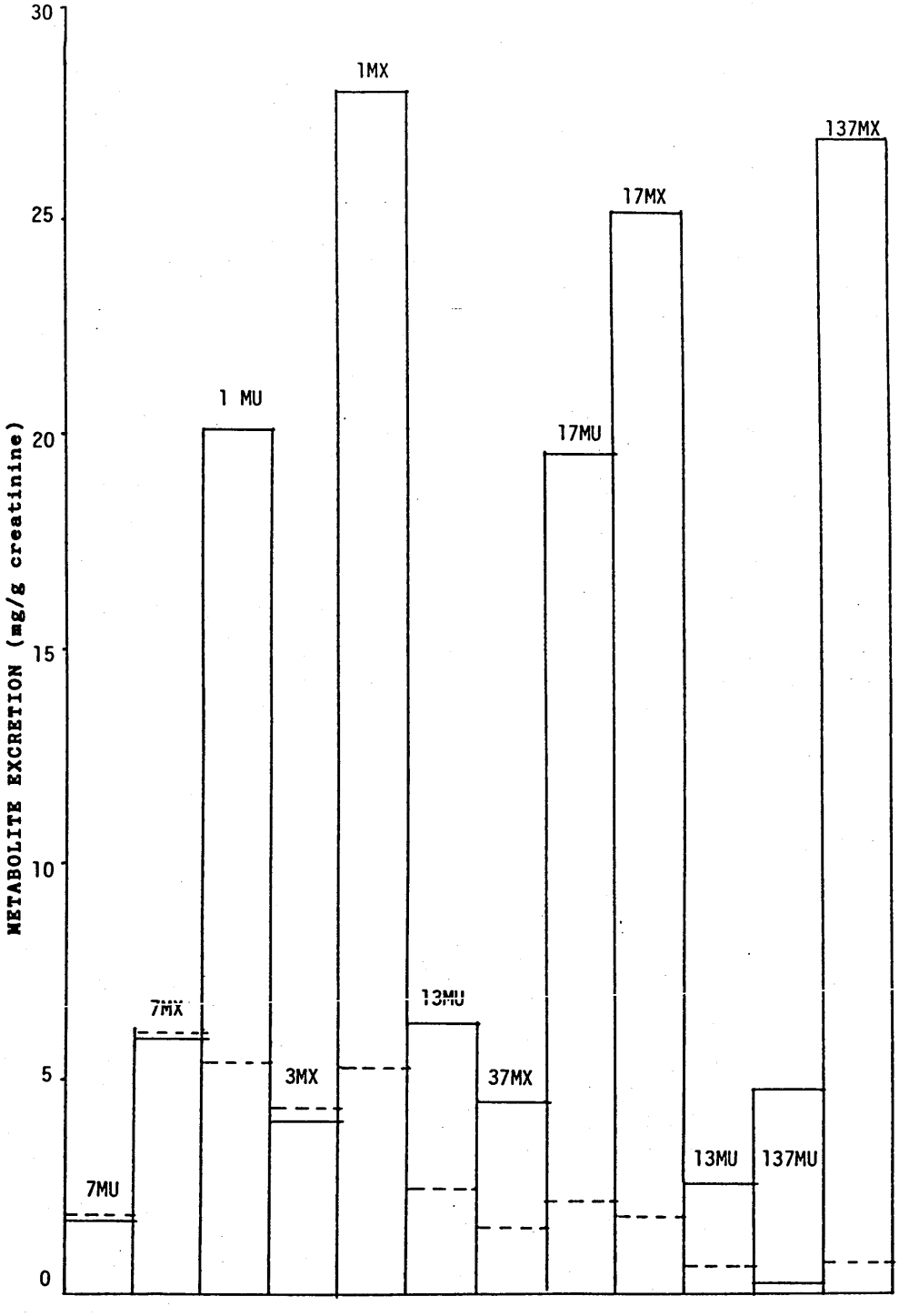


Fig 6.2 Mean methylxanthine metabolite values (mg/g/creatinine) in basal urine samples (---) and in samples collected for a 5h period following oral ingestion of caffeine (500mg).

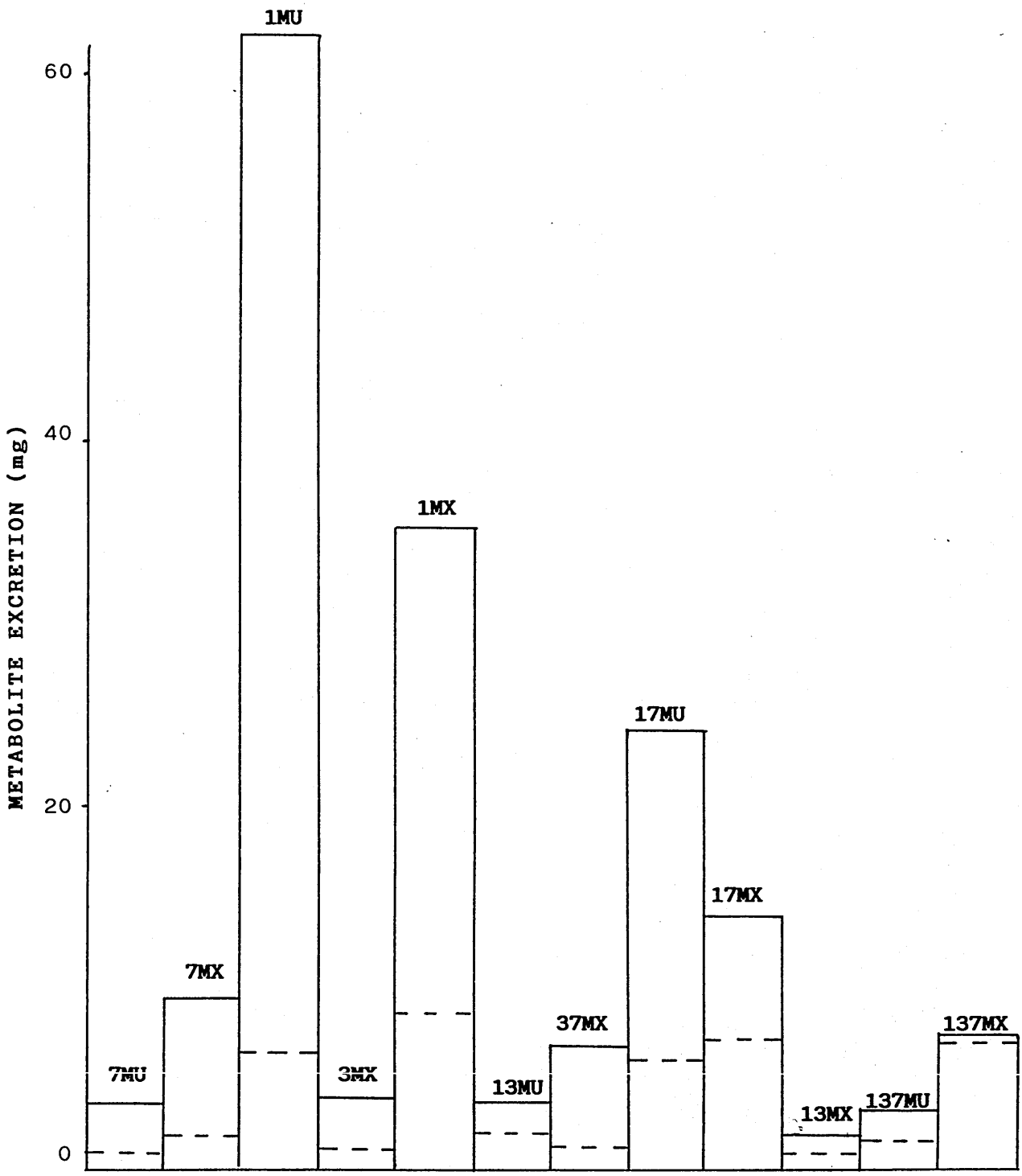


Fig 6.3 Mean methylxanthine metabolite ouput in urine samples (--) collected for a 5hr period (mg/5-h vol) from normal subjects following an oral dose caffeine (500mg). This is compared with output from normal subjects (mg/24-h vol) following an oral dose of 400mg caffeine.

For key to abbreviations refer to page 3.

500 mg caffeine is consistent with a report by Newton et al. (1981) confirming that caffeine obeys linear pharmacokinetics.

b) Urine metabolite excretion

The limited period of urine collection resulted in a low recovery of methylxanthine metabolites; the mean recovery for the 5 hour period following the ingestion of 500mg caffeine being only 8.3% (SD = 2.9) of the administered dose. However, in comparison with pre-drug values, methylxanthine metabolite levels (expressed as mg/g creatinine) in urine samples collected during this period showed significant increases in 1-methylxanthine, caffeine, 1,7-dimethylxanthine, 1-methyluric acid, 1,7-dimethyluric acid, 1,3,7-trimethyluric acid, 1,3-dimethyluric acid, theobromine and theophylline (Fig 6.2 and Fig. 6.3). The patterns of excretion were similar to those found previously (Section 5.) for 24 hour urine collections obtained from normal subjects, receiving either a single bolus dose of caffeine (400 mg) or a controlled daily dietary caffeine of 450 mg.

c) Physiological measurements

Electroencephalogram. Log "theta" power was decreased by caffeine in a dose-related manner (Fig. 6.4), with the effect persisting to 5 hours, especially with the high dose. Log "alpha" power was also decreased (Fig. 6.5), the doses initially having similar effects but the lower dose wearing off quicker. Log "beta" showed paradoxical effects, the 500 mg dose having less effect than the 250 mg dose (Fig. 6.5). The total power however, reflects drug effects on the slower

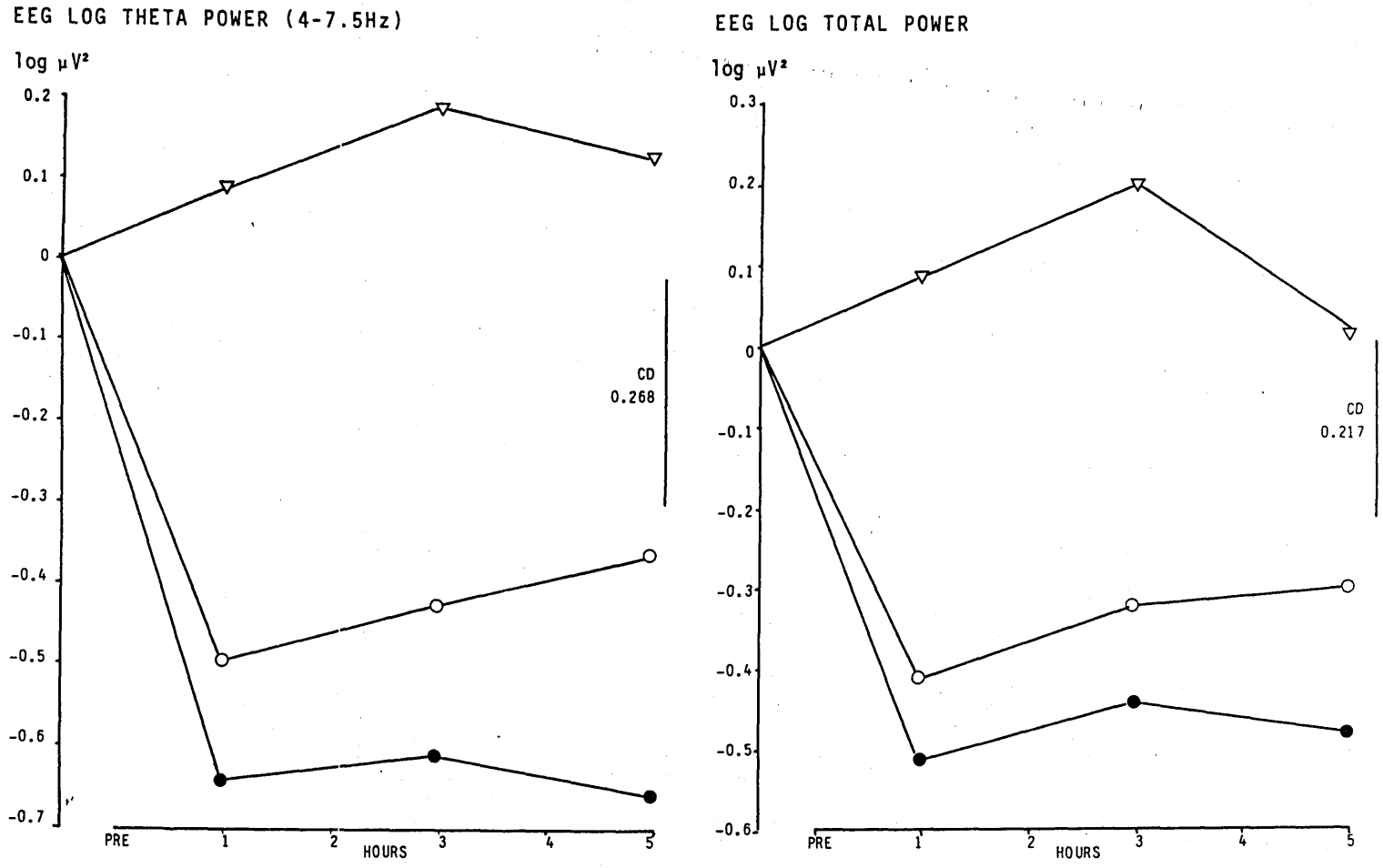


Fig 6.4 Mean EEG log power in 9 subjects after placebo (▽), 250mg caffeine (○) and 500mg caffeine (●). The vertical bar represents the critical difference (CD) at the 0.05 level of probability. Points further apart than that shown by the bar are significantly different.

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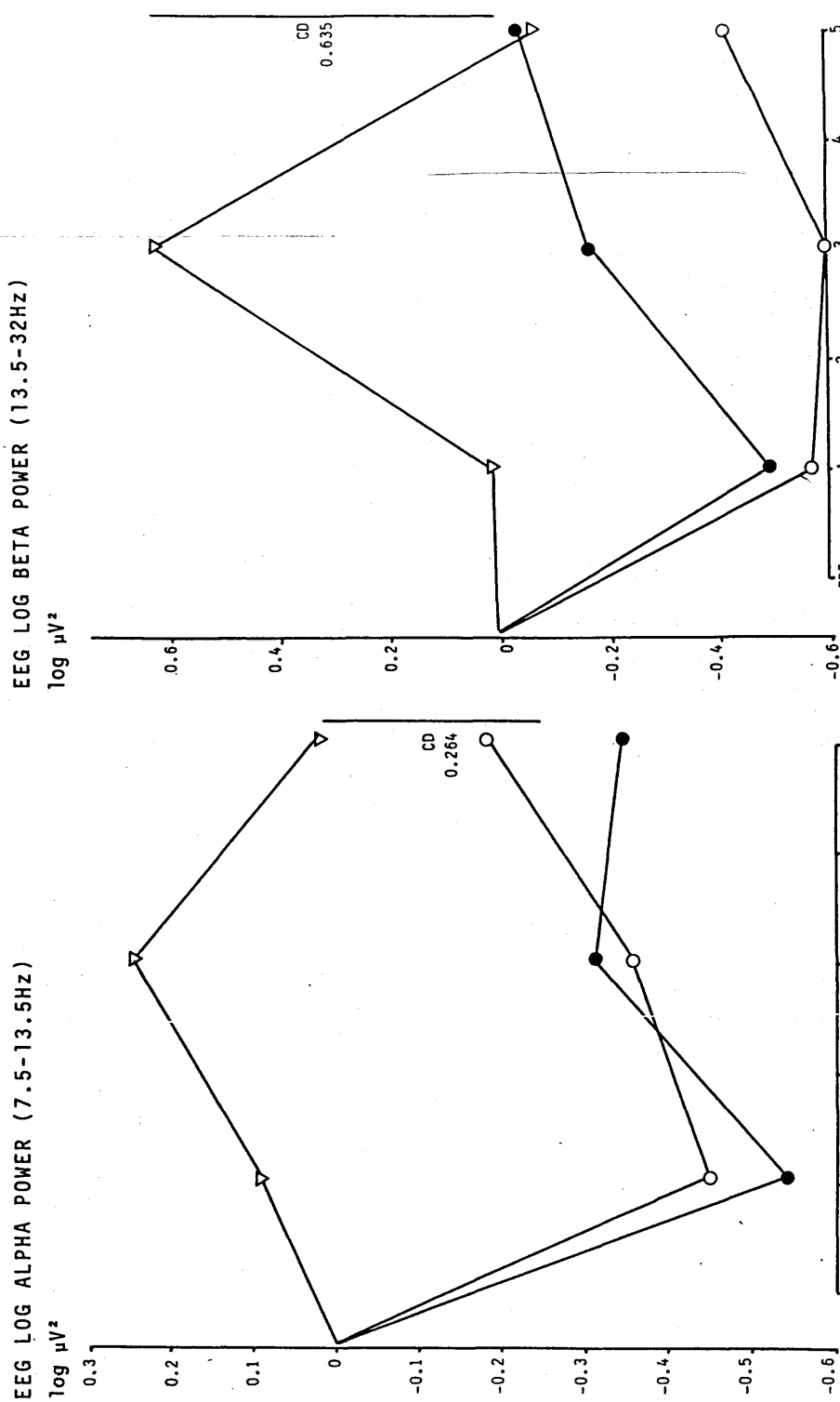

Fig 6.5 Mean EEG log power in 9 subjects after placebo (▽), 250mg caffeine (○) and 500mg caffeine (●). The vertical bar represents the critical difference (CD) at the 0.05 level of probability. Points further apart than that shown by the bar are significantly different.

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frequencies (Fig. 6.4).

Skin conductance. Caffeine was associated with a significant dose-related increase in skin conductance level (sweat gland activity) (Fig. 6.6), the effects persisting over the five hour period. There was no drug effect on the number of fluctuations.

Pulse and blood pressure were unaffected by either dose of caffeine.

Critical flicker fusion. Caffeine tended to increase CFF as compared with placebo but this effect marginally failed to reach significance ($P < 0.052$).

Tremor Caffeine produced a non-significant increase in the amount of tremor.

d) Psychological measures.

Tapping rate, reaction time, digit-symbol substitution (DSST), symbol copying (SCT) and number cancellation time (CT) all failed to show significant differences between the drug and placebo.

e) Self ratings

Mood rating scale. Caffeine was associated with a significant dose-related increase in alertness which persisted for 5 hours (Fig 6.7). The significant sub scales were: alert-drowsy ($P < 0.01$); energetic-lethargic ($P < 0.02$); quick witted-mentally slow ($P < 0.03$); and attentive-dreamy ($P < 0.04$). In general, the effects were dose-related; they began within the first hour, and tended to diminish thereafter, except on the alert-drowsy sub scale where the effect persisted. Other mood factors did not significantly distinguish drug from placebo.

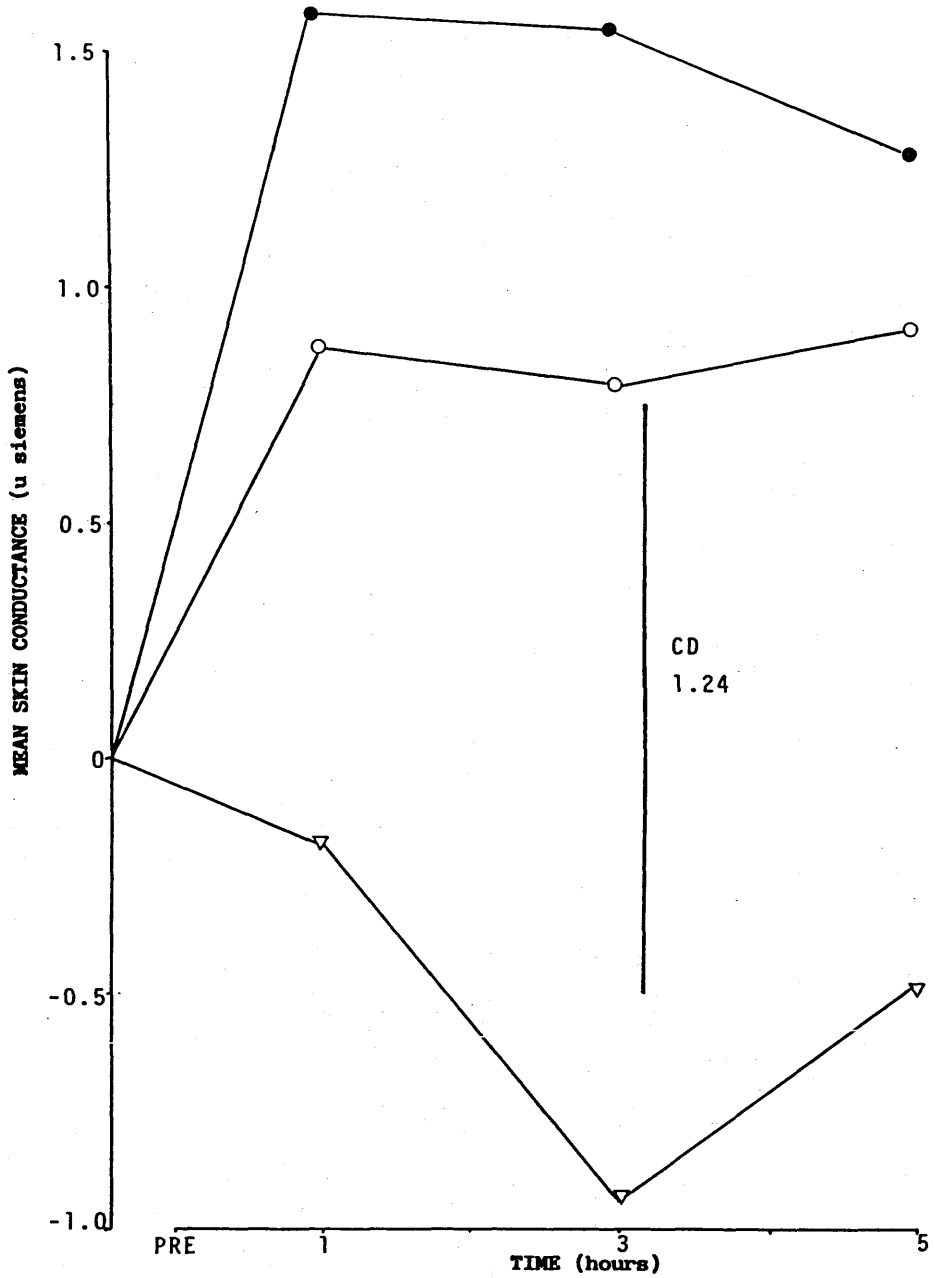

Fig 6.6 Mean skin conductance levels following placebo (▽), 250mg caffeine and 500mg caffeine (●).
CD=critical difference at the 0.05 level of probability.
(See Fig 6.4)

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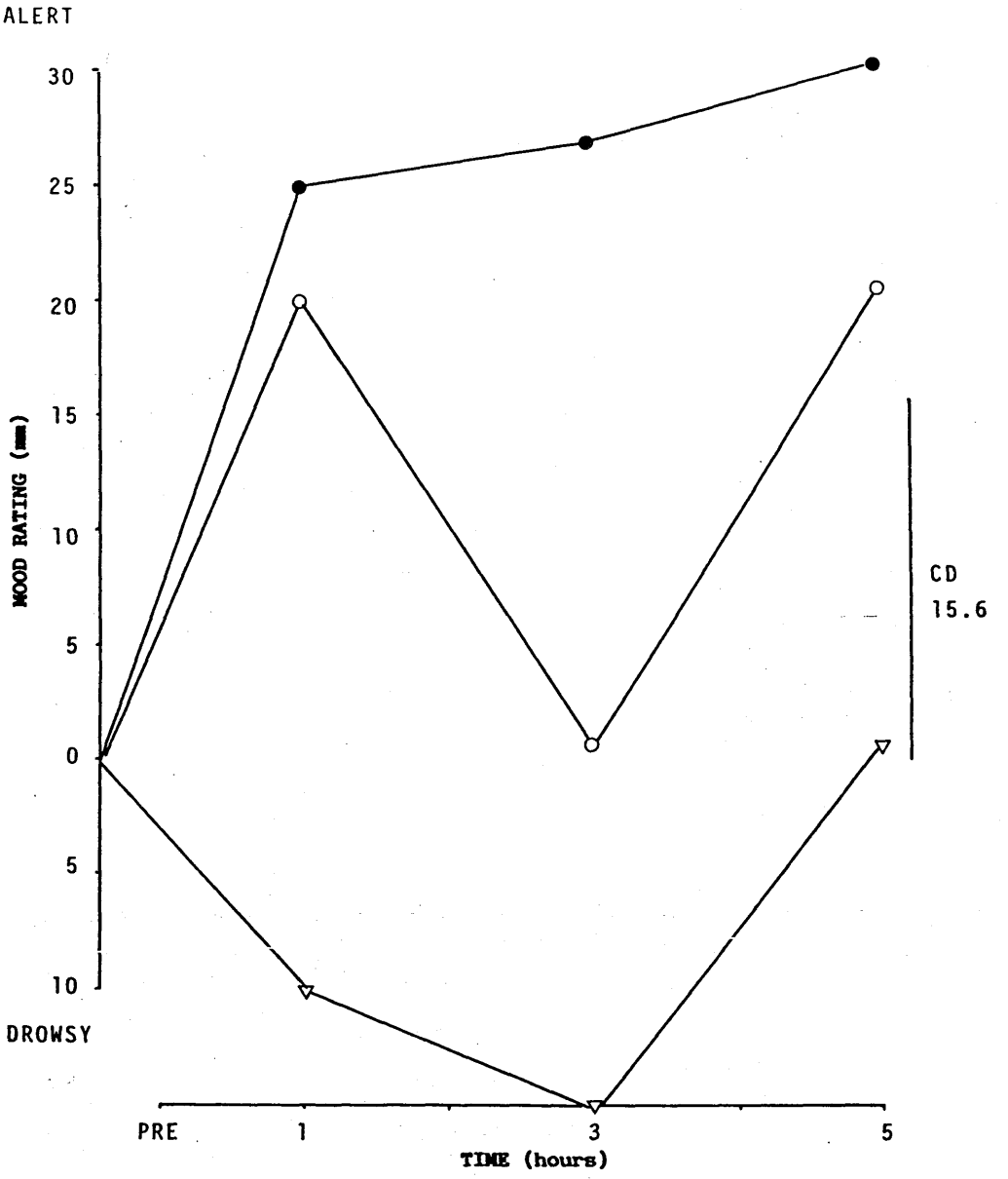

Fig 6.7 Mean mood rating for "alert-drowsy" after placebo (▽), 250mg caffeine (○) and 500mg caffeine (●).
CD=critical difference at the 0.05 level of probability.
(See Fig 6.4)

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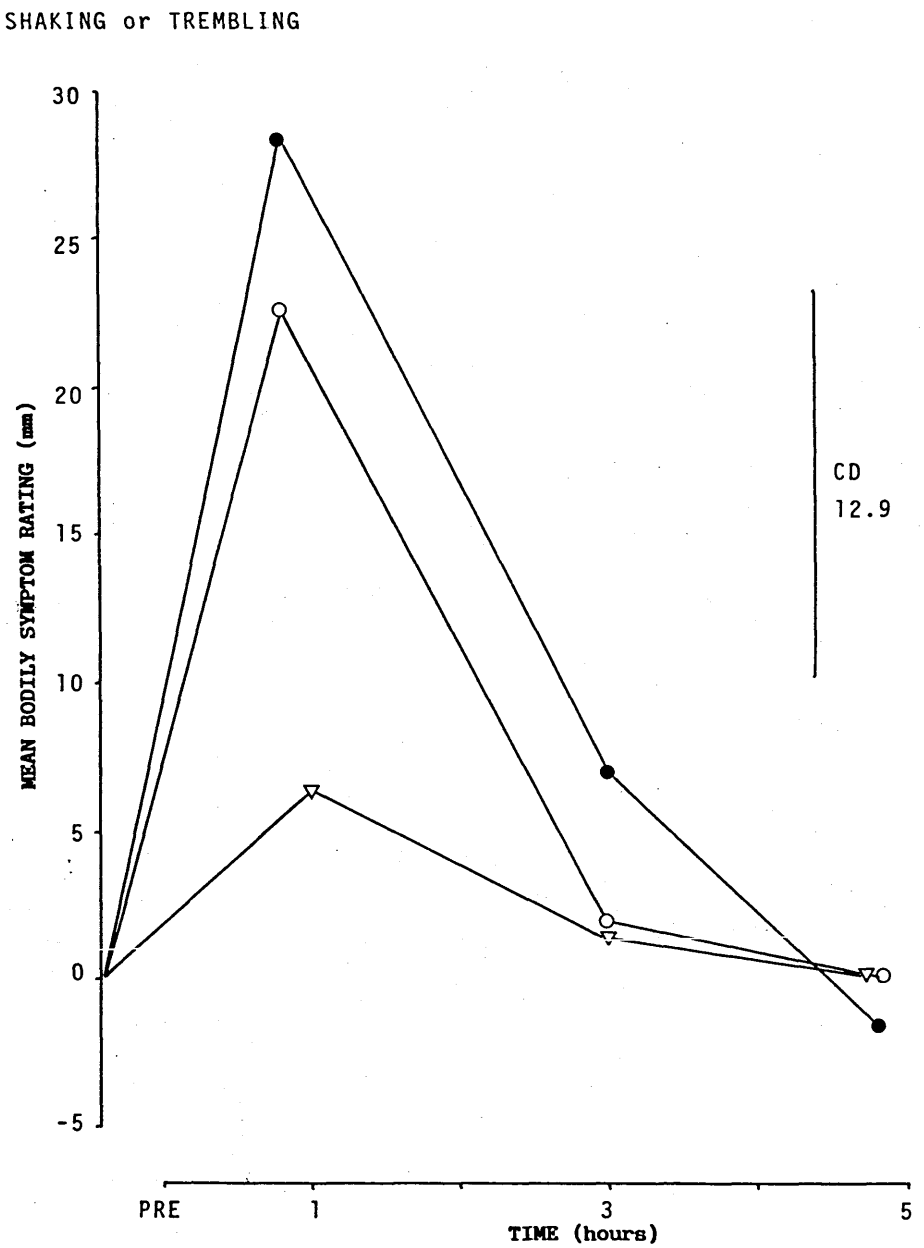

Fig 6.8 Mean bodily symptom rating "shaking or trembling" following placebo (▽), 250mg caffeine (○) and 500mg caffeine (●).
CD=critical difference at the 0.05 level of probability.
(See Fig 6.4)
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Bodily symptoms. The subjects reported more severe shaking and trembling (Fig. 6.8), maximal at one hour, and diminishing rapidly thereafter. There were also fewer complaints of headache ($P < 0.05$) and less tiredness ($P < 0.01$) after caffeine than after placebo. State anxiety inventory failed to show any difference between drug and placebo effects.

DISCUSSION

The precise calculation of pharmacokinetic data for his study was impeded by the limited number of subjects and the short period of sample collection. In addition there was a lack of control of relevant factors such as dietary caffeine consumption, the amount of smoking, female menstrual status and the taking of oral contraceptives. However the calculated values for elimination half-life and apparent volume of distribution were consistent with those found previously for normal healthy volunteers (Section 5.) In a study of random caffeine levels in hospital outpatients (Smith et al., 1982), 5% of the population were shown to have values in excess of 5.6 mg/l. This level is comparable to the average peak caffeine level following the 250 mg dose of caffeine. The gradual increase in 1,7-dimethylxanthine (paraxanthine) levels during the period of investigation is consistent with previous studies (Callahan et al., 1983; Arnaud 1986) indicating that within two hours following caffeine intake, the formation of this metabolite by 3-N demethylation accounts for 75% of the administered dose.

The limited period of urine collection resulted in a low

recovery of methylxanthine metabolites. However, the pattern of excretion after the 500 mg dose resembled closely the metabolite output estimated in normal subjects receiving a controlled dietary caffeine intake of 450 mg/24 hours. In agreement with data from previous studies (Callahan et al., 1983, Blanchard et al., 1985), the principal urinary metabolite were 1-methyluric acid, 1-methylxanthine and 1,7-dimethyluric acid.

An important consideration in this study was the effectiveness of quantitative EEG measuremnts as empirical dose/time markers of the reputed stimulant effects of caffeine. Evidence for the stimulatory action of caffeine was provided by the highly significant dose-related decrease in the slow-wave (theta) activity and a similar, but not dose related, decrease in alpha wave activity. The paradoxical responses observed with fast wave (beta) activity suggest that, whilst caffeine may have a similar direct (but dose related) effect in decreasing EEG power, a concomitant dose-related increase in beta activity may occur as a direct result of the stimulant action. Similar complex effects are occasionally observed in the opposite directions with sedatives such as premazepam (Golombok and Lader, 1984).

The failure to show significant changes in both systolic and diastolic blood pressures is in contrast with a report by Robertson et al. (1978) which showed an increase of 14/10 mm Hg one hour after a single dose of caffeine (250 mg). Although their subjects were normally abstainers of caffeine, the significance of this factor is doubtful since similar, but

less pronounced increases have been recently shown in habitual caffeine consumers (Smits et al., 1985).

The dose-related increase in skin conductance indicated an increase "arousal" in accordance with the known stimulatory effects of caffeine (Veleber and Templer, 1984). This was also demonstrated by the self-rating mood scale where the effects of caffeine included increased measures of alertness, energy, quick wittedness and attentiveness. Self rating bodily symtoms also indicated less tiredness. Whilst increases were observed in the measure of tremor by the accelerometer and the degree of shaking and trembling by the bodily symptom scales, only the latter showed significant changes. This would suggest that tremulousness is mainly subjective in nature.

Although the sensitivity of the psychological tests used in this study has been previously established in studies on the depressant effects of a wide range of psychotropic drugs, no significant effects were observed for this study. However, it is probable that the subjects were well motivated and performed the tasks to their maximum abilities thus leaving limited scope for drug induced improvements. Since the effects of caffeine will counter decline in repetitive performance tasks, the use of longer term tasks such as a standard vigilance task should be more sensitive.

As a result of the ubiquitous nature of caffeine consumption, often in almost toxic doses, prolonged periods of withdrawl are required before true baseline readings can be attained. In agreement with earlier reports (Section 5), the period of caffeine withdrawl for this study was in some

instances associated with the onset of headaches similar to those previously reported by Dreisbach and Pfeiffer (1943) and Greden et al. (1980). The effectiveness of a caffeine load in counteracting this effect was confirmed by a decrease in the self rating bodily symptom of caffeine.

The aim of this study was essentially of a pilot nature to assess the suitability of the test battery for use in assessing the psychophysiological effects of caffeine. The results obtained generally support the effectiveness of these investigations for use in future studies of this complex drug-subject interaction. The state anxiety inventory in normal subjects has previously been shown not to distinquish high caffeine consumption (Boulenger and Uhde, 1982); this is supported by the findings of this study.

Since the pharmacological properties of the dimethyl-xanthine metabolites of caffeine are reported to be similar to those of caffeine (Williams et al., 1978), the presence of significant plasma levels of paraxanthine may potentiate the effects of caffeine. The application of this battery of tests in monitoring the psychophysiological responses to a paraxanthine load would therefore be of considerable interest.

7. CAFFEINE IN PREGNANCY AND ITS EFFECTS ON THE NEWBORN INFANT

7. CAFFEINE IN PREGNANCY AND ITS EFFECTS ON THE NEWBORN INFANT

7.1 INTRODUCTION

7.1.1 THE METABOLISM OF CAFFEINE DURING PREGNANCY

Studies on the elimination of caffeine in pregnancy have consistently shown a substantial and progressive decrease in its clearance during the second and third trimesters (Aldridge et al.,1981; Knutti et al., 1982). A study of the salivary kinetic profiles, following an oral dose of caffeine (177 mg) in the form of instant coffee, in eight subjects during and after pregnancy (Aldridge et al., 1981), showed that the mean clearance of caffeine fell from 69.6 ml/hr/kg at 11 weeks gestation to 23.5 ml/hr/kg at 36 weeks. Corresponding salivary elimination half-life values increased fourfold from 5.3 to 18.1 hours. These changes disappeared within a few days post partum. The reductions in clearance were reflected more by changes in the elimination constant than in the apparent volume of distribution. Kinetic profiles of caffeine during the first trimester of pregnancy were similar to that of non-pregnant women not receiving oral contraceptives. These findings were confirmed by Knutti et al. (1982) who, in a study involving 15 pregnant women, found no correlation between the delayed clearance of caffeine and age, body weight, consumption of caffeinated beverages or changes in smoking habits.

Similar impairements in the elimination of caffeine have been observed in women receiving oral contraceptives. Following oral administration of radiolabelled caffeine to three non-smoking populations, Callahan et al. (1983) observed

that women taking oral contraceptives for at least 6 months had a lower mean plasma clearance (37 ml/hr/kg) compared to normal male subjects (83 ml/hr/kg) and ovulating females (109 ml/hr/kg). Respective caffeine elimination half life values were 10.4 hr (SD = 2.4), 4.5 hr (SD = 1.1) and 3.1 hr (SD = 0.9). The reduced plasma clearance of caffeine and its extended half-life in women receiving oral contraceptives were mainly reflected in lower amounts of metabolites in urine and a slower rate of metabolite formation. However, the pattern of metabolite excretion was similar for all three groups, the major urinary metabolites being 5-acetylamino-6-amino-3-methyluracil, 1-methyluric acid and 1-methylxanthine. In 1980, Patwardhan and colleagues used similar groups of subjects in a study restricted to the clearance of caffeine from plasma. Although their results showed a significantly prolonged half-life for caffeine in 9 females receiving oral contraceptives (10.7 hrs SD = 3.0), the apparent volumes of distribution and levels plasma protein binding were similar to those observed in 9 control females not receiving oral contraceptives showed. In comparison with results obtained for 13 healthy males, the pharmacokinetic data was similar with the exception that values for the apparent volume of distribution were significantly larger in both the female groups ($P < 0.05$).

The impaired elimination of caffeine during pregnancy may be explained by changes in the hormonal milieu (increased oestrogens and progesterone) which decrease the hepatic metabolism of certain drugs (Patwardhan et al., 1980). The

evidence that progesterone, oestrogens and their metabolites can induce, repress, or directly inhibit selective mono-oxygenase catalysed processes is abundant (Feuer and Kardish, 1975; Krauer and Krauer, 1977; MacKinnon et al., 1977). However these investigatons have been mainly descriptive with little attempt to determine the cause of the changes in drug metabolism. In a study of factors affecting hepatic drug metabolism in the pregnant rat, Turcan (1981) showed that elevated levels of placental lactogen caused a significant decrease in maternal mixed function oxidase activity when expressed as per gram of liver. Drug enzyme activity, determined with the substrates aniline (p-hydroxylation), ethylmorphine (N-demethylation) and p-nitrobenzoic acid (reduction) decreased progressively during gestation to 53-73% of the non-pregnant control level by day 20 of gestation. However, the total activity of the liver to metabolize drugs remained unchanged or increased because liver weight was elevated by up to 40% during pregnancy. The author concluded that, in the pregnant rat, changes in drug metabolism during gestation were not related to alterations in the concentration of cytochrome P-450. The rise in circulating steroids did not appear to be an important factor of mixed function oxidase activity. However it was suggested that oestrogens may induce hepatic growth hormone receptors which are important mediators of placental lactogen activity.

The low molecular weight of caffeine facilitates transport across the placenta (Goldstein and Warren, 1962) and evidence suggests that it may accumulate in foetal brain tissue (Galli

et al., 1975). Other facors which aid in placental transfer include its high lipid solubility and its low ionization at physiological pH. Caffeine is also secreted into breast milk and enters gonadal tissue (Goldstein and Warren, 1962).

7.1.2. The metabolism of caffeine in the newborn

Caffeine is eliminated remarkedly slowly by both premature and full-term newborn infants (Parsons et al., 1976; Aranda et al., 1981). The mean plasma caffeine half life approximates 100 hours in the preterm newborn infant (Aranda et al., 1981) which represents a 16-fold prolongation relative to the non-smoking adult whose mean half-life is about 6 hours. In full term infants, there is an 11-fold decrease in the plasma clearance of caffeine relative to that of the non-smoking adult. The decreased clearance is mainly due to slow elimination of caffeine as the apparent volumes of distribution are similar for both neonate and adult. In a study of the maturation of caffeine elimination in infancy (Aranda et al., 1979) the plasma clearance of caffeine at the age of 3 to 4 months was estimated to be 105 ml/hr/kg, a rate similar to that of the non-smoking adult value. No changes in caffeine elimination seemed to occur during the neonatal period itself. In addition, the clearance or half-life of caffeine during this period was not be related to indices of maturity such as birth weight, gestational age, and postnatal age.

The urine methylxanthine metabolite patterns in 10 infants, aged 8 days to 8 months, receiving therapeutic treatment with

caffeine (Aldridge et al., 1979) showed that during the first month of life, caffeine accounted for more than 85% of the identifiable products in urine. Caffeine remained the predominant component for the first 3 months before gradually decreasing to the adult value of less than 2% by the age of 7 to 9 months. This coincided with the attainment of normal adult metabolite patterns. The data suggests that the impaired clearance of caffeine in the newborn is mainly the result of a generalised deficiency in the oxidative metabolism of drugs handled by the cytochrome P-450 mono-oxygenase system. In particular, Pelkonen et al. (1973) observed that human fetal liver is much more deficient in aryl hydrocarbon hydroxylase activity (2% of the adult value) than in other catalytic functions of the mono-oxygenase complex (25-50% of the adult values).

7.1.3. Disposition of caffeine in human breast milk

Caffeine from dietary sources has long been known to enter milk from lactating mothers. Early studies by Irvin and Schumaker (1936) demonstrated the presence of caffeine in milk after tea or coffee consumption, with mean levels of approximately 8.2 mg/l being reached 4 hours after the ingestion of coffee containing 100 mg caffeine. However these studies used techniques lacking in specificity and sensitivity. Tyrala and Dodson (1979) studied the disposition of caffeine in maternal serum and milk in 4 lactating mothers from 4 to 12 months. Following oral ingestion of 150 mg caffeine sodium benzoate, peak levels in serum occurred by 60 minutes in all patients

and ranged from 2.4-4.0 mg/l. Caffeine appeared in both serum and milk by 30 minutes and the milk/serum ratio was constant, the average ratio being 0.52. Since approximately 75% of caffeine in serum is free, this ratio is less than the theoretical value suggesting that additional factors other than free diffusion determines the distribution across the mammary alveolar cell into milk. On the assumption that the infants would nurse 90 ml at 1 and 2 hours after the maternal dose, the total amount of caffeine ingested by the infant was estimated to be 170 ug or 0.11% of the maternal dose.

In a similar study, Berlin et al. (1984) investigated the disposition of caffeine in milk and saliva following the consumption of a caffeine containig beverage equivalent to their usual amount (conc. range = 35-336 mg). Caffeine was detected by 15 minutes in both milk and saliva; peak levels in milk (2.09 - 7.17 mg/l) and saliva (1.24 - 9.22 mg/l) were reached within 1 hour. Respective elimination half-life values for milk and saliva were 6.1 (SD = 4.4) and 4.0 (SD = 3.7) hours. Using similar criteria to Tyrala and Dodson (1979), they estimated that the amount of caffeine available for infant absorption ranged from between 0.01-1.64 mg or 0.06% to 1.5% of the maternal dose. In 1982, Bailey et al. monitored the caffeine concentration in milk of one woman over a 10.5 hour period during which she drank 12 cups of weak coffee (33.2 mg caffeine per cup). The total amount of caffeine consumed was 398 mg. Concentrations in milk ranged from 0 to 1.5 mg/l. A more recent study by Ryu (1985), which determined caffeine concentrations in milk of 9 lactating

women, reported a mean value for 6 of the subjects of 3.8 mg/l; the average values for the remaining 3 subjects were 13.4, 15.7 and 28.6 mg/l. However, whilst these values clearly indicate that breast fed infants may be exposed to high levels of caffeine, it is questionnable whether the results are representative of a normal pregnant population since the subjects were all receiving a high dietary caffeine intake of 750 mg per day.

Although the above mentioned studies reflect minimal exposure to caffeine following the maternal consumption of 1-2 cups of coffee, the delayed clearance in young infants may result in cumulative blood levels being reached.

7.1.4. Caffeine levels in umbilical cord blood

In 1976 Parsons et al. determined the concentration of caffeine in cord blood samples collected from 15 full term infants in Montreal, using a RIA procedure specific for caffeine (Cook et al., 1976), and found a mean value of 1.4 mg/l. Similar values were found by Soyka and colleagues (1981) in a more extensive study (n=30) of an infant population in Vermont USA (Soyka et al., 1981). Using the same analytical procedure they reported a median value of 1.0 mg/l (range = 0.01-5, n=329). This group also studied a population of 69 infants born in Marburg, West Germany in which they found a mean value of 1.6 mg/l (range = 0.2-8.9). A report by Van't Hoff (1981) suggests that cord blood caffeine levels in the United Kingdom may be somewhat higher than estimates from other countries. In a study involving 55 full term infants at

Guy's Hospital, London, he observed cord blood caffeine levels up to 12.6 mg/l.

7.1.5. Effects of caffeine consumption on the outcome of pregnancy

Concern about the possible harmful effects of caffeine in pregnancy have evolved mainly from animal studies which have associated caffeine intake with decreased intrauterine fetal growth, a lower birth weight and skeletal abnormalities. However, the implications of these findings in humans is unclear due to differences in the mode of exposure to caffeine, the amounts consumed and the species variations in metabolism of the drug.

In a retrospective survey by Weathersbee et al., (1977) the daily caffeine intakes of women who had recently been pregnant were estimated and analysed in relation to the outcome of pregnancy. In a subgroup of 16 women with a caffeine intake of a least 600 mg/day, 15 of the pregnancies ended in spontaneous abortion, stillbirth or premature births. However it is doubtful whether the consumption estimates accurately reflected the intake during pregnancy and the study failed to consider the effects of other risk factors such as age, smoking habits and alcohol consumption. More recently, Linn et al., (1982), in a study of the outcome of 12,205 pregnancies reported that lower birthweights and shorter periods of gestation occurred more often among the offspring of women who drank four or more cups of coffee per day. However, after controlling for smoking, alcohol consumption, demographic

characteristics, and medical history by standard logistic regression, they concluded that coffee consumption was not related to low birth weight, short gestation, or the presence of malformations. Cigarette smoking was one of the more prominent risk factors for a birthweight of less than 2.5 kg. Additional support for this is found in a study by Kuzma and Sokol (1982) which was primarily concerned with evaluating alcohol consumption and birthweight. The report concluded that women who consume the caffeine equivalent of 6 cups of coffee a day prior to delivery gave birth to babies weighing 1.5 oz less than babies born to women who consumed virtually no caffeine. In contrast, the birthweight reductions for consumption of two-thirds of a can of beer a day and smoking one or more packets of cigarettes per day were, 3.7 oz and 6.4 oz respectively.

The risk of congenital anomalies was evaluated by Rosenberg et al. 1982) in a case controlled study of 2,030 malformed infants. The focus of this study was two congenital anomalies which had been previously observed in rodents given high doses of caffeine. Cleft lip (with or without cleft palate) occurred in 299 babies, and isolated cleft palate was identified in another 120 babies. However, neither mothers who consumed more than 200 mg per day nor those who consumed more than 400 mg of caffeine per day were at significantly increased risk of delivering a baby with either of these clefts. Similar negative correlations were observed for infants with inguinal hernia, cardiac defects, pyloric stenosis and neural tube defects. The study concluded that caffeine has no major

teratogenic effects.

In summary, the cumulative evidence derived from epidemiological studies suggests that caffeine consumption by pregnant women does not materially increase the risk of low birthweight, preterm birth or congenital malformations (Leviton, 1984).

7.1.6. Adverse effects of caffeine in the newborn infant

The adverse reactions of caffeine in the newborn infant are mainly exaggerations of the known pharmacological effects. These include tachycardia, tachypnoea, seizures, opisthotonus, tremors, jitteriness, increased reflexes, hypertension and gastro-intestinal disturbances (Howell et al. 1981). Whilst these effects are most likely to result from excessive use of caffeine in the treatment of neonatal apnoea, the cumulation of caffeine due to excessive maternal consumtion should not be ignored.

7.1.7. Introduction to experimental work

The studies summarised in Section 7.1 have provided a significant amount of information on the pharmacokinetics and pharmacological properties of caffeine in maternal serum, cord blood and breast milk. This data, supplemented with information on the dietary consumption of caffeine during pregnancy, has enabled assessments to be made of the degree of foetal exposure to caffeine and the subsequent effects on the outcome of pregnacy. However, there are still important areas that require further investigation. In particular, the

mechanisms responsible for the substantial and progressive decrease in the elimination of caffeine during the second and third trimesters of pregnancy (Aldridge et al., 1981; Knutti et al., 1982) are unknown, although they are generally considered to be hormonal in origin.

The following studies were performed to provide further information on the metabolism of caffeine during pregnancy and to investigate the relationship between exposure to caffeine and the onset of certain metabolic disorders in the newborn infant. All measurements of caffeine and its metabolites were performed using the HPLC procedures described in Sections 2 and 3. Prior to commencing these studies, protocols were submitted to and approved by the Bromley Health District Ethics Committee.

7.2. ASPECTS OF CAFFEINE METABOLISM DURING PREGNANCY

7.2.1. METHYLXANTHINE LEVELS IN MATERNAL SERUM AND SALIVA

A previous study by Tebbutt et al. (1984) concluded that dietary surveys of caffeine consumption during pregnancy were only moderately well correlated with plasma levels of caffeine. In view of the prolonged half-life of caffeine during pregnancy, the authors suggested that whilst interviews on diet provides a basis for advising the patient, the monitoring of plasma levels probably provides the best reflection of the patient's caffeine intake.

This study compares estimates of serum levels of caffeine and its dimethylxanthine metabolites in a group of pregnant females with values obtained for a non-pregnant population. In addition possible alterations in the extent of protein binding during pregnancy are investigated by comparing serum/salivary caffeine ratios in the two populations.

Methods and materials

Levels of caffeine and its dimethylxanthine metabolites (theobromine, theophylline and paraxanthine) were measured in serum samples obtained from 63 randomly selected patients attending anti-natal clinics for routine assessment. In 17 of the subjects, methylxanthine levels were also determined in samples of saliva collected simultaneously at the time of venepuncture.

A statistical analysis of the random serum caffeine levels and the salivary versus serum concentration ratios were compared with results obtained previously for a non-preganant

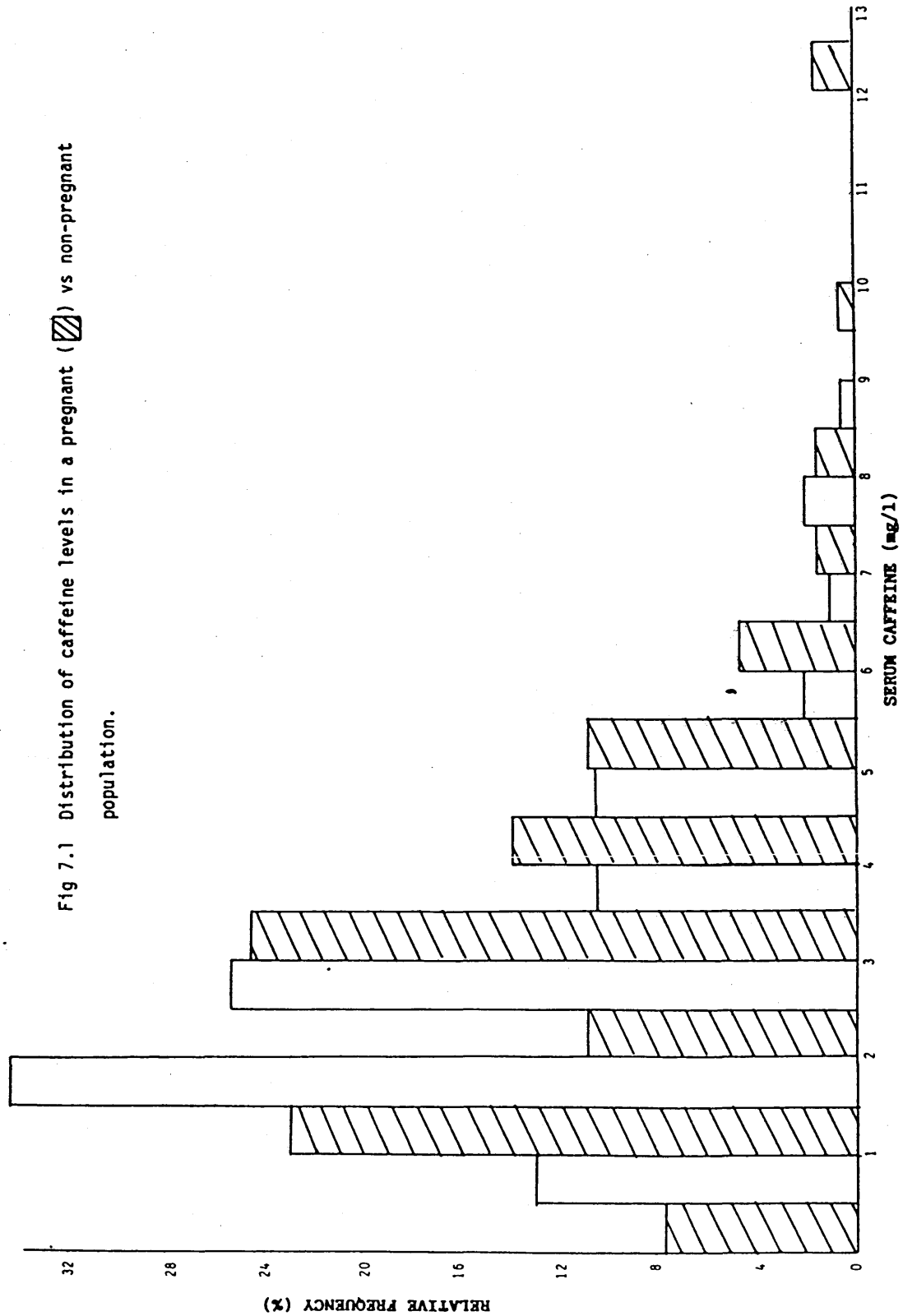

Fig 7.1 Distribution of caffeine levels in a pregnant (▨) vs non-pregnant population.

population (Section 5.).

Results

The levels of caffeine, theobromine, theophylline and 1,7-dimethylxanthine (paraxanthine), estimated in serum samples collected at random from a population of pregnant females, are summarized in Table 7.1. The values for caffeine are compared in the form of a histogram, with those obtained from random sampling of non-pregnant volunteers (Fig 7.1) .

	Theobromine	Paraxanthine	Theophylline	Caffeine	Total
Mean	1.05	0.89	0.40	3.45	5.74
S.D.	0.84	0.64	0.24	2.16	3.03
Range	0 - 3.89	0 - 4.40	0 - 1.12	0 - 12.1	0.65 - 15.7
(n = 63)					

Table 7.1 Summary of methylxanthine levels in maternal serum

The mean caffeine value for maternal serum samples (3.45 ± 2.43) was significantly higher ($P < 0.001$) than that previously obtained for samples collected from a non-pregnant population (2.43 + 1.63). Regression analysis of the values for caffeine in samples of serum and saliva collected from the pregnancy group showed a linear relationship. The coefficient of correlation was calculated to be 0.979 with a regression line of $y = 0.806x - 0.217$, where y = saliva and x = serum. (p.268)

Discussion

Previous studies on the elimination of caffeine during pregnancy have consistently shown a substantial and progressive decrease in its clearance during the second and third trimesters (Aldridge et al., 1981; Knutti et al., 1982). A result of this is that peak concentrations following a

caffeine load and the fluctuation in caffeine levels that are characteristic of usual consumption habits become less pronounced. The regular consumption of caffeinated beverages during pregnancy may therefore produce steady state maternal serum caffeine levels which may be used as an index of dietary caffeine intake. Since caffeine equlibrates rapidly across the placenta, the monitoring of caffeine levels in maternal serum may also provide an indication of foetal exposure.

The finding in this study of increased caffeine levels in maternal serum samples is not unexpected in view of known impairments in the elimination of caffeine during pregnancy However, completion of questionnaires by a limited number of the volunteers suggested that the consumption of caffeinated beverages decreased during pregnancy, the mean intake being approximately 260 mg/day. Since it has been reported that the apparent volume of distribution for caffeine is unaltered in pregnancy (Aldridge et al., 1981; Knutti et al., 1982), the increased concentrations of caffeine in maternal serum must be attributed to a delay in the clearance of caffeine. As the slope of the regression line for the ratio of levels in maternal serum and saliva samples was similar to that for the non-pregnant population, changes in the extent of protein binding can be eliminated. Subsequently it is probable that the delayed clearance of caffeine is the result of altered or impaired metabolism by the maternal liver.

An analysis of the histogram distribution of caffeine concentrations in maternal serum indicates that up to 10% of a pregnant population may have circulating levels in excess of 6

mg/l. Since the low molecular weight of caffeine freely facilitates transport across the placenta foetal exposure to caffeine may be significant. In addition many of the metabolites of caffeine have similar pharmacological properties (Williams et al., 1978) indicating a requirement to assess exposure in terms of total methylxanthine levels.

7.2.2. URINARY METABOLITES OF CAFFEINE IN PREGNANT WOMEN

The metabolism of caffeine has received little attention, due mainly to the natural reluctance of pregnant women to ingest large single doses of caffeine and the ethical problems of using radiolabelled compounds. The following study investigates the biotransformation of caffeine in pregnancy by comparing urine metabolite profiles of a group of pregnant women, receiving a controlled dietary intake of caffeine, with those of a group of non-pregnant healthy volunteers under similar conditions.

Methods and materials

A group of 15 pregnant women of between 34-36 weeks gestation and a control group consisting of 9 female volunteers not receiving oral contraceptives were enrolled after careful explanation of the study. A questionnaire was completed for each subject giving details of their normal dietary caffeine consumption, smoking habits and any current drug therapy. Each subject was given a series of pre-weighed amounts of coffee, of known caffeine content, equivalent to their normal consumption of caffeinated beverages, and requested to refrain from consuming any other form of caffeinated beverage, chocolate and caffeine containing analgesics. Using this protocol the 24 hour caffeine intake for the pregnancy group and the non-pregnant control group ranged from between 123-369 mg and 300-750 mg, respectively. Following a 24 hour period of controlled caffeine intake, participants were requested to collect urine samples for a 24

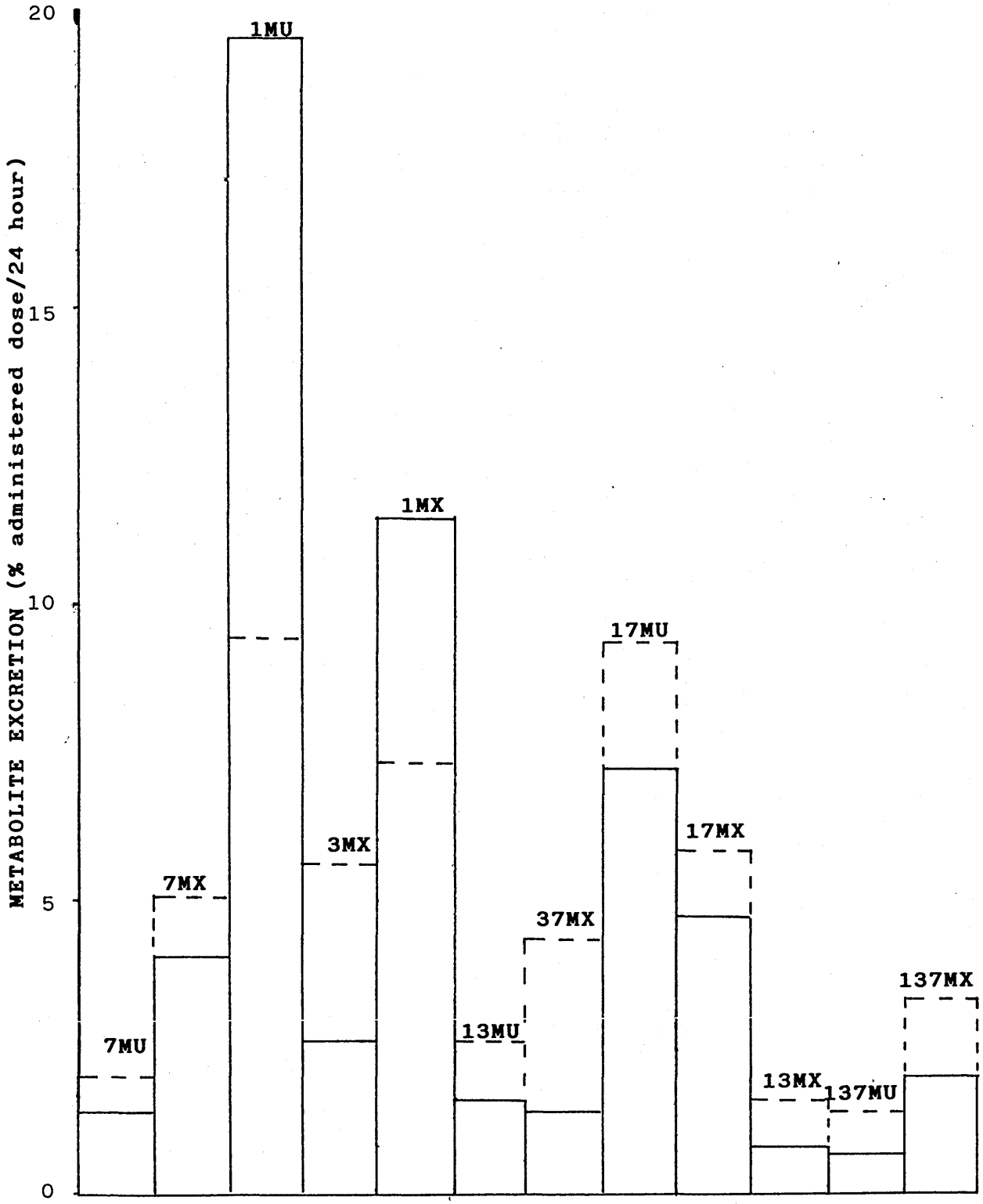

Fig 7.2. Urinary methylxanthine metabolite excretion (% administered dose/24 h in pregnant (--) and non-pregnant (—) healthy subjects receiving a controlled 24 h dietary caffeine intake.

For key to abbreviations refer to page 3.

METABOLITE	RECOVERY (% administered dose) Non-pregnant Mean SD	Pregnant Mean SD	Student's t-Test (P)
7-methyluric acid	1.4 ± 0.5	2.0 ± 0.6	< 0.05
7-methylxanthine	4.0 ± 0.3	5.0 ± 2.6	< 0.25
1-methyluric acid	19.5 ± 5.3	9.4 ± 3.7	< 0.001
3-methylxanthine	2.6 ± 0.7	5.6 ± 3.2	< 0.05
1-methylxanthine	11.4 ± 2.1	7.3 ± 3.4	< 0.02
1,3-dimethyluric acid	1.6 ± 0.5	2.6 ± 0.9	< 0.02
3,7-dimethylxanthine	1.4 ± 0.6	4.3 ± 3.4	< 0.05
1,7-dimethyluric acid	7.2 ± 2.1	9.3 ± 2.9	< 0.1
1,7-dimethylxanthine	4.7 ± 0.9	5.8 ± 1.1	< 0.05
1,3-dimethylxanthine	0.8 ± 0.4	1.6 ± 0.5	< 0.001
1,3,7-trimethyluric acid	0.7 ± 0.2	1.4 ± 0.6	< 0.01
1,3,7-trimethylxanthine	2.0 ± 1.1	3.3 ± 1.4	< 0.05

Table 7.2 Urinary metabolites of caffeine in healthy subjects receiving a controlled 24 hour dietary caffeine intake.

hour period whilst maintaining the same caffeine intake. The urine volumes were recorded and aliquots stored at -20 C until required for analysis. Following the quantitation of the urinary methylxanthine levels by HPLC, a statistical comparison was made of the metabolite profiles of the 2 groups studied.

Results

The urinary recoveries of caffeine metabolites obtained in the the two groups of volunteers are summarized in Fig. 7.2 and Table 7.2. Details of the individual results for each volunteer are enclosed in Appendix 4. The total 24 hour urine volumes were similar for both groups. Despite a variation in the 24 hr caffeine intake, the % recovery of the administered dose in 48 hours for the pregnancy group and the non-pregnant control group was similar, the mean values being 56.8 (+ SD. 8.9) and 56.8 (± SD. 8.6) respectively.

Compared with the non-pregnant subjects,the pregnancy group showed a marked decrease in the excretion of 1-methylxanthine (11.4% ± SD. 1.9 to 7.3% ± SD. 3.4) and 1-methyluric acid (19.5% ± SD. 5.3 to 9.4% ± SD. 3.7) with a general increase in the recoveries of the remaining metabolites, in particular those of 3,7-dimethyl xanthine (1.4% ± SD. 0.6 to 4.3% ± SD. 3.4) and 3-methylxanthine (2.6% ± SD. 0.7 to 5.6% ± SD. 3.2).

Discussion

The metabolic fate of caffeine (1,3,7-trimethylxanthine) is primarily demethylation and oxidation in the liver with unchanged caffeine accounting for only 1-3% of the

administered dose in the urine (Fig.7.3). In man, the metabolic stability of the methyl groups increases in the order 3,1 and 7 (Arnaud and Welsch, 1981). Although initial demethylation may occur at any of these three positions, yielding formaldehyde as a secondary product, the importance of the reactions, in terms of urinary metabolite recovery, are 3-N demethylation, 72%; 1-N demethylation, 20% and 7-N demethylation, 8%.

The metabolite recoveries for the non-pregnant control group in this study are similar to those found previously following a single oral dose of caffeine (Bonati et al., 1982; Callahan et al.,1983; Blanchard et al.,1985), indicating that the majority of administered caffeine undergoes 3-N-demethylation to form 1,7-dimethylxanthine, the primary metabolite found in serum. The major metabolites excreted in urine are 1-methyluric acid, 5-acetylamino-6-formylamino-3-methyl uracil, and 1-methylxanthine (Fig 7.3).

The significant decrease in the production of 1-methyl metabolites, in the pregnancy group, with relatively normal values of 1,7-dimethyl metabolites, suggests that during pregnancy the further metabolism of 1,7-dimethylxanthine may be impaired. This could be the result of an impairement in 7-N demethylation with respect to 1,7-dimethylxanthine as a substrate, although the absence of a concomitant increase in 1,7-dimethylxanthine excretion would tend to refute this possibilty. Alternatively the regulation may be located at the level of 3-N demethylation of caffeine. It is of significance that the clearance of theophylline (1,3-dimethylxanthine),

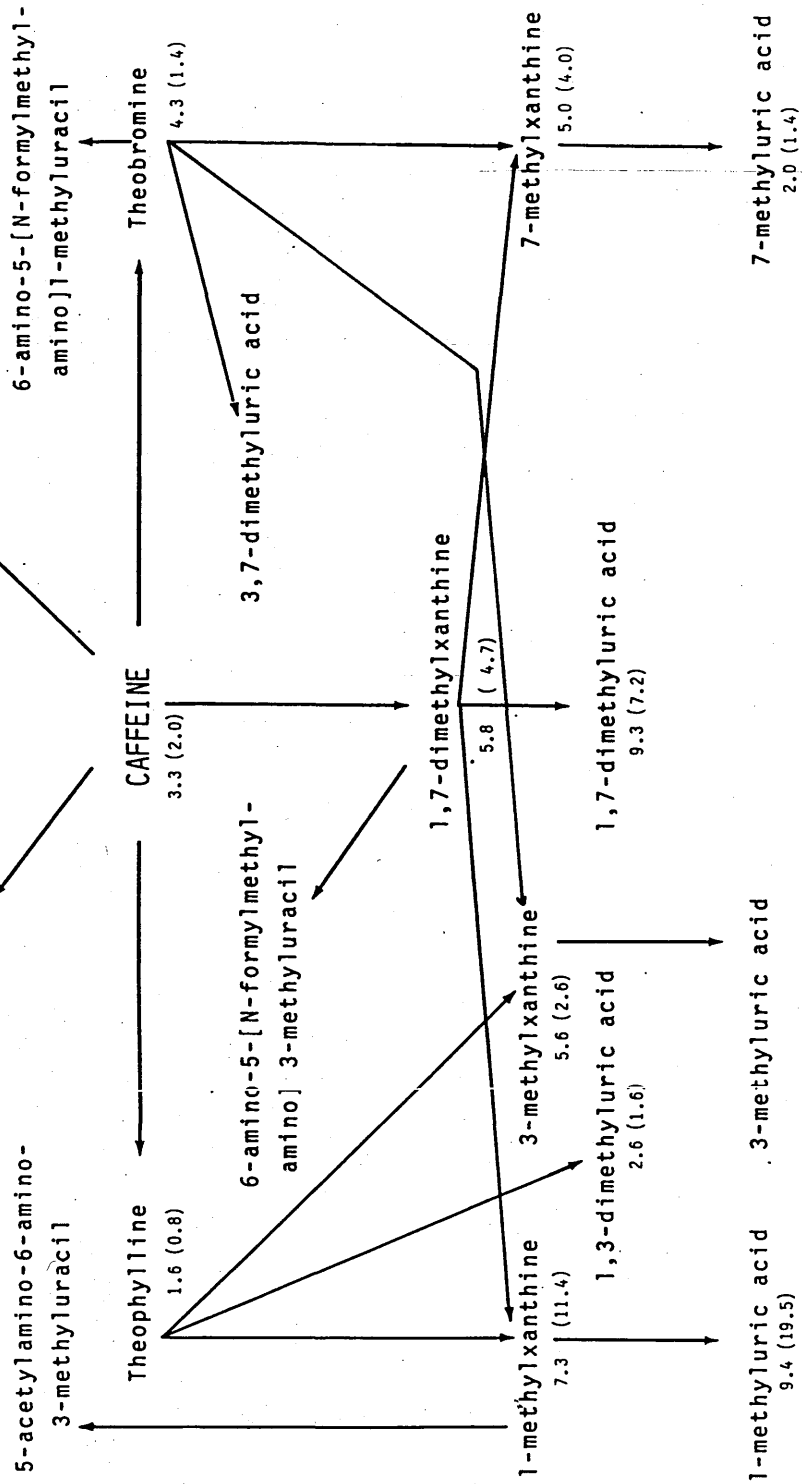

Fig 7.3 Quantitative metabolic pathways of caffeine in pregnancy.

Numbers refer to 24h urinary excretion as a percentage of dietary intake of caffeine. Values obtained from pregnant subjects are followed by those (in brackets) from non-pregnant subjects.

which does not involve 7-N demethylation, is not altered during pregnancy (Sutton et al., 1978). The increased recoveries of metabolites possessing a 3-methyl group suggests that, during pregnancy, the route of caffeine metabolism is altered in favour of an initial 1-N demethylation of caffeine to theobromine followed by 7-N demethylation to 3-methyl-xanthine. To a lesser extent, the initial 7-N demethylation of caffeine to form theophylline may also be increased during pregnancy, since the recoveries of 1,3-dimethyluric acid and theophylline are both significantly increased.

A previous study in which caffeine metabolite recoveries from normal ovulating females were compared with those obtained from women receiving oral contraceptives (Callahan et al., 1983), showed for the latter group, decreases in 1-methyl uric acid excretion from 16.14% to 11.06%, 1-methylxanthine excretion from 12.28% to 9.32%, and 5-acetylamino-6-amino-3-methyl uracil excretion from 11.69% to 8.61%. The excretion of 1,7-dimethyluric acid excretion was increased from 6.05% to 9.0% but no significant differences were observed for the excretion of 3,7-dimethylxanthine or 3-methylxanthine. The authors concluded that, whilst the reduced plasma clearance of caffeine and its extended half life in women on oral contraceptives was reflected in reduced amounts and a slower rate of metabolite formation; the pattern of excretion was similar to that of ovulating females and normal male volunteers. Although no attempt has been made to measure the acetylated uracil metabolites of caffeine in this study, it is probable that the unaccounted metabolites in both groups (ca.

43%) represents the formation of such compounds, in particular, 5-acetylamino-6-formylamino-3-methyluracil. The majority of reactions involved in the metabolism of caffeine require catalysis by the hepatic microsomal cytochrome P-450 mono-oxygenase system. Although pregnancy in animals has been associated with a progressive decrease in hepatocellular cytochrome P-450 mediated oxidative drug metabolism, in man the effect appears to depend on the particular drug in question. For example, the oxidative biotransformation of promazine and meperidine decreases during pregnancy (Rudofsky et al., 1966), whilst phenobarbital and phenytoin metabolism is increased (Mygind et al., 1976). The steroid hormones of pregnancy, progesterone, oestrogens and their metabolites, have been shown to induce, repress, or directly inhibit selective mono-oxygenase reactions (Feuer et al., 1975; MacKinnon et al., 1977; Field et al., 1979). These modulations in hepatic metabolism in pregnancy have been suggested to be a conseqence of changes in microsomal membrane phospholipids and/or the cytochrome P-450 spin state equilibrium (Symons et al., 1982). The apparent impairment of 7-N demethylation of 1,7-dimethylxanthine but not of 3,7-dimethylxanthine may reflect heterogeneity of the response of hepatic cytochrome P-450 haemoproteins to pregnancy hormones.

7.3. ADVERSE EFFECTS OF CAFFEINE IN THE NEWBORN INFANT

Reports from epidemiological studies (Linn et al., 1982; Rosenberg et al., 1982; Leviton et al., 1984) on the effects of caffeine consumption during pregnancy, have found no association between caffeine intake and an increased risk of low birthweight, preterm birth or congenital abnormalities in newborn infants. However the presence of high caffeine levels in maternal serum indicates that in some cases foetal exposure to caffeine may be sufficient to produce overt pharmacological effects. The extent of this exposure has been investigated by monitoring caffeine levels in umbilical cord blood (Parsons et al., 1976; Soyka et al., 1981; Van't Hoff, 1982; breast milk (Horning et al., 1975; Berlin et al., 1984), and amniotic fluid (Horning et al., 1986).In particular, a report by Van't Hoff (1982) has indicated that umbilical cord blood caffeine levels in the United Kingdom may be higher than those reported elsewhere in the world.

The adverse reactions of caffeine in the newborn infant are mainly exaggerations of the known pharmacological effects. These include tachycardia, tachypnoea, seizures, opisthotonus, tremors, jitteriness, increased reflexes, hypertension and gastrointestinal disturbances (Howell et al., 1981). However, reports on the occurrence of adverse effects of caffeine in the neonate have been restricted to those resulting from its therapeutic application in the treatment of neonatal apnoea. Aranda et al., (1981) found no obvious cardiovascular, neurological or gastrointestinal toxicity with plasma concentrations up to 50 mg/l and only transient jitteriness

was observed with concentrations from 50 to 84 mg/l. Kulkarni and Dorand (1979) reported a case where marked jitteriness and tremors with increased reflexes and tachypnoea developed in a full term infant with asphyxia treated with 125 mg of caffeine sodium benzoate intramuscularly, producing caffeine plasma concentrations of 55 mg/l. Toxic reactions occurring in 4 other cases of asphyxia with doses of 36-136 mg/kg were described by Banner and Czajka (1980). The adverse effects included seizures, tremors, tachypnoea, hypertension, tachycardia (hypotension and bradycardia in one) and fever.

The objective of this study was to further assess foetal exposure to caffeine in the UK and to test a hypothesis that high caffeine levels in the newborn infant may be a causative factor in the aetiology of certain metabolic disorders eg. neonatal apnoea and jitteriness. Information is provided on the levels of caffeine and its dimethylxanthine metabolites in maternal serum, cord blood and breast milk and the values are compared with the results of previous studies performed in the USA and W. Germany. In addition an assessment is made of the relationship between caffeine levels in the newborn infant and the onset of attacks of neonatal apnoea and jitteriness. A clinical assessment of the infants studied was achieved by completion of a questionnaire using a score rating system of bodily symptoms.

7.3.1. Population study of caffeine levels in cord blood and breast milk.

Determinations were made of the concentrations of caffeine and its dimethylxanthine metabolites (theophylline, theobromine and 1,7-dimethylxanthine) in samples of umbilical cord blood and breast milk, obtained at random from newborn infants and nursing mothers, using the HPLC procedure previously described.

Results

The values obtained for caffeine in cord blood are compared in Table 7.3. with results obtained from other studies. In agreement with previous reports from the United Kingdom, the cord blood caffeine levels found in this study (mean = 3.8 ± SD. 3.15) were significantly higher than reported values for both the USA and West Germany; 5 % of the values being in excess of 10 mg/l. Significant levels of theobromine, 1,7-dimethylxanthine and theophylline were detectable in samples of cord blood, breast milk and maternal serum (Table 7.4), producing, in some cases, cumulative values of pharmacological significance.

7.3.2. Relationship between cord blood caffeine levels and symptoms related to caffeine toxicity.

Reported toxic effects of caffeine include tachycardia, agitation, tremors, jitteriness, convulsions and gastric disturbances. Whilst these sypmtoms have been described in neonates receiving caffeine therapeutically for neonatal apnoea, the possiblity of caffeine toxicity occurring as a

	Vermont USA	Marburg W.Germany	London U.K.	Leeds U.K.	Present study
Mean value (mg/l)	*	1.6	*	2.85	3.8
Range (mg/l)	0.01 - 5	0.2 - 8.9	up to 12.6	0 - 11	0.4 - 22.7
Number samples	327	69	55	113	101
% conc. > 3 mg/l	13	8	31	*	51
% conc. > 10 mg/l	0	0	1.8	0.9	5
Reference		Soyka et al. (1981)	Van't Hoff (1982)	Hartley et al. (1984)	

Table 7.3 Summary of reported surveys on caffeine concentrations in umbilical cord plasma.

	Theobromine mg/l	Paraxanthine mg/l	Theophylline mg/l	Caffeine mg/l	Total mg/l
a) Cord blood					
Mean	0.85	0.77	0.40	3.80	5.75
S.D.	0.52	0.46	0.25	3.15	3.72
Range (n = 101)	0 - 2.83	0 - 2.88	0 - 1.12	0.43 - 22.7	1.13 - 25.7
b) Breast milk					
Mean	0.78	0.77	0.19	2.80	3.92
S.D.	0.46	0.47	0.35	2.18	2.99
Range (n = 31)	0 - 2.39	0 - 1.75	0 -1.60	0 - 8.69	0.52 - 11.4

Table 7.4. Methylxanthine levels in cord blood and breast milk

result of excessive foetal exposure has recieved little attention. Since these symptoms are frequently present in newborn infants, often with unknown aetiology, the following study was performed to investigate their relationship to circulating levels of caffeine.

Methods

Infants were selected for this study by Paediatric medical staff and were divided into those with symptoms of caffeine toxicity (ie. jittery infants, n=12) and a control group of healthy newborn infants (n=10). Timed blood samples were collected and assayed for caffeine by the previously described techniques. An estimate of the infants caffeine level at birth was obtained by assuming a half-life of 80 hours (Aranda et al., 1981) and extrapolating the results. These values were then compared, for each infant, with information obtained from the completion of a questionnaire giving details of Apgar scores (Appendix 5.) and a rating score (1-5) for the degree of jitteriness, tachycardia, tachypnoea, and gastrointestinal disturbances. Any additional information considered relevant to the study was also recorded.

Results

A summary of the relationship between estimates of neonatal caffeine levels at birth and the presence of symptoms of caffeine toxicity is shown in Table 7.5. The mean caffeine level for the group of jittery infants was 4.83 mg/l (SD = 4.68) compared to 3.05 mg/l (SD = 2.98) for the control group. However this difference was not statistically significant using a Student's T-test ($P < 0.5$). No significant degree of

CODE	CAFFEINE mg/l	APGAR SCORE 1 min	APGAR SCORE 5 min	BODILY SYMPTOMS RATING Jit	Tcard	Tpnoea	GI	Total	Comments
1	0	9	9	0	0	0	0	0	Prem
2	0.25	-	-	0	0	4	0	4	RDS
3	0.65	2	7	0	0	3	0	3	Prem
4	0.70	6	8	3	0	2	0	5	Irritable
5	0.95	3	10	0	0	3	0	3	Prem
6	1.03	8	10	0	0	0	0	0	Prem SFD
7	1.12	3	8	3	0	0	0	3	
8	1.3	9	10	3	0	2	0	5	Prem
9	1.84	5	9	4	0	0	0	4	Prem
10	2.21	-	-	3	0	0	0	3	
11	2.6	8	9	3	0	1	0	4	Prem SFD
12	3.2	8	10	4	0	4	0	8	
13	3.38	7	9	0	0	5	0	5	Prem RDS
14	3.52	9	10	3	0	0	1	4	
15	3.68	9	10	0	0	4	0	4	
16	5.3	-	-	0	0	4	0	4	
17	6.92	9	10	2	0	4	0	6	
18	7.2	9	10	0	0	0	0	0	
19	8.1	9	10	0	0	0	0	0	
20	8.2	3	8	3	1	0	0	4	
21	10.24	7	9	Fits	0	1	0	?	Fits x 4
22	16.1	8	9	3	4	0	0	7	

Table 7.5 Relationship between serum caffeine levels at birth and symptoms of caffeine toxicity

Abbreviations: Jit = Jitteriness; Tcard = Tachycardia; Tpnoea = Tachypnoea
GI = Gastrointestinal; Prem = Premature delivery;
RDS =Respiratory distress syndrome; SFD = Small for dates.

For Apgar scoring system refer to Appendix 5.

correlation was found between caffeine levels and the ratings for jitteriness, tachypnoea or gastrointestinal disturbances. However, symptoms of tachycardia were present only in infants with caffeine levels in excess of 8 mg/l. There is some evidence to suggest from the results that low caffeine levels are associated with low 1 minute apgar scores. The mean scores for caffeine levels of less than 3 and greater than 3 were 6.18 (SD = 2.6) and 7.5 (SD = 2.0) respectively. However the majority of infants with caffeine levels of less than 3 mg/l were premature births.

DISCUSSION

The cord blood caffeine levels found in this study were significantly higher than those previously reported by Soyka et al.(1981); 5% being in excess of 10 mg/l. This confirms earlier reports from Britain (Van't Hoff, 1982; Hartley et al., 1984) suggesting that foetal exposure to caffeine may be higher than previously supposed and may lead to the manifestation of adverse effects in the newborn infant. These include tachycardia, tachypnoea, seizures, tremors, jitteriness, increased reflexes, hypertension and gastrointestinal disturbances. However, the limited report in this study, on the relationship between caffeine levels at birth and a score rating assessment of bodily symptoms, showed no evidence to implicate circulating levels of caffeine with attacks of either jitteriness or neonatal apnoea. Since it has been suggested that overt CNS stimulation of caffeine is negligible when caffeine concentrations are below 15-20 mg/l (Howell et

al., 1981), it may be argued that the levels found in this study were below those required to produce significant effects. The apparent correlation between the incidence of caffeine levels of less than 3 and low 1 minute Apgar scores is in agreement with a previous report by Soyka et al. (1981) although it is probable that the low Apgar scores found in this study were mainly attributable to premature births.

Whilst adverse effects from caffeine may be regarded as negligible when caffeine concentrations are below 15-20 mg/l, the delayed clearance of caffeine in the newborn infant (T 1/2 = approx. 100 hours) may result in these levels being achieved by an accumulation of caffeine as a result of breast feeding. The caffeine concentrations for breast milk are similar to those described in 1984 by Berlin et al. (0-7.2 mg/l) indicating that in some infants, dietary intake of caffeine from breast feeding may be 4 mg/day or more. This value approaches the therapeutic maintenance dose (2.5 mg/kg) used for the treatment of neonatal apnoea and suggests that caffeine withdrawal from intrauterine habituated infants, especially where breast feeding is delayed or artificial feeding instituted, could play a part in the aetiology of neonatal apnoea. This concept is supported by a recent report by Khanna and Somani (1984) of a premature infant (31 weeks gestation) who developed respiratory distress ascribed to transient tachypnoea of the newborn. The apnoea was first noted 4 days after birth and a blood sample collected on the fifth day, prior to caffeine therapy, showed a caffeine level of 40.3 mg/l. On the assumption that the average half life of

caffeine in the newborn infant is 100 hours, the authors calculated that caffeine levels in this infant at birth would have approximated 80 mg/l. Following a loading dose of 10 mg/kg caffeine, and with maintenance doses of 5 mg/kg every 12 hours, there were no further episodes of apnoea after the 6th postnatal day. A detailed history revealed that the mother had consumed up to 24 cups of coffee per day for several weeks prior to delivery.

Since the dimethylxanthine metabolites of caffeine are all pharmacologically active (Williams et al., 1978), the presence of significant levels of these compounds in maternal serum, cord blood and breast milk, indicates a requirement to assess the effect of total methylxanthine levels in pregnancy and in the newborn infant. The results for cord blood are in close agreement with those recently reported by Hartley et al. (1984) and indicate that total methylxanthines may reach clinically significant proportions. As this study shows some evidence to suggest that the ratio of caffeine and its metabolites in maternal serum or saliva are consistent with values found in cord blood and breast milk, the screening of maternal serum or saliva for caffeine content may therefore provide a useful index to increased foetal exposure, especially in cases where the dietary consumption by the mother is known to be high.

8. THE PHARMACOKINETICS AND METABOLISM OF CAFFEINE IN CHRONIC LIVER DISEASE

8.1. CHRONIC PARENCHYMAL LIVER DISEASE.

Chronic parenchymal liver disease may be broadly classified into chronic hepatitis and cirrhosis. In chronic hepatitis, the zonal architecture of the liver is preserved and is essentially reversible, whilst in cirrhosis, nodular regeneration leads to an irreversible loss of the essential hepatic arcitecture.

8.1.1. Chronic hepatitis.

By definition, this is a chronic inflammatory reaction in the liver continuing without improvement for at least 6 months. Histologically, chronic hepatitis is divided into chronic persistent hepatitis, chronic lobular hepatitis and chronic active hepatitis. Since necrosis is minimal in persistent and lobular hepatitis, the prognosis of these conditions is good. However, chronic active hepatitis is marked by the presence of portal tract infiltration of mononuclear cells, extending irregularly into the surrounding parenchyma so that swollen liver cells become isolated in the inflammatory cell infiltrate. This process of hepatocyte destruction is termed "piecemeal necrosis" and leads to septum formation linking portal tracts and central veins. The ensuing disruption of lobular architecture is accompanied by the development of regenerative nodules and thereby cirrhosis.

The characteristic histological changes of chronic active hepatitis may be seen following viral infection of the liver, in Wilson's disease, alcoholic liver disease, in association with the administration of certain drugs eg. methotrexate and methyldopa and in "lupoid hepatitis", an autoimmune disorder

associated with other auotimmune diseases. Diagnosis of chronic active hepatitis can only be made with confidence following biopsy of the liver. Standard liver function tests are of little value as plasma levels of bilirubin, aspartate transaminase and alkaline phosphatase show considerable variation. Gamma globulin levels may be significantly increased, particularly in lupoid hepatitis, but similar increases are also seen in a number of other chronic parenchymal diseases. In autoimmune chronic active hepatitis, the diagnosis is questionable unless there is a high titre (1:80 or more) of antinuclear factor or smooth muscle antibodies. Antinuclear antibodies to double stranded DNA and smooth muscle antibodies to actin are also of diagnostic value. Chronic active hepatitis due to viral infections eg. hepatitis B, can also usually be diagnosed on immunological grounds.

8.1.2. Cirrhosis

Cirhosis is defined as a diffuse process with widespread fibrosis and nodule formation following hepatocellular necrosis. Distortion of the liver architecture occurs resulting in portal-systemic vascular shunts. Predominant clinical features include portal hypertension, splenomegaly, ascites, jaundice, endocrine abnormalities, haemorrhagic tendancies and hepatic encephalopathy. Classification may be made into three types, namely micronodular, macronodular and mixed cirrhosis. Micronodular cirrhosis is characterised by the presence of thick, regular connective tissue septa; the

small regenerative nodules varying little in size to the original lobules, but involving every lobule. The micronodular liver may represent impaired capacity for regrowth as in alcoholism, malnutrition, old age or anaemia. In macronodular cirrhosis the connective tissue septa vary in thickness and the nodules show marked differences in size, the larger ones containing histologically normal lobules. Mixed cirrhosis shows features of both micronodular and macronodular cirrosis. The aetiology of cirrhosis may be summarised as follows:-

1. Viral hepatitis - types B; non-A, non-B.
2. Alcohol
3. Metabolic eg. haemachromatosis, Wilson's disease, alpha 1 anti-trypsin deficiency, diabetes mellitus, galactosaemia, congenital tyrosinosis, type IV glycogenosis.
4. Prolonged cholestasis - intra and extra-hepatic.
5. Hepatic-venous outflow obstruction eg. veno-occlusive disease, Budd-Chiari syndrome, constrictive pericarditis.
6. Disturbed immunity - lupoid hepatitis.
7. Toxins and therapeutic agents eg. methotrexate, amiodarone.
8. Intestinal bypass.
9. Indian childhood cirrhosis.
10. Cryptogenic cirrhosis.

The diagnosis of cirrhosis can often be made confidently on

purely clinical grounds. Biochemical investigations are of little diagnostic value and, in the absence of classical clinical features, a liver biopsy is required to establish a firm diagnosis. Liver biopsy also allows identification of the cause of cirrhosis in haemochromatosis, Wilson's disease, alpha-1-antitrypsin deficiency etc. In decompensated cirrhosis serum bilirubin levels are increased, albumin is depressed and gamma-globulin raised. Alkaline phosphatase activity is usually raised to about twice normal although occasionally very high readings may be found. Serum transaminase levels may also be increased; prothrombin times are prolonged and do not return to normal with vitamin K therapy. There is usually a mild normocytic (occasionally macrocytic), normochromic anaemia; gastrointestinal bleeding may produce a hypochromic anaemia. Leucocyte and platelet counts are reduced and the bone marrow is macronormoblastic. Excess urobilinogen is present in the urine and bilirubin may also be detecetd if the patient is jaundiced. In the presence of ascites, urinary sodium excretion is diminished and in severe cases, less than 4 mmol/l are excreted daily.

8.1.3. Primary Biliary Cirrhosis.

This condition is associated with progressive granulomatous destruction of intra-hepatic bile ducts, leading eventually to the development of cirrhosis. Pruritis is the most common initial complaint and may precede jaundice by months or even years. The disease predominantly affects women, usually in middle age. Whlist the aetiology remains unknown, the disease

is associated with a profound immunological disturbance which has been related to the bile duct destruction. The final event appears to be an attack by cytotoxic lymphocytes on biliary epithelium. Hepatomegaly is virtually constant and splenomegaly occurs as portal hypertension develops. The disease usually lasts for 5 to 10 years terminating in liver failure or alimentary bleeding.

In its early stages, before the onset of jaundice, patients may have an isolated elevation of alkaline phosphatase and liver biopsy reveals the characteristic granulomata and bile duct damage. Eventually there may be deep jaundice, a moderate elevation of aminotransferases, raised alkaline phosphatase activity and a severe hypercholesterolaemia. Whilst the diagnosis is frequently made on clinical grounds, the condition may mimic extrahepatic biliary obstruction. The finding of a mitochondrial antibody is of significant value in establishing a diagnosis since it is found in almost all cases. Serum IgM levels are usually elevated in patients with primary biliary cirrhosis. IgM levels tend to be higher than IgG levels, which is reversal of the findings in patients with autoimmune chronic active hepatitis. However, this finding is of limited diagnostic value as IgM increases may show considerable variation.

8.1.4. The pathology of alcoholic liver disease.

The major factor in the pathogenesis of alcohol-related liver injury is the direct toxic effect of ethanol and its metabolites on the liver cell. Alcohol cannot be stored and

obligitaory oxidation must take place, predominantly in the liver where it is coverted to acetaldehyde, catalysed by the enzyme alcohol dehydrogenage. Acetaldehyde is then converted to acetyl CoA with acetaldehyde dehydrogenase acting as a coenzyme. This may then be further metabolized to acetate and oxidised to carbon dioxide and water in the citric acid cycle. The oxidation of ethanol is associated with a number of metabolic changes within the liver resulting in the generation of an excess of reducing equivalents, particularly the reduced form of nicotinamide adenine dinucleotide (NADH). The increased NADH:NAD ratio results in alterations in lipogenesis and lipid oxidation which favour hepatic triglyceride accumulation. Indirectly, the activity of the citric acid cycle is depressed resulting in decreased adenosine triphosphate (ATP) levels. In addition, acute ethanol intoxication causes abnormal fragility of cell lysosomes resulting in the release of lysosomal enzymes into the serum.

In Western countries the incidence of cirrhosis can be directly related to the amount and duration of alcohol ingested. There also appears to be an increased susceptibility of alcohol-induced damage in females (Morgan and Sherlock, 1977). Using the criteria that a bottle of spirits contains 240g of alcohol, the safe limit for daily consumption is now considered to be 60g in men and as little as 20g in women. Alcoholics with cirrhosis usually show a history of consuming about 190 g of alcohol daily for at least 10 years. The type of alcohol is not an important factor in determining the type and extent of damage. Although the total alcohol intake may be

similar, the steady daily imbiber is much more at risk than the spree drinker, who probably allows some liver repair to take place between bouts of drinking.

Hepatic injury resulting from long term excessive ingestion of alcohol may range from trivial accumulation of fat to irreversible cirrhosis. The classification of alcohol-induced lesions is based primarily on histological features (Sherlock, 1982; Maddrey, 1984) and includes fatty liver, alcoholic hepatitis, and alcoholic cirrhosis. Although a combination of these elements is present in most individuals, the necrosing and inflammatory lesions of alcoholic hepatitis are accepted as being the most importnat precursors to the development of cirrhosis.

Alcohol-induced fatty liver

The consumption of alcohol leads to a rapid accumulation of fat in liver cells but it is considered to be a reversible lesion and not a precursor for a more severe disease. Although many patients with alcohol-induced fatty liver show no symptoms, hepatomegaly is usually present and some patients may develop jaundice. Nausea, vomiting, ascites, oedema, and a tendancy to bleeding may also be present.

Alcoholic hepatitis

This is an inflammatory lesion characterized by alterations in cell size, infiltration of the liver with polymorphonuclear leucocytes, cell necrosis, and in many patients the presence of alcoholic hyalin. It frequently appears as severe hepatic decompensation after particularly heavy drinking, perhaps precipitated by vomiting, diarrhoea, an acute infection, or

prolonged anorexia. In its most florid form, alcoholic hepatitis presents as an overwhelming illness with fever, leucocytosis, abdominal pain, nausea, vomiting, bleeding, ascites and hepatic encephalopathy. Portal hypertension may occur even in patients in whom there is little or no evidence of cirrhosis. This may be reversible if and when the acute injury resolves. Nutritional changes of alcoholism, including peripheral neuritis, folic acid deficiency and scurvy may also be found.

Biochemically, serum transaminase and alkaline phosphatase levels are elevated. The concentration of serum albumin is usually low whilst gamma globulins levels are raised; these return to normal on improvement of the patients condition. Serum cholesterol levels may be very high and the liver serum lipaemic, often coinciding with pancreatitis. Hypokalaemia may also be prominant, epecially if there is diarrhoea.

Alcoholic cirrhosis

Cirrhosis in alcoholics results mainly from a progression of alcoholic hepatitis although the possiblity that it might follow alcohol induced fibrosis has also been investigated (Van Waes and Lieber, 1977). Furthermore, Goldberg (1977) has suggested that liver biopsy in alcoholics may reveal types of hepatitis other than alcoholic hepatitis. These include chronic active hepatitis and viral hepatitis which may provide additional routes to cirrhosis. Primary hepatocellular carcinoma (PHC) is predominantly associated with hepatitis B virus related cirrhosis but it is reported to occur in 3-15% cases of alcoholic cirrhosis (MacSween, 1982).

Laboratory investigation of alcoholic liver disease

The usual biochemical investigations are of little value in the detection and assessment of severity of alcoholic liver disease. Subsequently early diagnosis often depends on the patient's history which is notoriously unreliable, frequently resulting in a denial of excessive alcohol intake or considerable underestimation of the amount consumed. Elevated blood alcohol levels will only indicate recent excessive ingestion and are subsequently only of value as a follow up investigation for non-compliance with abstinence. Serum aspartate transaminase (AST) or alanine transaminase (ALT) are useful screening tests in assessing liver disease but they show poor correlation with the extent of injury. The AST:ALT ratio is useful in differentiating alcoholic from viral hepatitis, where 70% of patients with alcoholic liver disease show ratios greater than 2. This may be related to alcoholic damage to mitochondria or smooth muscle, with predominant release of AST.

Gamma glutamyltranspeptidase(γGT) levels are frequently used in the screening for alcohol abuse (Rosalki and Rau, 1972). However, there are conflicting reports on their degree of correlation with the extent of alcohol-related liver damage. Serum glutamate dehydrogenase is also a reliable marker for liver-cell necrosis but it is not specific for alcoholism. Similarly, the serum ratio of alpha-aminobutyric acid to leucine is elevated in chronic alcoholics, but it is a relatively insensitve index of hepatocellular damage rather than alcoholism. Serum IgA and alkaline phosphatase values may

also be markedly increased. Prolongation of the prothrombin time is perhaps the most useful index of the severity of alcoholic liver injury. A prothrombin value greater than 5 seconds over the control value after vitamin K therapy is an important indicator of severe damage.

8.1.5 Quantitative assessment of hepatocellular function

In spite of the existence of a number of quantitative tests of liver function, they have failed to gain wide acceptance and are restricted mainly to research applications. This is mainly because they tend to be cumbersome, expensive, time consuming and frequently employ toxic test substances.

Quantitative assessment of hepatocellular function may be achieved by measuring the galactose elimination capacity (Tygstrup, 1964). This is impaired in hepatocellular disease, and unlike glucose tolerance tests, the results are independent of insulin secretion. Galactose removal by the liver has also been used to measure hepatic blood flow. An index of the functional microsomal mass and viable hepatic tissue can be assessed by orally administering carbon-14 labelled aminopyrine and monitoring, for intervals over a two hour period, the amount of expired labelled carbon dioxide (aminopyrine breath test). Aminopyrine undergoes oxidative demethylation by the liver microsomal cytochrome p450 system. The test is mainly used to assess therapy and the effects of drugs on the hepatic microsomal enzyme system rather than for screening or diagnosis (Bircher et al., 1976). Labelled caffeine, galactose and phenacetin have also been used as

breath test substances, but the complexity and cost of these tests has limited their application.

Hepatocellular damage in viral hepatitis or chronic liver disease is associated with an increase in the concentration of serum bile acids (Ferraris et al., 1983). The measurement of these compounds eliminates the requirement for ingestion of potentially toxic substances. In addition, they may be easily determined by the use of radioimmunoassay procedures and require only a single fasting blood sample. However, bile acids are endogenous compounds and their fate depends on many variables, including hepatic uptake, conjugation, biliary excretion, gallbladder emptying and the influence of gut flora. The importance of these factors on serum bile acid levels is unclear and the lack of simple pharmacokinetic modelling prevents a rational interpretation of their results.

8.1.6. Effects of liver disease on drug disposition

It is well established that the clearance of a number of drugs in cirrhosis may be significantly impaired (Shand, 1975; Wilkinson and Schenker, 1976; Pessayre et al., 1978). Although the mechanisms responsible are not well established, the major biological determinants of hepatic drug clearance are hepatic blood flow, the activity of the overall hepatic elimination process (intrinsic clearance), the extent of protein binding of the drug in plasma, and the anatomical arrangement of the hepatic circulation (Wilkinson and Shand, 1975). The relative significance of these factors on the elimination of a particular drug depends largely on the hepatic extraction

ratio which may be estimated using the equation: E = (Cp-Ch)/Cp, where Cp and Ch are the concentrations in the peripheral and hepatic venous blood. Hepatic clearance is defined as the product of the extraction ratio (E) and the liver blood flow (Q). By combination of these two values, the intrinsic hepatic clearance (IHC) may be calculated which is a mathematical concept expressing the maximal ability of the liver to remove a drug.

$$IHC = E.Q/(1-E)$$

Calculation of the liver blood flow, hepatic extraction ratio and hepatic clearance may be achieved by catheterization of the hepatic vein followed by infusion of indocyanine green (ICG). In order to compensate for a reduction in hepatic extraction ratio of ICG in cirrhotics, constant infusion methods are generally employed.

The elimination of highly extracted drugs, such as propanolol and chlormethiazole (Pessayre, 1978), is influenced mainly by liver blood flow rather than intrinsic clearance. These drugs exhibit significant presystemic (or first-pass) hepatic elimination, during their passage from the gut to the systemic circulation in portal venous blood, and a reduction in intrinsic clearance, or extrahepatic shunting of blood in the mesenteric vein, will greatly increase their bioavailability after oral administration. For poorly extracted drugs, such as theophylline, antipyrine, tolbutamide and phenytoin, hepatic clearance is independant to liver blood flow but highly sensitive to changes in intrinsic clearance rate. First pass elimination effects of these drugs are minimal after oral

administration and most of the administered dose will reach the systemic circulation intact. Variations in the intrinsic clearance will not significantly affect this bioavailabilty.

The effect of the extent of protein binding of a drug also depends on the extraction ratio and the fraction of drug present in the blood in the unbound form. If the extraction ratio of total drug exceeds the free-fraction in the blood, elimination will be non-restrictive. The systemic clearance of total drug will be unaffected by alterations in binding whilst that of the free drug will be increased as binding increases, resulting in a tendancy for the half-life to decrease. On the other hand, if elimination is restrictive, total systemic clearance will be dependent on the extent of binding whereas free drug levels will not. Subsequently half life will be prolonged as binding is increased.

It is frequently assumed that altered elimination of drugs in liver disease is associated with an impaired activity of the cytochrome P-450 drug metabolizing system. However, a study by Farrell et al. (1979), using hepatic tissue obtained at biopsy from 69 patients, clearly established that the concentration of cytochrome P-450 and the activity of hydrocarbon hydroxylase are normal in mild liver disease and only decreased in the presence of extensive hepatocellular necrosis or active cirrhosis. In addition, the activity of another microsomal oxidase enzyme, ethylmorphine demethylase, remained unaltered even with severe liver disease.

In chronic liver disease there is a roughly parallel reduction in the clearance of both poorly and effeciently

extracted drugs.

8.1.7. The metabolism of caffeine in liver disease

Caffeine disposition in man occurs almost exclusively by hepatic demethylation and oxidation with unchanged caffeine forming less than 2% of the urine metabolites. Studies have shown (Arnaud and Welsch, 1981; Callahan et al., 1982) that during the first two hours after caffeine administration approximately 72 % undergoes initial demethylation to form 1,7- dimethylxanthine (paraxanthine). Demethylation also occurs at positions 1-N and 7-N and these reactions, catalysed by one or more of the cytochromes P-450 yield formaldehyde as secondary product. The effects of liver disease on caffeine elimination have been studied by monitoring plasma clearance following oral ingestion of caffeine (Statland et al., 1980; Desmond et al., 1980), and by measurement of $^{14}CO_2$ in breath following I.V. injection of [3-methyl-^{14}C] labelled caffeine (Wietholtz et al., 1981; Renner et al., 1984). Studies have shown that in patients with cirrhosis, there is a highly significant reduction in the clearance of caffeine with a prolongation of half life. Statland et al. (1979) described serum caffeine half-lives of 60 and 168 hours in two patients with alcoholic liver disease, compared to a mean value of 5.7 hours in 3 healthy subjects. In addition they investigated the effects of diazepam on caffeine metabolism which is also metabolized by the liver via demethylation and may compete with caffeine for metabolism. However, no difference was observed in the half life values of the normal subjects

measured with and without diazepam ingestion. Desmond et al. (1980) compared plasma elimination half-lives of caffeine in 15 normal subjects and in 10 patients with cirrhosis. In the normal subjects, peak caffeine levels appeared 30-60 minutes ater oral ingestion of caffeine (250 mg), after which, the concentration declined momo-exponentially with a mean half life of 5.2 hours. There were no age related differences in the half life, volume of distribution or clearance values. In the cirrhotic group, the half life of elimination was longer, although not statistically significant. This could not be accounted for in terms of altered pharmacokinetic parameters as the peak levels and time taken to reach peak levels were similar in both groups. Two of the cirrhotic patients had caffeine present in their zero sample, collected 3 days following caffeine withdrawl, and although their levels rose after administration of caffeine, they did not decline significantly over 48 hours. The total clearance of caffeine was reduced by 35% in the cirrhotic group, compared to the controls.

An assessment of the cytochrome P-448 dependent liver enzyme system was shown by Wietholtz et al. (1981) using a caffeine breath test, involving the combined administration of [7-methyl-^{14}C] and [3-methyl-^{14}C] caffeine. $^{14}CO_2$ exhalation curves in cirrhotics were clearly different from those in normal volunteers, being characterized by a slower rise and lower specific activity. The highest yield of $^{14}CO_2$ was obtained following administration of [3-methyl-^{14}C] caffeine and the average $^{14}CO_2$ rate was doubled in smokers. In a more

recent study, Renner et al. (1984) investigated the effects of liver disease on caffeine clearance and the exhalation of $^{14}CO_2$ following I.V. ingestion of 2 μCi of [3-methyl-^{14}C] caffeine together with 125 mg of unalbelled caffeine in 15 patients with cirrhosis, 11 subjects with miscellaneous liver disease and 10 normal volunteers. Their results confirmed the contention that caffeine breath tests closely reflect the plasma clearance of the compound, the cummualtive $^{14}CO_2$ production and specific activity decreasing in parallel with clearance. The cirrhotic group were characterized by a highly significant reduction in clearance and prolongation of half life although the volume of distribution was virtually unchanged.

	Normal group	Cirrhotic group
Half life (hours)	3.8 + 0.9	13.7 + 13.0
Clearance (ml/min/kg)	2.02 + 0.68	0.76 + 0.4
Vol.distribution (l/kg)	0.64 + 0.13	0.57 + 0.16

Patients with miscellaneous liver disease eg. non-cirrhotic alcoholic liver disease, chronic persistent hepatitis, non-cirrhotic chronic active hepatitis, haemaochromatosis and Gilbert's syndrome, exhibited only small changes in clearance and half life. This study also demonstrated a surprisingly close hyperbolic relationship between clearance and fasting plasma concentrations, suggesting that the latter may serve as a guide to the severity of liver disease.

There have been no reported studies on the urinary excretion of caffeine metabolites in liver disease. However, a

study on metabolite excretion following theophylline administration (Staib et al., 1980) demonstrated altered patterns in acute hepatitis, cholestasis, compensated and decompensated cirrhosis. Compared to a control group, there was an increase in the excretion of 1-methyluric acid and a concomitant decrease of 1,3-dimethyluric acid and 3-methyl xanthine. This suggests that in liver disease the 1-N demethylation of theophylline is inhibited and is compensated by a shift towards 1-methyluric acid formation. A recent report by Jost et al. (1985) described the use of measuring overnight salivary caffeine clearance (SCC) for assessing the severity of liver diseases in children. Following a dose of 4 mg/kg b.w., the SCC was significantly reduced in 33 patients with documented cirrhosis of alcoholic or post hepatic origin (0.58 ± 0.4 ml/min/kg) compared with values obtained from a group of 20 non-smoking adult volunteers (1.65 ± 0.4).

8.1.8. Outline of study

The aims of this study were as follows :-

1. To investigate the elimination of caffeine in compensated and decompensated chronic liver disease by comparing pharmacokinetic data, obtained from the measurement of caffeine levels in serum and saliva following oral ingestion of anhydrous caffeine (400 mg), with values previously obtained for a group of normal healthy volunteers (Section 5.).
2. To provide information on the rate of appearance and elimination of the dimethylxanthine metabolites of caffeine in serum and saliva
3. To investigate the biotransformation of caffeine in chronic liver disease by comparing urinary metabolite excretion patterns in patients with compensated and decompensated liver disease, with those found in normal subjects.
4. To assess the suitability and sensitivity of caffeine pharmacokinetic studies and/or the measurement of urine metabolites for assessing the severity of hepatocellular dysfunction.
5. To determine the value of monitoring overnight salivary caffeine clearance as a sensitive index of hepatic function.

Prior to commencing this study, a protocol was submitted to and approved by the Royal Free Hospital Medical Ethics Committee.

8.2. METHODS AND MATERIALS

Subjects selected for this study were divided into two categories based on a clinical assessment of their condition. Group 1 consisted of patients with well compensated cirrhosis (n=10) and Group 2 consisted of patients with clinically decompensated cirrhosis (n=8). Classification was made using a score rating of liver dysfunction based on a system previously described by Pugh et al., (1973). The parameters considered were the extent of encephalopathy and ascites and the values obtained for serum bilirubin, albumin and prothrombin time. A weighting system was used whereby 1,2, or 3 points were scored for increasing abnormality of each of the five parameters (Appendix 6.). Thus for patients with good hepatic function the total score was 5 points whilst those with poorer hepatic reserve had scores of up to 15 points. An allowance was made for patients with primary biliary cirrhosis where the level of bilirubin is normally out of proportion with other evidence of hepatic failure. An additional statistical analysis of the results was performed by dividing the subjects into 3 Pugh score groups: Group A, 5-6 points; Group B, 7-9 points; and Group C, 10-15 points.

Patients with alcoholic liver disease had all previously abstained from alcohol consumption, the minimum periods being 1 year in the compensated group and 3 weeks in the decompensated group. Patients with non-alcoholic liver disease and the control subjects consumed less than 5g alcohol per day. On enrollment into the study, a questionnaire was completed giving details of the subject's age, sex, nationality, weight,

Subject	Sex	Age (yrs)	Diagnosis	Drug therapy	Bili 5-17	AST 30-50	Alb 30-50	Creat 60-120	PT 11-14	Ascites 0;+;++	PSE I-IV	Pugh grade	Pugh score
Compensated liver disease patients													
JC	F	52	AC	1	6	14	47	70	12	0	0	A	5
IC	F	56	AC	2	7	20	46	64	12	0	0	A	5
PI	M	52	AC	3	18	76	45	85	13	0	0	A	5
EM	F	40	AC	4	19	24	40	71	17	0	0	A	5
EP	F	43	AC	5	8	27	46	50	12	0	0	A	5
AS	F	54	AC	5	7	17	46	65	15	0	0	A	5
BW	M	60	AC	8,9,10	15	15	44	82	15	0	0	A	5
NW	F	68	AC	1,7,11	5	33	39	69	14	0	0	A	5
HO	F	71	AC	5,8,20,23,24,25	21	27	38	70	15	0	0	A	5
AC	M	53	AC	1,6,7	52	45	43	57	17	0	0	B	7
Decompensated liver disease patients													
MS	F	42	AAC	7,5,21,24	67	100	29	62	15	0	0	B	8
FC	M	68	PBC	1,5,12-16	67	64	33	98	12	+	II	B	8
GG	F	63	PBC	4,7,13,15,26-30	228	152	34	151	12	0	I-II	B	9
BJ	F	41	PBC	5,7,15,17,21,22	396	247	36	90	12	+	I-II	B	9
FF	M	62	AC	1,13,14,28,31	33	27	33	157	18	++	I	C	10
VD	M	64	AC	5,15	312	103	28	104	19	+	I-II	C	11
CB	M	78	PBC	5,13,15,32,33	132	214	23	100	14	++	III	C	12
DH	M	64	AC	1,13,14,17-20	41	48	27	105	20	++	II	C	12
AS	F	59	PBC	5,13,15,24,34,35	444	150	38	120	19	+++	II-III	C	12

Table 8.1 Pugh Score Rating for Liver Disease Patients

Key to drug therapy

1. Folic acid	2. Vitamin E	3. Indocid	4. Lorazepam	5. Spironolactone	6. Glibenclamide	
7. Orovite	8. Slow K	9. Allopurinol	10. Tranxene	11. Malotilate	12. D-penicillamine	
13. Lactulose	14. Thiamine	15. Ranitidine	16. De-Nol	17. Cholestyramine	18. Frusemide	
19. Amiloride	20. Ferrous SO4	21. Ossopan	22. Nystatin	23. Cimetidine	24. Prednisolone	
25. Motilium	26. Dibenyline	27. Stanozolol	28. Paracetamol	29. Mag.trisilicate	25. Dorbanex	
31. Ampicillin	32. Nitrofurantoin	33. Quinine SO4	34. Cefoxitin	35. Metronidazole		

Key to abbreviations used AC - Alcoholic cirrhosis PBC - Primary biliary cirrhosis
Bili - Bilirubin, AST - Aspartate transaminase, Alb - Albumin, Creat - Creatinine, PT - Prothrombin time, PSE - Portal systemic encephalopathy

clinical history, normal dietary caffeine intake, present drug therapy, smoking habits and any other information considered relevant to the study (Appendix 6). A summary of the subject characteristics and Pugh score ratings is shown in Table 8.1. Subjects were requested to refrain from consuming all forms of caffeinated beverage, chocolate, caffeine containing foods and all but essential medications for a period of 72 hours before the study and for its duration.

On day 1 of the investigation, basal samples of serum, saliva and urine were collected prior to the administration of an oral dose of anhydrous caffeine (400 mg contained in a gelatine capsule), taken together with approximately 150 ml of water. Additional samples of blood and saliva were then collected concurrently at 20 mins, 40 mins, 1hr, 2hr, 3hr, 4hr, 6hr, 8hr, 12hr, 24hr and 48hr post caffeine ingestion. Total urine outputs were collected for the time intervals 0-24 hours and 24-48 hours. In anticipation of a delayed clearance of caffeine in patients with decompenstaed liver disease, further samples of blood and saliva were taken from these subjects at 72hr and 96hr and where possible, additional collections of urine were obtained for the periods 48-72 hr and 72-96 hr. Following centrifugation of the blood samples, the serum was separated and stored along with the saliva samples at - 20^{o} C. Total urine volumes were recorded and aliquots (30 ml) were stored in a similar manner.

Using the analytical procedures previously described, the levels of caffeine and its dimethylxanthine metabolites were determined in the samples of serum and saliva and the urine

samples were assayed to quantitate the excretion of methyl-xanthine metabolites. The serum/salivary pharmacokinetic data, derived using a computer based non-linear kinetics programme, and metabolite excretion profiles, for both groups of liver disease patients were compared with results obtained for a group of normal volunteers previously studied using a similar protocol. Statistical analysis was made using a Student's t-test for unpaired data; $P < 0.05$ was regarded as being statistically significant.

RESULTS

a) Serum/saliva kinetic data for caffeine

The caffeine levels in serum and saliva showed a linear relationship for both groups of liver disease patients. The mean coefficient of correlation for the compensated group was 0.953 (SD. 0.0056) compared to 0.928 (SD. 0.037) for the decompensated group. The mean saliva/serum concentration ratios of caffeine for the compensated and decompensated cirrhotic groups were 0.84 and 1.07 respectively. In comparison with values obtained for a group of normal volunteers (mean ratio = 0.71), the ratios for the compensated cirrhotic subjects were not statistically different ($P < 0.1$), whilst those for the decompensated subjects were significantly higher ($P < 0.01$). Elimination curves of the mean salivary caffeine levels for both groups of patients are compared in Fig 8.2 with mean values previously obtained from normal volunteers receiving a similar oral dose of caffeine.

The pharmacokinetic data, derived from elimination curves

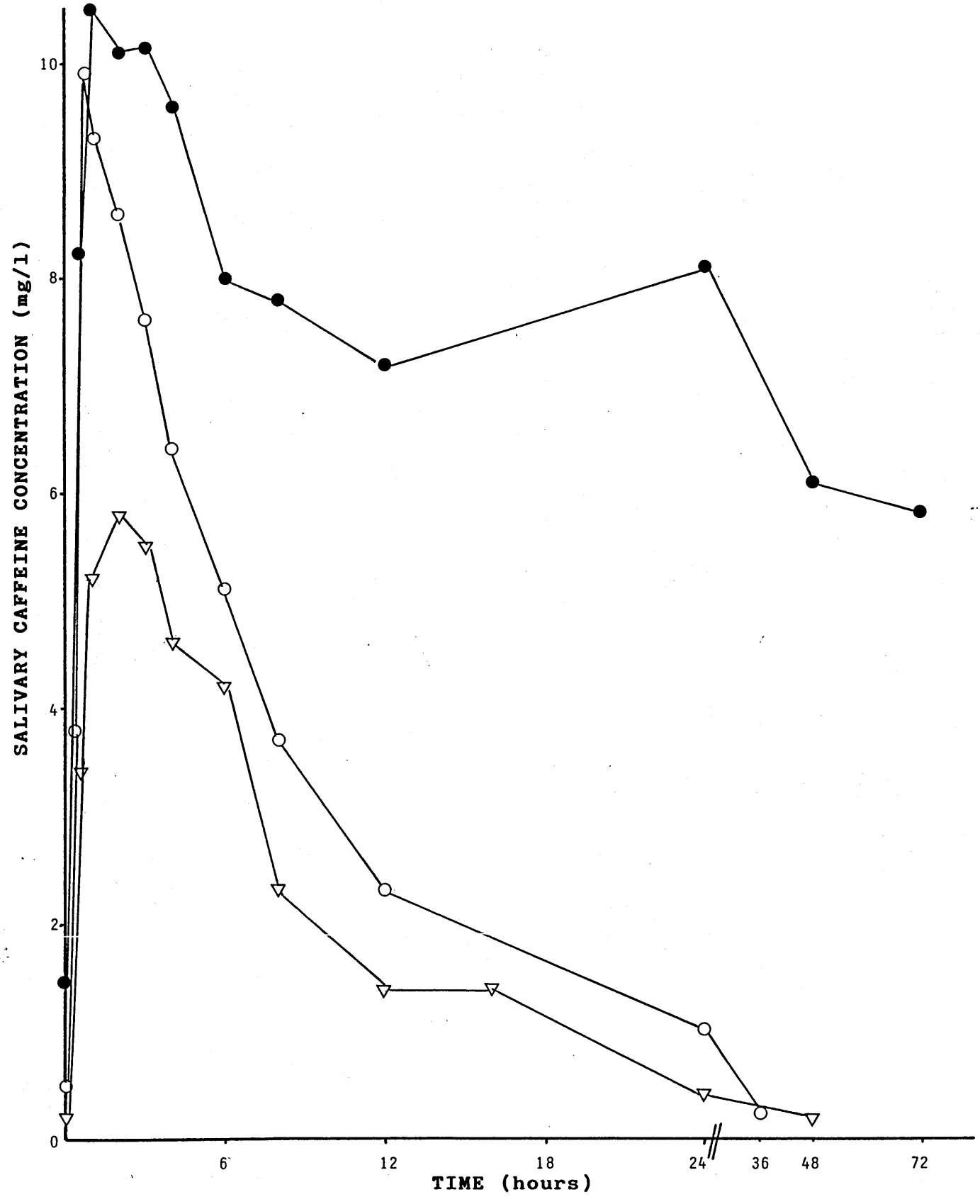

Fig 8.1 Mean caffeine levels in saliva following an oral dose of caffeine (400mg) in normal volunteers (▽), compensated (○) and decompensated (●) alcoholic cirrhosis patients.

Subject	Pugh Score (5 - 15)	Cmax mg/l		Cmax Time(h)		T1/2 (h)		k.el ($h.^{-1}$)		Vd ($ml.kg.^{-1}$)		AUC ($mg.l.^{-1}$ $h.^{-1}$)		Cl ($ml.kg.^{-1}$ min^{-1})	
		Ser	Sal	Ser	Sal	Ser	Sal	Ser	Sal	Ser	Sal	Ser	Sal	Ser	Sal
a) Compensated Cirrhotics															
JC	5	13.1	9.7	2	2	2.9	2.6	0.24	0.27		0.23	78.2	57.6	1.56	2.13
IC	5	14.1	12.1	0.33	0.33	3.0	2.9	0.23	0.24	0.49	0.47	74.3	60.8	1.89	2.13
PI	5	11.8	10.9	1	1	5.3	3.3	0.13	0.21	0.44	0.38	99.4	76.2	0.99	1.19
EM	5	8.0	8.2	2	3	4.6	3.9	0.15	0.18	0.33	0.19	89.1	83.4	1.07	1.04
EP	5	8.4	10.7	0.66	0.66	1.2	1.1	0.59	0.61	0.26	0.32	27.2	29.3	4.55	4.52
AS	5	13.1	-	0.66	-	4.8	-	0.15	-	0.36	-	96.6	-	0.87	-
BW	5	9.4	9.1	3	3	9.8	8.4	0.07	0.08	0.47	0.49	159.6	135.9	0.62	0.73
NW	5	14.1	15.4	0.66	0.66	5.7	4.1	0.12	0.17	0.41	0.39	128.9	97.4	0.66	0.88
HO	5	16.5	16.5	0.66	0.66	9.3	7.0	0.07	0.10	0.32	0.35	255.8	201.7	0.36	0.46
AC	7	10.0	9.8	1	1	5.0	4.3	0.14	0.16	0.46	0.46	86.0	75.1	1.17	1.35
Mean		11.85	11.38	1.20	1.37	5.16	4.18	0.189	0.224	0.39	0.36	109.5	90.8	1.49	1.6
S.D.		2.81	2.83	0.85	1.04	2.68	2.24	0.152	0.157	0.08	0.10	61.9	50.8	1.22	1.24
b) Decompensated Cirrhotics															
MS	8	9.0	9.2	2	2	14.1	25.8	0.05	0.03	0.42	0.48	204.1	333.4	0.36	0.22
FC	8	9.0	8.2	2	2	13.1	-	0.05	-	0.49	-	206.5	-	0.47	-
GG	9	14.3	16.4	2	1	9.7	11.0	0.07	0.06	0.63	0.57	213.1	262.6	0.76	0.61
BJ	9	12.9	-	4	-	10.8	-	0.06	-	0.47	-	244.5	-	0.50	-
FF	10	-	18.1	-	0.5	-	9.8	-	0.07	-	0.35	-	226.7	-	0.49
VD	11	14.9	-	4	-	100.0	150.5	0.01	-	0.18	-	>2000>	>2000	0.04	-
DH	12	-	12.4		0.5		44.5		0.016		0.58		626		0.16
CB	12	14.6	-	6	-	60.2	-	0.01	-	*	-	>2000		0.02	-
AS	12	*	*	*	*	>100.0	>100.0	0.005	0.005	*	*	>2000	>2000	0.01	0.01
Mean		12.45	12.86	3.33	1.2			0.036	0.031	0.47	0.47			0.360	0.252
S.D.		2.76	4.33	1.63	0.76			0.027	0.028	0.10	0.11			0.288	0.248

Table 8.2 Pharmacokinetics of caffeine in compensated and decompensated cirrhosis.

Key to abbreviations

Cmax - Peak caffeine concentration, T1/2 - Half-life, k.el. - Elimination constant, Vd - Apparent volume of distribution, AUC - Area under elimination curve, Cl - Clearance

of caffeine in serum and saliva, for both groups of patients is summarized in Table 8.2 and in Fig 8.2 and Fig 8.3. Statistical analysis of the results for serum vs saliva using Student's t test showed no significant differences between the values obtained for the elimination constants ($P < 0.9$), apparent volumes of distribution ($P < 0.9$) and total body clearance ($P < 0.8$). Corresponding coefficient of correlation values were 0.985, 0.935 and 0.985 respectively.

Basal salivary caffeine levels for the compensated cirrhotic group (0.49 + SD. 0.78) were similar to those found for normal controls (Less than 0.3 mg/l). Whilst the mean value for patients with decompensated liver disease was higher (1.46 + SD. 2.12), the values were not significantly different ($P < 0.1$). However, three of the decompensated group (J.B, V.D, and A.S) had levels in excess of 2 mg/l. Compared to the normal control group, the peak levels of caffeine in saliva for both groups of liver disease subjects were significantly higher ($P < 0.01$). Although the time required to reach peak levels was lower in the liver disease patients, the differences between the three groups were not statistically significant. For one subject (A.S), salivary caffeine levels remained in excess of 12 mg/l from 2 hr post dose, reaching a maximum value of 16.5 mg/l after 72 hours; data on this subject was excluded from the calculation of peak caffeine values.

Compared to the group of normal volunteers, patients from the compensated cirrhotic group showed a significant decrease in the apparent volume of distribution ($P < 0.002$) assessed from the salivary data. However, the salivary pharmacokinetic

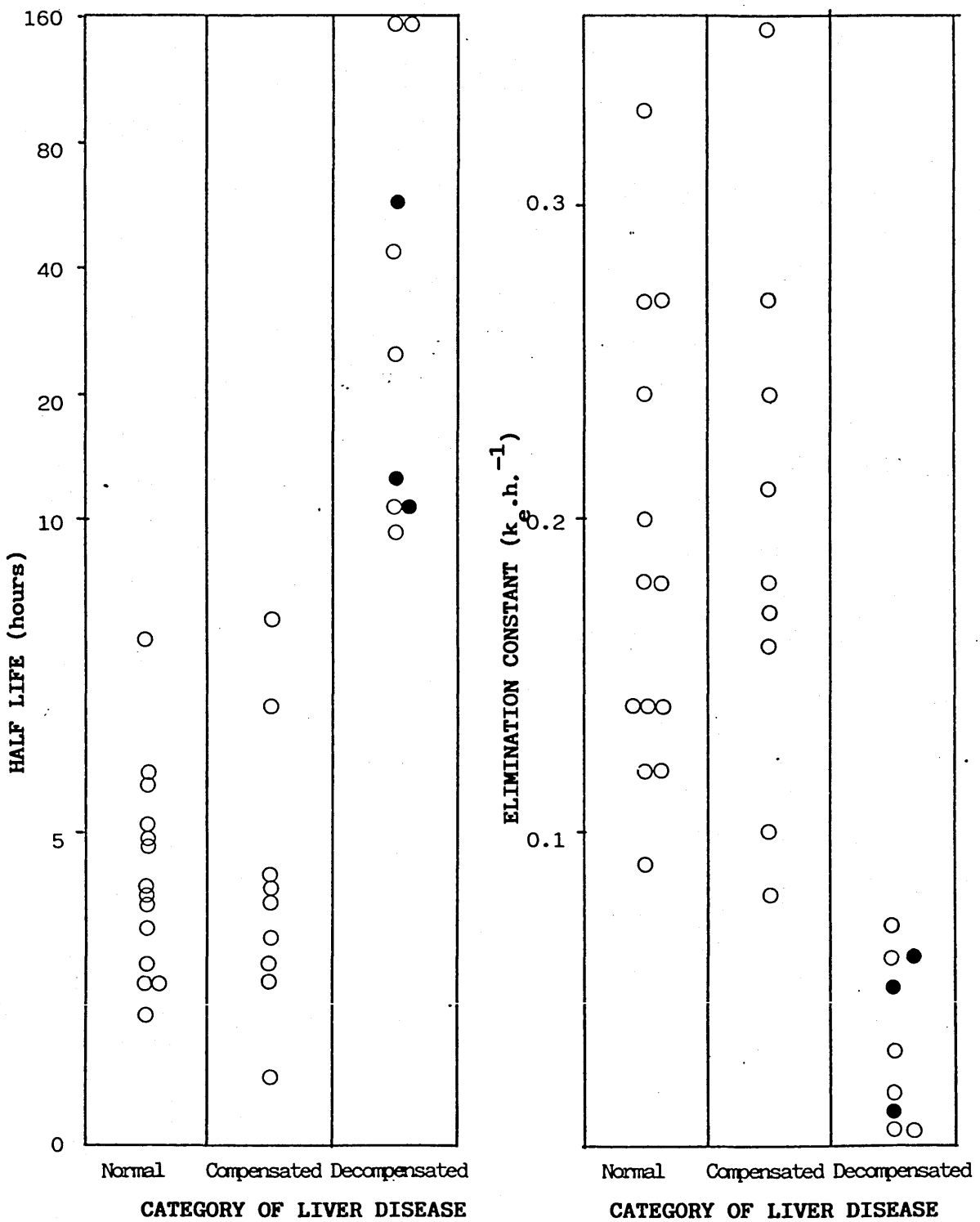

Fig 8.2 Pharmacokinetics of caffeine in normal volunteers and patients with compensated and decompensated liver disease.

○ = Saliva ● = Serum

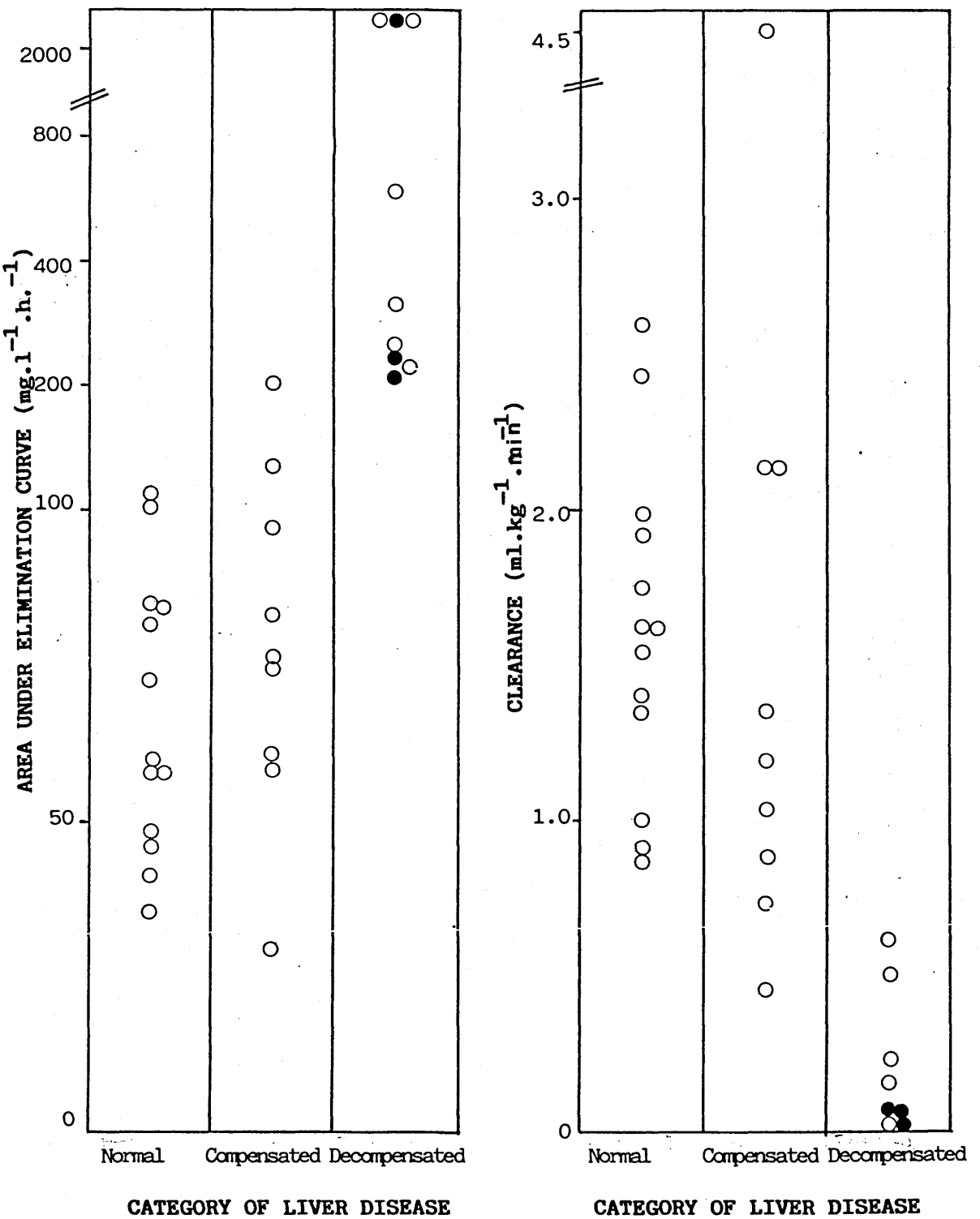

Fig 8.3 Pharmacokinetics of caffeine in normal volunteers and patients with compensated and decompensated liver disease.

○ = Saliva ● = Serum

Group	T1/2 (hr)		k.el ($hr.^{-1}$)		Clearance ($ml.kg^{-1}min^{-1}$)	
a) Mean values	Mean	SD	Mean	SD	Mean	SD
A	4.24	2.25	0.22	0.16	1.55	1.26
B	12.99	7.89	0.073	0.051	0.63	0.43
C	48.76	25.70	0.024	0.026	0.14	0.20
b) Student's t-test (P)						
A vs B	<0.02		<0.1		<0.2	
B vs C	<0.05		<0.1		<0.05	
A vs C	<0.001		<0.05		<0.05	
c) Mann Whitney U-test (P)						
A vs B	0.011		0.011		0.083	
B vs C	0.095		0.095		0.037	
A vs C	0.003		0.003		0.005	
d) Linear Regression analysis						
Coefficient of correlation (r)	0.844		0.640		0.592	
Significance (P)	<0.01		<0.01		<0.01	

Table 8.3 Statistical analysis of the relationship between caffeine clearance and Pugh score ratings of liver disease.

For details of Pugh score system refer to Appendix 6.
Abbreviations: T1/2 - half life, k.el - elimination constant
P = Level of significance

values for this group were similar to those previously obtained for normal healthy volunteers. Respective probability values for the elimination constant and salivary clearance were <0.9, and <0.5. In contrast, the decompensated group showed a significant decrease in the elimination constant (P <0.001) and the total salivary clearance (P <0.001) although the apparent volumes of distribution were similar (P <0.9).

A statistical analysis of the relationships between salivary pharmacokinetic data for caffeine and the assigned Pugh scores/group classification was made by linear regression analysis (Table 8.3). In subjects where saliva samples were not obtained, substitutions were made using data calculated from serum. Linear correlations existed between the Pugh score ratings and values for elimination half-life, elimination constant and total salivary clearance. The significance (P) of these correlations, determined by variance ratio (F-test) distribution, was in all cases <0.01. The ability of caffeine clearance studies to differentiate between subjects in the Pugh score groups was demonstrated by Student t-test analysis. The values for elimination half-life were significantly different for all possible group combinations. Significant differences were present between groups A and C (P <0.05) for the elimination constant values and between groups B versus C (P <0.05) and A versus C (P <0.05) for levels of the total salivary caffeine clearance.

Additional statistical analysis of the Pugh score/caffeine clearance relationships, using a Mann Whitney U-test showed similar levels of significance.

b) Dimethylxanthine metabolites in serum/saliva

The concentrations of both theobromine and theophylline in samples of serum and saliva showed, for the majority of subjects investigated, only minimal increases over basal levels. However three of the decompensated subjects (J.B, V.D, and D.H) showed significant levels of theobromine (More than 1.5 mg/l) in both basal and post caffeine samples.

The levels of 1,7-dimethylxanthine in serum and saliva showed a linear relationship for both groups of liver disease patients. The overall mean coefficient of correlation was 0.89 (SD. 0.084) with values ranging from 0.763 to 0.992. The ratio of concentrations in saliva versus serum were similar for both groups ($P < 0.5$); respective mean values for the compensated group and decompensated group were 0.74 (SD. 0.14) and 0.83 (SD. 0.19). The mean peak values for 1,7-dimethylxanthine (paraxanthine) are summarised in Table 8.4. Compared to the normal control group, the peak values of paraxanthine in saliva for the compensated cirrhotic group were significantly higher ($P < 0.05$) whilst those for the decompensated group were significantly decreased ($<P < 0.05$). In addition, the mean time for peak paraxanthine levels to be reached for subjects in the decompensated group (26.6 hr + SD. 21.2) was significantly longer ($P < 0.01$) than that for the normal volunteers (5.9 hr ± SD. 4.1) or the compensated cirrhotic subjects (6.9 hr ± SD. 3.3). Only minimal levels of paraxanthine were present in samples obtained from the decompensated subjects D.H. and V.D. In one subject (H.O), significant levels of an eluted

SUBJECT	SERUM Cmax mg/l	SERUM Time hr	SERUM AUC	SALIVA Cmax mg/l	SALIVA Time hr	SALIVA AUC
Normal volunteers						
N14	*	*	*	1.2	6	39.2
N15	*	*	*	3.6	4	34.3
N16	*	*	*	2.2	6	31.3
N17	*	*	*	1.8	4	40.2
N18	*	*	*	2.0	6	39.0
N19	*	*	*	1.2	12	34.8
N20	*	*	*	1.8	6	28.8
N21	*	*	*	1.7	3	13.8
N22	*	*	*	1.6	2	24.2
Mean				1.9	5.44	31.7
SD				0.7	2.87	8.5
Compensated cirrhotic group						
JC	4.4	6	64.9	3.1	6	42.9
AC	2.8	8	68.9	2.6	6	57.5
IC	4.2	8	56.6	4.0	6	55.1
PI	2.8	8	54.7	1.5	12	25.0
EM	3.0	12	50.0	2.5	8	35.5
EP	4.2	3	25.4	2.8	2	21.8
AS	3.7	6	88.3	3.1	4	53.2
BW	2.6	24	74.5	2.4	12	64.8
NW	3.0	12	70.6	2.5	6	39.4
Mean	3.41	9.4	61.5	2.72	6.9	43.9
SD	0.71	6.2	17.8	0.67	3.3	14.9
Decompensted cirrhotic group						
CB	2.0	24	41.4	*	*	*
FC	2.6	12	53.6	Insufficient samples		
DH	*	*	*	1.2	48	30.4
GG	1.8	12	47.3	1.1	12	30.4
MS	1.8	24	66.5	1.5	24	58.3
AS	**	**	**	**	**	**
VD	0.1	72	0.1	0.5	1	0.2
FF	*	*	*	1.2	48	45.8
Mean	1.66	28.8	41.8	1.10	26.6	33.0
SD	0.93	24.9	25.1	0.37	21.2	21.8

Table 8.4 Paraxanthine kinetics for serum and saliva samples collected from normal volunteers and chronic liver disease patients following an oral dose of 400 mg caffeine.

* Samples not collected. ** High magnitude peaks found during chromatography, possibly resulting from drug interference.

compound with a similar retention time to paraxanthine were present in both basal and post caffeine samples of serum and saliva. However by using the more specific chromatographic conditions previously described for urine metabolite analysis, it was possible to resolve this compound from paraxanthine and identify it as a non-specific compound. The chromatographs obtained from the subject A.S. also contained high magnitude peaks with retention times similar to those of theobromine and paraxanthine. These were probably representative of non-specific interference from concurrently administered drugs or their metabolites, although the possibility of caffeine metabolite accumulation in this subject cannot be completely ignored.

The bioavailablity of paraxanthine, assessed by AUC (area under curve of concentration vs time) values for the period of 0-24 hr post caffeine ingestion is summarized in Table 8.4. The correlation between values for serum and saliva was favourable ($r = 0.965$, $y = 0.978x + 1.44$, where y and x represent values for saliva and serum respectively). Whilst the AUC values for the compensated group were statistically higher ($P < 0.05$) than those found for the normal control group, this was mainly a result of their higher peak values, the overall rate of formation and clearance being similar. For the decompensated group, with the exception of H.O and A.S the AUC values generally reflected an impaired rate of formation of paraxanthine with significant values still detectable after 48 hours.

Additional statistical analysis using a non-parametric

Mann-Whitney rank test showed similar levels of probability to those reported.

c) Urinary methylxanthine metabolite profiles

The caffeine metabolite profiles of the two groups of liver disease subjects are compared in Table 8.5 and in Fig 8.4, Fig 8.5 and Fig 8.6 with data obtained from a group of normal volunteers treated in a similar manner. Basal urine samples, where collected, contained no appreciable amounts of xanthine metabolites. Compared to the normal control group, the decompensated patients showed a significant reduction ($P < 0.01$) in total metabolite excretion during the first 24 hour period. This was largely reflected by a decreased recovery of 1-methyluric acid (15.61 ± SD. 7.37 to 6.69 ± SD. 6.03; $P < 0.05$) and 1-methylxanthine (9.23 ± SD. 4.40 to 3.37 ± SD. 2.83; $P < 0.01$). Whilst the recovery of unchanged caffeine was increased (1.90 ± SD. 1.48 to 3.03 ± SD. 1.65) in the decompensated group for this time interval, the differences were not statistically significant ($P < 0.2$).

The recovery data for the period 24-48 hours showed a generalised increase in metabolite excretion for the decompensated group, in particular, the dimethylxanthine metabolites where significant increases were found for 1,7-dimethyluric acid (1.29 ± SD. 1.12 to 3.65 ± SD. 2.67; $P < 0.05$) and 1,3-dimethylxanthine (0.19 ± SD. 0.26 to 0.80 ± SD. 0.69; $P < 0.05$). The recovery of unchanged caffeine for this period remained significantly increased (0.10 ± SD. 0.16 to 1.67 ± SD. 1.61; $P < 0.02$).

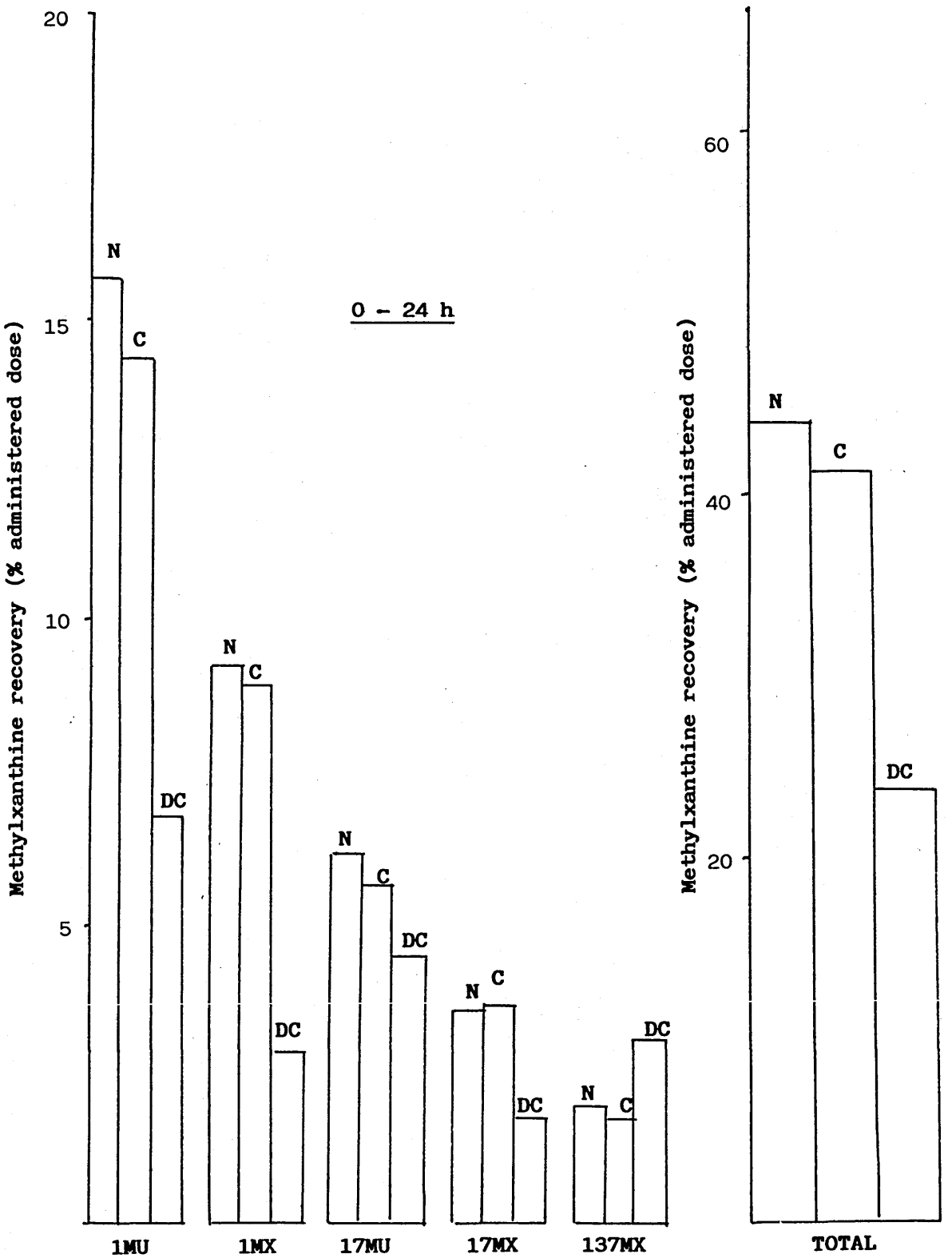

Fig 8.4 Methylxanthine excretion for the time interval 0 - 24 h following an oral dose of caffeine (400 mg) in normal volunteers and patients with compensated and decompensated liver disease.

N = Normal controls **C** = Compensated liver disease
DC = Decompensated liver disease.

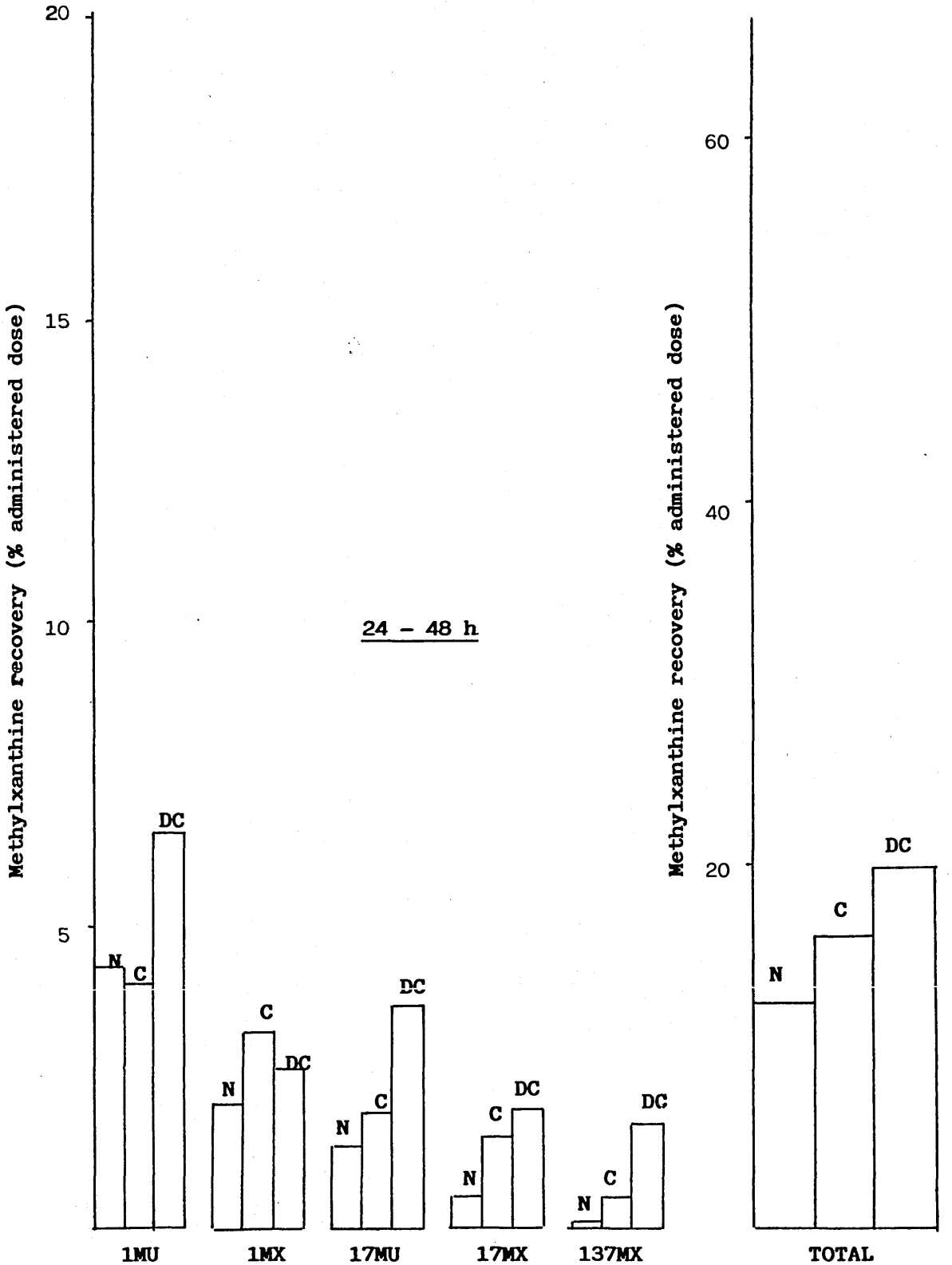

Fig 8.5 Methylxanthine excretion for the time interval 24 - 48 h following an oral dose of caffeine (400 mg) in normal volunteers and patients with compensated and decompensated liver disease.

N = Normal controls **C** = Compensated liver disease
DC = Decompensated liver disease.

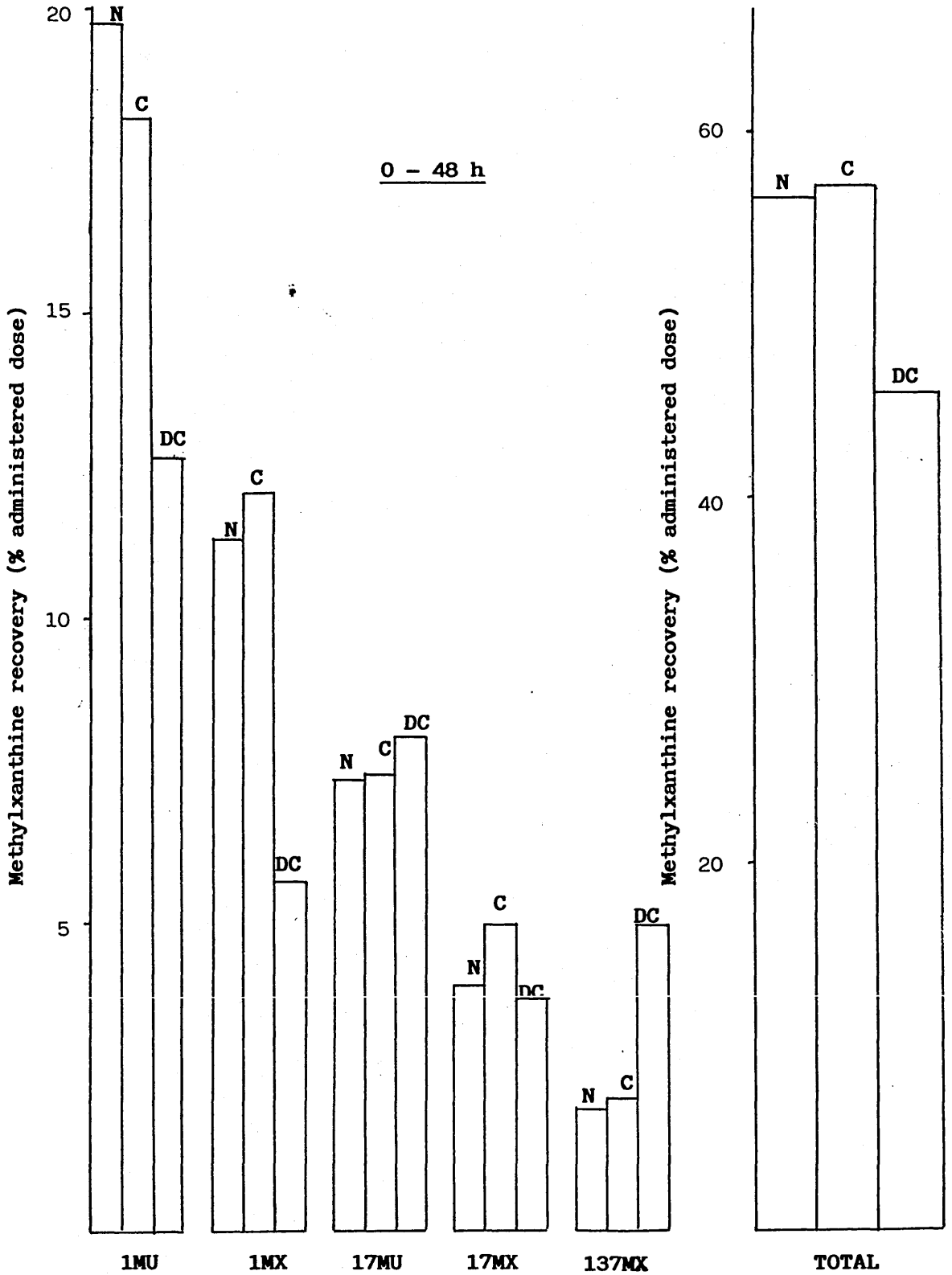

Fig 8.6 Methylxanthine excretion for the time interval 0 - 48 h following an oral dose (400 mg) of caffeine in normal volunteers and patients with compensated and decompensated liver disease.

N = Normal controls **C** = Compensated liver disease
DC = Decompensated liver disease.

Despite significant intra-group variations in the levels of metabolites, the mean total metabolite recoveries for the 48 hour period following caffeine ingestion were similar for all three groups. However, in patients with decompensated liver disease, the recovery of 1-methylxanthine for this period was significantly reduced (11.25 ± SD. 4.88 to 5.65 ± SD. 2.76; $P < 0.05$), whilst that of unchanged caffeine was significantly increased (2.0 ± SD. 1.42 to 5.00 ± SD. 2.75; $P < 0.02$). The mean metabolite recoveries for patients with compensated cirrhosis for both periods of urine collection were similar to those found for the group of normal controls.

For one compensated subject (BW) the low metabolite recovery and the significantly reduced excretion of 1-methyluric acid may be attributed to the concurrent administration of allopurinol, a known inhibitor of xanthine oxidase (Grygiel et al., 1979), the enzyme responsible for the conversion of 1-methylxanthine to 1-methyluric acid.

The relationship between metabolite recoveries and Pugh score assessments of the degree of hepatic insufficiency are summarised in Table 8.6. Whilst levels of serum creatinine were higher in the more decompensated subjects, there was no evidence to suggest overt impairment in renal function and 24 hour urine outputs were normal. The increased levels of creatinine in these patients was probably attributable to muscle wasting. An analysis of the individual Pugh score ratings versus the metabolite recovery for the time interval 0-24 hours showed a linear relationship. The coefficient of correlation (r), calculated by least squares linear regression

	NORMAL		COMPENSATED			DECOMPENSATED		
	Mean	SD	Mean	SD	P	Mean	SD	P
7MU	0.84	0.54	0.56	0.27	<0.2	0.96	0.60	<0.9
7MX	2.24	0.70	1.94	0.86	<0.5	1.39	0.73	<0.05
1MU	15.6	7.37	14.29	6.57	<0.7	6.69	6.03	<0.05
3MX	0.94	0.46	1.03	0.48	<0.7	0.54	0.53	<0.2
1MX	9.23	4.41	8.91	3.07	<0.9	2.83	2.13	<0.01
13U	0.94	0.41	0.81	0.36	<0.5	0.63	0.39	<0.2
37X	1.68	0.74	1.70	1.46	<0.9	1.01	0.68	<0.1
17U	6.08	1.25	5.60	2.14	<0.7	4.40	2.87	<0.2
17X	3.48	1.67	3.52	1.43	<0.9	1.69	1.55	<0.1
13X	0.44	0.40	0.55	0.15	<0.5	0.26	0.26	<0.5
137U	0.86	0.73	0.52	0.39	<0.25	0.49	0.47	<0.3
137X	1.90	1.48	1.70	0.67	<0.8	3.03	1.65	<0.2
TOTAL	44.14	12.41	41.26	11.42	<0.7	23.76	10.75	<0.01
Caffeine metabolite excretion (% dose) 0 - 24 hour								
7MU	0.39	0.38	0.59	0.62	<0.5	0.60	0.53	<0.5
7MX	1.15	0.79	1.43	0.59	<0.5	1.27	0.53	<0.8
1MU	4.30	2.88	4.03	2.57	<0.9	6.48	4.22	<0.3
3MX	1.05	0.64	0.80	0.29	<0.3	0.60	0.34	<0.2
1MX	2.03	1.37	3.19	2.46	<0.25	2.55	1.73	<0.7
13U	0.65	0.45	0.61	0.72	<0.9	0.53	0.40	<0.7
37X	0.73	0.45	0.97	1.22	<0.7	1.13	0.42	<0.2
17U	1.29	1.12	1.86	1.51	<0.3	3.65	2.67	<0.05
17X	0.50	0.64	1.44	1.48	<0.1	1.93	2.24	<0.2
13X	0.19	0.26	0.39	0.32	<0.2	0.80	0.69	<0.05
137U	0.09	0.15	0.36	0.55	<0.2	0.48	0.62	<0.2
137X	0.10	0.16	0.46	0.40	<0.05	1.67	1.61	<0.02
TOTAL	12.29	7.78	15.94	10.91	<0.5	20.93	10.46	<0.1
Caffeine metabolite excretion (% dose) 24 -48 hour								
7MU	1.23	0.63	1.15	0.77	<0.9	1.53	0.48	<0.5
7MX	3.39	1.24	3.37	0.86	<0.9	2.63	1.01	<0.25
1MU	19.83	8.50	18.32	5.95	<0.7	12.73	9.08	<0.2
3MX	1.99	0.96	1.81	0.55	<0.7	1.18	0.50	<0.1
1MX	11.25	4.88	12.10	2.83	<0.7	5.65	2.76	<0.05
13U	1.59	0.63	1.42	0.90	<0.7	1.20	0.79	<0.5
37X	2.40	0.58	2.67	1.71	<0.7	2.21	0.88	<0.7
17U	7.36	2.04	7.46	2.96	<1.0	8.11	4.96	<0.7
17X	3.98	2.00	4.96	2.13	<0.5	3.80	3.81	<0.9
13X	0.63	0.45	0.94	0.38	<0.2	1.08	0.65	<0.2
137U	0.95	0.72	0.88	0.74	<0.9	1.05	1.04	<0.9
137X	2.00	1.42	2.16	0.93	<0.8	5.00	2.75	<0.02
TOTAL	56.43	16.47	57.2	11.45	<0.9	45.85	15.40	<0.25
Total caffeine metabolite excretion (% dose) 0-48 hour								

Table 8.5 Caffeine metabolite excretion in normal volunteers and chronic liver disease patients following an oral dose of caffeine (400 mg).

Subject	Pugh Rating Score	Grade	Urine volume (l) 0-24h	24-48h	Metabolite recovery 0-24h	24-48h	Serum creatinine
JC	5	A	1.22	0.96	58.8	6.3	70
IC	5	A	1.58	2.00	49.8	6.8	64
PI	5	A	1.70	2.00	46.3	12.4	85
EM	5	A	1.40	0.75	31.8	9.1	71
EP	5	A	1.05	1.75	45.6	4.5	50
AS	5	A	1.90	1.55	51.0	18.4	65
BW	5	A	1.45	1.00	20.0	19.9	82
NW	5	A	1.90	1.38	41.7	19.6	69
HO	5	A	1.30	2.05	34.0	41.3	70
AC	7	A	1.10	1.20	33.6	21.1	57
FC	8	B	0.25	0.90	43.3	10.2	98
MS	8	B	1.90	1.60	22.4	28.7	62
BJ	9	B	1.82	3.00	30.7	36.9	90
GG	9	B	0.70	0.80	25.6	22.2	151
FF	10	C	1.05		16.0		157
VD	11	C	1.00	0.90	11.6	16.4	104
DH	12	C	1.70	1.50	16.7	11.2	105

Table 8.6 Urine metabolite recoveries in alcoholic cirrhosis subjects following an oral dose of caffeine (400 mg)

analysis, was 0.738. Whilst there were no significant differences between the metabolite recoveries for this period between subjects in Pugh score groups A versus B ($P < 0.2$), significant differences were found between groups A versus C ($P < 0.01$) and B versus C ($P < 0.05$).

Additional statistical analysis of the three groups of subjects using a non-parametric Mann-Whitney rank test showed similar levels of significance to those reported.

8.3 Salivary caffeine clearance in chronic liver disease.

Although the previous report has clearly demonstrated the value of caffeine elimination studies in differentiating comensated and decompensated forms of chronic liver disease, the requirement for multiple sampling of blood or saliva imposes limitations on their application to the routine assessment of hepatocellular function. In attempt to simplify the procedure, the following study investigates the value of measuring the 24 hour salivary clearance of caffeine, using a 4 point salivary caffeine analysis, following an oral dose of anhydrous caffeine (400 mg).

Methods

The groups of liver disease patients selected for this study and the conditions of enrollment and preparation were as described in Section 8.2. Following the collection of a basal sample of saliva, an oral dose of 400 mg anhydrous caffeine was administered to all participants and additional samples of saliva were collected after periods of 4 hr, 12 hr and 24 hr. Salivary caffeine concentrations were determined using HPLC

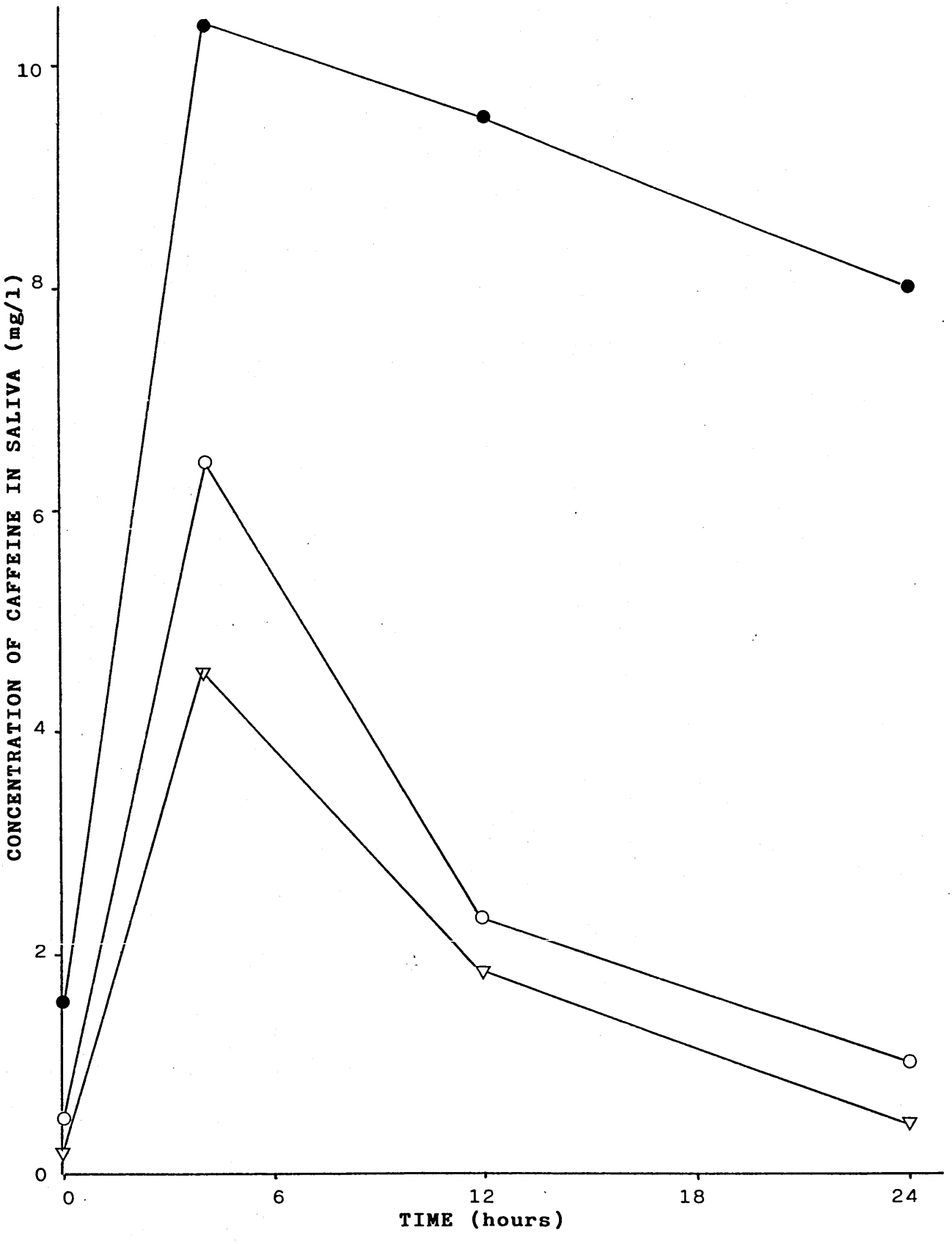

Fig 8.7 Elimination of caffeine in saliva of normal controls (▽), compensated (○) and decompensated (●) liver disease patients following an oral dose of 400mg anhydrous caffeine.

and the clearance of caffeine in the compensated and decompensated subjects was compared with values obtained for a group of normal volunters (Section 5).

Results

The salivary caffeine levels for the three groups studied are shown in Table 8.7. Compared to the normal control group, the salivary caffeine concentrations for the compensated cirrhotic group were similar in both basal samples ($P < 0.25$) and samples collected 12 hr ($P < 0.5$) and 24 hr post caffeine ($P < 0.25$). In contrast, the decompensated group showed significant increases in caffeine concentrations ($P < 0.001$) for samples collected at 4 hr, 12 hr and 24 hr post caffeine administration. Although the basal caffeine levels for the decompensated group were higher than those found in both the normal controls and compensated liver disease patients, the increases were not significant ($P < 0.1$). Statistical analysis of the results for the compensated group versus the decompensated group showed, for the latter, significant differences in caffeine concentrations at 4 hr ($P < 0.01$), 12 hr ($P < 0.001$) and 24 hr ($P < 0.001$).

In agreement with the results obtained for the more detailed pharmacokinetic study, the salivary caffeine clearance values clearly differentiate decompensated liver disease patients from normal controls and patients with compensated liver disease. This suggests that measurement of overnight salivary caffeine clearance, following an oral dose of 400mg caffeine, may provide a simpler alternative means of assessing hepatocellular function. However, since the procedure was unable to

SUBJECT	PUGH SCORE	SALIVARY CAFFEINE (mg/l) Basal	4 h	12 h	24 h
Normal control group					
N13	-	0	4.0	1.5	04
N14	-	0	2.7	1.2	1.5
N15	-	0	3.8	0.33	0
N16	-	0.2	7.1	1.99	0.2
N17	-	0.2	4.5	1.62	0.3
N18	-	0	3.8	1.95	0.4
N19	-	0.3	6.3	2.75	0.5
N20	-	0.3	4.0	2.88	0.5
N21	-	0.5	4.1	1.29	0.2
Mean	-	0.17	4.48	1.72	0.43
S.D.	-	0.18	1.36	0.78	0.43
Compensated liver disease patients					
JC	5	0	6.9	1.2	0
IC	5	0.7	6.7	1.8	0
HO	5	0	9.7	5.6	3.0
PI	5	0.3	5.2	2.3	0.4
EM	5	0.2	7.1	3.4	0.6
EP	5	2.6	2.0	0.2	0
AS	5	0.3	5.8	0.9	2.9
BW	5	0.2	7.8	4.2	2.4
NW	5	0.6	6.7	2.3	0.6
AC	7	0	6.0	1.2	0
Mean	-	0.49	6.39	2.31	0.99
S.D.	-	0.78	1.98	1.67	1.26
Decompensated liver disease patients					
MS	8	0.9	8.2	6.1	5.7
FC	8	0.2	7.6	*	2.7
GG	9	0	12.0	7.4	6.4
FF	10	0.2	7.6	5.5	2.7
VD	11	5.8	-	14.5	14.9
CB	12	2.2	14.1	*	11.8
AS	12	2.6	12.6	12.0	12.2
Mean	-	1.55	10.32	9.10	7.98
S.D.	-	1.98	2.92	3.95	4.45

Table 8.7. Salivary caffeine levels at times 0, 4, 12 and 24 h in normal control subjects and chronic liver disease patients following an oral dose of caffeine (400 mg)

differerentiate compensated liver disease patients from normal controls, further investigations are required in order to assess its sensitivity.

DISCUSSION

Since coventional liver fuction tests, with the possible exception of prothrombin times and serum albumin levels, are of limited value in the detection and assessment of severity of alcoholic liver diesease (Sherlock, 1981), attempts have been made in recent years to develop more precise means of quantitating hepatocellular function. These have included the use of galactose elimination tests (Tygstrup, 1964), carbon-14 labelled aminopyrine (Bircher et al., 1976) or caffeine breath tests (Wietholtz et al., 1981) and the measurement of serum bile acid levels (Ferraris et al., 1983). However, the complexity and cost of these tests has resulted in their use being largely confined to clinical research. Whilst previous reports have suggested that the use of a simple oral caffeine loading test may provide an accurate means of measuring the degree of hepatic insufficiency, they fail to show conclusive evidence of its clinical significance. This study provides a detailed investigation of the clearance of caffeine in patients with chronic liver disease, of varying aetiology and severity, and correlates the serum and salivary clearance values with a clinical and biochemical assessment of the severity of their hepatic dysfunction.

The close correlation between caffeine levels in serum and

saliva for the two groups of liver disease patients was similar to that found for normal healthy volunteers (Section 5), confirming the suitability of salivary measurements for determining pharmacokinetic data. Peak caffeine levels for normal controls and both groups of liver disease patients were obtained within 3 hours after oral administration of caffeine (400 mg), after which the concentrations declined mono-exponentially. Although peak drug concentrations in serum or saliva following oral administration may be affected by numerous physiological factors, such as its rate and extent of absorption, first pass elimination effects and rates of metabolism, the statistically significant increase in peak levels of caffeine in saliva of the compensated liver disease patients, compared to the normal controls, was probably due to the observed decrease in apparent volumes of distribution. A similar, but statistically insignificant decrease in apparent volume of distribution in liver disease patients has been previously reported by Renner et al. (1984). In contrast, the increased peak values in the decompensated patients are more likely to be explained by a decrease in the extent of protein binding in blood and the development of mesenteric porta-caval anastamoses which may significantly reduce first pass elimination effects. However, since the hepatic extraction of caffeine is less than 10% (Bircher, 1986), the resulting increase in bioavailablity would be minimal.

Analysis of the kinetic data on a group basis shows that, compared to normal volunteers, the clearance of caffeine is relatively unchanged in compensated cirrhosis. In contrast,

the reduction in mean values for both the elimination constants and clearance values in patients with decompensated liver disease suggests that, in these subjects, the drug metabolizing capacity of the liver is severely impaired. Although the kinetic results are in close agreement with previously published studies (Desmond et al., 1980; Renner et al., 1984), a strict comparison is difficult owing to variations in experimental design.

The urine metabolite recoveries obtained from this study showed a similar configuration of results; significant impairements in metabolite excretion being apparent only in subjects with decompensated liver disease. However, with the exception of a decreased recovery of 1-methyl derivatives, the overall pattern of metabolite excretion for this group was not significantly different. This suggests that in decompensated liver disease there is a generalised impairement in the clearance of caffeine rather than specific alterations in its biotransformation. Although a study by Staib and his colleagues (1980) has indicated that the impaired metabolism of theophylline in liver disease may be associated with a decrease in 1-N demethylation, the failure to find an increased recovery of 1-methyl metabolites with concomitant decrease in 3-methyl and 7-methyl metabolites in this present study would tend to refute this possibility for caffeine.

The presence of significant amounts of paraxanthine (1,7-dimethylxanthine) in serum and saliva is in agreement with reports by Arnaud et al. (1981) and Callahan et al. (1981) which indicate that approximately 75% of administered caffeine

undergoes an initial 3-N demethylation. Compared to the normal control group, the level of this metabolite in the serum/ saliva of the compensated cirrhotic subjects was significantly higher. However, since the total recovery of this metabolite in urine was similar for both groups, these differences are probably attributable to the corresponding decreases in apparent volume of distribution for this group. The reduction in peak levels of paraxanthine in the decompensated subjects in the absence of a corresponding decrease in bioavailability (AUC) is consistent with an impaired but normal disposition of caffeine in these subjects.

In agreement with previous reports (Desmond et al., 1980; Renner et al., 1984), the results of conventional liver function tests showed no apparent relationship to either the severity of liver disease or to the rate of elimination of caffeine. Although serum albumin levels in the decompensated group were significantly lower ($P < 0.001$) than those found in compensated liver disease patients, the corellation between albumin levels and caffeine clearance was poor ($r = 0.670$). However, the close relationship between Pugh score ratings of liver dysfunction and caffeine clearance indicates that the monitoring of caffeine elimination in serum or saliva and/or the measurement of urine metabolite recoveries may be used as a sensitive means of assessing the severity of hepatocellular dysfunction. By relating the salivary pharmacokinetic data to the severity of liver disease, clear cut of points are apparent which may be used to differentiate compensated and decompensated conditions. More patient studies are required to

clarify these points, but it is possible that an indication of decompensated liver disease could be based on the finding of a half-life of greater than 10 hours, an elimination constant of less than 0.8 and a salivary clearance of less than 0.4 ml/kg/min. Although the inability to differentiate between caffeine elimination rates in compensated cirrhotics and normal volunteers suggests that caffeine clearance studies may lack sufficient sensitivity to detect mild impairments in hepatocellular function, it is possible that the patients selected for this study may have resumed normal liver function since they were regarded as being "well compensated".

The use of a simplified procedure, involving a 4 point determination of salivary caffeine clearance, showed a similar differentiation of the severity of liver disease to that obtained using the more detailed pharmacokinetic and urine metabolite studies. Further investigations are required to define the sensitivity of the procedure and to provide suitable reference ranges, but, the preliminary results suggest that 24 hour or overnight measurement of salivary caffeine clearance may provide a simple means of assessing hepato-cellular function. In addition, it has been reported by Renner et al. (1984) that a close hyperbolic relationship exists between caffeine clearance and caffeine concentrations determined in serum samples collected following an overnight fast. Although the increases in basal caffeine concentrations for the decompensated patients in this study failed to reach significance, they were measured after a 3 day abstinence from caffeinated beverages. Since commencing this study, a report

by Wang et al. (1985) has suggested that the discrimination between patients with both cirrhotic and non-cirrhotic forms of liver disease and healthy volunteers, is best achieved by measuring caffeine concentrations in samples collected 12 hours after oral administration of a caffeine dose. However, the significance of this is questionable since the present study was unable to differentiate between the clearance of caffeine in compensated liver disease patients and normal controls

Chronic liver diseases, such as alcoholic cirrhosis, generally pass through a long period of minimum, non-specific symptoms ("compensated") until reaching the final stages in which ascites, jaundice, encephalopathy and pre-coma ("de-compensated") are common features. In addition to damage caused to the hepatic cells and architecture, the various pathophysiological disturbances may lead to a reduction in overall hepatic blood flow, the presence of intra- or extra-hepatic shunting and alterations in plasma protein synthesis. Although the total body clearance of a drug is a product of both metabolic and renal clearance, the previously described urine metabolite studies in normal volunteers have shown that renal excretion of unchanged caffeine, accounts for only 1-3% of an orally administered dose. The results of this present study indicate that even in patients patients with chronic liver disease, the excretion of unchanged caffeine is minmal. Subsequently, since caffeine elimination occurs almost entirely by the liver, the rate of hepatic clearance may be regarded as being equvalent to the total clearance of caffeine

calculated from pharmacokinetic data. A reduction in hepatic clearance, as observed in the decompensated group of patients is probably the result of combination of a) disordered hepatocyte function, b) shunting of blood away from the liver due to the presence of portasystemic anastamoses and c) a decrease in hepatic blood flow. However, since the hepatic extraction of caffeine is estimated to be about 10 per cent in normal subjects (Bircher, 1986), the effects of liver blood flow are minimal. The delayed clearance of caffeine is therefore more likely to be due to a reduction in intrinsic clearance resulting from a reduced mass of viable hepatocytes.

In this particular study, the absence of significantly elevated levels of aspartate transaminase suggests that it is unlikely that the extent of hepatic necrosis in the decompensated group would be sufficient to cause a profound reduction in microsomal drug metabolizing activity. In addition, although the concurrent administration of additional drugs to some of the patients may have enhanced microsomal drug metabolism, induction effects were assumed to be minimal. This is supported by a report by Farrell et al (1979) which demonstrated that patients with severe liver disease fail to show the normal increase in levels of cytochrome P-450 and microsomal oxidase activities associated with the ingestion of strong inducing drugs such as phenobarbital, phenytoin and glutethimide.

9.GENERAL DISCUSSION AND CONCLUSIONS

The ubiquitous consumption of caffeine, in the form of caffeinated beverages and various medications, has prevailed world-wide for many years. Whilst moderate doses of caffeine produce minimal pharmacological effects, consumption estimates in this study indicate that approximately 20% of the U.K. population have a dietary caffeine intake in excess of 500 mg per day. Despite this widespread usage, the scientific and medical literature has, until recently, contained only limited information on its pharmacokinetics, metabolism and pharmacological properties. This has been largely due to the absence of sensitive and specific methodologies for the measurement of caffeine and its metabolites in biological fluids. The present study describes the development and application of such procedures using high performance liquid chromatography.

Measurement of caffeine and its dimethylxanthine metabolites in serum and saliva.

Using a reverse phase chromatography system, incorporating a 100 x 4.5 mm i.d. Hypersil octadecysilane column with an elution system of acetonitrile/tetrahydrofuran/ 50 mM acetate buffer, pH 4.0 (4:1:95, v/v), levels of caffeine and its dimethylxanthine metabolites have been measured in serum, saliva (p.35) and breast milk (p.168). Eluted peaks were detected by monitoring their UV absorption at a wavelength of 280 nm. The incorporation of tetrahydrofuran into the mobile

phase has produced significant improvements in specificity over previously described methods by resolving theophylline from 1,7-dimethylxanthine (paraxanthine), the primary metabolite of caffeine found in these body fluids. Sample preparation using organic extraction with a chloroform/ isopropanol mixture has been adopted in order to eliminate the specificity problems associated with deproteinisation techniques. The recovery values and precision of the extraction procedure were very satisfactory and results suggest that aqueous standards may be suitably employed as an alternative to those prepared in drug-free serum. Caffeine levels determined by HPLC agreed well with those obtained using a radioimmunoassay (RIA) procedure (p.56). Whilst RIA procedures allow a large throughput of samples for analysis, the ability of the described HPLC procedure to simultaneously determine the concentrations of dimethylxanthine metabolites of caffeine in serum or saliva, and its high specificity, makes it ideal for precise pharmacokinetic studies. The method has also been satisfactorily applied to the determination of methylxanthine concentrations in beverages by injection of diluted samples directly into the system.

Determination of the urinary metabolites of caffeine.

The development of a chromatographic procedure for the separation of methylxanthine metabolites using a Hypersil octadecylsilane column with a simple concave gradient elution programme of 0-12.75% acetonitrile in 1% tetrahydrofuran, pH 4.8, produced significant improvements in specificity over

previously described procedures (p.60). In particular, the ability to resolve 7-methylxanthine from 1-methyluric acid, theophylline from 1,7-dimethylxanthine or 3,7-dimethylxanthine from 1-methylxanthine has permitted more precise studies to be made on the biotransformation of caffeine. In order to optimise sample preparation in terms of selectivity, recovery, precision and preparation time, a critical assessment has been made of the use of direct injection of diluted urine samples and a procedure involving the formation and extraction of an ion-pair complex. The extraction procedure showed excellent selectivity and much improved recovery from previous techniques based on chloroform/isopropanol extraction (p.73). Whilst the direct injection technique (p.68) significantly reduces sample preparation time and eliminates variations in recovery, its susceptibilty to high blank values and the occasional presence of an interfering peak impose limitations on its use. However, providing suitable cut off values are applied to compensate for these factors, experience has shown that the convenience of this technique is of value where quantitation is only required for the major metabolites. Such applications include screening for toxicity or detecting alterations in the pattern of metabolite excretion resulting from diseased states such as chronic liver disease.

Population studies related to dietary caffeine intake.

An assessment of dietary caffeine consumption, obtained using a retrospective questionnaire (p.91), showed an overall mean daily intake of 359 mg (SD = 189). This value is

significantly higher than previously reported intake levels for both American and Canadian populations. Whilst there was no significant variation between caffeine consumption and age, the consumption of caffeinated beverages in smokers was significantly higher than that found for non-smokers. For the total population studied, coffee drinking was found to be the major source of dietary caffeine, accounting for approximately 55% of per capita intake. The consumption of tea accounted for 44%, whilst caffeine intake from chocolate and cola flavoured drinks was minimal. In a separate survey, the mean caffeine concentration in serum samples collected from a non-pregnant population was 2.42 mg/l (SD = 1.59) with values ranging from 0 - 9.2 mg/l (p.108). In agreement with a similar study reported previously by Smith et al. (1982), 6% of the population studied were shown to have circulating levels in excess of 5 mg/l; this is comparable to the mean peak caffeine found following 250 mg oral doses of caffeine. However, no association was found between symptoms of anxiety and levels of caffeine in serum (p.110).

Pharmacokinetics and metabolism of caffeine in healthy human subjects.

The non-invasive nature of saliva sampling and the ability to obtain multiple samples, prompted further investigation into the suitability of salivary caffeine determinations for establishing pharmacokinetic data. A satisfactory correlation was found between concentrations of caffeine in samples of serum and saliva, collected simultaneously and assayed using

HPLC (p.53). Data derived from caffeine determinations in samples of serum and saliva, collected from normal volunteers following oral doses of caffeine, showed good correlation and enabled "reference ranges" to be established for use in future studies (p.101). The calculated mean values for elimination half-life (4.35 h ± SD. 1.95), elimination constant (0.185 h^{-1} ± SD. 0.07) and total salivary clearance (1.605 $ml.kg^{-1} min^{-1}$ ± SD. 0.55) are similar to those previously reported by Parsons and Neims (1978) and Zylber-Katz et al. (1984). Determination of the dimethylxanthine metabolites of caffeine showed that measurable concentrations of 1,7-dimethylxanthine were present in samples of serum and saliva 4-6 hours following an oral dose of caffeine (p.111). This is in agreement with a previous study by Arnaud and Welsch (1980) which, by monitoring the fate of radiolabelled caffeine, confirmed that 72% of an administered dose of caffeine undergoes an initial 3-N demethylation. However, levels of theobromine and theophylline, dimethylxanthine metabolites, produced by respective 1-N and 7-N demethylation of caffeine were minimal.

In normal volunteers, the urine methylxanthine metabolite recoveries for the 48 hour period following an oral dose of 400 mg caffeine (p.130) accounted for 56% (SD = 16.4) of the administered dose. The principal urinary products of caffeine were 1-methyluric acid (19.9% ± SD. 8.5), 1-methylxanthine (11.3% ± SD. 4.9) and 1,7-dimethyluric acid (7.4% ± SD. 4.9); the recovery of unchanged caffeine accounted for only 2% (SD = 1.4) of the administered dose. These findings are in agreement with earlier reports by Arnaud and Welsch (1981) and Callahan

et al. (1982) which investigated the metabolic fate of radiolabelled caffeine. Since the completion of this study, further reports on the biotransformation of caffeine (Callahan et al.,1983; Tang et al., 1983; Blanchard et al., 1985; Grant et al., 1985) have conistently shown similar methylxanthine recovery patterns for both oral and intravenous doses of caffeine. Measurement of the acetylated uracil metabolites of caffeine, such as 5-acetylamino-6-amino-3-metyluracil (AAMU) and 5-acetylamino-6-formylamino-3-methyluracil (AFMU), has not been possible in this study due to the inability to obtain pure standards and the requirement for additional methodology. However, it is probable that the unaccounted metabolites for the normal volunteers (ca. 44%) represents the formation of such compounds.

Psychopharmacological and electrophysiological effects of caffeine.

In collaboration with the Institute of Psychiatry, an assessment has been made of the psychophysiological and pharmacological effects of caffeine of caffeine in healthy human subjects. Quantitative EEG measurements provided evidence for the stimulatory effects of caffeine by showing highly significant dose-related decreases in slow-wave (theta) activity and similar, but not dose related decreases in alpha wave activity (p.130). The results also suggested that a concomitant dose-related increase in beta activity may occur as a direct result of the stimulant action. Further evidence for the stimulatory action of caffeine was provided by dose-

related increases in skin conductance and from self-rating mood scales which demonstrated increased measures of alertness, enery, quick wittedness and attentiveness (p.133). Self-rating bodily symptoms indicated less tiredness and increases in the degree of shaking and trembling following ingestion of caffeine. However, since changes in tremor, measured using an accelerometer, were not significant, the tremulousness was probably mainly subjective in nature. Whilst the design of the study permitted only a limited period for collection of serum and urine samples, the pharmacokinetic data and urine metabolite recoveries were similar to those found previously in more prolonged studies. It is anticipated that an understanding of the described physiological, psychological and subjective measures, combined with the ability to determine caffeine and its metabolites in body fluids, will enable the implications of caffeine in patients with chronic anxiety states to be assessed and also the effects of caffeine withdrawl.

Caffeine metabolism during pregnancy and its exposure effects on the newborn infant.

Compared to a normal non-pregnant population, the mean levels of caffeine in serum samples collected at random from a series of pregnant females (p.153) were significantly higher (3.45 mg/l ± SD. 2.43 versus 2.43 mg/l ± SD. 1.63). However this finding is consistent with reports from previous studies on the elimination of caffeine which have shown a substantial and progressive decrease in its clearance during the second

and third trimesters (Aldridge et al., 1981; Knutti et al., 1982). The increased serum levels of caffeine were not related to changes in dietary consumption of caffeinated beverages or to alterations in the extent of protein binding (p.155). In fact the completion of a retrospective questionnaire by a limited number of volunteers suggested that the consumption of caffeinated beverages is considerably decreased during pregnancy.

Whilst the impaired clearance of caffeine during pregnancy may be the result of alterations in metabolism, studies into this possibilty have been restricted by the natural reluctance of pregnant women to ingest large single doses of caffeine and the ethical problems of using radiolabelled compounds. These problems have been overcome in the present stuby by comparing the excretion of caffeine and its metabolites in urine samples collected from pregnant and non-pregnant women receiving a controlled dietary intake of caffeine. This has provided valuable information on the biotransformation of caffeine during pregnancy (p.158). The mean percentage of the administered dose of caffeine recovered in the urine was 56.8% in both groups. However compared to the non-pregnant group, the pregnant subjects excreted smaller amounts of 1-methylxanthine and 1-methyluric acid, whereas the excretion of most of the other metabolites, particularly 3,7-dimethylxanthine and 3-methylxanthine tended to be greater. Since the values for the 1,7-dimethyl metabolites were similar in both groups, it is suggested that during pregnancy the further metabolism of 1,7-dimethylxanthine may be impaired. This could result

from an impairment in 7-N demethylation with respect to 1,7-dimethylxanthine as a substrate or alternatively regulation may be located at the level of 3-N demethylation of caffeine. The increased recoveries of metabolites possessing a 3-methyl group suggests that, during pregnancy, the route of caffeine elimination is altered in favour of an initial 1-N demethylation of caffeine to theobromine followed by 7-N demethylation to 3-methylxanthine. To a lesser extent, the initial 7-N demethylation of caffeine to form theophylline may also be increased during pregnancy, since the recoveries of 1,3-dimethyluric acid and theophylline were shown to be significantly increased. In view of previous reports relating to the effects of steroid hormones on drug metabolism in pregnancy, it was concluded that the altered biotransformation of caffeine in pregnancy may be the result of hormonal influences on the hepatic drug metabolizing enzymes.

The estimates of cord blood caffeine levels in this study were significantly higher than those previously reported for the USA and W.Germany; 5% of the values being in excess of 10 mg/l (p.168). This suggests that foetal exposure to caffeine in the U.K. may be higher than previously supposed and may lead to the manifestation of adverse effects in the newborn infant. These include tachycardia, tachypnoea, seizures, tremors, jitteriness, increased reflexes and gastro-intestinal disturbances. Whilst it is unlikely that such effects occur at concentrations below 15-20 mg/l (Howell et al., 1981), these levels could be attained as a result of caffeine intake from breast feeding which may be 4 mg/day or more. Caffeine

withdrawl from intrauterine habituated infants, especially where breast feeding is delayed or artificial feeding instituted, could play a part in the aetiology of neonatal apnoea. Although no correlation could be found between caffeine levels and score ratings for jitteriness, tachypnoea or gastrointestinal disturbances (p.169), the caffeine levels in the group of infants studied were all below those required to produce significant effects. Since the dimethylxanthine metabolites of caffeine are all pharmacologically active, the presence of significant levels of these compounds in maternal serum (p.158), cord blood and breast milk (p.168), indicates a requirement to assess the effects of total methylxanthine levels in the newborn infant. As there is some evidence to suggest that the ratio of caffeine and its metabolites in maternal serum or saliva are consistent with values found in cord blood or breast milk, maternal screening may provide a useful index to increased foetal exposure, especially in cases where the dietary consumption of caffeine by the mother is known to be high.

<u>The pharmacokinetics and metabolism of caffeine in liver disease.</u>

Although the total body clearance of a drug is a product of both metabolic and renal clearance, the described urine metabolite studies in normal volunteers have shown that renal excretion of caffeine is minimal. Consequently, since caffeine metabolism occurs almost entirely in the liver, the total clearance of caffeine calculated from pharmacokinetic data,

may be regarded as being equivalent to the actual hepatic clearance. On the basis of these assumptions, this study has investigated the suitability of caffeine clearance studies as a means of assessing the extent of hepatocellular damage in patients with chronic liver disease. Following oral doses of caffeine, the salivary pharmacokinetic data (p.197) and urine metabolite recoveries (p.208) for patients with compensated liver disease were shown to be similar to values found for normal healthy volunteers. In contrast the salivary clearance of caffeine in patients with decompensated liver disease was significantly impaired. Whilst the excretion of urinary methylxanthine metabolites for this group was also reduced, the absence of significant variations in the pattern of metabolite excretion suggested that the biotransformation of caffeine in chronic liver disease is unaltered.

It is frequently assumed that the impaired clearance of drugs in liver disease is related to a reduction in the activity of the cytochrome P-450 drug metabolizing system. However, evidence for this is largely indirect and reports have shown that the microsomal enzymes show functional heterogeneity and are influenced to a different extent according to the form of liver disease (Farrell et al., 1979). In the absence of significantly elevated levels of aspartate transaminase in the decompensated subjects it is unlikely that the extent of hepatic necrosis in these patients was sufficient to cause a profound reduction in the degree of cytochrome P-450 related drug metabolism. Subsequently, since the low hepatic extraction of caffeine minimises the effects

of altered hepatic blood flow on caffeine clearance, the observed impaired clearance of caffeine in chronic liver disease is probably associated with a reduction in its systemic clearance due to the presence of intra- or extra-hepatic shunts.

Although the severity of liver disease and the rate of caffeine elimination were poorly reflected by the results of conventional liver function tests (p.195), a close correlation was shown between Pugh score ratings of liver dysfunction (Appendix 6) and caffeine clearance in serum or saliva (p.203) and the recovery of urine metabolites (p.214). This confirms the suitability of caffeine elimination studies as a means of distinquishing between compensated and decompensated conditions of cirrhotic liver diease. Whilst the inablity to differentiate compensated cirrrhotic patients from a normal control group suggests that these studies lack sufficient sensitivity to detect mild impairements in hepatocellular function, it is possible that the patients selected for this study may have resumed normal liver function, since they were regarded as being "well compensated". A simplified protocol measuring the 24 hour or overnight salivary caffeine clearance enabled similar assessments to be made of the extent of hepato -cellular dysfunction (p.215), although further investigations are required to determine fully the sensitivity of this test as a means of assessing functional hepatic reserve. However, the indications are, that salivary caffeine clearance studies may provide a safe and non-invasive method for the initial assessment and monitoring of treatment in patients with

chronic liver disease. In addition its use as an index of prognosis may be of assistance in the selection of patients for transplant surgery.

In conclusion, this study has provided sensitive and specific methodolgies for the simultaneous analysis of caffeine and its metabolites in body fluids. The establishment of "reference ranges" for pharamcokinetic data and urine metabolite recoveries, in normal healthy volunteers following oral doses of caffeine, have enabled comparisons to be made between the metabolism of caffeine in pregnancy and in alcoholic liver disease, both of which are associated with a delayed clearance of caffeine. Whilst there is direct evidence to implicate hormone induced alterations in the biotransformation of caffeine during the third trimester of pregnacy, the impaired clearance of caffeine in decompensated alcoholic liver disease is probably related to a reduction in hepatic uptake caused by altered architecture of the hepatocyte. However, further investigations are required to investigate the effects of different forms of liver disease on caffeine elimination. In particular, it would be of interest to examine the effects of conditions associated with hepatocellular damage, such as active hepatitis and hepatic necrosis, where alterations in the biotransformation may be apparent.

Since overt responses to caffeine withdrawl have been found both in this study and in previous reports by Dreisbach and Pfeiffer (1943) and Greden, (1980), a study is being prepared in which responses to the described physiological and

psychological measurements during periods of withdrawl will be related to subjective reports and to bodily concentrations of caffeine and its metabolites. Additional studies are also required to clearly establish the effects of foetal exposure to caffeine and also to assess the implications of caffeine in chronic anxiety states.

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APPENDIX 1 Urine "clean-up" procedures for HPLC analysis.

a)C-18 Sep-Pak Cartridges (Waters Associates Ltd, Hartford, Cheshire)

Columns were conditioned by attaching a 5 ml syringe on the column inlet and flushing through 2 ml methanol followed by 4 ml 0.2mM acetate buffer, pH 4.2. A 1 ml aliquot of standard or urine (diluted 1:10 with 20mM acetate buffer, pH 4.2) was then applied to the column inlet and allowed to permeate through the column bed. The solutes were eluted from the column by flushing with 1 ml acetate followed by 1 ml 1% acetonitrile in acetate buffer, pH 4.2 and finally 2 x 1 ml of 30% acetonitrile/1% tetrahydrofuran, pH 4.2. The collected fractions were then analysed to determine the methylxanthine recovery and the efficiency of the columns in removing non-specific substances.

b)Merck Extrelut columns (BDH Ltd, Poole, Dorset).

Columns were prepared using glass Pasteur pipettes filled with approximately 0.5g of Extrelut. A 0.6 ml aliquot of standard or urine (diluted 1:10 with 20mM acetate buffer, pH 4.2) was then added to the surface of the column and allowed to permeate for 10 minutes before purging with 5 ml chloroform /isopropanol to elute the methylxanthines. Following evaporation to dryness, under air at 45 C, the residue was dissolved in 1 ml of the mobile phase prior to chromatography. The columns were assessed in terms of their methylxanthine recovery and efficiency in removing non-specific substances.

MATERIAL REDACTED AT REQUEST OF UNIVERSITY

APPENDIX 3.

Test sequences for monitoring the psychopharmacological and electrophysiological effects of single doses of caffeine.

Physiological measures.

a) Electroencephalogram and Evoked Response. These were monitored in parallel using the same EEG recording obtained from vertex and left temporal electrodes (Cz and T3). After amplification the signal was passed through a 2-32 Hz bandpass filter before undergoing a Fourier Transform and power-spectral density analysis. Subjects were requested to press a key in response to each of a series of click stimuli. Thirty two samples each of 4.8s, were taken during the reaction time task (commencing at least 3s after each click), subject's eyes open and the spectral data pooled. The spectra were divided forconvenience into the clinical wavebands: "theta" 4-7.5; "alpha"7.5-13.5; and "beta" 13.5-32 Hz. The mean log power in each waveband was computed.

b) Skin Conductance level. Skin conductance was measured from a marked area on the left thumb. The mean level and number of fluctuations per minute were monitored during the reaction time task

c) Pulse rate and blood pressure. These were measured using a "Copal" Auto-inflation digital sphygmomanometer with counter.

d) Tremor was measured using an accelerometer attached to the middlefinger, extended horizontally with the arm supported from theelbow to the wrist. The output was amplified analysed

in the computer using a Fourier power-spectral density programme.

e) Critical flicker fusion. Subjects held a monocular device with Maxwellian view optics to the dominant eye. The stimulus was a red light-emitting diode activated by the computer. Background illumination was excluded. Three runs from flicker to fusion alternated with three from fusion to flicker to allow computation of a mean of the six values.

Psychological measures

a) Tapping rate. The subject tapped a key with two fingers of his preferred hand as quickly as possible for 60s. The mean inter- tap-interval was calculated.

b) Reaction time. 32 auditory clicks were presented to which the subject was asked to respond as fast as possible.

c) Digit-symbol substitution (DSST). This was a coding task in which the subject had to code symbols for digits. The score was calculated from the number completed in 90s.

d) Symbol Copying (SCT). The same symbols were used as for the DSST but the subject had to copy and not code them. It was scored similarly to the DSST.

e) Number cancellation time (CT). As a means of a measuring the levels of attention subjects were instructed to cancel 4's in a series of numbers. The time to complete the task and the number of errors were noted.

Parallel versions of the DSST, SCT, and CT were used on each occasion.

Self-ratings.

a) Mood rating scale. Feeling at the time of each testing was measured on a series of sixteen analogue scales (Bond and Lader, 1974). This mood rating scale has been previously subjected to a principal component analysis which yielded three factors. The first factor is one of alertness and consists of nine scales; alert-drowsy, strong-feeble, muzzy-clear-headed, well coordinated -clumsy, lethargic-energetic, mentally slow-quick- witted, attentive-dreamy, incompetent-proficient and interested-bored. The second factor measures contentedness and the five scales which load on it are; contented-discontented, troubled-tranquil, happy-sad, antagonistic-amicable and withdrawn-gregarious. The third factor, calmness, is composed of two scales; calm-excited and tense-relaxed. On each scale, the subject marked the point along a 100 mm line that represented how they felt.

b) Bodily symptom scale. A similar scale has been constructed to measure bodily symptoms. It has 13 items which have been used in the investigation of anxiety; anxiety, sweating, shaking or trembling, palpitations, nausea or sickness, dizziness, irritability, loss of appetite, muscular tension, indigestion or stomach trouble, physical tiredness, headache and concentration. The subject rated them along a 100 mm line between absent and severe.

c) State anxiety inventory (Spielberger , 1970) consists of a 20-item self-report scale designed to measure the current level of tension and apprehension. Widely used in a variety of research settings, the STAI is easily administered and scored.

BODILY SYMPTOMS SCALE 4

AME :.....

ATE.....

1. Please rate the way you feel in terms of the dimensions given below
2. Regard the line as representing the full range of each dimension
3. Rate your feelings as they are AT THE MOMENT
4. Mark clearly and perpendicularly across each line

ɔ anxiety	_____	very severe anxiety
ɔ sweating	_____	very severe sweating
ɔ shaking or embling	_____	very severe shaking or trembling
o palpitations or art beating fast	_____	very severe palpitations or heart beating fast
ɔ nausea or sickness	_____	very severe nausea or sickness
ɔ dizziness	_____	Very severe dizziness
ɔ irritability	_____	very severe irritability
ɔ loss of appetite	_____	very severe loss of appetite
ɔ muscular tension	_____	very severe muscular tension
ɔ indigestion or ɔmach trouble	_____	very severe indigestion or stomach trouble
ɔ physical tiredness	_____	very severe tiredness
ɔ headache	_____	very severe headache
ɔ loss of concentration	_____	very severe loss of concentration

SELF-EVALUATION QUESTIONNAIRE

Developed by C. D. Spielberger, R. L. Gorsuch and R. Lushene

STAI FORM X-1

NAME ..... DATE .....

DIRECTIONS: A number of statements which people have used to describe themselves are given below, Read each statement and then circle the appropriate number to the right of the statement to indicate how you *feel* right now, that is, *at this moment*. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe your present feelings best.

	NOT AT ALL	SOMEWHAT	MODERATELY SO	VERY MUCH SO
1. I feel calm	1	2	3	4
2. I feel secure	1	2	3	4
3. I am tense	1	2	3	4
4. I am regretful	1	2	3	4
5. I feel at ease	1	2	3	4
6. I feel upset	1	2	3	4
7. I am presently worrying over possible misfortunes	1	2	3	4
8. I feel rested	1	2	3	4
9. I feel anxious	1	2	3	4
10. I feel comfortable	1	2	3	4
11. I feel self-confident	1	2	3	4
12. I feel nervous	1	2	3	4
13. I am jittery	1	2	3	4
14. I feel "high strung"	1	2	3	4
15. I am relaxed	1	2	3	4
16. I feel content	1	2	3	4
17. I am worried	1	2	3	4
18. I feel over-excited and rattled	1	2	3	4
19. I feel joyful	1	2	3	4
20. I feel pleasant	1	2	3	4

MOOD RATING SCALE

NAME :..... .....

DATE.....

1. Please rate the way you feel in terms of the dimensions given below
2. Regard the line as representing the full range of each dimension
3. Rate your feelings as they are AT THE MOMENT
4. Mark clearly and perpendicularly across each line

ALERT	_____	DROWSY
CALM	_____	EXCITED
STRONG	_____	FEEBLE
MUZZY	_____	CLEAR-HEADED
WELL-COORDINATED	_____	CLUMSY
LETHARGIC	_____	ENERGETIC
CONTENTED	_____	DISCONTENTED
TROUBLED	_____	TRANQUIL
MENTALLY SLOW	_____	QUICK WITTED
TENSE	_____	RELAXED
ATTENTIVE	_____	DREAMY
INCOMPETENT	_____	PROFICIENT
HAPPY	_____	SAD
ANTAGONISTIC	_____	AMICABLE
INTERESTED	_____	BORED
WITHDRAWN	_____	GREGARIOUS

_____

_____

_____

APPENDIX 4

Procedure for the investigation of caffeine metabolism during pregnancy

The collection of samples for this study may be linked to the collection of 24h urine samples for oestriol estimations, if applicable. Patients must adhere to a controlled dietary intake of coffee for 48 hours and other related compounds must be excluded from the diet. These include tea, cocoa, chocolate, coca-cola and the following drugs - theophylline and caffeine containing analgesics.

Day 1 Commence controlled intake of caffeine by selecting a daily consumption of either 4,6,8 or 10 cups of coffee. This will be supplied in pre-weighed aliquots and should ideally be consumed at approximately the same times on each day.

Day 2 Continue with controlled caffeine intake

0800 Empty bladder and discard urine. Collect total urine output for the following 24h period including sample voided at 0800 on day 3.

N.B. 0800 is merely a suggested starting time and may be altered for individual convenience providing a 24h cycle is maintained.

Name: ..... Gestational dates: .....

Coffee intake throughout study:

4 cups 6 cups 8 cups 10 cups per day

Normal daily consumption of tea/coffee

Do you smoke? (If yes, please give details)

Please give brief details of any drugs taken during this study.

	CAFFEINE INTAKE mg	METABOLITE EXCRETION (% Dietary intake per 24 hours) 7MU	7MX	1MU	3MX	1MX	13MU	37MX	17MU	17MX	13MX	137MU	137X
1	123	2.9	5.0	8.0	5.0	4.6	3.2	0	8.1	5.8	2.6	2.4	7.0
2	123	2.1	4.4	12.4	3.2	8.5	2.2	2.1	8.6	5.4	1.5	1.2	4.4
3	184	1.9	8.1	12.9	9.5	13.0	2.6	3.9	10.3	6.3	1.5	1.0	2.6
4	246	2.5	6.4	9.5	6.9	5.6	1.9	5.5	8.7	6.1	0.9	0.8	3.3
5	246	1.9	4.1	9.7	4.7	9.2	2.8	2.0	16.3	5.4	1.9	2.0	4.4
6	246	2.5	2.7	5.6	2.7	4.3	1.3	4.1	5.6	7.3	1.7	0.9	4.2
7	246	1.2	3.5	7.1	5.2	6.9	3.7	4.7	8.0	7.4	2.0	2.2	4.2
8	246	0.6	2.0	14.4	4.1	10.8	2.2	3.5	11.0	5.7	1.1	2.0	2.4
9	246	1.6	2.1	4.3	2.9	5.9	1.5	1.7	6.8	5.0	0.8	1.0	2.8
10	246	3.0	5.9	9.2	7.5	6.3	2.1	5.0	9.0	5.4	2.1	0.9	2.7
11	246	2.6	5.6	9.8	5.6	15.0	2.7	4.6	9.0	6.1	1.9	0.8	1.4
12	308	2.1	4.1	14.6	2.9	6.1	4.5	1.8	12.8	7.1	1.7	1.8	3.1
13	369	2.0	3.8	13.8	2.4	5.7	3.7	2.5	13.0	7.1	1.5	1.7	2.9
14	369	1.9	12.5	3.4	14.6	3.6	2.2	8.7	6.8	3.4	0.9	0.9	1.1
15	369	1.9	5.2	6.1	7.1	3.4	2.0	13.9	5.9	4.2	2.0	1.3	3.3
Mean values		2.0	4.9	8.9	5.6	7.3	2.4	4.8	8.9	6.0	1.6	1.3	3.5
S.D		0.6	2.7	3.4	3.3	3.4	0.8	3.2	2.8	1.3	0.5	0.6	1.5

Mean caffeine methylxanthine metabolite excretion in urine samples obtained from pregnant women receiving a controlled dietary intake of caffeine.
(Results are obtained from a single 24 hour urine collection.)

For key to abbreviations refer to page 3.

	Caffeine Intake mg	24 hour methylxanthine excretion. % Dietary intake											
		7MU	7MX	1MU	3MX	1MX	13MU	37MX	17MU	17MX	13MX	137MU	137MX
1	300	2.3	4.5	23.7	2.9	14.0	2.0	2.2	8.5	6.0	1.3	0.7	1.3
2	300	1.9	3.9	20.8	2.9	10.2	1.0	2.0	5.4	5.0	0	0.9	4.8
3	300	2.2	4.0	16.5	3.3	7.5	2.8	1.2	11.1	5.3	1.3	0.9	2.4
4	450	1.2	3.8	25.1	2.2	10.9	1.3	1.1	5.2	3.1	0.9	0.4	1.6
5	450	0.8	3.5	9.5	1.3	9.4	1.5	0	8.8	3.9	0.4	0.7	1.8
6	450	1.2	3.9	21.0	3.8	13.5	1.5	1.7	7.0	4.5	1.1	0.5	1.1
7	450	1.2	4.0	23.2	2.4	12.7	1.5	1.5	6.0	4.5	0.5	0.6	1.5
8	600	1.1	4.3	22.6	3.0	12.9	1.8	1.6	8.3	4.9	0.8	0.8	1.8
9	750	1.3	3.7	13.5	2.1	11.4	1.3	1.4	4.7	5.3	0.7	0.4	1.4
Mean value		1.4	4.0	19.5	2.6	11.4	1.6	1.4	7.2	4.7	0.8	0.7	2.0
S.D.		0.5	0.3	5.3	0.7	2.1	0.5	0.6	2.1	0.9	0.4	0.2	1.1

Methylxanthine excretion in non-pregnant normal volunteers receiving a controlled dietry intake of caffeine.

(Results are expressed as the mean values of two consecutive 24 hour urine collections).

APPENDIX 5 - Study of caffeine levels in jittery infants

SURNAME ..... FIRST NAME ..... Unit No. .....

CONSULTANT .....Ward/Dept .....Age .....

Date and time of collection of blood sample .....

Clinical details

a) Apgar score at birth: 1 min ..... 5 min .....

b) Bodily symptom ratings (Please mark perpendicularly across each line)

	0 1 2 3 4 5	
Not jittery		Severely jittery
No palpitations or tachycardia		Severe palpitations or tachycardia
No sickness or diarrhoea		Severe sickness or diarrhoea
No sweating or fever		Very severe sweating or fever

c) General comments

	SCORE		
	0	1	2
Heart rate	absent	less than 100 per min	more than 100 per min
Respiratory effort	absent	gasping or irregular	crying or rhythmic breathing
Muscle tone	flaccid	some flexor tone	good with movement
Resonse to	none	poor (with a facial grimace)	good with a cry
Colour of abdominal skin or tongue	pallor	cyanosis	pink

Apgar scoring system

Re: Kelnar CJH, Harvey D. The sick newborn baby. Bailliere Tindall, 1981, London.

Subject	Dietary intake	CAFFEINE CONCENTRATION mg/l Maternal serum	Saliva	Cord blood	Breast milk
YP	90	3.4	2.8	1.8	-
TB	120	2.7	1.9	5.3	7.2
YR	210	1.4	1.2	1.5	-
TW	240	2.4	1.8	-	-
HC	240	2.2	1.6	1.6	1.2
TH	280	5.7	4.7	4.6	-
HP	280	5.9	4.5	-	-
CB	280	12.1	10.0	12.7	8.7
GM	300	4.4	6.1	-	-
JC	300	6.9	4.9	9.6	8.5
SW	360	1.1	0.6	7.5	-
MC	360	3.6	2.8	1.4	-
TB	?	6.6	3.9	-	1.9
TT	?	5.3	4.8	2.7	2.7
HJ	?	3.7	2.4	-	2.1
ME	?	2.1	1.2	-	-
EA	?	3.0	2.6	4.4	-

Relationship between caffeine levels in maternal serum and saliva, at 34-36 weeks gestation, and corresponding samples of cord blood and breast milk collected post-natally.

Caffeine Metabolism in Alcoholic Liver Disease

Protocol for sample collection

Name : ..... Unit No. .....

Date of study : ..... Caffeine dose (mg).....

	Time of Collection	
Sample Time	Plasma	Saliva
0 min		
30 min		
60 min		
2 hour		
3 hour		
4 hour		
6 hour		
8 hour		
12 hour		
24 hour		
48 hour		
72 hour		
96 hour		

Urine collections

Basal urine Time of collection : .....

Sample 1. (0-24 hours)

Time started ..... Time finished ..... Vol. .....

Sample 2. (24-48 hours)

Time started ..... Time finished ..... Vol. .....

APPENDIX 6

Caffeine Metabolism in Alcoholic Liver Disease

Name: ..... Unit No. .....

Age: ..... Sex: M/F Nationality: .....

Weight (kg): .....

Clinical history

Liver Function Tests

Bilirubin(umol/l)	AST(U/l)
Gamma GTP(U/l)	Alk.phos(U/l)
Albumin(g/l)	Prothrombin time(secs)

Daily caffeine intake

Coffee a) instant	b) percolated
Tea	Drinking chocolate
Coca-cola	

Present drug therapy (including oral contraceptives)

Daily alcohol consumption

Smoking habits

Any other relevant information

Adverse effects noted during study

Clinical and Biochemical Measurements	Points scored for increasing abnormality		
	1	2	3
Encephalopathy grade*	None	1 and 2	3 and 4
Ascites	Absent	Slight	Moderate
Bilirubin (umol/l)	<33	34-51	>51
PBC patients **	<68	69-171	>171
Prothrombin time (sec prolonged)	1-3 sec	4-6 sec	>6 sec
Albumin (g/l)	>35	28-35	<28

Grading system for severity of liver disease (Pugh et al., 1973)

* According to grading of Trey, Burns and Saunders (1966)
** An allowance is made in the grading of patients with primary biliary cirrhosis in whom the level of serum bilirubin is usually out of proportion to other evidence of hepatic failure.

Category A = 5-6 points
Category B = 7-9 points
Category C = 10-15 points

	% administered dose												
	7MU	7MX	1MU	3MX	1MX	13U	37X	17U	17X	13X	137U	137X	Total
J.C.													
0-24 hr	0.4	3.1	25.2	1.2	13.7	1.2	5.5	4.1	0.4	0.5	1.4	1.4	58.8
24-48 hr	0.1	0.8	2.3	0.5	0.9	0	0.5	0.4	0.7	0.1	0.1	0	6.3
A.C.													
0-24 hr	0.2	1.2	9.6	0.7	10.6	0.8	1.0	4.9	3.0	0.6	0.2	1.0	33.6
24-48 hr	0.4	1.9	5.0	0.8	5.8	0.7	0.8	2.8	2.0	0.2	0.3	0.5	21.1
I.C.													
0-24 hr	0.8	2.3	15.5	2.0	10.3	1.2	2.4	7.7	4.7	0.7	0.4	1.8	49.8
24-48 hr	0.1	1.2	1.5	1.0	0.9	0.4	0.5	0.5	0.5	0.2	0	0	6.8
P.I.													
0-24 hr	0.5	2.5	13.4	1.3	10.2	1.0	1.4	7.5	5.7	0.6	0.8	1.5	46.3
24-48 hr	0.2	1.6	3.3	0.9	2.6	0.5	0.4	1.1	1.2	0	0.3	0.6	12.4
E.M.													
0-24 hr	0.1	1.5	11.5	0.7	5.5	0.6	0.5	4.9	4.2	0.4	0.3	1.8	31.8
24-48 hr	0.6	1.1	2.1	0.8	1.1	0.3	0.6	0.9	0.9	0.3	0.2	0.3	9.1
E.P.													
0-24 hr	0.8	2.8	20.0	1.5	10.5	0.3	1.5	4.3	2.9	0.3	0.2	0.5	45.6
24-48 hr	0.5	1.0	0.9	0.2	0.3	0.3	0.3	0.3	0.5	0.1	0.1	0	4.5
A.S.													
0-24 hr	0.9	1.5	20.5	1.1	11.5	0.5	1.5	6.4	3.5	0.8	0.2	2.7	51.0
24-48 hr	1.2	2.3	4.7	1.3	3.6	0.4	0.6	2.5	0.8	0.7	0.3	0.5	18.4
B.W.													
0-24 hr	0.5	2.4	3.7	0.5	5.3	0.3	0.4	1.4	2.7	0.6	0.3	2.0	20.0
24-48 hr	0.3	0.8	4.3	0.8	7.1	0.5	0.8	2.4	1.7	0.8	0.1	0.6	19.9
N.W.													
0-24 hr	0.6	0.2	16.3	0.8	6.6	1.0	1.7	6.1	4.2	0.4	0.6	1.7	41.7
24-48 hr	0.4	1.2	7.3	0.9	3.5	0.4	0.8	2.6	0.7	0.8	0.3	1.0	19.6
H.O.													
0-24 hr	0.8	1.9	7.2	0.5	4.9	1.2	1.1	8.7	3.9	0.6	0.8	2.6	34.0
24-48 hr	2.1	2.4	8.9	0.8	6.1	2.6	4.4	5.1	5.4	0.7	1.9	1.1	41.3
48-72 hr	0.6	3.3	3.1	0	0	0.7	0.7	1.1	1.8	2.1	1.0	0	14.3

Methylxanthine metabolite excretion in compensated cirrhotic patients following an oral dose of caffeine (400 mg)

	% administered dose												
	7MU	7MX	1MU	3MX	1MX	13U	37X	17U	17X	13X	137U	137X	Total
F.C.													
0-24 hr	1.1	2.8	18.3	0.9	7.3	0.4	2.1	8.2	1.1	0.1	0	1.1	43.3
24-48 hr	0.2	1.2	4.0	0.7	1.4	0.2	0.6	1.4	0.3	0.1	0.1	0.2	10.2
G.G.													
0-24 hr	0.6	1.0	9.4	0.5	1.5	0.3	1.5	6.8	1.3	0.5	0.5	1.8	25.6
24-48 hr	0.5	0.7	7.8	0.8	0.9	0.3	1.7	6.0	2.1	0.8	0.3	0.5	22.2
48-72 hr	0.4	0.5	3.3	0.6	0.2	0.2	0.8	0.8	0	0.5	0.1	0.1	7.4
72-96 hr	0.6	0.3	1.8	0.2	0.5	0.6	0.2	0.6	0.4	0.2	0	0.1	5.5
D.H.													
0-24 hr	1.9	0.6	1.8	1.5	1.4	0.3	1.3	2.1	1.0	0	0.2	4.7	16.7
24-48 hr	0	1.0	1.9	0	0.7	0.3	1.4	2.3	0.7	0.7	0.1	2.2	11.2
B.J.													
0-24 hr	1.4	1.6	8.0	0.6	3.2	0.7	0.8	6.6	2.6	0.6	1.3	3.5	30.7
24-48 hr	0.8	2.1	13.6	1.0	4.4	0.6	1.3	7.2	2	0.7	1.7	1.5	36.9
48-72 hr	2.9	1.7	4.8	1.0	2.9	0.6	0.6	1.3	0	0.8	0	0	16.4
V.D.													
0-24 hr	0.2	1.4	0.6	0	2.9	1	0	0.4	0.4	0.1	0.7	3.9	11.6
24-48 hr	1.5	0.9	0.5	0.5	4.2	0.5	0.7	0.5	0.3	2.1	0.2	4.6	16.4
48-72 hr	1.2	1.0	0.5	0.3	1.9	0.3	0.3	0.4	0.3	0	0.5	3.0	9.6
72-96 hr	1.2	0.6	0.9	0.2	0.4	0.5	0.2	0.4	0.3	0	0.6	3.4	8.6
F.F.													
0-24 hr	1.1	1.5	5.4	0.3	1.1	0.4	0.6	4.0	0.6	0	0	1.2	16.0
72-96 hr	1.1	1.1	5.0	0.3	0.4	0.2	0.4	2.0	0.2	0	0	0.2	10.6
M.S.													
0-24 hr	0.4	0.8	3.3	0	2.4	1.3	0.8	2.7	4.8	0.5	0.7	5.0	22.4
24-48 hr	0.6	1.7	7.2	0.6	3.7	1.3	1.1	4.5	6.2	0.4	0.5	1.0	28.7

Methylxanthine metabolite excretion in decompensated cirrhotic patients following an oral dose of caffeine (400 mg)