ANALYTICAL AND CLINICAL STUDIES OF OXALATE IN STONE FORMERS

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Summary.

The metabolic processes involved in the production of oxalate from its major precursors were reviewed, and the analytical techniques used for the analysis of oxalate in biological material examined.

An automated enzymic procedure for urinary oxalate utilising oxalate decarboxylase and formate dehydrogenase was developed and evaluated using the reference method. The chosen procedure was subsequently employed for the determination of oxalate in urine obtained from a group of volunteers free of a history of stone formation and other disorders. A wide variation of oxalate excretion both within and between individuals was noted.

The reference procedure was used for urine oxalate analyses of 24 hour collections from 3 groups of stone formers ascribed one of two therapies; allopurinol or bendrofluazide. Biochemical tests of serum (calcium, magnesium, zinc and urate concentrations) and urine (calcium, magnesium creatinine and urate excretion) were also undertaken.

Efficacy of the above therapies in reducing stone recurrence rates was implied in this study although the reasons for these effects were not clear.

The use of allopurinol and bendrofluazide as effective agents in reducing urinary oxalate was not confirmed by this work.

CHAPTER 1. GENERAL INTRODUCTION.

1. UPPER URINARY TRACT STONE DISEASE.

1.1.Significance .

Upper urinary tract stone disease which occurs within the kidney and ureter has apparently been known since the times of the Ancient Egyptians and Hippocrates. Currently, in the western countries, some 2 - 3 % of people will develope a urinary tract stone at some time in their lives (Nordin and Hodgkinson 1972). It has been suggested that stones occur equally in both the sexes (Lonsdale 1968) although most workers have found that symptomatic stones are between 2 and 5 times as common in men as in women (Hodgkinson 1977, Robertson et al 1980a).

The appearance of concretions within the urinary tract may not in themselves be life threatening. However loss of kidney function may occur due to damage to the kidney caused by chronic obstruction, infection or surgical intervention; moreover although the disease itself may be managed successfully, the treatment necessary, such as surgical removal of a stone, may carry serious risks.

The pain associated with kidney stones, 'renal colic', is extreme; 'in all of clinical practice there is no worse pain encountered' (Coe 1980).

The peak age for the occurrence of stones has been

shown to be 35 years in men. In women a double peak has been observed at 30 and 55 years; there was a low incidence of stones in the elderly and in children, in developed societies (Robertson et al 1980a).

The disease is of a recurrent nature: some 15 % of stone formers were seen to have had a second stone incident within a year of the first episode, 42 - 48 % of the same group within 4 years and within 9 years 61 - 67 % were found to have had a recurrence (Coe 1980).

Stone disease is therefore likely to afflict a small but economically important group of society. An active therapeutic scheme to reduce the recurrence of this condition must be considered as worthwhile.

1.2. Types of stone.

In a review of clinical stone disease by Coe (1980) 4 types of stone were recognised, containing predominantly calcium, struvite (magnesium ammonium phosphate, $MgNH_4PO_4.6H_2O$), uric acid or cystine. The percents occurrence of each type from a total of 1870 stones primarily from the United States and Europe are shown in table 1.1. Although these are not absolute values they are in general agreement with the values suggested by Nordin and Hodgkinson (1972) from a study of 700 calculi and Hodgkinson (1977) from a study of 157 calculi.

The calcium stones may be further subdivided into

Table 1.1. Types of stone and percents occurrence (Coe 1980).

Stone type.	% of all stones.
Calcium	70.6
Struvite	21.5
Uric acid	5.4
Cystine	3.5

those that contain predominantly calcium oxalate, a mixture of calcium oxalate and calcium phosphate, and predominantly calcium phosphate stones. The percents occurrence of these stone types, displayed in table 1.2. (Coe 1980) show that, next to calcium, oxalate is the most frequently occurring component of renal stone and may be expected to be seen in over 60 % of all calculi.

Xanthine stones and 2,8, dihydroxyadenine stones have also been recognised but the occurrence rate of these types was less than 1 % (Silcock 1980).

1.3. Major causes of renal stone.

The underlying cause of all urinary tract stone formation is supersaturation of the urine with a poorly soluble material modified, in the case of calcium stones, by the presence or absence of crystallisation inhibitors and sources of seed crystals and in the case of struvite stones by the presence or absence of a urinary tract infection (Coe 1980).

The rare 2,8,dihydroxyadenine stones occur due to a congenital enzymatic abnormality in which there is a complete lack of adenine phosphoribosyltransferase (E.C. 2.4.2.7.). In this disorder adenine is converted to 2,8, dihydroxyadenine by xanthine oxidase (E.C. 1.2.3.2.). The congenital enzymatic deficiency of xanthine oxidase leads to xanthinuria and subsequent xanthine stone formation (Silcock 1980). A congenital disorder of cystine,

Table 1.2. Types of calcium stone and percents occurrence (Coe 1980).

Stone type.	% Occurrence of all Stone.
Mixed oxalate plus phosphate	37.8
Predominantly oxalate	25.4
Predominantly phosphate	7.4

arginine, lysine and ornithine transport affecting the epithelial cells of the renal tubules and the gastrointestinal tract leads to 'cystinuria' and the formation of cystine containing stones (Halperen and Thier 1980).

Uric acid stones occur when there is over-saturation of the urine with urate due to disorders of uric acid metabolism, reduced urine volume or a low urinary pH.

Struvite or 'infection' stones are considered to be the result of urinary infection with bacteria that possess urease (E.C. 3.5.1.5.). This enzymic splitting of urea leads to a combination of high urinary pH and ammonium concentration. Such stones also contain calcium phosphate (Silcock 1980, Coe 1980).

Calcium phosphate stones tend to occur in urine which is abnormally alkaline due to an acidification defect or a urinary tract infection (Silcock 1980). Calcium oxalate stones, in the pure or mixed forms, may be due to disturbances in calcium and / or oxalate metabolism leading to hypercalciuria and hyperoxaluria with subsequent stone formation. As mixed stones may contain significant amounts of urate, hyperuricosuria may also induce calcium stones (Silcock 1980, Coe 1980).

1.4. Idiopathic stone disease.

When there is no recognisable clinical pathology

to account for the formation of renal stones, as is the case for the majority of calcium stone formers, such subjects have been described as 'idiopathic' stone formers (Silcock 1980). However, a number of 'risk factors' relating to urinary composition, which predispose subjects to form stones, have been recognised.

Robertson et al (1980a), describing calcium stone formation as a multifactorial disorder, recognised six main urinary risk factors: low volume, high pH, increased excretion of calcium, increased excretion of oxalate, increased excretion of uric acid and the decreased excretion of calcium oxalate crystallisation inhibitors, particularly glycosaminoglycans (acid mucopolysaccharides).

These factors influenced the relative saturation of urine with stone forming constituents, and the major risk factor was considered to be the saturation of urine with oxalate (Robertson et al 1980a, Robertson et al 1980b, Robertson et al 1981), as a small change in the urinary oxalate would be responsible for a relatively large change in the overall 'risk' of forming stones.

2. SOURCES OF OXALATE IN MAN.

Oxalic acid is the simplest of the dicarboxylic acids (figure 1.1.). In nature, it rarely occurs as the free acid, but is more often seen as some form of oxalate salt. In this work, the term "oxalate" will be used

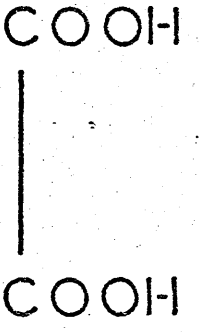


FIGURE 1.1. STRUCTURAL FORMULA OF ANHYDROUS OXALIC ACID

generally to describe the acid and its salts, unless a more specific name is required to differerentiate a particular form of oxalate.

The excretion of oxalate from the animal body has been recognised to be important in a number of pathological conditions, but in general, studies have been hindered by the lack of a suitable routine oxalate assay.

There are two sources of oxalate in man, direct dietary intake, and endogenous synthesis. Each will be described separately.

2.1. Direct dietary intake.

2.1.1. Oxalate in the diet.

Several estimates have been made of the oxalate content of the diet (Kohmann 1939, Andrews and Viser 1951, Zarembski and Hodgkinson 1962, Kasidas and Rose 1980). The values are in general aggreement with each other, although the use of a more recent method of analysis employing the enzyme oxalate decarboxylase (E.C.4.1.1.2.) (Kasidas and Rose 1980) produced results which were generally higher than those in the earlier reports. Particular foodstuffs, such as spinach, parsley, beetroot, rhubarb and chocolate may be considered rich in oxalate, whilst meat and dairy products contain only low concentrations. Tea, with

moderately elevated levels of oxalate, has been considered to be a major source of dietary oxalate for the English (Hodgkinson 1977) and the specific oxalate concentration for a particular tea has been shown to increase with the time of infusion (Kasidas and Rose 1980).

Oxalate occurs in plants in two main forms; the water soluble fraction, predominantly the sodium and potassium salts with some as the ammonium salt, and the water insoluble fraction, mainly the calcium salt with small amounts as the magnesium salt. The free acid, as such, rarely occurs in plants, and then only in trace amounts. The oxalate content of a particular plant has been shown to depend on the variety, age, climate, soil conditions and the parts analysed (Oke 1969).

The average oxalate content of the English diet has been estimated to be ^{1080} umol/d (range$_{780}$ - 1670 umol/d) predominantly from tea with bread and potatoes as other major sources (Zarembski and Hodgkinson 1962).

2.1.2. Intestinal absorption.

In vitro studies of rabbit intestinal mucosa and everted gut sacs from the rat, have shown oxalate uptake to be a passive, non energy dependant, non saturatable process (Binder 1974).

For absorption to occur, the oxalate must be in an available ie. soluble, form, but in the normal subject this is not generally the case. Absorption of oxalate from

the intestine has been shown to be poor, some 5 % or less of dietary oxalate being excreted in the urine of normal subjects following a normal diet (Hagler and Hermann 1973b). Under normal conditions, a high proportion of the dietary oxalate is precipitated in the lumen by dietary calcium, but if calcium intake is restricted then the oxalate excretion in the urine has been shown to rise in man (Zarembski and Hodgkinson 1962, Hodgkinson 1977), and in rats (Ribaya and Gershoff 1982). After an oral dose of soluble sodium oxalate, subjects following a normal diet demonstrated a rise in urinary oxalate of 2.3 - 4.5 % of the administered dose (Archer et al 1957, Zarembski and Hodgkinson 1962) whereas subjects who had been fasted overnight showed an increase of 13.6 $\pm$ 5.9 % of the administered dose (Tiselius et al 1981). The marked difference between the two studies was explained by the inhibitory effect of dietary calcium on oxalate uptake, which occurred due to the precipitation of insoluble calcium oxalate at pH above 3.Ø (Oke 1969) under which conditions both the calcium and oxalate were unavailable for absorption. Further evidence of this effect was shown by Chadwick, Modha and Dowling (1973) who demonstrated that non fasted human subjects absorbed 6.Ø $\pm$ Ø.9 % of an oral supplement of [14-C] labelled oxalate whereas fasted subjects absorbed 28.Ø $\pm$ 3.2 %. This may explain the observation of a negative correlation ($r = -Ø.6$) between the frequency of urolithiasis and the hardness of the water shown in a number of regions in the United States

(Dobson and Finlayson, 1973).

In man, the ingestion of oxalate rich foods impaired the intestinal absorption of calcium from other dietary sources, presumably due to the information of insoluble calcium oxalate (Pingle and Ramasastri, 1978). In the rats on high dietary oxalate intake mortality was significantly increased. The observed softening of the bones and reduced calcification of teeth suggested a marked bone resorption. No bone changes were noted in the control group (Kohman 1939).

In patients with ileocecal resection, absorption of an oxalate supplement has been show to be raised to 18.3 $\pm$ 7.0% and to 36.8 $\pm$ 14.0% in those with jejunoileal bypass. In the control group the absorption was 13.6 + 5.9% of the administered dose (Tiselius et al 1981). The reason for such an increase may be the presence of additional sites of oxalate absorption along the intestinal tract. Long chain

fatty acids have been shown to increase the absorption of oxalate possibly by chelating calcium (Binder 1974). The calcium was therefore no longer free to complex with oxalate and the free, soluble oxalate was therefore available for passive absorption.

2.2. Endogenous synthesis.

A number of oxalate precursors have been recognised in man and in animals. Compounds such as glycine, gelatin, glyoxylic acid, glycollic acid, ethylene glycol, ascorbic acid, certain amino acids (tryptophan, hydroxyproline, phenylalanine, tyrosine, threonine, aspartic acid, and asparagine), creatinine, purines, glucose and other carbohydrates have all been suggested as precursors of oxalate (Hagler and Herman 1973a, Hodgkinson 1977).

However, only two routes of production appear to be of significant importance in the human; these are the oxidative metabolism of ascorbic acid and the direct oxidation of glyoxylic acid.

2.2.1. From ascorbate.

Oxalate has been shown to be a metabolite of L-ascorbic acid (vitamin C) in the rat (Curtin and King 1955, Gamberdella and Richardson 1977, Hodgkinson 1978), guineapig (Banay and Dimant 1962), monkey (Tillotson and

McGown 1981), and man (Hellman and Burns 1958, Baker et al 1962, Abt, von Schuching and Enns 1963, Atkins et al 1964, Baker et al 1966a). Some 17 to 40 % of labelled L-ascorbic acid was excreted as labelled urinary oxalate, accounting for some 40 to 50 % of the total urinary oxalate excretion in normal adults (Baker et al 1962, Atkins et al 1964, Baker, Saari and Tolbert 1966b).

Radiotracer studies using [14-C] and [13-C] labelled ascorbate have shown that the oxalate was derived from carbon atoms one and two of the ascorbate molecule (Banay and Dimant 1962), presumably by the cleavage of the carbon number two to the carbon number three bond, but the pathway by which this was reached is not fully understood, and may vary between animal species (Hellman and Burns 1958).

The two isomers of ascorbic acid have been shown to behave differently in the animal body. The retention of intraperitoneally administered D-ascorbic acid has been shown to be considerably less than that of the L-isomer in the guineapig and the rat (Dayton and Burns 1958). This finding may explain the lack of vitamin C "activity" of the D-isomer. The vitamin C "activity" of certain other analogues of L-ascorbic acid in the guineapig has been shown to be dependent upon their retention (Zilva 1935).

The oxidation of labelled carbon atoms in L-ascorbic acid to respiratory carbon dioxide has been demonstrated in the guineapig and the rat (Dayton and Burns 1958, Hellman and Burns 1958, Dayton, Eisenberg and

Burns 1959, Gambardella and Richardson 1977). A similar finding has been shown in man (Abt et al 1963), but on further investigation was not reproduced (Hellman and Burns 1958, Baker et al 1966a). Baker et al (1966a) attributed these contradictory findings of carbon dioxide production from ascorbate by Abt et al (1963) to impurities in the ascorbic acid used by the latter. Baker et al (1966a) further proposed that the mechanism for the breakdown of ascorbic acid in man was as shown in figure 1.2., where the ascorbate was enzymatically reduced, in the presence of oxygen, to dehydroascorbic acid which remained bound to the enzyme; the carbon number two to carbon number three cleavage occurred whilst the lactone ring was still intact. It has been shown in rats that neither glycollate nor glyoxylate was involved in the breakdown of ascorbic acid (Gambardella and Richardson 1977), and in man that diketogulonic acid, an intermediate in the non-enzymic oxidative decomposition of ascorbic acid in aqueous solution, was not a urinary metabolite (Baker et al 1971).

2.2.2. From glyoxylate.

Glyoxylate may be metabolised via a number of pathways, to a number of intermediates / products. However, it is the metabolism of glyoxylate to oxalate that will be reviwed here.

Glyoxylate has been shown to be a precursor of

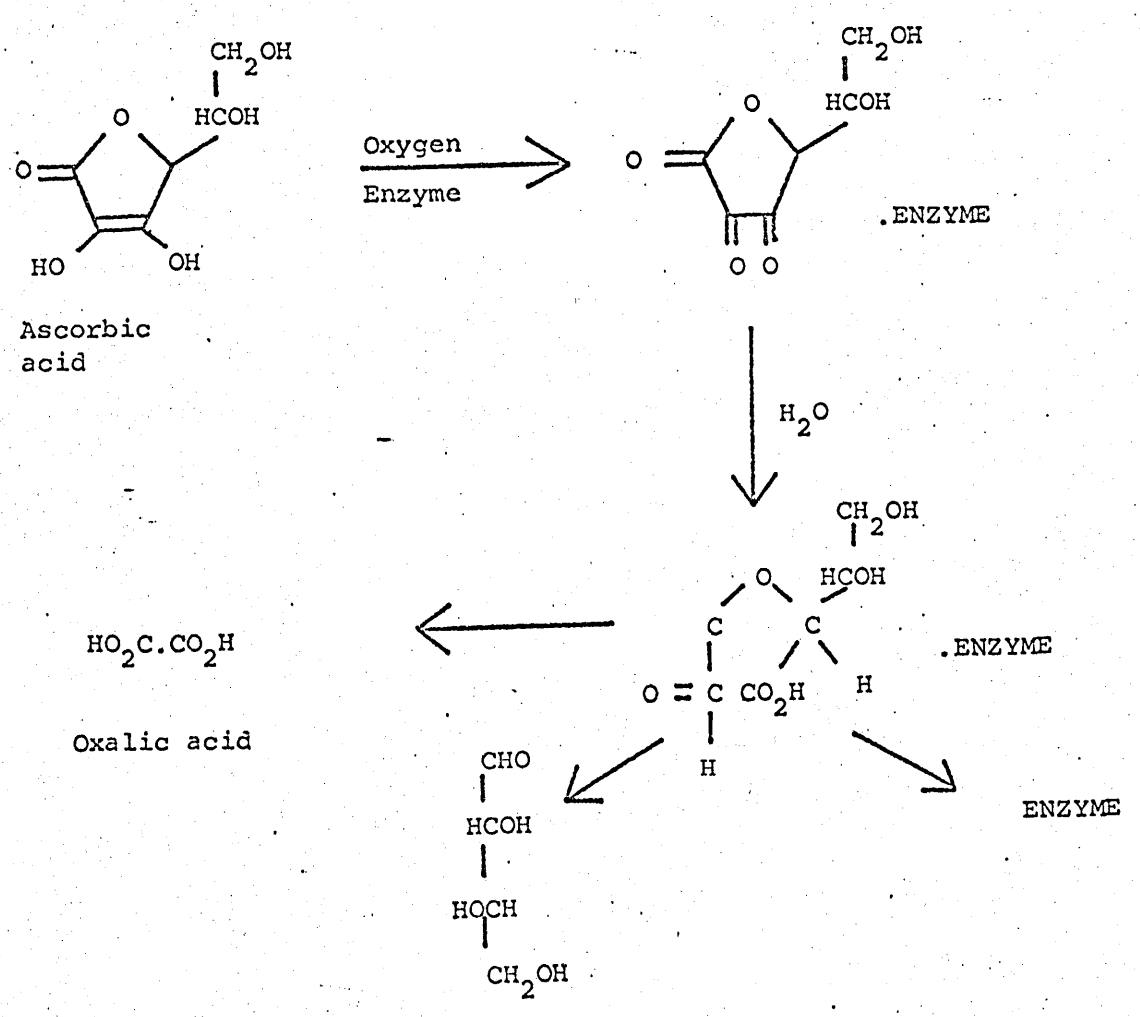


FIGURE 1.2. PROPOSED ENZYMIC CLEAVAGE OF ASCORBATE (BAKER et al 1966a)

oxalate in the rat (Weinhouse and Freidman 1951, Nakada and Weinhouse 1953, Hodgkinson 1978, Gambardella and Richardson 1978) and in man (Dean, Watts and Westwick 1968, King and Wainer 1968) using radiolabelled glyoxylate. The contribution of glyoxylate metabolism to the total oxalate excretion will vary depending on the presence of a number of glyoxylate precursors and glyoxylate forming reactions, but has been estimated to account for some 60 % of the urinary oxalate in a normal subject receiving a normal diet (Hodgkinson and Zarembski 1968).

Three enzymes, lactate dehydrogenase (E.C.1.1.1.27.), glycollate oxidase (E.C.1.1.3.1.) and xanthine oxidase (E.C.1.2.3.2.), isolated from human and animal tissues, have been shown to be able to catalyse the oxidation of glyoxylate to oxalate.

Lactate dehydrogenase appears to be, quantitatively, the most important. A preparation from pig heart has been shown to catalyse the reduction of glyoxylate to glycollate, requiring NADH at a pH optimum of 6.9, and the oxidation of glyoxylate to oxalate, requiring NAD at a pH optimum of 9.3. Oxalate itself was a non-competitive inhibitor of the glyoxylate reduction, and a competitive inhibitor of the oxidation (Warren 1970). The activity of lactate dehydrogenase from the soluble fraction of liver tissues, has been shown to be 100,000 times greater in affecting the oxidation of glyoxylate than that of either xanthine oxidase or glycollate oxidase

from similar preparations of human liver and heart tissue (Gibbs 1971).

Glycollate oxidase, a flavoprotein, catalyses the conversion of glycollate to glyoxylate by molecular oxygen (Kun, Dechary and Pitot 1954), as well as the oxidation of glyoxylate to oxalate. Again, there was product inhibition of this oxidation by the oxalate (Richardson and Tolbert 1961). Although in the human system, lactate dehydrogenase was considered to be the most important enzyme, it has been shown, in isolated perfused rat liver, using specific inhibitors, that glycollate oxidase was of greater significance in this system, as well as being involved in the synthesis of oxalate from ethylene glycol, glycine, serine, and ethanolamine (Liao and Richardson 1973).

Xanthine oxidase, another flavoprotein, also catalyses the conversion of glyoxylate to oxalate. In vitro studies have shown inhibition of oxalate production by the xanthine oxidase inhibitor allopurinol, although this inhibition was more extensive in enzyme preparations from milk than in those from the supernatant fraction of human liver (Gibbs and Watts 1966). However, the in vivo administration of allopurinol over a two week period of time did not reduce the oxalate excretion in four adult male stone formers (King and Wainer 1968), nor in two gouty subjects, and oxalate excretion was still evident in patients where the xanthine oxidase enzyme was congenitally absent. It therefore appears that in the human subject xanthine oxidase plays a minor role

(Gibbs and Watts 1966).

More recently a fourth enzyme, L-2-hydroxy acid oxidase (E.C. 1.1.3.15.) from rat kidney, has been shown to utilise a number of thiol glyoxylate adducts as substrates to produce oxalyl thioesters. These thioesters may eventually be broken down to produce oxalate, and this may be another pathway of oxalate formation from glyoxylate (Brush and Hamilton 1981).

The formation of oxalate from glyoxylate is not the major pathway of glyoxylate metabolism in normal subjects (King and Wainer 1968), and the alternate pathways are important, as they point to possible mechanisms for the *in vivo* accumulation of glyoxylate (Williams and Smith 1978).

The relevant metabolic pathways involved in the synthesis of oxalate and the metabolism of glyoxylate in man are shown in figure 1.3.

2.3.Increased endogenous synthesis.

2.3.1.Primary hyperoxaluria.

Primary hyperoxaluria is a general term for two genetic disorders of glyoxylate metabolism which are characterised by recurrent calcium nephrolithiasis, chronic renal failure and early death from uraemia (Williams and Smith 1978). The two discrete disorders have been classified as primary hyperoxaluria type I and type

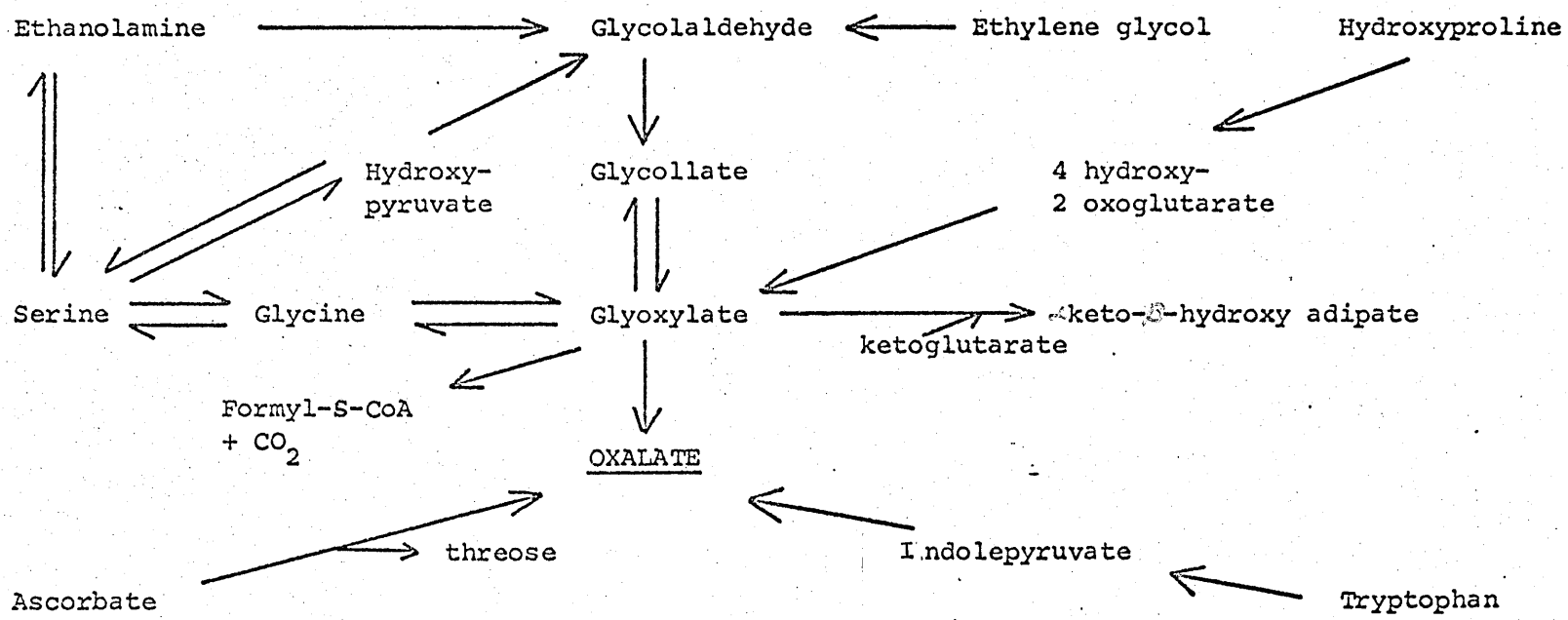


FIGURE 1.3. METABOLIC SYNTHESIS OF OXALATE IN MAN (HODGKINSON 1977)

II.

Primary hyperoxaluria type I (glycolic aciduria) has been shown to be due to an inherited defect of cytoplasmic 2-oxoglutarate:carboligase activity, which converts glyoxylate to keto- -hydroxy adipate (figure 1.3). This leads to an accumulation of glyoxylate and secondarily to an increased biosynthesis of oxalate and glycollate (Hodgkinson 1977, Williams and Smith 1978).

Primary hyperoxaluria type II (L-glyceric aciduria) is also an inherited disorder. The metabolic lesion has been less well defined, but it was suggested that a deficiency of D-glycerate dehydrogenase, which catalyses the interconversion of hydroxypyruvate and D-glycerate, as found in the leucocytes of patients with L-glyceric aciduria, was responsible for an increased hydroxypyruvate level and its subsequent increased reduction to L-glyceric acid (Williams and Smith 1978). The increase in the oxalate excretion was less clear, but Liao and Richardson (1972) have shown that hydroxypyruvate may be converted to oxalate therefore an increased level of hydroxypyruvate was responsible for an increased level of oxalate (Liao and Richardson 1978). Williams and Smith (1978) suggested that this increased oxalate synthesis was a result of the non specific reactivity of certain enzymes such as lactate dehydrogenase with hydroxypyruvate and glyoxylate; the reduction of hydroxypyruvate to L-glycerate by lactate dehydrogenase with the production of NAD favoured the oxidation of glyoxylate to oxalate

with the production of NADH by the same enzyme. However, more recently, hydroxypyruvate has been shown to inhibit the production of oxalate from glyoxylate by lactate dehydrogenase *in vitro* (Raghavan and Richardson 1983b).

The nonenzymic auto-oxidation of hydroxypyruvate has been sugested as another mechanism of oxalate formation (Raghavan and Richardson 1983a), as has the observation that hydroxypyruvate may bring about the decarboxylation of glyoxylate to oxalate by two non-enzymic mechanisms (Raghavan and Richardson 1983b).

2.3.2.Acquired hyperoxaluria.

Acquired hyperoxaluria may be regarded as 'secondary' to a 'primary' abnormal nutritional state (Williams and Smith 1978). As stated earlier, oxalate excretion may be increased by the ingestion or hyperabsorption of oxalate. Similarly, endogenous synthesis may be increased by the ingestion or hyperabsorption of oxalate precursors.

In section 2.2.1., ascorbate has been shown to give rise to oxalate. However, ethylene glycol, an anti-freeze agent (Parry and Wallach 1974, Gambardella and Richardson 1978) and methoxyflurane, a general anaesthetic (Frascino, Vanamee and Rosen 1971, McIntyre, Russell and Chambers 1973,) have also been shown to be metabolised to oxalate.

Vitamin B6 (pyridoxine) has been shown to be

required for the transamination of glyoxylate to glycine (Hodgkinson 1977) and pyridoxine deficiency has been shown to lead to hyperoxaluria in animals (Gershoff et al 1959) and man (Faber et al 1963).

3.ANALYTICAL METHODS.

Many procedures have been described for the analysis of oxalate, involving a variety of analytical techniques. Most of these procedures required preliminary separation of the oxalate from interfering substances; labelled oxalic acid has been frequently used as a recovery marker. This has increased the complexity of many of the procedures.

3.1. Atomic absorption.

Menache, (1979) described a procedure in which oxalate was precipitated with calcium chloride; the calcium remaining in the supernatant was determined by atomic absorption spectrophotometry. The amount of oxalate in the precipitate was then calculated indirectly, allowing for endogenous urinary calcium, obtained from 'reference' samples. The procedure was further optimised by Koehl and Abecassis (1976).

For colorimetric estimation of oxalate in urine it is necessary to separate the oxalate from interfering substances prior to analysis.

Separation has been affected by ion exchange chromatography (Dagneux, Ehlhorst and Olthius 1976, Olthius et al 1977), by precipitation as the calcium salt with calcium sulphate (Hodgkinson and Williams 1972) or calcium chloride (Hodgkinson and Zarembski 1961) and by extracting the urine with ether (Dempsey et al 196Ø, Hodgkinson and Zarembski 1961). The oxalic acid is then reduced to glycolic acid and is further reduced to formaldehyde which on addition of chromotropic acid forms a violet coloured p-quinoidal compound with a peak absorbance at 57Ø nm. Formaldehyde may also be reacted with 2,7, dihydroxynapthalene to give a red compound absorbing at 53Ø nm. This has been suggested as an alternative method for the estimation of oxalic acid (Calkins 1943, Snell and Snell 1953).

Hausman et al (1956) described a procedure in which, after precipitation as the calcium salt with calcium chloride, oxalate was decarboxylated to formate which then reacted with indole to form indylindolidinemethane which absorbs at 54Ø nm. The precipitation of oxalate as cerium oxalate and its subsequent oxidation by hydrogen peroxide to form a brown-coloured compound may also be used (Snell and Snell

1953).

Indirect colorimetry, in which oxalate reduced the colour intensity of the complex formed between ferric ion and 7-iodo-8-hydroxyquinoline-5-sulphonic acid or reduced the absorbance at 515 nm of the uranyl-4 (pyridyl-2-azo)-resorcin complex (Neas and Guyon 1972, Baadenhuijsen and Jansen 1975, Prenen et al 1983) has been described.

3.3. Titration.

The estimation of oxalate by direct titration with potassium permanganate after precipitation from urine as the calcium salt has been described by Archer et al (1957). After an ether extraction and calcium precipitation, Powers and Levatin (1944) back titrated an excess of potassium permanganate with sodium thiosulphate and after subtraction of a reagent blank calculated the oxalate concentration.

Cerate titration with nitroferroin as the indicator has been applied to the analysis of urinary oxalate (Koch and Strong 1969) by the modification of a procedure originally proposed for the estimation of oxalate in beer (Koch and Strong 1965).

More recently Giterson, Sloof and Schoufer (1970) precipitated oxalate from urine as the calcium salt with a known quantity of calcium chloride and subsequently estimated the calcium remaining in the supernatant by

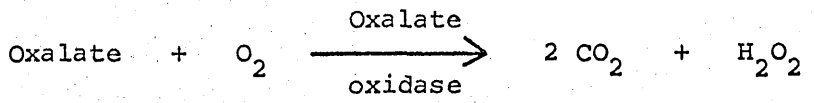
titration with EDTA using 'murexide' as an indicator. The urinary oxalate was indirectly calculated from this calcium estimation after subtraction of a 'blank' estimation of urinary calcium.

3.4. Enzymic methods.

Oxalate decarboxylase (E.C. 4.1.1.2.) and oxalate oxidase (E.C. 1.2.3.4.) have both been employed in the estimation of oxalate in urine. The reaction schemes for both enzymes are shown in figure 1.4.

In the oxalate oxidase system, the CO_2 produced has been quantified by following the pH change of the system caused by the diffusion of the CO_2 into an alkaline buffer (Kohlbecker, Richter and Butz 1979, Kohlbecker and Butz 1979, Boer, Leersum and Endeman 1984), and after immobilisation of the enzyme in an acrylamide gel over a CO_2 sensor to form an oxalate electrode (Sheldon et al 1983).

The hydrogen peroxide has been quantified colorimetrically using a peroxidase (E.C. 1.11.1.7.) enzyme system to bring about the formation of an indamine dye (Sagiura et al 1979, Laker, Hofmann and Meeuse 198Ø, Sagiura et al 198Ø, Buttery et al 1983, Crider 1983, Obzansky and Richardson 1983, Potezny et al 1983) as shown in figure 1.5., or by using a catalase (E.C. 1.11.1.6.) linked with an NADP-requiring aldehyde dehydrogenase (E.C. 1.2.1.5.) system to produce NADPH (Kohlbecker and Butz



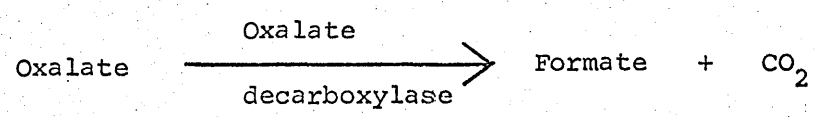
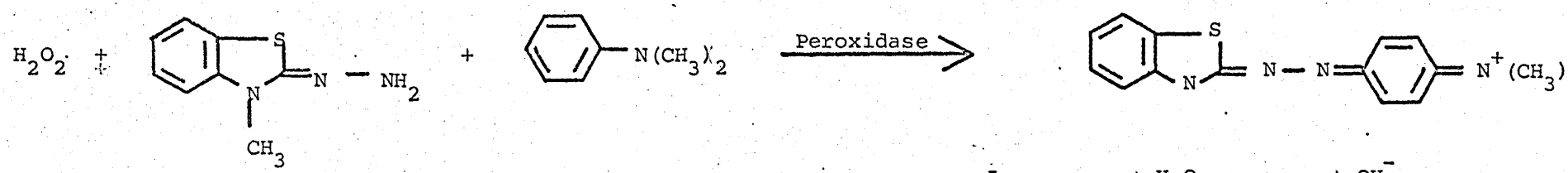


FIGURE 1.4. REACTION SCHEMES OF THE OXALATE OXIDASE AND OXALATE DECARBOXYLASE SYSTEMS



3-methyl-
2-benzothozlinone
hydrazone

N,N-dimethylaniline

Purple indamine dye

FIGURE 1.5. REACTION SCHEME OF THE PEROXIDASE / HYDROGEN PEROXIDE SYSTEM (LAKER 1980)

1979, Kohlbecker and Butz 1981) as shown in figure 1.6.

In the oxalate decarboxylase system, the CO_2 evolved has been measured manometrically in Warburg systems (Shimazono and Hayaishi 1957, Mayer, Markow and Karp 1963, Ribeiro and Elliot 1964), by following the pH change of an alkaline buffer (Hallson and Rose 1974 Ackay and Rose 1979), by following the change in conductivity of a solution of strontium hydroxide (Bishop et al 1982) and after immobilisation of the enzyme in an acrylamide gel over a CO_2 sensor to form an oxalate electrode (Sheldon et al 1983).

The formate has been quantified colorimetrically using an NAD-requiring formate dehydrogenase (E.C. 1.2.1.2.) system to produce NADH (Costello, Hatch and Bourke 1976, Hatch, Bourke and Costello 1977, Chalmers 1979, Beutler et al 1980, Yriberri and Posen 1980) as shown in figure 1.7.

3.5. Fluorimetric analysis.

The fluorimetric determination of oxalate in urine has been described in which the oxalate was first extracted with tri-n-butyl phosphate and subsequently precipitated as the calcium salt with calcium sulphate. The oxalate was then reduced to glyoxylic acid which formed a fluorescent complex with resorcinol, probably by the formation of a lactone of 2,2',4,4' tetrahydroxydiphenylacetic acid which had an excitation

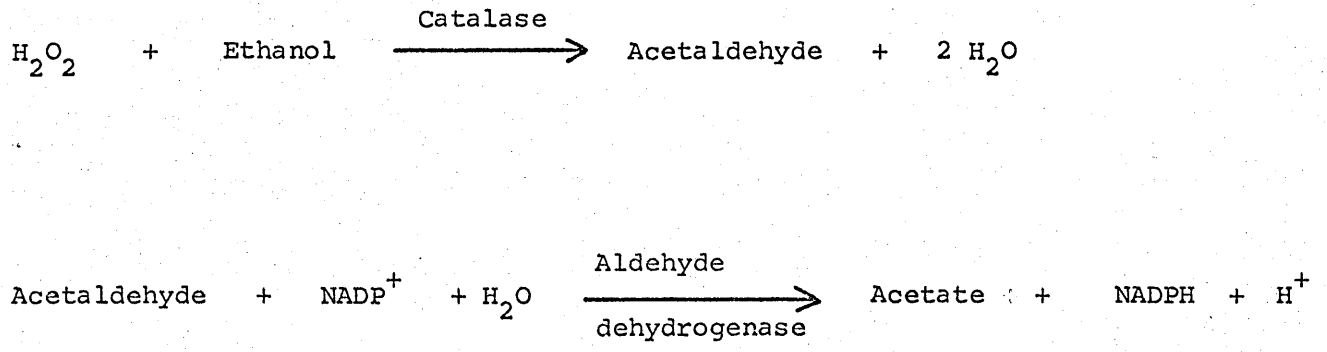
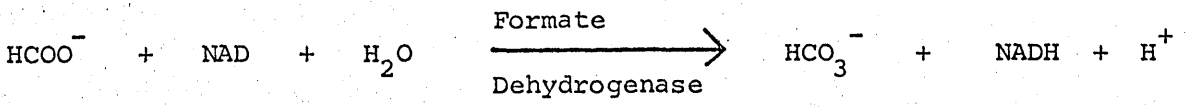


FIGURE 1.6. REACTION SCHEME OF THE CATALASE / ALDEHYDE DEHYDROGENASE SYSTEM (KOHLBECKER and BUTZ 1981)



formate bicarbonate

FIGURE 1.7. REACTION SCHEME OF THE FORMATE DEHYDROGENASE SYSTEM (BOEHRINGER 1982)

wavelength of 490 nm and an emission wavelength of 530 nm (Zarembski and Hodgkinson 1965a, Zarembski and Hodgkinson 1965b).

Alternatively, the oxidative power of oxalic acid, after precipitation with calcium chloride, has been used to reduce cerium (IV) to fluorescent cerium (III) which has an excitation wavelength of 260 nm and an emission wavelength of 350 nm. (Meola et al 1983).

3.6. Gas chromatographic methods.

Gas chromatography is a separation technique with which the compounds to be separated are partitioned between a mobile phase (the carrier gas) and a stationary phase (the liquid phase or adsorbent). The difference in partitioning of the components between the stationary and the mobile phase affects the separation.

The requirements for compounds to be analysed by gas chromatography are such that they are sufficiently volatile at temperatures below 400 ^{o}C and will not decompose at the working temperatures. If the compound itself does not fulfill these criteria, it may be possible to synthesise a stable volatile derivative.

The methods described for the analysis of oxalate all require initial derivatisation and usually some separation of the oxalate or its derivative from other interfering components of urine.

Columns packed with silicone (Tanaka et al 1980a,

Tanaka et al 1980b, Moye et al 1981, Moye et al 1983) or polyethylene glycol (Di-Corcia et al 1982) stationary phases, porous polymer packings (Chambers and Russell 1972, Charransol et al 1978, Park et al 1980) and capillary columns (Dosch 1979, Wolthers and Hayer 1982) have all been utilised in the analysis of oxalate.

Flame ionisation detectors have been used to detect the diethyl ester (Charransol 1978, Offner and Uring 1979) the dimethyl ester (Chambers and Russell1972, Farrington et al 1979, Park et al 1980, Di-Corcia et al 1982, Yanagawa, Ohkawa and Tada 1982), the dipropyl ester (Gelot et al 1979) and the trimethylsilyl derivative (Tanaka et al 1980a, Tanaka et al 1980b, Wolthers and Hayer 1982) using a variety of supports and liquid phases. The electron capture detection of the bis-2-chloroethyl ester (Tocco et al 1979, Moye et al 1981, Moye et al 1983) has also been reported. A capillary gas chromatographic method using a flame ionisation detector for the trimethylsilyl derivative has been described by Wolthers and Hayer (1982) and for the diethyl ester by Dosch (1979).

Duggan et al (1979) described a gas chromatographic mass spectrometer (GCMS) procedure for the analysis of oxalate as the di-n-propyl ester. A known amount of [1,2, 13C] oxalate was added to the sample prior to precipitation as the calcium salt and subsequent derivatisation. By the use of selective ion monitoring, the ratio of [12C] to [13C] and hence the initial

concentration of oxalate in the sample was determined.

The time of analysis for each sample has been as much as 8Ø minutes (Gelot et al 1979) but with more specific extraction prior to chromatography or by a technique known as 'backflushing' the analysis time has been reduced to between 6 and 8 minutes (Di-Corcia et al 1982, Dosch 1979).

The requirement for the formation of volatile derivatives, the extraction of either the oxalate or the oxalate derivative and the limited number of samples that may be run per day, make gas chromatography poorly suited for the routine estimation of oxalate.

3.7. High performance liquid chromatography. (HPLC).

HPLC is a separation technique similar to gas chromatography in which the compounds to be separated are partitioned between a mobile phase (the eluant) and a stationary phase (the column packing or column packing coating). The degree of partitioning between the stationary phase and the mobile phase affects the separation. The use of smaller particle sizes of the packing (eg 5 and 1Ø um) results in increased efficiency over classical liquid chromatography.

'Ion-pair' reversed phase chromatography is a form of partition chromatography which may be used to separate charged species, and as such may be used as an alternative to ion exchange chromatography. Counter ions are added to

the mobile phase to neutralise the charge of the solute ions and thus regulate their retention. The use of U.V.-absorbing or fluorescent counter ions may permit more convenient detection of the eluted solute and counter ion pair than of the solute alone.

Direct chromatography of oxalate using a reversed phase octasilyl column with a tetrabutylammonium hydrogen sulphate ion pair, which both increases the retention of oxalate on the column, and allows detection of the ion pair at a wavelength of 220 nm has been described (Libert 1981, Larsson, Libert and Asperud 1982). When the method was applied to the analysis of urinary oxalate (Larsson et al 1982) initial sample preparation was required and the analysis time for each sample was 20 minutes.

Imaoko et al (1983) described a procedure in which oxalate, after extraction with tri-n-butyl phosphate, was esterified with 9-anthryldiazomethane to form a fluorescent derivative which was subsequently chromatographed on a reversed phase octadecylsilyl column and detected fluorometrically. Although the retention time for the oxalate derivative was only 15 minutes, the need to allow for later eluting endogenous components increased the analysis time to 40 minutes per sample. Hughes, Hager and Sutton (1982) extracted oxalate with 1,2, diaminobenzene to form 2,3, dihydroxyquinoxaline which was subsequently chromatographed on a reversed phase octasilyl column with ultraviolet detection at 312 nm. A solvent program was used which included a gradient elution step to

rapidly clear the column of later eluting components; this reduced the analysis time to 21 minutes.

Anion exchange HPLC with electrochemical detection has been described using tetrabutylammonium tetrafluoroborate ion pair with isocratic elution (Mayer et al 1979a, Mayer et al 1979b) and with no ion pair agent but with gradient elution (Asper and Schmucki 1979). Seta (1980) described an anion exchange procedure utilising stopped flow ultraviolet scan detection with gradient elution.

3.8. Ion chromatography.

Ion chromatography was first described by Small, Stevens and Bauman (1975). It is a form of HPLC which utilises a low surface capacity ion exchange 'chromatographic' or 'separator' column to affect the separation coupled with a high capacity ion exchange 'suppressor' or 'stripper' column to reduce the background conductivity of the eluant by ion exchange action and therefore allow conductimetric detection of the eluted ions.

For oxalate analysis the 'separator' column is a low capacity anion exchanger, and the 'stripper' column a high capacity cation exchanger (H+ form).

The technique has been applied to the analysis of oxalate in unprocessed urine (Mahle and Menon 1982, Dionex 1982, Menon and Mahle 1983). Although the procedure has

been claimed to be accurate and precise (Mahle and Menon 1983) a single sample required 30 minutes analysis time and due to the need to regenerate the 'stripper' column after 2.5 hours of use the number of samples that may be analysed per day was limited.

3.9. Ion specific electrodes.

Ferrel, Blackburn and Vosburgh (1948) described a silver - silver oxalate and a mercury - mercurous oxalate electrode. The silver oxalate electrode system was further investigated by Mathur and Naqui (1968) who observed that the electrode system was not sensitive to wide variations in either temperature or electrolyte concentration and they suggested its use as a reference electrode. As such the application of this system to the estimation of oxalate was limited.

3.10. Isotachophoresis.

Isotachophoresis is an elecrophoretic technique which requires two electrolytes, the 'leading electrolyte', which contains an ion with highest electrophoretic mobility, and the 'terminating electrolyte', which contains an ion with the lowest electrophoretic mobility in comparison to the intermediate mobilities of the sample ions. The theory of elecromigration methods of separation has been described

by Prusik (1979).

A capillary isotachophoretic procedure for the estimation of oxalate in unprocessed urine has been described by Tschope, Bremen and Ritz (1981) in which the time for each analysis was between 20 and 60 minutes.

3.11. Isotope Dilution.

To overcome the problem of incomplete recovery or incomplete derivatisation of oxalate a number of workers have used isotope dilution techniques.

In the GC/MS method of Duggan et al (1979) a known amount of [13 C] labelled oxalate was added to the sample and, after derivatisation, the ratio of [12 C] to [13 C] was measured by selective ion monitoring and the total oxalate in the sample calculated by the dilution of the [13 C].

[14 C] labelled oxalate has been used where the total radioactivity added was known and after conversion to glycollate (Hockaday et al 1965) or reprecipitation as the calcium salt (Gibbs and Watts 1969), the specific activity of the product was estimated and the total oxalate content of the sample calculated.

3.12. Discussion.

The majority of the procedures so far described were not suited to the routine analysis of urinary

oxalate. Chromatographic procedures have been shown to be limited not only by the length of time required for each analysis but also by the need for initial sample preparation or derivatisation.

Spectrophotometric and oxidometric analyses were also restricted by the requirement for sample preparation and although recoveries may be monitored by the use of labelled oxalate and isotope dilution, this increases the complexity of the procedures.

Ion specific electrodes, although theoretically possible have not been applied to the direct estimation of oxalate in urine.

Of the procedures outlined in this section, the use of specific enzymes has attracted the greatest and most recent attention in the literature for the rapid and specific analysis of oxalate in unprocessed urine.

Table 1.3. shows the performance figures of a number of methods of urinary oxalate analysis, as quoted by the authors.

The lower limits of the cited reference ranges vary from Ø to 352 umol/d and the upper limits vary from 255 - 1Ø2Ø umol/d. This wide variation of reference range indicates the difficulties that the analysis of oxalate in urine has posed.

Methods which claim a lower reference limit of less than 1ØØ umol/d or an upper limit of less than 4ØØ umol/d probably underestimate the oxalate content of urine, and methods which claim a lower reference limit of

Table 1.3. Selected methods of urinary oxalate estimation.

METHOD	REFERENCE RANGE (umol/d)	RECOVERY (%)	PRECISION (CV%)	AUTHOR
Atomic Absorption	22 - 422	95	17.4	Koehl and Abecassis (1976)
Colorimetric (Chromotropic acid)	189 - 478	75 - 98	5.2	Hodgkinson and Williams (1972)
	126 - 505	98	5.3	Olthius et al (1977)
Colorimetric (Indirect)	194 - 618	93 - 104	12.0	Prenen et al (1983)
Back Titration	159 - 398	87 - 93	N/A	Powers and Levatin (1944)
Direct Titration	63 - 287	90 - 92	N/A	Archer et al (1957)
Indirect Titration	0 - 400	88 - 113	5.0	Giterson et al (1970)
Fluorometric	100 - 317	96 - 99	5.0	Zarembski and Hodgkinson (1965
Enzymatic (Oxalate oxidase/ CO_2)	53 - 418	92 - 109	7.4	Kohlbecker et al (1979)
Enzymatic (Oxalate oxidase/ H_2O_2)	up to 550	92 - 109	9.5	Buttery et al (1983)
	100 - 411	81 - 98	2.2	Crider (1983)
	311 - 478	95 - 109	11.0	Laker et al (1980)
	160 - 560	95.9	4.6	Potenzy et al (1983)
	254 - 512	95.2 - 100	5.5	Obsansky and Richardson (1983)
	Not stated	98	5.9	Kohlbecker and Butz (1981)
Enzymatic (Oxalate decarboxylase/ CO_2)	52 - 403	85 - 100	N/A	Mayer et al (1963)
	Not stated	76.8 - 117	7.2	Reiberio and Elliot (1964)
	150 - 500	93.3	12.6	Bishop et al (1982)
Enzymatic (Oxalate decarboxylase / formate)	155 - 418	99.2 - 107.6	3.4	Costello et al (1976)
	200 - 520	94	7.6	Yriberri and Posen (1980)
GC (FID)	80 - 500	101	8.3 - 10.0	Farrington et al (1979)
	100 - 255	98 - 100	N/A	Park et al (1980)
	75 - 475	65.3 - 100	11.3	Gelot et al (1980)
	352 - 1020	93.6 - 102.4	2.5	Di-Corcia et al (1982)
	126 - 586	98.3 - 101.2	2.19	Yanagawa et al (1983)
GC (ECD)	Not stated	84.2 - 111.3	10.3	Moye et al (1981)
GC (Capillary)	Not stated	99.6	1.6	Dosch (1979)
	50 - 410	101 - 112	8.3	Wolthers and Hayer (1982)
HPLC	Not stated	90.3 - 104.6	3.3	Larsson et al (1982)
	184 - 615	100.2	5.9	Hughes et al (1982)
	64 - 464	95 - 105	3.7	Imaoko et al (1983)
Ion Chromatography	200 - 677	97 - 105	6.1	Menon and Mahle (1983)
Isotachophoresis	190 - 815	80 - 111	3.2	Tschope et al (1981)

over 300 umol/d and an upper reference limit of over 700 umol/d probably overestimate the urinary oxalate. Nearly half of the methods cited in table 1. have reference ranges outwith these criteria.

From all of the above considerations, the need for an urinary oxalate assay which is simple to perform, rapid, precise and accurate is evident.

4. AIMS OF THE PRESENT STUDY.

4.1. Analytical.

In a recent study which compared six methods of urinary oxalate estimation, no single technique was found to be superior to the others. The methods investigated were colorimetric, enzymic, ion chromatographic, high performance liquid chromatographic and gas chromatographic procedures (Zerwekh et al 1983).

One of the aims of the present study has been to develop a procedure which was suitable for the routine analysis of a large series of samples.

4.2. Clinical.

The clinical aim of this study was to assess the effects of two prophylactic therapies, 300 mg allopurinol / day and 5 mg bendrofluazide / day, on the progress of stone forming patients presenting on an out patient basis

to the Urology Department of the Glasgow Royal Infirmary, both clinically by the interpretation of subjects' case notes and biochemically by the estimation of blood and urinary constituents, including urinary oxalate, before and during therapy (table 3.1.).

CHAPTER 2. METHODS.

1. REFERENCE URINARY OXALATE PROCEDURE.

The reference method of analysis used in this study was a colorimetric procedure based on the method described by Hodgkinson and Williams (1972), a development of two earlier procedures developed in the same laboratory (Hodgkinson and Zarembski 1961, Zarembski and Hodgkinson 1965) with some modifications suggested later by Husdan et al (1976). The method had been used in previous studies in the Urology Department (Scott et al 1978a, Scott et al 1978b).

The method relies on the separation of oxalate from interfering substances in urine by co-precipitation of oxalate from urine with calcium sulphate at pH 7. Subsequent reduction of the oxalate to glycollate by nascent hydrogen is brought about by boiling the precipitate with dilute sulphuric acid and a zinc pellet. The glycolic acid is then reduced to formaldehyde, which is believed to form a violet coloured p-quinoidal compound with chromotropic acid (Feigl 1966), by boiling with chromotropic acid and concentrated sulphuric acid. A similar reaction procedure has been described by Snell and Snell (1953). The probable reaction scheme is described in figure 2.1.

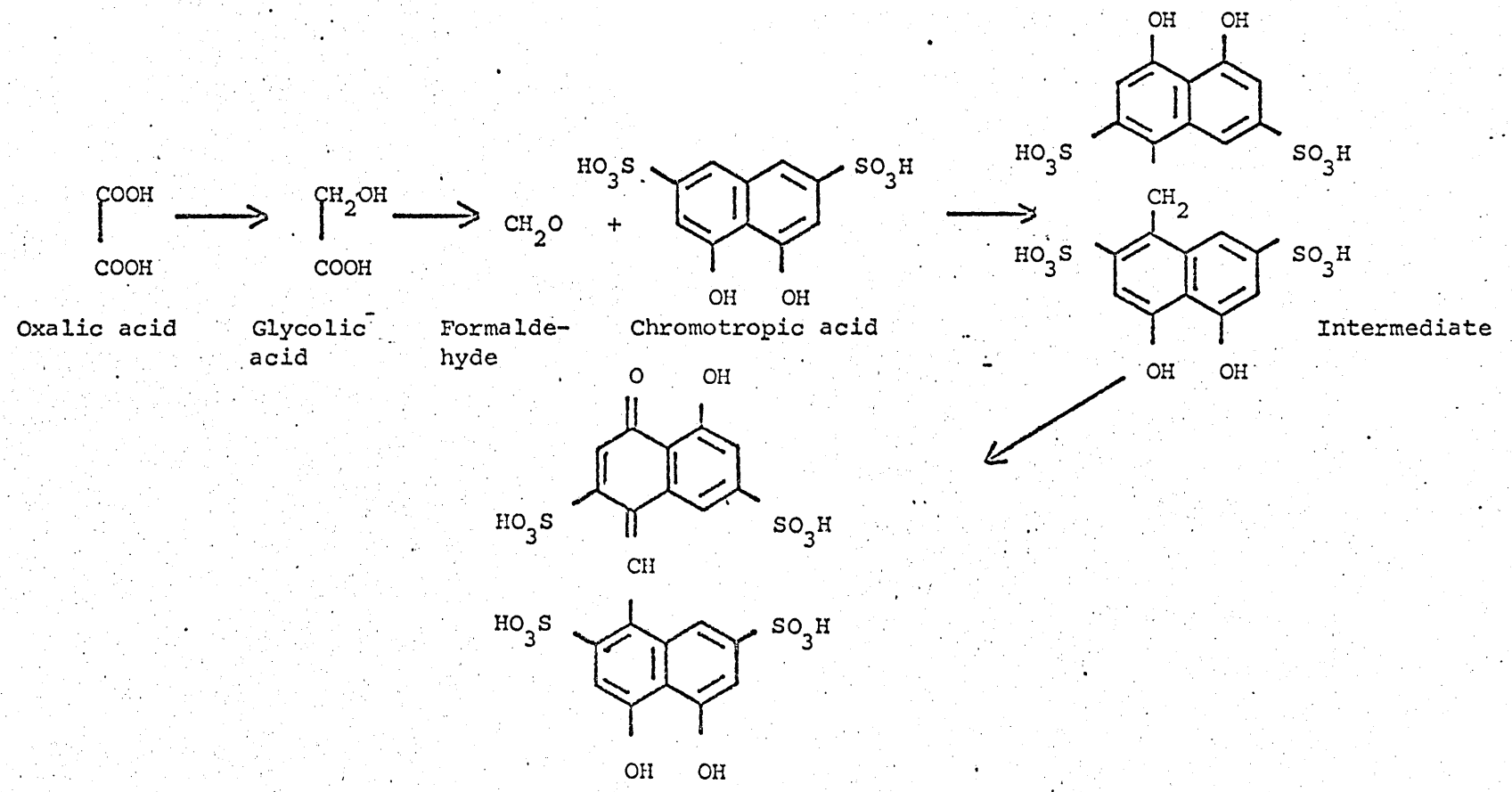


FIGURE 2.1. PROPOSED REACTION SCHEME FOR THE REFERENCE OXALATE PROCEDURE.

1.1. Sample Collection.

Twenty four hour urine samples were collected directly into polythene bottles containing 5 ml of concentrated hydrochloric acid which lowers the pH of the urine to between 1 and 3, with the object of preventing the oxalate precipitating before analysis. The solubility of calcium oxalate in aqueous solution has been shown to increase with increased acid concentration (Hodgkinson 1981).

The volumes of the urine collections were measured on receipt by weighing. 20 ml aliquots from the well mixed collections were taken for the analysis of calcium, magnesium, creatinine, urate and oxalate.

The aliquots for oxalate estimation were stored at +4 C until analysis, which was generally performed within one week of receipt. After analysis, the aliquots were stored at -20 ^{o}C to preserve the samples.

1.2. Materials.

Unless otherwise stated, all materials were of AnalaR grade, supplied by B.D.H. (England).

Centrifuge tubes

M.S.E. straight walled 112 x 28 x 20 mm internal diameter, scored at 20 ml level with a diamond.

Mean volume = 19.98 ml (CV = 1.32 %, n = 42).

1 % chromotropic acid

1 g 4,5-dihydroxynapthalene 2,7-disulphonic acid (Sigma Chemical Company) dissolved in 100 ml distilled water, stored at +4 C. Prepared fresh each week.

Stock standard oxalate. 60 mM.

2.7636 g Potassium Oxalate $K_2C_2O_4.H_2O$ made up to 250 ml with distilled water, stored at +4 C. Prepared fresh each month.

Working oxalate standard. 600 uM

1 ml of the stock standard oxalate diluted to 100 ml with distilled water. Prepared for each assay.

Zinc pellets

3.2 mm diameter zinc wire (Pierce and Warriner, England) cut into 5 mm lengths (300 mg) washed immediately before use in fresh 10 M nitric acid and rinsed in distilled water.

Calcium sulphate

Saturated aqueous solution prepared as required.

10 M nitric acid

158 ml of 70 % HNO_3 (m.wt.= 63.01) solution of specific gravity 1.42 made up to 250 ml with distilled water.

Ø.2 M sodium hydroxide

2 g NaOH (m.wt.=4Ø.Ø) dissolved in 25Ø ml distilled water.

Ø.2 M hydrochloric acid

4.4 ml of 35 % HCl (m.wt.= 36.46) solution of specific gravity 1.18 made up to 25Ø ml with distilled water.

Concentrated sulphuric acid

98 % H_2SO_4 of specific gravity 1.84.

5 M sulphuric acid

269 ml of concentrated sulphuric acid made up to 1 litre with distilled water.

2 M sulphuric acid

4ØØ ml of 5 M sulphuric acid made up to 1 litre with distilled water.

1 M sulphuric acid

5ØØ ml of 2 M sulphuric acid made up to 1 litre with distilled water.

Ethanol

Absolute alcohol C_2H_5OH. 99.7 %.

1.3. Procedure.

To 1.5 ml of urine or quality control samples, in

duplicate, was added Ø.5 ml of distilled water. The pH was then adjusted to pH 7 (6.8 to 7.2) by the dropwise addition of Ø.2 M NaOH or Ø.2 M HCl as necessary, the pH being monitored using a suitable pH electrode. 2 ml of saturated calcium sulphate was added, followed by 14 ml of ethanol. After mixing, the tubes were left at room temperature overnight. The samples were then spun at 1200 g for 1Ø minutes, the supernatant solution carefully decanted, and the tubes allowed to drain, in an inverted position, for a few minutes. The precipitate was then taken up in 2 ml of 1 M sulphuric acid.

Six standards were prepared by adding Ø, Ø.125, Ø.25, Ø.5, Ø.75 and 1.Ø ml of the 6ØØ uM working oxalate standard, to separate tubes, and making the final volume to 1 ml with distilled water. To each of these tubes was added 1 ml of 2 M sulphuric acid to provide standard values of Ø, 5Ø, 1ØØ, 2ØØ, 3ØØ, and 4ØØ uM oxalate.

A freshly cleaned zinc pellet was then added to each tube, and the tubes placed in a boiling water bath, in a fume cupboard, and evaporated to dryness.

After being allowed to cool, the zinc pellets were removed and washed with Ø.5 ml of 1 % chromotropic acid. (This was more easily achieved if the tube was clamped horizontally in a retort stand, and the zinc pellet drawn towards the mouth of the tube with a sealed and bent pasteur pipette, where it was washed with the chromotropic acid, and the washings passed into the tube, before final removal of the pellet.)

5 ml of concentrated sulphuric acid was then carefully added to each tube, with mixing, and the tubes were replaced in the boiling water bath for a further 30 minutes.

The tubes were removed and allowed to cool. 5 M sulphuric acid was added to a final volume of 20 ml in each tube and, after mixing, the absorbance was measured at 570 nm, using the zero standard as a blank.

The concentration of oxalate in the samples and quality controls was then calculated from the regression equation of the six standards.

1.4. pH of collected urine samples.

The pHs of 30 urine specimens, chosen at random, were measured. The mean value was 2.46 ($\pm$ 0.46) and the coefficient of variation was 18.8 %. The pH ranged from 1.70 to 3.80.

1.5. Linearity.

Aqueous standards up to 4 mmol/l were prepared. The results are shown in figure 2.2. The method was shown to be linear to a urinary oxalate concentration of 1500 umol/l. No specimens were found to be above this level.

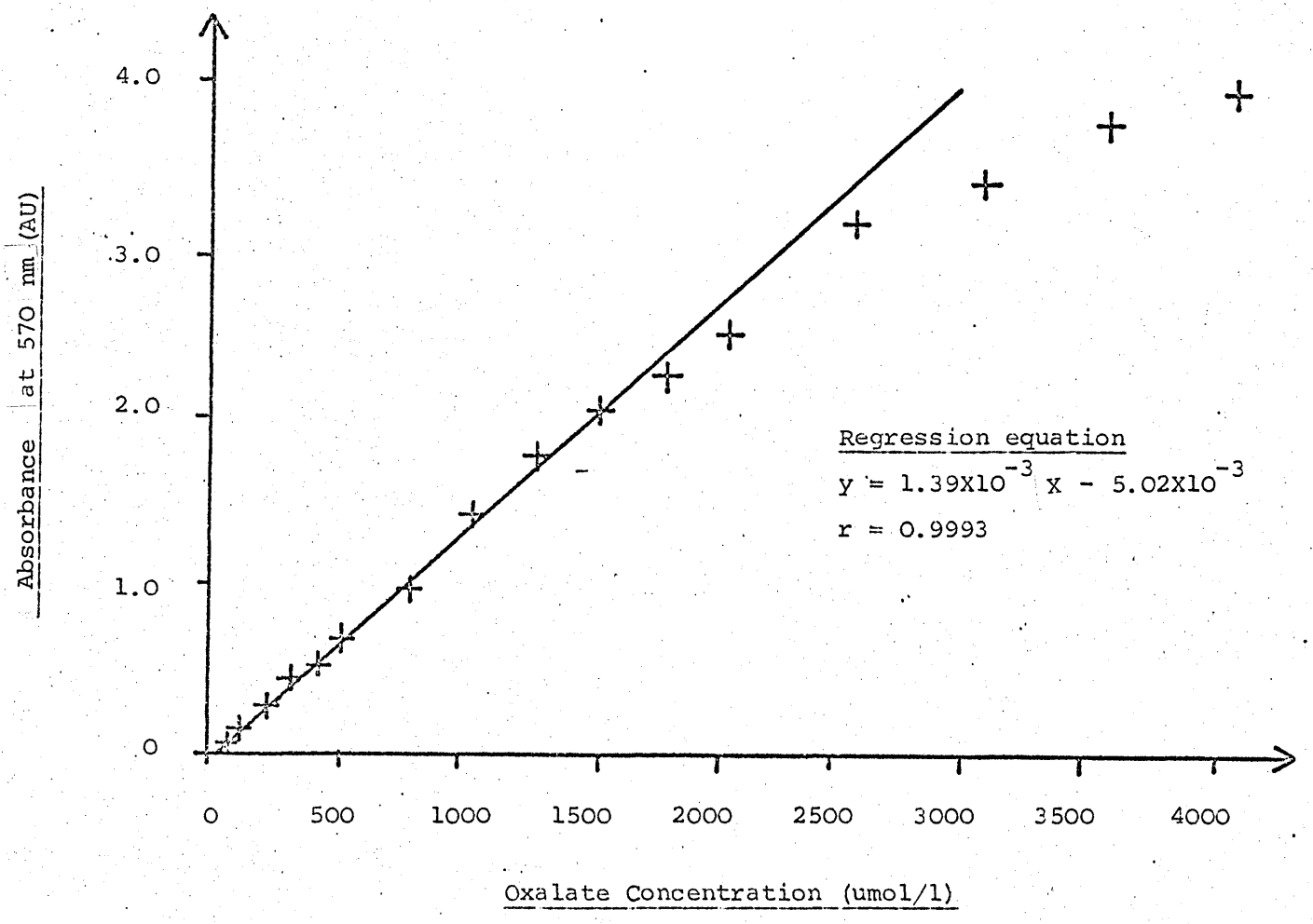


FIGURE 2.2. LINEARITY OF AQUEOUS OXALATE STANDARDS VERSUS OPTICAL DENSITY AT 570 nm

1.6. Precision.

1.6.1. Within run precision.

The within run precision was estimated using five urine specimens. The results are shown in table 2.1.

The coefficient of variation ranged from 2.13 to 9.62 %, and the mean values of the urine samples from 49.4 to 506 umol/l.

1.6.2. Between run precision.

Five urine specimens have been used as quality control material, prepared as shown in table 2.2., and to estimate the between run precision. The results are shown in table 2.3 and figure 2.3.

Sample number 4 was, in effect, an external quality control sample, and the target range for oxalate suggested by the manufacturers using a gas chromatographic method was 122 to 278 umol/l, and by an atomic absorbtion method 156 to 222 umol/l (Lyphocheck 1882). A mean value of 222 umol/l ($\pm$ 26.3) was found in this study.

The wide target range, quoted by the manufacturer for the gas chromatographic method, which was considerably different from the range quoted for the atomic absorption method limits the usefulness of this sample as an external quality control for any other method of analysis. This also underlines the overall problem of urinary oxalate

Table 2.1. Within run precision of the reference procedure.

SAMPLE	n	MEAN (umol /l)	S.D.	C.V.%
1	8	49.4	4.75	9.62
2	9	179	10.8	6.04
3	8	180	3.83	2.13
4	9	374	17.9	4.79
5	8	506	14.7	2.90

Table 2.2. Preparation of quality control samples.

SAMPLE	PREPARATION
1&2	Pooled urine specimen aliquoted (5ml) stored at -2ØC Thawed prior to use.
3&5	Pooled urine specimen, aliquoted (5ml) and lyophilised. Stored -2ØC. Reconstituted with 5ml distilled water prior to use.
4	'Lyphocheck Quantitative Control Urine 1 (Human)'. A lyophilised control urine. Supplied by Bio-Rad Laboratories (England) from Environmental Chemical Specialities, California.

Table 2.3. Between run precision of the reference procedure.

SAMPLE	n	MEAN (umol /l)	S.D.	C.V.%
1	34	99.2	16.6	16.8
2	18	131	12.1	9.25
3	30	177	19.2	10.9
4	26	222	26.3	11.9
5	12	341	25.4	7.46

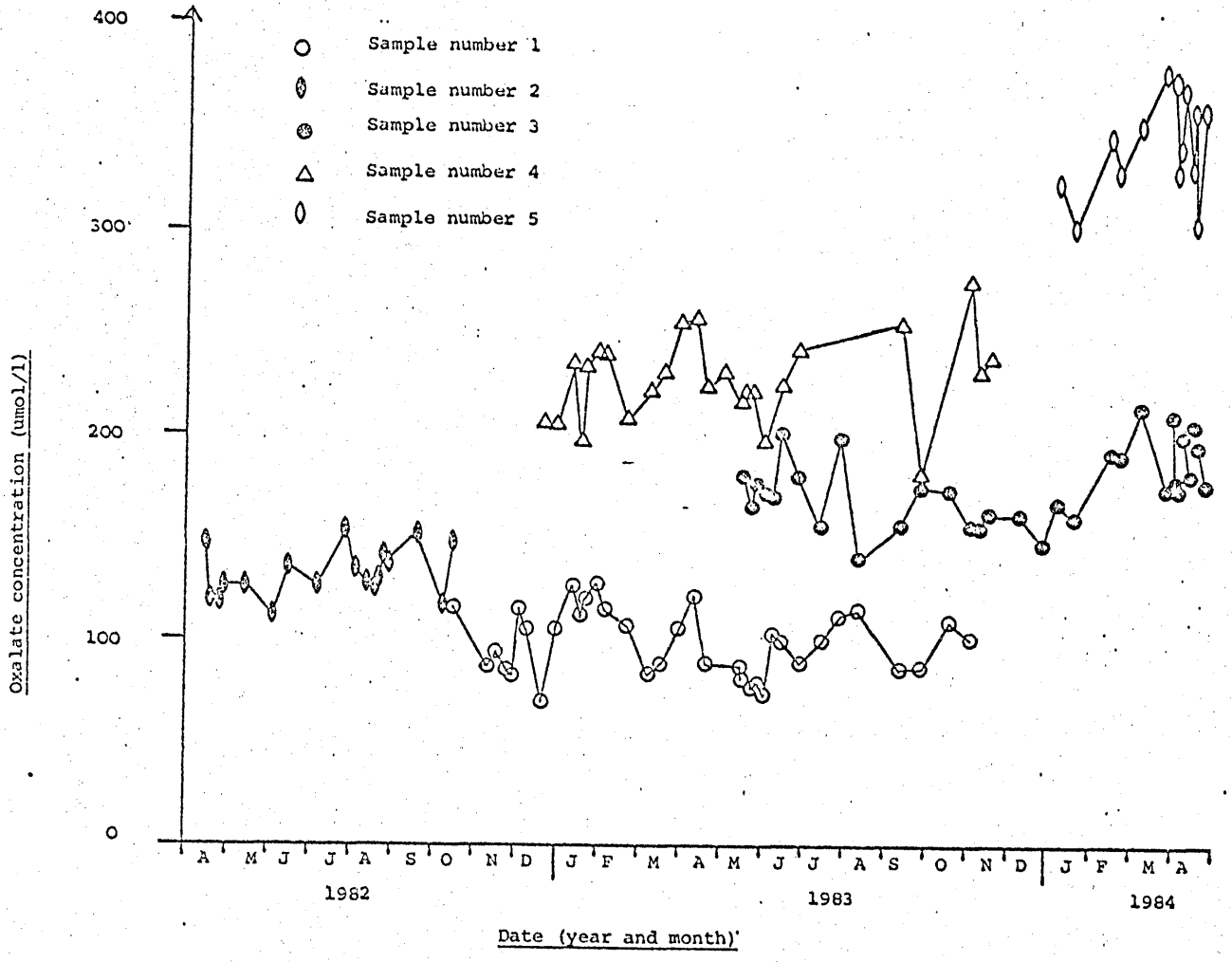


FIGURE 2.3. QUALITY CONTROL CHART FOR THE REFERENCE PROCEDURE

analysis of accuracy and precision.

The coefficient of variation ranged from 9.25 to 16.8 % for the quality control specimens, and the mean values ranged from 99.3 to 345 umol/l.

The quality control chart for the 2 year period of April 1982 to April 1984 is shown in figure 2.3.

1.7. Recovery.

1.7.1. Recovery from aqueous solution.

The recovery of oxalate from aqueous samples was estimated at five levels on a between run basis. The results are shown in table 2.4.

Overall, for 5Ø samples, the recovery was 1Ø3 % (± 9.45).

1.7.2. Recovery from urine.

A number of separate urine specimens were 'spiked' with aqueous oxalate at 3 levels, and the recoveries shown in table 2.5. were found.

Overall, for the 37 urine samples, the recovery ranged from 62.Ø to 91.5 % with a mean value of 75.4 % (± 7.91).

Table 2.4. Aqueous oxalate recovery in the reference procedure

SAMPLE	n	TARGET(umol/l)	Mean (umol/l)	S.D.	C.V.%	x RECOVERY%
1	13	50	50.8	7.22	14.2	102
2	9	74	79.1	7.39	9.34	107
3	6	148	151	7.88	5.24	102
4	12	370	379	16.0	4.24	102
5	14	400	407	26.7	6.57	102

Table 2.5. Oxalate recovery from urine in the reference procedure.

SPIKE (umol/l)	n	$\bar{x}$ RECOVERY%	C.V.%
100	5	70.9	8.56
200	16	77.4	11.1
300	16	74.9	10.1

1.7.3. Sample dilution.

The effect of sample dilution was investigated. A single urine specimen was diluted with distilled water and assayed 6 times at the following dilutions: undiluted, 1 in 2, and 1 in 5. The results are shown in table 2.6.

The mean recovery for all 18 estimations was 105 % (± 11.1).

1.8. Interferences.

Five potential precursors of oxalate were examined at levels that might be expected to occur in urine. A sixth component, glucose, was examined at a level which might be expected to occur in the urine from a poorly controlled diabetic subject, corresponding to between +++ (55 mM) and ++++ (>=111 mM) on Ames Multistix (Miles Laboratories, England). The mean recoveries and the results of paired t-tests for 5 urines are shown in table 2.7.

The only significant interference found was due to glucose, which produced a positive interference ($P < 0.01$).

1.9. Reference range.

The urinary oxalate excretion of 20 apparently healthy, non fasting adults was found to range from 191 to 454 umol/volume with a mean value of 318 umol/volume (±

Table 2.6. Effect of sample dilution in the reference procedure.

DILUTION	$\bar{x}$ CONCENTRATION (uM)	S.D.	RECOVERY %
Undiluted	85.7	12.4	100
1 in 2	41.9	5.16	97.8
1 in 5	20.2	3.63	118

Table 2.7. Interferences in the reference procedure.

COMPONENT	SPIKE	REFERENCE RANGE	$\bar{x}$ RECOVERY%	T-TEST	SIGNIFICANCE
Ascorbate	300uM	Not available	99.0	0.06	Not Significant
Glucose	100mM	Not available	142	5.07	P< 0.01
Glycine	300uM	128-4542umol/vol	95.1	1.76	Not Significant
Glycollate	300uM	Not available	99.3	0.28	Not Significant
Serine	300uM	174-479umol/vol	98.9	0.49	Not Significant
Tryptophan	300uM	26-235umol/vol	98.6	0.70	Not Significant

68.2).

1.1Ø. Discussion.

Hodgkinson (1981) has reported errors in the determination of urinary oxalate due to sampling errors caused by the variable precipitation of oxalate, related to pH, and advised that the pH of the urine should be lowered to between 1.6 and 1.8, in order to minimise these errors.

These errors were shown to occur in urine samples which were taken from the upper layers of 24 hour urine specimens which were agitated and subsequently allowed to stand. At pH 3.Ø the initial loss of oxalate was only 3 % per minute.

The mean pH value of a randomly chosen group of urine samples in this study was 2.46 (± Ø.46), and it was felt that the additional step of adding sufficient acid to bring the pH of the urine down to the advised level was not justified as the volume of acid required in some specimens was excessive, leading to sample dilution; Hodgkinson (1981) admits that these sampling errors may be reduced simply by thorough mixing.

In this study, the reference range for urinary oxalate was found to be 191 to 454 umol/volume, which is in agreement with that found by Hodgkinson and Williams (1972) of 189 to 478 umol/volume.

The within run precision (C.V.) ranged from 2.13

to 9.62 % over a concentration range of 49.4 to 506 umol/l, and the between run precision (C.V.) from 7.46 to 16.8 % over a concentration range of 99.3 to 341 umol/l.

Recovery from aqueous solution averaged 103 % (± 9.45) and from urine, 75.4 % (± 7.91), with a range in urine of 62.0 to 91.5 %. This urinary recovery was lower than that found by Hodgkinson and Williams (1972), of a mean value of 85.6 % and a range of 75 to 98 %. These higher recoveries were obtained from an oxalate 'spike' of 555 uM, whereas the 'spikes' used in this study ranged from 100 to 300 uM, which produced levels that were more likely to occur in urine specimens.

The effect of sample dilution was minimal, until a dilution factor of 1 in 5. By this stage, the lowest standard was over twice the concentration of the samples measured.

Of the substances investigated, only glucose caused a significant, positive, interference with the assay. This was found by Hodgkinson and Williams (1972), and has also been shown to occur in the estimation of formaldehyde by chromotropic acid (Pippenger et al 1983).

2.DEVELOPMENT OF ENZYMIC URINARY OXALATE PROCEDURE.

The method developed in this study was based on the double enzyme system using oxalate decarboxylase and NAD-requiring formate dehydrogenase, initially described by Beutler et al (1980) and later presented as provisional

working instructions for a U.V. method for the determination of oxalate in urine by Boehringer (1982). In this method, oxalate was converted to formate and CO_2 at pH 5 by oxalate decarboxylase. The formate formed was subsequently converted to bicarbonate at pH 7.5 by formate dehydrogenase with the production of NADH from NAD. The increase in optical density at 340 nm was proportional to the amount of oxalate present at the start, after subtraction of any increase in optical density due to endogenous formate in the sample by using an appropriate sample blank. The overall reaction scheme is shown in figure 2.4.

2.1. Materials.

All materials were of AnalaR grade, supplied by B.D.H. (England), unless otherwise stated.

Cuvettes

Sarsted (W. Germany) No. 67.741. 1 cm path length.

Micro cuvettes

Sarsted (W. Germany) No. 67.742. 1 cm path length.

Assay tubes

Sarsted (W. Germany) No. 55.484. 3.5 ml, 55 x 12 mm.

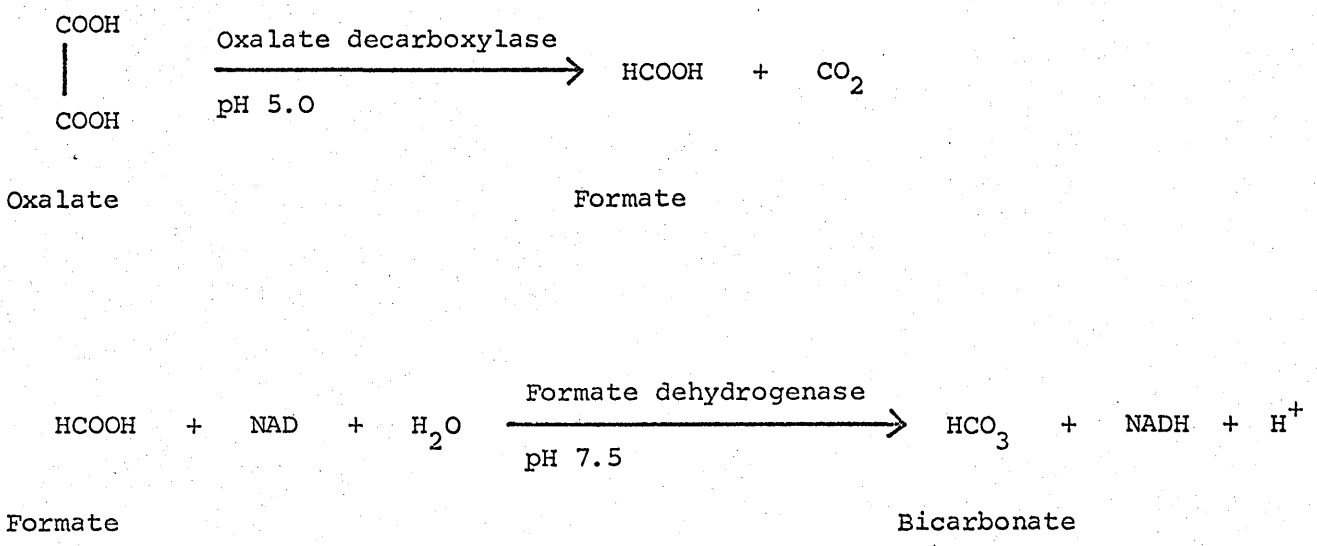


FIGURE 2.4. THE PRINCIPLE OF THE ENZYMIC ANALYSIS OF OXALATE (BEUTLER et al 1980)

Oxalate decarboxylase

Boehringer Corporation Ltd (BCL) London. From Aspergillus niger . 40 U per ml.

Formate dehydrogenase

BCL (London) from yeast. 0.4 U per mg of lyophylisate.

NAD

BCL (London). Crystallised B-nicotinamide-adenine dinucleotide as the monolithium salt. NAD-Li.$2H_2O$ (m.wt.=705.4).

50 mM NAD solution

420 mg NAD dissolved in 12 ml distilled water.

Pyrazole

Sigma Chemical Company, England. No.P2646 (m.wt. = 68.1).

100 mM stock standard oxalate

9.212 g potassium oxalate (m.wt. = 184.24) dissolved in 500 ml distilled water, stored at +4 C. Prepared each month.

100 mM stock standard formate

6.801 g sodium formate (m.wt. = 68.01) dissolved in 1 litre distilled water. Prepared each month.

500 uM working oxalate standard

0.5 ml of stock standard oxalate made to 100 ml with distilled water. Prepared each day.

Buffer 1. (pH 5.0)

0.13 M Na_2HPO_4 /0.07 M citric acid.

18.5 g disodium hydrogen orthophosphate dihydrate (m.wt. = 177.99) and 14.7 g citric acid monohydrate (m.wt. = 210.14) dissolved in 950 ml distilled water, adjusted to pH 5.0 with 5 M NaOH or 1 M orthophosphoric acid as required. Made up to 1 litre with distilled water.

Buffer 2. (pH 9.5)

0.15 M Na_2HPO_4

21.2 g disodium hydrogen orthophsphate monohydrate dissolved in 950 ml distilled water, adjusted to pH 9.5 with 5 M NaOH or 1 M orthophosphoric acid as required. 200 mg pyrazole added and made to 1 litre with distilled water.

Formate dehydrogenase solution

80 U formate dehydrogenase dissolved in 1 ml distilled water.

EDTA

Ethylene diaminetetra-acetic acid disodium salt (m.wt. = 372.24).

2.2. Instrumentation.

A Union Carbide CentrifiChem system 400 analyser (Union Carbide Corporation, New York) was used for this assay. It was a centrifugal spectrophotometric analyser and data processor, capable of processing up to 30 samples simultaneously.

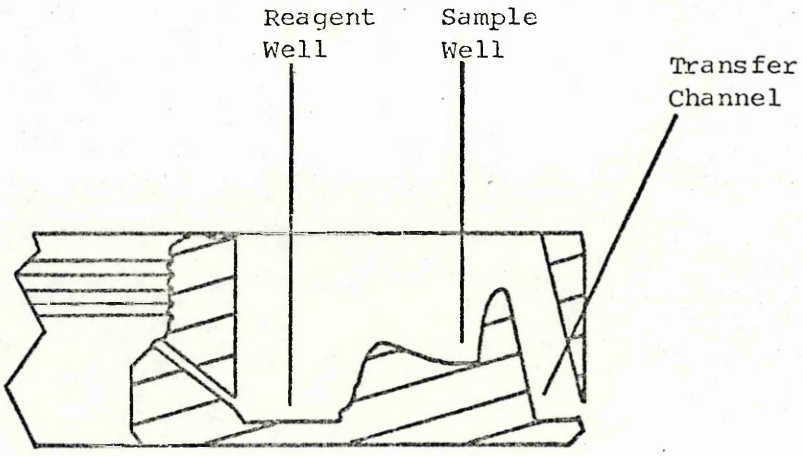
The Teflon transfer disc had 30 radial cavities, each contoured to form two separate wells for reagent and sample, as shown in figure 2.5., and was re-useable.

When the loaded transfer disc was placed in the environmentally controlled rotor of the analyser and spun, the reagent and sample were mixed and transferred from the disc, along the transfer channel, to corresponding optical cuvettes in the rotor and held there by centrifugal force.

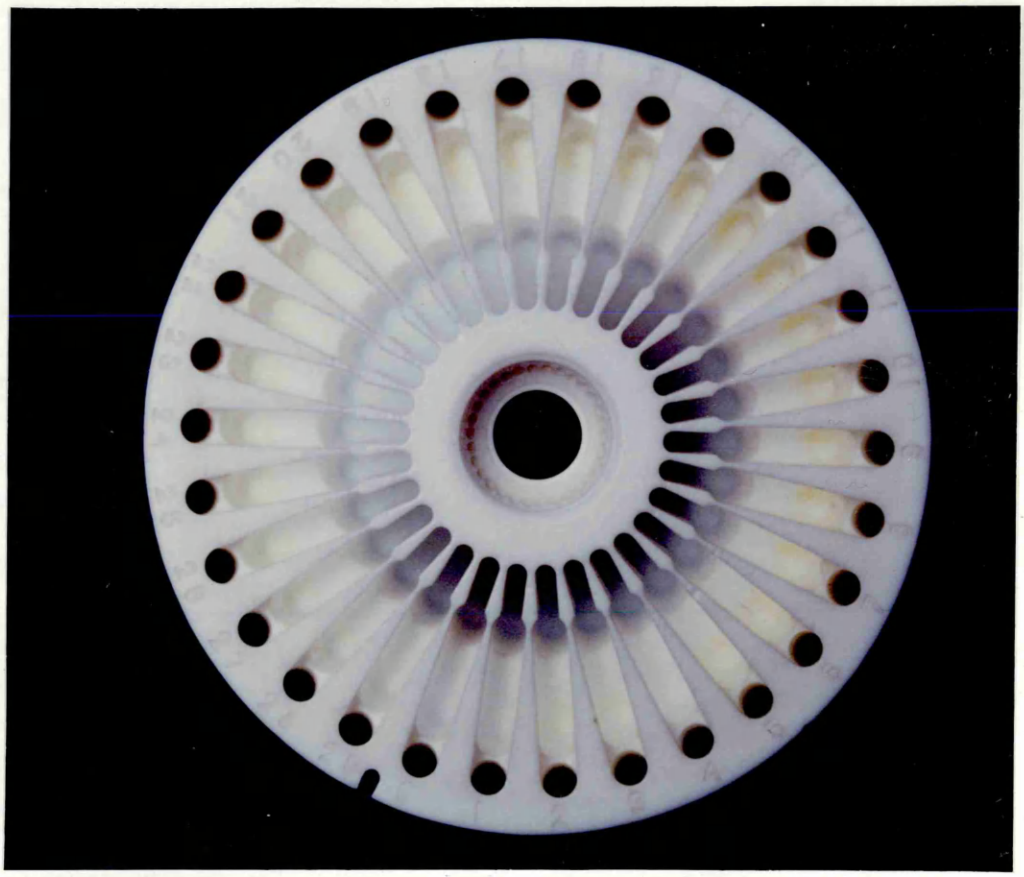
The optical system of the analyser was such that absorbance values for each of the cuvettes were obtained as the rotor spun. The absorbance changes were monitored visually on a cathode ray tube on the front of the analyser, as shown in figure 2.6.

The data processor had two 'channels' that stored absorbance values at one specified time, T0, and at any other specified time, DT, and the processor calculated results based on the difference between these two values for each cuvette.

The reaction conditions were preset before an analysis, on the front of the analyser. These parameters were temperature, wavelength, 'T0', 'DT', 'abnormal



Cross section



Plan

FIGURE 2.5. CROSS SECTIONAL DIAGRAM AND PLAN PHOTOGRAPH OF THE CENTRIFICHEM TRANSFER DISC.

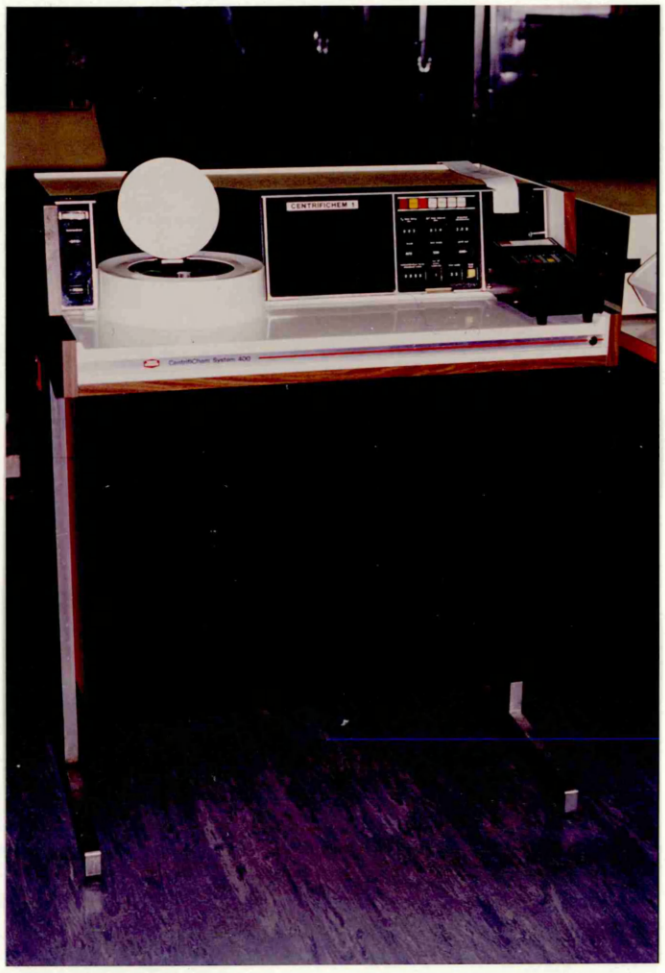


FIGURE 2.6. CENTRIFICHEM SYSTEM 400 ANALYSER

absorbance', 'blank', 'test mode', 'print out', 'standard value', 'number of prints', and 'test code'.

At the completion of a test, the transfer disc and the cuvettes were cleaned by initiating the 'clear' cycle.

For 'hold blank' analyses, it was necessary to process on the analyser a transfer disc loaded only with water. The values of the 'TØ' data channel of the processor were then arbitrarily fixed.

The other basic component of the CentrifiChem 4ØØ system was a pipettor. This allowed automated preparation of the transfer disc with pre-programmed amounts of sample, diluent and reagent. The reagent volume was set at either 25Ø or 35Ø ul and the sample volume was variable between Ø and 5Ø ul and the diluent volume between Ø and 55 ul, in steps of 1 ul.

2.3. Basic Aassay procedure.

To a series of 1 cm. cuvettes was added 2ØØ ul of sample and 2ØØ ul of buffer I. To each 'test' cuvette was added 2Ø ul of oxalate decarboxylase. The cuvettes were mixed and allowed to stand at room temperature for 2Ø minutes. To each cuvette was then added 1.Ø ml of buffer II and Ø.5 ml of 5Ø mM NAD solution. To each cuvette assigned as a 'test' was added 1.Ø ml of distilled water, and to each cuvette assigned as a 'blank' 1.Ø2 ml of distilled water. The cuvettes were mixed, and after 2 minutes the optical density at 34Ø nm was read (E1).

50 ul of the formate dehydrogenase solution was then added, the cuvettes mixed, and allowed to stand at room temperature for 20 minutes, after which the optical density at 340 nm was read (E2).

The optical density difference for each cuvette was calculated from E2-E1, and the difference due to oxalate, DE, was calculated by subtracting the optical density difference of the 'blank' cuvette from that of the coresponding 'test' cuvette.

The oxalate concentration in the sample was then calculated from the following equation:

$$c = \frac{V}{E.d.v.} \times DE$$

where; c = concentration

V = total volume

v = sample volume

d = light path

E = extinction coefficient of NADH

DE= absorbance due to oxalate

Under the above conditions;

$$c = 2.38.DE \text{ mmol/l}$$

This procedure was not evaluated, but used as a basis for the development of an automated micro-enzymatic method for urinary oxalate analysis.

It was both expensive, due to the volumes of reagent required, and complicated, due to the number of pipetting steps required for each sample.

A simpler and cheaper procedure was envisaged by reducing the reagent and sample volumes, the combination of reagents to allow single step reagent addition, and by some degree of automation of the formate dehydrogenase reaction step.

2.4. Reduction in sample and reagent volumes.

A single aqueous oxalate sample was analysed using the sample and reagent volumes shown in table 2.8.

Assays 1 and 2 were carried out in 1 cm disposable optical cuvettes and assay 3 was carried out in 1 cm disposable micro cuvettes.

The results are shown in table 2.9.

A single 24 hour urine sample was collected into a polythene bottle containing 5 ml of concentrated HCl, and immediately before assay, an aliquot was adjusted to pH 5 with 5 M NaOH or 1 M orthophosphoric acid as required.

The sample was assayed for oxalate 4 times as per assay 3 and a mean value of 124 uM ($\pm$ 6.19%) was found. The recovery of aqueous oxalate added to final

Table 2.8. Reagent and sample volumes (in ul) of the enzymic procedure.

Reagent	Assay 1	2	3
Buffer I	200	100	50
Sample	200	100	50
Decarboxylase	20	10	5
Buffer II.	1000	500	250
NAD solution	500	250	100
Water	1000	500	250
Formate Dehydrogenase	50	25	10
Total Volume	2970	1485	715

Table 2.9. Recovery of aqueous oxalate for 3 scales of enzymic assay

Assay	Standard Concentration (uM)	Oxalate Found (uM)	Recovery%
1	444	406	91.4
2	444	430	96.8
3	444	477	107

concentrations of +100, +200 and +300 umol/l in urine was 75.3, 86.8 and 78.5 % respectively. By comparison the recovery from the aqueous standards of 50, 100 and 200 uM oxalate was 97.9, 97.0 and 97.8 % respectively.

This showed that the scaling down of the method was at least feasible, and at this stage, the recovery of oxalate from urine was similar to that found using the reference method, but still well below 100 %.

2.5. Reagent combination.

In order to reduce the number of pipetting steps required in the assay, the reagents were combined, as far as possible.

For the first reaction, a single buffered oxalate decarboxylase reagent was prepared containing 4 units of oxalate decarboxylase per ml of buffer I. Addition of 50 ul of this reagent to 50 ul of sample produced the same ratio of sample volume to enzyme units, and a similar sample volume to total reaction volume ratio as in the basic assay procedure. For sample blanks, 50 ul of buffer I was used.

Due to the constraints of the reagent volumes available on the CentrifiChem 400 pipettor, and with a view to automation of the second reaction procedure, the formate dehydrogenase / NAD reagent volume was restricted to 250 ul only. This represented a reduction in volume by a factor of 10.2 in comparison to the basic assay

procedure.

A similar reduction in the sample size for the first reaction resulted in a final volume of 41.2 ul. A more convenient volume of 40 ul was selected.

The formate dehydrogenase / NAD reagent was prepared by adding 80 units of lyophylised formate dehydrogenase and 350 mg of NAD to 20 ml of buffer II. The final volume was made up to 50 ml with distilled water.

The sample volume to total reaction volume, the sample volume to enzyme units, and the sample volume to NAD ratios were 0.14, 0.1 ml/unit and 16 ul/umol respectively, which were similar to the ratios in the basic assay procedure.

2.6. Initial CentrifiChem procedure.

The oxalate decarboxylase and formate dehydrogenase reagents were prepared as described in section 2.5., and the following method was developed.

2.6.1. Method 1.

To a series of assay tubes was added 50ul of sample or standard. To each tube assigned as a 'test' was added 50 ul of the oxalate decarboxylase reagent, and to each tube assigned as a 'blank', 50 ul of buffer I. The tubes were mixed and allowed to stand at room temperature for 20 minutes, after which the contents of each tube were

transferred to the corresponding sample cup of the CentrifiChem 400 pipettor. The reagent boat of the pipettor was filled with the formate dehydrogenase reagent.

The following parameters were set on the pipettor; reagent volume = 250 ul; sample volume = 40 ul; sample and diluent volume = 90 ul. The pipetting cycle was then started.

Once the transfer disc had been prepared, the following parameters were loaded into the CentrifiChem 400 microprocessor:

Temperature	30 ^{o}
Filter	340 nm
T0	3 seconds
DT	2.5 minutes
Abnormal absorbance	2.0 U
Blank	AUTO
Test mode	TERM
Print out	ABSORBANCE
Standard value	0000
Number of prints	9
Test code	00

The loaded transfer disc was then placed in the analyser rotor and the analysis performed.

The change in absorbance due to oxalate was then calculated by subtracting the change in absorbance of the 'blank' cuvettes from the change in absorbance of the

coresponding 'test' cuvettes, after 15 minutes, and the oxalate concentration calculated from the regression equation of as series of standards.

2.6.2. Investigation of method 1.

As it was the second reaction step which was being investigated, aqueous formate standards rather than aqueous oxalate standards were prepared in order to reduce the reagent ie. oxalate decarboxylase requirement at this stage. The inclusion of a standard curve was considered appropriate, as this avoided any errors that might have occurred due to differences in absolute volumes in each sample if the results were calculated from the molar extinction value of NADH.

2.6.3. Estimation of formate standards.

A series of aqueous formate standards from Ø to 2Ø6Ø uM were prepared and assayed as per method 1.

As the absorbance values measured on the CentrifiChem were printed every 2.5 minutes (DT), the optical density against time for the four standards 294, 735, 147Ø, and 2Ø6Ø uM was plotted. These results are displayed in figure 2.7., and show that the reaction is complete within 15 minutes. For this reason the print out at 15 minutes was used for all further calculations.

The absorbance values at 15 minutes versus the

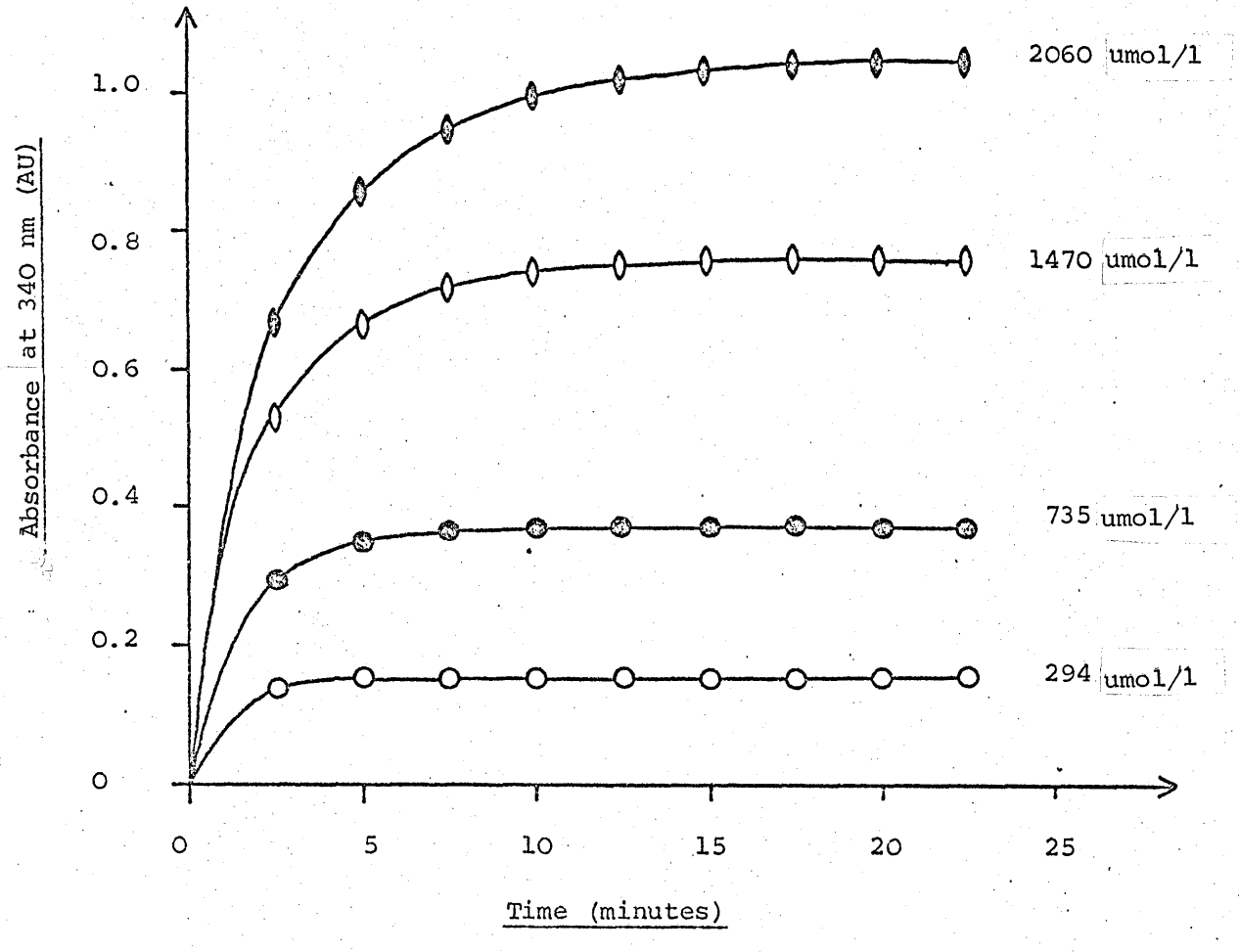


FIGURE 2.7. OPTICAL DENSITY VERSUS TIME FOR THE FORMATE DHYDROGENASE REACTION. METHOD 1

standard concentration are shown in figure 2.8.

These results show that for aqueous formate standards the method was linear to at least 2060 uM, and when the formate concentration was calculated from the extinction coefficient of NADH the mean recovery was found to be 95.8 % (± 4.11).

A number of standards were assayed to determine if there was any sample carry-over between high and low samples in the pipettor system. The results are shown in figure 2.9. From these results sample carry-over was evident.

A higher sample to diluent ratio of 35 ul sample to 55 ul diluent was investigated. The results, shown in figure 2.10., show that even at this increased sample to diluent ratio, sample carry-over was still occurring.

To overcome this problem, a transfer disc was prepared manually by the addition of 50 ul of the reagent mixture into the sample well of the disc, without diluent, and 250 ul of the formate dehydrogenase reagent to the reagent well. The results are shown in figure 2.11., and as was expected, no evidence of sample carry-over was noted.

2.7. Modified CentrifiChem procedure.

From the investigations in section 2.6. the following modifications to the initial CentrifiChem procedure were instigated.

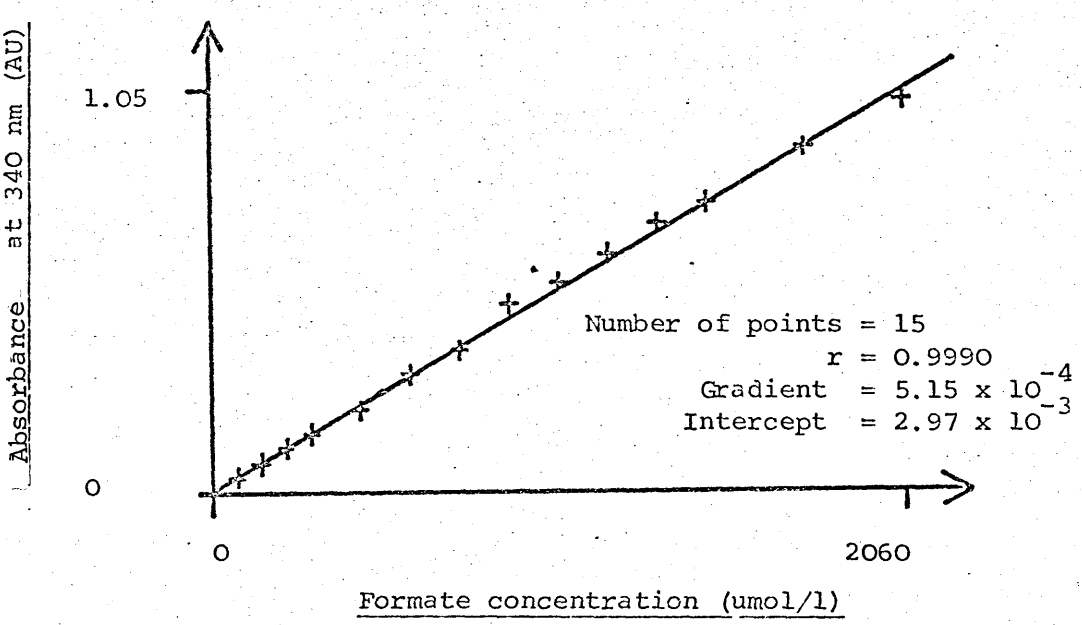


FIGURE 2.8. LINEARITY OF AQUEOUS FORMATE STANDARDS VERSUS OPTICAL DENSITY AT 340 nm. METHOD 1

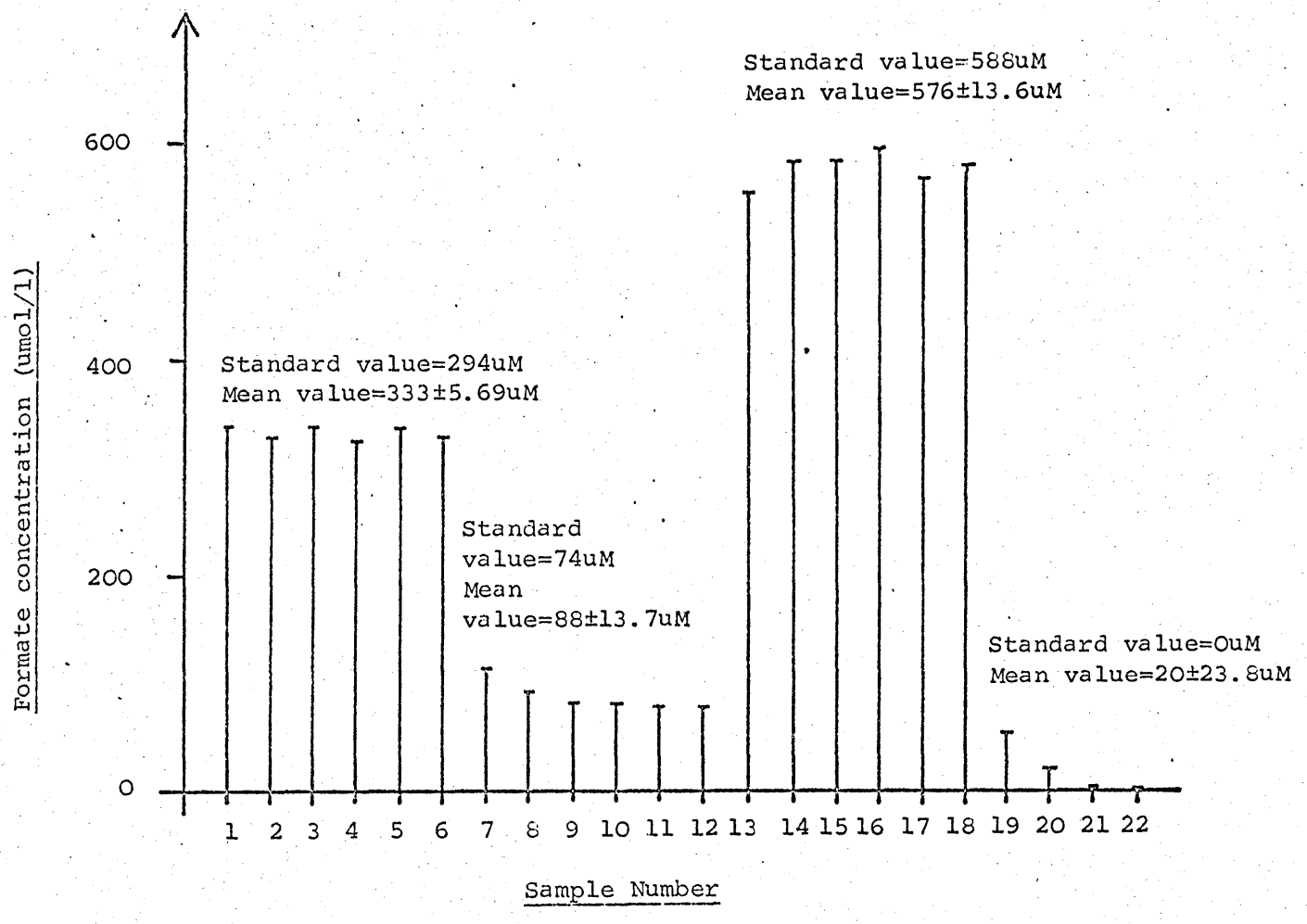


FIGURE 2.9. DEMONSTRATION OF PIPETTOR CARRY OVER. METHOD 1

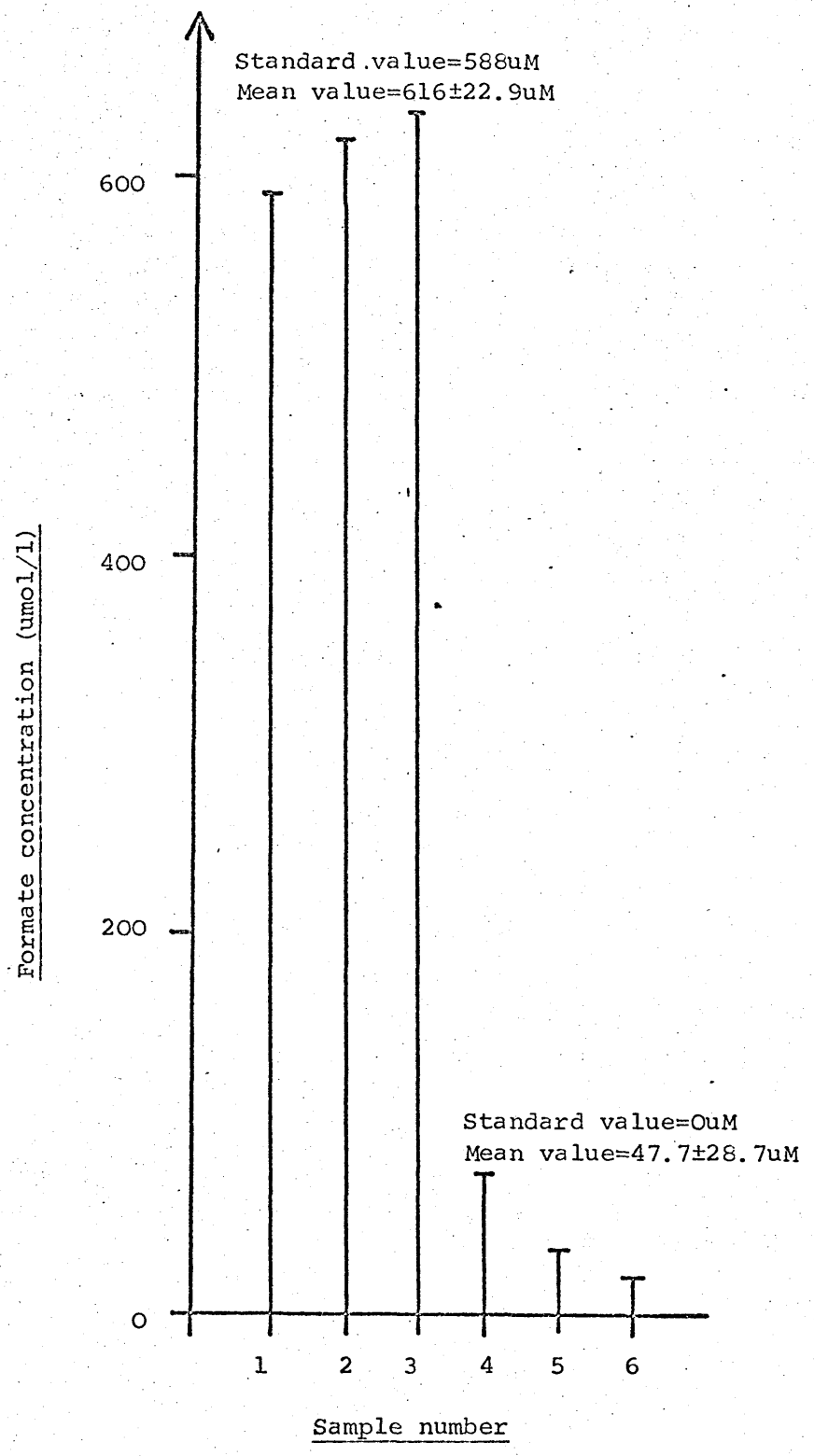


FIGURE 2.10. DEMONSTRATION OF PIPETTOR CARRY OVER AT INCREASED SAMPLE TO DILUENT RATIO. METHOD 1

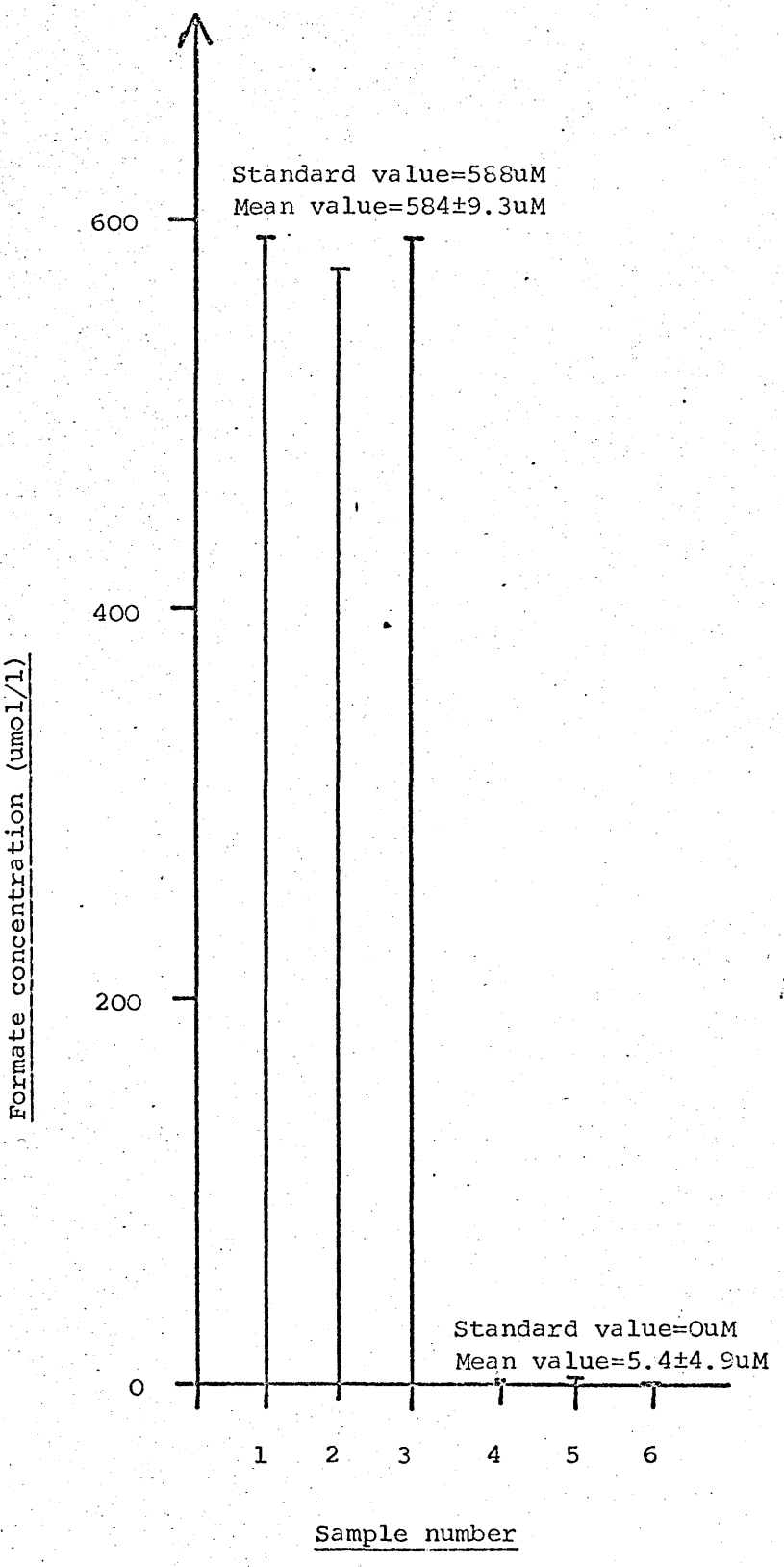


FIGURE 2.11. DEMONSTRATION OF NEGLIGIBLE OF CARRY OVER DUE TO MANUAL PREPARATION OF THE TRANSFER DISC. METHOD 1

2.7.1. Modification of procedure.

In order to increase the sensitivity of the assay, the sample volume for the oxalate decarboxylase reaction was increased from 50 to 100 ul, and the oxalate decarboxylase reagent volume maintained at 50 ul. To compensate for the increased amount of oxalate present in the reaction mixture, the incubation time was increased from 20 minutes to 1 hour.

The reagent and sample volumes for the formate dehydrogenase reaction were altered to 300 ul and 50 ul respectively since these volumes allow a more convenient manual preparation of the transfer disc, while maintaining a similar sample to total volume ratio.

In order to further reduce the cost of the assay, the concentrations of the formate dehydrogenase and the NAD in the formate dehydrogenase / NAD reagent were reduced.

The formate dehydrogenase reagent in section 2.5. was used such that 0.4 units of enzyme was added per cuvette. A combination of 8 units of formate dehydrogenase, 20 mg NAD, 3.8 ml buffer II and 5.6 ml distilled water produced a reagent that when used at a rate of 300 ul per sample delivered 0.31 units of enzyme per sample. To compensate for the reduced enzyme concentration, the incubation time on the CentrifiChem analyser was increased to 35 minutes. In this instance,

the time interval (DT) on the analyser was set to 5 minutes.

As the initial rate of reaction of the formate dehydrogenase reaction appeared to be rapid (figure 2.7.), the change in absorbance that occurred in the first few seconds of the reaction (TØ) was not measured and was not included in the measurement of the end-point absorbances. To overcome this, a separate 'hold blank' value was loaded into the CentrifiChem data processor as described in section 2.2., such that the end-point absorbances were compared to this preset value. As the initial time inteval, TØ, was no longer critical, it was increased to 5 seconds to ensure that the mixing of the reactants was complete before the first absorbance value was measured.

2.7.2. Investigation of modified procedure.

As the absorbance values measured on the CentrifiChem were printed every 5 minutes, a series of aqueous formate standards were run as per the modified procedure, and the absorbance values plotted against time. The results, shown in figure 2.12., confirmed that the formate dehydrogenase reaction was effectively complete within 35 minutes; the absorbance values at 35 minutes were used for all further calculations.

A linear response of absorbance to aqueous oxalate concentration was found (figure 2.13.).

Initial incubation periods for the oxalate

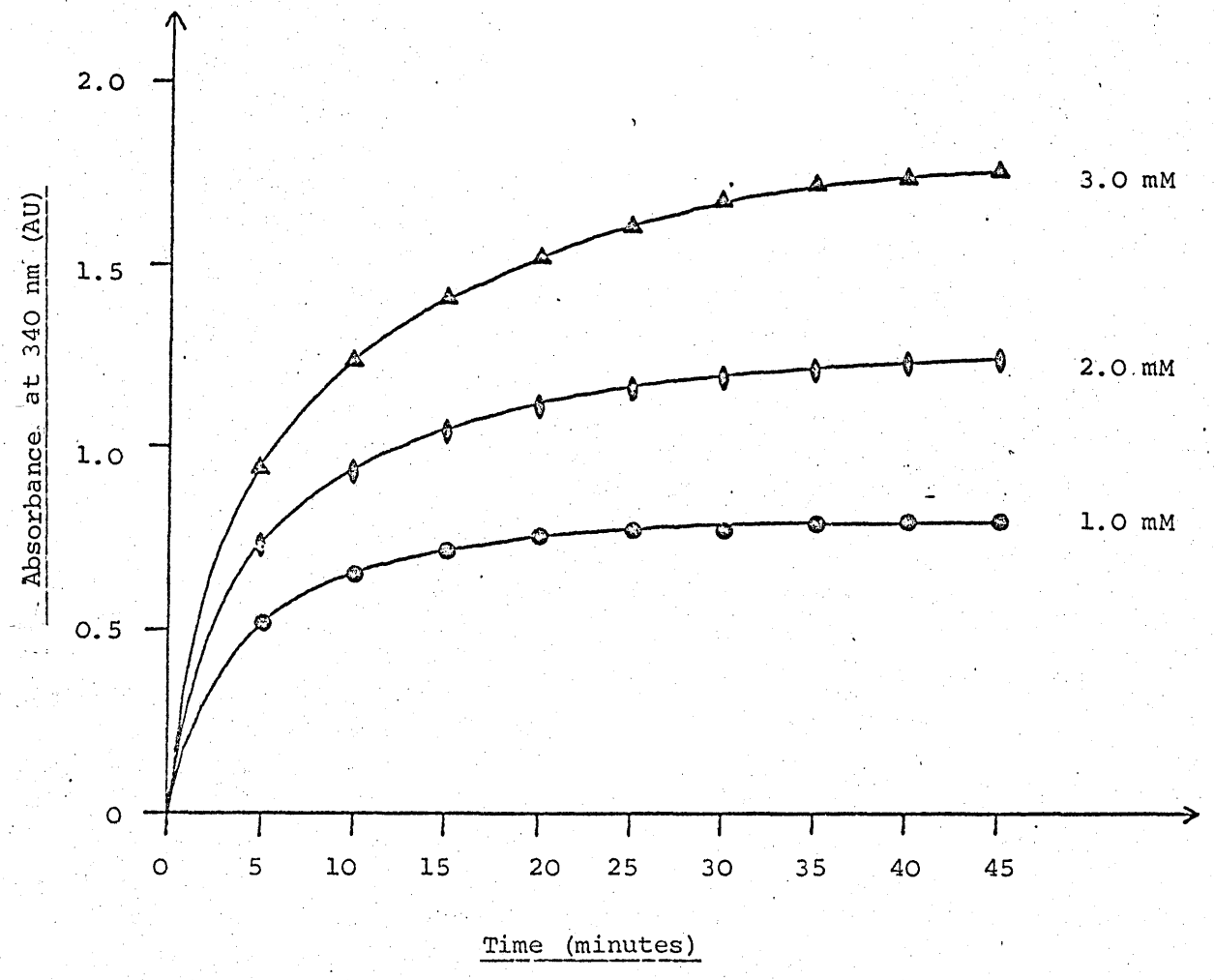


FIGURE 2.12. OPTICAL DENSITY VERSUS TIME FOR THE FORMATE DEHYDROGENASE REACTION. METHOD 1

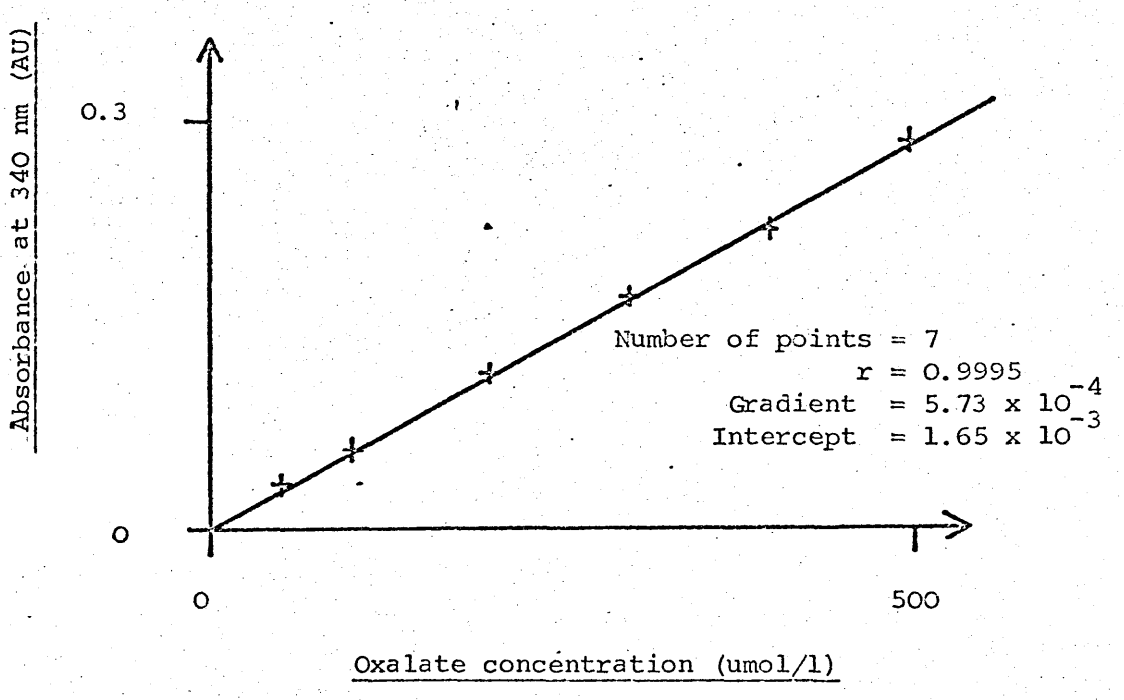


FIGURE 2.13. LINEARITY OF AQUEOUS OXALATE STANDARDS VERSUS OPTICAL DENSITY AT 340 nm. MODIFIED METHOD 1

decarboxylase reaction of Ø.5, 1 and 2 hours were investigated using two urine samples.

Immediately prior to estimation, an aliquot of the acidified 24 hour urine collection was brought to pH 5 by the addition of 5 M NaOH or 1 M orthophosphoric acid as required. The results, shown in table 2.1Ø., show that the oxalate decarboxylase reaction appeared to be incomplete at 3Ø minutes, but that it was complete within 1 hour.

At this stage, the recovery of a 3ØØ umol/l aqueous oxalate 'spike' to 4 urine samples was investigated. The results are shown in table 2.11.

The mean recovery was only 72 % ($\pm$ 15.6).

A number of studies, using the enzyme oxalate decarboxylase for the estimation of urinary oxalate, have included in the reagents ethylenediaminetetra-acetic acid (EDTA) (Mayer et al 1963, Rebeiro and Elliot 1964, Costello and Hatch 1976, Hatch et al 1977, Yriberi and Possen 198Ø), although only Mayer (1963) has noted that the EDTA increased the recovery of added oxalate.

Mayer (1963) suggested that the EDTA acted upon some enzyme inhibitor present in the urine, such as interference from anions, mainly phosphates, or that it may compete with the oxalate for cations such as Ca^{2+} or Mg^{2+} in the urine thus increasing the soluble oxalate fraction and improving the recovery.

To this end, to 5 ml aliquots of acidified 24 hour urine samples was added 15Ømg of disodium EDTA. The pH of the urines was then adjusted to 5 as described earlier and

Table 2.1Ø.Effect of incubation time on oxalate estimation.

SAMPLE	INCUBATION TIME		
	Ø.5h	1h	2h
1	122uM	146uM	147uM
2	172uM	195uM	191uM

Table 2.11.Oxalate recovery from urine in the enzymic procedure.

SAMPLE	SPIKE(uM)	RECOVERY%
1	300	81.3
2	300	48.7
3	300	76.0
4	300	81.3

the samples were placed in a 60 °C water bath for 20 minutes to ensure dissolution of the EDTA.

Four urine specimens were 'spiked' with aqueous oxalate, prepared in the above manner and then assayed as per the modified procedure. The results are shown in table 2.12.

The mean recovery was 100 % (± 6.8).

2.8. Further modifications.

From the limited investigations in section 2.7. the following modifications were made to the procedure before a detailed evaluation of the technique was performed.

2.8.1. Sample preparation.

The addition of EDTA to the urine sample prior to estimation was found to be necessary to ensure complete recovery of oxalate.

2.8.2. Two point standard curve.

As the method was shown to be linear, a two point standard curve with zero and 500 uM calibrators was required. The data processor of the CentrifiChem analyser was then be used to calculate and print out the oxalate concentrations in each cuvette by reference to these two

Table 2.12.Recovery of oxalate from urine.Effect of EDTA in the enzymic procedure.

SAMPLE	SPIKE(umol / l)	RECOVERY%
1	500	110
2	50	94.6
3	500	99.7
4	50	96.7

values.

2.9. Discussion.

The development of an automated micro-enzymic method of urinary oxalate analysis has been described.

For a single urinary oxalate analysis, consisting of a 'test' and a 'blank' estimation, an 88 % reduction in enzyme consumption and a 38 % reduction in the number of separate pipetting steps has been achieved.

As the formate dehydrogenase reaction has been adapted to run on the CentrifiChem 400 analyser, the need for 6 separate optical density measurements has been reduced and entirely automated, although the incubation times with both enzymes have been increased.

The overall procedure and the evaluation of the assay is described in section 3.

3.EVALUATION OF THE ENZYMIC URINARY OXALATE PROCEDURE.

The principle of this method, and the details of the instrumentation have been described in section 2. This section is concerned with the evaluation of the assay.

3.1. Materials.

All materials were of AnalaR grade, supplied by B.D.H. (England), unless otherwise stated.

Assay tubes

Sarsted (W. Germany) No. 55.484. 3.5 ml, 55 x 12 mm.

Oxalate decarboxylase

Boehringer Corporation Ltd (BCL) London. From Aspergillus niger . 40 U per ml

Formate dehydrogenase

BCL (London) from yeast. 0.4 U per mg of lyophilisate.

NAD

BCL (London). Crystallised B-nicotinamide-adenine dinucleotide as the monolithium salt. NAD-Li.$2H_2O$ (m.wt. = 705.4).

Pyrazole

Sigma Chemical Company, England. No.P2646 (m.wt. = 68.1).

100 mM Stock standard oxalate

9.212 g potassium oxalate (m.wt. = 184.24) dissolved in 500 ml distilled water, stored at +4 ^{o}C. Prepared each month.

500 uM Working oxalate standard

0.5 ml of stock standard oxalate made to 100 ml with distilled water. Prepared each day.

Buffer 1. (pH 5.0)

0.13 M Na_2HPO_4 /0.07 M citric acid.

18.5 g disodium hydrogen orthophosphate dihydrate (m.wt. = 177.99) and 14.7 g citric acid monohydrate (m.wt. = 210.14) dissolved in 950 ml distilled water, adjusted to pH 5.0 with 5 M NaOH and 1 M orthophosphoric acid as required. Made up to 1 litre with distilled water.

Buffer 2. (pH 9.5)

0.15 M Na_2HPO_4

21.2 g disodium hydrogen orthophsphate dihydrate (m.wt. = 177.99) dissolved in 950 ml distilled water, adjusted to pH 9.5 with 5 M NaOH or 1 M orthophosphoric acid as required. 200 mg pyrazole added and made to 1 litre with distilled water.

Working oxalate decarboxylase

100 ul oxalate decarboxylase (40 U/ml) mixed with 900 ul buffer 1 (pH 5.0). Prepared immediately before use.

Working formate dehydrogenase

8 U formate dehydrogenase, 40 mg NAD, 3.8 ml buffer 2, and 5.6 ml distilled water. Prepared 1 hour before use and allowed to stand at room temperature to ensure dissolution of the NAD.

EDTA

Ethylene diaminetetra-acetic acid disodium salt (m.wt. =

372.24)

3.2. Sample collection.

24 hour urine samples were collected as previously described in section 1.2. Immediately prior to assay, a 5 ml aliquot of the urine sample was brought to pH 5 by the dropwise addition of 5 M NaOH or 1 M orthophosphoric acid as required, after addition of 150 mg of EDTA. The samples were then placed in a water bath at 60 C for 20 minutes.

3.3. Procedure.

To a series of assay tubes was added 100 ul of the appropriate standard, quality control, or sample. To each tube assigned as a 'blank' was added 50 ul of buffer 1, and to each assigned as a 'test', 50 ul of the working oxalate decarboxylase. The quantity of working oxalate decarboxylase prepared in section 3.1. was sufficient for one batch of zero and 500 uM standards, 2 quality control urines and 11 patient urines plus blanks. The tubes were vortex mixed and allowed to stand at room temperature for 60 minutes. A transfer disc for the CentrifiChem 400 (Union Carbide Corporation, New York) was loaded manually by pipetting 50 ul of the reaction mixture from each numbered tube into the corresponding 'sample' position of the disc. 300 ul of the working formate dehydrogenase was then added to each corresponding 'reagent' position. 300

ul of distilled water was placed in reagent position 'zero' and if less than eleven patient samples were being estimated, to the remaining unoccupied reagent positions. The quantity of working formate dehydrogenase prepared in section 3.1. was sufficient for one complete transfer disc.

A 'water blank' was then loaded into the CentrifiChem using the following parameters.

Temperature	:30 ^{o}C
Filter	:340 nm
TØ	:5 seconds
DT	:15 seconds
Abnormal Absorbance	:2.Ø U
Blank	:AUTO
Test Mode	:TERM
Print Out	:Absorbance
Standard Value	:ØØØØ
Number of Prints	:1
Test Code	:ØØ

Once this blank had been successfully loaded, the parameter 'blank' was changed to 'HOLD', and the blank disc cleared. 'DT' was then changed to 35 minutes, 'Print Out' to 'CONCENTRATION' and 'Standard Value' to 'ØØØ'. These parameters were then loaded, the test disc placed in

the CentrifiChem rotor, and the analysis performed.

The oxalate concentrations in the quality controls and the patients' samples were calculated by subtracting the calculated 'blank' concentrations from their respective 'test' concentrations on the CentrifiChem print out.

3.4. Linearity.

Aqueous oxalate standards up to 1 mM and aqueous formate standards up to 3 mM were prepared and run as described in section 3.3. The results are shown in figures 2.14. and 2.15. respectively. The method was shown to be linear to at least 1 mM for aqueous oxalate and to 2 mM for aqueous formate. The formate or 'blank' concentration of 11 urine samples was estimated, and found to range between 1Ø8 and 56Ø uM (mean= 31Ø uM $\pm$ 153).

Overall the method was shown to be linear to a urinary oxalate value of at least 1 mM. Even where elevated levels of endogenous urinary formate were present, the results remained within the linear range of the method. No urine specimens were found that exceeded this range.

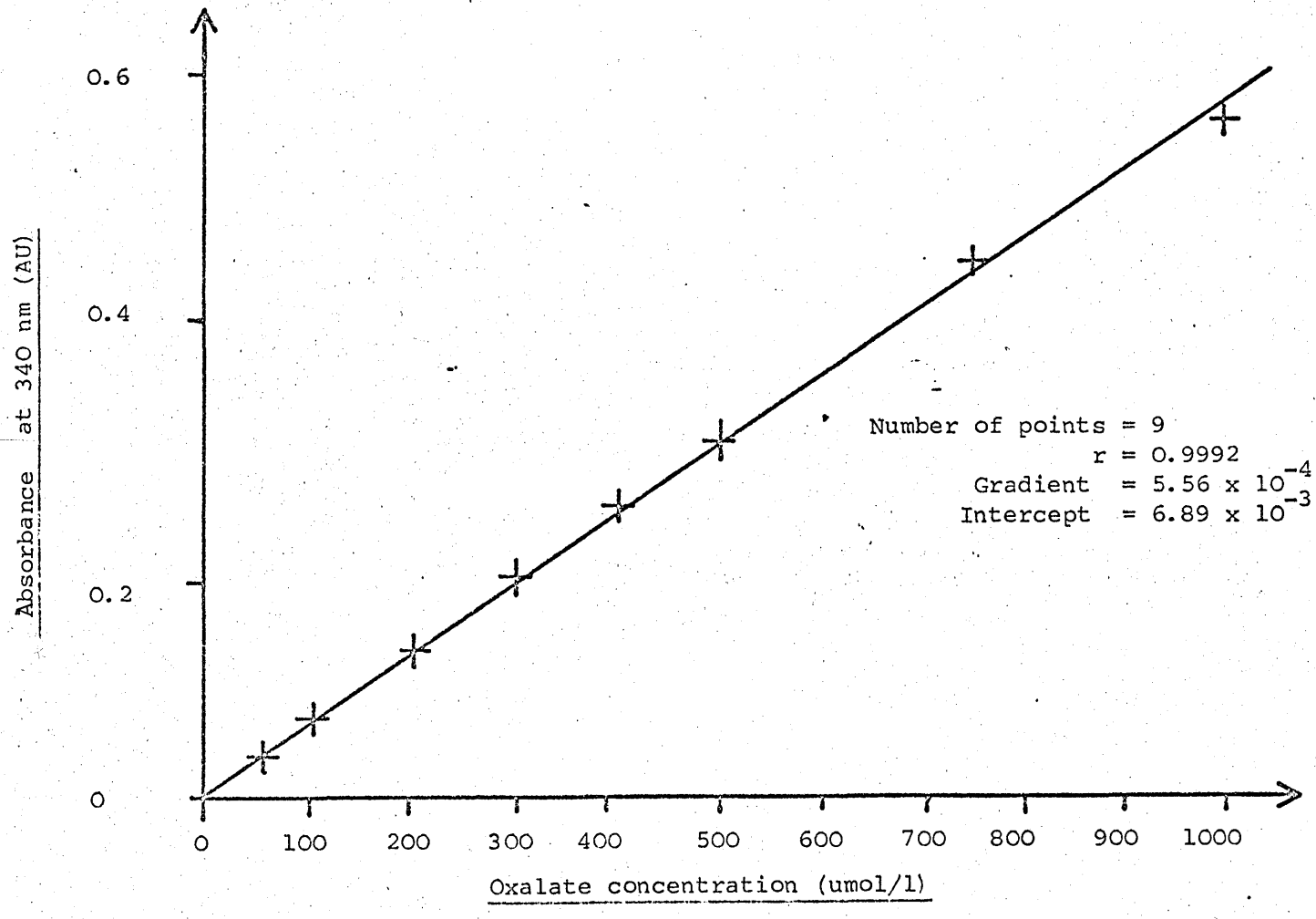


FIGURE 2.14. LINEARITY OF AQUEOUS OXALATE STANDARDS VERSUS OPTICAL DENSITY AT 340 nm

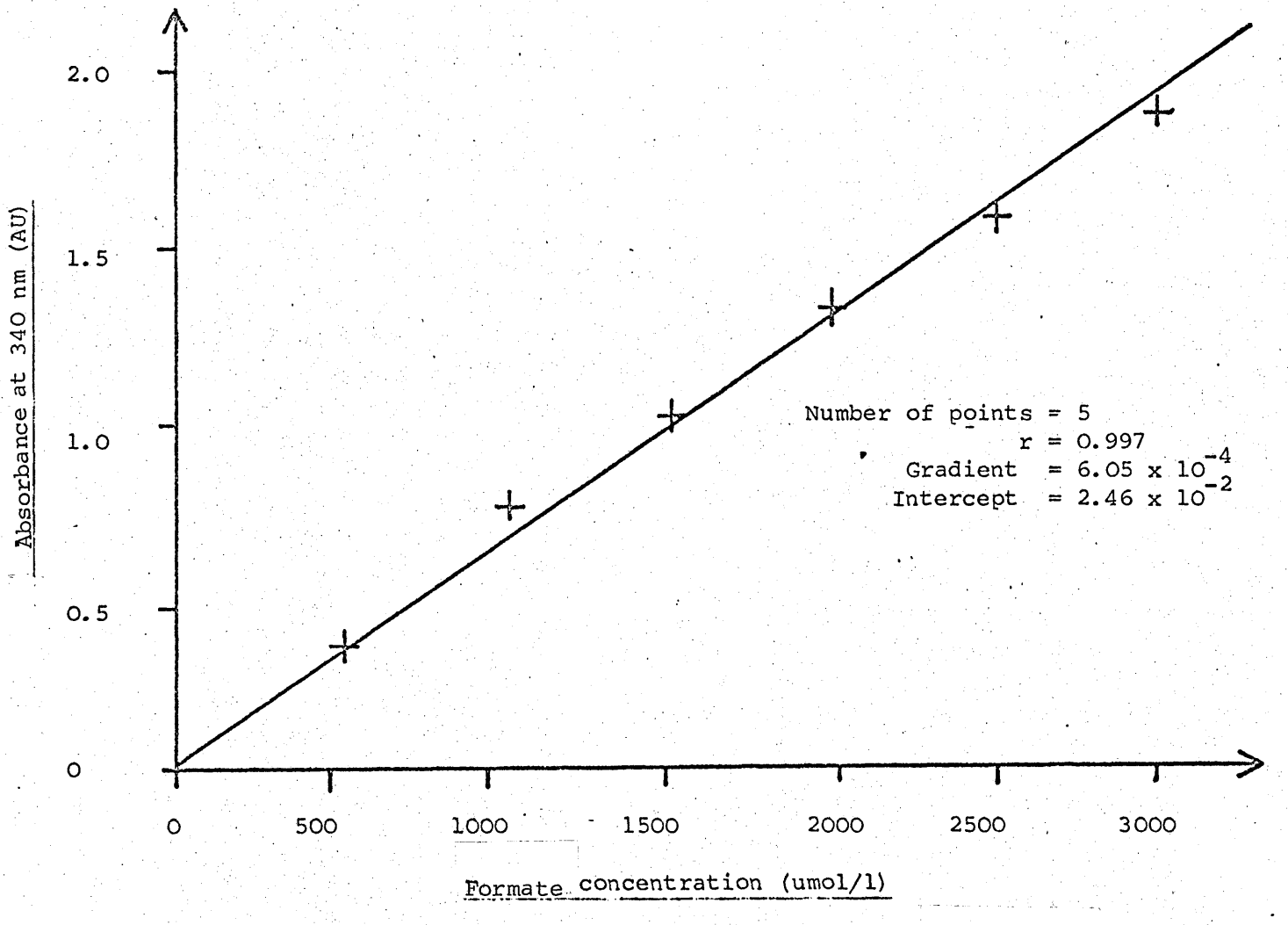


FIGURE 2.15. LINEARITY OF AQUEOUS FORMATE STANDARDS VERSUS ABSORBANCE AT 340 nm

3.5. Precision.

3.5.1. Within run precision.

The within run precision was estimated on four urine specimens. The results are shown in table 2.13.

The coefficients of variation' ranged from 3.47 to 7.61 %, and the mean values of the urine samples from 139 to 397 uM. This level of precision was comparable to the precision found with the reference method.

3.5.2. Between run precision.

Three urine specimens were used to estimate the between run precision. All were acidified pooled specimens, aliquoted, lyophilised and stored at -20 ^{o}C, reconstituted with distilled water prior to use. The results are shown in table 2.14.

The coefficients of variation' ranged from 6.23 to 9.47 %, and the mean values of the urine samples from 149 to 348 uM. This was an acceptable level of precision over a concentration range likely to be found in urine.

3.6. Recovery.

3.6.1. Recovery from urine.

Nine separate urine samples were 'spiked' with 300

Table 2.13. Within run precision of the enzymic procedure.

SAMPLE	n	MEAN (uM)	S.D.	C.V.%
1	6	139	10.6	7.61
2	6	204	9.97	4.88
3	6	357	9.86	6.76
4	6	397	13.8	3.47

Table 2.14. Between run precision of the enzymic procedure.

SAMPLE	n	MEAN (uM)	S.D.	C.V.%
1	22	149	14.1	9.47
2	12	210	18.6	8.86
3	13	348	21.7	6.23

uM oxalate. The recoveries obtained ranged from 92 to 107 % (mean = 97.8 % ± 5.57).

3.6.2. Sample dilution.

Three urine specimens were assayed undiluted and diluted 1 in 2 with distilled water. The results are shown in table 2.15.

The mean recovery from all three samples was 104 % (± 2.0).

3.7. Interferences.

The six potential interfering compounds ascorbate, glucose, glycine, serine, and tryptophan were investigated by addition to the assay medium for the enzymic assay. The mean recoveries and results of paired t-tests for 4 urines are shown in table 2.16.

No statistically significant interferences were found although tryptophan tended to produce slightly lower values for the urinary oxalate when compared to the 'unspiked' sample. The mean recovery in all these experiments was found to be 98.6 % (± 7.30).

3.8. Correlation of the enzymic and colorimetric procedures.

Forty five urine samples were assayed by both the

Table 2.15. Effect of sample dilution in the enzymic procedure.

SAMPLE	NEAT (uM)	DILUTED (uM)	RECOVERY %
1	128	68	1Ø6
2	175	89	1Ø2
3	318	165	1Ø4

Table 2.16. Interferences in the enzymic procedure. (n=4).

COMPONENT	SPIKE	REFERENCE RANGE	xRECOVERY%	T-TEST	SIGNIFICANCE
Ascorbate	300uM	Not available	108	0.95	Not Significant
Glucose	100mM	Not available	92.5	1.24	Not Significant
Glycine	300uM	628-4542umol/vol	89.0	1.75	Not Significant
Glycollate	300uM	Not available	102	0.49	Not Significant
Serine	300uM	174-479 umol/vol	104	-0.35	Not Significant
Tryptophan	300uM	26-235 umol/vol	95.8	2.34	Not Significant

reference method in section 1. and by the enzymic method described in this section. The results are displayed in figure 2.16. The correlation coefficient was found to be Ø.924.

A pair difference t-test of the reference method - the enzymic method gave the following results:

number of points	:45
mean difference	:-26.7
standard deviation	:55.7
standard error	:8.3Ø
students t value	:-3.216

The value of the students t-test indicated that the enzymic method produced results which were significantly higher than the reference method at a probability level of $P < Ø.Ø1$.

3.9. Reference range.

The urinary oxalate excretion of 2Ø healthy non fasting adults was found to range from 257 to 6Ø5 umol/vol (mean = 384 $\pm$ 22.4 umol/vol).

3.1Ø. Discussion.

The enzymic assay described would seem to provide considerable advantages over methods reported earlier. The recent introduction of commercially available oxalate

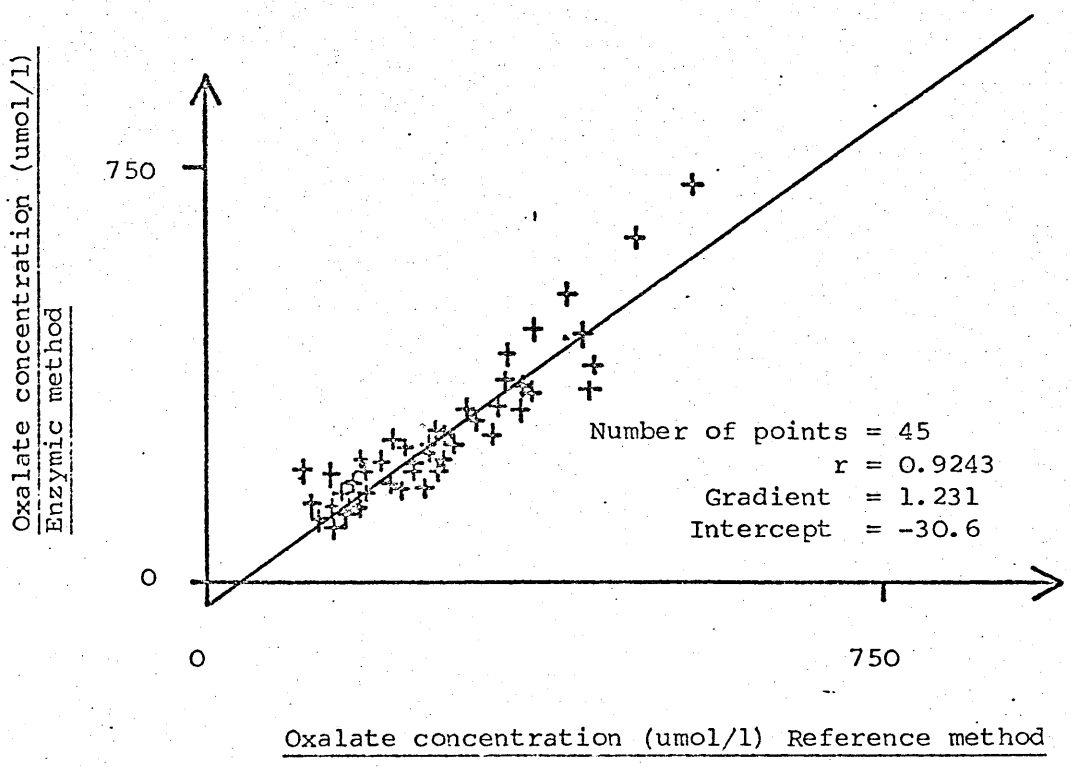


FIGURE 2.16. CORRELATION OF THE ENZYMIC AND THE REFERENCE OXALATE PROCEDURES

decarboxylase and formate dehydrogenase ensured a constant supply of standardised enzyme preparations. The preparation of reagents was therefore simpler and quicker since no lengthy enzyme preparations were required.

No sample extraction before assay was required and since sample preparation was kept to a minimum it was possible to use small volumes of urine (less than 5 ml).

The linearity of the method has been shown to be adequate for all urine samples encountered. The between run precision of this assay was better than the precision of the reference method at comparable oxalate concentrations, and the within run precision was similar for both methods.

No significant interference was found from the six components investigated and the mean recovery of oxalate from urine was 97.8 % $\pm$ 5.57.

The correlation between this method and the reference method described in section 1 was good (r = Ø.924). The generally higher results produced by the enzymic method would be expected due to the increased recovery of oxalate of 97.8 % compared to that of the reference method of 75.4 %. The reference range was accordingly higher when compared to the reference method.

The reagents used in this enzymic assay were considerably less hazardous than those required for the reference method, and the procedure was simple.

4. URINARY ALLOPURINOL AND OXIPURINOL SCREEN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

The procedure for the determination of urinary allopurinol and oxipurinol described by Miyazaki et al (1983) was used.

4.1. Materials.

Unless otherwise stated, all materials were of AnalaR grade supplied by BDH, England.

20 mM disodium hydrogen orthophosphate

3.56 g $Na_2HPO_4.2H_2O$ (m. wt. = 177.99)
dissolved in 1 litre of distilled water.

4 mM disodium hydrogen orthophosphate

0.71 g $Na_2HPO_4.2H_2O$ (m. wt. = 177.99)
dissolved in 1 litre of distilled water.

20 mM sodium dihydrogen orthophosphate

3.12 g $NaH_2PO_4.2H_2O$ (m. wt. = 156.01)
dissolved in 1 litre of distilled water.

4 mM sodium dihydrogen orthophosphate

0.62 g $NaH_2PO_4.2H_2O$ (m. wt. = 156.01)
dissolved in 1 litre of distilled water.

Diluent buffer

20 mM phosphate buffer, pH 8.0

20 mM sodium dihydrogen orthophosphate added slowly to 20 mM disodium hydrogen orthophosphate to reach pH 8.0.

Mobile phase

4 mM phosphate buffer, pH 6.0

4 mM disodium hydrogen orthophosphate added slowly to 4 mM sodium dihydrogen orthophosphate to reach pH 6.0.

Allopurinol / oxipurinol standard

27.2 mg allopurinol (Sigma Chemical Company) and 30.4 mg oxipurinol (Sigma Chemical Company) dissolved in 1 litre of distilled water.

HPLC column

25 cm x 4.6 mm stainless steel (Chrompack U.K.).

Stationary phase

5 u ODS Hypersil (Shandon Southern Products Limited, U.K.).

Pump

Gilson model 302 (Gilson Medical Electronics, U.S.A.).

Pulse dampener / pressure indicator

Gilson model 802, Manometric Module (Gilson Medical Electronics, U.S.A.).

Automatic sample injector

'WISP' model 710 B (Waters Associates, Cheshire, England).

Detector

Altex model 160 variable wavelength UV detector (Beckman, U.K.).

Recorder

Flat bed single pen recorder (Chessel Limited).

HPLC fittings

All fittings were of the 'Swagelok' type and all connecting tubing was of 1/16 " stainless steel (Scotlab Instrument Sales Limited, Scotland).

4.2. Procedure.

The mobile phase was pumped at a flow rate of 1 ml/minute which produced a back pressure of 1000 - 1500 psi. The detector sensitivity was set to 0.02 absorbance units full scale at a wavelength of 254 nm, and the autosampler programmed to inject 25 ul of sample and to allow a run time of 50 minutes per sample. The chart recorder was run at 1 mm/minute.

200 ul of urine sample or standard was added to 2 ml of the diluent buffer in a sample vial: standards were placed at intervals of 15 samples. Up to 48 samples and

standards could be processed in a single run.

The presence of allopurinol and oxipurinol was determined by comparing the retention times of the standard allopurinol and oxipurinol peaks with peaks observed in the sample chromatograms. The retention time of oxipurinol was 16.4 minutes and of allopurinol was 19.Ø minutes.

4.3. Discussion.

The method outlined above was used as a screening procedure to assess the presence of alloprinol and oxipurinol in urine samples. Urines were considered to be positive if peaks were noted for either oxipurinol or allopurinol or both in the sample chromatogram.

The procedure was essentially the same as that described by Miyazzaki et al (1983) with the exception of the column packing material which, in the original reference was 1Ø u in diameter and employed in a guard column, but in this instance was 5 u in diameter and no guard column was used. This modification did not appear to make any difference to the chromatographic system.

Figure 2.17. represents the chromatograms of an aqueous standard, a blank urine and a standard urine respectively. The retention times of 16.4 and 19.Ø minutes for oxipurinol and allopurinol were similar to the figures quoted by Miyazaki et al (1983) of 16.Ø and 18.8 minutes.

The use of the autosampler enabled a full sample

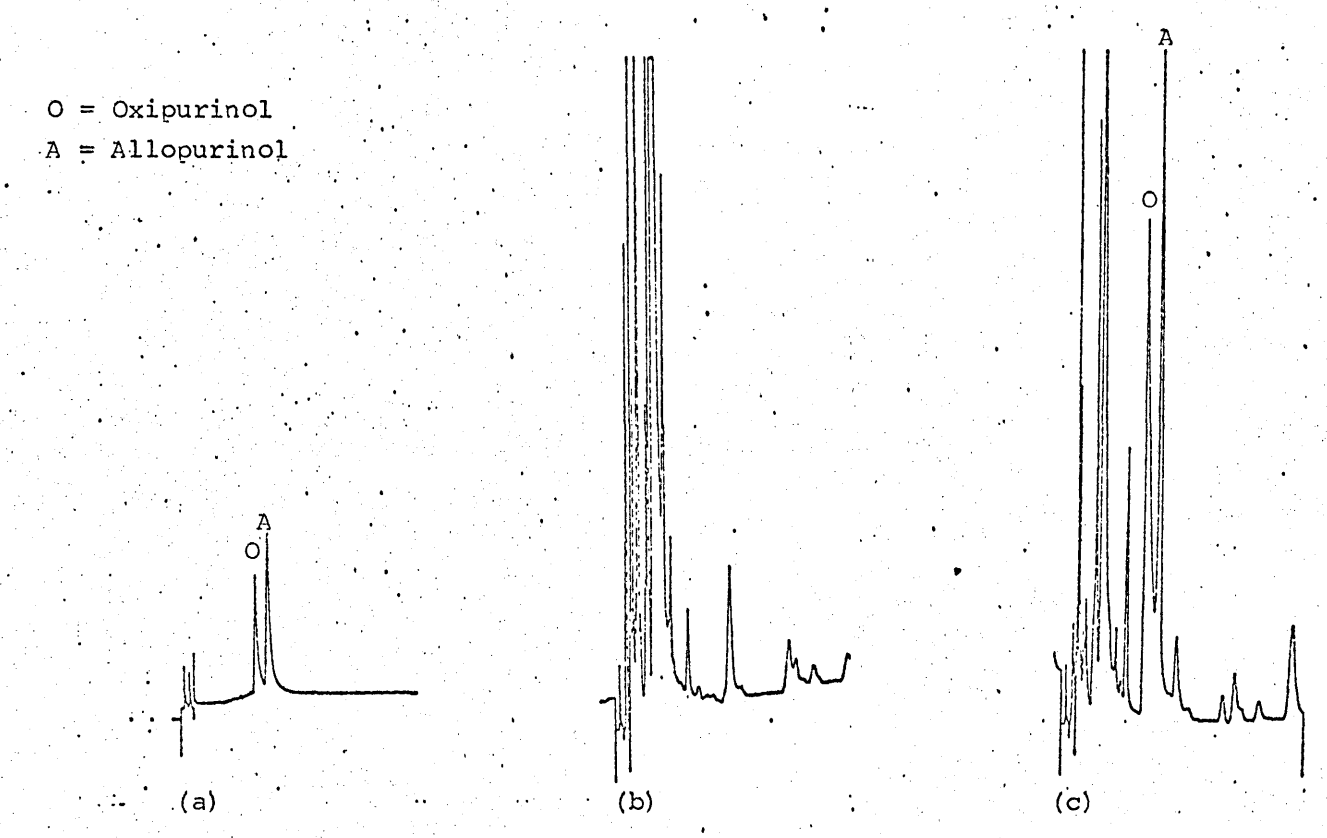


FIGURE 2.17. HPLC PROFILES OF: (a) 200 uM AQUEOUS STANDARD. (b) BLANK URINE. (c) STANDARD URINE (1mM)

rack to be processed over a 40 hour period.

5. URINARY BENDROFLUAZIDE SCREEN BY THIN LAYER CHROMATOGRAPHY (TLC).

The procedure for the detection of thiazide diuretics described by Sohn et al (1973) was used after adaption of the column extraction procedure to the 'Prep I' (Du Pont Instruments) automated sample processor.

5.1. Materials.

Unless otherwise stated all materials were of AnalaR grade, supplied by BDH, England.

TLC Plates

Silica gel 60 F_{254} precoated onto plastic sheets, 20 x 20 cm. Layer thickness 0.2 mm (E. Merck, Darmstandt).

Ethyl acetate

99.0 % $CH_3COOC_2H_5$ (m. wt. = 88.11).

Methanol

CH_3OH (James Burrough, England).

Ammonia

35 % NH_3, specific gravity = 0.88, (m. wt. = 17.03).

Acetone

99.5 % $(CH_3)_2.CO$, (m. wt. = 58.Ø8).

DMAB

Dimethylamino benzaldehyde (m. wt. = 149.19)

Extraction cartridges

'Type W' extraction cartridges, containing 9Ø mg cross linked styrenedivinylbenzene macroreticular resin, with activator (Du Pont Instruments).

Developing solvent

Ethyl acetate : methanol : ammonia, 17Ø : 2Ø : 1Ø ::V:V:V.

Revealing spray

Alkaline dimethylaminobenzaldehyde

2 g DMAB dissolved in 75 ml of 8Ø % acetone and 25 ml of ammonia.

Standard bendrofluazide

1Ø mg/litre bendrofluazide

One 5 mg bendrofluazide tablet (Berck) dissolved in 5ØØ ml of distilled water.

5.2. Instrumentation.

Sample preparation was carried out using a Prep I automated sample processor (figure 2.18.).

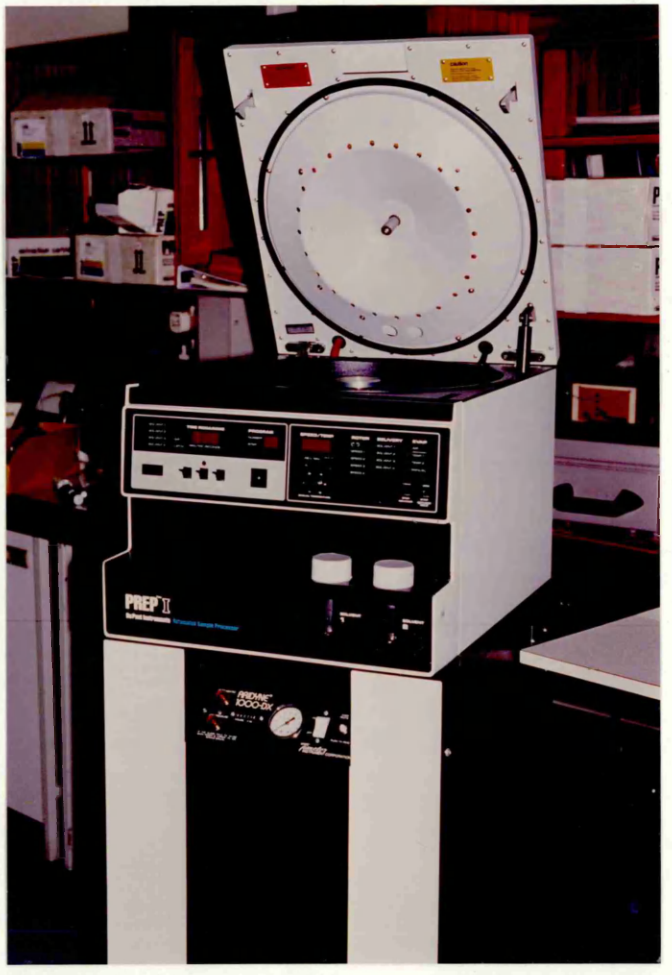


FIGURE 2.18. THE DU PONT 'PREP I' AUTOMATED SAMPLE PROCESSOR

This instrument was able to prepare liquid samples for analysis by extracting and, as a program option, concentrating components of interest. Up to 12 samples were able to be processed at one time to obtain either wet or dry extracts depending on the program selected.

Extraction was carried out under centrifugal force using a 12 position rotor attached to a bi - directional motor. Changes in rotor rotation rate and direction effected the required extraction and elution into the appropriate receivers.

During clockwise rotation, the extraction columns aligned with a waste receiver, the samples driven through the columns by centrifugal force and the columns washed with solvent from the first solvent reservoir. In the reverse direction, the columns re - aligned with the sample recovery cups and the components were eluted with the solvent from the second solvent reservoir. The samples were then dried down under a stream of warm air as the rotor spun.

The extraction cartridge assembly consisted of a resin column with reservoir, an effluent cup and a sample recovery cup which were placed in their respective positions within the rotor assembly.

5.3. Procedure.

4 ml of the urine sample or standard was added to the column reservoir and the extraction cartridge assembly

was placed in the rotor. Program number 15 was selected and, at this stage, program step 1 was selected manually and activated. After 3 minutes, the rotor was stopped and a further 4 ml of the sample was added to the column reservoir. To the first solvent reservoir was added 50 ml of distilled water as the column wash solvent, and to the second solvent reservoir was added 50 ml of ethyl acetate as the eluting solvent. Program number 15 was then reset and allowed to proceed automatically with the drying temperature set at 60 ^{o}C. Details of the program steps are shown in table 2.17.

After completion of the extraction program, the dried residue was redisolved in 50 ul of acetone. 25 ul of this solution was then spotted onto a tlc plate. A further 50 ul of acetone was added to the sample cup and a further 25 ul of this solution was loaded onto the same spot.

The tlc plate was then placed in a developing tank containing the developing solvent until the solvent front had progressed at least 10 cm from the origin after which the plate was removed and allowed to air dry.

The spots were revealed under a UV lamp at 254 nm and confirmation of the presence of thiazide was carried out by spraying the plate with the revealing agent alkaline DMAB to produce yellow spots.

5.4. Discussion.

A urine sample was considered to contain

Table 2.17. 'Prep I' extraction program.

STEP NUMBER	STEP FUNCTION	DURATION (Minutes)
1	Extract	3.ØØ
2	Extract	1.ØØ
3	Continue	Ø.3Ø
4	Dispense solvent(wash)	Ø.3Ø
5	Collect(waste)	2.ØØ
6	Spin	1.ØØ
7	Coast	Ø.3Ø
8	Reverse	Ø.Ø5
9	Reverse	Ø.1Ø
1Ø	Dispense solvent(elute)	Ø.3Ø
11	Collect(sample)	2.ØØ
12	Collect(sample)	1.ØØ
13	Evaporate	18.ØØ
14	Cool	Ø.15

bendrofluazide and therefore indicate compliance with therapy if under ultraviolet light a component was revealed in the sample which was at a similar Rf and of a similar blue colour as that of the standard which on subsequent spraying with DMAB produced a yellow colour.

The Rf of bendrofluazide was found to be Ø.7Ø and all spots revealed under UV produced the yellow colour reaction when sprayed with DMAB.

Under UV, two other components noted in the standard at an Rf of Ø.82 and Ø.43 respectively, which did not produce a yellow colour on spraying, were considered to be compounds used in the formulation of the bendrofluazide tablet used to prepare the standard.

6. ROUTINE LABORATORY ESTIMATIONS.

The following biochemical tests were performed through the routine biochemistry services at the Glasgow Royal Infirmary.

6.1. Estimations in blood.

Table 2.18. represents the methods of analysis used by the routine laboratory for the analysis of calcium (monthly CV = 1.4 % at 1.97 mmol / litre, n = 22), magnesium (monthly CV = 2.5 % at Ø.8Ø mmol / litre, n = 24), zinc (monthly CV = 1.9 % at 19.12 umol / litre, n = 22) and urate (monthly CV = 2.2% at 198 umol / litre, n =

Table 2.18. Routine serum analyses.

Test	Method	Reference Range
Calcium	Colorimetric (Cresolphthalein complexone)	2.2Ø - 2.6Ø mmol/l
Magnesium	Atomic Absorption	Ø.7Ø - 1.ØØ mmol/l
Zinc	Atomic Absorption	12 - 18 umol/l
Urate	Colorimetric (Phosphotungstic acid)	21Ø - 54Ø umol/l (M)
		15Ø - 36Ø umol/l (F)

M = Male. F = Female.

18) in serum with the departmental reference ranges.

6.2. Estimations in urine.

Table 2.19. represents the methods of analysis used by the routine laboratory for the analysis of calcium (monthly CV = 1.3 % at 3.43 mmol / litre, n = 21), magnesium (monthly CV = 2.4 % at 1.23 mmol / litre, n = 24), creatinine (monthly CV = 1.3 % at Ø.93 mmol / litre, n = 22) and urate (monthly CV = 1.8 % at Ø.6Ø mmol / litre, n = 18) in urine with the departmental reference ranges.

Table 2.19. Routine urine analyses.

Test	Method	Reference Range
Calcium	Colorimetric (Cresolphthalein complexone)	3.Ø - 6.Ø mmol/24h
Magnesium	Atomic Absorption	2 - 11 mmol/24h
Creatinine	Colorimetric (Jaffe Reaction)	9 - 18 mmol/24h
Urate	Colorimetric (Phosphotungstic acid)	2.8 - 4.4 mmol/24h

CHAPTER 3. CLINICAL STUDIES.

1. THE CLINICAL STUDY OF STONE FORMERS.

1.1. Protocol.

Patients presenting at the outpatient clinic of the Urology Department with a proven 'stone incident' and with no previous active therapy for renal stones were considered for this study. A 'stone incident' was defined as acute renal colic, spontaneous passage of a stone, radiological confirmation of a new stone or surgical removal of a new stone.

1.1.1. Initial patient work up.

Baseline investigations for each patient were as follows:

previous stone disease history

age

sex

biochemistry (table 3.1.)

bacteriology (mid stream specimen of urine)

radiology (intravenous urography)

1.1.2. Selection of treatment regime.

Patients, who on initial investigation, presented

Table 3.1. Biochemical investigations in stone formers.

Specimen	Test
Blood	Calcium concentration
	Magnesium concentration
	Zinc concentration
	Urate concentration
24 hour urine	Calcium excretion
	Magnesium excretion
	Creatinine excretion
	Urate excretion
	Oxalate excretion

with a 24 hour urinary calcium excretion of less than 8.0 mmol / volume were randomly ascribed allopurinol, 300 mg / day or bendrofluazide, 5 mg / day with potassium supplement. These subjects were designated treatment groups 1 and 2 respectively.

Patients, who presented with a urinary excretion of calcium of more than or equal to 8.0 mmol / vol were ascribed bendrofluazide, 5 mg/day with potassium supplementation. These subjects were designated treatment group 3.

1.1.3. Clinical follow up.

Patients were requested to attend the outpatient clinic on a 3 monthly basis, at which times the biochemical and bacteriological investigations were repeated; at yearly intervals, where possible, a plain abdominal radiograph was undertaken, observing the 10 day rule for female subjects.

1.1.4. Compliance.

The compliance of each subject with their respective therapy was monitored retrospectively by the procedures described in sections 2.4. and 2.5. For inclusion in the final analyses the presence of the respective drug in at least 66 % of the urine samples analysed for each subject was required.

1.2. Patients.

Eighty eight subjects were considered for the study. However, 8 male and 4 female subjects were excluded as shown in table 3.2. prior to any clinical follow up.

The previous medical histories and the presenting conditions of the 5Ø male and 26 female subjects that remained are shown in tables 3.3. and 3.4. repectively.

The mean age of all subjects was 49.4 ± 12.Ø years (n = 76) and there were no significant differences in the distribution of age between the three groups and sexes (unpaired t - test).

1.3. Results: Group 1.

1.3.1. Exclusions.

Thirty two subjects were assigned to this group. However, 2 male and 2 female subjects were excluded due to non compliance with therapy. A further 5 male subjects were excluded due to failure to attend or provide specimens at the clinic. 24 subjects, 17 males and 7 females remained in group 1.

The mean age was 51.6 ± 11.7 years (n = 24) and there was no significant difference in the mean age

Table 3.2. Patients excluded prior to follow up.

Reason	Male	Female
Reaction to allopurinol	3	1
Reaction to bendrofluazide	Ø	1
Defaulted from therapy	2	1
Incomplete work up	2	Ø
Pregnancy	-	1
Relocation	1	Ø
Total	8	4

Table 3.3. Previous renal stone histories of all subjects.

History	Male	Female
Nil	27	14
Renal colic	5	3
Renal stone	13	7
Operation		
Ureterolithotomy	2	Ø
Nephrectomy	1	1
Pyelolithotomy	2	1
Total	5Ø	26

Table 3.4. Presenting renal stone incident of all subjects

Condition	Male	Female
Renal colic	8	1
Renal stone	35	15
Operation		
Ureterolithotomy	6	5
Nephrectomy	Ø	1
Pyelolithotomy	1	4
Total	5Ø	26

between the sexes (unpaired t - test).

1.3.2. Clinical results.

The mean period of follow up for this group of patients was 19.7 ± 7.26 months (n = 24) which represented a total of 39.4 patient years. During this time 4 male subjects (16.7 %), one of whom presented with a recurrent urinary tract infection, suffered a total of 6 stone incidents (table 3.5.) with a mean period to the first recurrence of 8.75 ± 4.Ø3 months (n = 4). A single female subject presented with a recurrent urinary tract infection.

1.3.3. Biochemical results.

The mean urinary excretion of oxalate, calcium, urate and magnesium, pretreatment, after 3 months of therapy and after 9 to 15 months of therapy are shown in figures 3.1. and 3.2.

The mean serum concentration of calcium, magnesium, zinc and urate, for the same periods of therapy, are shown in figures 3.3. and 3.4.

The Wilcoxon paired signed rank test of the difference between two medians was applied to the pretreatment and subsequent values of the analytes mentioned above, and the following results were found:

Table 3.5. Stone incidents in group 1 subjects.

	Males	Females
Incident free	13	7
Single incident	3	Ø
Multiple incidents	1	Ø
Total	17	7

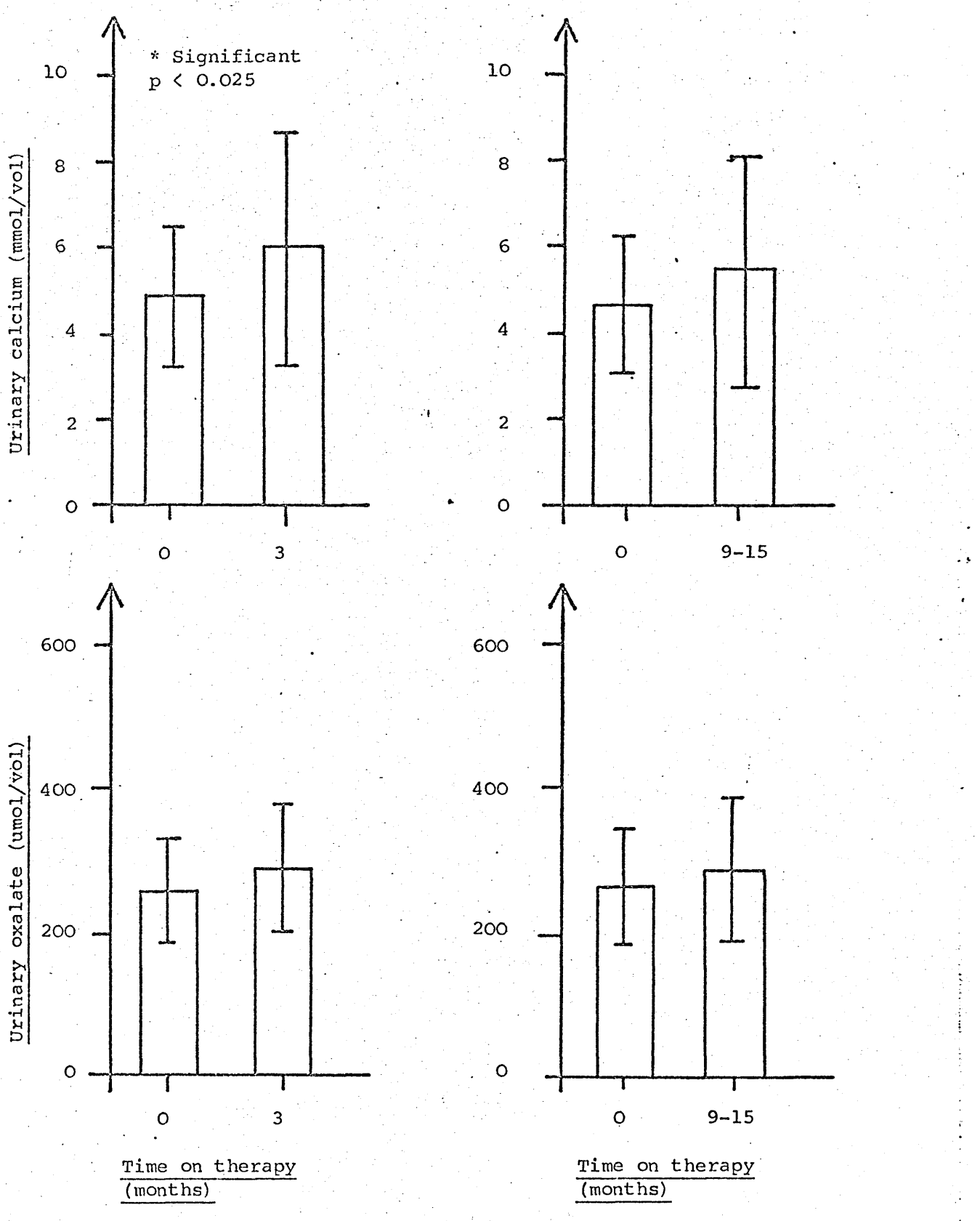


FIGURE 3.1. URINARY CALCIUM AND OXALATE EXCRETION DURING ALLOPURINOL THERAPY

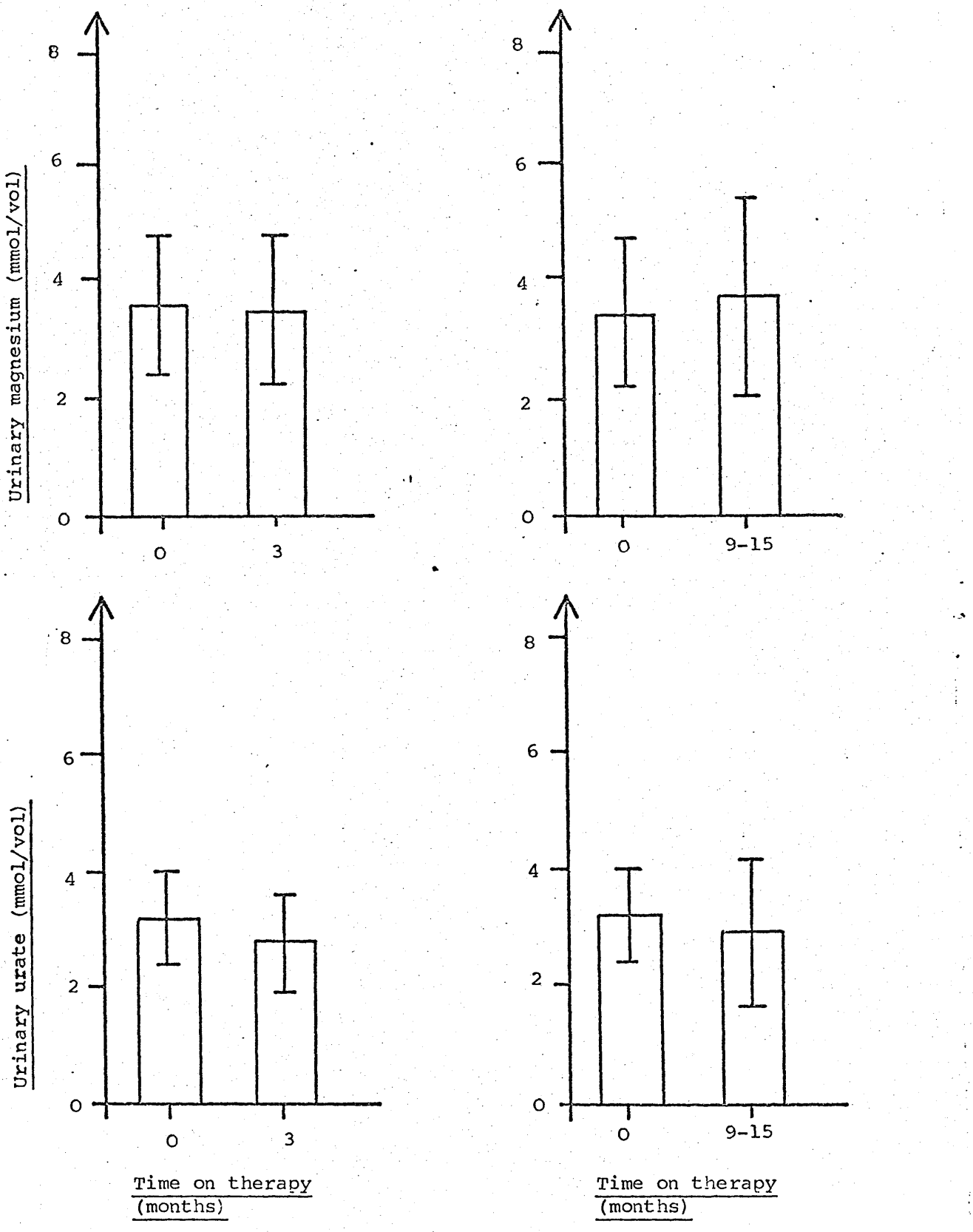


FIGURE 3.2. URINARY MAGNESIUM AND URATE EXCRETION DURING ALLOPURINOL THERAPY (GROUP 1)

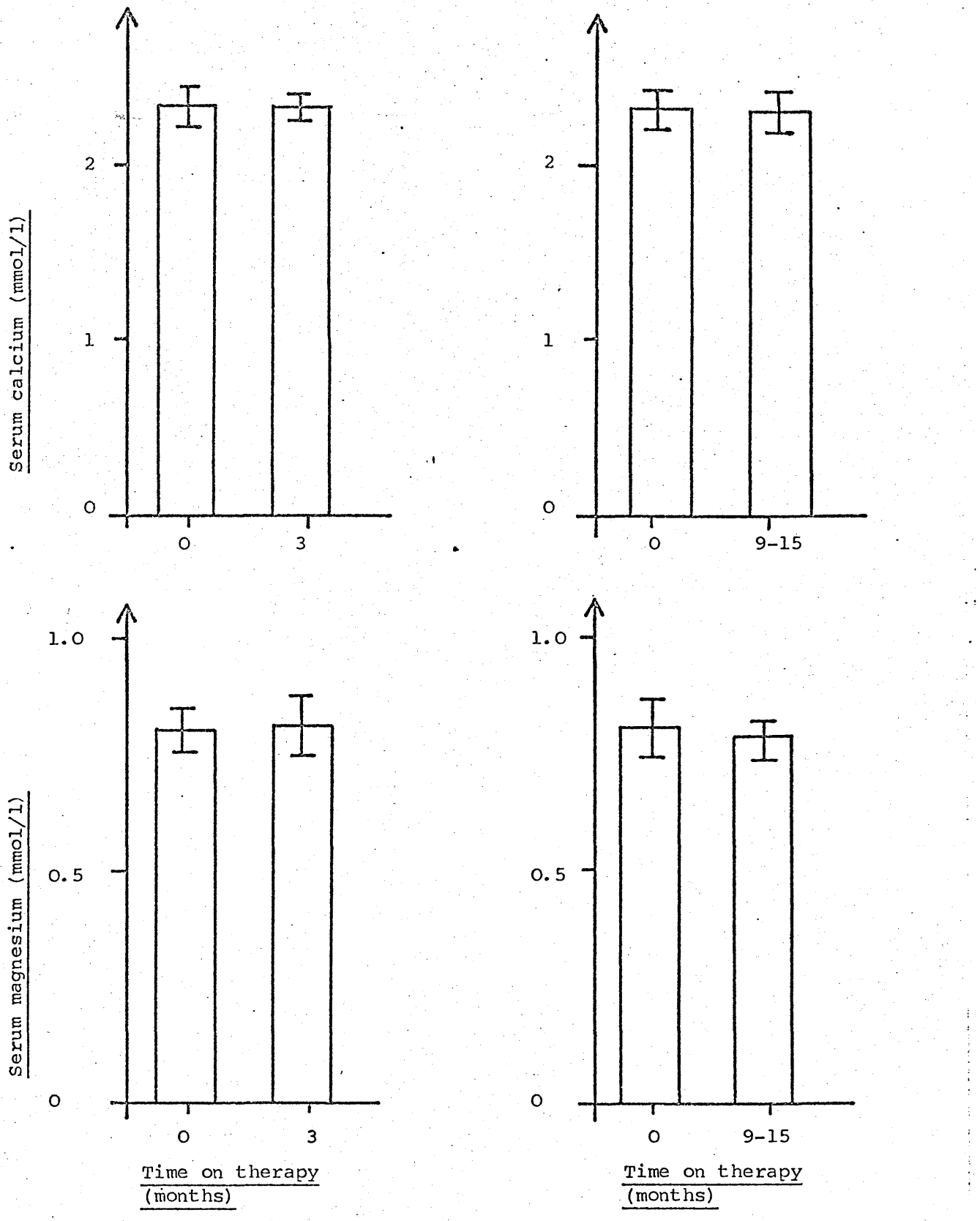


FIGURE 3.3. SERUM CALCIUM AND MAGNESIUM CONCENTRATIONS DURING ALLOPURINOL THERAPY. (GROUP 1)

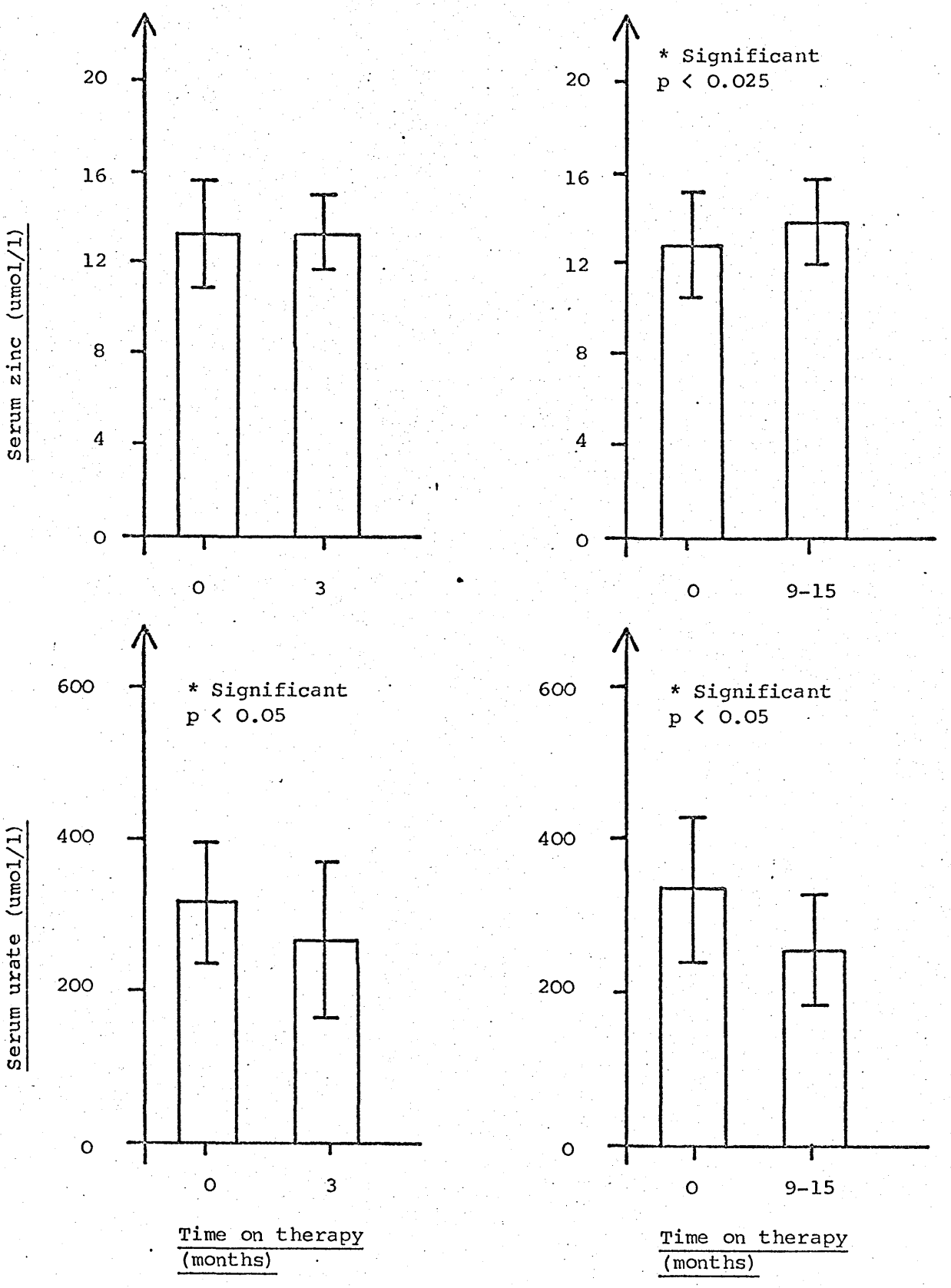


FIGURE 3.4. SERUM ZINC AND URATE CONCENTRATIONS DURING ALLOPURINOL THERAPY (GROUP 1)

a) There was a significant increase in the urinary calcium excretion after 3 months of therapy ($p < 0.025$) as shown in figure 3.1., the mean value rising from 4.93 $\pm$ 1.73 to 6.12 $\pm$ 2.83 mmol / volume (n = 22).

b) The serum zinc concentration was increased ($p < 0.025$) after 9 to 15 months of therapy from a mean value of 13.2 $\pm$ 2.43 to 14.2 $\pm$ 2.01 umol / litre (n = 11).

c) The serum urate concentration was significantly reduced after 3 months and after 9 to 15 months of therapy ($p < 0.05$ in both time intervals) as shown in figure 3.4., the mean value falling from 322 $\pm$ 76.1 to 275 $\pm$ 102 umol / litre (n = 13) and from 335 $\pm$ 91.8 to 255 $\pm$ 75.4 umol / litre (n = 15) respectively.

There were no significant changes noted in the urinary oxalate and magnesium excretions nor in the serum calcium and magnesium concentrations.

1.4. Results: Group 2.

1.4.1. Exclusions.

Thirty one subjects were assigned to this group. However, 3 male and 3 female subjects were excluded due to non compliance with therapy. A further 1 male and 1 female subject were excluded due to failure to attend or to provide specimens at the clinic. Finally, 23 subjects, 13 males and 10 females remained in group 2.

The mean age was 46.Ø ± 13.3 years (n = 23) and there was no significant difference in the mean age of the sexes (unpaired t - test).

1.4.2. Clinical results.

The mean period of follow up for this group was 23.2 ± 6.59 months (n = 23) which represented a total of 44.5 patient years. During this time 3 male and 2 female subjects (21.7 %) suffered a total of 18 stone incidents (table 3.6.) with a mean period to the first recurrence of 9.4Ø ± 5.18 months (n = 5). One other female subject presented with a recurrent urinary tract infection.

1.4.3. Biochemical results.

The mean urinary excretion of oxalate, calcium, urate and magnesium, pretreatment, after 3 months of therapy and after 9 to 15 months of therapy are shown in figures 3.5. and 3.6.

The mean serum concentrations of calcium, magnesium, zinc and urate, for the same periods of therapy, are shown in figures 3.7. and 3.8.

When the Wilcoxon paired signed rank test was applied the following results were found:

a) There was a significant decrease in the serum magnesium concentration after 3 months and after 9 to 15

Table 3.6. Stone incidents in group 2 subjects.

	Males	Females
Incident free	1Ø	8
Single incident	2	1
Multiple incidents	1	1
Total	13	1Ø

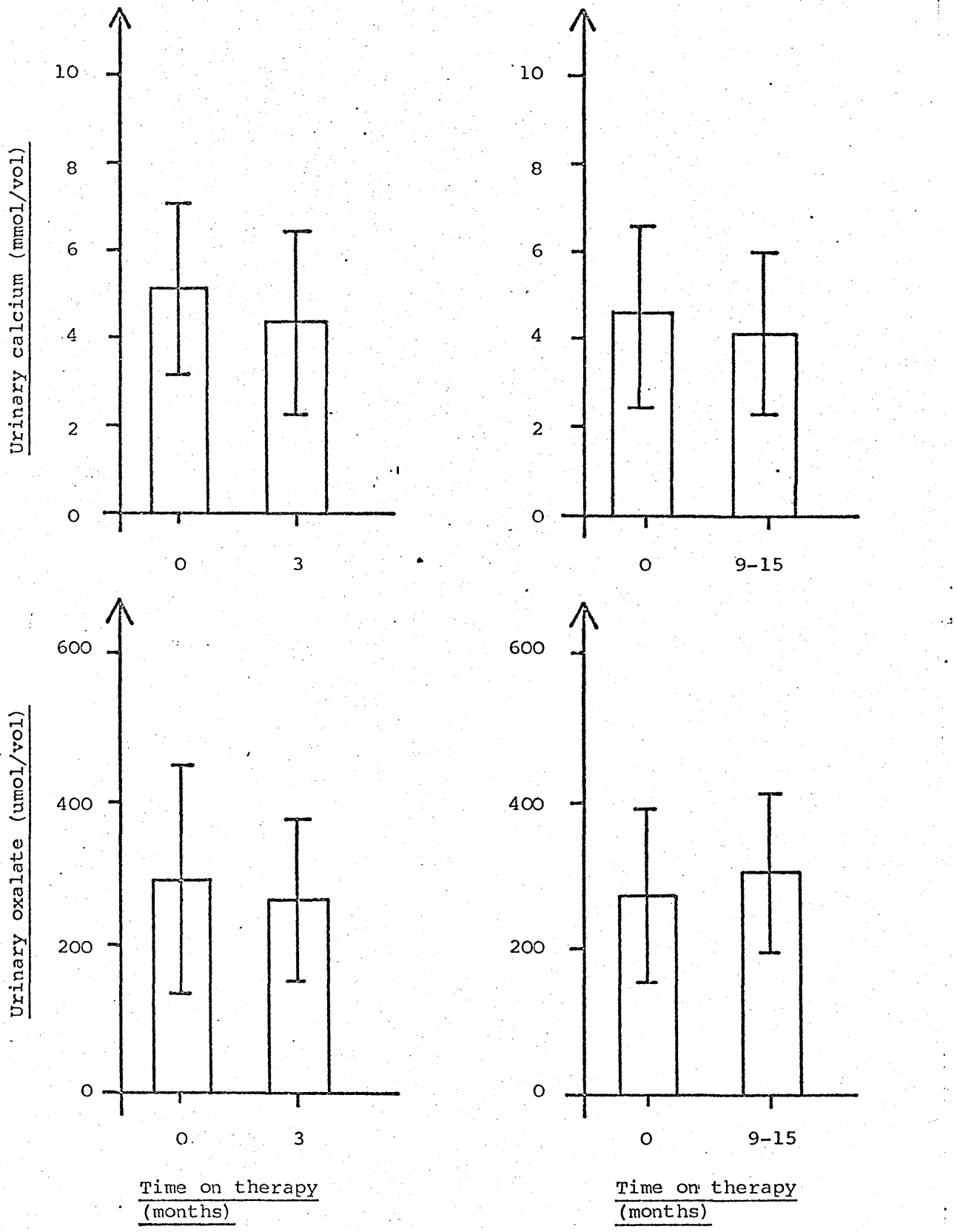


FIGURE 3.5. URINARY CALCIUM AND OXALATE EXCRETION DURING BENDROFLUAZIDE THERAPY (GROUP2)

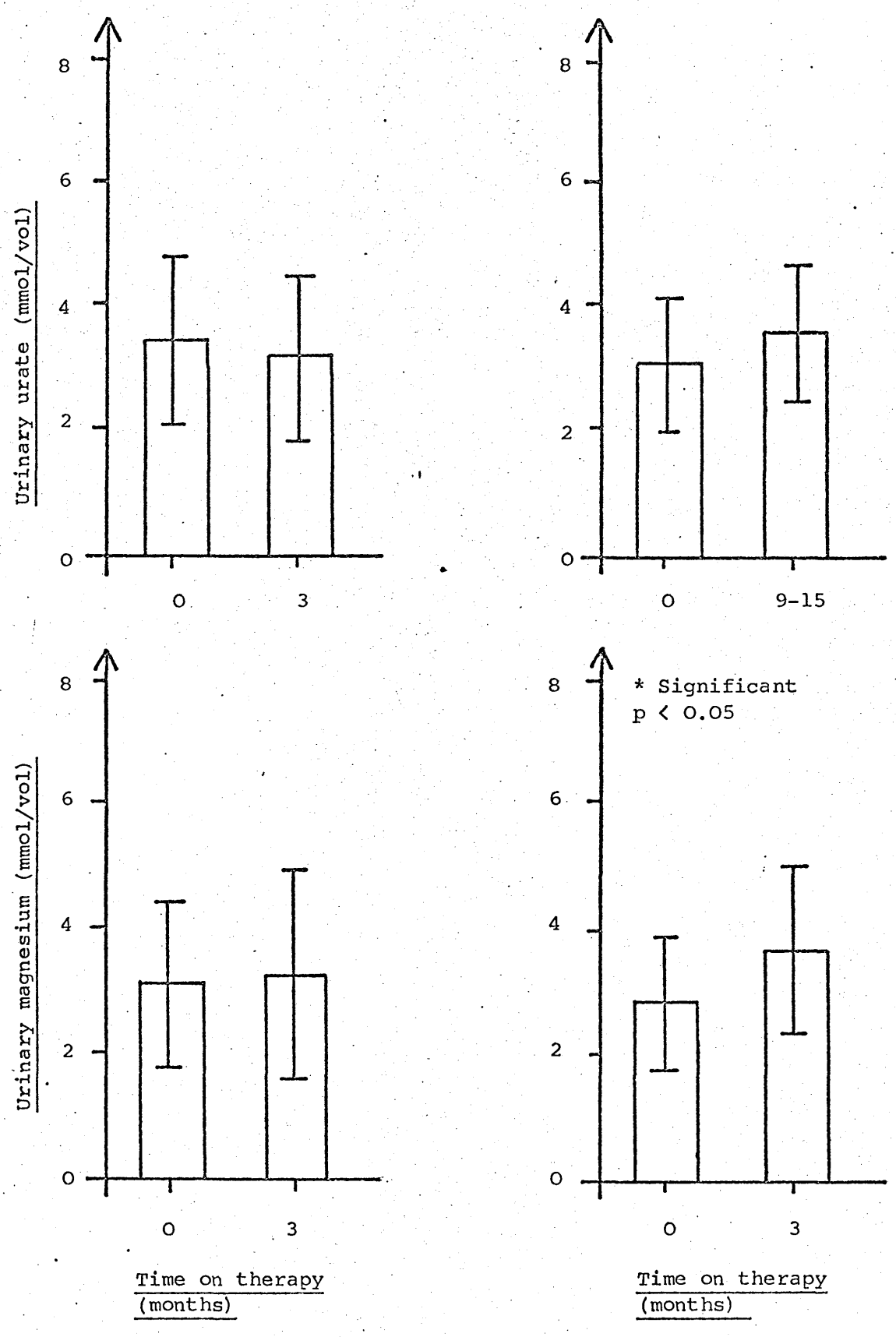


FIGURE 3.6. URINARY URATE AND MAGNESIUM EXCRETION DURING BENDROFLUAZIDE THERAPY (GROUP 2)

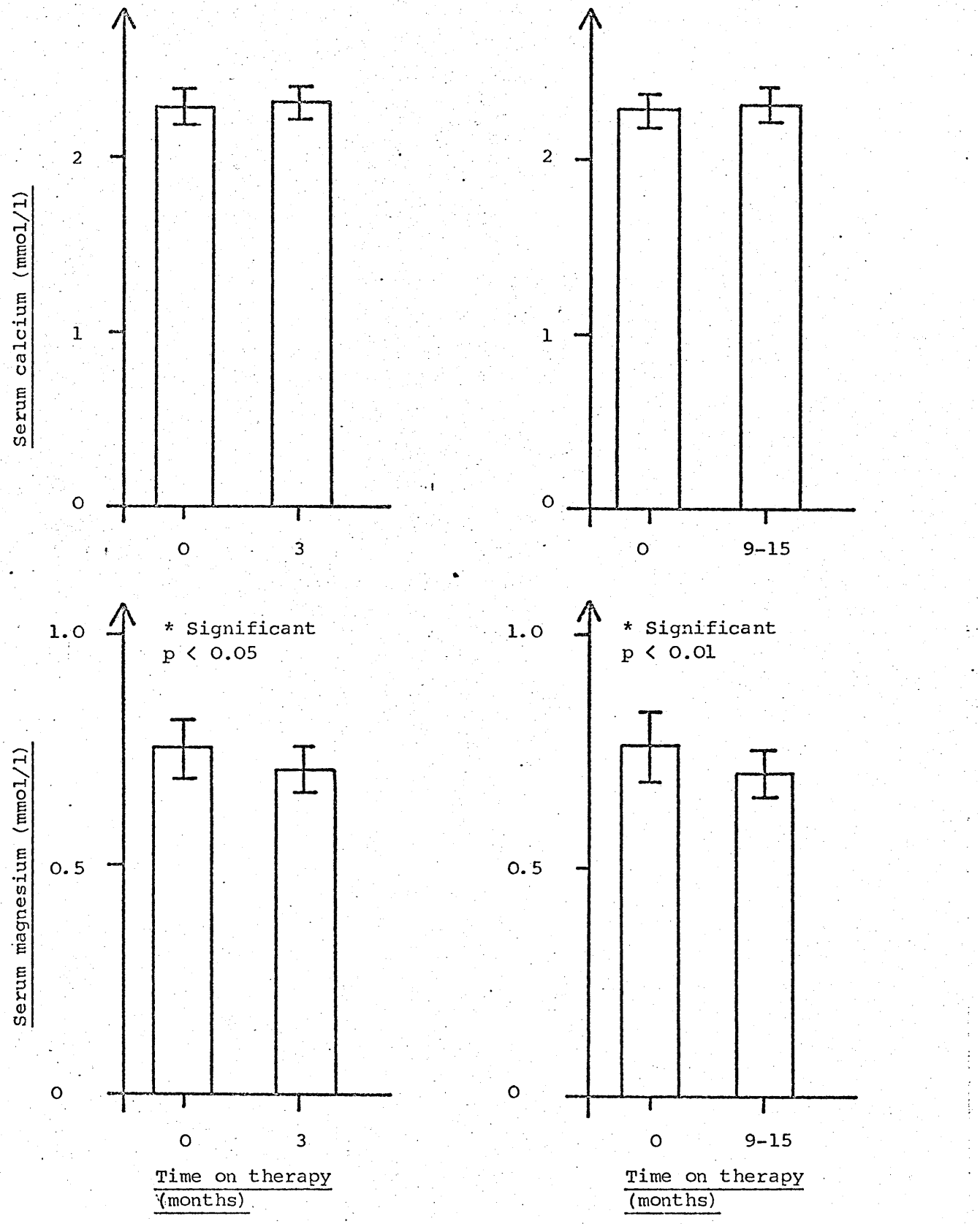


FIGURE 3.7. SERUM CALCIUM AND MAGNESIUM CONCENTRATIONS DURING BENDROFLUAZIDE THERAPY (GROUP 2)

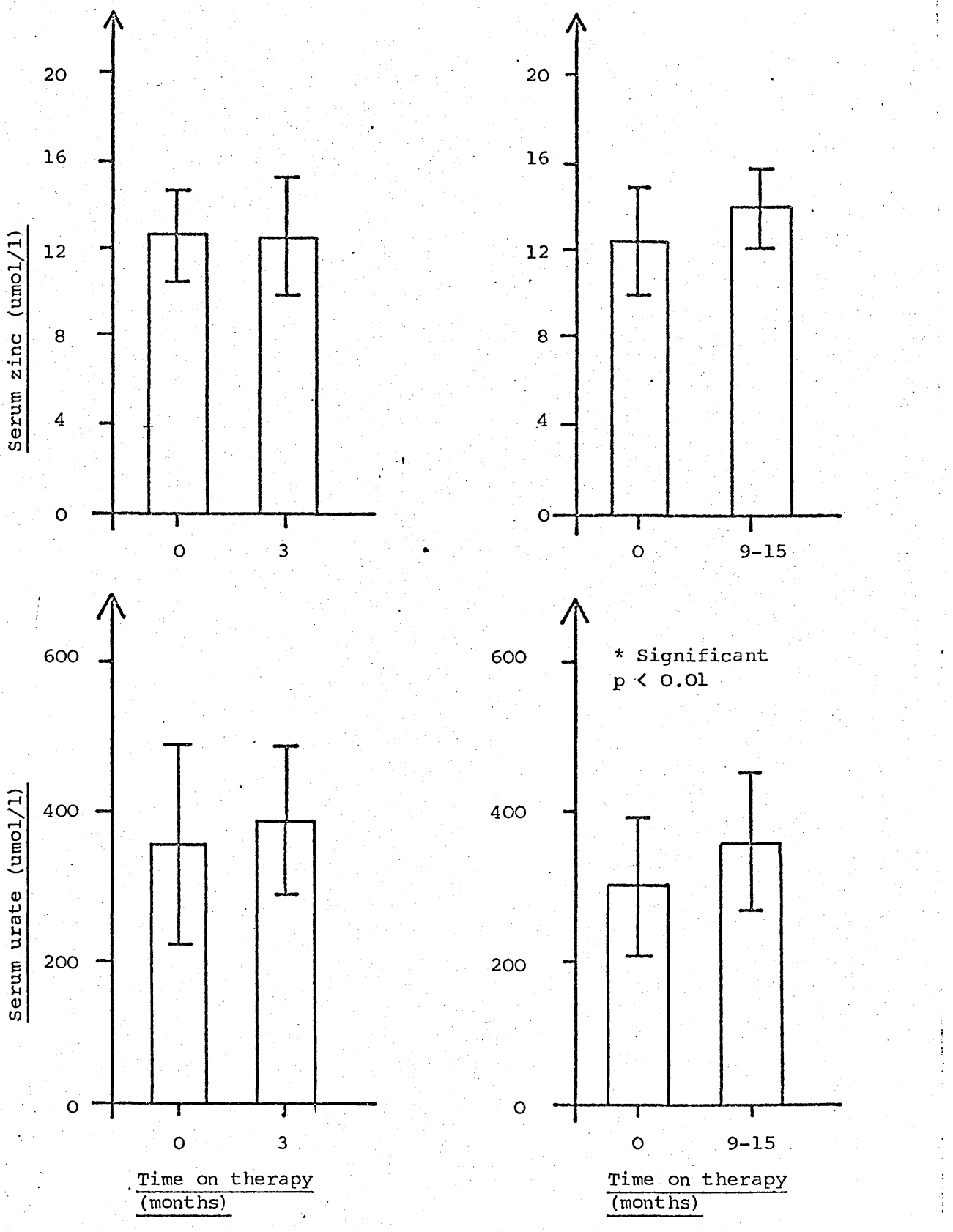


FIGURE 3.8. SERUM ZINC AND URATE CONCENTRATIONS DURING BENDROFLUAZIDE THERAPY (GROUP 2)

months of therapy ($p < 0.05$ and $p < 0.01$ respectively) as shown in figure 3.7., the mean values falling from 0.76 $\pm$ 0.066 to 0.72 $\pm$ 0.046 mmol / litre (n = 7) and from 0.78 $\pm$ 0.077 to 0.71 $\pm$ 0.051 mmol / litre (n = 9).

b) There was an increase in the urinary magnesium excretion after 9 to 15 months of therapy ($p < 0.05$) as shown in figure 3.6. the mean value rising from 2.94 $\pm$ 1.08 to 3.78 $\pm$ 1.41 mmol / volume (n = 17).

c) The serum urate concentration rose significantly ($p < 0.01$) after 9 to 15 months of therapy, as shown in figure 3.8. from a mean value of 307 $\pm$ 96.2 to 370 $\pm$ 95.6 umol / litre (n = 12).

There were no significant changes noted in the urinary calcium, oxalate, and urate excretions nor in the serum calcium and zinc concentrations.

1.5. Results: Group 3.

1.5.1. Exclusions.

Thirteen subjects were assigned to this group. However, 5 male and 1 female subjects were excluded due to non compliance with therapy. Finally, 7 subjects, 6 male and 1 female remained in group 3.

The mean age was 42.9 $\pm$ 9.8 years (n = 7) and there was no significant difference in the mean age between the sexes (unpaired t - test).

1.5.2. Clinical results.

The mean period of follow up for this group of patients was 20.3 ± 6.45 months (n = 7) which represented a total of 11.8 patient years. During this time 1 male subject (14.3 %), who also presented with a recurrent urinary tract infection, suffered a single stone incident after 14 months of therapy. The single female in this group also presented with a recurrent urinary tract infection.

1.5.3. Biochemical results.

The mean urinary excretion of oxalate, calcium, urate and magnesium, pretreatment, after 3 months of therapy and after 9 to 15 months of therapy are shown in figures 3.9. and 3.10.

The mean serum concentrations of calcium, magnesium, zinc and urate, for the same periods of therapy, are shown in figures 3.11. and 3.12.

When the Wilcoxon paired signed rank test was applied, the following results were found:

a) There was a significant fall in the urinary calcium excretion after 3 months of therapy ($p < 0.05$) from a mean value of 10.8 ±

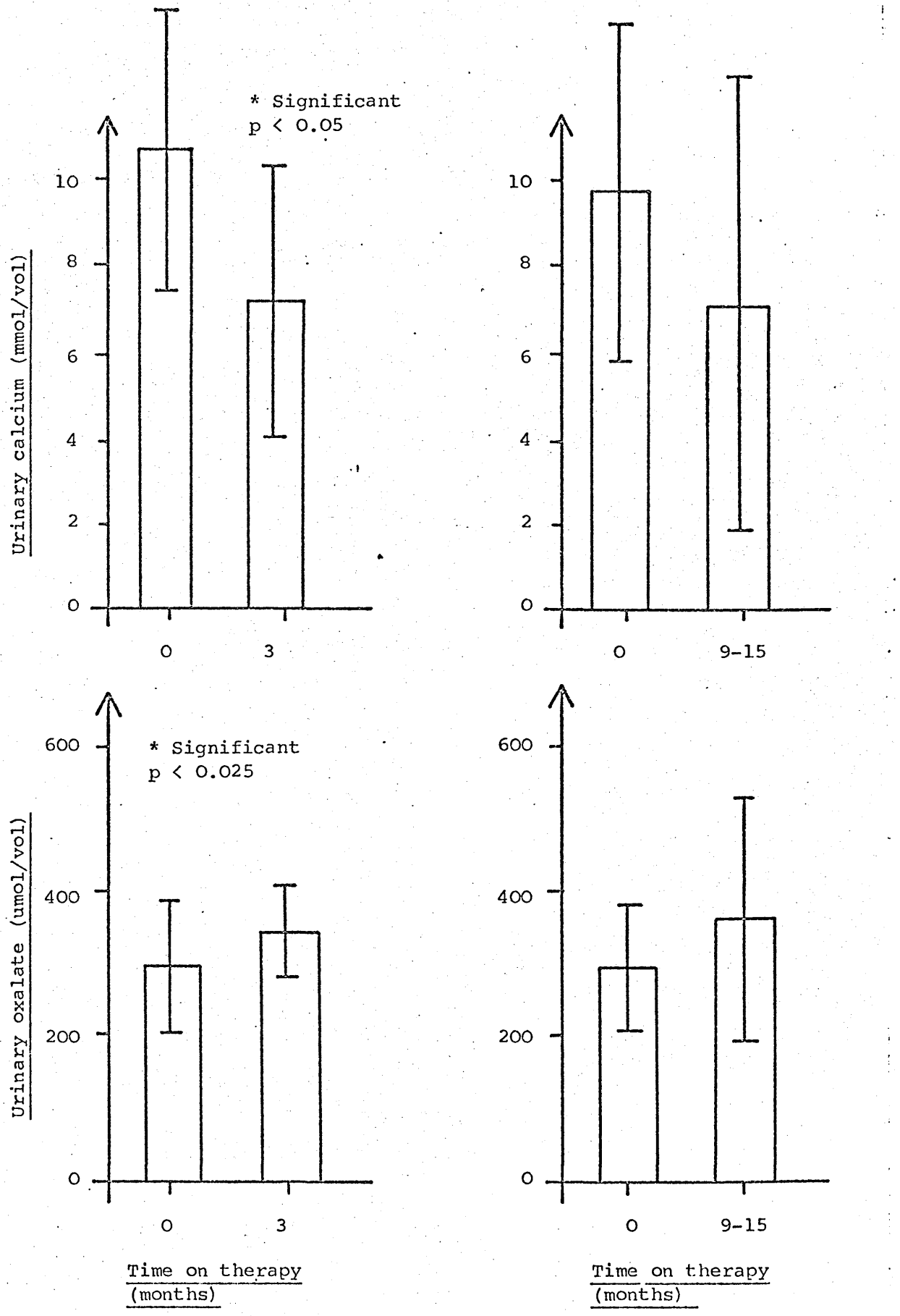


FIGURE 3.9. URINARY CALCIUM AND OXALATE EXCRETION DURING BENDROFLUAZIDE THERAPY (GROUP 3)

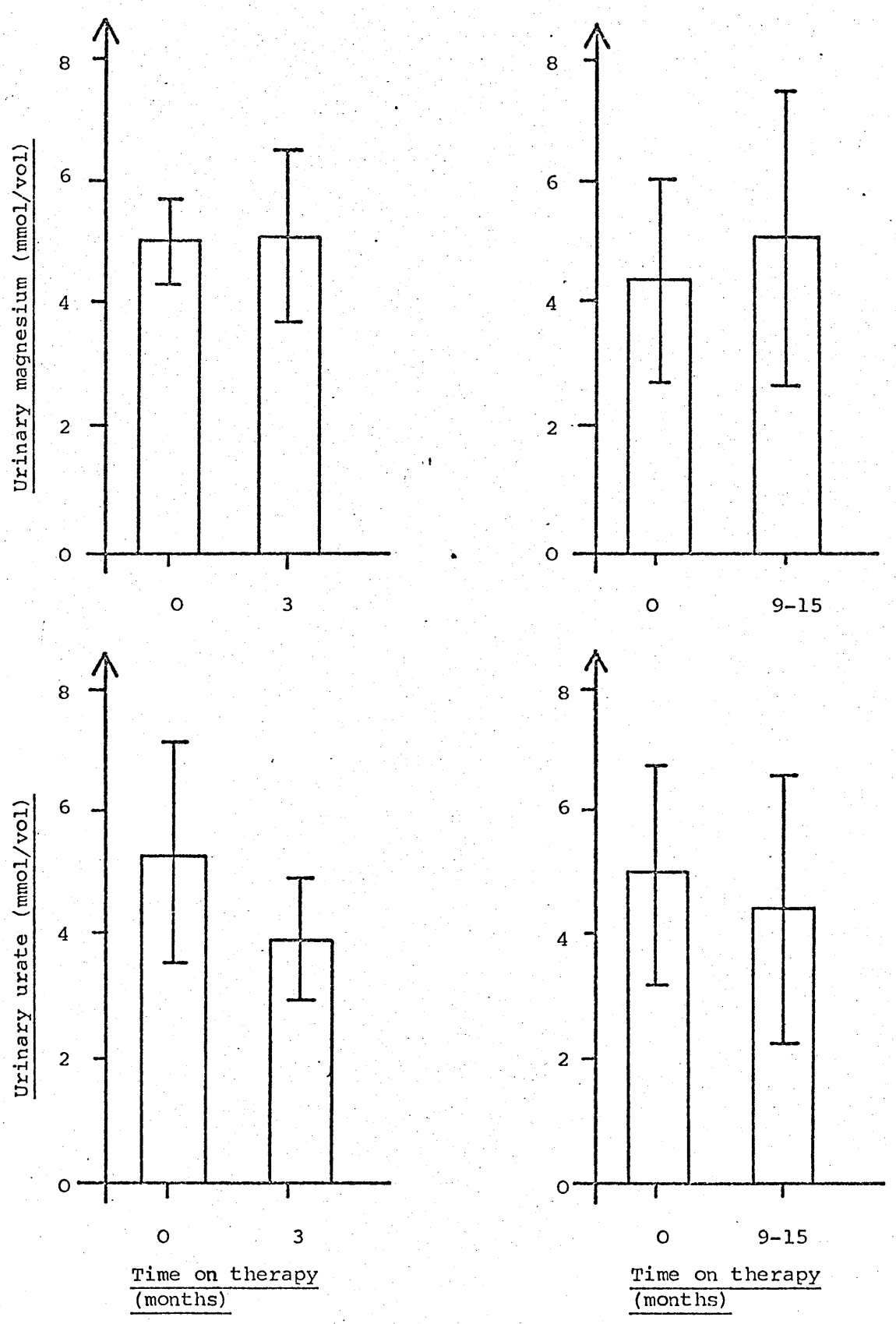


FIGURE 3.10. URINARY MAGNESIUM AND URATE EXCRETION DURING BENDROFLUAZIDE THERAPY (GROUP 3)

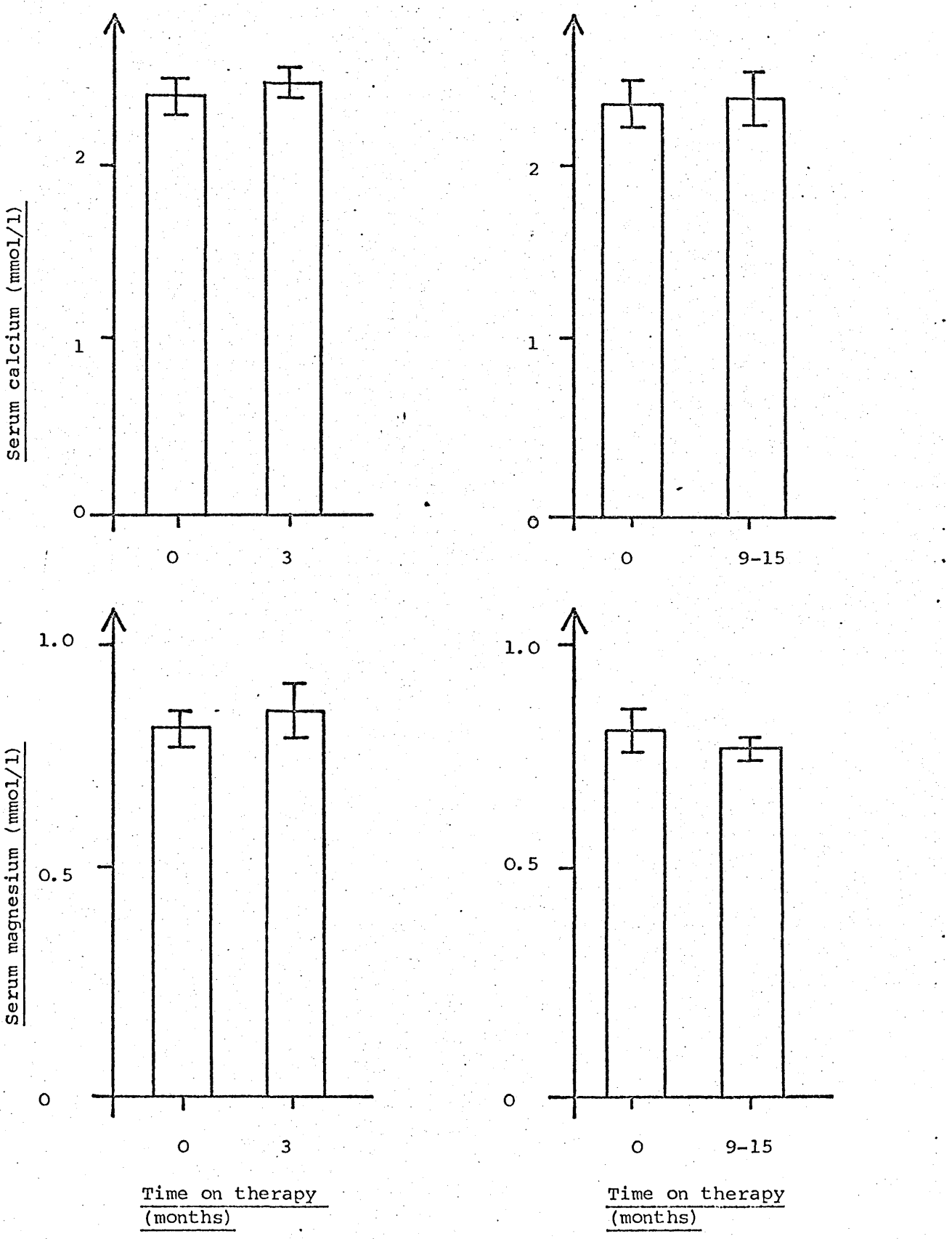


FIGURE 3.11 SERUM CALCIUM AND MAGNESIUM CONCENTRATIONS DURING BENDROFLUAZIDE THERAPY (GROUP 3)

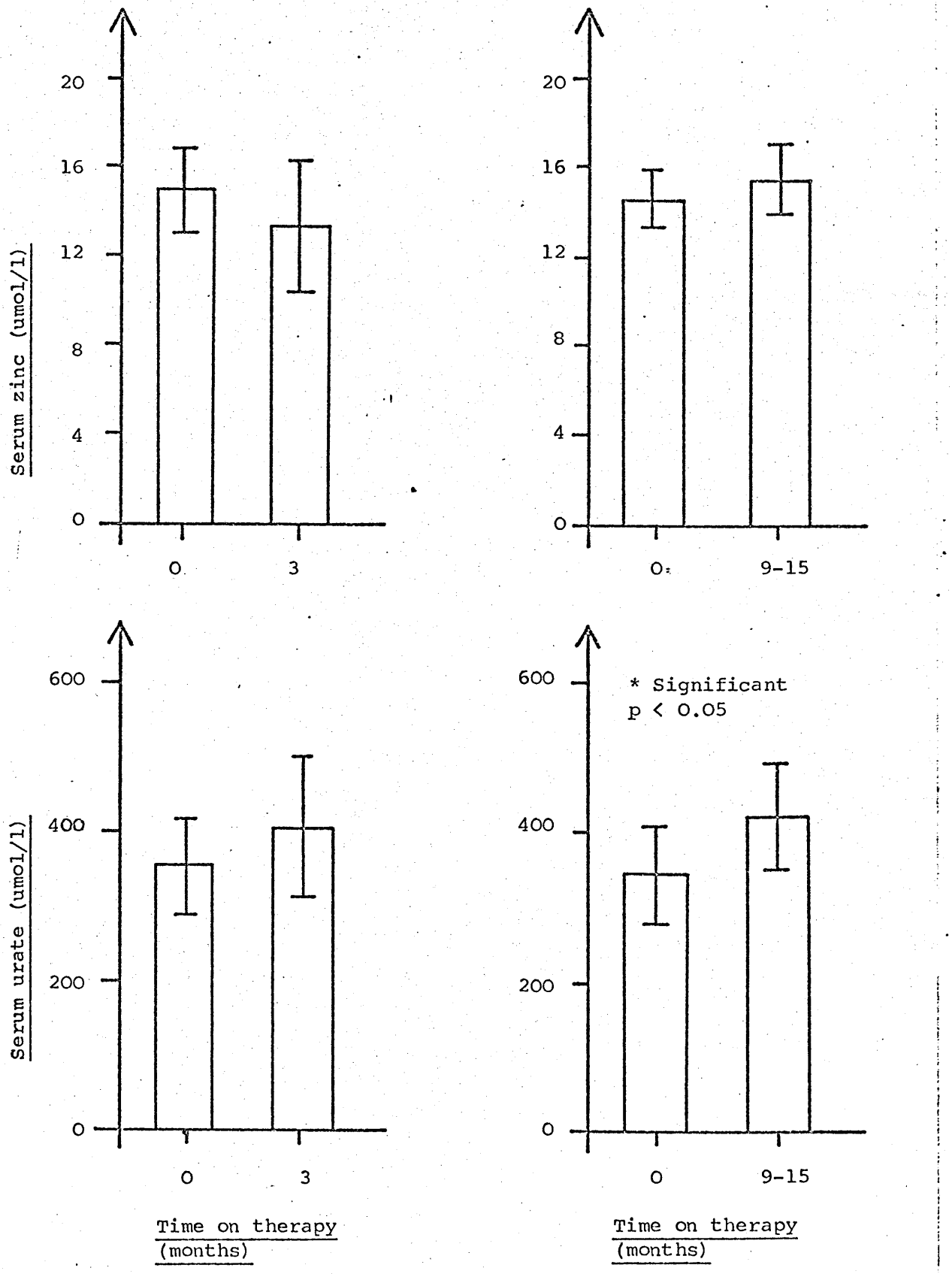


FIGURE 3.12. SERUM ZINC AND URATE CONCENTRATIONS DURING BENDROFLUAZIDE THERAPY (GROUP 3)

3.39 to 7.10 ± 3.23 mmol / volume (n = 6) as shown in figure 3.9.

b) There was a significant rise in the urinary oxalate excretion ($p < 0.025$) after 3 months of therapy, as shown in figure 3.9. from a mean value of 300 ± 95.2 to 354 ± 69.2 umol / volume (n = 6).

c) There was a significant increase in the serum urate concentration after 9 to 15 months of therapy ($p < 0.05$), as shown in figure 3.12., from a mean value of 354 ± 62.7 to 430 ± 68.9 umol / litre (n = 5).

There were no significant changes noted in urinary magnesium and urate excretion nor in serum calcium, magnesium and zinc concentrations.

When an unpaired t - test was applied to the mean values for each analyte mentioned above, from each of the 3 groups, the following results were found:

a) The mean urinary excretion of calcium for group 3 subjects of 9.91 ± 3.96 mmol / volume (n = 7) was significantly greater (unpaired t - test) than the mean values for group 1 subjects of 4.97 ± 1.66 mmol / volume (n = 24) and for group 2 subjects of 4.84 ± 2.02 mmol / volume (n = 22, $p < 0.001$ in both cases). A significant difference was observed between the values, after 3 months of therapy, for group 3 subjects of 7.19 ± 3.29 mmol / volume (n = 6) and for group 2 subjects of 4.47 ± 2.09 mmol / volume (n = 18, $p < 0.05$) but not between groups 3 and 1, and no significant differences were noted after 9 to 15 months of therapy.

b) The mean pretreatment urinary excretion of urate for group 3 subjects of 5.Ø4 ± 1.84 mmol / volume (n = 7) was significantly greater than the mean value for group 1 subjects of 3.4Ø ± 1.Ø3 mmol / volume (n = 22, p < Ø.Ø5). A significant difference was observed between the values after 3 months of therapy for group 3 subjects of 3.98 ± Ø.96 mmol / volume (n = 6) and for group 1 subjects of 2.86 ± Ø.81 mmol / volume (n = 22, p < Ø.Ø1), and after 9 to 15 months of therapy between the group 3 mean value of 4.54 ± 2.17 mmol / volume (n = 7) and the group 1 mean value of 3.Ø7 ± 1.29 mmol / volume (n = 2Ø, p < Ø.Ø5).

1.6. Discussion.

The overall ratio of male to female patients considered for this study was 1.93 to 1.Ø which was in general agreement with other studies, in which the incidence of symptomatic stone disease in males was considered to be greater than in females (Hodgkinson 1977, Robertson et al 198Øa).

The overall compliance to therapy of the subjects ascribed allopurinol was 87.5 % which was an acceptable level for a closely followed group taking a single daily dose. The overall compliance of the subjects ascribed bendrofluazide was slightly less, being 72.7 %, possibly due to the necessity of concurrent therapy with potassium

supplements which were required to be taken 3 time per day, and which may, on occassion, produce gastric upset (Data Sheet Compendium 1978a).

Adverse reactions attributed to bendrofluazide and necessitating withdrawal of therapy were rare and occurred in only 1 female subject representing 2 % of all subjects ascribed to the drug. Adverse reactions attributed to allopurinol were more common and occurred in 3 male and 1 female subject, representing 13 % of all subjects ascribed such therapy. Bendrofluazide has been recognised as having a low incidence of side effects (Data Sheet Compendium 1978b), and a similar incidence of reactions to allopurinol therapy of 9.8 % has been reported (Scott, Mathieson and McLelland 1979).

When the presenting renal stone incidents of all female subjects were considered, 38.5 % presented having required surgical intervention compared to 14.Ø % of all males. This implied that clinical stone disease, although rarer in females, was of a more severe nature than that observed in males, although no reason for this was apparent.

The incidence of urinary tract infection found during the present study was 7.7 % in females and 4.Ø % in males.

The presence of a urinary tract infection did not relate to the requirement for initial surgical intervention, and the infection rates were not sufficiently different to account for the apparent

difference in the severity of symptomatic stone disease observed between the sexes.

Figure 3.13. represents the percentage of subjects stone-free versus time, after an initial stone incident, which was found in an untreated group of subjects (Coe 1980). Also plotted in this figure are the percentages of subjects that were stone - free, at the end of the study, versus the mean time of follow up for each group. For each group, the recurrence of stone was less than was shown for the untreated group but, although this may have been the case and although it may be more pronounced after an increased period of follow up, only a general comparison between the treated and untreated groups may be made in this instance.

Allopurinol therapy has been shown to reduce the recurrence of stone disease in hyperuricaemic or hyperuricosuric stone formers alone (Coe and Raisen 1973, Smith 1977, Coe 1977) and in metabolically normal stone formers in conjunction with thiazide diuretics (Coe 1977).

The rationale behind the allopurinol regime was that, as a xanthine oxidase inhibitor, allopurinol would reduce the oxidation of xanthine and hypoxanthine to uric acid and may also reduce the urinary excretion of oxalate (Scott et al 1978a, Scott et al 1978b).

It has been suggested that uric acid or sodium urate crystals could promote calcium stone formation by providing a substrate for the growth of calcium oxalate crystals; ie. epitaxy (Lonsdale 1968), therefore a

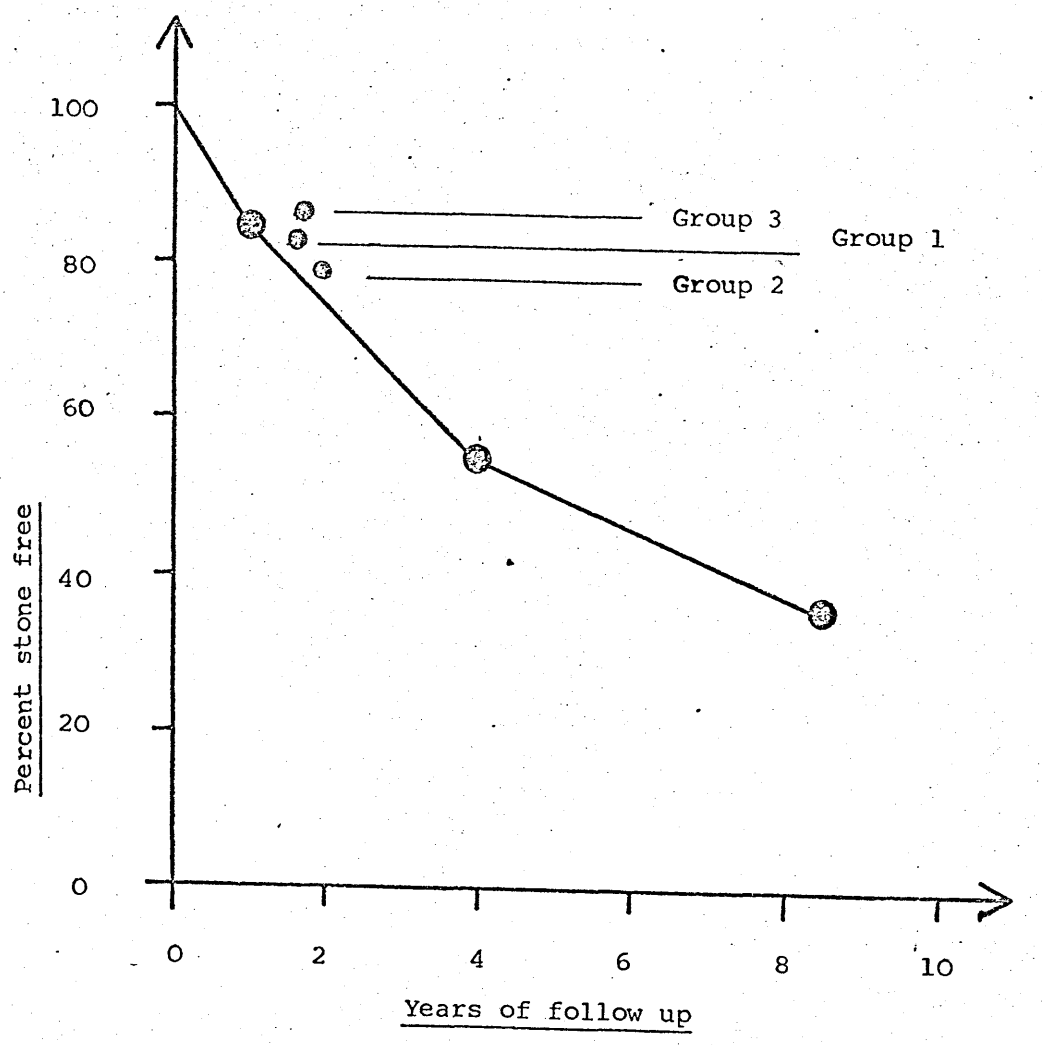


FIGURE 3.13. STONE RECURRENCES IN AN UNTREATED GROUP OVER 9 YEARS (Coe 1980): STONE RECURRENCES OBSERVED IN THE PRESENT STUDY

reduction in urinary uric acid would reduce the probability of forming stones.

More recently, Robertson et al (1976) suggested that urinary urate might interfere with crystallisation inhibitors (glycosaminoglycans) in urine. A decreased concentration of urate would therefore favourably affect the inhibition of calcium oxalate crystallisation. An increase in the inhibition of crystal growth in vitro during treatment with allopurinol has been shown (Tiselius 1980) but more recently Hallson, Rose and Sulaiman (1982) have shown that, in whole human urine, an increase in the urinary urate did not increase the calcium oxalate crystals formed and these workers did not support the suggestion that reducing urinary urate concentration may be of value in the treatment of patients with stone disease.

Urinary oxalate has been considered to be the major risk factor involved in renal stone formation (Robertson et al 1981) and the reduction of urinary oxalate excretion during allopurinol therapy has been shown by some (Scott et al 1978a, Scott et al 1978b) but not other workers (Tiselius 1980, Tiselius and Larsson 1982).

Urinary calcium has been shown to be unchanged during allopurinol therapy (Scott et al 1978a, Scott et al 1978b, Tiselius 1980, Tiselius and Larsson 1982) but in this study there was a significant increase in the urinary excretion of calcium after 3 months of therapy ($P <$

Ø.Ø25), the mean value rising outwith the reference range, although this increase was not maintained after 9 to 15 months of therapy.

The renal handling of calcium has been shown to involve almost complete reabsorption of filtered calcium; 5Ø to 55 % reabsorbed in the proximal tubule, 2Ø to 3Ø % in the loop of Henle and 12 to 23 % in the distal tubule (Goldberg, Agus and Goldfarb 1976).

As some 5Ø % of calcium is reabsorbed in the proximal tubule any changes effecting urate, which is also handled predominantly in the proximal tubule might be expected to influence the final excretion of calcium.

Alternatively, a number of varied adverse reactions attributed to allopurinol (Data Sheet Compendium 1983a) may be humoral in origin, and such effects may also influence the renal handling of calcium.

The finding that the calcium excretion was not significantly different from the baseline value after 9 to 15 months of therapy may indicate a chronic change in the calcium balance of the subjects being studied.

The effect of seasonal changes in dietary calcium intake was unlikely to have been responsible for the observed data as allopurinol therapy was initiated in different subjects at different times of the year.

Serum zinc levels, although shown to have increased after 9 to 15 months of therapy ($p < Ø.Ø5$) are subject to large diurnal variations and should be estimated on fasting blood samples. Although all blood

samples in this study were taken between 11.ØØ amd and mid - day, fasting samples were not specified and combined with the low significance of this finding makes the interpretation of this change, if any, difficult, although a chronic change in the serum zinc levels similar to that of the renal handling of calcium may have occurred. Urinary zinc excretion was not measured in this study due to the problem of sample contamination.

In this study group randomly ascribed allopurinol therapy there was a significant fall in the blood urate concentration after 3 months and after 9 to 15 months of therapy ($p < Ø.Ø5$ in both cases) due to the inhibitory effect of allopurinol on xanthine oxidase, with the mean value remainig within the reference range. Although there was no significant change in the urinary excretion of urate in this group, which was a similar finding from previous work in this department (Scott et al 1978) a significant fall during therapy has been observed by other workers (Tiselius 198Ø, Tiselius and Larsson 1982).

The renal handling of urate in the rat has been shown to involve complete glomerular filtration and bidirectional transport limited to the proximal nephron. Reabsorption was almost complete during tubular passage therefore excreted urate was almost entirely derived from tubular secretion. (Lang et al 1979).

Although no significant decrease of urinary urate excretion was shown in this group a reduced serum urate concentration migh be expected to produce a fall in the

urinary urate excretion due to reduced tubular secretion.

Urinary oxalate excretion has been shown to be reduced in stone formers (Scott et al 1978) and in normal male subjects receiving a high purine diet (Simmonds et al 1981) during allopurinol therapy, but no significant difference in oxalate excretion was shown in this study which was in agreement with other studies (Tiselius 1980, Tiselius and Larsson 1982).

The role of xanthine oxidase in the endogenous synthesis of oxalate has been shown to be small (Gibbs and Watts 1966) so the inhibition of this enzyme by allopurinol was unlikely to have had a significant effect on oxalate excretion.

The renal handling of oxalate in the rat has been shown to be similar to that of urate in that it involved virtually complete glomerular filtration with tubular reabsorption and tubular secretion located in the proximal nephron (Lang et al 1981). Tubular secretion was shown to be inhibited by urate (Greger et al 1978); this implied that the effect of allopurinol therapy, if any, would be to increase rather than to decrease urinary oxalate excretion.

The lack of any significant change in urinary oxalate excretion during allopurinol therapy may therefore be caused by a combination of reduced endogenous synthesis due to xanthine oxidase inhibition and an increased renal clearance due to the reduced inhibition of tubular secretion of oxalate by urate.

Bendrofluazide therapy has been successfully used to treat stone disease when given continuously (Backman et al 1979, Ljunghall 1981a), intermittantly (Rundle and Scott 1980) or in combination with magnesium oxide (Ahlstrand and Tiselius 1983). Other thiazide diuretics such as hydrochlorothiazide (Klein and Griffith 1981, Scholz, Schwille and Sigel 1981, Cohanim and Yendt 1981, Maschio et al 1981), trichlormethiazide (Coe 1977) and chlorthiazide (Klein and Griffith 1981) have also been shown to be of benefit in the prevention of the recurrence of kidney stone disease. However, in a report of a double blind controlled clinical trial of bendrofluazide versus a placebo, Brocks, Dahl and Wolf (1981) showed that although the thiazide group demonstrated a similar reduction in stone recurrence to that found by other workers, the placebo group had a reduction of the same magnitude, over a mean period of follow up of 1.6 years. In conclusion, Brocks et al (1981) concede that the period of follow up during therapy was short and a statement as to the long term benefits of thiazide therapy to these patients cannot be made.

Urinary calcium has been considered to be a significant risk factor involved in renal stone formation (Robertson et al 1981) and urinary magnesium has been shown to reduce calcium oxalate crystal formation in human urine (Hallson, Rose and Sulaiman 1982).

In the current study, 2 groups of patients were ascribed bendrofluazide with potassium supplements; a

normocalciuric and a hypercalciuric group. In both groups, the serum urate concentration was shown to have risen significantly after 9 to 15 months of therapy, although the mean levels remained within the reference range, and there were no significant differences in the urinary excretion of urate during therapy.

Increased serum urate is a documented side effect of bendrofluazide therapy (Data Sheet Compendium 1978a) and has been reported by a number of workers during prolonged therapy (Jorgensen and Brunner 1974, Backman et al 1979, Ljunghall et al 1981b) and in this instance was probably caused by the inhibition of tubular secretion of urate by competition at the tubular transport step from the diuretic agent (Lang et al 1979).

Changes in the serum magnesium concentration with and without changes in the urinary magnesium excretion during bendrofluazide therapy have been demonstrated (Jorgensen and Brunner 1974, Backman et al 1979, Ljunghall et al 1981b)

In the present study, during bendrofluazide therapy, the normocalciuric subjects demonstrated a significant fall in the serum magnesium concentration after 3 months and after 9 to 15 months of therapy, and a significant increase in the urinary excretion of magnesium after 9 to 15 months of therapy. This represented a chronic renal loss of magnesium during therapy, probably caused by an effect of the bendrofluazide at the proximal end of the distal convoluted tubule, decreasing magnesium

reabsorption.

Although no statistically significant changes on the serum concentration nor the urinary excretion of magnesium were seen in the hypercalciuric subjects during the present study, this was probably due to the limited size of the group.

The reduction of urinary calcium during bendrofluazide therapy has been shown in normocalciuric and in hypercalciuric subjects (Jorgensen and Brunner 1974, Backman et al 1979, Ljunghall et al 1981b). A number of mechanisms accounting for this fall have been suggested including a decreased intestinal absorption of calcium (Yendt and Cohanim 1978), humoral effects particulary due to parathyroid hormone (Brickman, Massry and Coburn 1972) and a direct renal effect (Costanzo and Weiner 1974).

From the current data, only the hypercalciuric subjects showed a statistically significant fall in urinary calcium, after 3 months of therapy to a mean value which was still outwith the reference range. However, no significant difference was observed after 9 to 15 months of therapy probably due to the limited number of subjects within the group.

As the serum calcium concentration in this study was not significantly changed, the calcium balance of the subjects may have been changed, although the mechanism by which this was reached was unclear.

Yendt and Cohanim (1978) have shown a significant reduction in urinary oxalate excretion during chronic

hydrochlorothiazide administration after at least 1 year of therapy, and it has been suggested that this was due to a decreased intestinal absorption of calcium caused by chronic hydrochlorothiazide therapy (Yendt, Gagne and Cohanim 1965).

In the present study, a significant increase in the urinary oxalate excretion after 3 months of bendrofluazide therapy was observed in the hypercalciuric subjects. Although this was contradictory to the observations of Yendt and Cohanim (1978), it has been shown that there was a reduction in the renal excretion of oxalate during volume expansion (Greger et al 1978). During thiazide therapy a degree of volume contraction might be expected and this in turn could produce an increased renal excretion of oxalate and therefore an increased urinary excretion.

The hypercalciuric group in the current study was selected only on the basis of an elevated urinary calcium excretion, and as such the mean pretreatment value of urinary calcium was significantly higher in this group than in the other two.

After 3 months of therapy, due to the hypocalciuric effect of bendrofluazide, there was no significant difference between the mean calcium excretion of the initially hypercalciuric group and the allopurinol group. However, there was still a significant difference between the two bendrofluazide groups, due to the limited reduction in urinary calcium excretion in the

normocalciuric group.

As well as an elevated urinary calcium, there was a significantly higher urinary urate excretion, the mean value being outwith the reference range, in the hypercalciuric subjects when compared to the other two normocalciuric groups. Although this difference was resolved between the bendrofluazide groups during therapy, the mean urinary excretion of urate remained significantly higher in the initially hypercalciuric group than in the group randomly ascribed allopurinol. This was due to the reduced excretion of urate in the group receiving allopurinol.

2. INVESTIGATION OF URINARY OXALATE VARIATION IN NORMAL SUBJECTS USING THE ENZYMIC METHOD OF OXALATE ESTIMATION.

The importance of urinary oxalate excretion in stone disease has already been stated in chapter 1. and the possible existence of a diurnal variation in the urinary excretion or urinary concentration of oxalate has been suggested by some (Hodgkinson 197Ø, Tiselius and Almgard 1977, Bach et al 1979, Vahlensieck, Bach and Hesse 1982)but not all workers (Tocco et al 1979).

It has been suggested that such a diurnal rhythm might lead to situations in which the 'risk' of stone formation may be increased at a specific time of day (Tiselius and Almgard 1977).

In this study the variation of oxalate excretion,

oxalate concentration and urine flow rate was examined in healthy volunteers diurnally and on a daily basis over 3 consecutive days.

The subjects involved were also being followed to asses the pharmacokinetics of a single dose of the antibiotic 'Tinidazole'. This drug was not known to have any effect on the excretion of oxalate (Data Sheet Compendium 1983b).

2.1. Subjects and Methods.

5 male and 3 female laboratory staff aged between 19 and 40 years with no known pathological condition were recruited to the study.

Subjects were requested to remain fasted from 10.00 pm the previous evening and urine collection was commenced at 9.00 am on 'day 1' and continued as shown in table 3.7. until the end of 'day 3'.

The subjects remained fasted until 13.00 h on 'day 1' after which there were no dietary restrictions. During the fasting period, free access to drinking water was permitted to ensure an adequate urine output.

After sample collection, the volume of urine passed was noted and an aliquot of the well mixed sample was acidified by the addition of concentrated HCl to a pH of less than 3, and stored at -20 ^{o}C until analysis.

The oxalate estimation was performed using the

Table 3.7. Sample collection periods.

Sample		Collection Period
Day 1	1.	09.00 - 11.00 h
	2.	11.00 - 13.00 h
	3.	13.00 - 15.00 h
	4.	15.00 - 17.00 h
	5.	17.00 - 19.00 h
	6.	19.00 - 21.00 h
	7.	21.00 - 09.00 h
Day 2		09.00 - 09.00 h
Day 3		09.00 - 09.00

enzymatic procedure described and evaluated in chapter 2.

2.2. Results.

2.2.1. Specificity of the Enzymatic Assay.

A 2 mM aqueous solution of the antibiotic 'Tinidazole' when estimated as per the assay procedure did not differ from the 'zero standard' value.

2.2.2. Exclusion of Subjects.

Of the 8 subjects recruited to the study 1 male and 1 female subject were excluded due to incomplete sample collection. The age range of the remaining 6 subjects was 22 to 40 years.

2.2.3. Urinary Oxalate Excretion.

The diurnal variation of urinary oxalate excretion rate for the 6 subjects is shown in figure 3.14. The rate of excretion varied throughout the 24 hour period and was highest between 5.00 pm and 7.00 pm and generally lowest during the night through to mid morning (9.00 pm to 11.00am).

When a paired t-test was applied to the data no statistically significant difference between any of the time periods was shown. The mean excretion rate over the 24 hour period was 13.8 uM/h ($\pm$ 2.32).

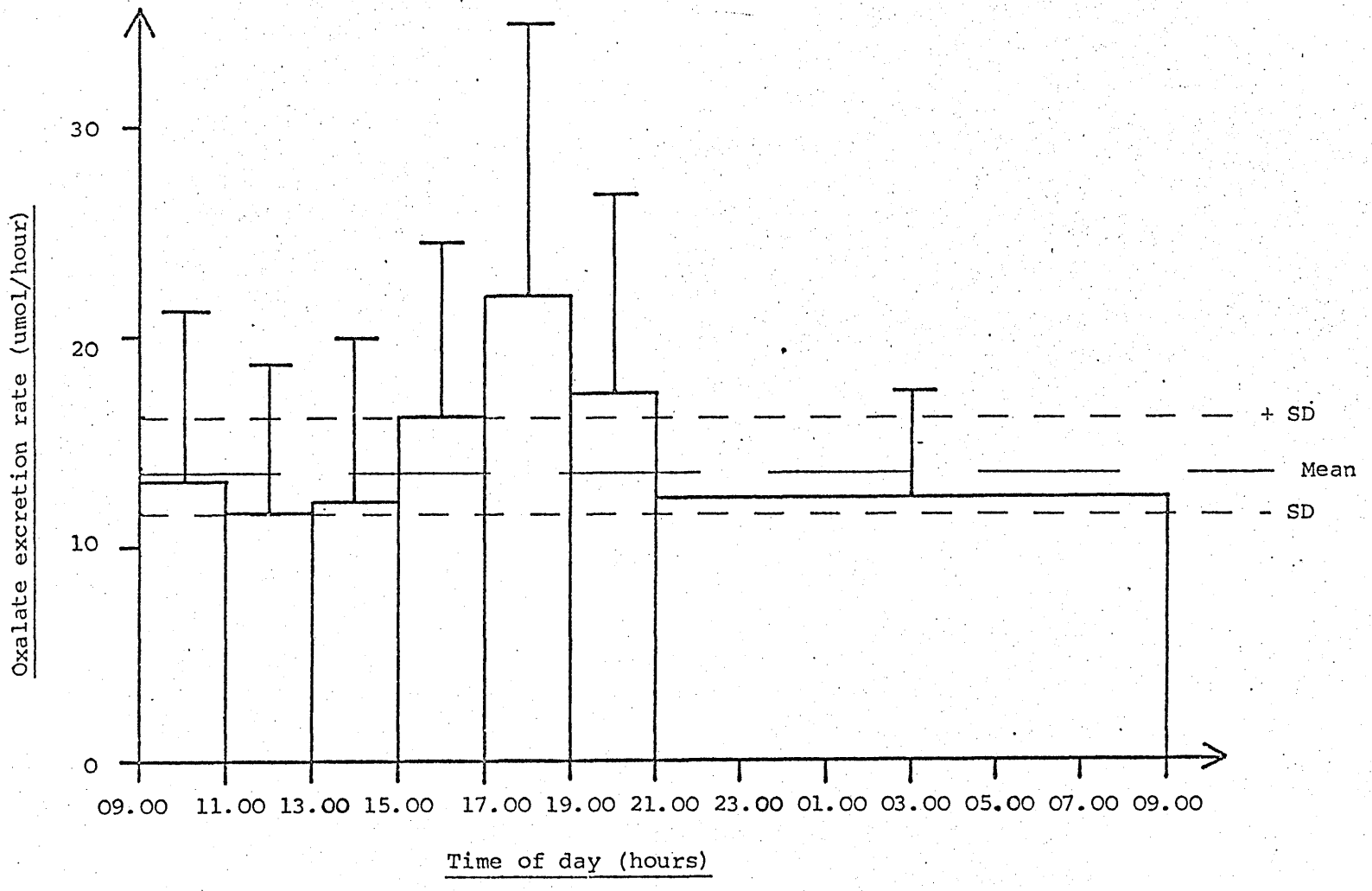


FIGURE 3.14. DIURNAL VARIATION OF URINARY OXALATE EXCRETION RATE

The variation of the daily oxalate excretion rate over 3 consecutive days is shown in figure 3.15. No statistically significant differences, using a paired t-test was shown between the days. The mean excretion rate over the 3 day period was 12.9 umol/h (± 4.59).

The interindividual variation of the daily oxalate excretion over the 3 day study period shown in table 3.8. ranged from 6.5 to 64.7 % and the intraindividual variation shown in table 3.9. ranged from 17.Ø to 33.9 %.

2.2.4. Urinary Oxalate Concentration.

The diurnal variation of urinary oxalate concentration is shown in figure 3.16. No statistically significant difference was found between any of the time periods using a paired t-test. However, the general impression obtained from the histogram was of a lower oxalate concentration in the morning through to the mid afternoon (9.ØØ am to 3.ØØ pm) and a higher concentration from the mid afternoon and throughout the night (3.ØØ pm to 9.ØØ am). The mean oxalate concentration over the whole day was 245 umol/l (± 66.Ø).

The variation of the daily oxalate concentration over the 3 day study period is shown in figure 3.17. No statistically significant difference was found (paired t-test) and the mean oxalate concentration for all 6 subjects was 244 umol/l (± 91.5).

The coefficient of interindividual variation of the daily urinary

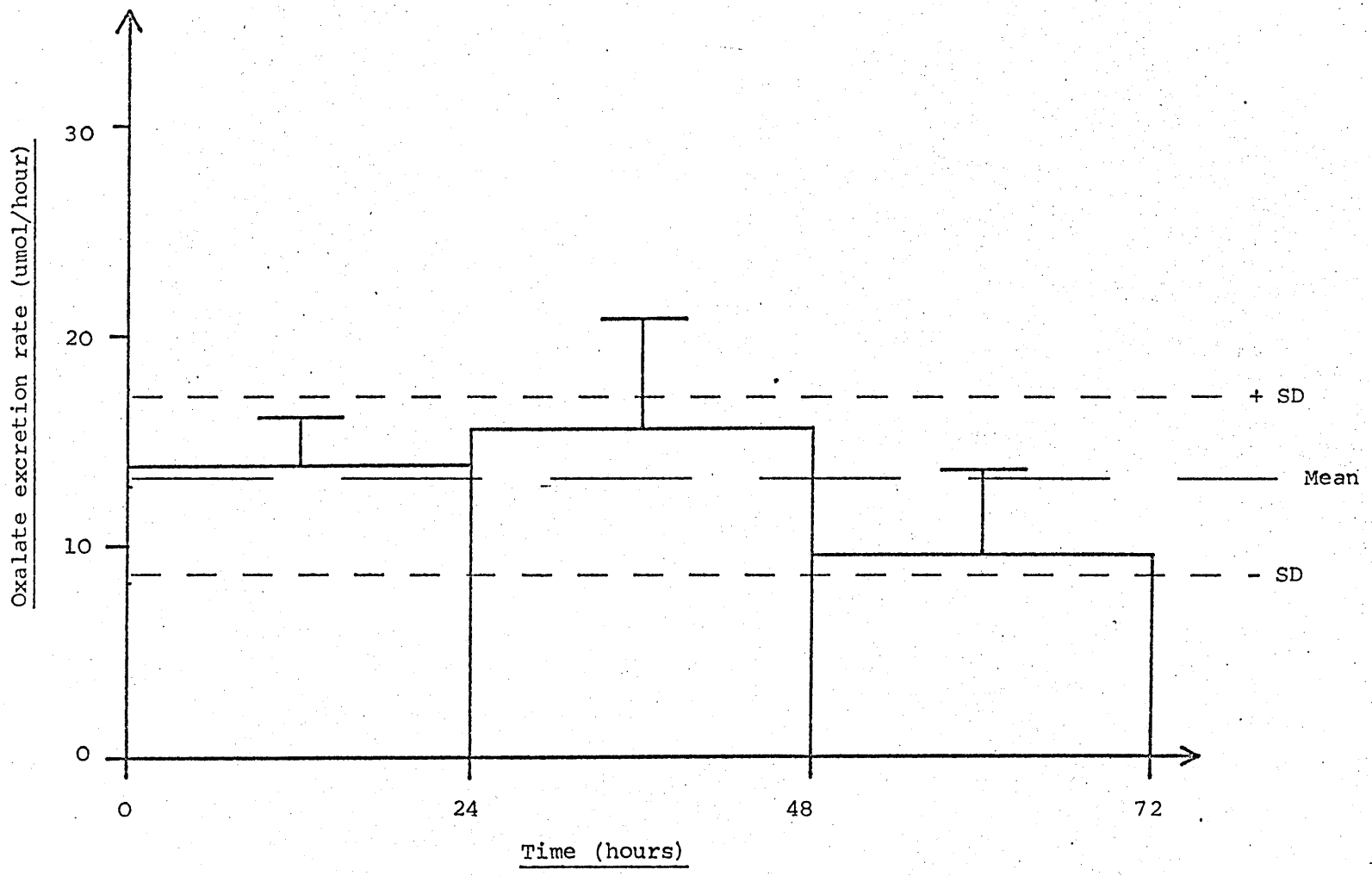


FIGURE 3.15. VARIATION OF 24 HOUR URINARY OXALATE EXCRETION OVER 72 HOURS

Table 3.8.Interindividual variation of oxalate excretion over 3 consecutive days.

Subject	Mean excretion umol/d (± S.D.)	Interindividual CV%
1.	336 (± 217)	64.7
2.	248 (± 160)	6.5
3.	314 (± 61.6)	19.6
4.	324 (± 187)	57.8
5.	277 (± 18.5)	6.7
6.	360 (± 62.2)	17.3

Table 3.9.Intraindividual variation of oxalate excretion for 6 subjects over 3 consecutive days.

Day	Mean excretion umol/d (± S.D.)	Intraindividual CV%
1.	330 (± 56.2)	17.0
2.	372 (± 126)	33.9
3.	228 (± 94.4)	41.5

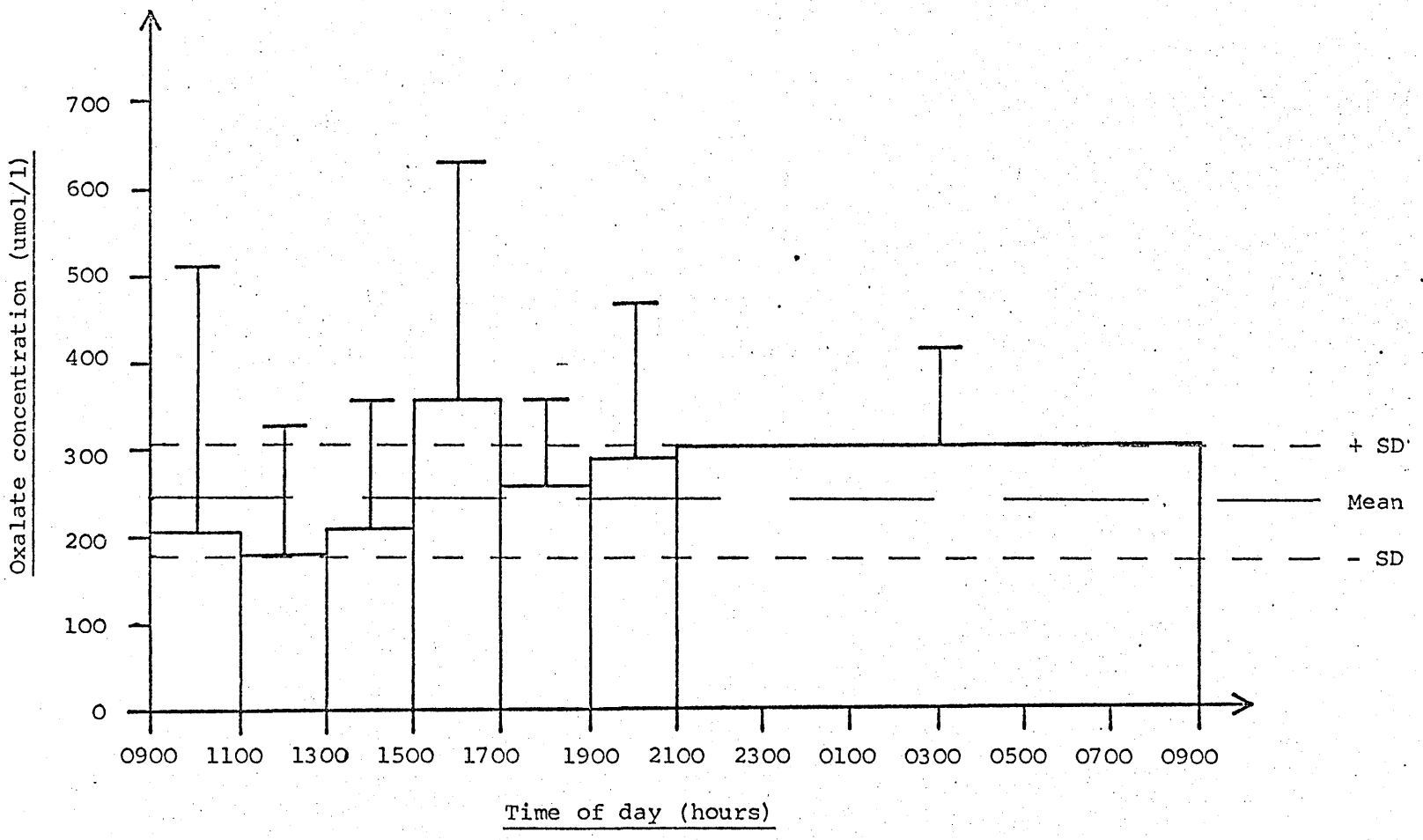


FIGURE 3.16. DIURNAL VARIATION OF URINARY OXALATE CONCENTRATION

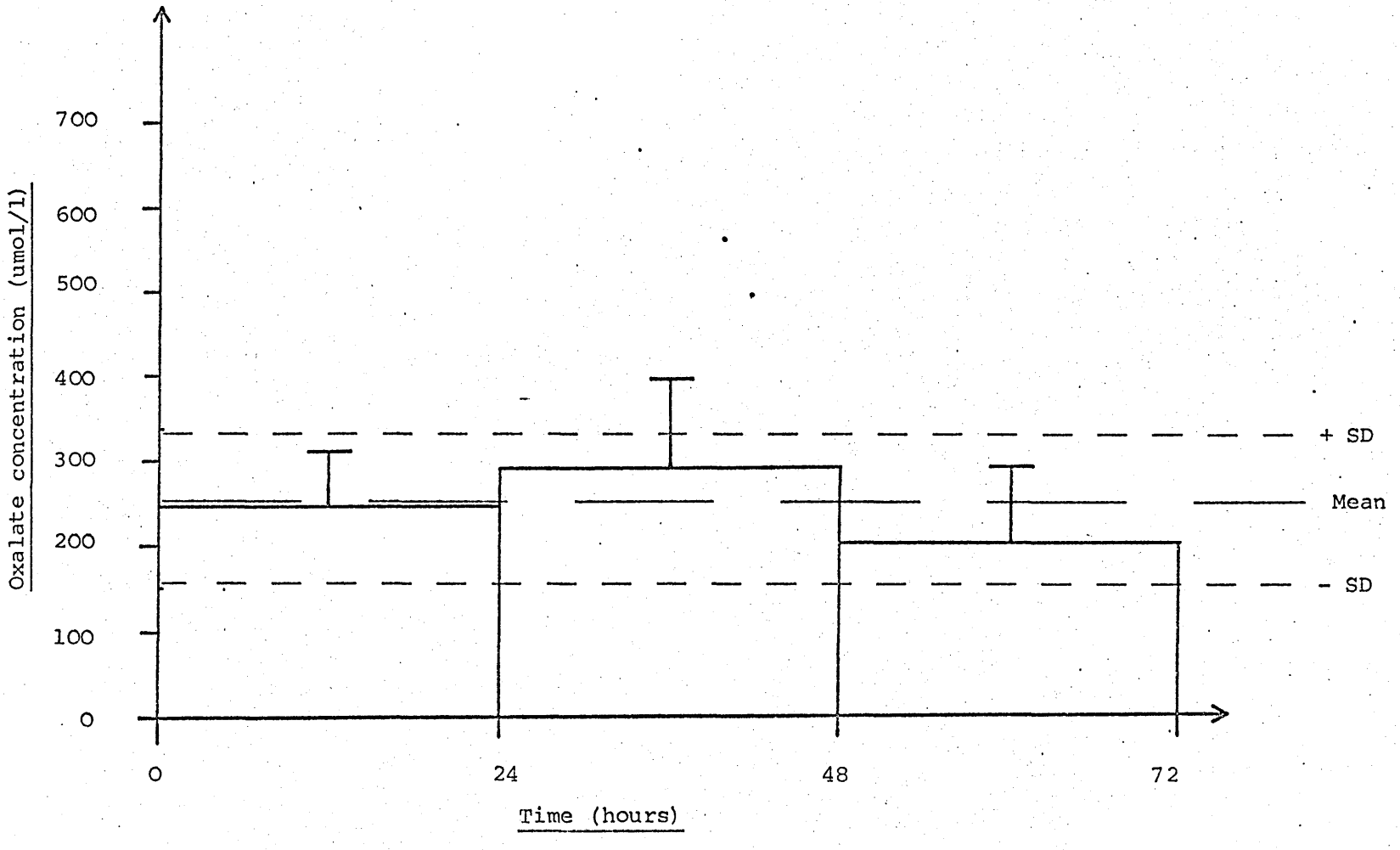


FIGURE 3.17. VARIATION OF 24 HOUR URINARY OXALATE CONCENTRATION OCER 72 HOURS

oxalate concentration over the 3 day study period shown in table 3.10. CV_{inter} ranged from 4.8 to 68.6 % and the intraindividual CV_{intra} variation shown in table 3.11. ranged from 27.0 to 45.4 %.

2.2.5. Urine Flow Rate.

The diurnal variation of urinary flow rate is shown in figure 3.18. There was a tendancy for urine flow rate to be reduced during the evening and night (7.00 pm to 9.00 am) and increased during the rest of the day. A paired t-test showed a significant fall in the urine flow rate between the 7.00 pm to 9.00 pm sample period and the 9.00 pm to 9.00 am sample period at the 5 % probability level. The mean urine flow rate over the 24 hour period was 58.5 ml/hour ($\pm$ 11.5).

The variation of the daily urine flow rate over the 3 day period is shown in figure 3.19. Using the paired t-test, no statistically significant differences were noted between the days and the mean daily urine flow rate was 54.8 ml/hour ($\pm$ 13.3).

The interindividual CV_{inter} variation of the daily urine flow rate over the 3 day period shown in table 3.12. ranged from 2.3 to 27.0 % and the intraindividual CV_{intra} variation, shown in table 3.13. ranged from 19.7 to 24.9 %.

Table 3.10. Intra individual variation of oxalate concentration over 3 consecutive days.

Subject	Mean concentration umol/l (± S.D.)	Intra individual CV%
1.	241 (± 165)	68.6
2.	139 (± 6.7)	4.8
3.	245 (± 53.9)	22.0
4.	279 (± 113)	40.5
5.	302 (± 55.6)	18.4
6.	259 (± 36.4)	14.0

Table 3.11. Inter individual variation of oxalate concentration for 6 subjects over 3 consecutive days.

Day	Mean concentration umol/l (± S.D.)	Inter individual CV%
1.	245 (± 66.Ø)	27.Ø
2.	289 (± 1Ø6)	36.6
3.	2ØØ (± 9Ø.7)	45.4

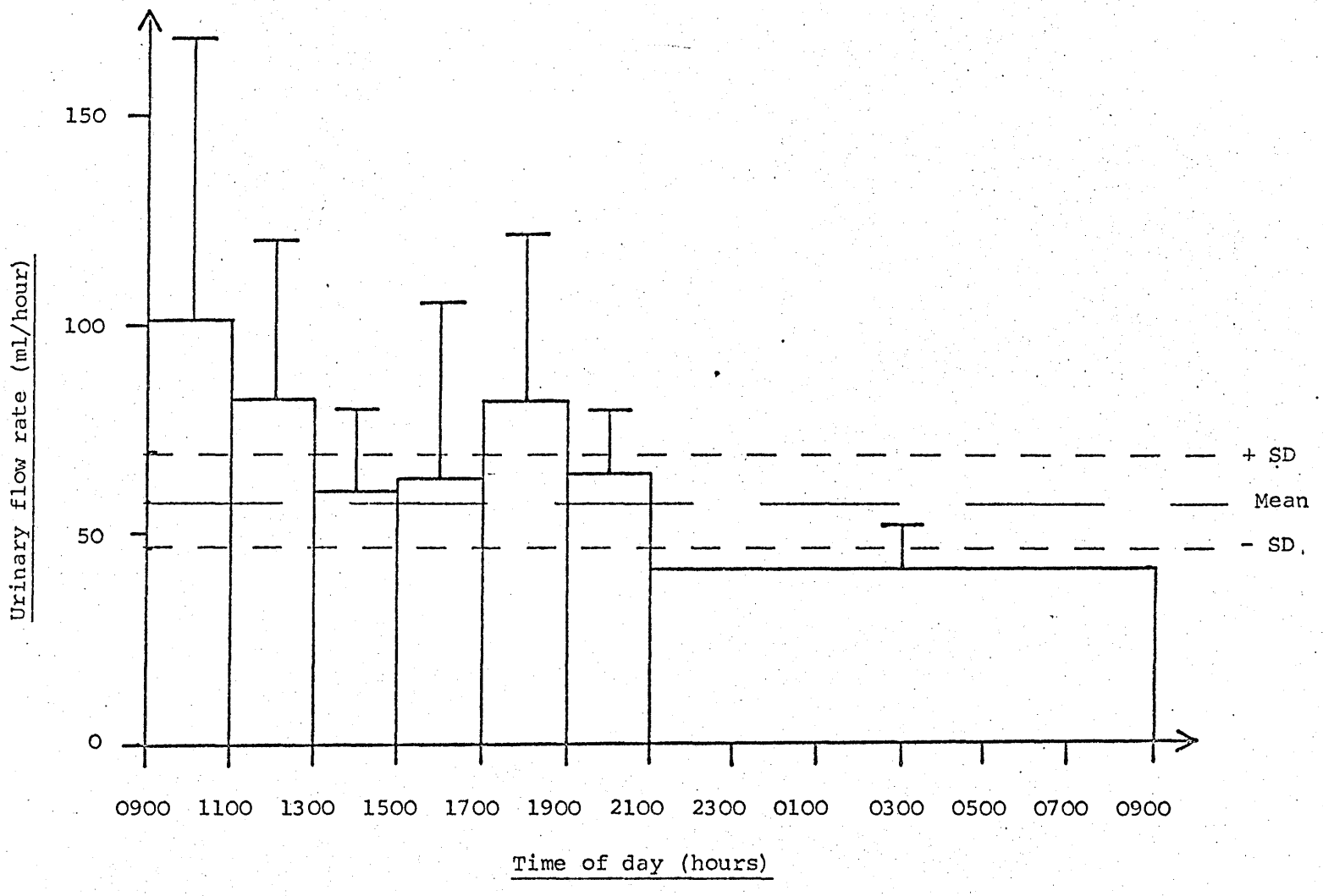


FIGURE 3.18. DIURNAL VARIATION OF URINARY FLOW RATE

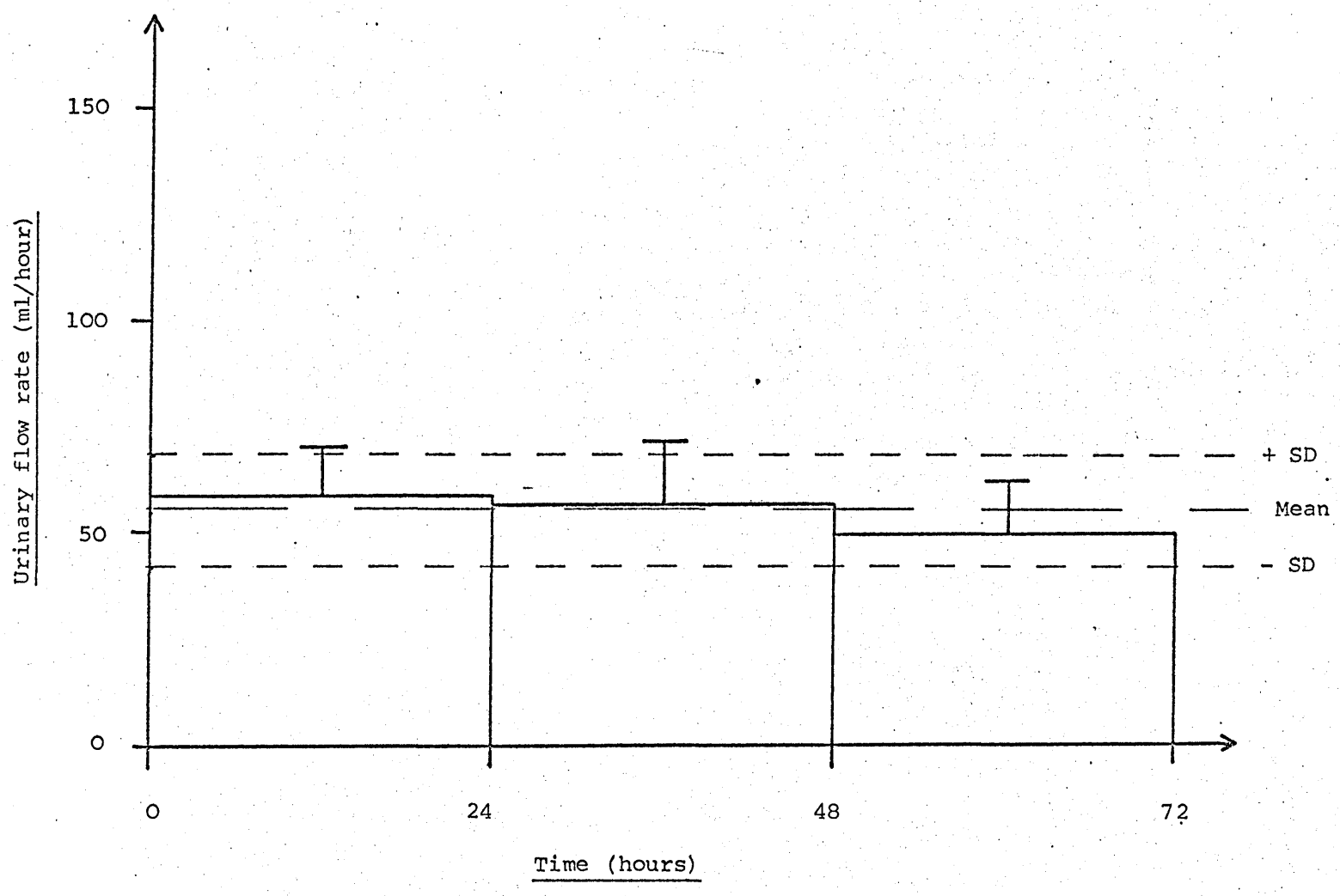


FIGURE 3.19. VARIATION OF 24 HOUR URINARY FLOW RATE OVER 72 HOURS

Table 3.12. Intra individual variation of daily urine flow rate over 3 consecutive days.

Subject	Mean flow rate ml/hour (± S.D.)	Intra individual CV%
1.	57.7 (± 12.7)	22.Ø
2.	74.Ø (± 1.7)	2.3
3.	53.7 (± 4.9)	9.2
4.	46.Ø (± 11.5)	25.Ø
5.	39.7 (± 1Ø.7)	27.Ø
6.	57.7 (± 5.5)	9.6

Table 3.13. Interindividual variation of daily urine flow rate for 6 subjects over 3 consecutive days.

Day	Mean flow rate ml/hour (± S.D.)	Inter individual CV%
1.	58.5 (± 11.5)	19.7
2.	56.5 (± 14.1)	24.9
3.	49.3 (± 12.3)	24.9

2.2.6. Correlation of Results.

Linear regression analysis showed that there was a very highly significant correlation between the diurnal variation of oxalate concentration and the diurnal variation of oxalate excretion as shown in figure 3.20. A correlation coefficient of 0.6950 was found ($n=42$, $p<0.001$).

A similar correlation was found between the mean daily oxalate concentration and the mean daily oxalate excretion rate over the 3 day study period as shown in figure 3.21. A correlation coefficient of 0.7869 was found ($n=18$, $p<0.001$).

A highly significant negative correlation was found between the diurnal urine flow and the diurnal oxalate concentration as shown in figure 3.22. A correlation coefficient of -0.4653 was found ($n=42$, $p<0.01$).

2.3. Discussion.

With the development of the enzymatic procedure for urinary oxalate estimation described in chapter 2.3. oxalate determinations in a large series of samples was possible.

No statistically significant diurnal variation of urinary oxalate excretion or urinary oxalate concentration was found due to the wide range of values

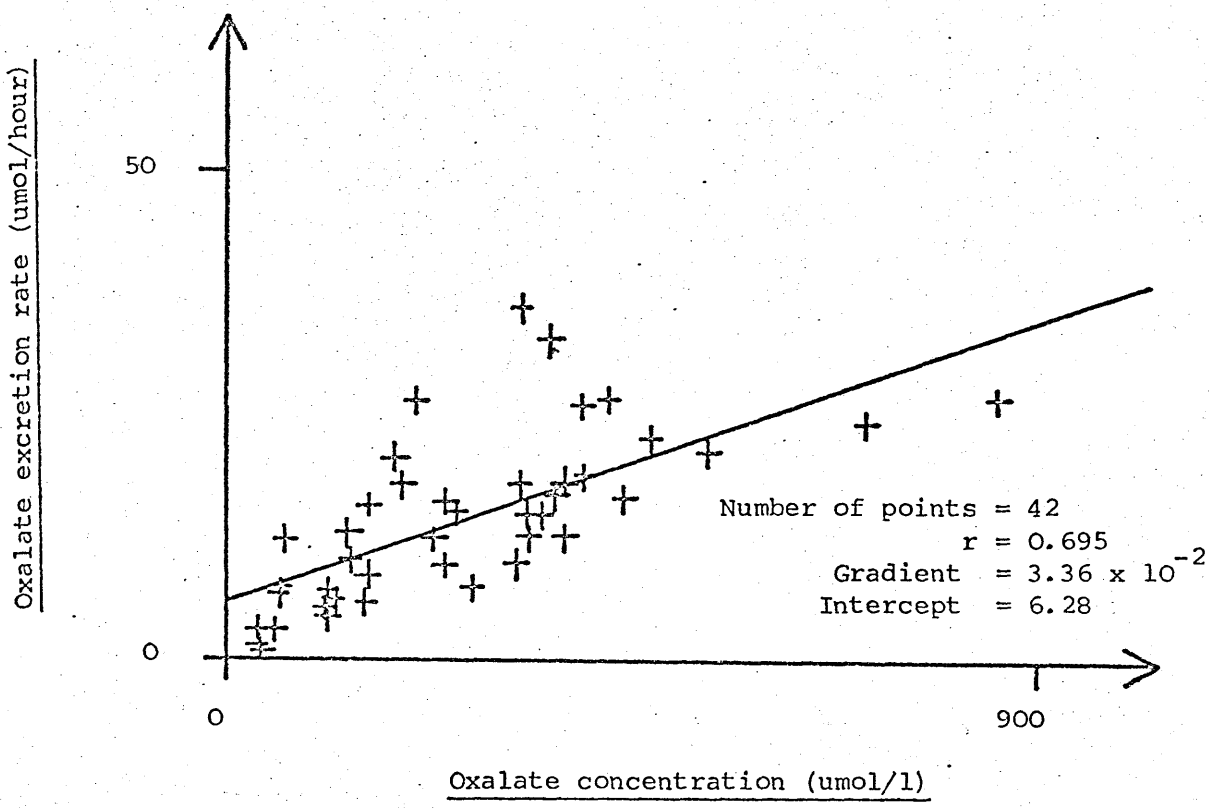


FIGURE 3.20. DIURNAL OXALATE CONCENTRATION VERSUS DIURNAL OXALATE EXCRETION RATE

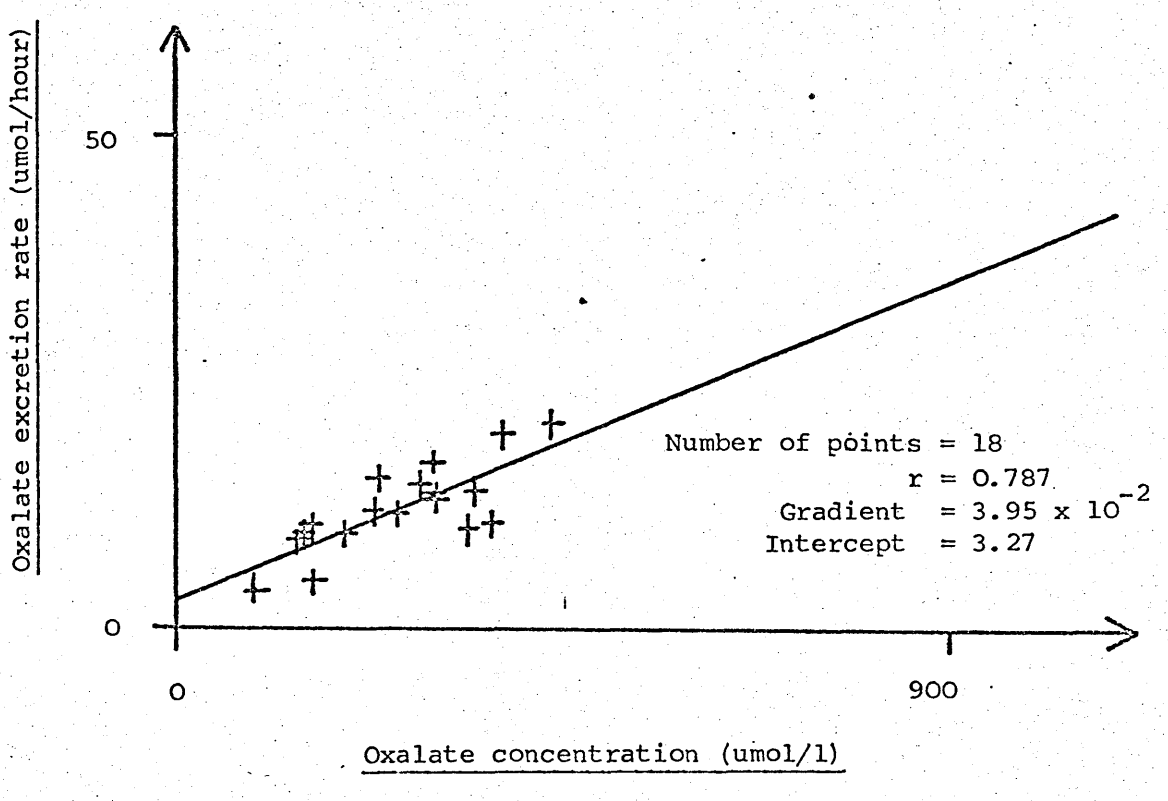


FIGURE 3.21. MEAN DAILY OXALATE CONCENTRATION VERSUS MEAN DAILY OXALATE EXCRETION RATE

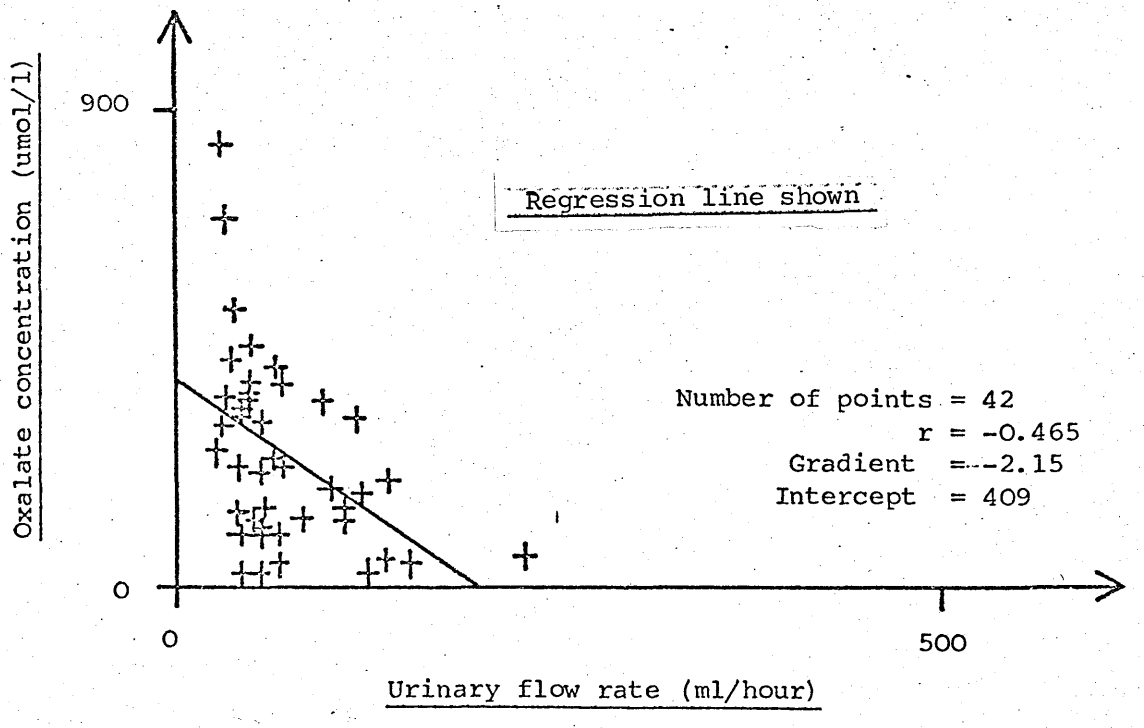


FIGURE 3.22. DIURNAL URINARY FLOW RATE VERSUS DIURNAL OXALATE CONCENTRATION

from individuals for each time period as shown by the large error bars, representing 1 standard deviation, in figures 3.14 and 3.16. However, general diurnal trends in oxalate excretion, concentration and urine flow rate were evident in figures 3.14, 3.16 and 3.18.

As subjects were fasted from 10.00 pm the previous evening until 1.00 pm on the day of the study, the generally low mean oxalate excretion rate observed between 9.00 am and 1.00 pm may represent the basal endogenous excretion of oxalate. The mean excretion rate subsequently increased until 7.00 pm and this may be associated with the dietary intake and absorption of oxalate and oxalate precursors. Although the sampling periods were too long during the night and early morning to provide detailed results, the fall in oxalate excretion rate over this period may be related to reduced dietary intake and absorption.

Increased urinary oxalate excretion associated with dietary intake has been shown in non stone forming subjects, as in this instance, and in stone forming subjects (Tiselius and Almgard 1977, Hargreave et al 1977, Vahlensieck, Bach and Hesse 1982).

Urinary oxalate concentration tended to be lower between 09.00 - 15.00 h and higher between 15.00 - 09.00 h (figure 3.16.) although no statistically significant differences were shown. Conversely, urine flow rate tended to be lower between 21.00 - 09.00 h and higher between 09.00 - 21.00 h (figure 3.18.). The fall in urine flow

rate between the 7.00 pm to 9.00 pm and the 9.00 pm to 9.00 am time periods was significant at the 5 % probability level.

A highly significant negative correlation ($p<0.001$) was found between these two components (figure 3.22.) which was similar to the relationship found by Tiselius and Almgard (1977). This indicated that at periods of low urine flow rate, the urinary oxalate concentration tended to be higher.

A very highly significant positive correlation was observed between the diurnal oxalate concentration and the diurnal oxalate excretion rate ($n=42$, $r=0.6950$, $p<0.001$) as shown in figure 3.20., and between the mean daily oxalate concentration and the mean daily oxalate excretion rate ($n=18$, $r=0.7869$, $p<0.001$) as shown in figure 3.21.

Although the urinary concentration of oxalate was related to the urinary flow rate, these results confirmed the concept of Hargreave et al (1977) in which the variation of oxalate excretion was not related directly to the rate of urine flow as had been suggested by others (Tiselius and Almgard 1977, Vahlensieck et al 1982) and that the urinary oxalate excretion was influenced more by other factors such as dietary intake and absorption. However, outwith these influences, an increase in the total oxalate excretion related to an increase in the urine flow has been shown by Zarembski and Hodgkinson (1969).

Over the 3 day study period there was no

significant difference between the mean daily excretion rate of oxalate (figure 3.15.), the mean daily oxalate concentration (figure 3.17.) or the mean daily urine flow rate (figure 3.19.).

For all 6 subjects, the mean interindividual variations of the mean daily urinary oxalate excretion rate, the mean daily oxalate concentration and the mean daily urine flow rate were 28.8 % $\pm$ 25.8 umol/h 28.1 % $\pm$ 23.1 umol/l and 15.9 % $\pm$ 10.1 ml/h, and the mean intraindividual variations were 30.8 % $\pm$ 12.5 umol/h, 36.3 % $\pm$ 9.2 umol/l and 23.2 % $\pm$ 3.0 ml/h respectively.

In all 3 instances, the value for the interindividual variation was lower than the corresponding value for the intraindividual variation, although the difference was not statistically significant.

These values, in aggreement with those stated by Tocco et al (1979), emphasised the wide variations both within and between subject that were encountered which subsequently made the clinical interpretation and significance of the results possible in only general terms.

CHAPTER 4. CONCLUSIONS.

1. The analysis of urinary oxalate.

The clinical interest in urinary oxalate is increasing, with a number of oxalate kits, based on enzymes, becoming available, and a wide variety of analytical techniques have been applied to the analysis of oxalate.

The analytical procedure developed during the current work has been shown to be precise accurate and, compared to the reference procedure, safe and easy to perform.

Had there been time, further development of the procedure might have led to suitable method for the routine estimation of both serum and urinary oxalate.

The interpretation of urinary oxalate analyses has been shown to be complicated by the large inter and intraindividual variation of oxalate excretion in normal subjects and the possibility of a diurnal variation of oxalate excretion has been confirmed.

2. The effects of therapy in stone formers.

The mechanisms by which allopurinol may have reduced the formation of stones were obscure but may have been related to a reduction in the urinary urate excretion increasing the crystallisation inhibitory properties of

urine, or reducing the initiation of calcium oxalate crystal growth due to epitaxy. The novel observation of an increase in the urinary calcium excretion during allopurinol therapy may be considered to have been an adverse effect as far as stone disease was concerned, but the effect was only temporary.

Bendrofluazide therapy was shown to favourably alter the urinary excretion of both calcium and magnesium, with respect to the risk of forming renal stone, although, in the hypercalciuric group, the urinary excretion of oxalate was increased.

The mode of action of bendrofluazide, particularly the hypocalciuric effect, is still obscure, and its influence on other factors related to the formation of stone was possible.

Although a reduction in the stone recurrence rate during therapy with allopurinol and bendrofluazide has been implied, the period of follow up has been too short to draw any definite conclusions as to the clinical effects of these therapies.

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