THE CIRCADIAN RHYTHM OF SERUM INORGANIC SULFATE LEVELS AND ACETAMINOPHEN CHRONOPHARMACOKINETICS

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To my parents,

ABSTRACT

Conjugation with inorganic sulfate is an important route of metabolism for many xenobiotics and endogenous compounds. The inorganic sulfate concentration in blood is one of several factors that might limit the rate of substrate sulfation *in vivo*. Factors affecting the physiological availability and disposition of inorganic sulfate therefore may be important in sulfoconjugation processes. This study confirmed a small but consistent circadian variation of serum inorganic sulfate levels in healthy men. Acetaminophen, a common analgesic/antipyretic, can cause a gradual depletion of inorganic sulfate in the body when administered in a variety of doses by different routes. The administration of acetaminophen in subchronic doses decreased the serum inorganic sulfate levels but did not abolish the circadian rhythm. Decreased renal clearance of inorganic sulfate at depressed serum sulfate levels (during acetaminophen administration) is consistent with conservation of inorganic sulfate by increased tubular reabsorption. No significant chronopharmacokinetic changes in the sulfation of acetaminophen at steady state between the AM and PM administration periods were noted.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iii

ABSTRACT v

LIST OF FIGURES ix

LIST OF TABLES xi

LIST OF APPENDICES xiii

1.0 INTRODUCTION 1

1.1 Sulfoconjugation 1

1.1.1 Supply of Inorganic Sulfate 1

1.1.2 Sulfate Activation and Transfer 4

1.1.3 Substrates 6

1.1.4 Competition with Glucuronidation 6

1.1.5 Extrahepatic Sulfation 7

1.2 Inorganic Sulfate 9

1.2.1 Analytical Methods 9

1.2.2 Regulation of Inorganic Sulfate Levels *In vivo* 11

1.2.3 Factors Affecting the Serum Sulfate Levels 14

1.2.3.1 Age 14

1.2.3.2 Sex 15

1.2.3.3 Diet 16

1.2.3.4 Pregnancy 19

1.2.4 Species Differences 21

1.2.5 Chronobiological Considerations 22

1.3 Acetaminophen 26
1.3.1 Analytical Methods 27
1.3.2 Absorption 28
1.3.3 Distribution 30
1.3.4 Elimination 31
1.3.5 Chronopharmacokinetics 38
1.4 The Present Investigation 40
2.0 METHODOLOGY 42
2.1 Analysis of Inorganic Sulfate 42
2.1.1 Sample Preparation 42
2.1.1.1 Serum Analysis 42
2.1.1.2 Urine Analysis 43
2.1.2 Construction of Calibration Curves . . . 43
2.1.3 Analytical System 44
2.2 Analysis of Acetaminophen and Metabolites . . . 45
2.2.1 Sample Preparation 45
2.2.1.1 Serum Analysis 45
2.2.1.2 Urine Analysis 46
2.2.2 Construction of Calibration Curves . . . 46
2.2.2.1 Serum 46
2.2.2.2 Urine 47
2.2.3 Analytical System 48
2.2.3.1 Serum 48
2.2.3.2 Urine 49

2.3 Pharmacokinetic Studies 50

2.3.1 The Preliminary Investigation 50

2.3.2 Phase 1: Circadian Rhythm of Inorganic Sulfate 50

2.3.3 Phase 2: Chronopharmacokinetics of Acetaminophen 51

2.4 Data Analysis 53

2.4.1 Inorganic Sulfate Data 53

2.4.2 Acetaminophen Data 54

2.4.3 Statistical Procedures 56

3.0 RESULTS AND DISCUSSION 57

3.1 Analytical Methods for Inorganic Sulfate . . . 57

3.1.1 Serum Samples 57

3.1.2 Urine Samples 62

3.2 Analytical Methods for Acetaminophen and Metabolites 67

3.2.1 Serum Samples 67

3.2.2 Urine Samples 72

3.3 Inorganic Sulfate Disposition: Phase 1 78

3.4 Inorganic Sulfate Disposition: Phase 2 87

3.5 Acetaminophen Disposition: Phase 2 96

4.0 SUMMARY 110

5.0 CONCLUSIONS 113

6.0 LIMITATIONS OF THE INVESTIGATION 114

REFERENCES 116

LIST OF FIGURES

Page

Figure 1: The metabolic conversion of methionine and L-cysteine into inorganic sulfate in man 3

Figure 2: The enzymatic reactions associated with sulfoconjugation (formation of active sulfate and transfer to a substrate) 5

Figure 3: Structure of acetaminophen 26

Figure 4: The metabolism of acetaminophen in humans . . . 33

Figure 5: Representative chromatograms for the analysis of inorganic sulfate in serum and urine. A: Blank, B: Standard (0.10 mM), C: Serum, D: Urine. (1, Inorganic sulfate; 2, System Peak) 59

Figure 6: Representative calibration curve for the analysis of inorganic sulfate in serum and urine 60

Figure 7: Representative chromatograms for the analysis of acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in serum. A: Blank serum, B: Blank serum with internal standard C: Serum standard of acetaminophen glucuronide (1) (8 μg/mL), acetaminophen sulfate (2) (8 μg/mL), acetaminophen (3) (2 μg/mL),and internal standard (4) (20 μg/mL). D: Serum sample 1.0 h following ingestion of 650 mg acetaminophen at steady state 69

Figure 8: Typical calibration curve for the analysis of acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in serum 70

Figure 9: Representative chromatograms for the analysis of acetaminophen, acetaminophen glucuronide and acetamiophen sulfate in urine. A: Blank urine, B: Blank urine with internal standard C: Urine standard of acetaminophen glucuronide (1) (10 μg/mL), acetaminophen sulfate (2) (5 μg/mL), acetaminophen (3) 1 μg/mL), and internal standard (4) (10 μg/mL). D: Urine sample (0-6h) following ingestion of 650 mg acetaminophen at steady state 74

Figure 10: Typical calibration curve for the analysis of acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in urine (The top concentration scale is for AG and AS while the bottom scale is for Acetaminophen) 75

Figure 11: Circadian variation in serum inorganic sulfate levels in healthy male volunteers (Mean ± S.E.M.; Phase 1 n=7 and Phase 2 n=6) 81

Figure 12: Circadian variation in urinary excretion rate of inorganic sulfate in 7 healthy male volunteers (Mean ± S.D.; Phase 1, 24 h control study) . . . 83

Figure 13: Circadian variation in inorganic sulfate renal clearance in 7 healthy male volunteers (Mean ± S.D.; Phase 1, 24 h control study) . . . 84

Figure 14: Effect of multiple dose acetaminophen administration on the mean (± S.D.) 0-12 h urinary excretion rate of inorganic sulfate in healthy male volunteers (Phase 1 n=6 and Phase 2 n=6; * p < 0.05 paired t-test) 91

Figure 15: Effect of multiple dose acetaminophen administration on the mean (± S.D.) 0-12 h inorganic sulfate renal clearance in healthy male volunteers. (Phase 1 n=6 and Phase 2 n=6; * p< 0.05 paired t-test) 92

Figure 16: Serum concentrations of acetaminophen, acetaminophen glucuronide and acetaminophen sulfate following administration of 650 mg acetaminophen at steady state. (Subject DM, semi-log plot) 97

Figure 17: Serum concentrations of acetaminophen , acetaminophen glucuronide and acetaminophen sulfate following administration of 650 mg acetaminophen at steady state. (Mean ± S.E.M., n = 6) 98

LIST OF TABLES

Page

Table 1: Coefficients of variation of peak heights for inorganic sulfate in serum 61

Table 2: Coefficients of variation of peak heights for inorganic sulfate in urine 64

Table 3: Coefficients of variation of peak height ratios for acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in serum 71

Table 4: Coefficients of variation of peak height ratios for acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in urine 76

Table 5: Serum inorganic sulfate levels in healthy men (mean ± S.D.) during Phase 1 (n=7, 24 h control study) 80

Table 6: Renal clearance and excretion rate of inorganic sulfate in healthy men (mean ± S.D.) during Phase 1 (n=7, 24 h control study) 82

Table 7: Serum inorganic sulfate levels (mean ± S.D.) in healthy men during Phase 1 and Phase 2 of the study 89

Table 8: Inorganic sulfate urinary excretion rates (mean ± S.D.) in healthy men during Phase 1 and Phase 2 of the study 90

Table 9: Pharmacokinetic characteristics of acetaminophen following multiple dose administration of 650 mg to six subjects; Comparison for two consecutive dosing intervals (mean ± S.D.) 100

Table 10: Pharmacokinetic characteristics of acetaminophen glucuronide and acetaminophen sulfate following multiple dose administration of 650 mg to six subjects; comparison for two consecutive dosing intervals (mean ± S.D.) . 101

Table 11: Urinary recovery of acetaminophen and metabolites following multiple dose administration of 650 mg acetaminophen to six subjects. Mean (± S.D.) of recoveries expressed as percent of recovered dose for two consecutive dosing intervals 103

LIST OF APPENDICES

		Page
Appendix I	Apparatus and Equipment; Materials and Supplies	129
Appendix II	Volunteer Consent Forms	134
Appendix III	Volunteer Information Sheets	138
Appendix IV	Study Protocol	141
Appendix V	Glossary of Chronobiological Terminology .	144
Appendix VI	Standard Meal Composition	147
Appendix VII	Data: Serum Inorganic Sulfate Levels for Individual Subjects	150

1.0 INTRODUCTION

1.1 Sulfoconjugation

The existence of metabolically formed sulfate esters was first documented in 1876 when Baumann detected phenyl sulfate in urine collected from horses given phenol. Sulfoconjugation, credited with being the first discovered conjugation process (Brzeznicka et al., 1987), is a major pathway for the biotransformation of many xenobiotics in man and other animal species. Sulfoconjugation is an important feature of in vivo chemical defence. A number of factors are involved in the regulation of in vivo sulfation but the relative importance of each of these factors is not known. In man there has been relatively little investigation of the pharmacokinetics of sulfation in vivo. A complete description of sulfation pharmacokinetics requires knowledge of the pharmacokinetics of the substrate, its metabolites and the availability of inorganic sulfate.

1.1.1 Supply of Inorganic Sulfate

For many years it was thought that the sulfate ion was absorbed slowly and incompletely from the gastrointestinal tract. Krijgsheld et al.'s (1979) investigation of sulfate absorption in the rat provided the first data on oral absorption of sulfate in this species. Their data suggested a rapid and almost complete

absorption of orally administered sulfate in the rat. In a later report Cocchetto and Levy (1981) demonstrated that a large oral dose administered to humans in divided portions over three hours was well tolerated and absorbed to a significant extent. This evidence refuted the common view that sulfate was poorly absorbed -- a factor felt responsible for its laxative action.

Although dietary inorganic sulfate can be absorbed, it supplies only a small fraction of the intracellular pool (Bidlack et al., 1986). The oxidation and desulfuration of cysteine supplies the majority of cellular sulfate, the main source being L-cysteine in food (Mulder, 1984). L-cysteine is converted in vivo to many metabolites one of which is inorganic sulfate. D-cysteine, the less important isomer, is not used for anabolic functions and this compound has been used as a sulfate source to selectively enhance the availability of sulfate in the rat (Glazenburg et al., 1984).

In vivo, the first step in the production of sulfate from L-cysteine is enzymatic oxidation of the sulfhydryl group followed by enzymatic transamination (Figure 1). The sulfite product is converted by sulfite oxidase to oxidized sulfate. The reaction is usually rapid. Methionine can be utilized in the L-cysteine cascade after enzymatic conversion to cysteine.

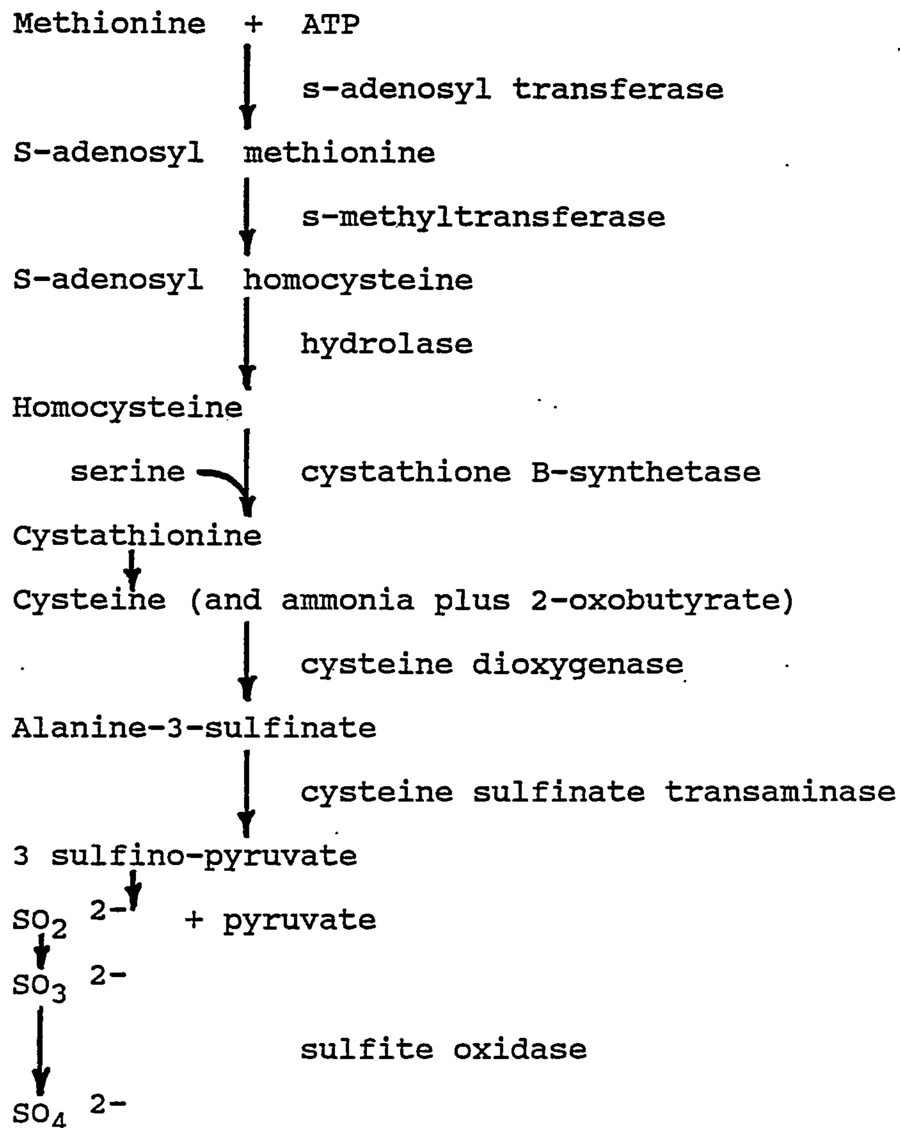

FIGURE 1: The metabolic conversion of methionine and L-cysteine to inorganic sulfate in man.

1.1.2 Sulfate Activation and Transfer

Inorganic sulfate is not directly transferred to the acceptor substrate, but must first be activated. The large number of metabolically sulfated substances led researchers to propose there was a common metabolic carrier for activated sulfate (Lipmann 1958). The identity of the cosubstrate for sulfation, adenosine-3'-phosphate-5'-sulfatophosphate (PAPS), and its role in this conjugation process was elucidated in the late 1950's (Lipmann, 1958). The unexpected finding of two separately linked phosphates indicated PAPS was likely synthesized as a two step reaction (Lipmann 1958). PAPS is synthesized in a two step enzyme catalyzed reaction from inorganic sulfate and ATP (Brzeznicka et al., 1987). The two enzymes involved are: ATP-sulfurylase (sulfate adenyltransferase; EC 2.7.7.4) and adenosine-5'-phosphosulfate [APS] -kinase (adenylsulfate kinase; EC 2.7.1.25). APS, the product of the first enzymatic reaction is entirely inactive as a sulfate donor (Lipmann, 1958). The formation of PAPS, the active sulfate donor is primarily regulated by the availability of the starting components, ATP and inorganic sulfate. Transfer of the sulfate from PAPS to the acceptor substrate (be it endogenous or exogenous) is catalyzed by one or more of the ubiquitous (Brzeznicka et al., 1987), cytosolic (Bock, 1977) sulfotransferases.

There is a distinct lack of data in the literature regarding PAPS levels in tissues. In a recent report Brzeznicka et al. (1987) examined the PAPS concentration in tissues of

various laboratory animals and found the liver had the highest PAPS level in all species examined. Both species and tissue differences were described but no sex differences within a species were noted for the PAPS concentrations. Correlation of serum sulfate levels and hepatic PAPS concentrations (Hjelle et al., 1985) supports the hypothesis PAPS biosynthesis may be dependent on the inorganic sulfate available from the serum. Figure 2 summarizes the reactions associated with sulfoconjugation.

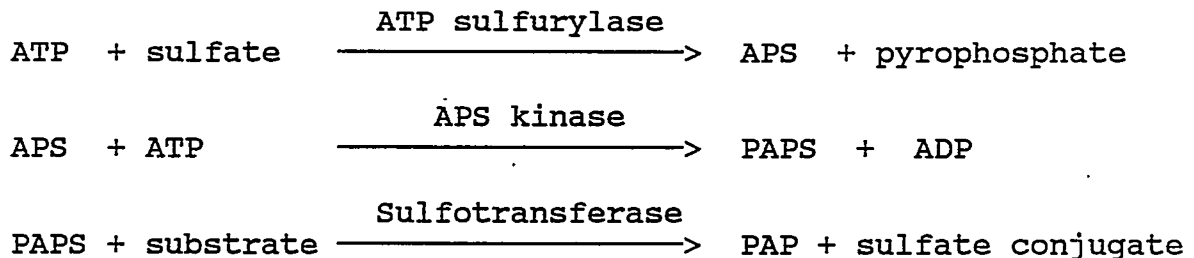

Figure 2: The enzymatic reactions associated with sulfoconjugation (formation of active sulfate and transfer to a substrate).

Three factors are considered to influence the sulfoconjugation process -- inorganic sulfate supply, PAPS concentrations and sulfotransferase activity. The relative importance of these factors is still the subject of debate.

1.1.3 Substrates

Many endogenous compounds (e.g., norepinephrine) are substrates for sulfoconjugation as part of the normal physiologic processes _in vivo_. Additionally, many exogenous compounds are metabolized to sulfoconjugates as part of the body's detoxification mechanisms.

The hydroxyl group in the molecular structure is the most common functional group involved in sulfate conjugation. This group can be a phenolic, alcoholic, hydroxylamine or hydroxamic acid hydroxyl group (Mulder 1984). Compounds which contain one or more hydroxyl groups are particularly good substrates for sulfoconjugation (Dawson _et al_., 1983). The hydroxyl group may be introduced into the molecule by a phase I metabolic pathway.

Other functional groups can also undergo sulfoconjugation. For example, amines can form sulfamates and thiols can form thiosulfates -- a rare conjugate (Mulder 1984).

1.1.4 Competition with Glucuronidation

Many drugs are metabolized simultaneously by more than one pathway. Sulfation and glucuronidation are two major pathways of xenobiotic metabolism that compete for many substrates. Glucuronidation and sulfation are the major conjugation reactions in xenobiotic metabolism with

glucuronidation usually the more dominant of the two (Bock, 1977).

The transferases for glucuronidation and sulfation are present in almost all mammalian cells (Bock, 1987) and often compete for the same substrate (Mulder et al., 1984a). Sulfation frequently predominates at low substrate doses, but glucuronidation at higher substrate doses. (Koster and Mulder, 1982). Several explanations have been offered to account for this phenomenon. These include depletion of inorganic sulfate at higher substrate doses (Koster et al., 1981), differences in the affinity of the substrate for the two pathways (Mulder et al., 1984a), nonhomogeneous distribution of the transferases in the liver (Morris and Pang, 1987) and the limited availability of the cosubstrate for sulfation (Bock, 1977 ; Koster et al., 1981). Sulfation has been termed a high affinity, low capacity route; glucuronidation a low affinity, high capacity route.

1.1.5 Extrahepatic Sulfation

The liver is not the only site where conjugation reactions can occur (Cassidy and Houston, 1980). However, there is relatively little data available on extrahepatic sulfoconjugation of xenobiotics. Studies in rats and dogs indicate the kidney (Chen and Schwartz, 1985; Dawson et al.,

1983; Tremaine et al., 1984; Wong and Yeo, 1982), lung (Cassidy and Houston, 1980; Mistry and Houston, 1985; Wong and Yeo, 1982) and the small intestine (Cassidy and Houston, 1980; Chen and Schwartz, 1985; Dawson et al., 1983; Mistry and Houston, 1985; Wong and Yeo, 1982) are capable of sulfoconjugation.

Numerous exogenous compounds are substrates for sulfoconjugation (Dawson et al., 1983) and in general the vast majority of the sulfates are toxicologically inert (Mulder and Meerman, 1983). Because the liver in general is the organ with the highest capacity for sulfate metabolism (Chen and Schwartz, 1985), the contribution of extrahepatic sites to xenobiotic catabolism is often neglected and underestimated. The importance of extrahepatic sulfation however should not be overlooked. Wong and Yeo (1982) have proposed that extrahepatic sites, in both man and other animals, may provide a sink into which endogenous and exogenous compounds alike can be distributed and disposed of by sulfoconjugation. The changes observed in the sulfation pattern (as is often seen by change in the sulfate/glucuronide metabolite ratio) may in part be due to changes in extrahepatic metabolism (Mulder et al., 1984b). Very few studies have examined the importance of extrahepatic clearance to the overall *in vivo* clearance of substrates undergoing sulfation.

1.2 Inorganic Sulfate

1.2.1 Analytical Methods

A number of methods have been developed to determine the concentration of inorganic sulfate in biological fluids -- mainly serum and urine. The number of methods attest to the inherent difficulties in quantitating this anion (Tallgren, 1980). Large amounts of sulfate can be determined quite reliably with several methods but the assay of lower concentrations is more difficult. This is a fundamental problem when attempting to develop procedures for use with human biological fluids because sulfate levels in man are among the lowest levels in any of the species examined (Krijgsheld et al., 1980).

Some of the techniques used in the past include gravimetry (Loeb and Benedict, 1927), colorimetry (Hakkinen and Hakkinen, 1959) , flame (Nachtrieb, 1950) and atomic absorption (Michalk and Manz, 1980) spectrophotometry and radiometric procedures (Miller et al., 1961). Turbidimetric procedures based on the measurement of precipitated barium sulfate by ultraviolet spectrophotometry have been most commonly used. The original turbidimetric method of Berglund and Sorbo (1960) has been modified by Krijgsheld et al. (1979), Lundquist et al. (1980) and Pascoe et al. (1984).

In more recent years, liquid chromatography has been used in the determination of inorganic sulfate. Reversed-phase high performance liquid chromatography with ultraviolet detection

has been used to measure serum sulfate levels (Koopman et al., 1985). However, a more suitable and novel method for the determination of anions (including sulfate) is ion chromatography with electrical conductivity detection. Ion chromatography, a technique based on the principles of ion exchange, was first described by Small et al. (1975). The original procedure involved the use of two columns -- the first column to separate the ions to be analyzed and the second column, the suppressor column, to remove the background elements in the eluent (Anderson 1976; Lee 1984). The sample ions are then measured-- usually by electrical conductivity. Now, single column or nonsuppressed ion chromatography (avoiding the use of the suppressor column by using low capacity resins for the exchange column and eluents of low conductivity) has been introduced and is now a widely used technique (Morris,1984; Schmuckler et al., 1986). Whether the dual column or single column methodology is employed in the analysis of inorganic sulfate in biological fluids, ion chromatography offers certain advantages. The methodology is more accurate than previous assays (Cole and Scriver, 1981), as well as fast, simple (Lee, 1984) and sensitive. Concentrations otherwise undetectable can be measured (Cole and Landry, 1985), and smaller sample volumes usually suffice (de Jong and Burggraaf, 1983). Because of these advantages, ion chromatography is becoming a routine method for measuring inorganic sulfate in a variety of biological fluids (Reiter et al., 1987).

1.2.2 Regulation of Inorganic Sulfate Levels In vivo

The main source of free inorganic sulfate in body fluids is the catabolism of sulfur containing amino acids (mainly cysteine and methionine). An adequate supply of these amino acids is primarily responsible for one component of sulfate homeostasis even though small fractions of inorganic sulfate in the body are derived from gut absorption and hydrolysis of sulfoesters. In man, the total amount of freely available noncompartmentalized sulfate is calculated to vary from 1.5 to 7 mmoles (Tallgren, 1980). Free sulfate can be well absorbed by rats (Krijgsheld et al., 1979) and man (Morris and Levy, 1983).

Walser et al. (1953) first used ^{35}S labelled inorganic sulfate to estimate the volume of extracellular fluid. The distribution space of sulfate, which is between 20 and 30 % of the body volume for several species (Mulder, 1984), approximates the volume of extracellular fluid. This suggests that sulfate is capable of passing through the capillary membranes with ease but can also be effectively excluded from crossing the cell membrane (Walser et al., 1953). However, studies in rats have indicated (Mulder and Scholtens, 1978) that the inorganic sulfate in blood (serum) is available for sulfation that occurs within liver cells. These researchers indicate their data from rats shows the pool of PAPS in the liver is very small and that perhaps it is synthesized only when it is needed. Based on their data, Mulder and Scholtens (1978) conclude the sulfate concentration in serum

is a good reflection of the amount of sulfate immediately available for conjugation.

The uptake of inorganic sulfate has been studied in a few types of cells and the uptake is believed to be catalyzed by an anion carrier that also transports chloride (Mulder, 1984). With rat hepatocytes the intracellular concentration of sulfate is approximately equal to the concentration outside the cell (Mulder, 1984). In 1984, Morris and coworkers reported lower levels of sulfate in the cerebrospinal fluid (CSF) than the plasma of rats. The authors suggest that sulfate is transferred by a specialized and possibly saturable process from the plasma to the CSF. The initial studies with rats were conducted to examine the role sulfoconjugation may have in regulating the levels of neurotransmitters. Dosing the animals with acetaminophen decreased the sulfate levels in both serum and the CSF. In subsequent experiments in man (Morris et al., 1986), CSF levels were lower than serum levels, but, unlike the data obtained for rats, dosing with acetaminophen did not cause a change in the CSF sulfate levels. A variety of explanations were suggested for this variation, the most interesting being the possibility of a proximal-distal concentration gradient for inorganic sulfate in the CSF of man.

Serum sulfate levels are thought to reflect the overall equilibration between intake, distribution to tissue pools and renal excretion of in vivo inorganic sulfate regulation (Cole and Scriver, 1980). Serum inorganic sulfate

levels are usually between 0.24 and 0.42 mmol/L in healthy humans. Inorganic sulfate is eliminated from the body largely by renal excretion (Lin and Levy, 1982; Cole and Scriver, 1980). The kidneys serve as a main regulator of serum sulfate levels (Mulder, 1984). Sulfate is freely filtered by the glomerulus and is actively reabsorbed in the proximal tubule of the kidney (Cole et al., 1986) probably with the co-transport of sodium (Mulder, 1984). The reabsorbed fraction varies with nutritional state and age of the individual.

Approximately 60 to 80 % of the sulfate load filtered by the kidneys represents the excess production of sulfate from the metabolism of cysteine and methionine (Cole et al., 1986). This excess sulfate is the amount which exceeds the immediate body requirements (Mulder, 1984). Under normal physiological conditions, the renal clearance of sulfate in various species, including man, is about 10 - 35 % of the GFR (Lin and Levy, 1983). Although some studies have reported the presence of a tubular secretory process for sulfate in mammals, the clearance pattern is consistent with glomerular filtration and partial tubular reabsorption by a capacity limited process (Lin and Levy, 1983; Morris and Levy, 1983). With reduced renal function, the ability to excrete sulfate is compromised resulting in retention of sulfate and an increase in serum sulfate levels (Cole et al., 1986; Lin and Levy, 1983; Pillion et al., 1984; Tallgren, 1980). As renal function declines, serum sulfate levels rise at a similar rate (Cole and Scriver, 1980). The elevated levels of

sulfate presumably represent the retention of larger amounts of free sulfate in the body. Serum levels of creatinine and sulfate are positively correlated in both rats (Lin and Levy, 1982) and humans (Lin and Levy, 1983).

Goudsmit and Keith (1962) note that in 1921 Denis reported the accumulation of sulfate in human serum in advanced stages of renal disease. These same authors report that in many cases of early renal dysfunction, inorganic sulfate can be the first substance to accumulate in the blood. Sulfate can account for a considerable fraction of the anions (anion-gap) that accumulate in end-stage renal disease (Cole and Scriver, 1980; Cole et al. 1986). In humans with end-stage renal disease, inorganic sulfate can be efficiently removed by hemodialysis or continuous ambulatory peritoneal dialysis (Cole et al., 1986). Cole et al., (1986) speculate serum sulfate levels of up to 10 mmol/L may not have acute adverse effects, but long term effects of excessively high levels are unlikely to be benign.

1.2.3 Factors Affecting the Serum Sulfate Levels

1.2.3.1 Age

In man, age has been identified as a factor influencing serum levels of inorganic sulfate. In 1978 Meier and Schmidt-Kessen reported that fasting blood inorganic sulfate levels were

approximately 50 % higher in adults aged 55 - 74 years (n=243) than in adults aged 21-28 years (n = 89). Based on mean serum sulfate levels Tallgren (1980) grouped adults (149 females, 70 males, aged 15 - 93 years) in four categories: 15-24 years (0.22 ± 0.08 mM); 25-44 years (0.26 ± 0.09 mM); 45-74 years (0.30 ± 0.09 mM); and 75-93 years (0.34 ± 0.09 mM). Tallgren also analyzed males and females separately and found men reach an "old age" level at about 45 years and women reach similar levels much later, at about 75 years.

Serum sulfate levels are reported to be higher in infants (Cole and Scriver, 1980) than older children and adults. In a study of 46 infants and children under the age of three years, the serum sulfate levels were 0.47 mM in newborns and 0.33 mM in three year olds. Beyond three years of age, levels remain relatively constant in the child, adolescent and young adult (Cole and Scriver, 1980). Pascoe et al. (1984), Pillion et al. (1984) and Tallgren (1980) have reported results similar to those of Cole and Scriver (1980).

1.2.3.2 Sex

In humans, numerous researchers have reported there is no indication of a sex difference in the concentration of serum inorganic sulfate (Cole and Scriver, 1980; Krijgsheld et al., 1980; Pillion et al., 1984; Reiter et al., 1987). The lack of a

difference in sulfate levels between males and females would appear to apply to all age groups examined (Cole and Scriver, 1980) with the exception of levels in later life, when males reach "old age" levels prior to females (Tallgren 1980).

Although there does not appear to be any significant difference in serum inorganic sulfate levels between males and females, males excrete approximately 60 % more sulfate per day than females (Mulder 1984).

1.2.3.3 Diet

Nutritional effects on xenobiotic metabolism in man are hard to evaluate since most investigations have followed changes in metabolism only after short term dietary modification (Bidlack and Smith, 1984; Bidlack et al., 1986). Investigations of the interaction between nutritional factors and drug biotransformation have primarily been concerned with the effect of the diet on oxidative metabolism. Substantially less information is available on the effect of diet on conjugation metabolism, including sulfoconjugation (Mulder et al., 1982). Relatively little is known about the influence a diminished supply of sulfur containing amino acids may have on the concentrations of essential cofactors (Glazenburg et al., 1983). In addition, the role of free inorganic sulfate in human nutrition has not been studied (Greer et al., 1986). In light of

these considerations, it is not surprising that in a recent review of the effects of nutritive factors on xenobiotic metabolism there was virtually no mention of sulfoconjugation (Guengerich, 1984).

Diet may affect both cofactor availability and transferase activity (Mulder et al., 1982). In vitro, sulfotransferase activity in protein deficient rats is normal (Woodcock and Wood, 1971). Other researchers (Mulder and Krijgsheld, 1986) have reported starvation decreases the activity of hepatic ATP sulfurylase in the rat. However, the activity of this enzyme is not a rate limiting step and may therefore have no effect on in vivo sulfation. In contrast with findings of Mulder et al., (1982), Mulder and Krijgsheld (1986) and Krijgsheld et al.,(1982) report serum sulfate levels decrease when rats are fed a low protein diet.

Sulfur amino acid metabolism is one of several factors contributing to inorganic sulfate homeostasis (Tallgren 1980). The inorganic sulfate pool can be depleted when food is deficient in sulfate and sulfur containing amino acids (Krijgsheld et al., 1982). Decreased availability of cysteine (or methionine) limits the supply of sulfate for PAPS synthesis (Bidlack et al., 1986). Methionine can be rapidly converted to cysteine (Figure 1) and it is cysteine which ultimately plays a role in xenobiotic metabolism as a precursor for sulfate which in turn is required for cofactor synthesis (Glazenburg et al., 1983).

In an early study, Sabry and coworkers (1965) were able

to demonstrate a strong correlation between the urinary excretion of inorganic sulfate and the dietary intake of methionine and cysteine in humans. Eight diets, with protein content providing between 0.8 and 4.0 grams of methionine and cysteine per day, had been tested. The dietary intake of methionine plus cysteine closely correlated (r=0.98) with urinary sulfate excretion. Unfortunately the serum sulfate levels were not monitored. Very few studies of dietary influence on sulfate status have measured both the urinary excretion and serum levels of inorganic sulfate. Tallgren (1980) reports data for one male subject in which the sulfate levels in fasting morning samples were not significantly different when protein intake was varied from 20 g daily for 10 days to 120 g daily for 10 days.

If extended over a period of several days, fasting is the most drastic nutritional change (Mulder _et al_., 1982). However, data on the _in vivo_ sulfoconjugation of drugs during the fasting state has only rarely been reported (Mulder and Krijgsheld, 1986). What data is available is conflicting. Mulder _et al._ (1982) report rats fasted for 72 hours did not have a reduced serum sulfate level. They felt this was due to the resultant catabolic state and sulfate retention by the kidneys. When the fasted rats were given harmol intravenously the sulfate/glucuronide ratio in urine was the same as in control animals but the total rate of conjugation had decreased. Mulder _et al_., (1982) concluded the sulfate and glucuronide conjugating pathways were less easily disrupted than _in vitro_ data would

indicate. In man, some variation in serum sulfate levels within a given day may be due to dietary protein intake (Cole and Scriver, 1980). In adults who fasted overnight, sulfate levels were 0.29 ± 0.03 mM but random daytime values were significantly higher (0.33 ± 0.06 mM) and more variable. After the ingestion of a large amount of protein or sulfur containing amino acids, the serum sulfate level probably reaches a maximum in 6 to 8 hours post-ingestion (Tallgren, 1980).

In 20 children with kwashiorkor (severe malnutrition) urinary excretion of sulfate was found to be about 33 % of the normal value (Ittyerah, 1969). The decreased urinary excretion appeared to be due primarily to the low dietary protein intake and hence a reduced sulfur amino acid intake. When the malnourished state was corrected the urinary excretion of sulfate increased and was comparable to values for normal children. Urinary excretion of sulfate in adults correlates with protein intake. However,in very low birth weight (VLBW) premature infants urinary excretion of inorganic sulfate correlates with methionine intake but not total protein intake (Greer *et al.*,1986).

1.2.3.4 Pregnancy

The physiological process of sulfation is important for many biosynthetic and detoxification functions. In the

developing fetus, sulfation is an important step in the synthesis of glycosaminoglycans and cerebroside sulfate (Morris and Levy, 1983). Cole et al. (1985) suggest the changes in sulfate disposition are a natural physiological process designed to enhance the availability of sulfate to the developing fetus and placenta. Nonrenal mechanisms, including secondary active transport of sulfate by the placenta, may be involved in sulfate regulation during pregnancy (Cole et al., 1985).

The plasma/serum concentrations of most electrolytes tend to decrease slightly during pregnancy but this is not the case with inorganic sulfate (Morris and Levy, 1983). Goudsmit and Keith (1962) referred to a study conducted in 1932 by Anderson and Tompsett which reported the inorganic sulfate levels to be normal in cases of uncomplicated pregnancy. However, more recent reports disagree. Serum sulfate levels in females in the third trimester of pregnancy are higher than in age matched controls (0.592 ± 0.275 mM, n=118 vs. 0.263 ± 0.091 mM, n=42) (Tallgren, 1980). Levels during the third trimester (Cole et al., 1985; Morris and Levy, 1983; Pascoe et al., 1984) are higher than at the time of delivery (Cole et al., 1984). There is some evidence that levels in pregnant women increase throughout gestation resulting in a 39 % higher value by the middle of the third trimester (Cole et al., 1985). The fetal to maternal concentration gradient for serum sulfate is 6.6.

1.2.4 Species Differences

Sulfoconjugation occurs in every species examined thus far, including mammals, fish, amphibia, birds, reptiles and invertebrates (Mulder 1984). There is a wide variation between different species in serum inorganic sulfate levels (from 0.3 mM in mammals to 2.5 mM in birds) (Krijgsheld et al., 1980). At the lower limit are man and monkey with average levels of 0.27 mM and 0.39 mM respectively; then the rat with levels of about 0.8 mM and the highest levels in the goat (2.26 mM) and rooster (2.41 mM). Within a given species the reported values are relatively consistent and levels in different rat strains are similar. Lin and Levy (1983) have speculated that the two most likely reasons for the observed species differences are variations in the sulfate formation rate (related to dietary intake) and the renal clearance of inorganic sulfate. Since the inorganic sulfate distribution space is between 20 and 30 % of the body volume in all species (approximating distribution in the extracellular fluid) (Mulder, 1984) and inorganic sulfate is not appreciably protein bound, variation in volume of distribution is not likely to account for the observed species differences (Lin and Levy, 1983).

The renal clearance of sulfate normalized to body weight is similar in man, rats, rabbits and dogs under normal physiological conditions (Lin and Levy, 1983). This data suggests the lower serum sulfate levels found in man may be due

to a lower intake of dietary precursors.

Glucuronidation is the main conjugation pathway "competing" with sulfation. In some species sulfation is the predominant conjugation pathway and in others it is glucuronidation. For example, cats are almost exclusive sulfators whereas the pig and rabbit principally conjugate xenobiotics with glucuronic acid but have some capacity for sulfation. There seems to be a slight preference for sulfoconjugation in man; but, glucuronidation can be as important for some drugs (Mulder, 1984). Although the serum inorganic sulfate levels may contribute to the species differences in sulfoconjugating capacity other factors such as variable enzyme kinetics and even deficiencies in specific sulfotransferases also play an important role.

1.2.5 Chronobiological Considerations

Chronobiology, defined as "that branch of science which objectively quantifies and explores mechanisms of biological time structure including the important rhythmic manifestations of life", originated around 1950 primarily through the studies of Franz Halberg (Scheving et al., 1974). Almost every physiological or behavioral variable which can be measured is subject to some rhythmicity in the healthy organism (Scheving et al., 1974). All levels of biological organization, from the

whole organism to the subcellular level in all living beings (from nucleated unicellulars to man), demonstrate rhythms (Reinberg and Smolensky, 1982). Studies on the rhythms demonstrated by human body functions have revealed rhythms with cycle frequencies of about 24 h (circadian), seven days and one month (Halberg et al., 1980). (A glossary of chronobiological terms is included as Appendix V). Ritschel (1984) has identified that more than 100 physiological and psychological parameters are controlled or influenced by daily rhythms.

Although rhythms can have a multitude of frequencies and occur at different levels of organization in the living system, biological rhythms demonstrate some basic characteristics. The four basic characteristics have been identified as (1) a genetic origin; (2) persistence in the absence of time clue or cue; (3) species specificity but with interindividual variability; and (4) an influence of certain environmental factors termed synchronizers (Reinberg and Smolensky, 1982).

Of the many different types of rhythms, circadian rhythms have received the most attention in the literature. In humans, many body functions such as cardiac output, pH and rate of secretion of various body fluids, renal and liver blood flow rates exhibit circadian rhythms. Relatively little attention has been paid to the influence these rhythms may have on the disposition of xenobiotics. Although the available information is limited, over the course of about the last twenty years the

influence of rhythms on the disposition of many xenobiotics has resulted in the formation of a new area of study termed chronopharmacokinetics. Chronopharmacokinetics is concerned with the changes of plasma concentration - time profiles and pharmacokinetic parameters of xenobiotics as administration times are varied (usually during a 24 h period) (Ritschel, 1984; Reinberg and Smolensky, 1982). Usually, rhythmic changes in the bioavailability, metabolism and excretion pattern are studied by administering a single dose at four different times separated by appropriate washout periods. The chronopharmacokinetics associated with multiple dose administration have only rarely been investigated.

The chronopharmacokinetics of several xenobiotics have been evaluated in man. These include analgesics, non-steroidal anti-inflammatory agents, theophylline, digitalis, propranolol, antibacterials, anticancer agents and antipsychotics. Reinberg and Smolensky's (1982) first review on circadian changes of xenobiotic disposition in man has been followed by others (Cherrah et al., 1985; Ritschel, 1984).

Absorption, distribution, metabolism and excretion of xenobiotics can exhibit circadian variation. Ritschel (1984) has identified elimination via the kidney as the most important single disposition process to be influenced by circadian rhythms. A number of parameters related to kidney function, such as renal plasma flow, glomerular filtration rate and urinary pH, exhibit pronounced circadian rhythms. The apparent volume of

distribution is also influenced by many factors which show a circadian rhythm - the plasma volume, blood flow rate, and pH. In addition, variation in plasma protein concentration of alpha-1-acid glycoprotein during a 24 h period (Yost and DeVane, 1985) may influence the distribution volume of certain drugs.

In man, a variety of experiments have identified circadian rhythms associated with select inorganic ions. The urinary excretion patterns of potassium, sodium and calcium ions demonstrate oscillatory properties (Wever, 1975). Circadian variation in the composition of whole saliva is demonstrated most consistently for sodium, chloride and phosphate ions (Ferguson and Botchway, 1980). Neither of these studies examined the possibility of circadian variation in inorganic sulfate levels.

In 1978 Meier and Schmidt-Kessen demonstrated a circadian rhythm of serum inorganic sulfate levels in man. Levels were at a minimum before noon and a maximum in the afternoon and evening. In 1980 Krijgsheld and colleagues identified a circadian rhythm of serum inorganic sulfate levels in the rat. Levels were highest during the rest cycle and stayed relatively low during the activity cycle.

Since inorganic sulfate is of fundamental importance in metabolic sulfoconjugation, inorganic sulfate circadian variation must not be overlooked as a variable which may influence the chronopharmacokinetic profile of xenobiotics metabolized by this route.

1.3 Acetaminophen

Acetaminophen (paracetamol, N-acetyl-para-aminophenol, 4-hydroxy-acetanilide) (Figure 3), is a white, crystalline odorless powder with a slight bitter taste. The compound is a weak organic acid with a pKa of 9.5 and a molecular weight of 151.2. It has moderate water and lipid solubility and is largely non-ionized over the physiologic pH range. Acetaminophen was first used in medical therapy in 1893 by von Mehring (Forrest _et al_., 1982) but was not recommended for widespread use due to side effects. The product used by von Mehring was most likely very impure (Savides and Oehme, 1983). Today, acetaminophen is routinely used as an analgesic and antipyretic in adults, children and infants.

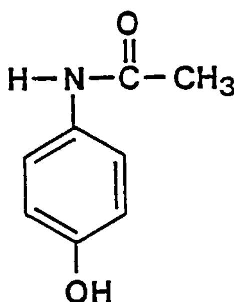

Figure 3: Structure of Acetaminophen

Acetaminophen has antipyretic and analgesic actions similar to acetylsalicylic acid (ASA), but has only weak anti-inflammatory actions. A major advantage over ASA is the fact acetaminophen causes no gastric irritation or bleeding. Adverse effects associated with the use of acetaminophen are only rarely reported. In recommended therapeutic doses acetaminophen is considered to be one of the safest of all minor analgesics and antipyretics (Savides and Oehme, 1983). However, overdoses can be fatal. When taken in large doses (>10 grams), toxicity can occur largely as a result of glutathione depletion. The LD 50 in humans has been estimated as 500 mg/kg (Savides and Oehme, 1983). However, susceptibility to acetaminophen hepatotoxicity varies between individuals. There are no immediate specific clinical manifestations of hepatotoxicity; maximal liver dysfunction is delayed for about three days. Prolongation of the acetaminophen plasma half-life and an increase in the ratio of unchanged to conjugated drug affords a poorer prognosis.

1.3.1 Analytical Methods

Acetaminophen and its metabolites can be quantitated in serum/plasma and urine by a variety of analytical techniques. Many of the earlier methods demonstrate poor sensitivity and are subject to assay interference. The methods include colorimetric (Brodie and Axelrod, 1948), thin layer chromatography (Jollow *et*

al., 1974), gas chromatography (Chan and McCann, 1979) and high performance liquid chromatography techniques (Adriaenssens and Prescott, 1978; Ameer et al., 1981; Buskin et al., 1982; Colin et al., 1986; Howie et al., 1977; Ladds et al., 1987; O'Connell and Zurzola 1982; Sood and Green, 1987; Wilson et al., 1982).

Chromatographic methods, primarily high pressure liquid chromatography (HPLC), are the favored method of analysis. The HPLC methods offer specificity, sensitivity and simple, rapid sample handling. The majority of the HPLC procedures utilize a reverse-phase analytical column in conjunction with ultraviolet detection at a wavelength of 249 nm (range 240 - 254 nm). The parent compound can be extracted by organic solvents from the serum/plasma but extraction of the more polar conjugates is difficult and not routinely done. The parent compound and metabolites can be quantitated following precipitation of plasma proteins, with subsequent injection onto the HPLC system. For determination of acetaminophen and metabolites in urine, direct injection of a sample (usually diluted) is most often used.

1.3.2 Absorption

Following oral administration, acetaminophen is rapidly absorbed from the gastrointestinal tract (Forrest et al., 1982) and has an analgesic effect in 15 - 30 minutes (Savides and Oehme, 1983). In humans, absorption from the stomach is negligible (Prescott, 1980). The rate limiting step in the

absorption of acetaminophen is reportedly the rate of gastric emptying (Heading et al., 1973). Absorption of acetaminophen from the small intestine is rapid with an estimated half-time for absorption of 6.8 minutes (Savides and Oehme, 1983). Absorption of acetaminophen is by passive transport (Prescott, 1980) and there appears to be no site of preferential absorption within the small intestine (Forrest et al., 1982).

A variable portion of the absorbed dose is lost to first pass metabolism and consequently the drug is not completely available to the systemic circulation following oral administration (Rawlins et al., 1977). The proportion of the dose reaching the systemic circulation ranges from 76 % (Prescott, 1980) to 92 % (Levy and Yamada, 1971). Rawlins and co-workers (1977) report a dose dependent availability, increasing from 68 % after 0.5 grams to 90 % after 1 - 2 grams. However, in 1984 Clements and colleagues reported the oral systemic availability was 80 % for both a 5 mg/kg and 20 mg/kg dose.

Absorption of acetaminophen from a solution in healthy, fasting subjects is very rapid with peak plasma levels occuring within 15 - 30 minutes (Prescott, 1980). Absorption from solid dosage forms (namely tablets) is usually slower (Prescott, 1980) but it is unclear to what extent tablet formulation influences the absorption (Forrest et al., 1982). Following oral administration of tablets to healthy, fasting subjects, the mean maximum plasma level has been noted at 20 min,

60 min and 1.4 h (Forrest et al., 1982). Under practical clinical conditions there may be as much as an 80 fold range in the plasma concentrations one hour after a therapeutic dose (Prescott, 1980).

The absorption of acetaminophen can be delayed by factors altering gastric emptying rate such as food (increased carbohydrate but not high lipid or protein content), posture (less rapid during sleep or when laying on left side), disease or drugs (gastrointestinal motility modifiers such as propantheline) (Prescott, 1980; Forrest et al., 1982). Usually the total amount absorbed is not decreased by altering the rate of absorption.

1.3.3 Distribution

Acetaminophen is rapidly distributed through most tissues and fluids. A tissue:plasma concentration ratio of approximately unity is reached in all tissues. The short time of 3 to 19 minutes for initial distribution following intravenous administration is indicative of rapid tissue distribution (Rawlins et al., 1977). The apparent volume of distribution is approximately 0.9 L/kg in man (Prescott, 1980; Rawlins et al., 1977). The distribution volume is similar in patients with epilepsy, Gilbert's syndrome, the elderly, anephrics and healthy controls (Forrest et al., 1982).

Acetaminophen does not bind appreciably to plasma proteins at therapeutic concentrations. At high levels (as can be seen in overdoses) the binding is between 15 and 21 % (Gazzard et al., 1973; Morris and Levy, 1984). In 1976, Lowenthal reported the sulfate and glucuronide conjugates of acetaminophen did not bind to plasma proteins even at the high concentrations seen in anephric patients. However, Morris and Levy (1984), using equilibrium dialysis and ultrafiltration, found the sulfate conjugate was > 50 % bound. The binding of the glucuronide conjugate was minor (< 10 %).

Acetaminophen crosses the placenta (Savides and Oehme, 1983) and enters the fetal circulation. Teratogenic effects have not been reported. The compound is not extensively excreted in human breast milk.

1.3.4 Elimination

Acetaminophen is extensively metabolized, mainly in the liver, with only 2 - 5 % of a therapeutic dose excreted unchanged in the urine. As was initially proposed by Cummings et al. (1967), acetaminophen is primarily metabolized to the glucuronide and sulfate conjugates (Levy et al., 1982). The urinary excretion profile of acetaminophen and its main metabolites has been characterized for man and several animals including mice, rats, cats and dogs. In most species studied,

four main metabolites have been identified. These include acetaminophen glucuronide (AG), acetaminophen sulfate (AS), acetaminophen cysteine (AC), and acetaminophen mercapturate (AM) (Savides and Oeheme, 1983) (Figure 4).

In man, usually 85 to 90 % of the administered dose is excreted in the urine within 24 hours (Forrest et al., 1982). Although the metabolic profile can vary somewhat, the relative proportions excreted in the urine are: AG 55 - 75 %, AS 20 - 40 %, AC and AM combined 5 - 10 %, and the parent drug 2 - 5 % (Levy and Yamada, 1971; Prescott, 1980). Other minor metabolites, including five detected in the urine of overdose patients, have been reported (Knox and Jurand, 1977). These minor metabolites usually constitute less than 1 % of the dose (Forrest et al., 1982). In neonates and young children, glucuronidation is deficient and the formation of acetaminophen sulfate is the predominant metabolic pathway (Miller et al., 1976; Prescott, 1980). Although there can be considerable variation in metabolism between individuals, fractional urinary recovery of each conjugate is remarkably constant within a given subject (Clements et al., 1984).

The cysteine and mercapturate conjugates are formed from a minor fraction of acetaminophen converted by a cytochrome P - 450 dependent mixed function oxidase system to a reactive metabolite (Savides and Oehme, 1983). The reactive metabolite, N-acetyl-p-benzoquinoneimine (Newton et al., 1986; Peggins et al., 1987; Savides and Oehme, 1983) is normally rapidly

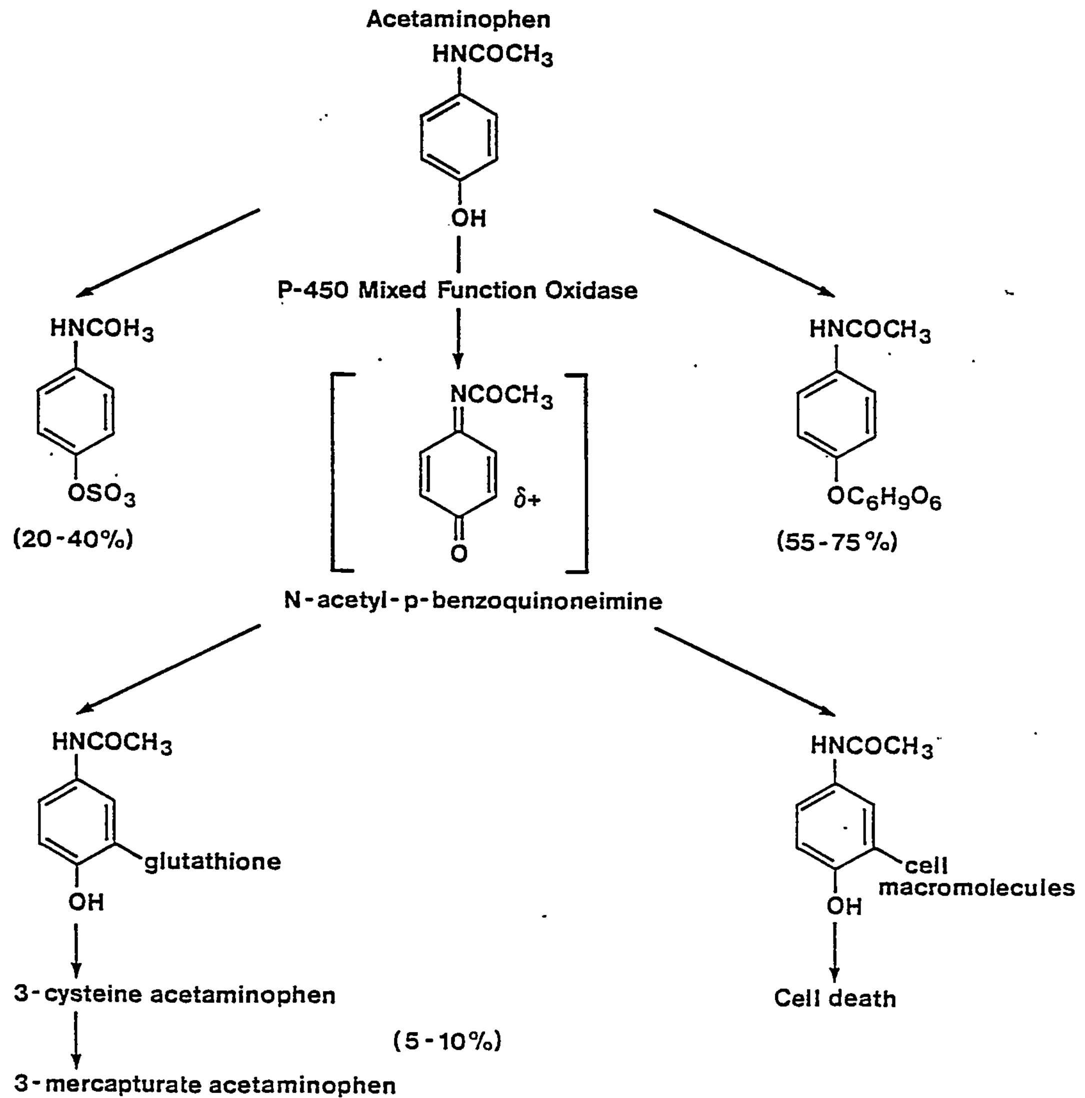

Figure 4: The metabolism of acetaminophen in humans

detoxified by conjugation with glutathione. The reactive metabolite may also bind to cell macromolecules producing nephrotoxicity and hepatotoxicity. It is generally agreed that the toxic metabolite in the liver is the semiquinonimine. However, the identity of the nephrotoxic metabolite is still the subject of debate. It may be PAP, the deacetylated metabolite of acetaminophen, or it may be similar to the hepatotoxic metabolite (Peggins et al., 1987). Most investigators agree the metabolite is formed locally in the kidney and not transported from the liver (Peggins et al., 1987).

In 1963 Nelson and Morioka published the first report on the kinetics of the metabolism of acetaminophen in man. In their report only urine samples were assayed for unchanged and total acetaminophen; there was no urinary conjugate data and no serum data. In 1967 Cummings and coworkers first reported separate rates of excretion for acetaminophen and the two major conjugates in urine. After many other reports on the kinetics of acetaminophen, Slattery and Levy (1979) developed a pharmacokinetic model for acetaminophen elimination in man. The model was based on data obtained from both therapeutic doses in healthy adults and overdoses in cases of poisoning. The model consists of four parallel pathways -- the formation of AG and AS which follow Michaelis-Menten kinetics and two apparent first order processes -- the renal excretion of acetaminophen and the formation of the reactive metabolite.

The plasma elimination half-life of acetaminophen shows

considerable variability between individuals but is usually in the range of 1.5 - 3.5 hours (Nelson and Morioka, 1963; Prescott et al., 1968; Prescott, 1980; Slattery et al., 1987). The half-life in elderly patients may be slightly longer than in young adults but is not outside the normal range. There does not appear to be a detectable difference in the half-life after oral and intravenous administration (Rawlins et al., 1977). There is some indication that the pharmacokinetics of acetaminophen are more complicated than originally supposed. Rawlins et al. (1977) report the kinetics following IV administration are incompatible with a one compartment open model. The IV data is felt to be more consistent with the two compartment open model (Prescott,1980).

The total body clearance values usually average about 5 mL/min/kg (Forrest et al., 1982). The mean renal clearance of acetaminophen is 13 mL/min. The renal clearances of the acetaminophen conjugates AG and AS are much higher: 130 mL/min and 166 mL/min respectively (Prescott, 1980).

Acetaminophen undergoes glomerular filtration with subsequent passive tubular reabsorption (Forrest et al., 1982). The highly polar glucuronide and sulfate conjugates are both filtered at the glomerulus and appear to be actively secreted by the tubules. These two conjugates are rapidly excreted in the urine and the rate of their appearance in the urine is limited by their rate of formation. Prescott and Wright (1973) found the urine pH does not affect the clearance of acetaminophen or its

conjugates. However, there is a positive correlation between urine flow rate and the clearance of unchanged acetaminophen (Prescott and Wright, 1973).

There is very little data available on the biliary excretion of acetaminophen and its metabolites in man. The major form of acetaminophen found in the bile is the glucuronide conjugate (Hjelle and Klassen, 1984). The glutathione conjugate is also excreted into the bile (Forrest et al., 1982). In rats, the conjugates can be hydrolyzed in the gut allowing enterohepatic circulation (Watari et al., 1983).

In anephric patients there are apparently no significant differences in the biologic half-life or the volume of distribution for acetaminophen when compared with healthy control subjects (Lowenthal et al., 1976). In contrast to a control group, anephric patients show substantial accumulation of the AG and AS in the plasma -- concentrations which can be four times those seen in healthy subjects (Lowenthal et al., 1976).

Hepatitis does not affect the metabolism of acetaminophen. Peak plasma concentrations and clearance values in patients with hepatitis are similar to controls (Jorup-Ronstrom et al., 1986). In mild liver disease the metabolism of acetaminophen, as indicated by the plasma half-life, is not affected (Forrest et al., 1979). In more severe liver disease, the half-life is prolonged indicating slight impairment of metabolism (Forrest et al., 1979).

Reports of the drug's metabolism in patients with

Gilbert's syndrome are conflicting. Douglas and coworkers (1978) claim acetaminophen elimination is impaired in this syndrome and attribute the effect to a decrease in hepatic glucuronyl transferase activity. However, Ullrich and colleagues (1987) suggest the metabolism of acetaminophen is normal in Gilbert's patients.

In a study designed to detect ethnic differences in acetaminophen metabolism, Critchley et al. (1986) report the mercapturate and cysteine conjugates accounted for a total of 9.3 % of the recovered dose in Caucasians compared with only 5.2 % and 4.4 % in West and East Africans respectively. Although no ethnic differences in sulfate conjugation were detected, the mean fractional recovery of AG was significantly less in Caucasians than in Africans. In a similar study no significant differences in metabolic profiles were detected between the groups tested (Sommers et al., 1987).

With the concomittant administration of other therapeutic agents the metabolism of acetaminophen can be altered. For example, oral contraceptives increase the clearance of acetaminophen by 49 % (Miners et al., 1983). In patients on anticonvulsants, clearance is 46 % greater than in controls (Miners et al., 1984a). This change in metabolism is a result of altered glucuronidation and oxidation (Bock et al., 1987; Miners et al., 1983; Miners et al., 1984a).

A variety of xenobiotics including ascorbic acid (Houston and Levy, 1976), salicylamide (Levy and Yamada, 1971),

and fenoldopam (Ziemniak et al., 1987) interact through a metabolic basis with acetaminophen. The mechanism of the interaction involves competition for available inorganic sulfate.

1.3.5 Chronopharmacokinetics

The limited information on the chronopharmacokinetics of acetaminophen mainly derives from studies in laboratory animals. A few studies have been reported in man but the results are conflicting.

In adult male mice the plasma half-life of acetaminophen is significantly shorter at 10:00 am than at 6:00 pm (Schnell et al., 1983). In the same report these researchers found a circadian rhythm associated with the lethality of acetaminophen. Lighting schedules and feeding altered circadian rhythms of both acetaminophen toxicity and hepatic glutathione levels in male mice (Schnell et al., 1984). In rats, extrahepatic metabolism appears to be prominent at 11:00 pm but not at 9:00 am indicating there are temporal variations in the disposition of acetaminophen in the rat (Belanger et al., 1987).

Temporal changes in the disposition of acetaminophen have been studied in man on only a few occasions. Shively and Vesell (1975) reported the terminal half-life of acetaminophen in

normal volunteers was significantly longer when given as a single dose at 6:00 am than at 2:00 pm. The 15 % increase in the half-life was presumed to be due to a decrease in the apparent volume of distribution. In a study using a single dose administered at three different times on three separate occasions, the time of drug administration did not seem to cause significant changes in either the absorption or disposition of acetaminophen (Malan et al., 1985).

In a recent investigation of temporal variation as a factor affecting acetaminophen toxicity, Kamali and colleagues (1987) report significantly more of the glucuronide conjugate in the 0 - 4 h morning (8:00 am oral dose) urine collection compared with five other administration times. The fraction excreted as the glucuronide was smallest after the 8:00 pm dose. The acetaminophen clearance, half-life and volume of distribution did not differ when the drug was given at 8:00 am or 8:00 pm by the intravenous route. Their results indicate a circadian variation in the rate and/or extent of absorption as evidenced by the change in the glucuronide conjugate elimination (the urine contained almost twice the amount of AG after the 8:00 am dose as compared with the 8:00 pm dose). Although this study cannot be compared directly with the study of Shively and Vesell (1975), both studies detected some circadian variation in the disposition of acetaminophen. Further work is required to characterize the chronopharmacokinetics of acetaminophen.

1.4 The Present Investigation

Serum inorganic sulfate has long been recognized as an important component of the overall sulfation process. It is reasonable to assume that any physiological factor which influences the body level of inorganic sulfate may in turn modify the disposition of agents which undergo sulfoconjugation _in vivo_. A circadian rhythm of serum inorganic sulfate may be one such factor. There is a report of a circadian rhythm for serum inorganic sulfate levels in man (Meier and Schmidt-Kessen, 1978). Acetaminophen was chosen as a "model" compound to investigate both the effect of sulfate circadian variation on the sulfoconjugation process and also the effect of xenobiotic administration on the sulfate levels. Acetaminophen, a commonly used analgesic/antipyretic, is metabolized mainly to sulfate and glucuronide conjugates. The half-life is conveniently short so that elimination of the drug should occur within a daily cycle. A multiple dose regimen was chosen to parallel the common usage pattern of the drug. Important facets of the relationship between inorganic sulfate and acetaminophen remain unexplored. Acetaminophen administered in a variety of dosages and routes can cause a gradual depletion of inorganic sulfate in the body (Lin and Levy, 1982). A single oral dose of 1.5 grams causes a decrease in the body stores of inorganic sulfate (Morris and Levy, 1983) and it has been estimated that 12 grams of acetaminophen administered over a 24 hour period in divided doses

should lead to almost complete depletion of inorganic sulfate in man (Galinsky and Levy, 1981). No studies investigating the possible connection between the circadian rhythm of serum inorganic sulfate and acetaminophen disposition are available. With these considerations in mind two questions were formulated: is the circadian rhythm of inorganic sulfate one of the factors influencing the disposition of acetaminophen ? and does the administration of acetaminophen alter or disrupt the circadian rhythm of inorganic sulfate ?

The purpose of the present investigation was, therefore,to examine the interaction between the circadian rhythm of serum inorganic sulfate and the administration of a substrate for sulfation -- namely acetaminophen. The specific objectives of the project were:

1. To develop appropriate analytical methodology to quantitate inorganic sulfate in serum and urine and also to measure acetaminophen and its metabolites (the glucuronide and sulfate conjugates) in serum and urine.

2. To confirm and characterize the circadian rhythm of serum inorganic sulfate in man.

3. To examine the disposition of acetaminophen at steady state during two consecutive dosing intervals and characterize any chronopharmacokinetic patterns evident under these conditions.

2.0 METHODOLOGY

2.1 Analysis of Inorganic Sulfate

2.1.1 Sample Preparation

2.1.1.1 Serum Analysis

An Amicon MPS-1 Micropartition system device (Amicon Corporation, Danvers,MA) fitted with an Amicon type YMT Ultrafiltration membrane (Amicon Corporation, Danvers, MA) was assembled and visually inspected. A 500 µL aliquot of water suitable for HPLC analysis (Milli-Q water system, Millipore Corporation, Milford, MA) was introduced into the device. The entire device was centrifuged for 17 minutes (2,000 x G, ambient temperature) using a fixed angle rotor in an IEC HN S11 centrifuge (International Equipment Company, Needham Heights, MA). The teflon filtrate cup of the MPS-1 device was changed and a 500 µL aliquot of serum (thawed at room temperature and mixed for 30 seconds) was introduced into the sample reservoir. The entire device was centrifuged again for 17 minutes at 2000 x G. The ultrafiltrate was diluted appropriately (usually a dilution factor of eight) with distilled, deionized water and a 50 µL sample was injected onto the HPLC system.

2.1.1.2 Urine Analysis

A sample of urine (3 to 4 mL), was allowed to thaw at room temperature, mixed (Vortex Deluxe Mixer, Canadian Laboratory Supplies, Toronto, ONT) for 60 seconds and then placed in a glass tube (16 x 100 mm) fitted with a teflon lined screw cap (Kimble products of Owens - Illinois, Toledo, OH). The sample was centrifuged at 2000 x G, 4°C for 10 minutes in a swinging bucket centrifuge (Accuspin - FR Centrifuge, Beckman Instruments Inc., Palo Alto, CA). An aliquot of the centrifuged urine was diluted appropriately (80 to 100 fold) with distilled, deionized water and a 50 μL sample injected onto the HPLC system.

2.1.2 Construction of Calibration Curves

Varying amounts of sodium sulfate (0 μmoles to 0.25 μmoles) in aqueous solution (diluted from a 0.001 M stock solution) were placed in glass tubes (16 x 100 mm) and sufficient distilled, deionized water added to make a total volume of 1.0 mL. The standard solutions were then mixed for 60 seconds. Seven standard solutions ranging from 25 μM to 250 μM plus a blank (0 μM) were routinely prepared. These solutions were injected directly onto the analytical system (50 μL volume). Calibration curves were constructed by plotting peak height vs. concentration of inorganic sulfate. A quality control standard (100 μM) was injected at the end of each day of analysis.

2.1.3 Analytical System

Serum and urine samples were analyzed on an isocratic HPLC system consisting of a LKB Model 2150 HPLC pump (LKB, Broma, Sweden), a Waters Model 430 Conductivity detector (Waters Associates, Milford, MA) and a Model 7125 injector equipped with a 500 µL loop (Rheodyne Inc., Cotati, CA). Samples (50 µL) were injected using a Hamilton 100 µL # 710 glass syringe (Hamilton Co., Reno NV). The signal from the detector (set at 2.5 µS.f.s.) was monitored by a linear strip chart recorder Model 255 (Canadian Laboratory Supplies, Toronto, ONT) with a fixed input voltage of 10 mV. The chart speed was 10 cm/h.

The anion exchange column (Waters Anion IC - Pak, 4.5 mm x 50 mm, Waters Associates, Milford, MA) was protected by an IC - Pak Anion Guard Column insert (Waters Associates, Milford, MA) and a Rheodyne Model 7302 column inlet filter. The mobile phase consisted of 4 mM potassium hydrogen phthalate adjusted to pH 4.5 with 1 N sodium hydroxide. The mobile phase was sonicated (Branson Ultrasonic Cleaner B-220, Branson Cleaning Equipment Company, Shelton CT) and degassed (Millipore Solvent Filtration System, 0.45 µm filters) prior to use. The flow rate was 1.00 mL/min. The temperature of the detector cell was maintained at 35°C by the temperature compensator of the conductivity detector.

2.2 Analysis of Acetaminophen and Metabolites

2.2.1 Sample Preparation

2.2.1.1 Serum Analysis

The internal standard (3-acetamidophenol, 5 μg) was added to the Reacti-vial (1.0 mL, Pierce Chemical Co., Rockford, IL) from a methanolic stock solution (0.1 mM) and the methanol was removed under a gentle stream of nitrogen gas (Meyer N-Evap Analytical Evaporator, Organomation Associates, Inc., Northborough, MA).

The set of serum samples for one volunteer was allowed to thaw at room temperature. Each sample was then mixed for 30 seconds. A 250 μL aliquot of serum was added to a Reacti-vial containing the internal standard (3-acetamidophenol). The contents of the Reacti-vial were mixed for 30 seconds. Acetonitrile (375 μL) was added (serum:acetonitrile ratio of 1 / 1.5) and the liquid vigorously mixed for 60 seconds using a Vortex Deluxe Mixer. Following protein precipitation, the Reacti-vials were centrifuged at 2000 x G for 10 minutes at 4°C (Accuspin-FR Centrifuge). A 100 μL aliquot of the clear supernatant was transferred to a 12 x 75 mm Falcon polypropylene tube (Becton Dickinson Canada Inc., Mississauga, ONT) and the solvent was evaporated using a water bath (40°C) and a gentle stream of nitrogen gas. The residue was dissolved in 100 μL of distilled, deionized water and mixed for 30 seconds. An aliquot of this mixture was injected onto the HPLC system.

2.2.1.2 Urine Analysis

The urine samples were allowed to thaw at room temperature. Each sample was mixed for 30 seconds. Approximately 1.0 mL of urine was transferred to a polyethylene microcentrifuge tube using a disposable pipette. The urine samples were centrifuged (5000 x G, 10 minutes) at ambient temperature (Fisher Model 59 Centrifuge, Fisher Scientific Ltd., Fairlawn NJ). The internal standard, 3-acetamidophenol (20 μg), was added as a methanolic solution to 16 x 100 mm glass tubes (Kimble products of Owen-Illinois, Toledo,OH). The methanol was then removed under a gentle stream of nitrogen. An aliquot (10 - 20 μL) of freshly centrifuged urine was diluted 100 - 200 fold by adding sufficient distilled, deionized water to yield a final volume of 2.0 mL. The tubes were mixed for 30 seconds. An aliquot of this mixture was injected directly onto the HPLC analytical system.

2.2.2 Construction of Calibration Curves

2.2.2.1 Serum

The calibration curves for the determination of acetaminophen, acetaminophen glucuronide and acetaminophen sulfate were constructed using varying amounts of acetaminophen (0 - 5 μg), acetaminophen glucuronide (0 - 7.5 μg), and acetaminophen sulfate (0 - 7.5 μg) and 250 μL of blank serum (pooled from study volunteers). This yielded concentrations of 0 to 20 μg/mL for acetaminophen and 0 - 30 μg/mL for both the

glucuronide and sulfate conjugates.

To facilitate preparation of standard solutions, the drug and metabolites were added to pooled blank serum (from the volunteers participating in the study) to yield the specifications just described. Acetaminophen, acetaminophen glucuronide and the acetaminophen sulfate were added as methanolic solutions to 16 x 100 mm glass tubes and the solution was evaporated to dryness under a gentle stream of nitrogen. Aliquots (2 mL) of the pooled blank serum were then added to prepare a bulk supply of the serum standards. These serum standards were frozen in portions and a 250 μL volume was used to prepare the standards. The standards were treated as described earlier in Section 2.2.1.1 . Five different concentrations plus a blank were routinely used to construct the calibration curves.

2.2.2.2 Urine

The calibration curves for acetaminophen and its two major conjugates (the glucuronide and sulfate) were constructed using 20 μL blank urine, 3-acetamidophenol as the internal standard (20 μg), varying amounts of acetaminophen (0 - 8.0 μg) and the two conjugates, acetaminophen glucuronide (0 - 60 μg) and acetaminophen sulfate (0 - 40 μg). Appropriate aliquots of methanolic solutions of the three compounds plus the internal

standard were added to 16 x 100 mm glass tubes and evaporated to dryness (under a gentle stream of nitrogen). The blank urine and sufficient distilled, deionized water to make all volumes equal to 2.0 mL were mixed for 60 seconds. An aliquot (10 μL) was injected directly onto the HPLC system. Six different concentrations plus a blank were used to construct each calibration curve.

2.2.3 Analytical System

2.2.3.1 Serum

Serum samples were analyzed on an isocratic HPLC system consisting of a LKB Model 2150 HPLC pump (LKB, Broma, Sweden) and a Waters Model 490 Programmable Wavelength Detector (Waters-Millipore, Milford, MA). The signal from the detector (operated at 0.03 a.u.f.s.) was monitored using a Linear Chart Recorder Model 255 (Canadian Laboratory Supplies, Toronto, ONT) with a fixed input voltage of 10 mV and a chart speed of 10 cm/h. Injections were made onto an Altex Ultrasphere ODS HPLC column (250 x 4 mm id, 5 μm particle size, Beckman Instruments Inc., Berkley, CA) with a Hamilton 100 μL # 710 injection syringe (Hamilton Co., Reno,NV) through a Model 7125 injector equipped with a 500 μL loop (Rheodyne Inc., Cotati, CA). The analytical column was protected by a guard column and Rheodyne Model 7302 column inlet filter.

The mobile phase consisted of 0.015 M potassium phosphate monobasic adjusted to pH 2.65 with 0.9 M phosphoric

acid and acetonitrile in a ratio of 90:7. The mobile phase was degassed prior to use utilizing a microfiltration apparatus fitted with 0.45 μm filters (Millipore Corp., Milford, MA). The flow rate was 1.2 mL/min and the absorbance of the column eluent was monitored at 249 nm. Analysis was conducted at ambient temperature.

2.2.3.2 Urine

The urine samples were analyzed on the HPLC system described in Section 2.2.3.1 with the following modifications. The mobile phase consisted of 0.015 M potassium phosphate monobasic (adjusted to pH 3.00 with 0.9 M phosphoric acid) and acetonitrile in a ratio of 90:5. The 490 detector was programmed as follows: at the start of each injection run the a.u.f.s. was set at 0.10, changed to 0.05 at 5 minutes, 0.01 at 9 minutes and back to 0.05 at 13 minutes. The a.u.f.s. of the detector was returned 0.10 to reinitiate the sensitivity - time program. The injection volume was 10 μL.

2.3 Pharmacokinetic Studies

2.3.1 The Preliminary Investigation

To test the analytical procedures for inorganic sulfate, serum and urine levels of inorganic sulfate were measured in a healthy male volunteer. On two separate occasions, with and without a 53 mg/kg dose of sodium sulfate (the dose used by Morris and Levy, 1983), several blood samples were collected during the course of a 24 h period. Total urine output was collected in fractions for the 24 hours.

2.3.2 Phase 1 : Circadian Rhythm of Inorganic Sulfate

The proposal for the research study ("The Circadian Rhythm of Serum Inorganic Sulfate and Relationship with Acetaminophen Chronopharmacokinetics"; Project # 87 - 80) was approved by the President's Advisory Committee on Ethics in Human Experimentation. Healthy, non-smoking males between the ages of 20 and 28 were recruited for the study. All subjects were Caucasians who maintained their normal diet.

All participants underwent a physical examination and blood and urine tests to ensure they were healthy and laboratory values were within normal limits. Seven subjects entered the study. All participants gave informed consent (See Appendix II).

The participants were supplied with a Volunteer Information Sheet (See Appendix III) which was supplemented with

verbal instructions. The participants were required to be medication free two weeks prior to and during the study. Ethanol consumption was prohibited one week prior to the study and caffeine was restricted the day before and the day of the study.

All of the participants were required to fast starting at midnight on the day of the study (day of sampling). Small amounts of water were allowed. On the day of the study standardized meals (Appendix VI) were provided at: 7:30 am, 12 noon, and 6:00 pm.

Ten blood samples (7 mL each) were obtained from each volunteer during a 24 h period: at 7 am, 9 am, 11 am, 1 pm, 3 pm, 5 pm, 7 pm, 9 pm, 11 pm and 7 am. All blood samples were collected by separate venipuncture into evacuated plain (serum) tubes (Venoject[R]). Phlebotomy was performed by a Registered Laboratory Technician. Total urine output over the 24 h period was collected in the following fractions: 7 am - 11 am, 11 am - 3 pm, 3 pm - 7 pm, 7 pm - 11 pm, and 11 pm - 7 am. Aliquots of serum and urine samples were stored at - 20°C until analysis.

2.3.3 Phase 2 : Chronopharmacokinetics of Acetaminophen

Six of the seven subjects who completed Phase 1 entered Phase 2 of the study. Restrictions on medication, ethanol and caffeine use were as outlined for Phase 1. Each subject received a random number of excess acetaminophen

tablets to facilitate a compliance check. The importance of the dosage regimen and punctual administration times were stressed.

Each volunteer ingested 650 mg of acetaminophen (Tylenol[R]) every six hours (8 am, 2 pm, 8 pm and 2 am) for three days. On day four the 2 am, 8 am and 2 pm doses were consumed, and all of the participants were requested to fast from midnight until the morning meal provided. Standardized meals (the same composition as in Phase 1) were provided on day four (sampling day) at 7 am, 11:30 am and 5:30 pm. The meals were provided half an hour earlier in Phase 2 so as to minimize any interference with absorption of acetaminophen.

On day four blood samples were collected during two consecutive dosing intervals at the following times: 8 am, 8:30 am, 9 am, 9:30 am, 10 am, 11 am, 12 noon, 2 pm, 2:30 pm, 3 pm, 3:30 pm, 4 pm, 5 pm, 6 pm, and 8 pm. All blood samples were obtained by separate venipuncture and collected in evacuated plain (serum) tubes (Venoject[R]). Total urine output was collected during each dosing interval. ie) 8 am - 2 pm and 2 pm - 8 pm. Aliquots of serum and urine samples were stored at - 20°C until time of analysis.

2.4 Data Analysis

2.4.1 Inorganic Sulfate Data

Pharmacokinetic parameters were calculated using the following equations:

$$\text{Urinary excretion rate} = \frac{\text{urine volume x urine concentration}}{\text{collection interval}}$$

$$\text{Renal clearance} = \frac{\text{urinary excretion rate}}{\text{serum level at mid point of collection interval}}$$

Parameters were normalized to body weight and the values expressed per kilogram body weight.

The area under the curve (AUC) from the first sampling point to the last sampling point was calculated using the trapezoidal rule. The average serum sulfate level (C_{av}) over a specified time interval was calculated as the AUC of the serum level vs. time plot during one dosing interval divided by the time of the interval. Other values including the maximum serum inorganic sulfate level (C_{max}), the time at which C_{max} occurred (t_{max}) and the minimum serum inorganic sulfate level (C_{min}) and the time at which C_{min} occurred (t_{min}) were recorded for specified time intervals. The difference between the C_{max} and C_{min} was expressed as a percent (ΔC) and calculated as:

$$\frac{C_{max} - C_{min}}{C_{max}} \times 100$$

2.4.2 Acetaminophen Data

Pharmacokinetic parameters were calculated using the following equations:

$$t_{1/2} = \frac{0.693}{\lambda z}$$

$$V_d/F = \frac{D}{AUC_{ss} \times \lambda z}$$

$$CL/F = \frac{D}{AUC_{ss}}$$

$$CL_{A-AG} = CL/F \times f_{AG}$$

$$CL_{A-AS} = CL/F \times f_{AS}$$

$$CL^{A}{}_{r} = \frac{U^{A}{}_{0-\tau}}{AUC^{A}{}_{0-\tau}}$$

$$CL^{AG}{}_{r} = \frac{U^{AG}{}_{0-\tau}}{AUC^{AG}{}_{0-\tau}}$$

$$CL^{AS}{}_{r} = \frac{U^{AS}{}_{0-\tau}}{AUC^{AS}{}_{0-\tau}}$$

Where $t_{1/2}$ = terminal half-life

λz = terminal elimination rate constant

D = dose of acetaminophen

CL = total body clearance

F = oral availability

V_d = apparent volume of distribution

AUC_{ss} = area under the serum concentration vs. time curve for a dosing interval at steady state

CL_{A-AG} = partial metabolic clearance to AG

CL_{A-AS} = partial metabolic clearance to AS

f_{AG} = fraction of recovered dose excreted as AG

f_{AS} = fraction of recovered dose excreted as AS

$CL^{A}r$ = renal clearance of acetaminophen

$CL^{AG}r$ = renal clearance of AG

$CL^{AS}r$ = renal clearance of AS

$U^{A}0\text{-}\tau$ = the amount of A excreted in the urine during a dosing interval at steady state

$U^{AG}0\text{-}\tau$ = the amount of AG excreted in the urine during a dosing interval at steady state

$U^{AS}0\text{-}\tau$ = the amount of AS excreted in the urine during a dosing interval at steady state

$AUC^{A}0\text{-}\tau$ = area under the A serum concentration - time curve for a dosing interval at steady state

$AUC^{AG}0\text{-}\tau$ = area under the AG serum concentration - time curve for a dosing interval at steady state

$AUC^{AS}0\text{-}\tau$ = area under the AS serum concentration - time curve for a dosing interval at steady state

The elimination rate constant (λz) was calculated by log linear least squares regression analysis of the terminal phase of the serum concentration - time curve. The number of time points used in the calculation was determined by the linearity of the curve, and in most cases the last 4 - 6 points were used. In a few cases 3 points were used. The area under the serum concentration - time curve was calculated using the trapezoidal rule. The fraction excreted as acetaminophen or conjugates was calculated in acetaminophen equivalents of the total amount recovered.

Since the oral availability of acetaminophen was not determined in this study, the clearance and volume of distribution terms reported are in actuality CL/F and Vd/F.

2.4.3 Statistical Procedures

Data were analyzed using the paired Student t-test. For all statistical tests a p - value of 0.05 or less was considered significant. All results are expressed as the mean ± standard deviation (unless standard error of the mean indicated).

3.0 RESULTS AND DISCUSSION

3.1 Analytical Methods for Inorganic Sulfate

Methods were developed for the analysis of inorganic sulfate in both serum and urine. Although the initial preparation and handling of these two biological fluids varied prior to analysis, the HPLC system using single column ion chromatography with conductance detection was the same. This technique is more specific and precise than the traditional turbidimetric methodology.

3.1.1 Serum Samples

The method developed in our laboratory for the analysis of inorganic sulfate in serum was fast, reliable, reproducible and sensitive. The retention time for sulfate was 6.8 minutes and a system peak eluted at 10.5 minutes (Figure 5). The overall run time was about 13 minutes.

Calibration curves were linear and reproducible over the concentration range utilized (Figure 6). Although no internal standard was used, there was an excellent correlation between the peak height of standards and their respective concentrations; correlation coefficients for the curves were always > 0.99. The C.V. for the slopes of the curves was 4.78 % (n=22) and the y-intercept was only slightly greater than zero (0.1669 ± 1.8163). The C.V.s for the peak heights of inorganic

sulfate, calculated from the peak heights of standards used to construct the calibration curves (n=22), were less than 6.5 % for all concentrations (Table 1). The minimum detectable concentration was 6 μmol/L. The concentration of sulfate in all diluted samples was between 25 - 250 μmoles/L.

One of the most frequently cited advantages of ion chromatography is the minimal workup required for sample preparation prior to analysis (Morris, 1984). The sample preparation for this method of analysis is easier and requires less technical manipulation than the turbidimetric method. Serum is usually treated in one of two ways prior to injection on the column -- dilution with water (Cole and Scriver, 1981; deJong and Burggraaf, 1983) or precipitation of proteins with acetonitrile. However acetonitrile precipitation did not produce "clean" chromatograms or resolution acceptable for routine use. Other chemical methods of protein precipitation were not investigated.

Ultrafiltration proved to be a better method of protein removal prior to analysis. The Amicon micropartition system (MPS - 1, Amicon Corporation) used generates an ultrafiltrate by centrifugation partition. The integrity of the membrane was routinely checked by testing the ultrafiltrate with Albustix^R protein detection strips. The YMT cellulostic filters contained some residual inorganic sulfate. When membranes were rinsed with distilled/deionized water, the filtrate produced samples with levels of sulfate near the assay detection

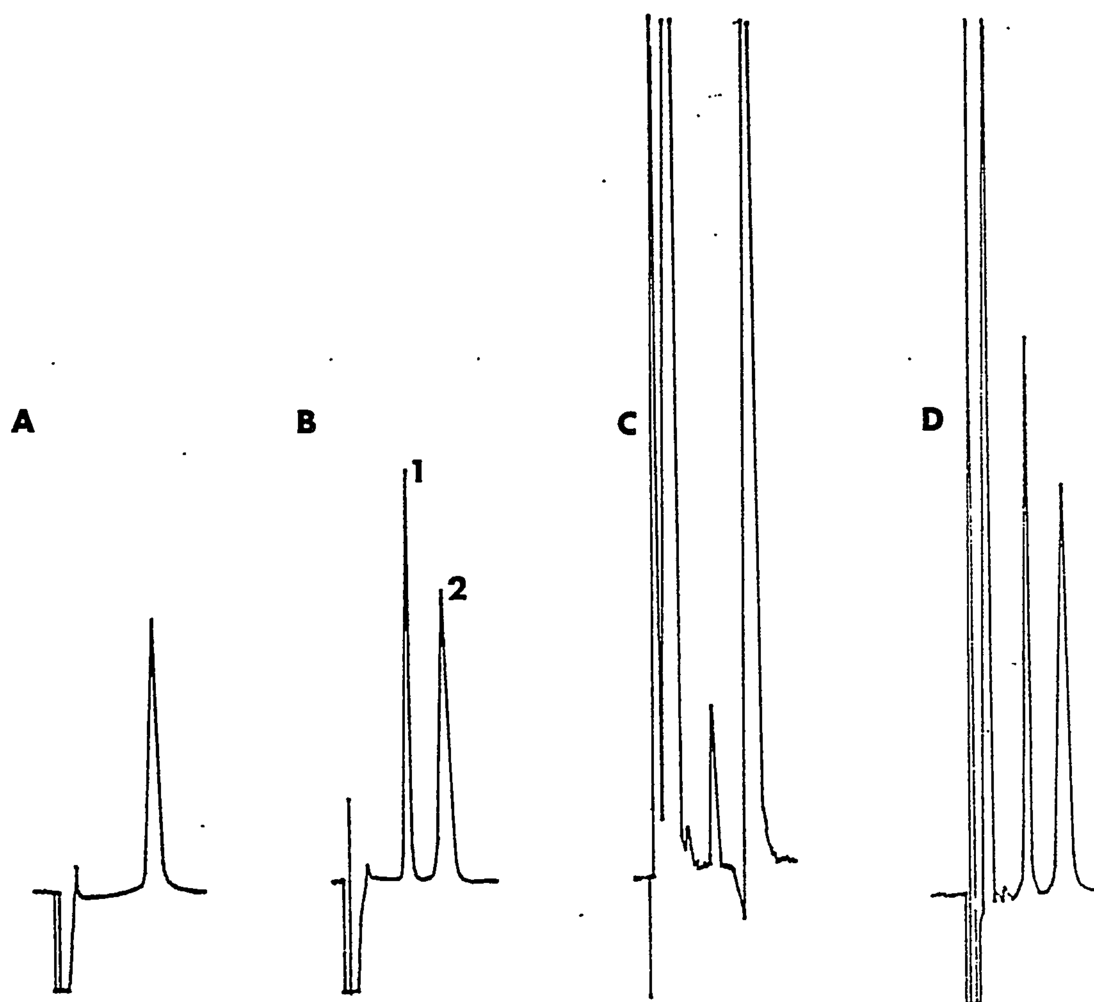

Figure 5: Representative chromatograms for the analysis of inorganic sulfate in serum and urine. A: Blank, B: Standard (0.10 mM), C: Serum, D: Urine. (1, Inorganic sulfate; 2, System Peak)

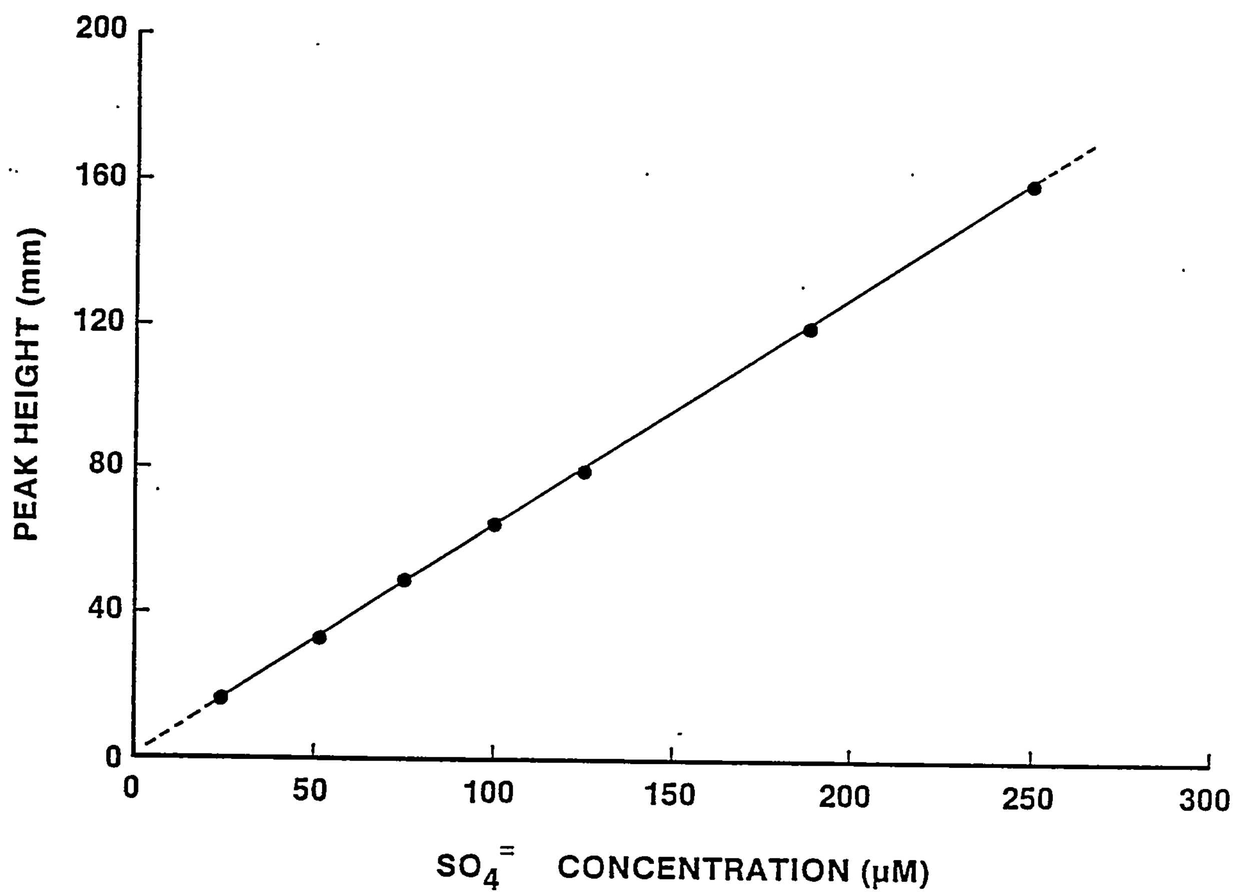

Figure 6: Representative calibration curve for the analysis of inorganic sulfate in serum and urine.

Table 1 Coefficients of variation of peak heights for inorganic sulfate in serum. *

CONCENTRATION (μmoles/L)	C.V. (%)
25	4.54
50	4.37
75	3.10
100	2.42
125	2.79
187.5	2.38
250	6.10

* peak height determined from samples used to construct daily calibration curves (n=22).

NOTE: The quality control standard (100 μmoles/L) was injected at the end of each day of analysis. (102.3 ± 2.8 μmoles/L; mean ± S.D., n=22)

limits. After one rinsing with water, no additional sulfate was collected (as indicated by the analysis of the ultrafiltrate) when further volumes (up to five) were used to rinse the membrane. Standards subjected to the ultrafiltration procedure did not differ from those which were untreated and directly injected. All membranes were rinsed once with water prior to processing a serum sample. A 500 μL aliquot of serum was routinely used; however, smaller volumes of 250 μL produced results identical to those obtained with a larger serum aliquot. The 500 μL volume provided a larger volume of ultrafiltrate.

Cole and Landry (1985) used ultrafiltration to remove proteins prior to analysis on an ion chromatographic system. This report was found after we had developed our procedure.

3.1.2 Urine Samples

Prior to chromatographic analysis urine samples were centrifuged and diluted. The same chromatographic system was utilized for both serum and urine samples. The system peak did not interfere with the baseline separation routinely obtained (Figure 5). Sensitivity, retention times and the run time were identical to those obtained for the serum assay.

Calibration curves for urine analysis were linear over the concentration range of 25 to 250 μmoles/L and the correlation coefficient was always > 0.99 (Figure 6). The

coefficient of variation for the slope of the curves (n=11) was 5.80 %. The y-intercept was -0.3200 ± 2.3666. Measurement of inorganic sulfate in urine was consistent. The C.V.s for the peak height of inorganic sulfate, calculated from the peak heights of standards used to construct eleven calibration curves were less than 7.0 % for all concentrations (Table 2).

In 1960 Berglund and Sorbo published a turbidimetric assay procedure for the determination of inorganic sulfate in urine and serum. For many years this was considered the standard method of analysis. The turbidimetric methodology has always been complicated by nonspecificity and imprecision. Consequently, it is understandable that the introduction of ion chromatography by Small et al. in 1975 was a milestone in the analysis of inorganic ionic species, including inorganic sulfate. The original methodology involved the suppressed ion or dual column technique. However, the use of nonsuppressed or single-column ion chromatography has become increasingly popular. With single column ion chromatography chromatographic efficiency is higher but the sensitivity is lower, often by an order of magnitude (Morris, 1984). The sensitivity demonstrated is more than sufficient for the determination of inorganic sulfate in biological fluids. Single column anion chromatography is a simple, relatively economic and accurate method for the determination of inorganic sulfate in biological media.

Various combinations of the mobile phase components,

Table 2: Coefficients of variation of peak heights for inorganic sulfate in urine. *

CONCENTRATION (μmole/L)	C.V. (%)
25	2.57
50	2.58
75	2.07
100	2.48
125	2.47
187.5	2.51
250	6.56

* peak height determined from samples used to construct daily calibration curves (n=11).

NOTE: The quality control standard (100 μmoles/L) was injected at the end of each day of analysis. (100.8 ± 2.6 μmoles/L; mean ± S.D., n=11).

the molar strength and pH of the mobile phase were tested to develop the chromatographic system. The mobile phase components used by Morris (1984) -- potassium hydrogen phthalate (KHP) 4 mM adjusted to pH 4.5 with 1 N sodium hydroxide -- proved to be suitable. This mobile phase produced acceptable run times and good chromatography. However, with the analysis of serum samples a "negative" inflection similar to that reported by Morris (1984), was noted after the sulfate peak. This peak did not interfere with the baseline chromatography achieved (Figure 5).

A system peak appeared routinely on all chromatograms. System peaks have been the subject of numerous reports in the literature (Erkelens et al., 1987; Jackson and Haddad, 1985; Levin and Grushka, 1986; Okada and Kuwamoto, 1984). Levin and Grushka (1986) note that chromatograms frequently show more peaks than the number of compounds one is expecting to identify. These resulting peaks are termed many things, such as pseudo peaks or vacancy peaks, but are most frequently referred to as system peaks. In initial attempts to identify this peak, changing the pH or molar concentration of the KPH eluent caused the system peak to migrate in its position on the chromatogram or, with extreme modifications, to disappear completely. The retention time of the system peak increased as the pH increased, a result similar to that reported by Jackson and Haddad, (1985). At a pH of about 6.5 no system peak was observed. Erklens et al. (1987) explain that the system peak results from protonated phthalate which is retained on the column by a reversed phase mechanism.

This is supported by Jackson and Haddad (1985) who indicate that their research suggests both eluent anion and neutral eluent molecules can be displaced from the column upon injection of a sample. Eluent anions are not retained on the column and elute with the injection peak. The neutral or protonated eluent molecules of the KHP system elute slowly by a reversed phase mechanism and appear as either positive or negative peaks. This theory supports findings at a pH of 6.5 where the KHP eluent is completely ionized and the system peak is not found. Many ions have a tendency to exhibit retention times similar to that of a system peak. Sulfate is identified as an example under a wide range of mobile phase conditions (Jackson and Haddad, 1985). The system peak in our assay did not interfere with the analysis of the sulfate ion and hence was considered of no major consequence.

Because inorganic sulfate is always present in serum and urine blank biological samples were impossible to obtain. Consequently, standards and blanks were produced from Milli-Q grade water to which was added varying amounts of sodium sulfate. These aqueous standards closely resembled the diluted urine samples and the ultrafiltrate of serum samples. The reproducibility, sensitivity and selectivity of the method made the use of an internal standard unnecessary.

Freezing biological samples at -20°C did not change sulfate concentrations during storage. This has previously been reported by numerous investigators (Cole et al., 1982; deJong and Burggraaf, 1983; Reiter et al., 1987). In addition, Pascoe

et al., (1984) measured sulfate levels in samples which underwent numerous freeze/thaw cycles and reported no change in the sulfate concentrations.

3.2 Analytical Methods for Acetaminophen and Metabolites

Methods were developed for the analysis of acetaminophen and its two major metabolites -- the glucuronide and sulfate conjugates in both serum and urine. Although the treatment of the two biological fluids prior to analysis was not the same, both analyses were performed using isocratic reversed-phase HPLC with a UV detector.

3.2.1 Serum Samples

The parent compound, glucuronide and sulfate conjugates were quantitated in serum. Because of differences in the polarities of the three compounds acetonitrile precipitation, rather than extraction, was used to prepare serum samples for analysis. Various serum:acetonitrile ratios were tested. A ratio of 1/1 did not provide adequate precipitation of proteins or adequate resolution on HPLC analysis. A ratio of 1/1.5 provided acceptable results. By increasing the ratio to 1/2 the sample did not show remarkable improvement in chromatographic resolution. Vigorous mixing of the serum samples after the

addition of the acetonitrile was essential to ensure maximal protein precipitation. The time of this mixing was increased (from 10 seconds to 60 seconds) and standardized, resulting in no further complication.

Blank serum did not produce any chromatographic peaks that interfered with the peaks for the drug, its metabolites or the internal standard 3-acetamidophenol. The retention times were: acetaminophen glucuronide 3.9 min, acetaminophen sulfate 5.0 min, acetaminophen 7.9 min and the internal standard 13.7 minutes (Figure 7). The overall run time was 18-19 minutes to allow elution of a late endogenous peak.

Calibration curves for acetaminophen and the two conjugates (glucuronide and sulfate) were linear in the ranges studied and the correlation coefficient was always greater than 0.99 (Figure 8). The slopes of the curves were consistent from day to day (C.V.s were: AG 2.31 %, AS 7.20 %, and A 2.33 %) and the y-intercept routinely approached zero (y-intercepts were: AG 0.0524 ± 0.0134, AS 0.0056 ± 0.0083, and A 0.0038 ± 0.0072). The coefficients of variation for peak height ratios were determined for acetaminophen, acetaminophen glucuronide and acetaminophen sulfate from ten calibration curves with five different concentrations (Table 3).

The method developed for the analysis of acetaminophen and conjugates was relatively fast and reproducible.

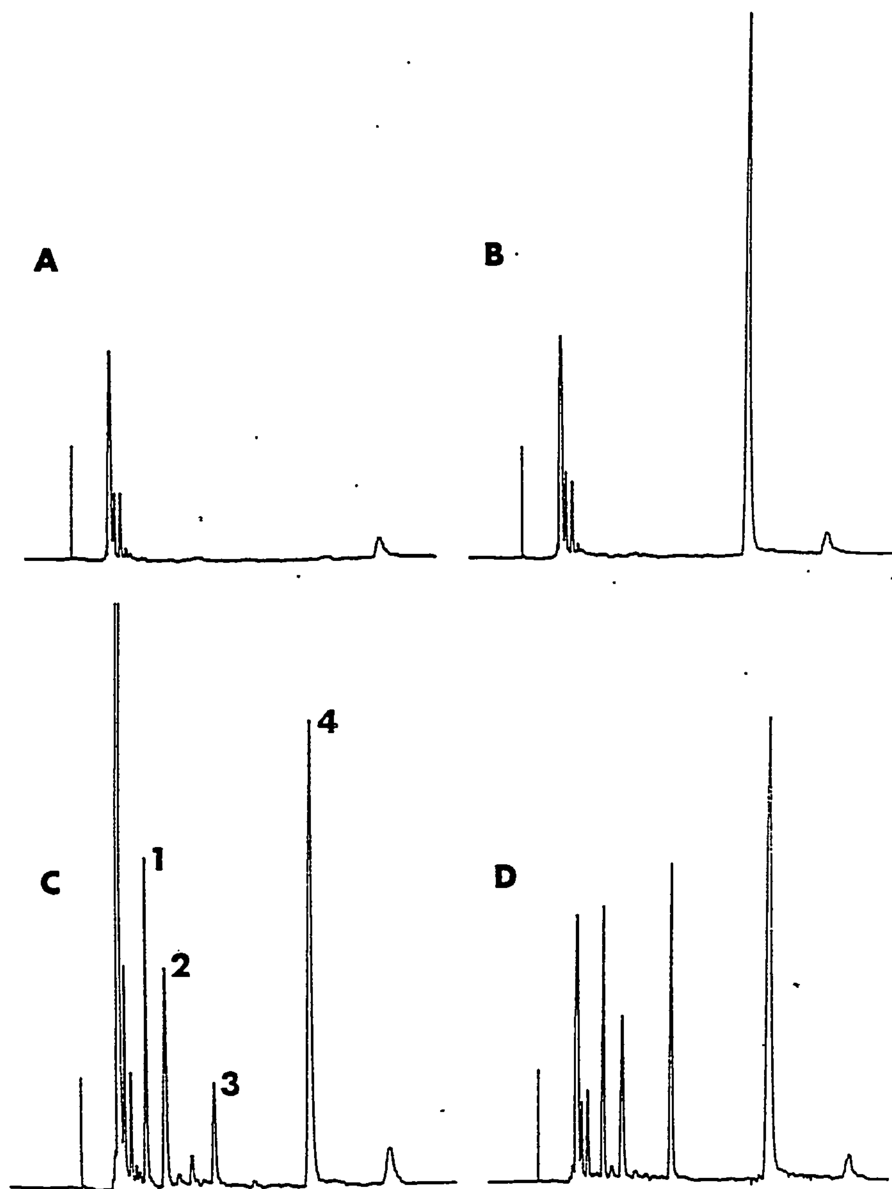

Figure 7: Representative chromatograms for the analysis of acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in serum. A: Blank serum, B: Blank serum with internal standard C: Serum standard of acetaminophen glucuronide (1) (8 μg/mL), acetaminophen sulfate (2) (8 μg/mL), acetaminophen (3) (2 μg/mL), and internal standard (4) (20 μg/mL). D: Serum sample 1 h following ingestion of 650 mg acetaminophen at steady state

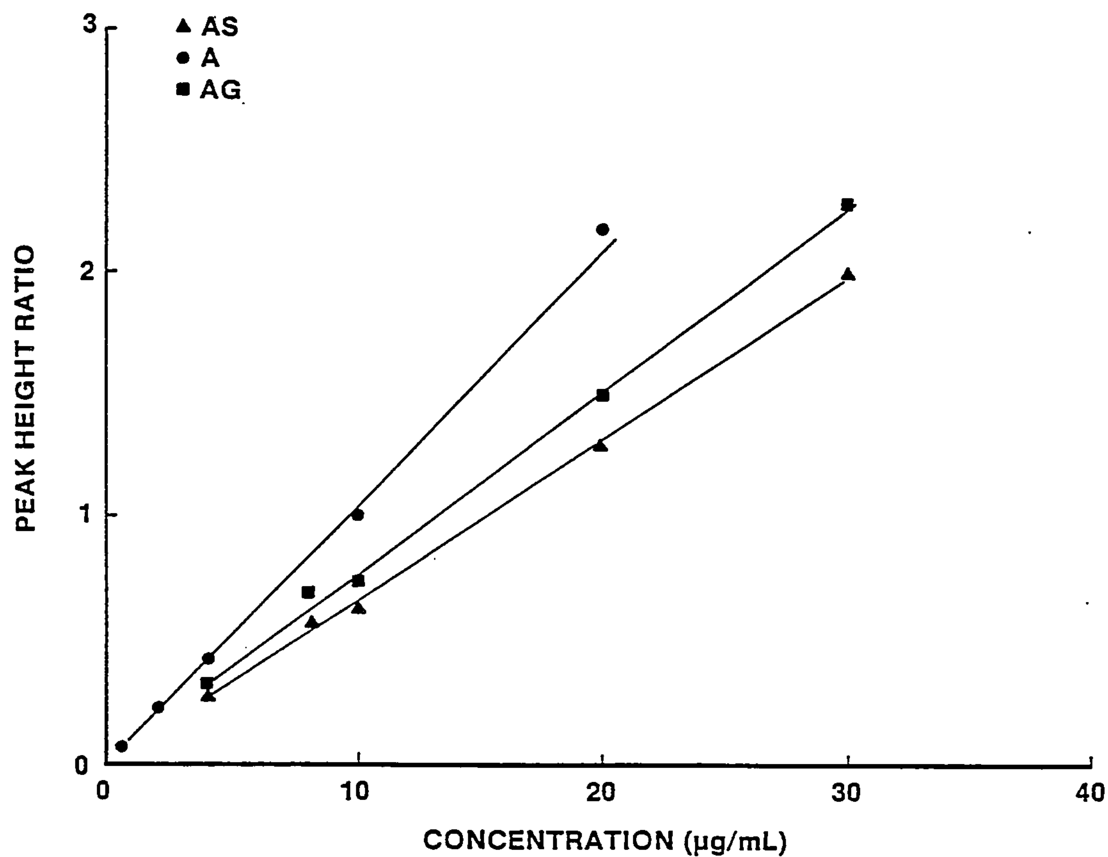

Figure 8: Typical calibration curve for the analysis of acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in serum.

Table 3: Coefficients of variation of peak-height ratios for acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in serum. *

COMPOUND	CONCENTRATION ($\mu g/mL$)	C.V. (%)
Acetaminophen	0.5	5.67
	2.0	3.53
	4.0	1.46
	10.0	1.55
	20.0	1.42
Acetaminophen	4.0	3.92
glucuronide	8.0	2.56
	10.0	2.83
	20.0	3.25
	30.0	2.25
Acetaminophen	4.0	9.36
sulfate	8.0	8.08
	10.0	7.99
	20.0	8.25
	30.0	7.19

* based on ten measurements at each concentration.

3.2.2 Urine Samples

Acetaminophen and its two major conjugates were quantitated by direct injection of diluted urine using an analytical system similar to that of Howie *et al*. (1977) and Sood and Green (1987). Methods which utilized enzyme hydrolysis were avoided because we wanted to measure the conjugates as separate entities not indirectly by hydrolysis. Two other metabolites, the cysteine and mercapturate conjugates were not quantitated due to their instability in solution and lack of authentic samples of pure metabolites.

Blank urine samples did not produce any interfering peaks on the chromatogram. The retention times were: acetaminophen glucuronide 4.2 min, acetaminophen sulfate 6.1 min, acetaminophen 10.6 min and the internal standard, 3-acetamidophenol 18.8 minutes (Figure 9). The overall run time was approximately 23 minutes.

Calibration curves of peak height ratio vs concentration were linear over the concentrations studied (Figure 10). Correlation coefficients were always greater than 0.998. Inter-assay variation was small as shown by similar slopes (C.V.s not more than 1.0 %, n=6). The y-intercept approached zero for each compound (y-intercepts were: AG -0.0206 ± 0.0081, AS -0.0021 ± 0.0083, and A 0.0526 ± 0.0139). The C.V.s of peak height ratios were determined for acetaminophen and its glucuronide and sulfate conjugates from six curves which utilized six different

concentrations (Table 4).

The analytical method developed was similar to that of Sood and Green (1987). Identifying a reversed phase chromatographic system which gave good peak symmetry and adequate resolution while at the same time minimizing interference from endogenous components in the biological samples was difficult. The choice of the pH of the mobile phase was crucial. The mobile phase for both serum and urine analysis consisted of a 0.015 M phosphate buffer and acetonitrile. To achieve adequate baseline separation while avoiding endogenous peaks, a buffer to acetonitrile ratio of 90:7 adjusted to pH 2.65 was used for serum; a ratio of 90:5 adjusted to pH 3.00 for urine. By making these adjustments in the mobile phase, the parent compound, the two metabolites of interest and endogenous components in both serum and urine were successfully resolved.

Numerous compounds were evaluated for use as an internal standard. Several compounds were discarded due to interference by an endogenous peak; e.g., 2-hydroxyacetamido-phenol had an adequate retention time but could not be utilized because it coeluted with an endogenous peak. The retention time for fluorophenol was too long (34 minutes). 3-Acetamidophenol had an acceptable retention time and baseline resolution and was selected as the internal standard for use in the assay procedure. This internal standard had also been used by other researchers (Buskin et al., 1982; Lo and Bye, 1979; Hendrix-Treacy et al., 1986).

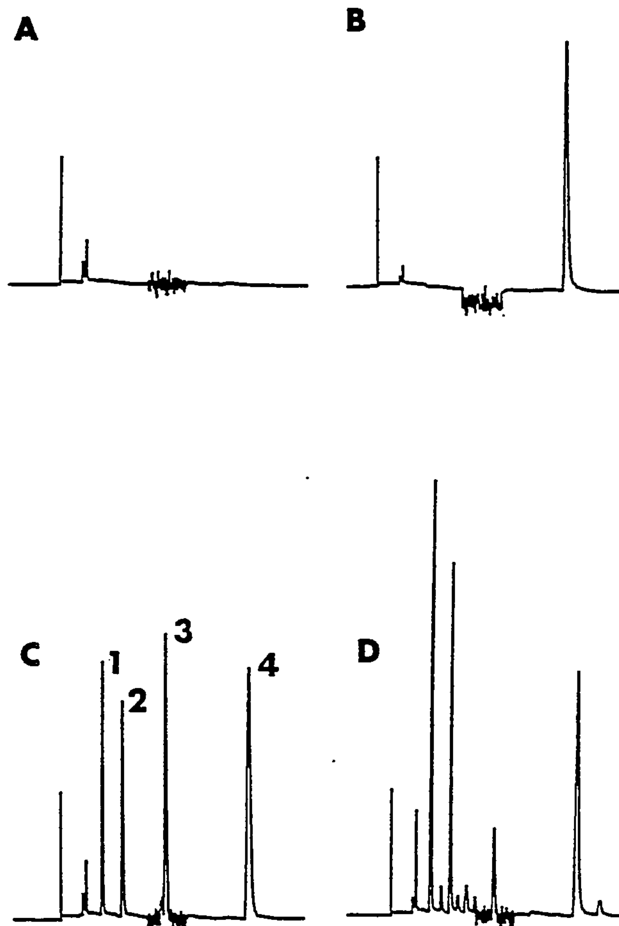

Figure 9: Representative chromatograms for the analysis of acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in urine. A: Blank urine, B: Blank urine with internal standard C: Urine standard of acetaminophen glucuronide (1) (10 μg/mL), acetaminophen sulfate (2) (5 μg/mL), acetaminophen (3) (1 μg/mL), and internal standard (4) (10 μg/mL). D: Urine sample (0-6h) following ingestion of 650 mg acetaminophen at steady state.

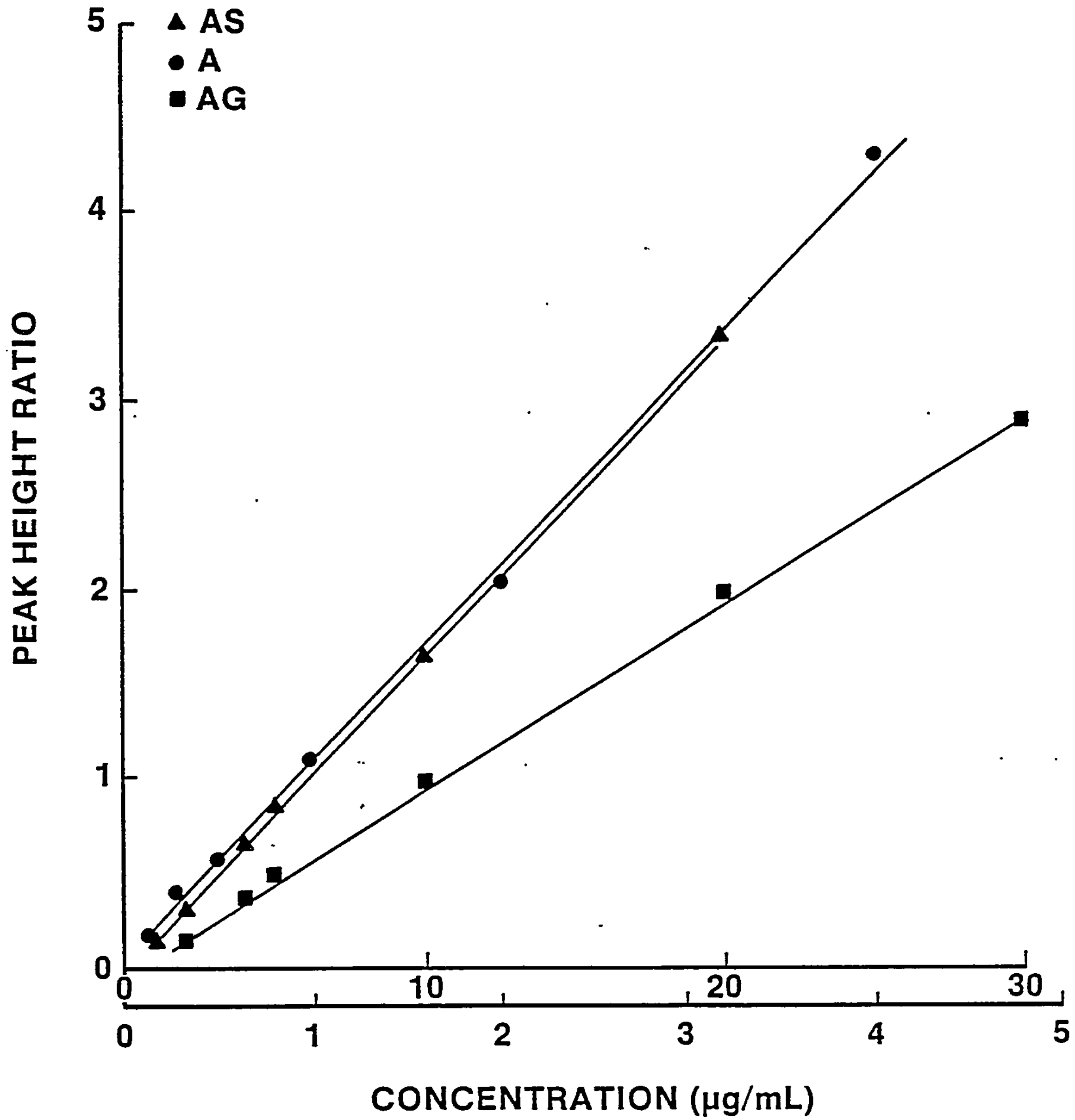

Figure 10: Typical calibration curve for the analysis of acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in urine. (The top concentration scale is for AG and AS while the bottom scale is for acetaminophen).

Table 4 Coefficients of variation of peak height ratios for acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in urine. *

COMPOUND	CONCENTRATION (μg/mL)	C.V. (%)
Acetaminophen	0.125	8.54
	0.25	2.96
	0.50	3.60
	1.00	6.11
	2.00	1.36
	4.00	0.69
Acetaminophen glucuronide	2.0	9.09
	4.0	1.84
	5.0	1.87
	10.0	1.67
	20.0	1.21
	30.0	1.13
Acetaminophen sulfate	1.0	8.98
	2.0	2.44
	4.0	1.49
	5.0	1.75
	10.0	0.90
	20.0	1.05

* based on six measurements at each concentration.

The detection limits of the assay procedures were established on the basis that a signal-to-noise ratio of less than two was unacceptable. Visual observation of the analytical signal (peak height) is difficult when the ratio is smaller than two. The detection limit for acetaminophen and acetaminophen sulfate in serum was approximately 0.2 μg/mL, for the glucuronide conjugate, 0.4 μg/mL. In urine, detection limits for both conjugates were similar to the respective serum values. For acetaminophen a higher sensitivity of 0.03 μg/mL was obtained.

A variety of sensitivities for the various assay procedures have been reported. Sensitivities have commonly been calculated with reference to a signal-to-noise ratio (S/N) of 2 or 3. Detection limits of about 1 μg/mL for the assay of acetaminophen in plasma/serum samples of about 1 μg/mL were reported by Adriaenssens and Prescott (1978) and O'Connell and Zurzola (1982). Other researchers have reported slightly greater sensitivity (Ameer et al., 1981; Buskin et al., 1982; Hendrix-Treacy et al., 1986; Lo and Bye 1979). In authentic urine samples Wilson et al. (1982) reported a sensitivity of 0.5 μg/mL for acetaminophen and 1.0 μg/mL for both AG and AS. Ladds et al. (1987) reported a detection limit of 10 ng for acetaminophen; Hendrix-Treacy et al. (1986), 1 μg/mL. Coefficients of variation for these assay procedures usually did not exceed 6.0 %.

The sensitivity, selectivity and reliability of our assays were similar to other procedures reported in the literature. The developed assays were successfully used in all

subjects for the routine analysis of A, AG and AS.

3.3 Inorganic Sulfate Disposition: Phase 1

Seven volunteers (all nonsmoking males) completed Phase 1 of the study. The mean age of the volunteers was 23.4 ± 2.6 years and the mean weight, 84.2 ± 13.3 kg. All participants were Caucasian and judged healthy by physical examination, blood and urine tests. All volunteers maintained their normal diet which was unrestricted except when fasting was required and when standard meals were supplied. Serum and urine samples were collected during the course of a 24 hour period (7 am - 7 am).

The average value for serum inorganic sulfate over a 24 h period (0.360 ± 0.029 mmoles/L) was in agreement with the literature values reported for humans (Cole and Scriver, 1980; Lin and Levy, 1983; Morris and Levy, 1983). However, the levels we report are lower than those previously reported for normal individuals in this geographic location. Hendrix-Treacy et al (1986) reported an average value over 6 hours of 0.550 ± 0.080 mmoles/L. The high natural sulfate content of soil and water in Saskatchewan was thought to contribute to an average serum sulfate level higher than that reported by other workers. Geographic locations can influence the sulfate content of the environment and thus the serum level of inorganic sulfate. This is the case in Rosetown, Saskatchewan where high sulfate levels

in drinking water result in higher average serum sulfate levels (D.Mayers, M.Sc. dissertation, 1988). However, since both the present investigation and the study of Hendrix-Treacy et al. (1986) were conducted in the same city (Saskatoon) the discrepancy in serum sulfate levels reported by Hendrix-Treacy et al. (1986) as compared to the values found in the present investigation is most likely attributable to the differences in analytical methodology utilized in the two studies. Hendrix-Treacy et al. (1986) obtained their values by use of a modified turbidimetric assay procedure; the present investigation utilized the more precise ion chromatography methodology.

During a 24 hour period from 7 am to 7 am the next day, the serum inorganic sulfate level averaged 360.5 ± 29.2 μmoles/L (Table 5). In individual 24 h profiles, the minimum serum level (302.3 ± 40.2 μmoles/L) occurred between 7 am and 3 pm (mean: 11 am ± 2.6h). The serum levels peaked between 3 pm and 11 pm (mean: 7 pm ± 3.3 h) at an average value of 407.8 ± 42.7 μmoles/L. The difference between the minimum and maximum values in individuals averaged 25.8 ± 6.3 %. The maximum and minimum values were significantly different (paired t-test, $p<0.05$). When the average serum inorganic sulfate concentrations were plotted against time the minimum of 328.1 ± 57.4 μmoles/L occurred at 11 am and the maximum of 394.5 ± 40.2 μmoles/L at 9 pm (Figure 11). In all subjects, serum sulfate levels fluctuated over the 24 hour period but the pattern of sulfate levels varied among individuals.

Table 5: Serum inorganic sulfate levels in healthy men (mean ± S.D.) during Phase 1 (n=7, 24 h control study)

PARAMETER	Phase 1 24h period (7am-7am)
C_{AVE} (μM)	360.0 ± 29.2
C_{MAX} (μM)	407.8 ± 42.7
C_{MIN} (μM)	302.3 ± 40.2
ΔC (%)	25.8 ± 6.3
T_{MAX} (h)	7:00pm ± 3.3
T_{MIN} (h)	11:00am ± 2.6

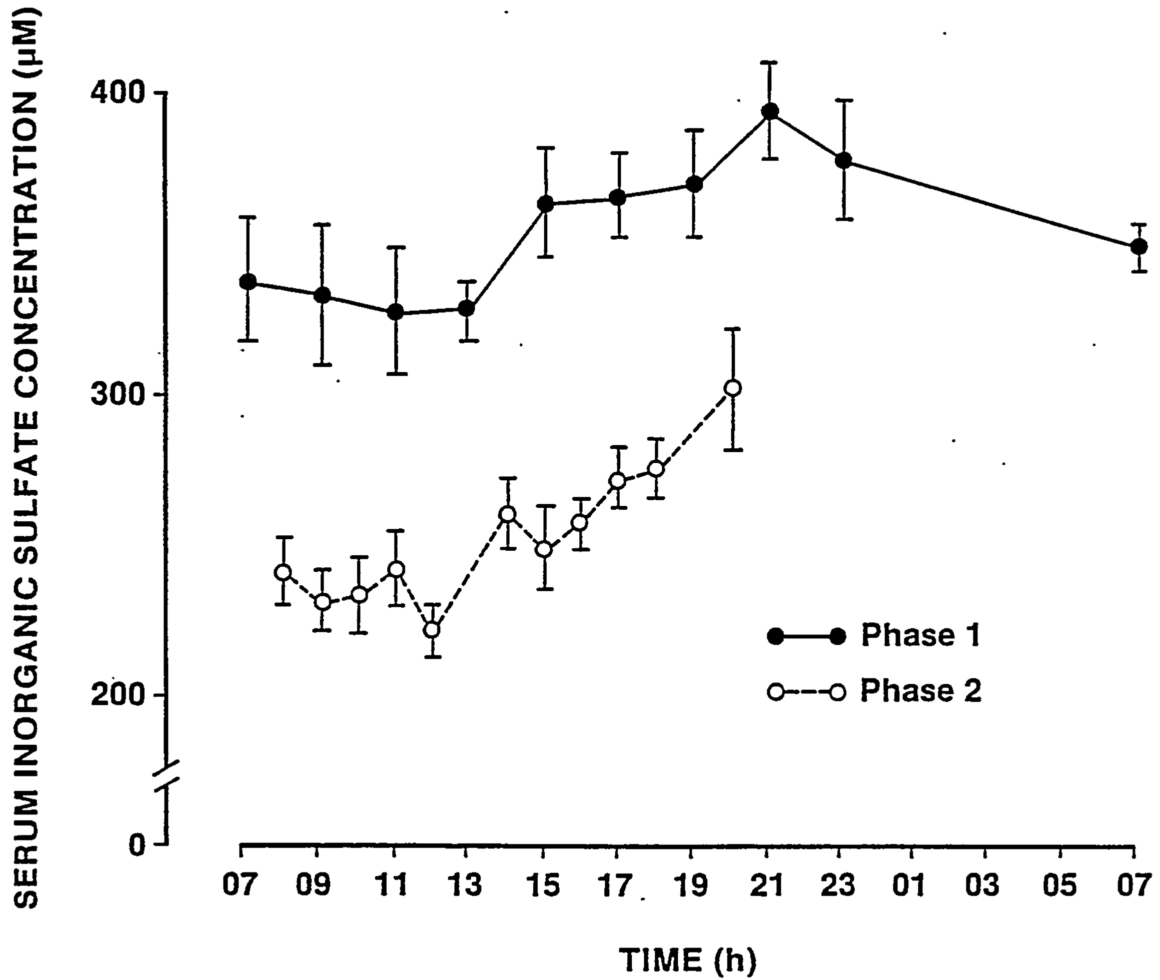

Figure 11: Circadian variation in serum inorganic sulfate levels in healthy male volunteers (mean ± S.E.M.; Phase 1 n=7 and Phase 2 n=6).

The average urinary excretion rate of inorganic sulfate over the 24 h period was 12.5 ± 2.4 μmoles/h/kg (Table 6, Figure 12). The renal clearance of inorganic sulfate was relatively constant over the 24 hour span (Figure 13) and averaged 0.58 ± 0.08 mL/min/kg (Table 6).

Table 6: Renal clearance and excretion rate of inorganic sulfate in healthy men (mean ± S.D.) during Phase 1 (n=7, 24 h control study)

PARAMETER	Phase 1 24 h period (7am-7am)
Urinary excretion rate of inorganic sulfate (umoles/h/kg)	12.5 ± 2.4
Renal clearance of inorganic sulfate (mL/min/kg)	0.58 ± 0.08

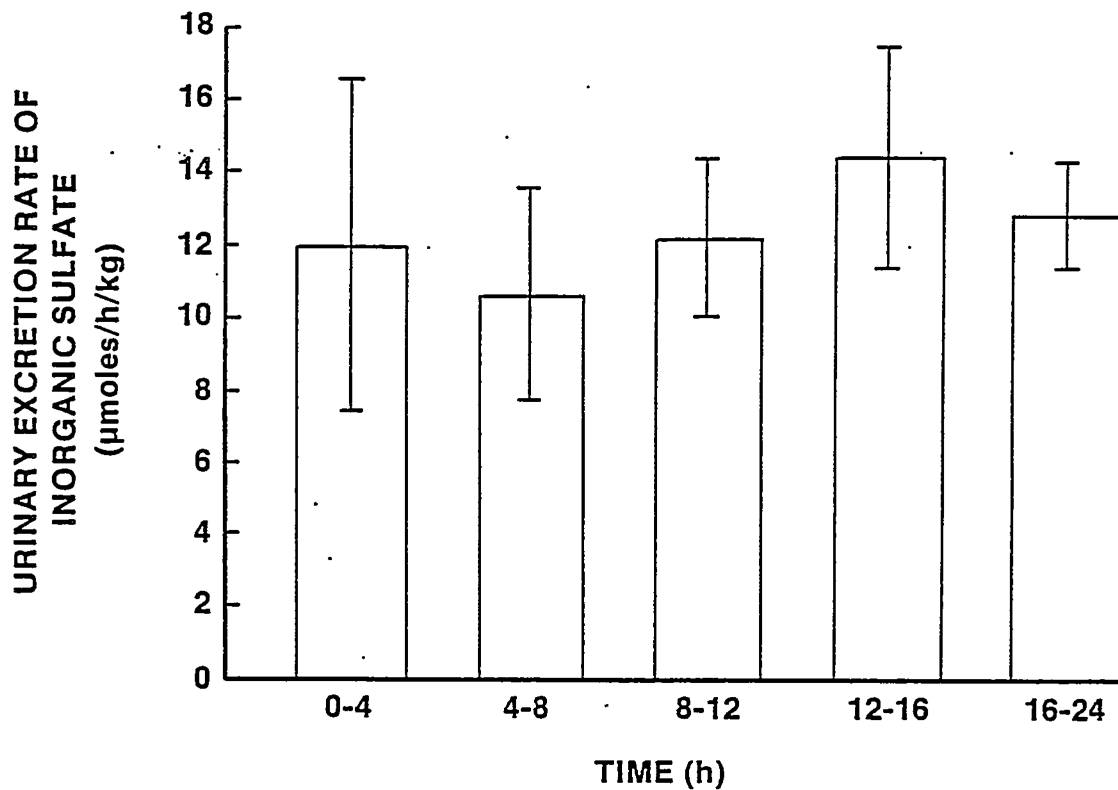

Figure 12: Circadian variation in urinary excretion rate of inorganic sulfate in 7 healthy male volunteers (Mean ± S.D.; Phase 1, 24 h control study).

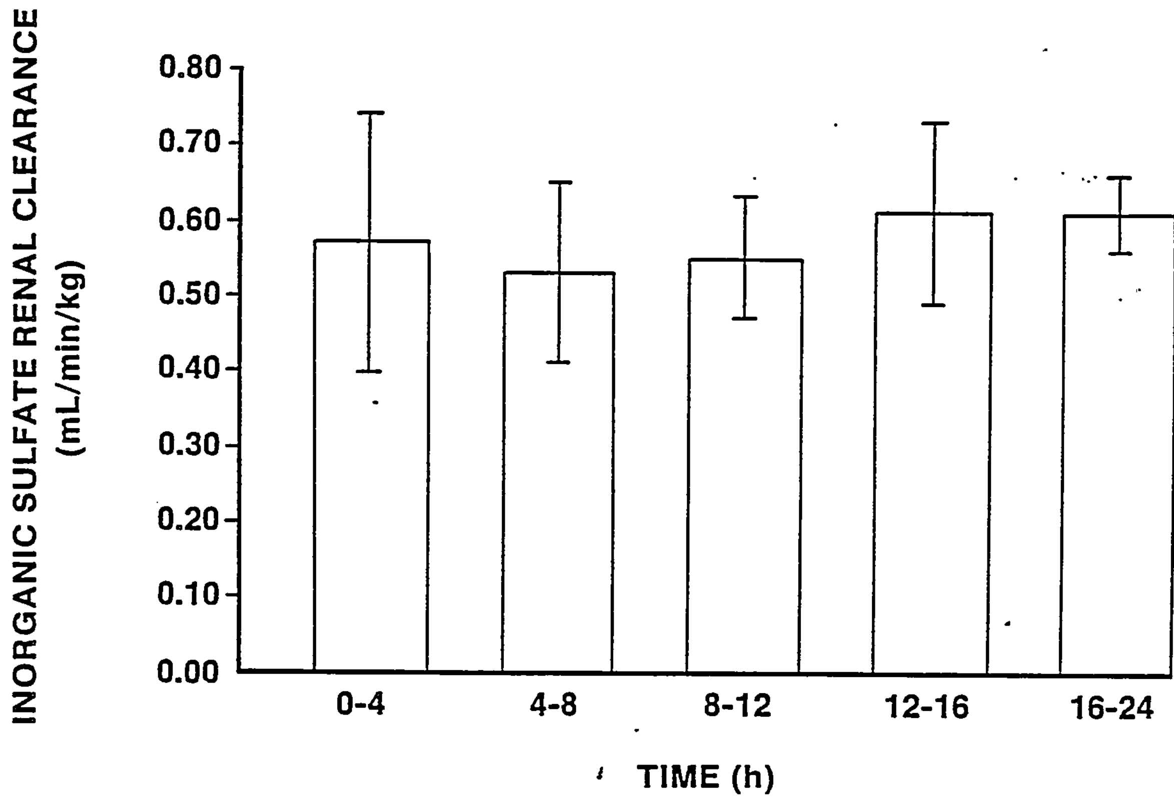

Figure 13: Circadian variation in inorganic sulfate renal clearance in 7 healthy male volunteers (Mean ± S.D.; Phase 1, 24 h control study).

To characterize the pharmacokinetics of a substance which is metabolized by sulfoconjugation the kinetics of endogenous inorganic sulfate should also be monitored. The disposition of compounds undergoing sulfoconjugation can be affected by factors influencing the physiological availability and disposition of inorganic sulfate (Levy, 1986). The availability of inorganic sulfate and cosubstrates such as PAPS are determined by supply and demand; variables which interrupt the equilibrium of the supply and demand chain can alter the sulfate homeostasis and may influence the overall sulfoconjugation process. The serum sulfate level is an important component of sulfoconjugation and this value reflects the equilibration between intake, distribution to tissue pools and renal excretion (Cole and Scriver, 1980).

Results of initial experiments indicated there was a circadian rhythm of serum inorganic sulfate levels. Reports in the literature offered support for our findings. Krijgsheld et al. (1980) reported a circadian rhythm for serum inorganic sulfate in the rat, and suggested a rhythm was expected since sulfate levels can be affected by many factors including the dietary consumption, the metabolic demand and the rate of urinary excretion. Although not referenced in the 1980 report by Krijgsheld et al., evidence of a circadian rhythm for inorganic sulfate in humans was published in 1978 by Meier and Schmidt-Kessen. A minimum level was noted before noon (11 am) and a maximum in the evening and late afternoon (6 pm). There was

approximately a 14.5 % difference between the average maximum serum value and the average minimum value, and a 23 % difference in urinary excretion rates over a 24 h period (Meier and Schmidt-Kessen, 1978). In rats, the serum level peaks in the afternoon (rest cycle) and when the lights are off (active cycle) it decreases staying relatively low during the night. This difference represents approximately a 30 % variation in serum levels (Krijgsheld et al., 1980). The activity - rest cycle and possibly the dietary pattern during these two periods may be major synchronizers (zeitgebers) in the circadian rhythm of serum inorganic sulfate in the rat.

During Phase 1 of the present investigation the percentage of change between minimum and maximum sulfate levels (approximately 18 %) and urinary excretion rate (approximately 29 %) were slightly higher than those reported by Meier and Schmidt-Kessen, (1978). The times and values of the average serum-concentration time curves were in general agreement with the only other published study in humans (Meier and Schmidt-Kessen , 1978) and confirms the circadian variation of serum inorganic sulfate in man. The reasons for the circadian rhythm in man remain unknown. Intake of food has been suggested as a factor contributing to the rhythm.

Dietary protein intake can contribute to some variation in serum sulfate (Cole and Scriver, 1980) and is one factor affecting sulfate homeostasis in man (Tallgren, 1980). Cole and Scriver (1980) report sulfate levels in adults who fasted

overnight were lower than random daytime values which were more variable and skewed upwards (0.29 ± 0.03 mmol/L versus 0.33 ± 0.06 mmol/L). This represents approximately a 12 % change between the averages. Tallgren (1980) has proposed the inorganic sulfate level in serum probably peaks 6 to 8 hours following the intake of a large amount of protein or amino acid sulfur. In one subject, varying the protein intake from 20 grams daily for 10 days to 120 grams daily for 10 days had no effect on the morning fasting serum sulfate levels (Tallgren, 1980).

Standard meals were utilized on the sampling days in both Phases of our study. During Phase 2 the meal administration times were all advanced one-half hour to minimize the interference with drug ingestion. Snack times were unaltered. Diet is difficult to control in man and the sulfate content of most foods is not known (P.Jeffries, personal communications, 1988) but, we decided the individual variability of diet was a possible systematic difference we would attempt to minimize with respect to timing and content on the sampling days in the event this variable is an important synchronizer in the inorganic sulfate circadian rhythm.

3.4 Inorganic Sulfate Disposition: Phase 2

Serum and urine samples were collected from six volunteers during two consecutive dosing intervals between

administration of acetaminophen. The intervals covered time periods of 8 am - 2 pm and 2 pm - 8 pm.

The mean serum inorganic sulfate level during the first six hour interval (236.0 ± 20.4 μmoles/L) was significantly lower ($p<0.05$) than during the second six hour interval (269.0 ± 24.1 μmoles/L) (Figure 11). The difference of serum inorganic sulfate between the two dosing intervals was on average 12.2 %. Over a 12 hour block, the minimum levels in each individual occurred between 10 am and 3 pm (mean 12 noon ± 2.4 h). The peak level occurred at 8 pm in all but two subjects. The average time of the maximum level was 6:50 pm ± 2.7 h. The minimum level (211.0 ± 16.6 μmoles/L) was significantly less than the maximum (308.9 ± 38.7 μmoles/L) ($p< 0.05$). The average difference between the minimum and maximum level was 31.3 ± 5.1 %. Based on the plot of average serum inorganic sulfate at each time, a minimum level of 220.1 ± 20.8 μmoles/L occurred at 12 noon; a maximum of 300.5 ± 47.8 μmoles/L at 8 pm.

The urinary excretion rates (5.3 ± 1.9 and 6.7 ± 2.2 μmoles/h/kg) and renal clearance (0.37 ± 0.12 and 0.41 ± 0.12 mL/min/kg) of inorganic sulfate for the two dosing intervals were not significantly different.

The results for the 12 h periods of Phase 1 (7 am - 7 pm) and Phase 2 (8 am - 8 pm), which coincided most closely, were compared (Tables 7 and 8; Figures 14 and 15).

Table 7: Serum inorganic sulfate levels in healthy men (mean ± S.D.) during Phase 1 and Phase 2 of the study

PARAMETER	Phase 1 12h period (7am-7pm)	Phase 2 12h period (8am-8pm)	p-value
C_{AVE} (μM)	352.3 ± 35.4	252.5 ± 18.9	**
C_{MAX} (μM)	403.9 ± 39.0	308.9 ± 38.7	**
C_{MIN} (μM)	319.9 ± 34.9	211.0 ± 16.6	**
ΔC (%)	20.5 ± 8.5	31.3 ± 5.1	*
T_{MAX} (h)	4:00pm ± 3.0	6:50pm ± 2.7	NS
T_{MIN} (h)	11:40am ± 1.0	12 noon ± 2.4	NS

Number of subjects = 6
Phase 1 (Control)
Phase 2 (Multiple dose acetaminophen administration)

* $p < 0.05$
** $p < 0.01$
NS no significant difference

Table 8: Inorganic sulfate urinary excretion rates in healthy men during Phase 1 (n=6) and Phase 2 (n=6) of the study (mean ± S.D.)

PARAMETER	Phase 1 12 h period (7am-7pm)	Phase 2 12 h period (8am-8pm)	p-value
Urinary excretion rate of inorganic sulfate (μmoles/h/kg)	12.2 ± 2.9	6.0 ± 1.9	**
Renal clearance of inorganic sulfate (mL/min/kg)	0.58 ± 0.11	0.39 ± 0.10	*

* $p < 0.05$

** $p < 0.01$

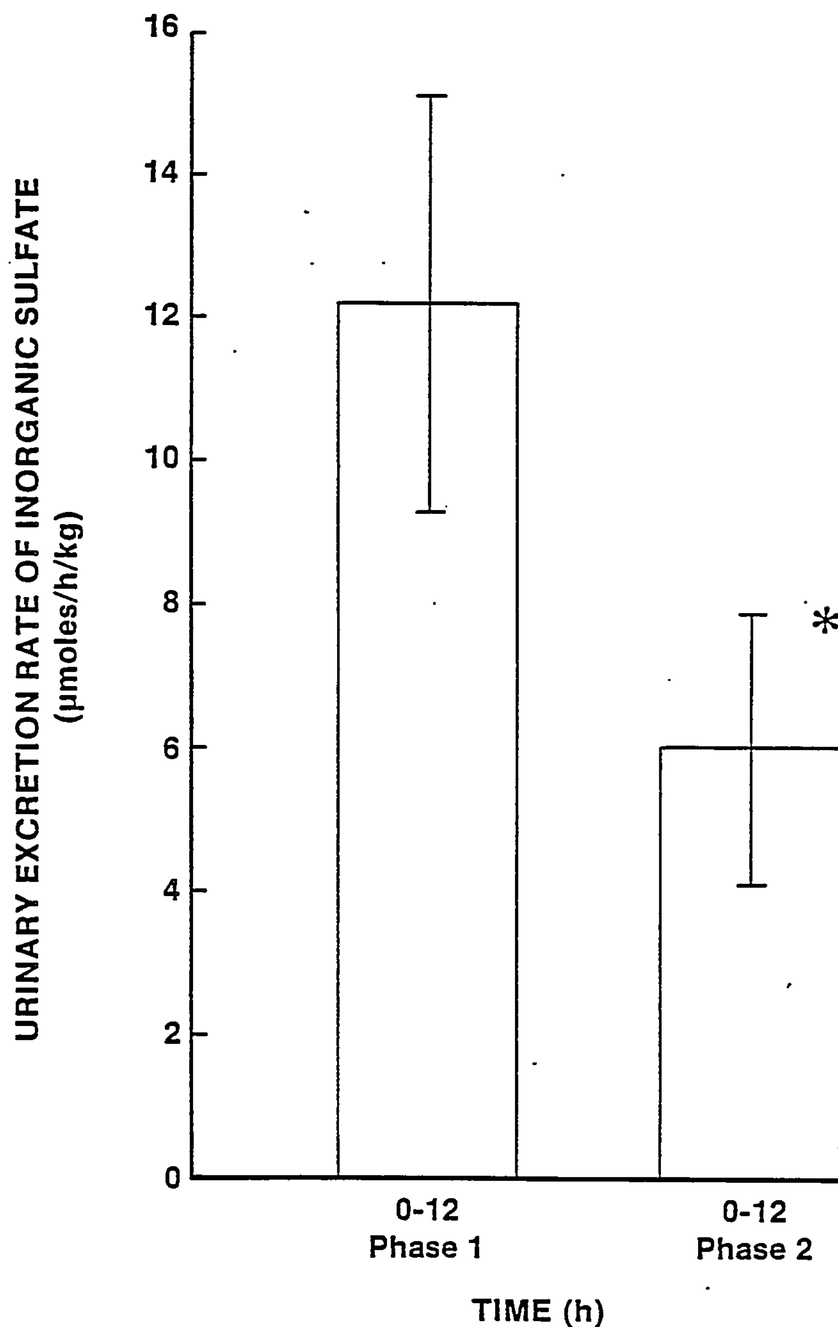

Figure 14: Effect of multiple dose acetaminophen administration on the mean (±S.D.) 0-12 h urinary excretion rate of inorganic sulfate in healthy male volunteers (Phase 1 n=6 and Phase 2 n=6; * $p<0.05$, paired t-test).

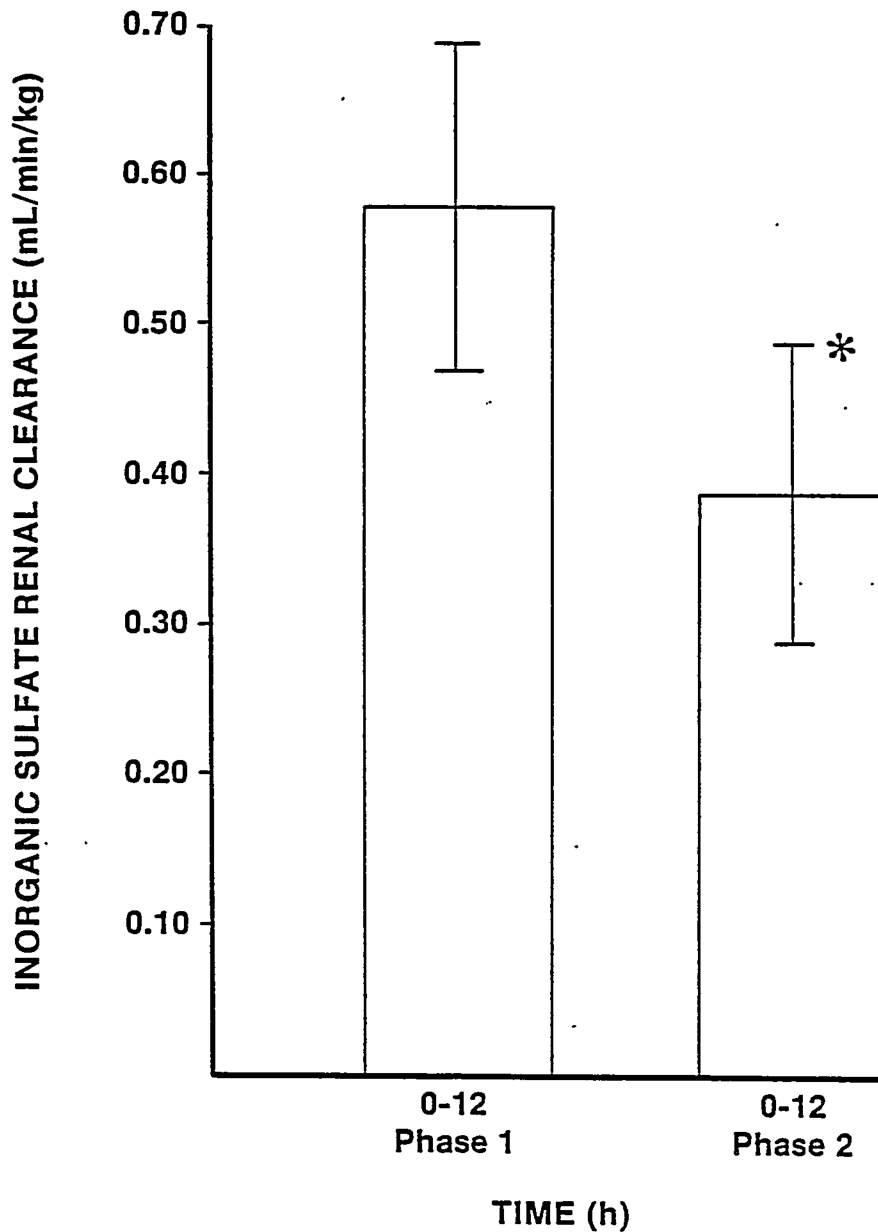

Figure 15: Effect of multiple dose acetaminophen administration on the mean (± S.D.) 0-12 h inorganic sulfate renal clearance in healthy male volunteers (Phase 1 n=6 and Phase 2 n=6; * $p<0.05$, paired t-test).

For the 12 h period of Phase 1 the mean ± standard deviation was calculated for the six subjects who completed both Phase 1 and Phase 2 of the study. The mean serum inorganic sulfate level for the 12 h period (7 am - 7 pm) (352.3 ± 35.4 μmoles/L) was not significantly different from the 24 h value. The minimum level (319.9 ± 34.9 μmoles/L) occurred between 7 am and 3 pm (mean 11:40 am ±1 h) and the maximum (403.9 ± 39.0 μmoles/L) at 4 pm ± 3 h. The average difference between the minimum and maximum values was 20.5 ± 8.5 %. The average plot of serum inorganic sulfate concentration versus time profiles showed a minimum level of 332.2 ± 61.8 μmoles/L at 11 am and a maximum at 7 pm (372.6 ± 46.8 μmoles/L).

The urinary excretion rate of inorganic sulfate over the 12 h period was 12.2 ± 2.9 μmoles/h/kg and very similar to the 24 h value. The renal clearance of inorganic sulfate (0.58 ± 0.11 mL/min/kg) was relatively constant over the 12 h period and virtually unchanged from the 24 h value.

In both Phase 1 and 2 the patterns of serum sulfate levels over time were remarkably similar. In Phase 1 the difference between the maximum and minimum levels was 25.8 ± 6.3 %; in Phase 2, 31.5 ± 5.1 %. The average Phase 2 levels were, however, 28.1 ± 4.7 % lower than the Phase 1 levels. The administration of multiple dose acetaminophen caused a decrease in the serum inorganic sulfate levels but did not abolish the circadian rhythm (Figure 11). There was still a sizeable fluctuation (31.3 ± 5.1 %) in the serum levels over the course of

the two acetaminophen dosing intervals (Phase 2).

The excretion rate (6.0 ± 1.9 µmoles/h/kg) (Figure 14) and renal clearance (0.39 ± 0.10 mL/min/kg) of inorganic sulfate (Figure 15) over the 12 h span were significantly lower than during Phase 1 when no acetaminophen was taken (12.5 ± 2.4 µmoles/h/kg and 0.58 ± 0.08 mL/min/kg respectively).

Levy _et al_. (1982) reported the maximum sulfate depleting effect of acetaminophen occurred about two hours after oral administration. Hendrix-Treacy _et al_. (1986) noted a net drop in serum sulfate levels 1 - 2 hours after a single dose. After multiple doses a drop in levels was seen extending over a period of 0.5 - 2 hours after the last dose. During their single dose study more than half of the subjects had a rise in levels 0.5 - 1 hour after the dose and prior to the drop -- something not previously reported. A mobilization of the sulfate into the blood in response to an increased need for sulfate in the metabolism of acetaminophen was offered as an explanation. A similar rise prior to the drop was not seen in the sulfate levels with multiple dose administration. Some of the fluctuation in serum inorganic sulfate levels noted after administration of acetaminophen by Hendrix-Treacy _et al_. (1986) may have been the natural circadian rhythm of inorganic sulfate. In Phase 2, serum sulfate levels decreased slightly at one hour (times are post-dose with drug administration at 0h and 6h) and again at four hours, (more pronounced than the decrease at 1 h) and rose again by six hours. There was also a slight decrease at seven

hours. In the control study (Phase 1) there was a slight decrease at the time corresponding to the decrease at four hours in Phase 2 but there was no decrease seen at the corresponding seven hour time (Figure 11). The more pronounced decreases observed at one and seven hours (one hour following the afternoon dose) in Phase 2 may correspond to the utilization of inorganic sulfate in forming the AS conjugate.

A decrease in the urinary excretion of sulfate does not necessarily indicate a decrease in the serum level, it may indicate that the sulfate is being used for metabolic processes (Lin and Levy, 1983). In our study, the excretion rate of sulfate was more than 50 % lower when acetaminophen was administered. The renal clearance of inorganic sulfate was substantially reduced in Phase 2. Lin and Levy, (1983) and Hendrix-Treacy et al. (1986) also reported a decrease in the clearance of inorganic sulfate after acetaminophen administration and speculated there is renal tubular reabsorption of this ion when it is depleted or there is an increased need for it in the metabolism of acetaminophen; i.e., a feedback mechanism can decrease the renal clearance of inorganic sulfate when there is an increased need for the ion. The results of the present investigation support this hypothesis.

3.5 Acetaminophen Disposition: Phase 2

The serum acetaminophen concentrations varied widely among the six volunteers. A representative (Figure 16) and the average (Figure 17) serum concentration - time profile during two consecutive dosing intervals at steady state are illustrated. The peak serum concentrations for the two dosing intervals were not significantly different. The average peak serum concentration occurred at one hour post drug ingestion for both intervals. The minimum acetaminophen level varied from 1.1 to 4.4 μg/mL during the two dosing intervals and always occurred immediately prior to the next dose (or six hours post-dose). The minimum level for both intervals averaged 2.2 ± 1.3 μg/mL. There was no significant difference between the minimum values obtained during the two intervals ($p > 0.05$, paired t-test).

The acetaminophen glucuronide and the acetaminophen sulfate levels also varied widely among the six volunteers (Figure 17). The times of minimum and maximum concentrations for the two conjugates were more variable among individuals than for acetaminophen. The maximum serum level of the glucuronide conjugate averaged 14.9 ± 12.0 μg/mL and 12.4 ± 8.1 μg/mL for the first and second dosing intervals respectively (NS, $p > 0.05$). During the first interval the maximum occurred at two hours following acetaminophen ingestion and at 1.5 h post-dose in the second interval. The minimum glucuronide level was 4.8 ± 4.1 μg/mL for the first interval and for the second, 5.8 ± 4.2 μg/mL

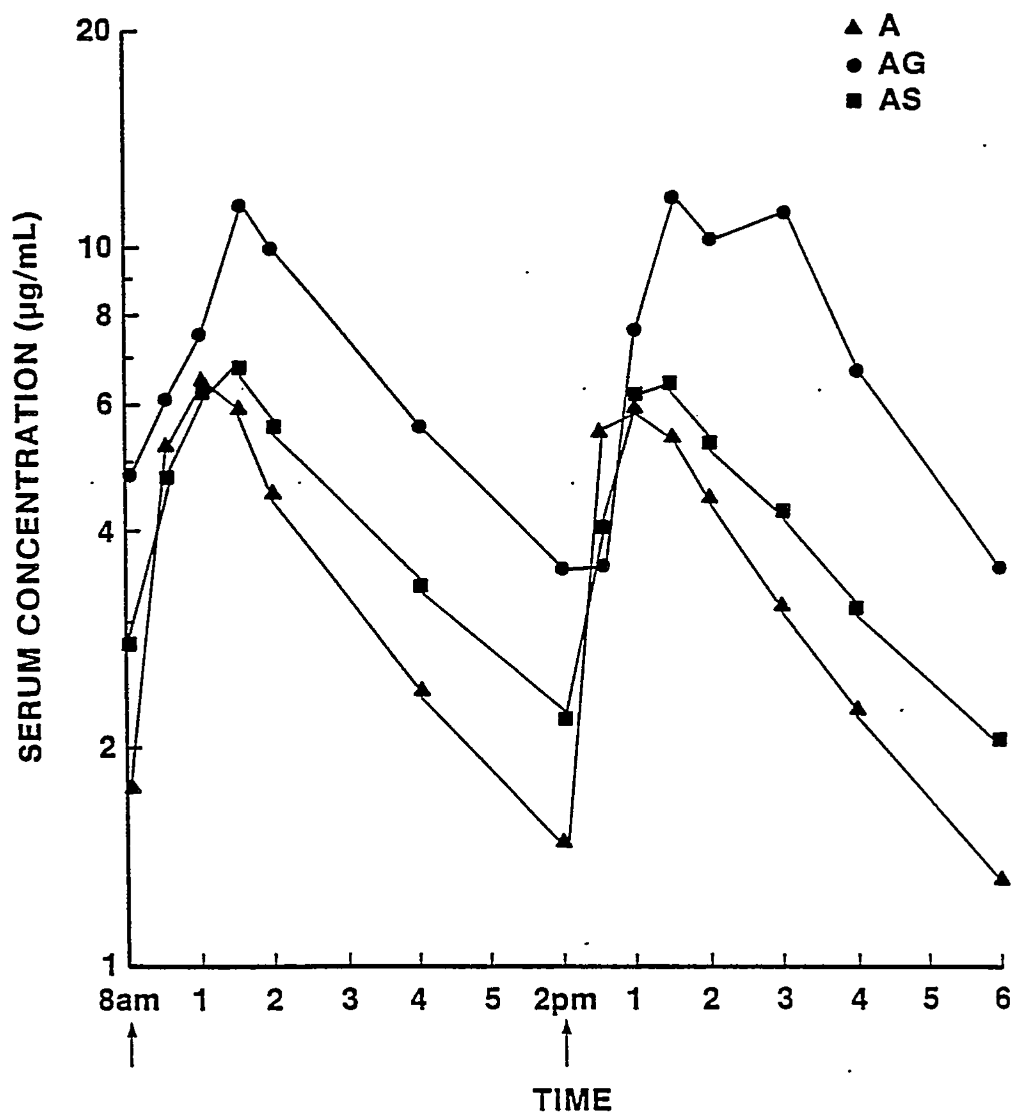

Figure 16: Serum concentrations of acetaminophen, acetaminophen glucuronide and acetaminophen sulfate following administration of 650 mg acetaminophen at steady state (Subject DM, semi-log plot).

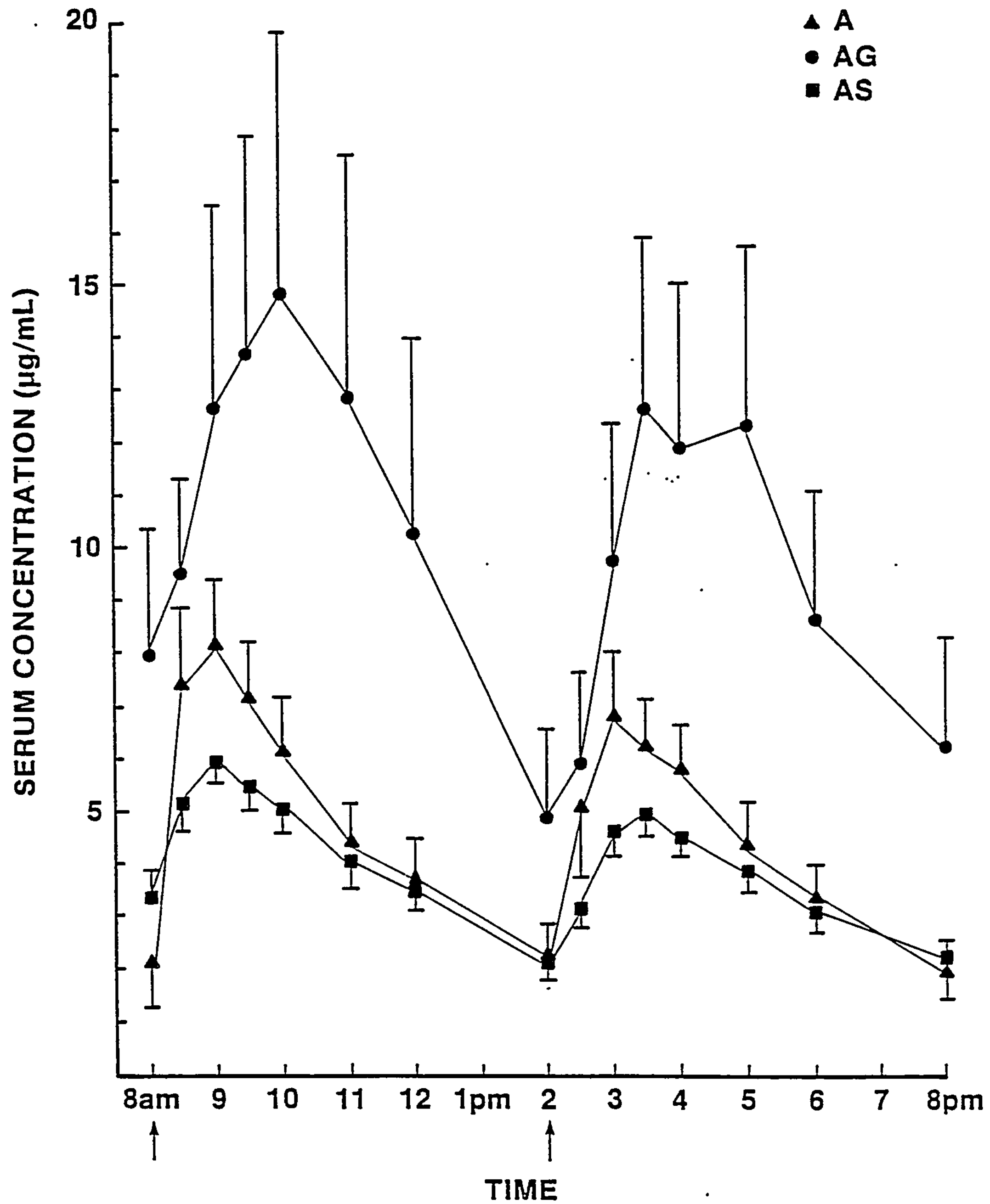

Figure 17: Serum concentrations of acetaminophen, acetaminophen glucuronide and acetaminophen sulfate following administration of 650 mg acetaminophen at steady state (means ± S.E.M. of six volunteers).

(NS, $p > 0.05$). For both intervals the minimum value occurred at six hours following drug ingestion (immediately prior to the next dose).

For the sulfate conjugate of acetaminophen the maximum serum values obtained were 5.9 ± 0.8 μg/mL and 4.8 ± 0.9 μg/mL for the first and second intervals respectively (NS, $p > 0.05$). The maximum was observed at one hour post-dose in the first interval; at 1.5 h in the second. The minimum levels averaged 2.1 ± 0.8 μg/mL and 2.2 ± 0.8 μg/mL for the two intervals (NS, $p > 0.05$). The minimum value occurred at six hours following drug ingestion.

The pharmacokinetic characteristics of acetaminophen are summarized in Table 9, the glucuronide and sulfate conjugates in Table 10. The terminal elimination half-life for acetaminophen was slightly but significantly longer in the second dosing interval than the first (2.38 ± 0.11 h versus 2.12 ± 0.17 h). In all but one subject (KH) the half-life of the sulfate conjugate was also longer in the second dosing interval, but the mean difference did not reach statistical significance ($0.10 > p > 0.05$). The half-life for AG was not significantly different between the first and second intervals ($p > 0.05$).

The AUC for acetaminophen and AS were similar but less than half the AUC for AG. Although the AUC for acetaminophen and AS were slightly higher for the 8 am - 2 pm interval than for the 2 pm - 8 pm interval in all subjects, the difference was not statistically significant. The AUC values for AG showed no

Table 9: Pharmacokinetic characteristics of acetaminophen following multiple dose administration of 650 mg to six subjects; Comparison for two consecutive dosing intervals (mean ± S.D.)

PARAMETER	Interval 1 (8am-2pm)	Interval 2 (2pm-8pm)	p-value
Half-life (h)	2.12 ± 0.17	2.38 ± 0.11	*
Elimination rate constant (h^{-1})	0.328 ± 0.026	0.292 ± 0.014	*
AUC (μg x h/mL)	28.64 ± 11.94	25.80 ± 10.71	NS
Volume of distribution Vd/F (L/kg)	0.99 ± 0.36	1.22 ± 0.38	NS
Total body clearance (CL/F) (mL/min/kg)	5.32 ± 1.60	5.89 ± 1.76	NS
Partial clearance as metabolites (mL/min/kg)			
A-->AG	3.48 ± 1.24	4.01 ± 1.36	NS
A-->AS	1.66 ± 0.58	1.76 ± 0.62	NS
Renal clearance of acetaminophen (mL/min/kg)	0.14 ± 0.03	0.12 ± 0.02	NS

* $p < 0.05$
NS no significant difference ($p > 0.05$)

Table 10: Pharmacokinetic characteristics of acetaminophen glucuronide and acetaminophen sulfate following multiple dose administration of 650 mg to six subjects; Comparison for two consecutive dosing intervals (mean ± S.D.)

PARAMETER	Interval 1 (8am-2pm)	Interval 2 (2pm-8pm)	p-value
Half-life (h)			
AG	2.34 ± 1.06	2.13 ± 0.61	NS
AS	2.43 ± 0.41	2.85 ± 0.29	NS*
Elimination rate constant (h^{-1})			
AG	0.384 ± 0.279	0.348 ± 0.101	NS
AS	0.292 ± 0.049	0.244 ± 0.022	NS*
AUC (μg x h/mL)			
AG	63.58 ± 45.73	55.99 ± 35.62	NS
AS	24.46 ± 4.64	21.16 ± 4.02	NS
Renal clearance (mL/min/kg)			
AG	4.33 ± 4.02	5.06 ± 3.54	NS
AS	2.34 ± 0.09	2.76 ± 0.38	NS

NS no significant difference
NS* p value between 0.05 and 0.10

pattern in individuals and mean values were not significantly different for the two intervals.

The apparent volume of distribution (Vd/F) for acetaminophen was slightly lower in each subject for the 8 am - 2 pm interval than the 2 pm - 8 pm interval (0.99 ± 0.36 L/kg and 1.22 ± 0.38 L/kg respectively). For each subject, the total body clearance of acetaminophen was higher during the second interval (5.89 ± 1.76 mL/min/kg) than during the first (5.32 ± 1.60 mL/min/kg). The partial clearances of the two conjugates were also higher in each individual (with the exception of the AS partial clearance in subject GG) during the second dosing interval. However, the difference in Vd/F, total and partial metabolic clearances were not statistically significant ($p > 0.05$, paired t-test).

The renal clearance of acetaminophen in all but one subject (KH) was lower in the second (0.12 ± 0.02 mL/min/kg) than the first interval (0.14 ± 0.03 mL/min/kg). There was no pattern among the subjects for the renal clearance of the glucuronide conjugate. On the other hand, the renal clearance of acetaminophen sulfate was lower in the earlier dosing interval for all subjects except one (BL). The observed differences in the renal clearances fail to reach statistical significance in all cases.

The total amount of acetaminophen and metabolites recovered in the urine represented 76.5 to 111.0 % of the administered dose (Table 11).

Table 11: Urinary recovery of acetaminophen and metabolites following multiple dose administration of 650 mg acetaminophen to six subjects. Mean (± S.D.) of recoveries expressed as percent of recovered dose for two consecutive dosing intervals

COMPOUND	Recovery (%) Interval 1 (8am-2pm)	Interval 2 (2pm-8pm)	p-value
Acetaminophen	3.85 ± 2.67	2.47 ± 1.15	NS
Acetaminophen glucuronide	64.65 ± 6.53	67.44 ± 6.37	NS
Acetaminophen sulfate	31.50 ± 5.99	30.10 ± 6.12	NS
TOTAL*	96.41 ± 15.74	93.40 ± 12.10	NS

NS no significant difference, $p > 0.05$

(Note: * for TOTAL recovery n=5 as subject KH was excluded from calculations due to an incomplete urine collection for Interval 1).

The recovery for subject KH was low due to an incomplete urine collection during the first interval. When data for this subject was excluded, the average recovery (n=5) for the first interval was 96.41 ± 15.74 % of the administered dose and 93.40 ± 12.10 % for the second interval. Total recovery for the two intervals was not significantly different. In all individuals, the glucuronide conjugate constituted a higher percent of the recovered dose in the second dosing interval, whereas the sulfate conjugate was higher in the first interval. The differences were not statistically significant. In all individuals the percent of recovered dose represented by unchanged acetaminophen was slightly higher during the first interval but the difference was not statistically significant.

Many reports on the disposition of acetaminophen in rats (Lin and Levy, 1981) and man (Morris and Levy, 1983) have been published. However, very few of these reports have dealt with disposition at steady state conditions or chronic administration (Hendrix-Treacy *et al*., 1986). We measured the serum concentrations of acetaminophen and the glucuronide and sulfate conjugates obtained at steady state and also examined the chronopharmacokinetics of acetaminophen at steady state. To our knowledge this is the first report of both the serum levels of conjugates at steady state and steady state chronopharmacokinetics.

The serum and urine levels of the compounds were measured in healthy volunteers. The various kinetic parameters

calculated were in agreement with those found in the literature. The C_{max} values for acetaminophen varied over a two fold range. The very high C_{max} values observed in a couple of cases may be explained in part by some accumulation of the drug or the result of slightly incorrect timing of doses for example, the inconvenience of the 2 am dose may have caused some subjects to deviate slightly from the exact six hour intervals between doses. During the course of our study the administration times were stressed and volunteers were asked to immediately report any late or missed dose. In order to confirm compliance the volunteers were supplied with extra tablets and at the end of the dosing regimen the extra tablets were counted to ensure the proper number had been utilized. No discrepancies were found with this compliance check.

The time for peak acetaminophen levels after oral administration has been reported as 20 minutes, 60 minutes and 1.4 hours for tablets (Forrest et al., 1982; Prescott, 1980), and was 1.0 hour in the present study. The average serum concentration-time profiles for the measured compounds (Figure 17) were similar in terms of relative concentrations to the curves reported by Prescott (1980)(i.e. AG concentrations were highest, A next and AS concentrations the lowest). Although the data reported by Prescott (1980) was based on administration of a single dose of acetaminophen, concentration curves were similar to those in the present study with slight variations in the time of average maximums. Prescott (1980) reported the AS curve

showed a peak level at 1.4 h which was earlier than the AG curve (2.4 h). We observed similar findings for the first interval (1.0 h for AS and 2.0 h for the AG curve). During the second interval both peak values occurred at 1.5 h (the AG value at 3 h was only slightly lower than that observed at 1.5 h).

The average half-life values reported for acetaminophen during the two intervals (2.12 h and 2.38 h respectively) were similar to other values in the literature (Hendrix-Treacy et al., 1986; Miller et al., 1976; Nelson and Morioka, 1963; Prescott, 1980; Rawlins et al., 1976; Slattery et al., 1987). The value for the volume of distribution is in good agreement with other reports in the literature (Lowenthal et al., 1976; Prescott, 1980; Rawlins et al., 1977) as are the total body clearance values (Clements and Prescott, 1976; Lowenthal et al., 1976; Rawlins et al., 1977).

The design of the present study allowed comparison of the disposition during two consecutive dosing intervals so chronopharmacokinetic considerations could be examined. Other chronopharmacokinetic studies on acetaminophen have been performed with single doses. The only pharmacokinetic parameter which was significantly different during the two intervals was the acetaminophen half-life (significantly longer in the second interval).

Shively and Vesell (1975) (using single doses) reported that the half-life of acetaminophen was significantly longer in normal volunteers when the drug was given at 6 am as opposed to 2

pm. The decrease in half-life was approximately 15 %. This change was presumed to be due to a change in the apparent volume of distribution -- which decreased by about 13 % after the 2 pm dose. We also noted a significant change in the acetaminophen half-life (a change of slightly over 12 %). However, the half-life was longer after the 2 pm dose rather than the early morning dose (8 am). Malan et al. (1985) used a one gram oral dose of acetaminophen in six volunteers and report no significant differences in the $t½$, C_{max}, t_{max} or AUC_{po} for three separate administration times -- 8 am, 2 pm and 8 pm. The time of administration did not cause significant changes in the absorption or disposition pattern of acetaminophen (Malan et al., 1985).

Kamali et al. (1987) using single 1.5 gram oral doses of acetaminophen, reported some changes in the disposition of acetaminophen over time. These changes centred on the glucuronide metabolite; more of this compound was found in the 0 - 4 hour urine fraction when the drug was given at 8 am compared with other times. The smallest percentage of AG was found with administration at 8 pm. There was no influence of time on acetaminophen sulfate disposition in their study. The total body clearance, half-life and volume of distribution also did not differ at 8 am or 8 pm. Prescott (1980) reported the AS conjugate initially accounted for a very high proportion of the total amount of the drug excreted but the proportion decreased with time. This is consistent with a limited and decreased

capacity for sulfate conjugation (Prescott, 1980).

The serum inorganic sulfate levels were, on average, 12.2 % lower in the first six hour dosing interval than the second interval. The excretion rate and renal clearance of sulfate were slightly higher during the second interval than during the first. On average AS recovery values were higher in the first than the second interval. The slightly lower renal excretion rate of inorganic sulfate during the first interval may, in part, be linked to an increased production of AS and utilization of the sulfate supply in sulfoconjugation processes, but is more likely due to the lower serum inorganic sulfate levels.

In 1986 Levy reported the proximate cause of nonlinear pharmacokinetics of sulfoconjugation for phenolic drugs as the limited availability and consequent depletion of inorganic sulfate. If the nonlinear pharmacokinetics are primarily influenced by this one variable, in the absence of enzyme kinetic involvement and other influencing factors, then one would have expected an influence of the serum inorganic sulfate circadian rhythm on acetaminophen sulfate formation. However several factors must be considered. Hendrix-Treacy <u>et al</u>. (1986) suggest that sulfate levels in the serum may not always be rate controlling or reflect the supply of activated sulfate for the sulfation of acetaminophen. Under the conditions of our study, the circadian variation of serum inorganic sulfate levels did not significantly influence the metabolism of acetaminophen. The

greater decrease in average serum inorganic sulfate levels produced by multiple dose acetaminophen administration than the normal fluctuation between the minimum and maximum serum sulfate levels provides further information on the sulfate depleting capabilities of acetaminophen.

4.0 SUMMARY

The Present Investigation consisted of two phases. In Phase 1 seven young, healthy men were used to confirm and characterize the circadian rhythm of serum inorganic sulfate over a 24 hour period. Our results are in good agreement with the only other report on the circadian variation of serum inorganic sulfate levels in man (Meier and Schmidt-Kessen, 1978). Serum levels fluctuated from a minimum value at between 7 am and 3 pm (11 am ± 2.6h) to a maximum at between 3 pm and 11 pm (7 pm ± 3.3h). Minimum and maximum serum levels averaged 302.3 ± 40.2 μmoles/L and 407.8 ± 42.7 μmoles/L respectively. Mean serum sulfate levels over the 24 h period were 360.0 ± 29.2 μmoles/L (mean ± S.D.). The urinary excretion rate of inorganic sulfate over the 24 h period was 12.5 ± 2.4 μmoles/h/kg. Renal clearance of inorganic sulfate was 0.58 ± 0.08 mL/min/kg and was relatively constant over the 24 h period. The serum sulfate levels, urinary excretion rate and renal clearance of inorganic sulfate were similar to values reported in the literature.

Six of the seven subjects who completed Phase 1 participated in Phase 2. Phase 2 examined the disposition of acetaminophen at steady state during two consecutive dosing intervals to determine if any chronopharmacokinetic patterns existed under these conditions. The mean serum sulfate level over the 12 h period (8 am - 8 pm) was 252.5 ± 18.9 μmoles/L. This value was significantly lower ($p<0.01$) than the value

obtained for the 12 h period of Phase 1 which most closely corresponded to Phase 2 (7 am - 7 pm; 352.3 ± 35.4 μmoles/L). Both the maximum and minimum serum sulfate levels obtained during Phase 2 were significantly lower ($p<0.01$) than the corresponding values obtained for the 12 h period of Phase 1. The times the maximum and minimum levels occurred were not significantly different between the two Phases. On average the serum sulfate levels were 28.1 ± 4.7 % lower during Phase 2 than during Phase 1. Although the serum levels were lower during multiple dose acetaminophen treatment, the circadian rhythm of serum inorganic sulfate was still evident. The pattern of the serum inorganic sulfate circadian rhythm was remarkably similar for the two phases with the exception of lower levels in Phase 2. The urinary excretion rate of inorganic sulfate was reduced by more than 50 % in Phase 2 (6.0 ± 1.9 μmoles/h/kg) ($p<0.01$). The renal clearance of inorganic sulfate was also significantly reduced ($p<0.05$) in Phase 2 (0.58 ± 0.11 mL/min/kg vs. 0.39 ± 0.10 mL/min/kg).

Glucuronide and sulfate conjugates as well as the parent compound acetaminophen were measured in serum at steady state during morning and afternoon dosing intervals. Calculated pharmacokinetic parameters were in agreement with literature values. Only one parameter (the acetaminophen $t_{\frac{1}{2}}$) was significantly different ($p<0.05$) between the morning (8 am) and afternoon (2 pm) dosing intervals. The acetaminophen half-life was approximately 12 % longer after the 2 pm dose (2.12 ± 0.17 h

vs 2.38 ± 0.11 h). This was presumed to be primarily due to a nonsignificant increase in the volume of distribution after the 2 pm dose. There were no significant differences in the urinary recovery profiles between the two administration times.

5.0 CONCLUSIONS

1. There is a small but consistent circadian variation in serum inorganic sulfate levels in healthy men.

2. Multiple dose acetaminophen (650 mg q.6.h. for 4 days) decreases the serum inorganic sulfate level by 28.1 ± 4.7 % but does not abolish the circadian rhythm.

3. The decrease in renal clearance of inorganic sulfate at depressed serum sulfate levels, i.e., following subchronic acetaminophen administration, is consistent with conservation of inorganic sulfate by increased tubular reabsorption.

4. Sulfation of acetaminophen at steady state is not significantly different between the morning (8 am) and afternoon (2 pm) administration periods.

6.0 LIMITATIONS OF THE INVESTIGATION

The purpose of this investigation was to examine the interaction between the circadian rhythm of serum inorganic sulfate and the administration of a substrate for sulfation -- namely acetaminophen. As with all scientific investigations, there are some theoretical and practical limitations associated with the present investigation which should be acknowledged:

Number of subjects: Although a larger sample size would have increased the statistical power of the study, numerous factors such as recruiting healthy volunteers and economic constraints dictated that a smaller, but reasonable, number of subjects participate. Choice of the model compound: Acetaminophen was chosen as the 'model' compound because of its common usage, metabolism mainly to glucuronide and sulfate conjugates and a half-life conveniently short so that elimination of the drug should occur within a six to eight hour period. An alternate 'model' compound with a more extensive elimination via sulfoconjugation may have produced a more dramatic effect on the circadian rhythm of serum inorganic sulfate; or, for that matter, an effect of the rhythm on the drug's metabolic pattern.

Administration of multiple doses: There are a number of limitations inherent with multiple dose studies. Timing of the administered doses is crucial and the dosage regimen must be rigidly adhered to. Slight deviations from the administration times (although not reported by any of the volunteers completing

the present study) may result in a "carryover" effect. This phenomenon is avoided during single dose administration studies. If smaller doses had been used in the multiple dose regimen or, if two separate single dose studies had been conducted a slightly different pattern of results may have emerged. However, the multiple dose regimen used in the present study is characteristic of the common usage pattern of the medication. Time of administration: In both single and multiple dose studies the timing of drug administration in relation to the serum inorganic sulfate circadian rhythm could influence the results. Should the compound be administered closer to the times that minimum and maximum sulfate levels occur? If so, how far in advance of these times in order to maximize the effects? These questions are not easily answered. At some point though, the manipulation of the administration times in relation to the circadian pattern may lose all practical significance and become entirely academic in nature. In our case, administration times were similar to those of regular use and the two study periods represented the low and high portions of the circadian variation curve; i.e., the average serum sulfate levels were significantly different for the morning and afternoon doses.

The present investigation will serve to facilitate the design of future studies in this area.

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