EFFECTS OF EXPOSURE TO POLYBROMINATED BIPHENYLS ON URINARY STEROID METABOLIC PROFILES IN MAN AND RATS AS DETERMINED BY CAPILLARY GC AND GC/MS/DS

By

John James Vrbanac, Jr.

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

1983

ABSTRACT

EFFECTS OF EXPOSURE TO POLYBROMINATED BIPHENYLS
ON URINARY STEROID METABOLIC PROFILES IN MAN AND RATS
AS DETERMINED BY CAPILLARY GC AND GC/MS/DS

By

John James Vrbanac, Jr.

Polyhalogenated organic compounds have been shown to be potent inducers of mixed-function oxidases (MFOs) responsible for the metabolism of a wide range of compounds including steroid hormones. Since induction of MFO enzyme systems increases the metabolism of steroids and since alterations in steroid hormone secretion and metabolism may be reflected by changes in the urinary steroid metabolic profile, the effects of exposure to PBBs (potent MFO inducers) on steroid hormone excretion were investigated by examining urinary steroid metabolic profiles in exposed humans and in an experimental model (rats).

Advantages and disadvantages of using capillary column GC/FID and packed and capillary columns GC/MS/MSSMET (an off-line reverse library search analysis) were investigated. Capillary GC/FID was adequate for quantitative analysis of most steroids isolated from human urine. The major drawback to using capillary GC/FID was the time involved in analysis of the GC traces. Generation of human urinary steroid metabolic profiles by GC/MS/MSS was faster, but a major drawback was the relative insensitivity of this method compared to capillary column GC/FID.

Exposure of male and female rats to PBBs etc was observed to alter the urinary steroid metabolic profile in the corticosteroid region of the profile.

Urinary 6β-hydroxycortisol, a well-characterized indicator hepatic cytochrome P-450 mixed function oxidase activity, was increased in the PBBs-exposed humans (non-smoking healthy adult male subjects with greater than 50 ppb PBBs in their serum). This effect was less than that seen following chronic exposure to high therapeutic doses of phenobarbital, but was similar in magnitude to increases in urinary 6β-hydroxycortisol excretion reported for workers exposed to DDT. Certain other steroids, all but one of adrenal origin, appeared to be elevated in the PBBs exposed population.

ACKNOWLEDGEMENTS

The author gratefully acknowledges the staff and students associated with the National Institutes of Health/Michigan State University Mass Spectrometry Facility, especially Millie Martin, Linda Seymour, Jennifer Valentine, Dave Pinkston, Lee Westover, Mike McPherson, Rick Chapman, Brian Musselman, Vicki McPharlin, Charles C. Sweeley, J. Throck Watson and John F. Holland. My gratitude is also extended to all committee members, Theodore M. Brody, W. Emmett Braselton, Jr., John F. Holland, J. Throck Watson and Jerry B. Hook, for extending to me their valuable time, and to Karen DeFord for preparation of this manuscript.

Special thanks to my major advisor, W. Emmett Braselton, Jr., and to Charles C. Sweeley, for their expert guidance throughout this project.

Finally, I extend my sincere gratitude to my wife, Patricia, and to Mary Ellen and J.D. for support and friendship.

TABLE OF CONTENTS

-PRELIMINARY PAGES-

Acknowledgements. ii

List of Tables. v

List of Figures vi

-TEXT PAGES-

SECTION I. General Introduction. 1

A. Objectives. 1

B. Background. 3

1. Mixed function oxidases 3

2. Effects of xenobiotics on steroid metabolism and related subjects 6

3. Metabolic profiling 16

C. Rationale 37

SECTION II. Development of Analytical Methods. . . . 38

A. Introduction. 38

B. Experimental. 40

C. Results and Discussion. 49

SECTION III. Animal Studies. 83

A. Introduction. 83

B. Experimental. 92

C. Results 93

D. Discussion. 121

Table of Contents (continued)

SECTION IV. Human Studies. 123

A. Introduction 123

B. Experimental. 130

C. Results 132

D. Discussion. 162

SECTION V. Conclusions and Direction 172

APPENDIX I. Michigan Department of Public Health, Division of Environmental Epidemiology Health Questions. 178

APPENDIX II. Individual Human Subject Health Information 186

APPENDIX III. Glossary of Terms. 190

LITERATURE CITED. 191

LIST OF TABLES

TABLE		PAGE
1	Precision: GC profiling of urinary steroids.	52
2	Precision: Capillary column GC/MS profiling of urinary steroids	68
3	Effect of chronic exposure to PBBs on female rat urinary steroids	96
4	Changes in urinary excretion of some corticosterone metabolites in female rats following acute exposure to PBBs.	99
5	Urinary metabolites of corticosterone in control and PBBs exposed adult male rats	114
6	Urinary steroid metabolic profiles of a normal adult male: 24 hour excretion patterns over a 6-day period . . .	133
7	Urinary steroid metabolic profiles of a normal adult male: Morning spot urine excretion patterns over a 6-day period	136
8	Average urinary excretion patterns over a 6-day period in absolute amounts for a 24-hour urine collection relative to creatinine and a morning spot urine collection	138
9	Determination of 6@-hydroxycortisol by automated reverse library search of selected mass chromatograms. . .	146
10	Human urinary steroid metabolic profiles of non-smoking male subjects: Average plus standard deviation, normalized to the internal standard and urinary creatinine	153
11	Unknowns elevated in the urine of PBBs exposed human subjects as determined by capillary GC with FID.	154
12	Human urinry steroids in controls, PBBs exposed and phenobarbital exposed individuals as determined by GC/MS/DS	156
13	Possible consequences of induction and/or inhibition of liver microsomal enzyme systems	166

LIST OF FIGURES

FIGURE		PAGE
1	GC/MS/DS analyses of urinary steroids from a normal female.	18
2	Description of MSSMET library files	22
3	Description of found file	25
4	Description of the normalized found file.	29
5	Example of a typical MSSMET quantitative printout (Found File) obtained for a normal female human urinary steroid sample.	30
6	Flow diagram for isolation and chemical derivatization of urinary steroids by a modification of the method of Leunissen and Thijssen (1978)	45
7	Urinary steroid metabolic profile of a post-puberal pre-menopausal female human obtained by capillary column GC with flame ionization detection.	50
8	Urinary steroid metabolic profile of a normal adult male subject obtained using a 60 m DB-1 bonded phase fused silica capillary column	54
9	GC/MS analysis of aliphatic hydrocarbon standards and urinary steroids using a 3 m, 3% OV-101 packed column . .	57
10	GC/MS analysis of urinary steroids in the region of RI 2500-3100 using a 3 m, 3% OV-101 packed column.	60
11	GC/MS analysis of urinary steroids using a 50 m (0.3 mm I.D.) OV-101 WCOT fused silica capillary column	62
12	GC/MS analysis of urinary steroids using a 50 m OV-101 WCOT fused silica capillary column.	65
13	Urinary steroid metabolic profile of a normal adult male subject	70
14	Changes in peak shape and number of points across a gas chromatogarphic peak following increasing amounts of n-pentadecane injected into a 25 m OV-101 fused silica capillary column (0.32 mm I.D., 150°C isothermal)	74

LIST OF FIGURES (continued)

FIGURE		PAGE
15	Total ion intensity v. scan number in the region of the chromatogram where 11β-hydroxyandrosterone, 11β-hydroxy-etiocholanolone and the internal standard elute	76
16	Urinary steroid metabolic profiles using a 25 m OV-101 fused silica capillary column with FID (top trace) and the same column with MS detection (repetitive scanning; 2 s scan cycle)	78
17	Urinary steroid metabolic profiles of female rats exposed to 100 ppm PBBs chronically (top trace) and of an appropriate control group.	94
18	Mass spectrum of the MO-TMS derivative of a tetrahydroxy-pregnane-one that increased in the urine of PBBs exposed female rats	100
19	Mass spectrum of the MO-TMS derivative of a tetrahydroxy-pregnane-one that decreased in the urine of PBBs exposed female rats	102
20	Mass spectrum of the MO-TMS derivative of a pentahydroxy-pregnane-one that decreased in the urine of PBBs exposed female rats	104
21	Capillary column GC traces obtained for three different fractions from Sephadex LH-20 column chromatography . . .	109
22	Capillary column GC traces obtained for two different fractions from Sephadex LH-20 column chromatography . . .	111
23	Mass spectrum of a 5α-pregnane-3,11,16,20,21-pentol . . .	116
24	Capillary GC trace of urinary steroids obtained for a group of four male rats before (control) and after ("germ-free") antibiotic treatment.	118
25	Electron impact mass spectrum of the methoxime-trimethyl-silyl derivative of 6β-hydroxycortisol.	145
26	Dot plots of urinary concentrations of 6β-hydroxycortisol in phenobarbital and control groups	148
27	Dot plots of urinary concentrations of 6β-hydroxycortisol in PBBs exposed and control groups.	150
28	Plots of Student's t-statistic when urinary steroid data are randomly reassigned	168

SECTION I

General Introduction

SECTION I

OBJECTIVES

The long range goal of this work was to develop a non-invasive procedure for the identification of individuals with significant xenobiotic-induced alterations in steroid metabolism. Initial efforts focused on development of capillary column gas chromatography (GC; a glossary of terms is listed in Appendix III) with flame ionization detection (FID) and gas chromatography/mass spectrometry/data system (GC/MS/DS) methods for qualitative and quantitative analysis of urinary steroids. It was believed that these methods would allow certain xenobiotic-induced changes in steroid hormone metabolism to be monitored using urine samples. It has been demonstrated that many environmental chemicals that either stimulate or inhibit mixed function oxidase (MFO) enzyme systems alter steroid hormone metabolism in a similar manner. For this project polybrominated biphenyls (PBBs) were chosen as a model to represent xenobiotic stimulators of MFO enzyme systems.

The immediate objectives of this project were to:

1) Develop a quantitative procedure for the isolation, purification and derivatization of steroids from urine and plasma.

2) Develop capillary column GC and an automated GC/MS/DS technique for the quantitative analysis of a mixture of steroids isolated from urine or plasma.

3) Use the rat as a model to examine the effects of PBBs on the urinary steroid metabolic profile and correlate these changes with known specific effects these chemicals have on steroid metabolism.

4) Extend the study to a human population by examining the steroid metabolic profiles of a group of subjects accidentally exposed to PBBs.

BACKGROUND

1. Mixed function oxidase enzyme systems:

The term "mixed-function" oxidase was first used by Mason (1957) to describe certain types of biological oxidations. This term describes particular enzyme complexes which require molecular oxygen and NADPH and which exhibit a wide range of substrate specificities. In these reactions one atom of oxygen appears in the metabolized substance and one atom of oxygen appears in water. Steroid hormone lyases and hydroxylases are part of this general enzyme system. The mixed-function oxidase sytem is also responsible for the oxidation of lipids and for the oxidative detoxication of a large number of harmful xenobiotics. In general, the mixed-function oxidase enzymes responsible for the oxidations of xenobiotics, lipids and steroids are associated with smooth endoplasmic reticulum, although their synthesis is apparently associated with the rough endoplasmic reticulum.

A carbon monoxide binding pigment in liver microsomes was first described by Klingenberg (1958) and Garfinkel (1958). This pigment was later named cytochrome P-450 since it was observed to have an absorption maximum at 450 nm when combined with carbon monoxide and was shown to be a protoporphyrin hemoprotein of the cytochrome b-5 type (Omura and

Sato, 1964). It is now recognized that the cytochrome P-450 system is involved in the transfer of electrons to oxygen to form water and oxidized product in the above mentioned mixed-function oxidase systems, and that there is a wide range of substrates of endogenous and exogenous origin that undergo oxidation, reduction or are otherwise metabolized (i.e., clevage reactions for example) by cytochrome P-450 complexes (Wiekramasinghe, 1975; Blumberg, 1978; Makin, 1975; Schulster, 1976). Cytochrome P-450 participation in steroid hydroxylation reactions has been shown to occur in the mitochondria and microsomes of those tissues associated with steroid synthesis (Wiekramasinghe, 1975; McIntosh et al., 1971 and 1973; Mason et al., 1973; Meigs and Ryan, 1968). The concentration and distribution of the various cytochrome P-450 enzyme complexes is quite dependent upon such variables as sex, age, metabolic state and environmental factors (McIntosh et al., 1973; McKerns, 1968).

It is well established that there are multiple forms of cytochrome P-450 for the metabolism of substances of both endogenous and exogenous origin (Wiekramasinghe, 1975; Blumberg, 1978). Sladek and Mannering (1966) were the first to obtain evidence for more than one form when they noticed that changes in the carbon monoxide difference spectrum occurred when animals were pretreated with polycyclic aromatic hydrocarbons. The carbon monoxide difference spectrum maximum had shifted from 450 mμ to 448 mμ in these experiments and this hemoprotein has come to be termed

cytochrome P-448. Two classical types of microsomal mixed-function oxidase enzyme induction have evolved using the above classification. Phenobarbital is the classical inducer of the P-450 type cytochrome and 3-methylcholanthrene (3-MC) is the classical inducer of the P-448 type cytochrome (Sladek and Mannering, 1969a, 1969b). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is an agent that also belongs to the 3-MC classification of inducer. The 3-MC type induction affects the metabolism of a smaller number of compounds than the phenobarbital type induction. In some cases, pretreatment of subjects with a specific inducer will affect the pharmacological/toxicological response to a second agent (the effect can be either augmented or attenuated). For example, barbiturate-induced sleeping time is reduced by pretreatment of subjects with phenobarbital.

Phenobarbital-like induction also results in proliferation of the endoplasmic reticulum. This type of induction has been characterized by a number of enzyme assays, aminopyrine-N-demethylase and epoxide hydrolase being two of the better known examples. 3-MC-like induction results in an abnormal liver pathology and has also been characterized using a number of specific enzyme assays (benzo[a]pyrene hydroxylase and UDP-glucuronyl-p-nitrophenol glucuronyl transferase for example).

2. Effects of xenobiotics on steroid metabolism and related subjects:

It is well established that many different drugs and environmental chemicals affect both hepatic and extrahepatic MFO activity (Conney, 1971, 1976; Conney et al., 1973). These effects can result in an alteration in the intensity and duration of action of drugs metabolized by the systems. For example, phenobarbital, benzo[a]pyrene, DDT, polychlorinated biphenyls (PCBs), phenylbutazone and aminopyrene stimulate oxidative drug metabolizing enzymes in liver microsomes while chloramphenicol, chlorthion, carbon monoxide, and bis-hydroxycoumarin inhibit these enzymes (Conney et al., 1973). Since the microsomal MFO system is important in steroid hormone biochemistry and xenobiotic induction of MFOs has been shown to affect steroid metabolism it follows that xenobiotic-induced alterations in MFO activity could influence endocrine balance to the extent that biological functions such as reproduction may be influenced.

Induction by PCBs. Contamination of much of the ecosystem by PCBs is considered to be a serious environmental concern. PCBs are lipid soluble compounds and are chemically stable. PCBs have been observed to cross the placental barrier and have been found in milk (Orberg, 1977; Allen and Barsotti, 1976; Kuratsune et al., 1972; Takagi et al., 1976). PCBs increased the hepatic metabolism of

testosterone, 4-androstene-3,17-dione and estradiol-17β in the chicken (Nowicki and Norman, 1972). This altered hepatic metabolism of steroids is believed to be the cause of PCBs-induced egg shell thinning in certain species of birds (Haegele and Tucker, 1974) since the plasma concentrations of estrogens and progesterones are believed to be critical in this process. Rats, mice and rhesus monkeys treated with PCBs showed elevated corticosterone concentrations and increased size of the adrenals (Wasserman et al., 1973; Sanders et al., 1974; Barsotti and Allen, 1975). Interestingly, in one study the "total plasma corticoids" in mice was reported to be slightly reduced (Sanders and Kirkpatrick, 1975). Exposure to PCBs resulted in decreased urinary dehydroepiandrosterone in the boar (Plantonow et al., 1972). PCBs also increased the catabolism of exogenously administered progesterone, estradiol-17β and testosterone in rodents, an effect attributed to PCBs-induced increases in microsomal enzyme activity (Orberg and Kihlstrom, 1973; Orberg and Lundberg, 1974; Orberg and Ingvast, 1977; Derr, 1978). In general, the highly chlorinated PCBs have a greater stimulatory effect on microsomal MFO enzymes than the lowly chlorinated PCBs (Bickers et al., 1974; Orberg, 1976). Thus, PCBs affect the metabolism of androgens, estrogens and corticosteroids and would therefore be expected to affect physiological processes regulated by these steroids.

Exposure to PCBs has been observed to result in a

variety of toxicities to the reproductive system. Women exposed to PCBs and other chlorinated hydrocarbons have shown menstral cycle irregularities and dysmenorrhea (Kuratsune et al., 1972) and rhesus monkeys exposed to PCBs showed lengthened menstral cycles (Allen and Barsotti, 1976). Female mice exposed to PCBs showed a reduction in the number of ova implanted following mating and a lengthened estrus cycle (Orberg and Kihlstrom, 1973; Orberg et al., 1972) and pregnant rabbits treated with PCBs showed reduced litter sizes (Villeneuve et al., 1971a and 1971b). Certain of the lowly chlorinated PCBs show estrogenic activity, an effect which is apparently obscured in complex PCBs mixtures by altered steroid metabolism and other factors (Nelson, 1974; Bitman and Cecil, 1970; Gellert, 1978). Thus, PCBs decrease the reproductive capability of mammals, an effect that at least, in part, probably relates to effects on steroid hormone metabolism.

Induction by PBBs. Polybrominated biphenyls (PBBs) were accidentally added to dairy cow feed in Michigan resulting in a contamination of the food supply (Dunckel, 1975). Like PCBs, PBBs accumulate in milk and fat tissues and are chemically stable (Fries and Marrow, 1974; Matthews et al., 1977, 1978; Rickert et al., 1978). Also, like PCBs, PBBs are potent stimulators of liver and extrahepatic microsomal MFO enzyme systems (Dent et al., 1976; McCormack et al., 1978, 1979; Arneric et al., 1980; Newton et al., 1980). Initial studies investigating PBBs indicated that they had

characteristics similar to the TCDD-type toxic polyhalogenated aromatic-hydrocarbons and also the classical inducer phenobarbital and therefore were classified as "mixed" inducers (Dent, 1976; Sladek and Mannering; 1969a, 1969b). PBBs were noted to be inducers of liver microsomal aryl hydrocarbon hydroxylase as well as enzymes which are induced by phenobarbital. PBBs exposure was observed to elicit a toxicity syndrome similar to TCDD in that PBBs caused 1) pathological changes in the liver similar to those seen following TCDD, 2) depressed body weight gains, 3) were immunotoxic, 4) and produced edema in chicks (Jackson and Halbert, 1974; Ringer and Polin, 1977). Because PBBs are a mixture of various congeners with different chemical properties, it was of interest to define the chemical nature of the mixture and the biological effects of the various PBBs congeners present in Firemaster BP-6 and other commercial PBBs preparations. The particular commercial mixture of PBBs which contaminated Michigan contained 10 components each of which made up greater than 1% of the mixture, with three of these components accounting for 75% of the mixture (Moore and Aust, 1978; Moore et al., 1978, 1980). These considerations are important since the chemical and toxicological properties of individual pure congeners are highly variable. In particular, those congeners with 2 or more ortho bromines are relatively non-toxic and have a phenobarbital-like effect in that they induce liver microsomal enzymes usually induced by phenobarbital and cause a

proliferation of hepatic endoplasmic reticulum. Congeners without bromines in the ortho position have a toxicity similar to TCDD and induce microsomal enzymes usually associated with 3-methylcholanthrene exposure. Those congeners with one ortho bromine show moderate toxicity and were "mixed" inducers of liver microsomal enzymes, having both 3-methylcholanthrene phenobarbital-like effects (Dannan et al., 1978; Moore et al., 1980). Individual components of the commercial mixture of PBBs which contaminated Michigan all contained at least one ortho bromine and 89% of the total mixture contained two ortho bromines. Thus, it would be reasonable to expect that one of the possible effects of this particular mixture of PBBs would be a "phenobarbital-like" effect in the exposed human population.

PBBs reduced the response to exogenous steroid sex hormones (McCormack et al., 1979). PBBs can undergo trans-placental movement and, being lipid soluble, are present in the milk of exposed lactating females. The offspring of rats fed a diet containing PBBs from the 8th day of pregnancy until 28 days postpartum had significant concentrations of PBBs in liver, kidney and fat tissues 300 days later (McCormack et al., 1980a, 1980b). The offspring also showed renal and hepatic enzyme stimulation, a reduction in pentobarbital sleeping time and histopathological alterations. Dietary PBBs administration to rats by the above regime altered in vitro metabolism of progesterone by hepatic microsomal MFOs in the offspring (Arneric et al., 1980).

In the same study PBBs were reported to increase the metabolism of progesterone to both 16α- and 6β-hydroxyprogesterone. This effect was sex dependent in immature animals and resembled the MFO stimulatory effects of phenobarbitol more that 3-methylcholanthrene in this system. Metabolism of estradiol, estrone and ethynyl estradiol by hepatic microsomes was also increased by exposure to PBBs (Newton et al., 1980; Bonhaus et al., 1982). The effects of PBBs treatment on the activities of testosterone 16α-hydroxylase, 7α-hydroxylase, 6β-hydroxylase and 17β-dehydrogenase were also studied using the above treatment protocol. 7α-Hydroxylase activity was stimulated by PBBs in males and females. This effect was more pronounced in females at 2 and 4 months of age. 16α-Hydroxylase activity was increased in females of all ages while only the immature males showed an increase in activity. 6β-Hydroxylase activity was increased in both sexes at all ages. 17β-Dehydrogenase activity was elevated at all ages in treated females and in one month old males. Interestingly, 10 ppm PBBs increased 16α-hydroxylase and 17β-dehydrogenase activities in 8 week old males but 100 ppm PBBs did not, suggesting that high concentrations of PBBs may actually inhibit steroid metabolism of these two enzymes In the same series of studies (Newton et al., 1980, 1982), PBBs also decreased the reductive metabolism of testosterone to the more potent androgens 5α-dihydrotestosterone and 5α-dihydroandrostenedione.

As one might expect, the reproductive toxicities associated with PCBs have also been seen following PBBs exposure. PBBs administration lengthened the estrus cycles in rats and rhesus monkeys (Johnston et al., 1980; Lambrecht et al., 1978). The effect in monkeys was associated with attenuated serum progesterone peaks (Lambrecht et al., 1978). Perinatal PBBs exposure delayed vaginal opening in rats (McCormack et al., 1980b; Harris et al., 1978). PBB exposure reduced spermatogenesis and caused testicular atrophy in bulls, and resulted in hypoactive seminiferous tubules in rhesus monkeys (Allen et al., 1978; Jackson and Halbert, 1974). Thus, like PBCs, exposure to PBBs stimulated the metabolism of androgens and estrogens and interfered with reproductive processes.

PBBs treatment has been observed to have significant effects on the metabolism of sex steroids. PBBs increased the conversion of progesterone to 16α- and 6β-hydroxy-progesterone and increased the oxidative metabolism of estradiol, estrone and ethynyl estradiol. PBBs treatment also increased testosterone metabolism and induced a reduction in androgenicity of testosterone, an effect which may be brought about in three ways: 1) decreased 5α-reduction of testosterone to 5α-dihydrotestosterone and 5α-dihydro-androsterone, 2) enhanced oxidation of testosterone by 6β-, 7α- and 16α-hydroxylases, 3) enhanced 17β-oxidation to androstenedione.

Since the major route of elimination of conjugated and unconjugated metabolites of steroid hormones is through renal excretion, the above xenobiotic-induced changes in steroid hormone metabolism should be reflected in the amounts of steroid metabolites in the urine. For example, increased urinary excretion of 16α- and 6β-hydroxylated metabolites of progesterone and 16α-, 7α- and 6β-hydroxylated metabolites of testosterone might be observed in PBBs-treated subjects. In general, one might expect that large increases in the metabolism of any steroid hormone should be reflected by increased urinary excretion of its metabolites.

Many compounds have been reported to inhibit microsomal MFO activity. For example, the halogenated hydrocarbons chlorthion and carbon tetrachloride decreased microsomal MFO activity (Conney et al., 1973). Certain heavy metals such as cadmium, lead and organic mercurial compounds also decreased MFO activity (Conney et al., 1973; Means and Schnell, 1979, 1980; Meredith et al., 1977; Ahotupa et al., 1979; Aitio et al., 1978). Cadmium treatment caused a marked inhibition of hepatic microsomal MFO drug metabolizing enzyme activity (Means and Schnell, 1980; Hadley et al., 1974; Krasny and Holbroot, 1977) and has reduced testicular synthesis of testosterone both in vivo and in vitro (Chandler et al., 1976; Gunn et al., 1965). However, perinatal cadmium exposure increased both adrenal steroidogenesis and the hepatic reduction of corticosterone in adult rats, an effect that was suggested to be due to a

general increase in the activity of hepatic steroid reductases (Grady et al., 1978). Such an effect is interesting in view of the fact that PBBs inhibited hepatic steroid reductases (McCormack et al., 1978; McCormack et al., 1979) In contrast to cadmium, chronic administration of methyl mercury resulted in a decreased hepatic metabolism of corticosterone and a decreased synthesis of steroids in the adrenal gland (Grady et al., 1978; Burton and Meikle, 1977) Cadmium, methyl mercury and lead were also toxic to both the male and female reproductive system (Lancranjan, 1975; Lucier et al., 1977; Harbison et al., 1978). Grady et al. (1978) showed that cadmium effects on corticosterone reduction and adrenal steroidogenesis were correlated with increased plasma corticosterone and adrenal weights. Ten ppm cadmium in drinking water was reported to completely prevent reproduction in a colony of mice (Schroeder and Mitchener, 1971).

Thus, many different xenobiotics can cause significant changes in both hepatic and extrahepatic enzyme systems that metabolize steroid hormones. It appears likely that although the mechanisms of action of these compounds are indeed very complex, at least part of their toxicities relates to altered metabolism of steroid hormones. This is especially true for some of the polyhalogenated hydrocarbons. In preliminary studies, PBBs treatment affected the profile of steroid metabolites in the urine of rats. These observations lead one to suspect that physiologically

significant changes in steroid metabolism caused by exposure to environmental chemicals should result in a significant alteration in the excretion of steroid metabolites and that such changes may be detected by examining urinary steroids.

Metabolism of PBBs. The endoplasmic reticulum is the site of metabolism for a large number of drugs and xenobiotics and is probably where metabolism of certain PBB congeners occurs. In vitro studies indicate that at least two of the major congeners in Firemaster BP-6 can undergo rapid metabolism in rat hepatic microsomal preparations isolated from subjects pretreated with phenobarbital. Microsomal preparations from nontreated and 3-MC pretreated subjects were ineffective (Dannan et al., 1978; Dannan and Aust, 1983). These particular congeners are the only two in the Firemaster BP-6 mixture known to have at least one unsubstituted para carbon. In these experiments the reaction rate was determined by substrate disappearance (measured by GC) and the chemical nature of the metabolite(s) was not determined (aromatic hydroxylation would be the most likely reaction to be occurring). Metabolism of some PBB congeners not found in the Firemaster BP-6 mixture has been investigated (Safe et al., 1976; Kohli and Safe, 1976; Kohli et al., 1978) but will not be discussed here.

3. Metabolic Profiling:

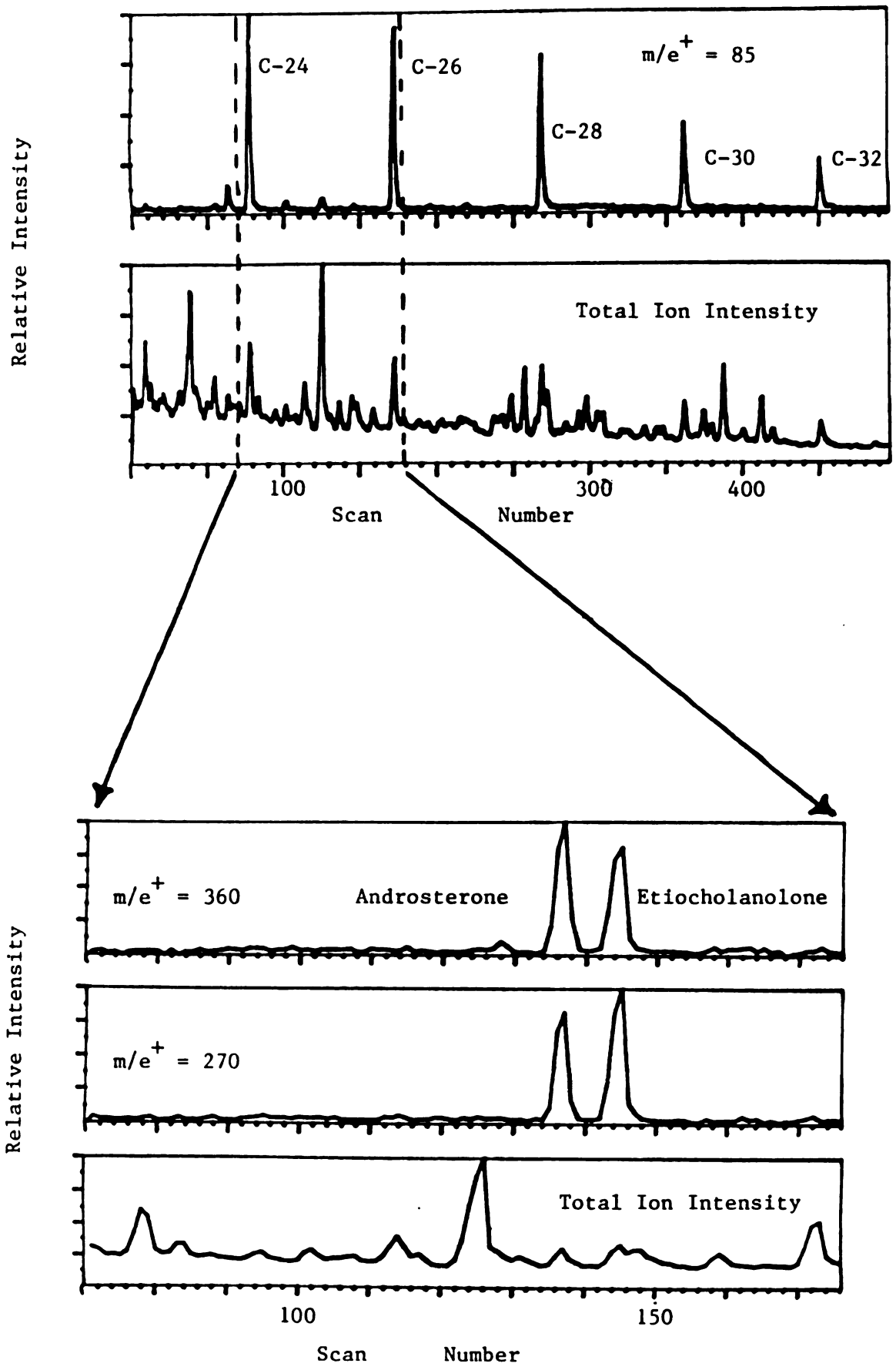
General Concepts. The concept that individuals have distinct metabolic patterns reflected by the constitutents of their biological fluids (i.e., urine, blood, amniotic fluid, cerebrospinal fluid, sweat, etc.) originated with the work of Roger Williams (1951). By utilizing the technique of paper chromatography, Williams showed convincingly that the patterns for a variety of compounds found in urine varied greatly from one individual to another but were relatively constant for any given individual. William's concept was not employed by others until the late 1960's when liquid and gas chromatographic techniques had become refined enough to allow for studies of this type with considerably less effort. The phrase most often used to describe multicomponent analysis of biological fluids, "metabolic profiling", was defined by Horning and Horning (1971a, 1971b) as "multicomponent GC analyses that define or describe metabolic patterns for a group of metabolically or analytically related metabolites". The Hornings also suggested that "profiles may prove to be useful for characterizing both normal and pathologic states, for studies of drug metabolism, and for human development studies."

Although metabolic profiling of biological mixtures is a relatively new technology, the analytical techniques that are used are not. These include paper chromatography, thin layer chromatography, column chromatography, gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS).

Of these techniques the most versatile and useful is GC/MS with computer assisted analysis of the data. Indeed, most of the ongoing research in the area of metabolic profiling presently uses the technique of reconstructing mass chromatograms by computer from arrays of complete mass spectra (Hites and Biemann, 1970). Briefly, this technique involves the taking of complete mass spectra repetitively throughout an entire GC analysis with subsequent computer storage of the data. The resulting information is a three dimensional array with axes of time, mass, and ion intensity. A plot of ion intensity vs. scan number for any particular mass is called a mass chromatogram. Examples of mass chromatograms can be seen in Figure 1. By examining the mass chromatogram of certain key ions, qualitative and quantitative information about specific compounds can be ascertained. Although most of the original work in the area of metabolic profiling was done using packed column GC separation, advances in capillary column technology in recent years, particularly in the area of column fabrication and coating techniques, have made this method very appealing as a separation vehicle for profiling analysis. Indeed, once experimental parameters are determined and stringently reproduced (i.e., sample preparation and GC parameters), capillary technology offers not only excellent qualitative but satisfactory quantitative analysis of complex mixtures.

MSSMET. During the past 5-6 years, a system has been developed at the MSU/NIH Mass Spectrometry Facility for

Figure 1. GC/MS/DS analyses of urinary steroids from a normal female. A mixture of straight-chain saturated alkanes (C-20,22,24,26,28,30,32, 34) has been co-injected for calculation of methylene unit retention indicies. Mass chromatogram $m/z^{+}=85$ shows where the various alkanes elute (top trace). The region around androsterone and etiocholanolone is expanded in the bottom panels, and selected ion chromatograms for androsterone and etiocholanolone shown.


Relative Intensity
C-24
C-26
C-28
C-30
C-32
m/e+ = 85
Total Ion Intensity
100
300
400
Scan Number
m/e+ = 360
Androsterone
Etiocholanolone
m/e+ = 270
Total Ion Intensity
Relative Intensity
100
150
Scan Number

automated simultaneous qualitative and quantitative anlaysis of complex organic mixtures by GC/MS/DS systems (Gates et al., 1976a, 1976b, 1978a, 1978b, 1978c; Sweeley et al., 1977; Gates and Sweeley, 1978; Sweeley, 1979). This technique uses methylene unit retention indices for the time dimension and an off-line reverse library search of the data obtained from GC/MS runs for qualitative and quantitative analysis of complex biological mixtures. The system is abbreviated as MSSMET (mass spectral metabolite search).

Figure 1 illustrates the type of mass spectral data from which the MSSMET data system identifies and quantifies a particular compound in a complex mixture of chemically-similar substances. Data shown are for a GC/MS determination of normal female urinary steroids. These data were obtained by repetitively scanning the mass spectrometer analyzer from 40 to 700 m/z every 4 seconds. The abscissa represents scan number and the ordinate represents ion intensity. The total ion intensity (TII) is the sum of the intensities of all ions recorded for any particular scan and is analogous to a GC trace. Also shown with the TII is the mass chromatogram for m/z = 85 which indicates where the co-injected straight chain saturated hydrocarbons elute.

The bottom of Figure 1 is an expanded display of the TII between scan 70 and 175. Also shown are mass chromatograms for m/z = 270 and 360, which are prominent ions in the electron impact mass spectrum of the methoxime-trimethylsilyl derivative of androsterone and etiocholanolone

(Table 6 lists the IUPAC name for trivial names and abbreviations used). In determining the presence or absence of a particular compound listed in the MSSMET library, the computer looks for the presence of a GC peak of a characteristic ion ("designate ion") within a time "window". The exact location of the window is defined by the scan numbers at which the co-injected hydrocarbons elute. If a GC peak for the designate ion is found within this window, then the computer examines for the occurrence of other characteristic ions for this particular compound ("confirming ions") with GC peaks that maximize within 1 or 2 scans of the peak maxima for the designate ion. The program then integrates peak areas for each GC peak found and calculates a correlation coefficient based upon the differences between the library and observed ratios of the designate and confirming ion intensities. If the correlation coefficient is greater than a value arbitrarily set by the operator, then the compound is considered "found" and relevant information concerning this compound (i.e., integrated area and peak height of the designate ion, retention index, and retention time of the compound, etc.) is stored in a "found-file". The correlation coefficient, peak detection and peak area integration algorithms are explained in greater detail later.

Figure 2 shows a typical entry into the MSSMET compound "library" used by the computer to identify and quantitate compounds from a repetitive scanning GC/MS data file. MSSMET is a highly interactive program. The operator has a

Figure 2. Description of MSSMET library files.

KEY TO MSSMET LIBRARY*

```
1     OPN:/WM 135,
      OPN:/CB 69,
     *ENTRYTYPE:MSSMET
2     NAM: * 1 ANDROSTERONE(3A-HYDROXY-5A-ANDROSTANE-17-ONE)
3     TMI:  2529
4     DIN:  270
5     CIN:  270,999,360,546
```

*Typical library entry for MSSMET analysis of urine samples, based on a reverse library search using GC retention indices and selected mass chromatograms.

1. Examples of options. MSSMET has 24 different options available to the user for interaction with the computer system. In the examples shown, the WM 135 option sets the "window" at 135 seconds around the retention index of a particular compound (androsterone, in this case). The computer will search all mass spectral data within the designated time frame for confirming ions of the selected compound. CQ is the value the correlation coefficient must equal or exceed for a particular compound to be printed out by the computer as a candidate.

2. Identifying compound number and name (common names and IUPAC).

3. Retention index (TMI). The retention index of androsterone, 2529, indicates that androsterone elutes between C-25 and C-26 (straight chain saturated hydrocarbons) at a time after the elution of C-25 that is 29% of the time interval between the elution of C-25 and C-26.

4. The designate ion in this case is at m/z 270. This ion is usually one of the more intense ions in the mass spectrum of androsterone and is also an ion that does not occur in the mass spectra of compounds that elute near the compound in question, if possible. The designate ion is also used for quantitation.

5. "CIN" refers to the confirming ion set. This set includes the designate ion (peak at m/z 270); the intensity of the designate ion is reported as 999 in this set. Thus, in this example the confirming ion set consists of two peaks at m/z 270 and 360 having relative intensities of 99.9% and 54.6%, respectively; this information is represented above by the sequence "CIN: 270, 999, 360, 546". The number of confirming ions used can be selected by the operator up to 8.

number of decisions to make when building a library that influence MSSMET analysis of a particular data set. These decisions include the choice of ions for each particular entry into the library and of values for various options that influence the analysis of the data. In the MSSMET library, a retention index and key ions with their relative intensities are listed for each compound. One ion, the "designate ion", is used for quantitation, and the other ions are used to confirm identification of the compound. In the example shown, the compound in question, androsterone, has a retention index of 2529 and key ions are m/z = 270 and 360, with the intensity of m/z = 360 equal to 54.6% the intensity of m/z = 270. M/z = 270 has been chosen as the designate ion.

Figure 3 shows a typical entry into a file generated by the MSSMET program which lists all the compounds found by the computer. The two most important pieces of information contained in the found file are the integrated areas of the designate ions for each compound and the correlation coefficient. In the example shown in Figure 3, androsterone has been identified with a correlation coefficient of 98 (100 is perfect) with the area of the designate ion being 43,317 (no units). In general, areas less than 1000 should be questioned. Also shown is the amount of androsterone present in relationship to the amount of an internal standard (comment number 8 in legend to Figure 3). This value is calculated by dividing the area of the designate ion of androsterone by

Figure 3. Description of found file. The found file lists those compounds identified by the computer as being present in the sample.

KEY TO TYPICAL "FOUND" FILE

```
1  2       3          4 5  6   7      8         9        10
*  1  ANDROSTERONE 5 + 98 43317 0.177E+00 21:53 - 0.09

                     + 99 20042 0.218E+00 PER DROP
                    16 17  18     19         20

11    12   13   14   15
2524  -5  134  137  141

MISSED 0 of 2
       21
```

a) The "found" file lists those compounds identified by the computer as being present in the sample. The following information is printed with each found file.

1. Off-scale indicator. Indicates when maximum peak height is above a designated value.
2. Compound identification number.
3. Compound name.
4. Number of the peak out of number of candidate peaks found in time window. This number represents the chronological order of a series of chromatographic peaks within the designated time window for androsterone detected by the computer during data analysis.
5. Match category for peak area as determined by the correlation coefficient and the difference between the actual and the calculated retention index. Match categories include + (high correlation coefficient), (intermediate range correlation coefficient) and - (poor match with library retention index).
6. Correlation coefficient for peak area.
7. Peak area of designate ion.
8. Relative amount of compound using peak areas (area of designate ion relative to the area of the designate ion of the internal standard, corrected for the amount of internal standard added, ml of urine used and mg/ml of creatine). Value is unitless. The letter E indicates that the value is written in exponential form (i.e., the value shown is 0.177). Values of E 00 are nearly always significant. Depending upon the amount of internal standard added, values of E-02 or less could also be significant.
9. Retention time (minutes:seconds).
10. Difference of retention time from that predicted by library (minutes:seconds).
11. Retention index (methylene units).
12. Difference of retention index from library value.
13. Starting scan number of peak.

Figure 3 (continued)

14. Scan number at apex of peak.
15. Ending scan number of peak.
16. Match category for peak height. Match category defined in comment 5.
17. Correlation coefficient for peak height.
18. Peak height of designate ion.
19. Relative amount of compound using peak height for quantitation (see comment 8).
20. Method of area calculation.
21. Number of confirming ions missed.

the area of the designate ion of the internal standard, correcting for the amount of internal standard added, the ml of urine used, and the mg/ml of creatine. This value is referred to as the "area internal standard".

After computer generation of a "found" file, MSSMET evaluates the data for quantitation purposes and prints this data in a "normalized found" file. A typical entry into this "normalized found" file is seen in Figure 4. The most important pieces of information contained in this file are the "area partial sum" and the "area internal standard" values. The "area internal standard" is calculated by dividing the integrated area of the designate ion for a particular compound by the integrated area of the designate ion for the internal standard. The "area partial sum" is calculated by dividing the integrated area of the designate ion for a particular compound by the sum of the integrated areas for the designate ions of all compounds found and multiplying this value by 10,000. Values for the "area partial sum" generally fall between 1 and 10,000. Values less than 1 should be questioned. Values for the "area internal standard" are entirely dependent upon the amount of internal standard added.

Figure 5 shows part of a typical quantitative printout that is obtained for a typical human urinary steroid profile. In the example shown, the first line identifies the date of the computer analysis (March 10th, 1981), the name of the GC/MS data file (I11118001.MSF), and the library

KEY TO NORMALIZED FOUND FILE*

1	2424	-5	ANDROSTERONE	98	281.445	0.117	99	317.620	0.218
2	2541	-6	ETIOCHOLANOLONE	99	380.289	0.239	99	395.004	0.271
3	2585	0	DHA	91	11.136	0.007	84	8.637	0.006
1	3	3	4	5	6	7	8	9	10

*The normalized found file contains quantitative information for those compounds listed in the found file. The following information is printed with each normalized found file.

1. Compound identification number.
2. Retention index.
3. Difference of retention index from library value.
4. Compound name.
5. Correlation coefficient for peak area.
6. Area partial sum. This value is calculated by dividing the integrated area of the designate ion for a particular compound by the sum of all integrated areas for the designate ions of all compounds found and multiplying this value by 10,000.
7. Height partial sum. Calculated in same manner as area partial sum but using peak heights instead.
8. Correlation coefficient using peak height.
9. Area internal standard. This value is calculated by dividing the integrated area of the designate ion for a particular compound by the integrated area of the designate ion for the internal standard.
10. Height internal standard. Calculated in the same manner as area internal standard but using peak heights of designate ion current responses.

Figure 4. Description of the normalized found file. The normalized found file contains quantitative information on those compounds listed in the found file.

Figure 5. Example of a typical MSSMET quantitative printout (Found File) obtained for a normal female human urinary steroid sample. Format is the same as described in Figure 4. Information relative to the sample and date(s) of analyses are contained in each printout. Various unknown compounds (designated as UN1, UN2, ... etc.) have also been identified by their characteristic retention indices and ion currents.

10-MAR-81 *COMPOUNDS FOUND* I11118001 MSF STER2091B LIB
3-DEC-80 BA 102 URINARY STEROIDS MO-TMS BREAST CANCER SAMPLE

1.000 ML
1.000 MG CREATININE
50.000 UG INTERNAL STANDARD

AREA SUM 14375759
HEIGHT SUM 2800457

	CPD NO	RI	DELTA RI	NAME	AREA MATCH	AREA PS	AREA IS	HEIGHT MATCH	HEIGHT PS	HEIGHT IS
&	96	3397	7	CHOLESTERYL BUTYRATE	86	857 054	0 000	86	631 465	0 000
*	98	1957	4	UN 1 (1953)	82	50 982	2 970	83	116 024	9 190
*	101	2035	-1	UN 4 (2036)	98	298 135	17 400	98	570 728	45 200
*	102	2053	-5	UN 5 (2058)	69	2 970	0 173	71	4 510	0 357
*	103	2071	-2	UN 6 (2073)	84	41 100	2 400	84	44 350	3 510
*	105	2139	4	UN 8 (2135)	72	17 952	1 050	70	14 287	1 130
*	106	2139	1	UN 9 (2138)	85	0 888	0 052	93	3 039	0 241
*	107	2192	3	UN 10 (2189)	98	145 186	8 470	98	238 900	18 900
*	108	2202	9	UN 11 (2193)	92	3 300	0 193	91	8 259	0 654
*	110	2211	3	UN 13 (2208)	82	78 093	4 560	77	77 395	6 130
*	111	2240	2	UN 14 (2238)	75	125 357	7 310	76	161 691	12 800
*	112	2248	1	UN 15 (2247)	54	0 899	0 052	78	2 528	0 200
*	114	2277	4	UN 17 (2273)	86	4 040	0 236	86	3 596	0 285
*	115	2308	3	UN 20 (2305)	96	77 789	4 540	96	83 090	6 580
*	116	2345	4	UN 22 (2341)	96	67 856	3 960	96	86 507	6 850
*	117	2351	7	UN 32 (2344)	79	10 955	0 639	97	9 941	0 787
*	118	2345	-2	UN 33 (2347)	91	13 889	0 810	99	13 912	1 100
*	119	2405	-3	UN 24 (2408)	84	3 364	0 196	84	6 178	0 489
*	121	2442	5	UN 26 (2437)	80	4 360	0 254	79	4 442	0 352
*	1	2526	3	ANDROSTERONE(3A-HYDROXY-5A-ANDROSTANE-17-ONE)	78	882 444	51 500	78	1340 724	106 000
*	2	2542	2	ETIOCHOLANOLONE(3A-HYDROXY-5B-ANDROSTANE-17-ONE)	84	590 497	34 400	84	1005 150	79 600
*	3	2586	-8	DHA(DEHYDROEPIANDROSTERONE - 3B-HYDROXY-5-ANDROSTENE-17-ONE	87	31 525	1 840	89	41 008	3 250
*	4	2589	-2	11-OXO-ANDROSTERONE(3A-HYDROXY-5A-ANDROSTANE-11,17-DIONE)	73	16 120	0 940	76	17 776	1 410
*	5	2638	9	11-OXO-AETIOCHOLANOLONE(3A-HYDROXY-5B-ANDROSTANE-11,17-DION	70	0 849	0 049	70	2 214	0 175
*	6	2709	1	11-BETA-HYDROXYANDROSTERONE(3A,11B-DIHYDROXY-5A-ANDROSTANE-	91	141 555	8 260	93	177 560	14 100
*	10	2771	-3	PREGNANEDIOL(3A,20A-DIHYDROXY-5B-PREGNANE)	93	3241 913	189 000	93	1739 227	138 000
*	12	2808	3	PREGNANETRIOL(3A,17A,20A-TRIHYDROXY-5B-PREGNANE)	92	1218 243	71 100	91	1438 062	114 000
*	13	2808	0	3B,20A-DIOH-5-PREGNENE	91	151 978	8 870	94	182 220	14 400
*	14	2821	2	16-OXO-ANDROSTENEDIOL(3B,17B-DIHYDROXY-5-ANDROSTENE-16-ONE)	81	0 853	0 050	79	1 793	0 142
*	16	2853	1	ANDROSTENETRIOL	88	34 541	2 020	89	37 544	2 970
*	18	2850	3	3-ALPHA,6-ALPHA-DIHYDROXY-5-BETA-PREGNAN-20-ONE	71	5 156	0 301	71	7 049	0 558
*	21	2885	-1	ESTRIOL(3,16A,17B-TRIHYDROXY-1,3,5(10)-ESTRATRIENE) (OV-101	53	5 743	0 335	76	6 685	0 529
*	27	2872	0	TETRAHYDRO-SUBSTANCE S(3A,17A 21-TRIHYDROXY-5B-PREGNANE-20-	84	34 567	2 020	84	36 537	2 890
*	28	2920	10	ALLO-TETRAHYDRO-SUBSTANCE S(3A,17A,21-TRIHYDROXY-5A-PREGNAN	83	10 575	0 617	77	11 941	0 946
*	30	2917	-2	3A,11B,20A-TRIOH-5B-PREGNANE	93	3 854	0 225	91	10 098	0 800
*	31	3086	3	ALPHA-CORTOLONE(3A,17A,20A,21-TETRAHYDROXY-5B-PREGNANE-11-O	86	120 062	7 000	86	132 603	10 500
*	33	3086	2	BETA-CORTOL(3A,11B,17A,20B,21-PENTAHYDROXY-5B-PREGNANE)	70	48 927	2 850	74	64 229	5 090
*	34	3069	0	18-HYDROXYTETRAHYDRO-CMPD A(3A,18,21,-TRIOH-5B-PREGNANE-11,	71	3 983	0 232	86	4 949	0 392
*	36	3077	1	HEXAHYDRO-COMPOUND A	80	16 182	0 944	85	20 018	1 590
*	43	3127	4	3A,11B,20A,21-TETRAHYDROXY-5A-PREGNANE	71	35 757	2 090	74	26 685	2 110
*	45	2944	6	16-ALPHA-HYDROXPREGNENOLONE(3B,16A-DIOH-5B-PREGNANE-20-ONE)	82	9 683	0 565	78	11 737	0 929
*	46	2946	4	3-BETA,16-ALPHA-DIHYDROXY-5-ALPHA-PREGNAN-20-ONE	75	11 803	0 689	76	11 566	0 916
*	50	2962	-2	3B,15B,16A,17B-TETRAHYDROXY-5-ANDROSTENE	93	1 551	0 090	93	5 581	0 442
*	52	2970	-8	(THE) TETRAHYDROCORTISONE(3A,17A,21-TRIOH-5B-PREGNANE-11,20	99	250 978	14 600	98	302 440	23 900
*	56	3016	0	ALLO-TETRAHYDROCORTICOSTERONE(3A,11B,21-TRIOH-5A-PREGNANE	67	47 818	2 790	76	61 854	4 900

that was used in the analysis (STER2091B.LIB). The second line lists the date the GC/MS file was transferred from the data system interfaced with the instrument used in the analysis to a PDP-11/44 which performs the MSSMET analysis. The rest of this line contains the first line of the "comments" for this particular data file. The next few lines list the values used by the computer in normalizing the "area internal standard" values per mg internal standard per ml urine per mg creatine. The sum of the peak area and heights is listed next. The remainder of the information is as previously described. In the example shown, cholesteryl butyrate is identified as the internal standard by the ampersand (&) at the far left. Note that besides compounds of known chemical structure, various unknowns (designated as UN1,2,...etc.) have also been identified by their characteristic retention indices and ion currents.

Using a MSSMET system developed for urinary organic acids and which employs the same operational principles described above, over 200 separate urinary acid components can be analyzed on a single GC run. This system was used in a study comparing organic acids in the urines of healthy adults, children with neuroblastoma, and children that did not have cancer (Gates et al., 1978b, 1978c). Neuroblastoma urines could easily be identified as different from other children's urines by exhibiting significantly lower levels of α-hydroxyisobutric, lactic, glycolic and two unidentified acids, while at the same time significantly higher levels of

tartaric, citric, homovanillic, vanillylmandelic, caffeic, m-hydroxyphenylhydracrylic and one unidentified acid.

Steroid hormones. Metabolic profiling of urinary steroids originated with the work of Gardiner and Horning (1966). Their work demonstrated that most major urinary steroids could be separated in a single gas chromatographic run as the methoxime-trimethylsilyl ether derivatives. The major advances since this time have involved improvements in the methods for isolation of steroids from urine (Leunissen and Thijssen, 1978; Thenot and Horning, 1972; Shackelton et al., 1970; Bradlow, 1968), the introduction of wall-coated open-tubular (WCOT) glass capillary columns (Novotny and Zlatkis, 1970; Novotny et al., 1976; Horning et al., 1974; Luyten and Rutten, 1974) and the use of computer-assisted GC/MS analysis of steroid profiles (Baty and Wade, 1974; Shackleton and Honour, 1976; Pfaffenberger and Horning, 1975; Setchell et al., 1976). In general, these methods involve the isolation of steroids and steroid conjugates from urine or blood by the use of a polystyrene non-ionic absorbent column, enzymatic hydrolysis of the steroid conjugates by β-glucuronidase and sulfatase, isolation of the steroids a second time either by adsorption on a column or by solvent extraction, and finally the formation of volatile derivatives. Some procedures include a solvolysis step for recovery of steroid sulfates not enzymatically liberated (Leunissen and Thijssen, 1978) while others

involve column separation of the steroids into different fractions on Sephadex LH-20 (Setchell et al., 1976).

Metabolic profiling of human urinary steroids has attracted much more attention in the past ten years, mostly from investigators in Europe, and the volume of literature on this subject is large. One of the more obvious applications of this technique is the study of individuals with defects in steroid metabolism (i.e., Cushing's syndrome, adrenal carcinoma, pituitary tumors, 21-hydroxylase deficiency, 17α-hydroxylase deficiency, etc.) and many examples of abnormal profiles have appeared in the literature (Leunissen and Thijssen, 1978; Novotny et al., 1976; Luyten and Rutten, 1974; Shackleton and Honour, 1976; Pfaffenberger and Horning, 1975; Spiteller, 1978; Honor et al., 1978). Steroid metabolic profiles have also been studied in pregnancy (Begue et al., 1978; Eriksson and Gustafsson, 1970), in newborns and infants (Shackelton et al., 1971; Shackelton and Taylor, 1975) and in conditions of emotional stress and physical exertion (Spiteller, 1978). For example, Ludwig et al. (1977) reported that emotional stress increased urinary dehydroepiandrosterone (DHEA, 3β-hydroxy-5-androsten-17-one) by up to 20-fold. Steroid metabolic profiles of neutral and acidic steroids have also been obtained for blood, amniotic fluid, bile and feces (Shackelton and Mitchell, 1967; Aldercreutz et al., 1978).

Two mass spectrometry techniques for the computer assisted GC/MS analysis of steroid profiles have been

reported in the literature. One technique used repetitive scanning of the MS-analyzer (usually from 40 to approximately 700 m/z) and computer storage of the data for later analysis (Baty and Wade, 1974; Setchell et al., 1976). Since these investigators report MS scan times in the order of 6 to 12 seconds, the MS data obtained cannot be used for quantitative analysis if a capillary column is used, and data obtained in this manner for a packed column is only semiquantitative (i.e., not enough points across a peak for satisfactory quantitation are obtained using a 10 second scan cycle). Another technique involved the use of WCOT capillary columns and the taking of a complete mass spectrum at the apex of each peak as seen by monitoring the total ion current (Spiteller, 1978; Ludwig et al., 1977). This technique used the mass spectrometry data only as a means of peak identification and made no attempt to quantitate.

Quantitative metabolic profiling is a technique that has only recently been developed and GC/MS computer assisted metabolic profiling is an even newer technology. The technology for quantitative metabolic profiling has been mostly used in the study of endocrine and metabolic disorders in humans and primates. Very little or no work has been done with other species nor has it been applied to other areas of scientific research. For example, urinary steroid and organic acid metabolic profiles for many common laboratory animals have never been reported. Quantitative metabolic profiling using GC/MS computer assisted technology has many

obvious scientific applications and its use will accelerate with with increased availability of the necessary instrumentation.

RATIONALE

A large number of environmental contaminants cause significant induction of hepatic and extrahepatic microsomal mixed-function oxidiase (MFO) enzymes, while other environmental contaminants inhibit these enzymes. Steroid hormones are metabolized by MFO enzyme systems and since induction of MFO enzyme systems affects the metabolism of steroids it is believed that at least part of the toxicity associated with exposure to polyhalogenated organic compounds may result from altered steroid metabolism. It is also known that specific inducers affect specific monoxygenase enzymes. In view of these observations, this study used PBBs as a model stimulator of MFO enzymes that affect steroid metabolism. The experiments described in this dissertation were designed to test the following hypotheses:

1) PBBs alter steroid metabolism in a characteristic manner and this altered metabolism is reflected by significant changes in the urinary steroid metabolic profile.

2) Humans accidentally exposed to high levels of PBBs will be distinguishable from non-exposed individuals by examination of their urinary steroid metabolic profiles.

SECTION II

Development of Analytical Methods

Section II

INTRODUCTION

This section concerns the development of methodologies for generation of steroid metabolic profiles by capillary column GC and GC/MS/DS using both packed and capillary columns. Metabolic profiling has already been discussed in the Introduction of Section I. However, it is instructive to review some of the analytical problems involved in developing these procedures. First, we are dealing with a mixture where minor components of interest may be 1000 to 5000 times less concentrated than the major component(s). The procedure must quantitatively extract all compounds of interest, in this case steroids, from a biological matrix (urine, blood, CSF, etc.). Chemical derivatization must also be quantitative. The analytical systems ideally should be able to quantitate all components of the mixture (within the limits of detection defined by signal to noise considerations). In addition, the methodology must provide for computation and manipulation of over 100 data points per analysis.

In general, analysis of steroid profiles by GC or GC/MS/DS involves three stages of analysis: (1) preparation of derivatized samples; (2) GC or GC/MS analysis and (3) manual

or computer analysis of the data. Extraction of steroids from urine, serum or tissues can be either general, or specific groups of steroids can be selectively isolated and purified. Introduction of the Amberlite XAD-2 extraction procedure by Bradlow (1968) greatly simplified the extraction of polar steroids and steroid conjugates. Various procedures for general extraction of steroids from tissues and body fluids using solids and non-polar solvents have appeared in the literature (Leunissen and Thijssen, 1978; Shackelton et al., 1970; Bradlow, 1968; Sjövall and Axelson, 1979). Selective isolation procedures for estrogens, 3-keto steroids, synthetic steroids, free steroids, steroid glucuronides, steroid sulfates and steroid disulfates using column liquid chromatography and solvent extraction procedures have appeared in the literature (Sjövall, 1975; Sjövall and Axelson, 1979; Novotny et al., 1976; Axelson and Sjövall, 1977; Cohen et al., 1978; Murphy and D'Aux, 1975). Sjövall, Axelson and co-workers have done much of the pioneering research on these purification procedures (Sjövall, 1975; Sjövall and Axelson, 1979; Setchell et al., 1976; Axelson and Sjövall, 1977) and have described an automated analysis using a forward library search technique for the qualitative identification of individual components of mixtures of steroids (Sjövall, 1975; Reimendal and Sjövall, 1972, 1973).

EXPERIMENTAL

Materials. All solvents were redistilled in glass. Enzymes used were β-glucuronidase from Helix pomatia [activity: 62880 Fishman units (F.U.) per vial, with one F.U. defined as that activity which will hydrolyze 1.0 mg of phenolphthalein glucuronide per hour at pH 5.0, 37°C; Calbiochem, La Jolla, CA, USA]. Amberlite XAD-7 (polystyrene non-ionic adsorbent; Mallinckrodt, Paris, KY, USA) was washed with ten volumes of methanol, acetone, methanol-distilled:water (1:1), ethanol and finally distilled water. O-Methoxyamine hydrochloride and Sylon BTZ [N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA)-trimethylchlorosilane (TMCS)-trimethylsilylimidazole, 3:2:3] were purchased from Supelco (Bellefonte, PA, USA). Reference steroids were purchased from Steraloids (Wilton, NH, USA). Lipidex-5000 was purchased from Packard Instruments (Downers Grove, IL, USA). Sep-Pac® C-18 cartridges were obtained from Waters Associates, Inc. (Milford, MA, USA).

Preparation of standards. Each steroid standard (2-5 mg) was weighed into a 2-dram screw-top vial with a PTFE cap liner. Methanol was then added to make a 1.0 µg/µl solution. Samples which contained undissolved steroids were

diluted with an equal volume of dimethylsulfoxide (DMSO), making a 0.5 µg/µl solution. An aliquot (250 µl) of each stock solution (500 µl for those with DMSO added, making a total of 250 µg per sample) was placed in a 10-ml test-tube with a screw-top cap (PTFE lined) and the solvent was evaporated with a stream of nitrogen at ambient temperature. Methoxime-trimethylsilyl (MO-TMS) derivatives were prepared as follows: 50 µl of a 100 µg/µl solution of O-methoxyamine hydrochloride in dry, redistilled pyridine were added and this mixture was heated at 60°C for 1 h. Following removal of excess reagent under a stream of nitrogen, 200 µl of Sylon BTZ were added and the samples were heated at 80°C for 24 h. Samples were placed in glass capillaries which had previously been sealed at one end and then sealed with a flame. Retention indices (i.e., methylene units) for each MO-TMS derivative were determined on a 25-m SP-2100 wallcoated open tubular capillary (WCOT) column (0.2 mm I.D.) and on a 3-m, 3% OV-101 [Supelcoport 80-100 mesh, 2 mm I.D.] packed column by co-injection of straight-chain saturated hydrocarbons. Each sample was also injected separate to insure that the derivatization procedure was quantitative (i.e., only one peak was observed).

Determination of Relative Response Factors. Equal amounts of each steroid and two internal standards (3β,17β-dihydroxy-17α-methyl-5β-androstane and cholesteryl butyrate) were dried and derivatized as described below. A volume containing 1 µg of each of the steroids was analyzed by a

packed column GC/MS data system using conditions described below. The relative response factor was determined by dividing the integrated area of a chosen ion in the mass spectrum of the internal standard, hereafter referred to as the 'designate' ion, by the integrated area of the designate ion of each steroid standard.

Isolation of steroids (Method I). Amberlite XAD-7 was packed in 1.0-cm I.D. glass columns to a height of 4 cm. Each column had a 100-ml reservoir. Columns were washed with 40 ml of distilled water before being used. Urine samples (20 ml each) were pipetted onto the columns. The loaded columns were washed with 2 x 5 ml of distilled water. Steroids were eluted with 3 x 5 ml of absolute ethanol, at a constant flow-rate (0.5-1.0 ml/min). Ethanol was evaporated from the eluate by aspiration using a rotary evaporator in a water-bath at 37°C. A sodium acetate buffer (0.5 M, pH 4.55) was added (3 ml, plus 1 ml to wash) and the samples were tranferred to small Erlenmeyer flasks. β-Glucuronidase derived from mollusk (Calbiochem) was reconstituted by adding 3 ml of distilled water to each vial, and was added (300 µl) to each sample. After incubation for 48 h in a 37°C water-bath, sodium chloride (1.5 g) was added. Liberated steroids were extracted by shaking with 25 ml of ethyl acetate for 60 min. The ethyl acetate layer was removed by pipet, and the aqueous phase was re-extracted two more times with 5 ml aliquots of ethyl acetate. The pH of the aqueous phase was adjusted to 1.0 with concentrated HCl

and the solution was again extracted three times with ethyl acetate. The combined acidic organic phases were heated at 45°C for 18 h and then combined with the first extraction phase. The recombined ethyl acetate phase was washed with 2 x 5 ml of 8% aqueous sodium bicarbonate then transferred to a small round-bottom flask (washing the aqueous phase three times with 2-ml aliquots of ethyl acetate). Steroids lost in the aqueous phase were recovered by XAD-7 extraction and elution with ethanol, as described above.

The combined ethyl acetate and ethanol phases were evaporated by aspiration using a rotary evaporator and a water-bath at 37°C. The samples were resuspended in 3 ml of ethanol and transferred to small screw-top test-tubes (washing with 2 x 1 ml ethanol). The internal standards were added at this time. Samples were then dried under a stream of nitrogen at 60°C, and 50 µl of a solution of O-methoxyamine hydrochloride (100 µg/µl) in dry pyridine, plus 100 µl of dry pyridine, were added to the dry residue. This solution was heated for 60 min at 60°C, excess pyridine was removed under nitrogen at 60°C, 100 µl of Sylon BTZ were added, and the sample was heated for 18 h at 80°C.

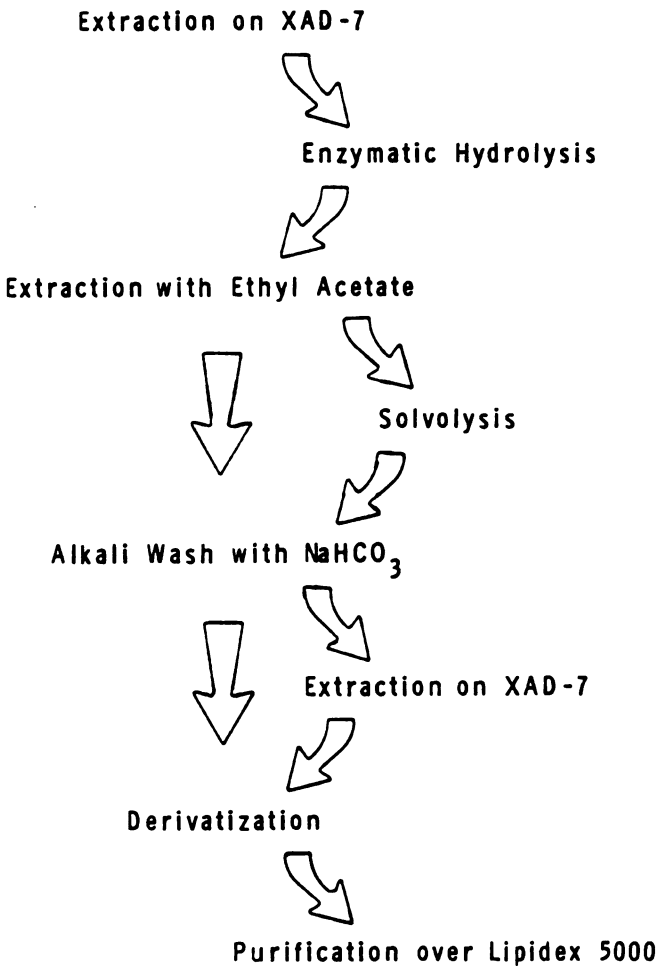
Excess silylation reagents and polar compounds were separated from the derivatized steroid fraction on a Lipidex-5000 column (70 x 5 mm) containing hexane-hexamethyldisilazane-pyridine-2,2-dimethoxypropane (97:1:2:10, v/v). The sample was transferred to the top of the column by adding 400 µl of the solvent, which was also

passed through the column. For rapid filtration nitrogen pressure was applied, resulting in a flow of 3 ml/min. Solvent (3.5 ml) was passed through the column to recover the derivatized compounds. Solvents were then evaporated under nitrogen at 60°C and the sample was redissolved in 1 ml of hexane containing 2% BSTFA. A flow diagram of the procedure is seen in Figure 6.

Isolation of steroids (Method II). Steroids were also isolated by the procedure described by Shackelton (1980). Sep-Pak® cartridges were pre-washed with 2 ml of methanol followed by 5 ml of distilled water. Silanized glass syringes were used at all times. An aliquot (20 ml) of urine was passed through the cartridge at a rate of one drop per second. Following a 5 ml distilled water wash, steroids and steroid conjugates were eluted with 2 ml of methanol into a 10 ml test tube with a screw cap. The methanol was dried under nitrogen and steroid conjugates were hydrolysed enzymatically as described above. Steroids were extracted from the enzyme buffer using Sep-Pak® cartridges. Cartridges were washed with 5 ml of distilled water and steroids were eluted with 2 ml of methanol. Cholesteryl butyrate (20 μg) was added at this time. The samples were dried under a stream of nitrogen and methoxime-trimethylsilyl (MO-TMS) derivatives were prepared as described above.

Gas chromatography. Gas chromatography was performed on a Hewlett-Packard 5840A equipped with a split and splitless

Figure 6. Flow diagram for isolation and chemical derivatization of urinary steroids by a modification of the method of Leunissen and Thijssen (1978). Following enzymatic hydrolysis, the freed steroids were extracted into ethyl acetate (Fraction 1). The aqueous phase was then acidified and again extracted with ethyl acetate (Fraction 2). Following solvolysis, Fraction 2 was washed with 8% aqueous sodium bicarbonate. Any steroids in the aqueous sodium bicarbonate phase were extracted using XAD-7 (Fraction 3). Fractions 1, 2 and 3 were combined, dried and MO-TMS derivatives were formed.


Extraction on XAD-7
Enzymatic Hydrolysis
Extraction with Ethyl Acetate
Solvolysis
Alkali Wash with $NaHCO_3$
Extraction on XAD-7
Derivatization
Purification over Lipidex 5000

capillary column injection port and a flame ionization detector. Aliquots (1 μl) of each sample were chromatographed on a SP-2100 wall-coated open tubular (WCOT) fused silica capillary column (25 m x 0.2 mm I.D.). Conditions of analysis were as follows: injection port temperature, 280°C; initial temperature, 180°C; temperature programming at 2°C/min to a final temperature of 280°C; 50 min isothermal period at the end of the run; detection signal attenuation, 1; hydrogen flow, 30 ml/min; and air flow, 200 ml/min; split ratio, 20:1; and μ = 25 cm/sec. In later experiments a 60 m DB-1 bonded phase fused silica capillary column (0.242 mm I.D., 0.1 μm film thickness; J and W Scientific) replaced the 25 m SP-2100 capillary column. Conditions of analysis were as follows: initial temperature of 180°C, temperature programming at 1.25°C/min, final temperature of 310°C, μ = 20 cm/sec. All other parameters were the same as previously stated.

Mass spectrometry. Mass spectral data were obtained on an LKB-2091 gas chromatograph-mass spectrometer with a dual Digital Equipment PDP-8e based foreground-background data system. The gas chromatograph contained a coiled glass column (3 m x 2 mm I.D.) packed with 3% OV-101 on Supelcoport (80-100 mesh), a 50-m and 25-m OV-101 WCOT fused silica capillary column (0.3 mm I.D.), a 25 m SP-2100 WCOT fused silica capillary column (0.2 mm I.D.) or a 60 m OV-1 fused silica capillary column (0.3 mm I.D., 1.0 μm film thickness). Conditions of operation were as follows: GC

oven temperature programming from 180 to 280°C at 2°C/min; ion source temperature, 280°C; GC injection port, 280°C; electron multiplier voltage, 1500 V; scans at 4-sec intervals at scan speed 3 (m/z 50-730) or 2-sec intervals at scan speed 2 (m/z 50-600); accelerating voltage, 3.5 kV; trap current, 50 μA; filament current, approximately 4 A; and ionizing voltage, 70 eV. Calibration of nominal mass was against reference ions of perfluorokerosine and instrumental performance was evaluated each day by inspection of reconstructed mass chromatograms. The packed column was pre-treated by two injections of BSTFA-TMCS silylation mixture at 280°C. An aliquot (0.5 μl) of a mixture of eight straight-chain saturated hydrocarbons (20,22,24,26,28,30,32 and 34 carbon atoms) in hexane was co-injected with each sample. After samples were analyzed under the above conditions, each run was validated by brief manual inspection of a few mass chromatograms and the data were then transferred to a PDP 11/44 computer (Digital Equipment) for subsequent analysis and storage on magnetic tape. The PDP 11/44 system consisted of 1 16-bit, 124 K-word memory minicomputer with two 1.2 million word removable disks, one CDC-9766 300 Mega byte disk, seven- and eight-track magnetic tape drives, DEC writer, Tektronix 4010 scope display unit, and a Tektronix 4610 hard copy unit.

RESULTS AND DISCUSSION

Various methods for the isolation and derivatization of urinary steroids were investigated for reproducibility. The aim of these investigations was to establish the simplest "general" extraction procedure that would give a high degree of reproducibility. The first procedure investigated used an XAD-2 extraction, followed by enzymatic hydrolysis and finally extraction of the steroids a second time with XAD-2. Trimethylsilylimidazole was used to derivatize the extracted steroids. A reproducible capillary column GC trace with or without a Lipidex-5000 "clean-up" step (see Experimental) could not be obtained using this procedure. Leunissen and Thijssen (1978) reported overall recoveries of 90% or better of the radioactivity when human subjects were administered labeled androstenedione, estrone, 3β-hydroxy-5-androstene-17-one (DHEA) and DHEA sulfate. They also reported a high degree of precision, which was confirmed in the present investigation (Figure 7 and Table 1). The present investigation also confirmed that the alkali wash and purification of the silylation mixture are important steps for obtaining reproducible GC chromatograms. The precision of this method was about the same with or without inclusion of the solvolysis step (only small amounts of material were

Figure 7. Urinary steroid metabolic profile of a post-puberal pre-menopausal female human obtained by capillary column GC with flame ionization detection. The top three traces (A, B and C) are for 3 separate preparations of the same urine sample and demonstrate the overall excellent precision of the method. The lower recordings are of sample plus a mixture of straight-chain hydrocarbons (D), and of a sample blank, using distilled water (E). Conditions of analysis are given in the text.

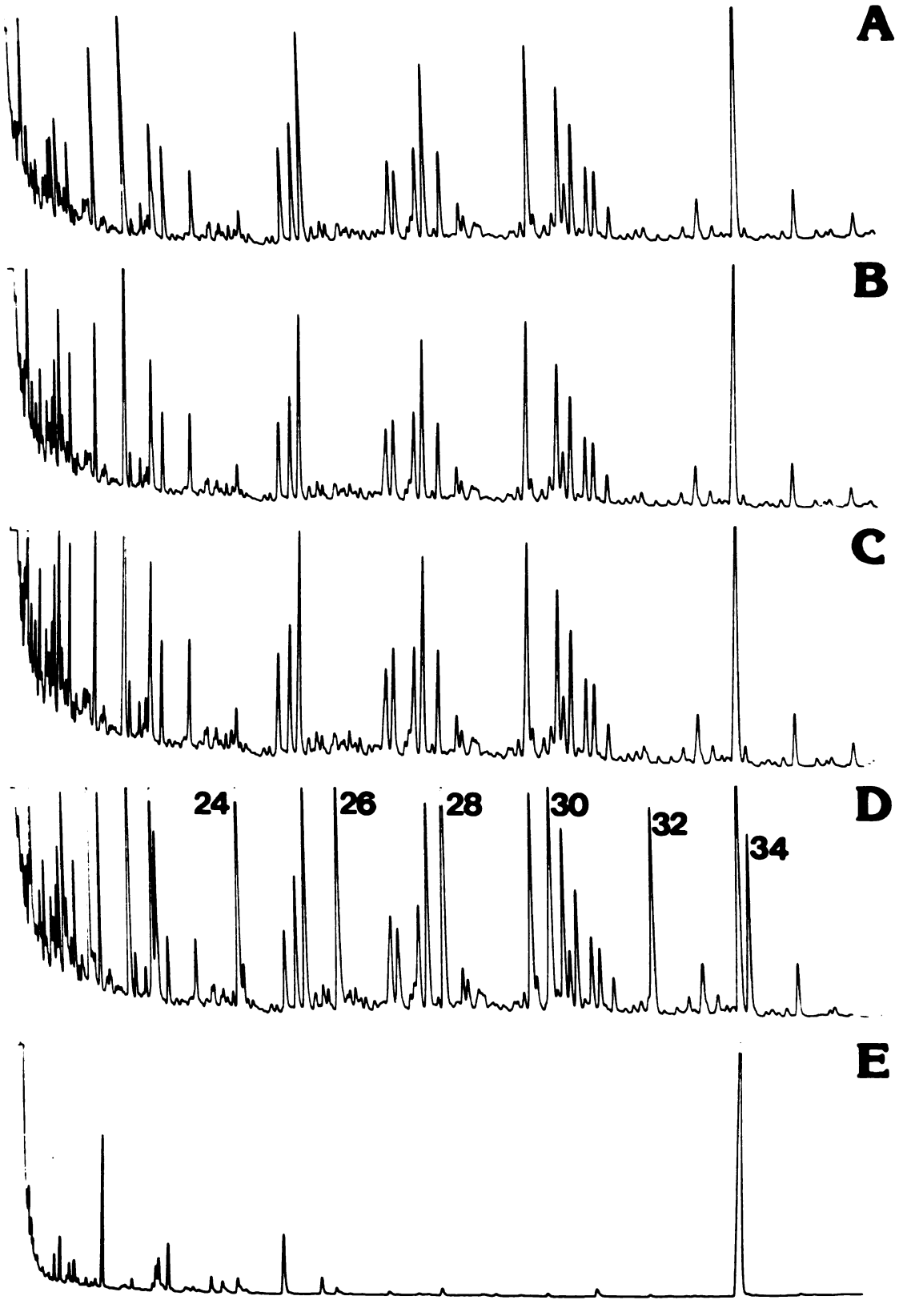

A
B
C
24
26
28
30
32
34
D
E

Table 1. Precision: GC profiling of urinary steroids[a]

Steroid	Sample Number[b] 1	2	3	Mean ± S. D.
Androsterone	19.70	21.45	21.26	20.82 ± .928
Etiocholanolone	21.48	23.15	22.89	22.50 ± .898
11β-hydroxyetiocholanolone	5.24	6.07	5.77	5.69 ± .420
Pregnanetriol	5.80	5.66	5.90	5.78 ± .120
THE	20.38	23.12	20.80	21.4 ± 1.475
THA	5.64	3.45	4.08	4.39 ± 1.127
THF	9.67	10.03	9.64	9.78 ± .217
α-Cortolone	5.37	5.61	5.39	5.45 ± .133

[a]Data obtained with HP5840A recording gas chromatograph; 25 meter SP-2100 fused silica capillary; programmed 180-280°C at 2°C per minute.

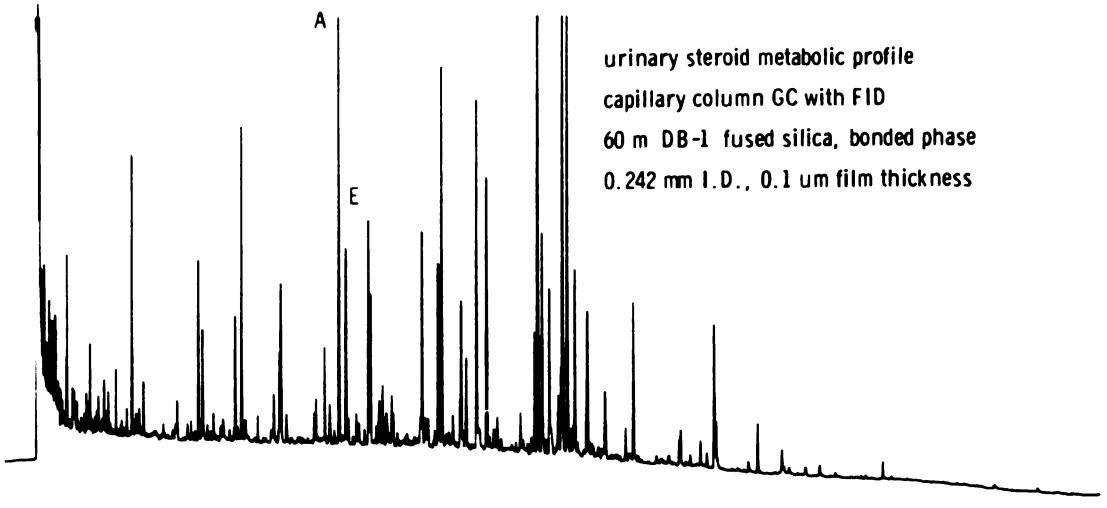
[b]Values are normalized and expressed as percentage of total for these eight urinary steroids.

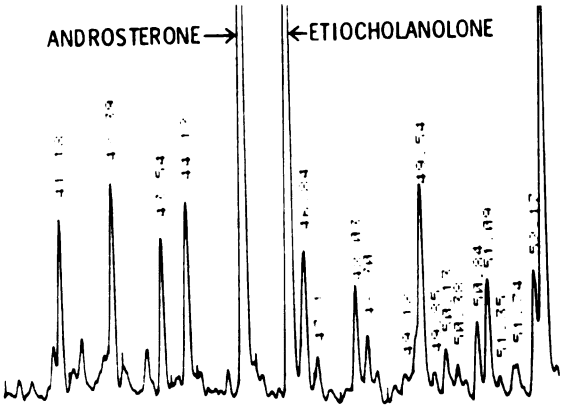
observed in this fraction). However, although we only found very small amounts of liberated steroids in the solvolysis phase in two different urine samples, this step was included in the procedure to minimize the effect of variations in enzyme activity from one batch to another, and because the sulfatases isolated from Helix pomatia reportedly are not able to hydrolyze 3αOH sulfate conjugates in 5α-steroids and 17- and 20-hydroxyl sulfate conjugates (Luenissen and Thijssen, 1978).

Figure 8 shows a urinary steroid metabolic profile of a normal adult male obtained with a 60 m DB-1 bonded phase fused silica capillary column (narrow base, thin film). Androsterone (A) and etiocholanolone (E) are labeled. The higher quality of the chromatogaphy is in contrast to that seen in Figure 7, which was obtained with a 25 m SP-2100 fused silica capillary column. The bottom trace, run at a faster chart speed, shows a smaller region of the chromatogram where androsterone and etiocholanolone elute. This column separated these epimers to such a degree that four small peaks could elute between them.

Quantitative metabolic profiling of volatile derivatives of steroids isolated from tissues or body fluids by GC/MS analysis using the repetitive scanning technique can be accomplished using either packed or capillary columns. Capillary columns offer two distinct advantages over packed columns, increased resolution and decreased sample loss due to adsorption during chromatography. The major disadvantage

Figure 8. Urinary steroid metabolic profile of a normal adult male subject obtained using a 60 m DB-1 bonded phase fused silica capillary column. The bottom trace, run at a faster chart speed, shows the region of the chromatogram where androsterone (A) and etiocholanolone (E) elute. Conditions of analysis: 180 to 310°C at 1.25°C/ minute, 50 minute isothermal period, split injection, μ = 20 cm/sec.

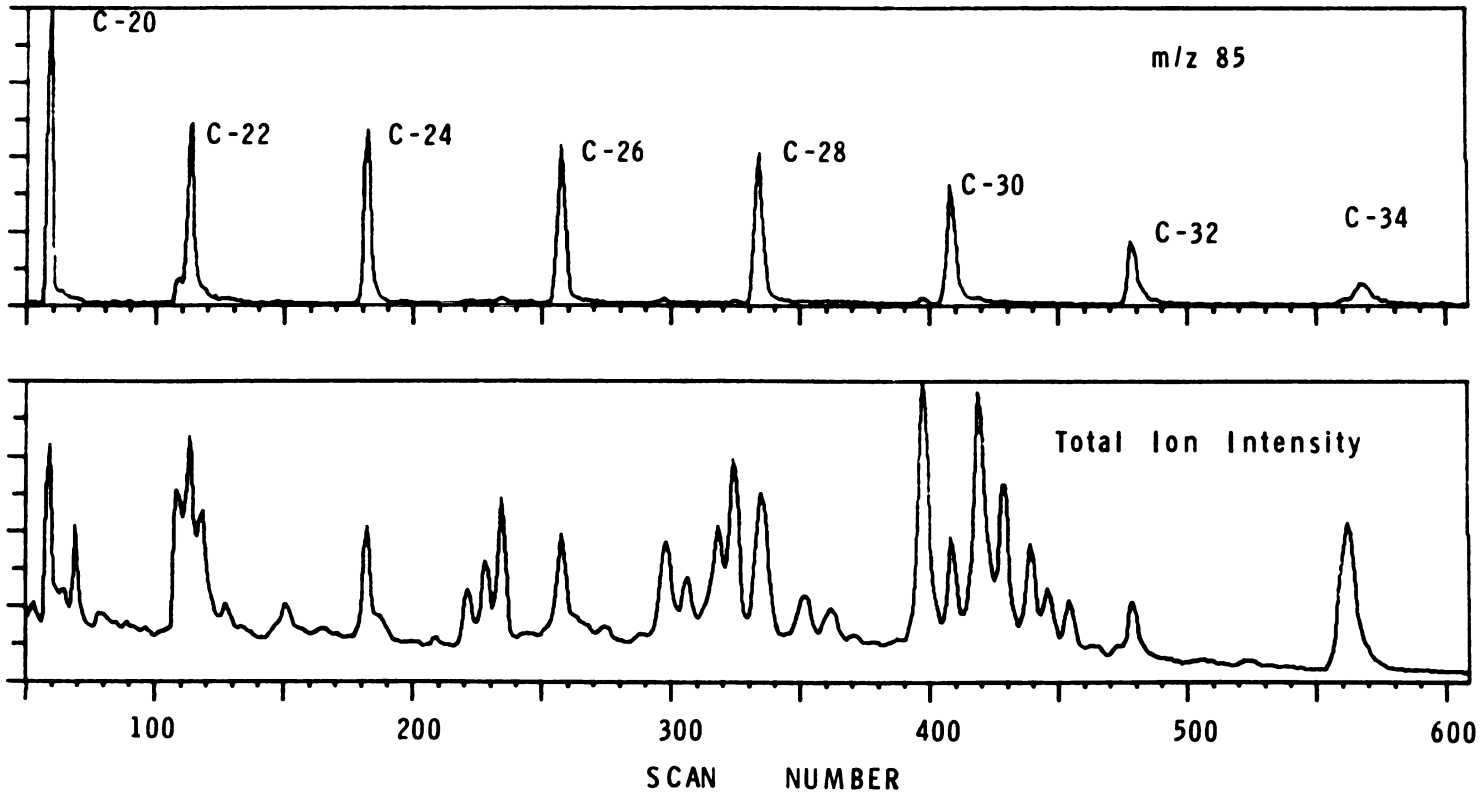

A
E
urinary steroid metabolic profile
capillary column GC with FID
60 m DB-1 fused silica, bonded phase
0.242 mm I.D., 0.1 um film thickness


ANDROSTERONE→
←ETIOCHOLANOLONE

of capillary columns is the necessity for shorter repetitive scan cycle times to establish a sufficient number of data points in reconstructed mass chromatograms. Quadrupole mass filters offer sufficiently short repetitive scan cycle times and have been used in the analysis of steroid profiles by capillary column GC/MS (De Weerdt et al., 1980; Maume et al., 1979). However, quadrupoles lack sensitivity in the high mass region of the mass spectrum necessary for steroid analysis. Magnetic sector instruments have the required sensitivity but have the disadvantage of slower repetitive scan cycle times. One way to circumvent partially this problem is to shorten the mass range over which the magnet is scanned. For example, Axelson and Sjövall (1977) reported using a 4-sec scan cycle time over m/z 250-480 in the analysis of steroids isolated from plasma.

Optimum qualitative and quantitative conditions for automated GC/MS/DS analysis of urinary steoids were established with a 3-m, 3% OV-101 packed column. Although it was known that many of the steroids do not separate satisfactorily by packed column chromatography, mass chromatograms were examined for their potential in quantitating poorly-resolved GC peaks. Figure 9 is a GC/MS total ion intensity trace of a sample of urinary steroids separated on the 3-m OV-101 packed column. Conditions of analysis are given in the Experimental. The mass chromatogram at m/z 85 shows where the co-injected hydrocarbons eluted. The broad shape of the peaks is typical of packed column analysis of derivatized

Figure 9. GC/MS analysis of aliphatic hydrocarbon standards and urinary steroids using a 3 m, 3% OV-101 packed column. Shown are the total ion intensity and the mass chromatogram for m/z 85 which shows where the co-injected hydrocarbons elute. Conditions of analysis are given in the text.


C-20
C-22
C-24
C-26
C-28
C-30
C-32
C-34
m/z 85
Total Ion Intensity
100
200
300
400
500
600
SCAN NUMBER

steroids. Steroids elute in the region between C-24 and C-34. Metabolites of testosterone elute in the first region of the chromatogram, metabolites of progesterone and estradiol elute in the middle region and metabolites of the adrenal steroids elute in the late region of the chromatogram. Figure 10 demonstrates that many urinary steroids can be easily quantitated by mass chromatography when packed columns are used, while poorly resolved steroids, which have nearly identical mass spectra, cannot be adequately analyzed by this approach. These results were anticipated from previous work by other investigators. For example, the steroid epimers THF and allo-THF shown by mass chromatogram m/z = 652 (M^+-31) are not separated to any appreciable extent by packed column GC. Neither are 11β-hydroxyandrosterone or 11β-hydroxyetiocholanolone (not shown).

An alternative approach, using capillary columns for the automated GC/MS/DS analysis of urinary steroids, was then investigated. To quantitate accurately the sharper capillary column peaks, the scan cycle time was shortened. Using a 25-m SP-2100 WCOT fused silica capillary column (0.2 mm I.D.) the ability of 1-, 2-, and 3-sec repetitive scan cycle times to reproducibly quantitate the much sharper peaks and give the desired reproducibility was investigated. A 3-sec cycle time was inadequate except for the largest peaks or when the amount of sample injected was sufficient to overload the column. Figure 11 shows the total ion intensity obtained using a 2-sec scan cycle time (m/z 50-600).

Figure 10. GC/MS analysis of urinary steroids in the region of RI 2500-3100 using a 3 m, 3% OV-101 packed column. Shown are the total ion intensity and an ion chromatogram of a characteristic ion for each of several steroids found in human urine. Conditions of analysis are given in the text.

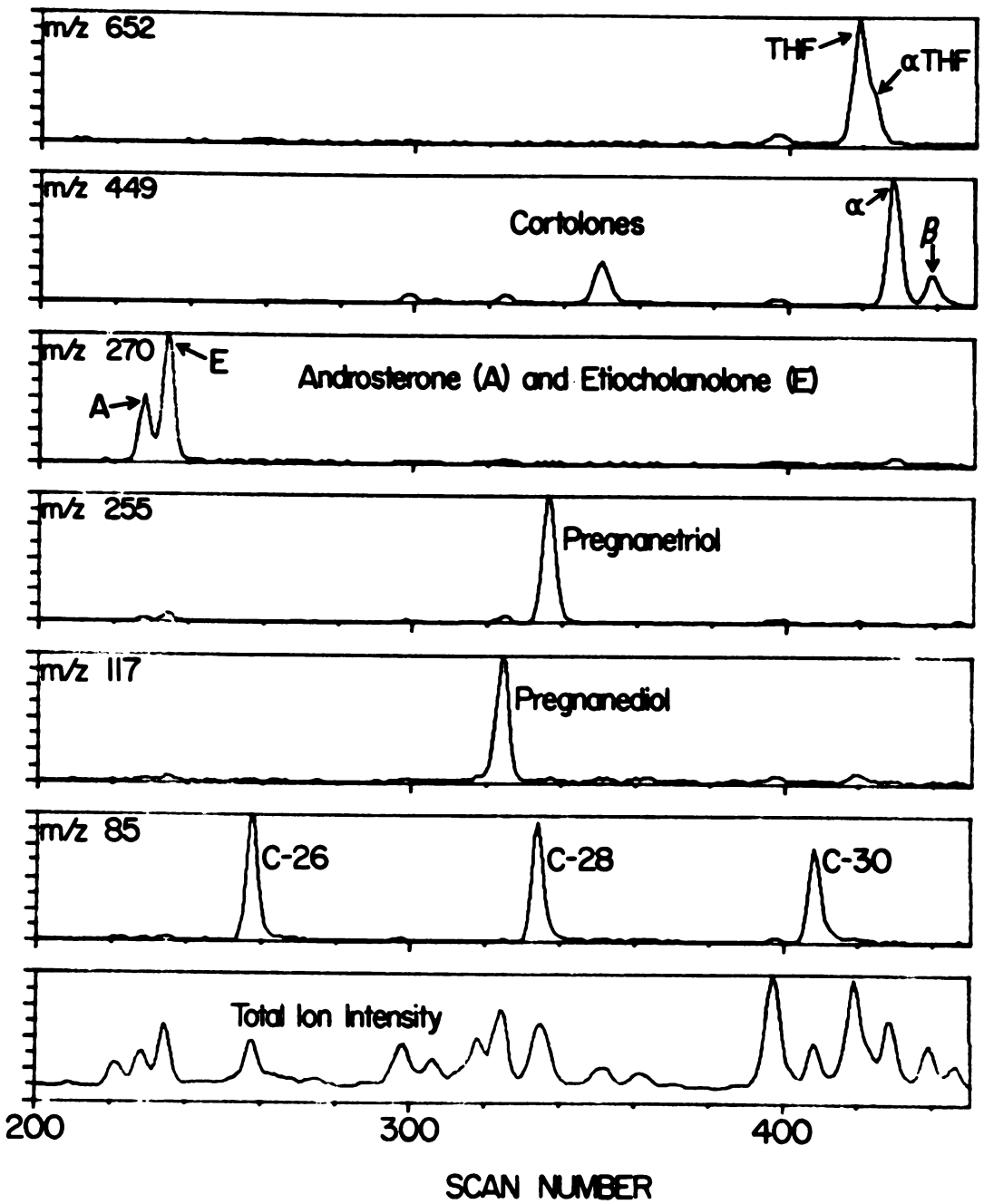
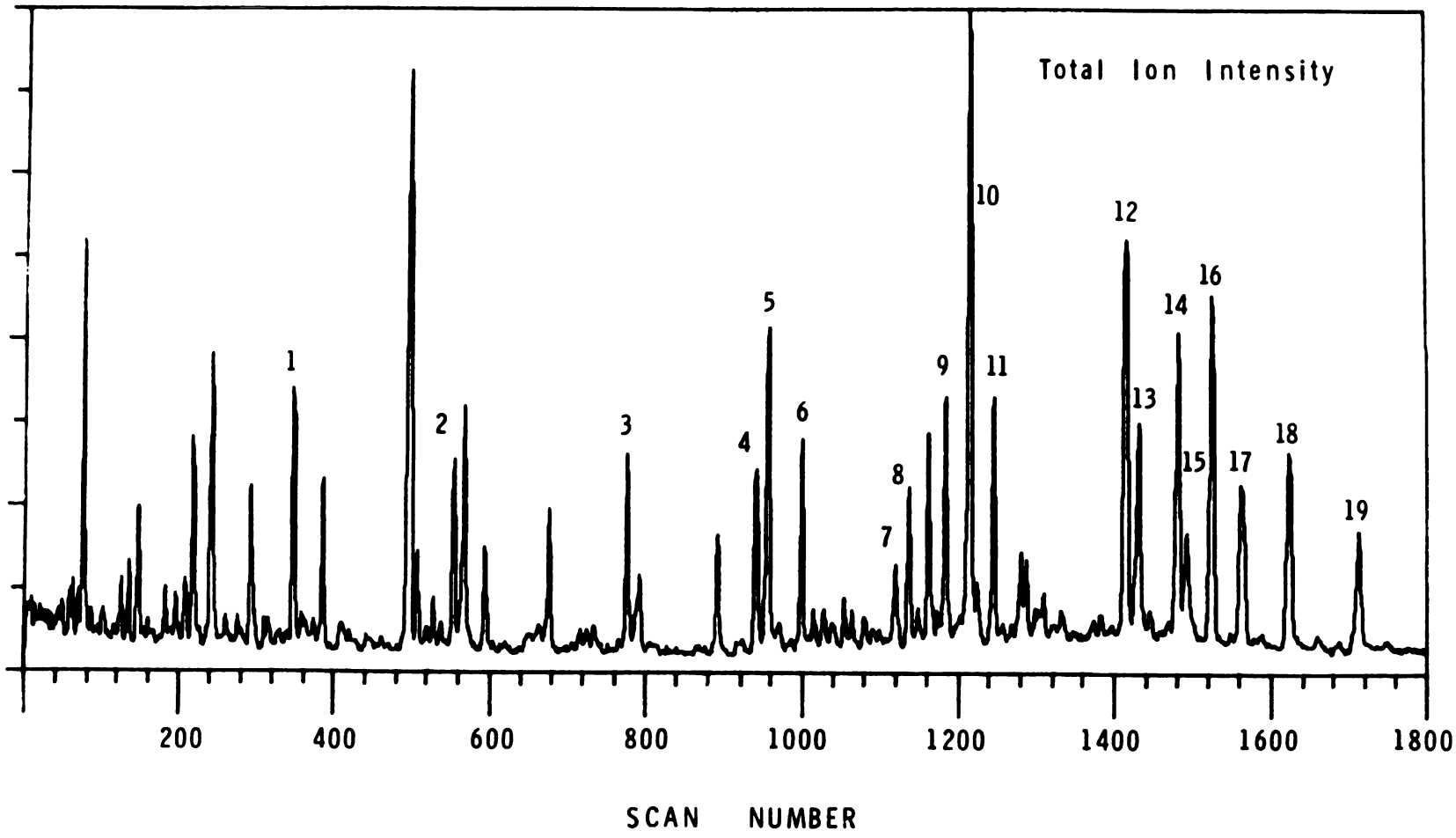

m/z 652
THF
αTHF
m/z 449
Cortolones
α
β
m/z 270
E
A
Androsterone (A) and Etiocholanolone (E)
m/z 255
Pregnanetriol
m/z 117
Pregnanediol
m/z 85
C-26
C-28
C-30
Total Ion Intensity
200
300
400
SCAN NUMBER

Figure 11. GC/MS analysis of urinary steroids using a 50 m (0.3 mm I.D.) OV-101 WCOT fused silica capillary column. Shown is the total ion intensity. Three µl of the sample was injected along with 1 µl of a mixture of straight chain saturated hydrocarbons (0.5 µg/µl). A 5:1 split ratio was used. Identity of peaks: 1) C-20 hydrocarbon, 2) C-22 hydrocarbon, 3) C-24 hydrocarbon, 4) androsterone, 5) etiocholanolone, 6) C-26 hydrocarbon, 7) 11β-hydroxy-androsterone, 8) 11β-hydroxy-etiocholanolone, 9) 16α-hydroxy-dehydroepiandrosterone, 10) pregnanediol and C-28 hydrocarbon, 11) pregnanetriol, 12) THE, 13) C-30 hydrocarbon, 14) THF, 15) allo-THF, 16) α-cortolone, 17) β-cortolone and β-cortol, 18) α-cortol, 19) C-32 hydrocarbon.

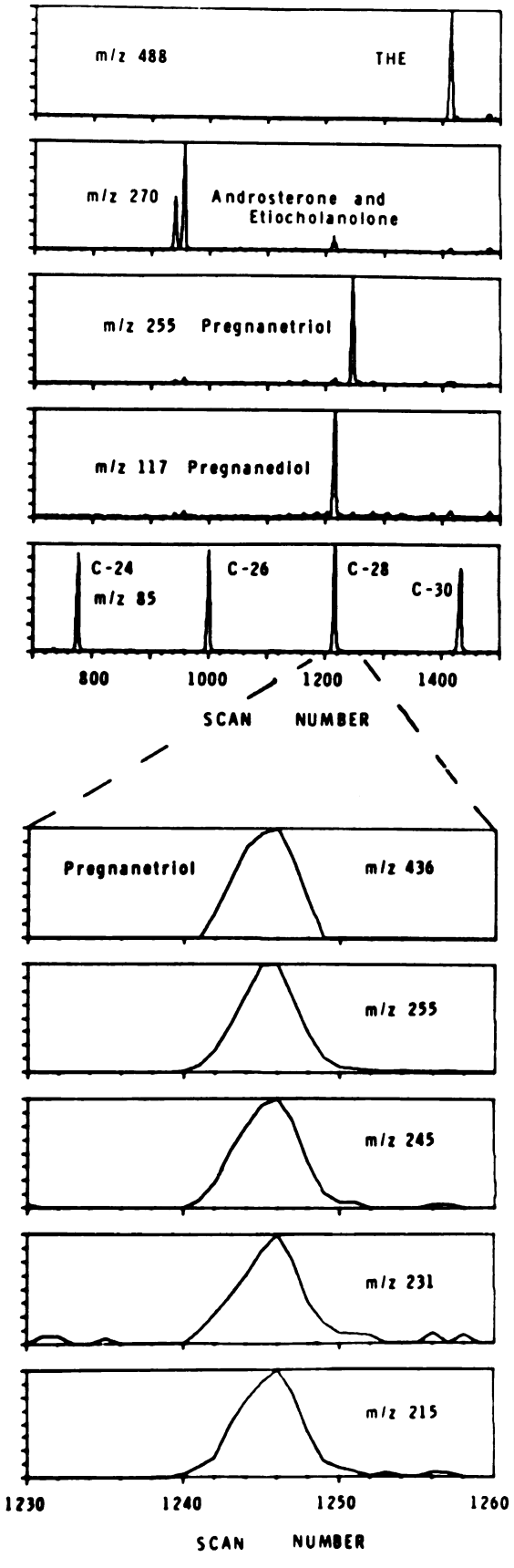

Total Ion Intensity
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
200
400
600
800
1000
1200
1400
1600
1800
SCAN NUMBER

This GC/MS trace is in dramatic contrast to the GC/MS trace shown in Figure 9 which was obtained using a packed column. Some of the peaks have been labeled (see Figure legend). The top of Fig. 12 shows the region between C-24 and C-30. Characteristic mass chromatograms of the co-injected hydrocarbons, androsterone, etiocholanolone, pregnanediol, pregnanetriol and 3α,17α-21-trihydroxy-5β-pregnane-11,20-dione (THE) are displayed. It is important to understand that these steroids were identified not only by their characteristic ion currents, but also by their characteristic retention indices.

The bottom of Fig. 12 shows the set of ions for pregnanetriol that the MSSMET program used to identify and quantitate this compound. For each compound searched for by the MSSMET program, one ion that is both characteristic and intense was used for quantitative purposes (the designate ion) and the presence and relative intensities of a few other ions produced during fragmentation were used to confirm the identity of the compound in question (confirming ion). Retention index as well as good ion statistics on the designate and confirming ions are crucial to the identification of each compound. In the example shown, m/z 255 is used to quantitate pregnanetriol and the other peak area intensities were used in the calculation of a confidence coefficient indicating the presence of this compound.

Reproducibility of the computer-assisted automated metabolic profiling analysis of urinary steroids using capillary

Figure 12. GC/MS analysis of urinary steroids using a 50 m OV-101 WCOT fused silica capillary column. Shown are characteristic mass chromatograms for the co-injected hydrocarbons, androsterone, etiocholanolone, pregnanediol, pregnanetriol and THE. The bottom graph shows the set of ions for pregnanetriol that the MSSMET program uses to identify and quantitate this compound. Conditions of analyses are given in the text. A K-factor (Gates *et al.*, 1978b, 1978c) was determined for pregnanetriol and the amount injected was calculated to be 18 ng.


m/z 488
THE
m/z 270
Androsterone and
Etiocholanolone
m/z 255 Pregnanetriol
m/z 117 Pregnanediol
C-24
m/z 85
C-26
C-28
C-30
800
1000
1200
1400
SCAN NUMBER
Pregnanetriol
m/z 436
m/z 255
m/z 245
m/z 231
m/z 215
1230
1240
1250
1260
SCAN NUMBER

column GC/MS is summarized in Table 2. Values are the integrated peak areas determined by the MSSMET program of a characteristic ion for each steroid expressed as a percent of the sum of the areas for these five urinary steroids. Data are from four separate GC/MS analyses of the same sample using a 25-m SP-2100 WCOT fused silica capillary column (0.2 mm I.D.). Data are given for the earlier eluting steroids since their GC peaks are much sharper than those occurring later in the GC run (the cortols and the cortolones, for example) and thus they are the most difficult to quantitate accurately by the repetitive scanning technique. The overall precision was 4.8% for these four separate GC/MS analyses (calculated by expressing the standard deviation as a percent of the mean and averaging these values for the five steroids shown). Also shown in Table 2 is the ratio of etiocholanolone to androsterone as determined by MSSMET. Using a K-factor (calculated by knowing the ratio of the intensities of ion currents for the internal standard, cholesteryl butyrate, and pregnanetriol for equal amounts of each compound [Gates et al., 1978b, 1978c]), the amount of pregnanetriol was calculated to be 18 ng injected (150 ng/ml urine). These results demonstrate that a 2-sec scan cycle time is sufficient to quantitate urinary steroids by capillary column GC/MS using reconstructed mass chromatograms from repetitive scanning data.

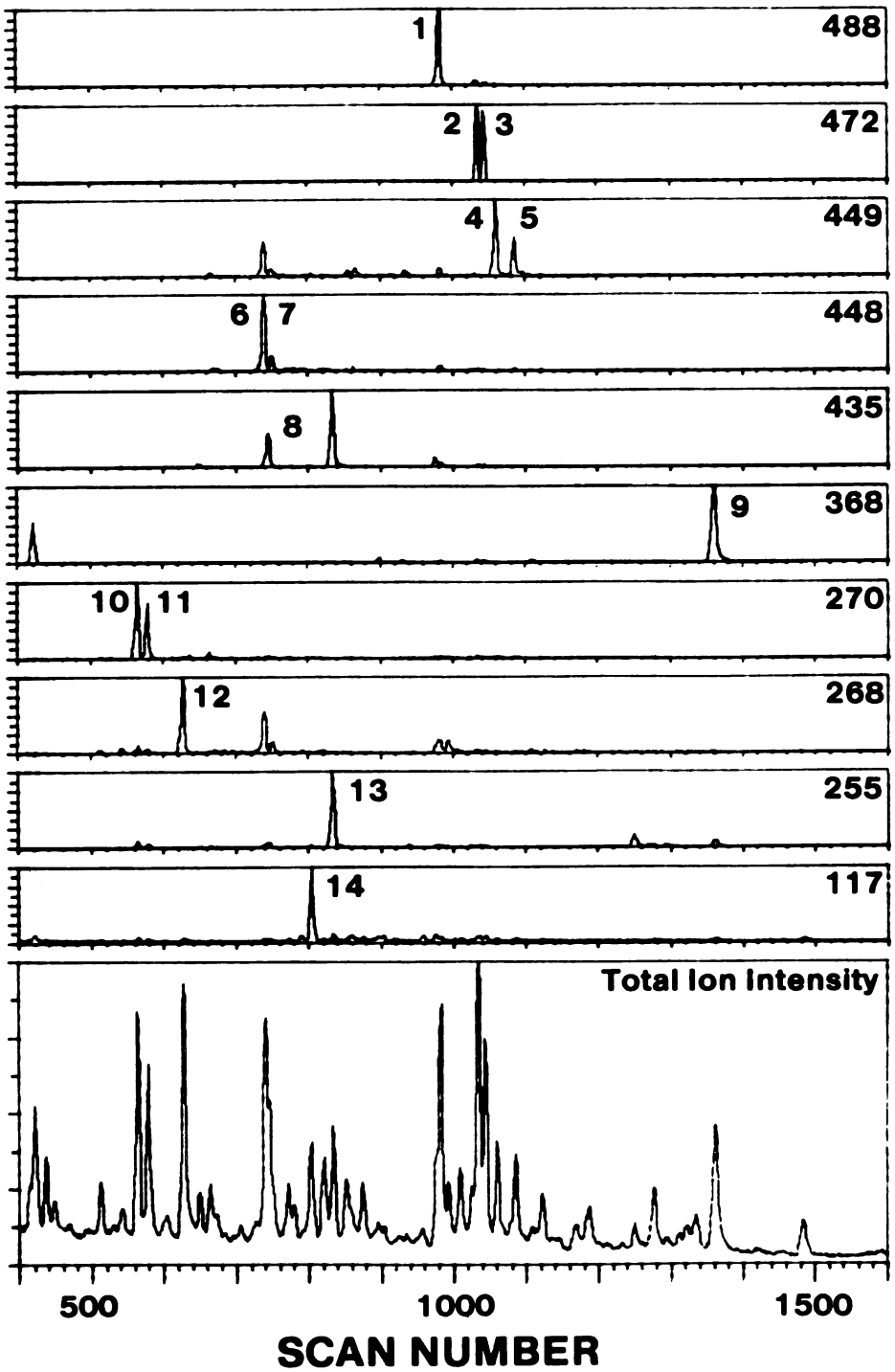
Table 2. Precision: Capillary Column GC/MS Profiling of Urinary Steroids[a]

Steroid	Run Number 1	2	3	4	Mean ± S.D.[b]
Androsterone	9.4	8.6	10	10	9.50 ± 0.7
Etiocholanolone	12	12	13	13	12.5 ± 0.6
Pregnanediol	59	59	57	56	57.8 ± 1.5
Pregnanetriol[c]	14	16	15	15	15.0 ± 0.8
THE	5.4	5.8	5.8	5.9	5.73 ± 0.22
Etio./Andro.[d]	1.2	1.3	1.3	1.3	1.28 ± 0.05

[a]Values are the integrated peak areas determined by MSSMET of a characteristic ion for each steroid expressed as a percent of the sum of the areas for these 5 urinary steroids. Data are from 4 separate GC/MS analyses of the same sample using a 25 M SP-2100 WCOT fused silica capillary column (0.2 mm I.D.).
[b]The average precision was ± 4.8 percent (calculated by expressing each S.D. as % of the mean for the 5 steroids shown).
[c]A K-factor (Gates et al., 1978b, 1978c) was determined for pregnanetriol and the amount injected corresponded to 18 ng.
[d]Ratio of etiocholanolone to androsterone.

Optimization of GC/MS conditions for automated reverse library search of selected mass chromatograms using capillary columns. We have found that two factors must be taken into consideration when developing optimal conditions for the quantitative analysis of complex mixtures by capillary column gas chromatography repetitive scanning mass spectrometry that are not considerations for capillary gas chromatography alone. First, capillary gas chromatographic peaks are much sharper than packed column peaks and require shorter scan cycle times to obtain an adequate number of data points for quantification. Thus, while sharp peaks are desirable for quantitative analysis of complex mixtures by capillary gas chromatography alone, this was detrimental when appropriate scan rates could not be realized with the instrument system available. The second important consideration was the detection and accurate quantification of minor components in a complex mixture. Compounds that were 100-1000 times less concentrated than the major components in a complex mixture were quantified relatively easily by capillary gas chromatography with FID, as long as they were well separated from nearby peaks. However, when repetitive scanning GC/MS was used, sensitivity problems occurred. Figure 13 shows the urinary steroid metabolic profile of a normal adult male subject. Selected mass chromatograms for a number of the major urinary steroids have been plotted above the total ion intensity (see Figure legend). The capillary

Figure 13. Urinary steroid metabolic profile of a normal adult male subject. The total ion intensity is plotted against scan number (2s scan cycle) at the bottom and selected mass chromatograms of designate ions for a few major steroids are plotted above. Labelled peaks are (1) THE, (2) THF, (3) allo-THF, (4) cortolone, (5) β-cortolone, (6) 11β-hydroxyandrosterone, (7) 11β-hydroxyetiocholanolone, (8) internal standard, (9) cholesterylbutyrate, added as a second internal standard, (10) androsterone, (11) etiocholanolone, (12) DHEA, (13) pregnanetriol (14) pregnanediol. Conditions of analysis are as follows: LKB-2091 GC/MS with a dual Digital Equipment Corp. PDP-8e based foreground-background data system (Martin et al., 1980); 25 M OV-101 fused silica capillary column, 0.32 mm I.D.; μ = 25 cm s^{-1}; split injection, 2 µl of sample injected; gas chromatographic oven temperature programming from 180 to 280°C at 2°C min^{-1}; El ionization at 70 eV; 2 s scan cycle interval, m/z 50-550.


1
488
2
3
472
4
5
449
6
7
448
8
435
9
368
10
11
270
12
268
13
255
14
117
Total Ion Intensity
500
1000
1500
SCAN NUMBER

gas chromatograph had to be overloaded with sample when using repetitive scanning mass spectrometry for detection and analysis of urinary steroids.

The lower limit of accurate quantification for any compound by mass chromatography from repetitive scanning GC/MS data is is determined by such factors as the percent total ionization of the ion used for quantification of a particular compound, scan speed of the mass spectrometer, cleanliness of the source and other factors. For full mass range repetitive fast scanning GC/MS, this limit is generally in the range of 1.0-10 ng injected for modern instruments operated in an ordinary manner. Thus, if minor components are 100-200 times lower in level than the major components of a mixture, several hundred nanograms of the major components must be injected for accurate quantification of these minor components. Unfortunately, this resulted in overloading of those capillary columns that offered sufficient column efficiency to separate steroid stereoisomers. Although this was not aesthetically pleasing, this condition did not present an analytical problem so long as compounds with virtually identical mass spectra were still chromatographically resolved. A moderate degree of overloading of the capillary column therefore provided a wider concentration dynamic range than would otherwise have been possible.

Optimal metabolic profiling analysis of urinary steroids requires determination of conditions which allow adequate chromatographic separation of major stereoisomers such as

androsterone and eticholanolone, THF and allo-THF. Although these peaks were somewhat distorted by overloading, the precision of quantification was not compromised so long as the chromatographic separation of these epimers was maintained.

These considerations are graphically illustrated in Figures 14, 15 and 16. Figure 14 shows the change in peak shape and the increase in number of data points across a peak that occur with increasing amounts of hydrocarbon, n-pentadecane, injected onto the capillary column. The exact sample size where a particular capillary column will become 'overloaded' is related to the internal diameter of the capillary column and the stationary phase film thickness. With capillary columns that offer sufficient performance to separate steroid stereoisomers we have observed that steroids overloaded when between 100 ng and 500 ng of each steroid are injected. Figure 15 shows total ion intensity v. scan number in the region where the internal standard elutes. Also shown are characteristic reconstructed mass chromatograms for one of the internal standards (m/z 435), 11β-hydroxyandrosterone (m/z 448; first peak) and 11β-hydroxyethiocholanolone (m/z 448; second peak). Although there is a slight overlap of the two m/z 448 peaks and the m/z 453 peak exhibits a moderate degree of 'fronting', each separate profile is well defined and the peak areas can be accurately integrated using a fully automated computer search of selected mass chromatograms.

Figure 14. Changes in peak shape and number of points across a gas chromatographic peak following increasing amounts of n-pentadecane injected into a 25 m OV-101 fused silica capillary column (0.32 mm I.D., 150°C isothermal). m/z 99 and 85 are plotted v. scan number for 5, 50, 200, 350, 600 and 2000 ng injected. A 2 s scan cycle was used.

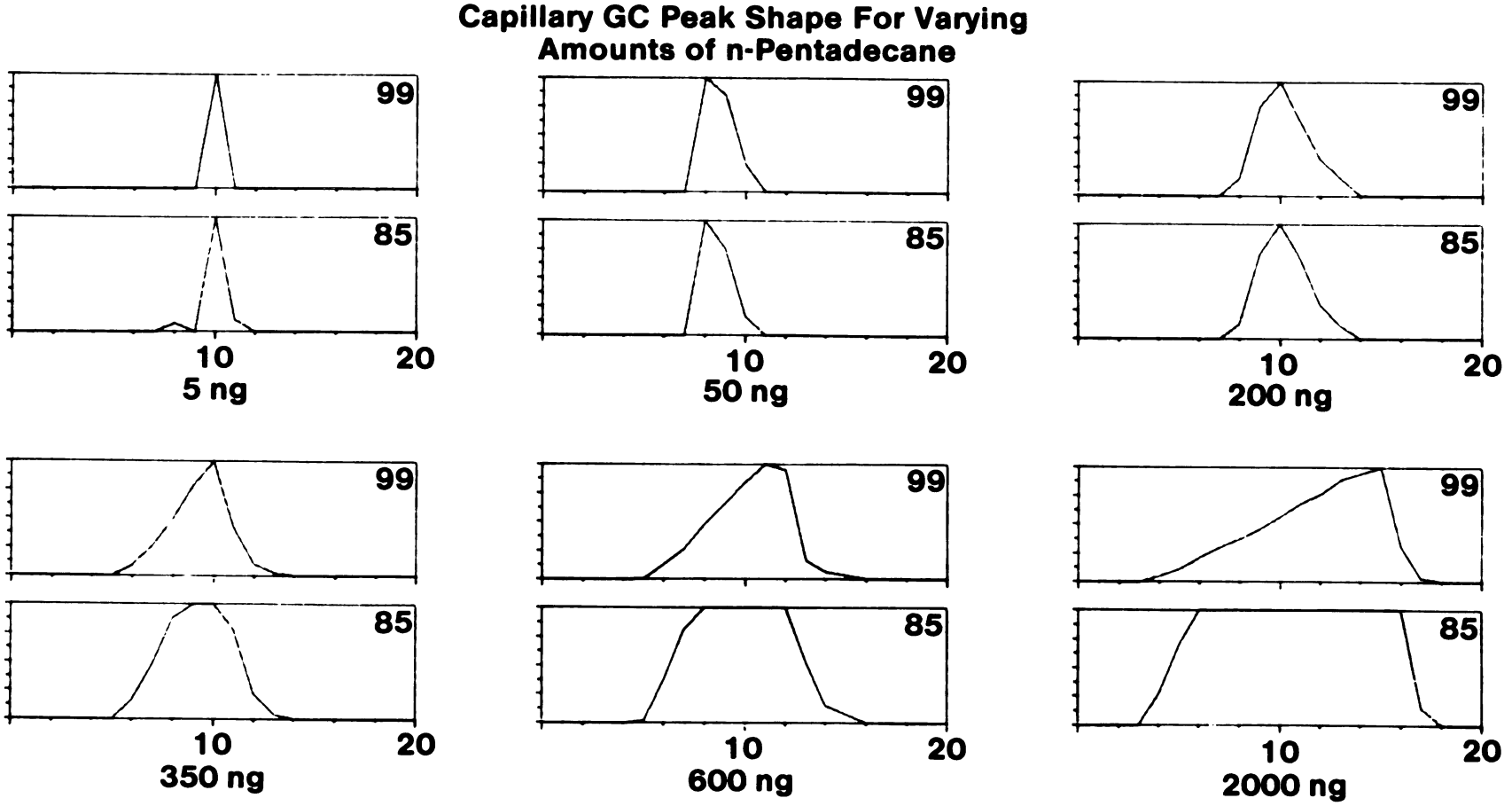

Capillary GC Peak Shape For Varying
Amounts of n-Pentadecane
99
85
10
20
5 ng
50 ng
200 ng
350 ng
600 ng
2000 ng
25 m OV-101 Fused silica capillary column,
0.32 mm I.D., 150° C isothermal, 2 sec. scan cycle

Figure 15. Total ion intensity v. scan number in the region of the chromatogram where 11β-hydroxyandrosterone, 11β-hydroxyetiocholanolone and the internal standard elute. Shown are characteristic mass chromatograms for the internal standard (m/z 435) and 11β-hydroxyandrosterone (m/z 448; first peak) and 11β-hydroxyetiocholanolone (m/z 448, second peak). Conditions of analysis are given in the text.

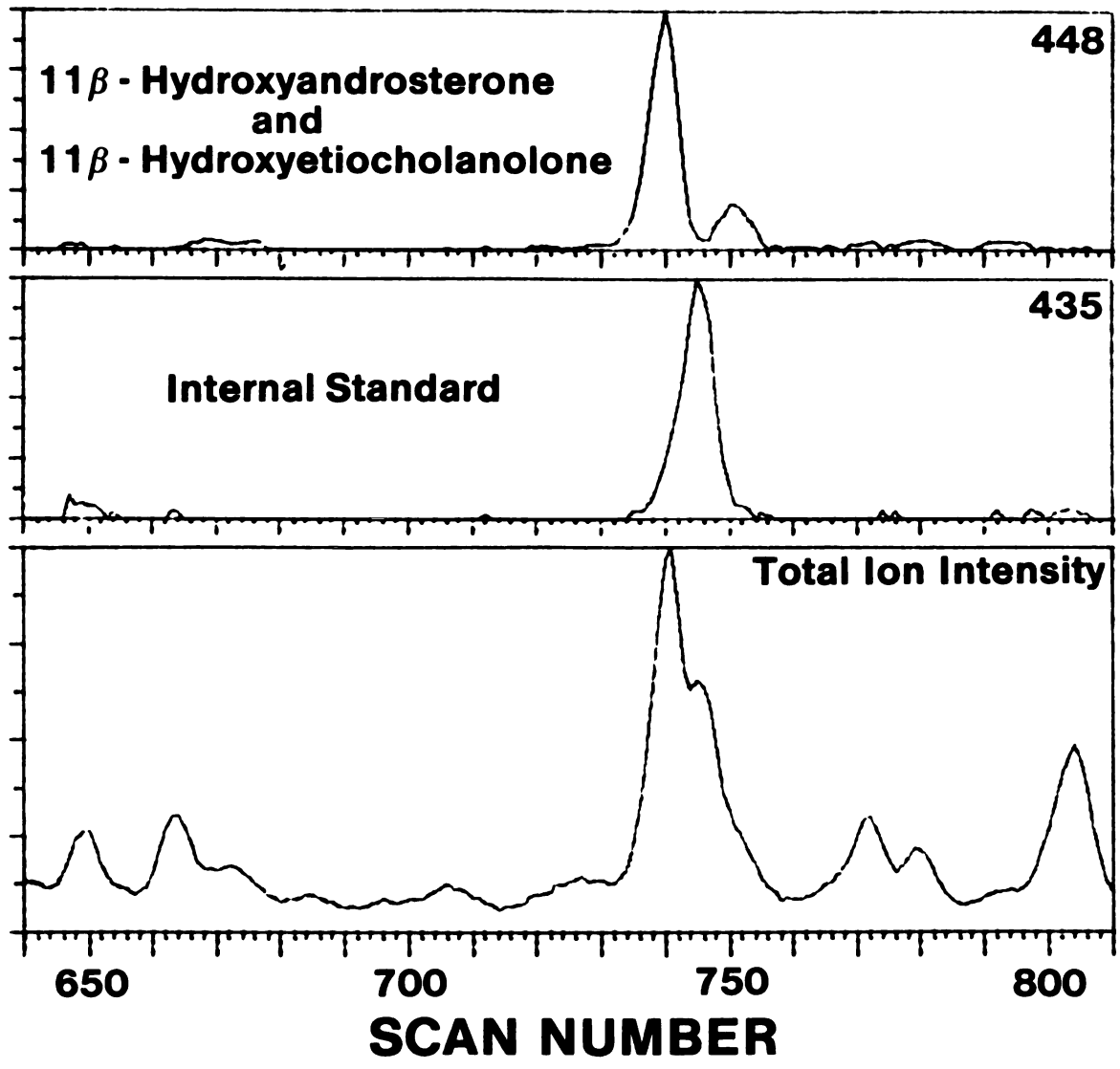
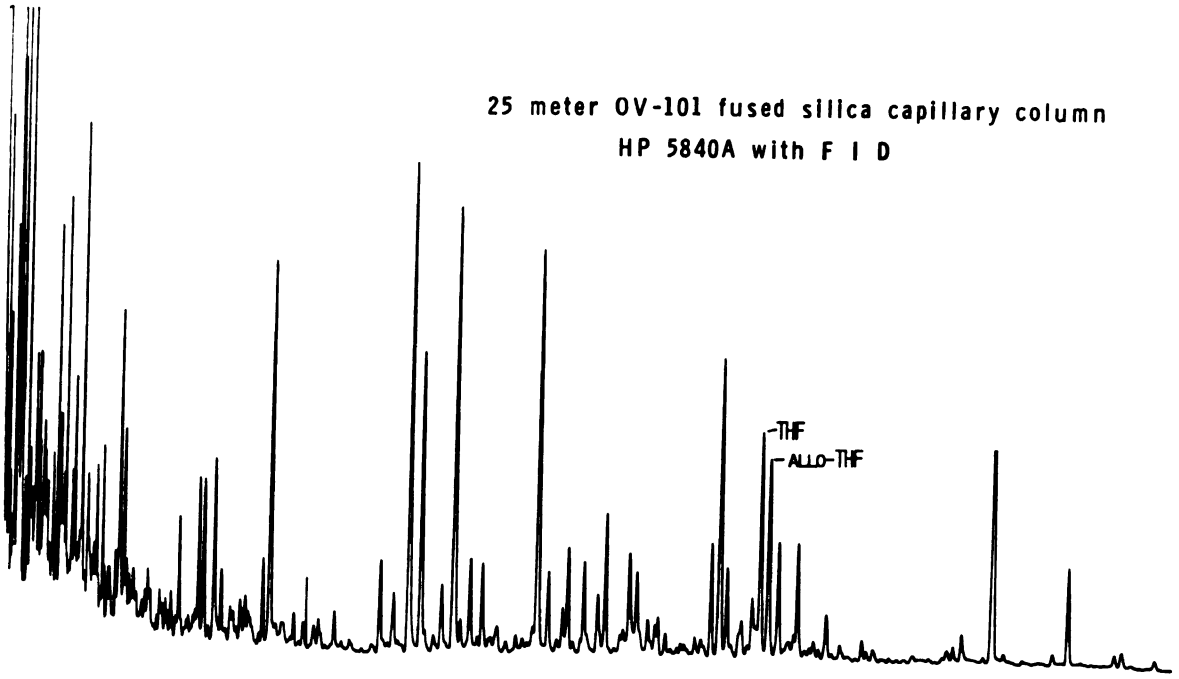
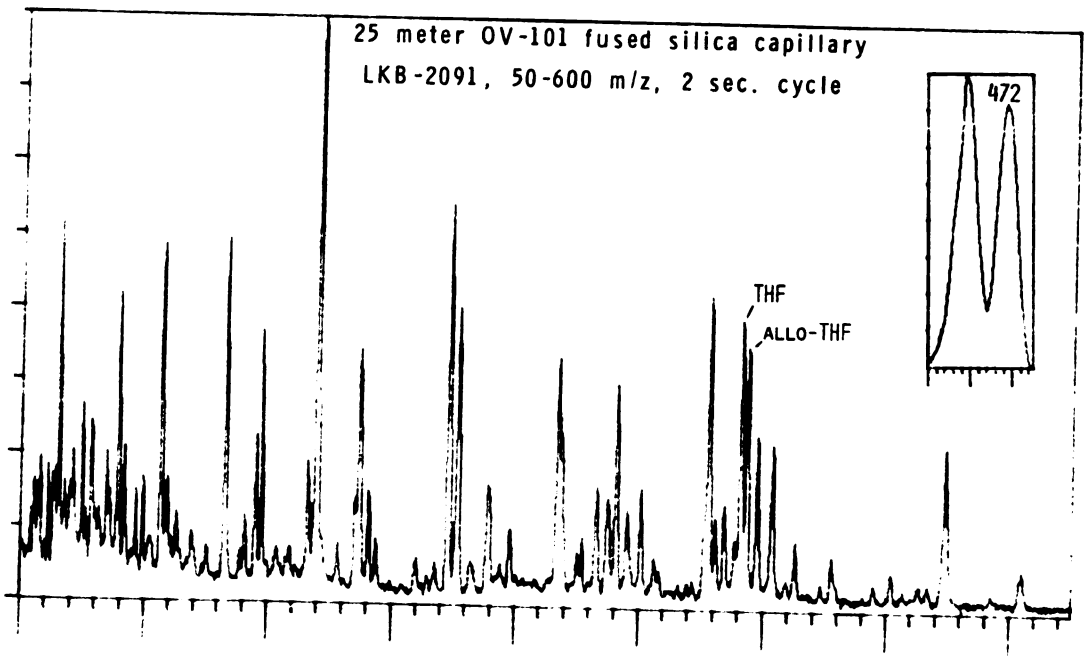

448
11β - Hydroxyandrosterone
and
11β - Hydroxyetiocholanolone
435
Internal Standard
Total Ion Intensity
650
700
750
800
SCAN NUMBER

Figure 16. Urinary steroid metabolic profiles using a 25 m OV-101 fused silica capillary column with FID (top trace) and the same column with MS detection (repetitive scanning; 2 s scan cycle). In the top trace THF and allo-THF are labeled to demonstrate the complete separation of these epimers using FID. The bottom trace shows a GC/MS analysis using the same column. A 2 second scan cycle was used.


25 meter OV-101 fused silica capillary column
HP 5840A with F I D
THF
ALLO-THF


25 meter OV-101 fused silica capillary
LKB-2091, 50-600 m/z, 2 sec. cycle
472
THF
ALLO-THF

The major steroid epimers that were the most difficult to separate were THF and allo-THF. The top trace of Figure 16 shows a urinary steroid metabolic profile obtained using a 25 m, 0.32 mm I.D., OV-101 fused silica capillary column with FID. THF and allo-THF are labeled to demonstrate the complete separation of these epimers. The bottom trace in Figure 16 shows a GC/MS analysis using the same column (TII is plotted against scan number). The column has been overloaded such that these epimers are no longer completely separated. However, the degree of separation is such that each epimer can still be independently quantitated well within the precision of the overall method (separation shown by mass chromatogram m/z = 472; insert).

It might be informative to consider further some of the advantages and disadvantages of packed and capillary columns for automated metabolic profiling analysis of urinary steroids by a GC/MS data system using reverse library search of selected mass chromatograms. Capillary columns are reported to offer several distinct advantages over packed columns, such as increased resolution, decreased sample loss due to adsorption during chromatography (Axelson and Sjövall, 1977), and potentially shorter analysis times. However, accurate quantification of minor components in complex mixtures by repetitive scanning GC/MS using capillary column chromatography requires high sensitivitiy and fast scan speeds to establish a sufficient number of data points in reconstructed mass chromatograms. Quadrupole mass

filters offer sufficiently short scan cycle times but exhibit a severe mass intensity discrimination problem above approximately m/z 500, where many of the qualitatively characteristic fragment ions of methoxime-trimethylsilyl derivatives of steroids occur. Although magnetic sector instruments do not have this problem they have the distinct disadvantage of limitations in the repetitive scan cycle times.

It was our experience that for many applications, packed column gas chromatography was adequate for metabolic profiling analysis of urinary steroids and was, in fact, quite superior in certain situations where differences in the levels of minor steroid metabolites were of interest. As an example, in the studies described in sections III and IV, certain differences that were detected by packed column GC/MS data systems were not observed by capillary column GC/MS data systems unless the columns were severely overloaded. Thus, in certain situations (metabolic profiling of body fluids, for example) it would be prudent to use both packed and capillary columns, taking advantage of the qualities each of these systems offers for optimal qualitative and quantitative analysis.

The investigations just described have led to the development of an automated GC/MS/DS procedure and a capillary column GC/FID procedure for reproducible quantitative analysis of complex mixtures of steroids. Important features of the GC/MS/DS system are the use of both packed

and capillary column chromatography, a non-mass discriminating mass analyzer and a fully automated reverse library search procedure using methylene unit retention indices and reconstructed mass chromatograms.

SECTION III

Animal Studies

SECTION III

INTRODUCTION

This section describes experiments, using the rat as a model, designed to examine the effects of PBBs on the urinary steroid metabolic profile, and to correlate differences in the profile with known specific effects these chemicals have on steroid metabolism in vitro (i.e., increased 6β, 7α and/or 16α-hydroxylations; see background in Section I). Since steroid metabolism and excretion in the rat is very complex and qualitatively different from man, an overview of the findings of the most relevant investigations into steroid hormone metabolism and excretion in rats is presented below.

Metabolism of steroid hormones in rats. Steroid hormones must be metabolized to more polar molecules in order to be excreted in the urine and feces. In general, this is accomplished by a series of oxidative, reductive and conjugation reactions in both man and rats. Although man and the rat have certain metabolic reactions in common, there are many species differences (see Introduction, Section IV, for a detailed discussion of steroid metabolism in man). For example, while both man and rats reduce the 4-ene-3-one A-ring configuration, the rat has extensive

further metabolism that occurs via gut microbes during enterohepatic circulation (Eriksson, 1971a). Some of the microbial metabolites are reabsorbed and may be excreted in the urine (Gustafsson, 1968; Eriksson and Gustafsson, 1970; Gustafsson, 1970) as well as the faeces. Also, there are marked sexual differences in the metabolism and excretion patterns of steroids which are in part due to differences in the oxidative metabolism of steroids in the livers of male and female rats as well as to differences in conjugation reactions (Eriksson et al., 1972).

Lisboa et al. (1968) showed that rat liver microsomes are capable of hydroxylation of testosterone to 3-keto-Δ^4-hydroxy metabolites. These metabolites were identified as 2β-, 6β-, 7α-, and 16α-hydroxytestosterone and 6β,16α,17β-trihydroxyandrost-4-ene-3-one. Gustafsson et al. (1968a) reported that saturated C-19 steroids with a 3-keto-5α-configuration were formed from rat liver microsomes. These metabolites were reported to be 2β-, 6β-, 7α- and 16α-hydroxy-5α-dihydrotestosterone. These compounds were also formed from 2β-, 6β-, 7α- and 16α-hydroxytestosterone precursors. When 5α-dihydrotestosterone was used as substrate, 2β-, 7α- and 6α-derivatives were formed but not 6β-hydroxy-5α-dihydrotestosterone. Gustafsson et al. (1968b) also reported the formation of several 5α-androstanetriols by rat liver microsomes. When testosterone was used as a substrate, 5α-androstane-2β,3α,17β-triol, 5α-androstane-3α, 7α,17β-triol and 5α-androstane-3α(and 3β),16α,17β-triol were

formed. 2β- and 7α-Hydroxytestosterone precursors gave androstane triols with a 3α-configuration while incubation with 6β- and 16α-hydroxytestosterone gave both 3α- and 3β-epimers. When 5α-dihydrotestosterone was used as a substrate, 5α-androstane-2β,3α(and 3β),17β-triol, 5α-androstane-3α(and 3β),7α,17β-triol and 5α-androstane-3α(and 3β), 16α,17β-triol were formed (no 6β-hydroxy steroids were formed). Only 5α-androstane-2β,3α,17β-triol was formed when 5α-androstane-3α,17β-diol was incubated. 5α-Androstane-3β, 17β-diol was not hydroxylated by the microsomal preparation. 6α-Hydroxylation of testosterone and androstenedione by rat liver microsomes has also been reported (Gustafsson and Lisboa, 1970a). Several metabolites of 4-androstene-3,17-dione, 5α-androstane-3,17-dione, 3α-hydroxy-5α-androstane-17-one and 3β-hydroxy-5α-androstane-17-one are formed in rat liver microsomes. These include 3α(and 3β),16α-dihydroxy-5α-androstane-17-one, 3α(and 3β),7α-dihydroxy-5α-androstane-17-one, 3β,7β-dihydroxy-5α-androstane-17-one and 3β,17β-dihydroxy-5α-androstane-16-one (Gustafsson and Lisboa, 1970b). 6α- and 6β-Hydroxylation of testosterone has also been reported to occur in rat testis preparations (Gustafsson and Lisboa, 1970c). A number of metabolites of testosterone have been identified in the feces of germ-free male and female rats. These include 3α,11β-dihydroxy-5α-androstane-17-one, 3α,7α-dihydroxy-5α-androstane-17-one, 3α,15α-dihydroxy-5α-androstane-17-one, 5α-androstane-3α,5α, 17β-triol and 5α-androstane-3α,17β-diol (Gustafsson and Sjovall, 1968).

Differences have been noted between male and female rats in the metabolism of corticosterone by the isolated perfused liver preparation. Metabolites in the male rats were 5α-pregnane-3α,11β,20β(and 20α),21-tetrol and 5α-pregnane-3β, 11β,20β,21-tetrol. Only small amounts of 5α-pregnane-3α, 11β,20β,21-tetrol were produced in female rat liver while the predominant metabolites were 3α(and 3β),11β,15α,21-tetrahydroxy-5β-pregnane-20-one and 3α(and 3β),11β,21-trihydroxy-5α-pregnane-20-one. Female rat livers also produced far greater amounts of mono- and disulfate conjugates than did the male rat livers (Eriksson and Gustafsson, 1971). Gustafsson and Sjovall (1968b) identified a number of 15α- and 21-hydroxylated C-21 steroids in germ-free rats that were not found in conventional rats. 3α,11β,15α,21-tetrahydroxy-5α-pregnane-20-one and 3α,15α,21-trihydroxy-5α-pregnane-11,20-dione were the major peaks while smaller amounts of 11β,21-dihydroxy-5α-pregnane-3,20-dione, 3α,11β, 21-trihydroxy-5α-pregnane-20-one, 3α,21-dihydroxy-5α-pregnane-11,20-dione and 3α,15α-dihydroxy-5α-pregnane-20-one were found. The absence of 21-hydroxylated steroids in feces from conventional rats has been explained by 21-dehydroxylation of 21-hydroxy-20-keto steroids by gut microbes (Eriksson et al., 1969).

Rat liver microsomes can hydroxylate progesterone at a number of positions. Gustafsson and Lisboa (1970d) reported the formation of 2α-, 6β-, 6α-, 7α-, 15α-, 15β- and 16α-hydroxyprogesterone in rat liver microsomes. Several

hydroxypregnanolones have been isolated from the feces of male and female germ-free rats. These include 3α,16α-dihydroxy-5α-pregnane-20-one, 3β,16α-dihydroxy-5-pregnene-20-one and 3β,16α-dihydroxy-5α-pregnane-20-one (Gustafsson et al., 1968a). When [4-^{14}C]pregnenolone was injected intraperitoneally in female rats, 70% of the radioactivity was recovered in the bile (via cannula), with 45% of this radioactivity being in the monosulfate fraction, 28% in the disulfate fraction, 22% in the glucuronide fraction and 2% in the free fraction (Cronholm et al., 1971). Metabolites of pregnenolone in the glucuronide fraction included 3α-hydroxy-5α-androstane-17-one, 3α-hydroxy-5α,17β-pregnane-20-one and 3α,16α-dihydroxy-5α-pregnane-20-one while 3α,7α-dihydroxy-5α-androstane-17-one, 3α,15α-dihydroxy-5α-androstane-17-one, 3α,11β-dihydroxy-5α-androstane-17-one and 3α,16β-dihydroxy-5α-pregnane-20-one were found in the monosulfate fraction and 3α,7α-dihydroxy-5α-androstane-17-one, 3α,11β-dihydroxy-5α-androstane-17-one, 5α-pregnane-3β,20β-diol and 3α,21-dihydroxy-5α-pregnane-20-one were found in the disulfate fraction. In these experiments the quantitatively most important steroid metabolites were the unlabeled corticosterone metabolites 3α(and 3β),11β,21-trihydroxy-5α-pregnane-20-one and 3α,11β,15α,21-tetrahydroxy-5α-pregnane-20-one.

Following intraperitoneal administration of [4-^{14}C] pregnenolone and [4-^{14}C]corticosterone to female germ-free rats the following steroids were recovered in the

feces: disulfate fraction; 3α(and 3β),11β,21-trihydroxy-5α-pregnane-20-one, monosulfate fraction; 3β,17α-dihydroxy-5α-pregnane-20-one, 5α-pregnane-3α,16α,20α-triol, 2α,3β,16α-trihydroxy-5α-pregnane-20-one and 3α,15β,16α-trihydroxy-5α-pregnane-20-one (Eriksson and Gustafsson, 1970b). Conventional rats showed a different distribution of radioactivity, in part due to the formation of 3β,11α-dihydroxy-5α-pregnane-20-one and 17α-pregnane derivatives of some of the above metabolites. The steroids identified in the feces of conventional rats were estriol, 3α,15α(and 15β)-dihydroxy-5α,17α(and 17β)-pregnane-20-one, 3α,19-dihydroxy-5α,17α-pregnane-20-one, 5α-pregnane-3α,16α,20-triol, 3β,11α-dihydroxy-5α-pregnane-20-one, 2α,3α-dihydroxy-5α,17α-pregnane-20-one and 3α,17α-dihydroxy-5α-pregnane-20-one.

In a subsequent experiment using the same protocol a large number of steroids were noted in the free fraction in conventional rats while only a small number could be found in the free fractions of germ-free rats (Eriksson, 1970). In the conventional rats, 3α,20β-dihydroxy-5α-pregnane-11-one and 3β,20β-dihydroxy-5α-pregnane-11-one were characterized as metabolites of corticosterone whereas 3α-hydroxy-5α,17α-pregnane-20-one, 3α-hydroxy-5α,17β-pregnane-20-one, 3β-hydroxy-5α,17α-pregnane-20-one, 3β,15β-dihydroxy-5α-pregnane-20-one, 3β,15α-dihydroxy-5α-pregnane-20-one and 3α,16α-dihydroxy-5α-pregnane-20-one were identified as metabolites of pregnanolone. No steroids were completely identified in the germ-free animals. Sulphohydrolase and

glucuronohydrolase activities have been characterized in the intestine of conventional and germ-free rats. The sulphohydrolase activity was present only in conventional rats (glucuronohydrolase activity being present in conventional and germ-free animals) and could hydrolyze sulfuric acid esters of the 3α,3β,17β and 21-hydroxyl groups (Eriksson and Gustafsson, 1970c).

Eriksson et al. (1971) reported the presence of eight 15-hydroxylated C-21, O-4 steroids in urine and feces of conventional female rats: 3α(and 3β),11β,15α-trihydroxy-5α,14β-pregnane-20-one, 3α(and 3β),15α-dihydroxy-5α,14β-pregnane-11,20-dione, 3α(and 3β),15α,20β-trihydroxy-5α,14β-pregnane-11-one and 5α,14β-pregnane-3α(and 3β),11β,15α,20β-tetrol. These were shown to be microbial metabolites of 15α, 21-hydroxylated C-21, O-5 steroids present in germ-free rats.

The occurrence of steroid monosulfates in the urine of conventional and germ-free rats was investigated by Gustafsson (1970). Steroid monosulfates were not present in the urine of male rats, conventional or germ-free. Female germ-free rats excreted 5α-androstane-3α,17β-diol, 3α,11β-dihydroxy-5α-androstane-17-one, 3α,7α-dihydroxy-5α-androstane-17-one, 5α-androstane-3α,7α,17β-triol, 3α,16α-dihydroxy-5α-pregnane-20-one, 3α,15α-dihydroxy-5α-pregnane-20-one, 3α(and 3β),17α-dihydroxy-5α-pregnane-20-one, 5α-pregnane-3α,16α,20α-triol, 11β,21-dihydroxy-5α-pregnane-3,20-dione, 3α,11β,21-trihydroxy-5α-pregnane-20-one, 3α,15α,21-trihydroxy-5α-pregnane-11,20-dione and 3α,11β,15α,21-tetrahydroxy-5α-pregnane-20-one monosulfates. Conventional

female rats excreted 3α,19-dihydroxy-5α-androstane-17-one, 3α-hydroxy-5α,17α-pregnane-20-one, 3α-hydroxy-5α-pregnane-20-one, 3α-hydroxy-5α-pregn-16-ene-20-one, 3α,19-dihydroxy-5α,17α-pregnane-20-one and 3β,15α-dihydroxy-5α-pregnane-20-one monosulfates in addition to the above steroid monosulfates present in the urine of germ-free rats. Eriksson (1971b) has examined steroids in the urine and feces of conventional and germ-free male rats derived from [4-^{14}C] pregnenolone and [4-^{14}C]corticosterone (intraperitoneal injection). In general, the metabolites found in both urine and feces were more polar than those derived from [4-^{14}C] pregnenolone and [4-^{14}C]corticosterone in female rats. A series of 5α-pregnane-3,11,16,20,21-pentol isomers were the major metabolites of corticosterone in the urine and feces of conventional male rats. 5α-pregnane-3α(and 3β),11β,20β, 21-tetrol and 3α(and 3β),20β-dihydroxy-5α-pregnane-11-one were excreted in the feces but not the urine. In the germ-free male rats 5α-pregnane-3α(and 3β), 11β,20β,21-tetrols and 3α(and 3β),11β,21-trihydroxy-5α-pregnane-20-one were the predominant species in the mono- and disulfate fraction from feces although minor amounts of C-19 O-3 and C-21 O-3 steroids were found in the monosulfate fraction. In the germ-free male rats all radioactive steroids in the urine were in the free raction. These compounds were C-19 O-5, C-21 O-4, C-21 O-5, C-21 O-6 and C-21 O-7 steroids that could not be fully characterized.

In summary, although man and the rat have a number of metabolic pathways in common, there are a large number of differences. In general, metabolites of steroids in the urine and feces of rats (both conventional and germ-free) are more polar than steroid metabolites in the urine and feces of man. Enterohepatic circulation in the rat is more important for steroid metabolism than it is in man. Ring hydroxylations are an important (major) route of steroid metabolism in the rat with the 2,6,7,15,16, and 19 positions being the most important. Steroids with the 3-keto-4-ene configuration can undergo hydroxylation before reduction of the A-ring. Steroids are excreted unconjugated or as glucuronides, monosulfates or disulfates in rats, with marked sexual differences in the distribution of metabolites in these fractions. In addition, urinary steroids in rats reflect metabolism of corticosterone, the major adrenal steroid in rats, while urinary steroids in man reflect metabolism of cortisone and cortisol, the major adrenal steroids in that species.

EXPERIMENTAL

Materials. All materials were obtained from the sources previously described in Section II.

Animals. Male and female Sprague-Dawley rats were purchased from Spartan Farms, Haslett, MI. Animals were housed four to a cage. A 12-hour day-night cycle was used.

Isolation of Steroids. Steroids were isolated by the procedure described in Section II.

Gas Chromatography. Urinary steroid metabolic profiles were generated using a 60 m DB-1 bonded phase fused silica capillary column as previously described in Section II.

Gas Chromatography/Mass Spectrometry. Urinary steroid metabolic profiles were generated by GC/MS/DS as previously described in Section II.

Collection of Urine Samples. Urine was collected using stainless steel metabolic cages. The collection mechanism allowed for separation of urine from feces. Urine was collected over a 24 hour period in a 50 ml Erlyenmeyer flask. Animals were allowed food (crushed) and water ad libitum during the collection period.

RESULTS

Initial studies focused on determining the effect of exposure to PBBs on the urinary steroid metabolic profile. Urine was obtained from two groups of adult female rats (4 animals per group, pooled sample collection) that had been exposed to 100 ppm PBBs in the diet from day 8 of gestation until 4 months of age when urine samples were collected. Urine was also obtained from two groups of control animals. Urinary steroids were isolated using Method I (see Section II). Urinary steroid metabolic profiles were generated by packed column GC/MS/DS. These pilot studies indicated that the urinary steroid metabolic profiles in PBBs-exposed and non-exposed groups were qualitatively the same. However, certain quantitative differences were observed. These observations are illustrated in Figure 17 and Table 3. Figure 17 shows the urinary steroid metabolic profiles obtained for one of the PBBs-exposed groups and the profile obtained for one of the control groups. Scan number is plotted against m/z = 73, a characteristic fragment ion of trimethylsilyl derivatives. Qualitatively the two profiles are very similar. However, a number of quantitative differences, or apparent quantitative differences, can be seen, especially in the later region of the chromatogram.

Figure 17. Urinary steroid metabolic profiles of female rats exposed to 100 ppm PBBs chronically (top trace) and of an appropriate control group. Urinary steroids were isolated using Method I as described in Section II, Development of Methods. Profiles were generated by GC/MS/DS. Scan number is plotted against m/z = 73, a characteristic fragment ion of TMS derivatives. Quantitative differences are seen in the later eluting compounds.

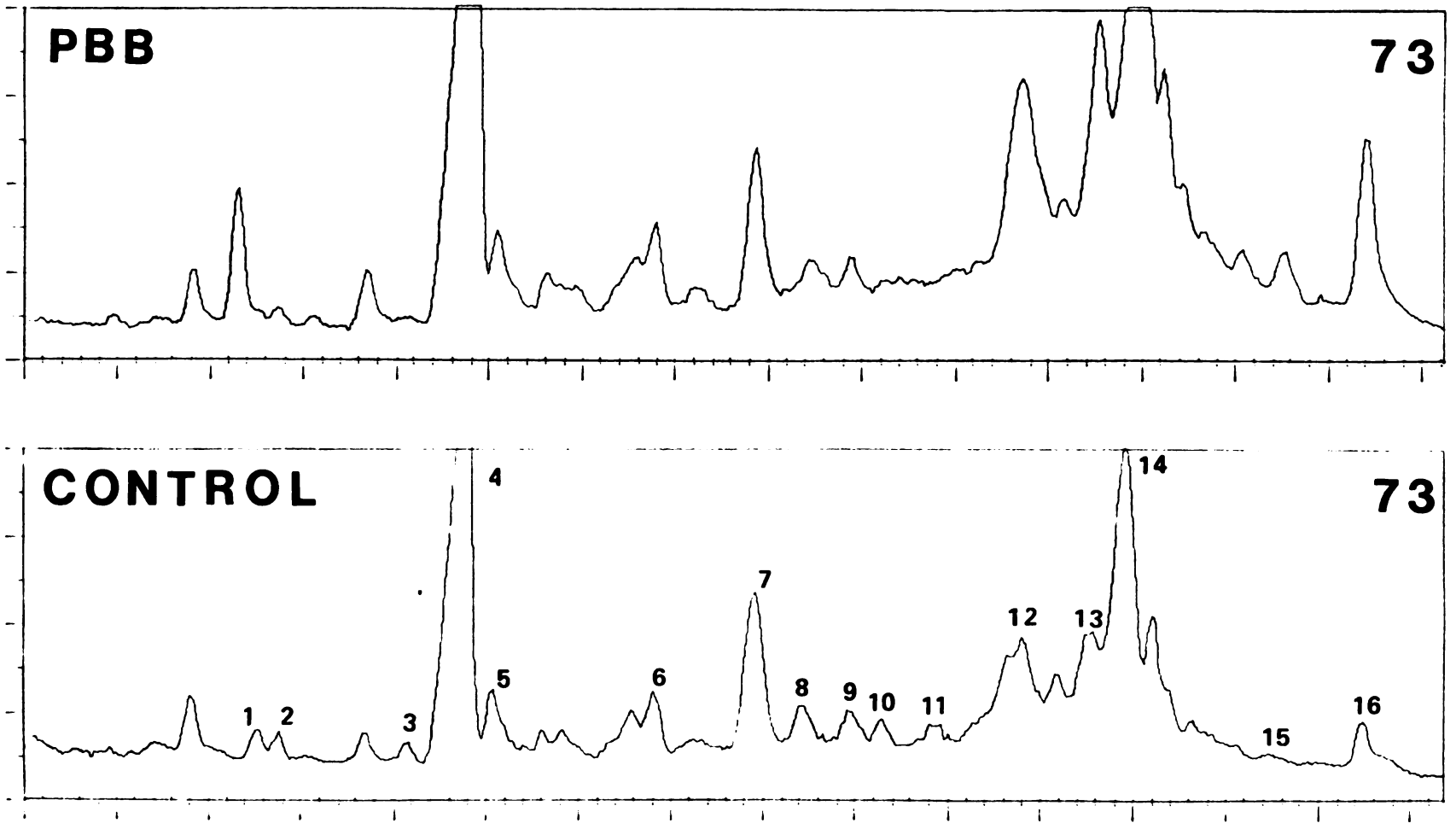

PBB
73
CONTROL
73
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

Table 3. Effect of Chronic Exposure to PBBs of Female Rat Urinary Steroids[1]

Peak Number	Retention Index	Control[2]	PBB[2]
3	2515	0.208	0.229
4	2568	6.67	14.6
6	2711	0.465	0.957
7	2797	0.615	1.52
9	2875	1.34	0.92
12	3017	1.69	18.4
14	3126	2.91	16.5
16	3331	2.05	14.5

[1] Female rats were exposed to 100 ppm PBs from day 8 of gestation until 4 months of age when urine samples were collected.

[2] Values are the average for 8 animals per group with 4 animals pooled per sample (n=2). Values were calculated using the following formula:

$$\frac{PA_{\mu}}{PA_{CB}} \times \frac{a(\mu g)}{b(ml)c(mg/ml)}$$

where,

PA_{μ} = peak area of designate ion of compound in question.

PA_{CB} = peak area of m/z=255 of cholesteryl butyrate.

a = µg of cholesteryl butyrate added.

b = ml of urine used.

c = mg creatinine per ml of urine.

This is the region of the chromatogram where MO-TMS derivatives of metabolites of corticosterone, cortisol and cortisone elute (retention index 2900-3300). Data for some of the peaks observed have been tabulated in Table 3. Values are the average for the two groups (i.e., two control pooled collections and two PBBs pooled collections). Each value was calculated as the peak area of an intense and characteristic fragment ion for each compound in question, divided by the peak area of m/z 255 of cholesteryl butyrate, the internal standard, times the amount of internal standard added, divided by the mls of urine extracted and the creatinine concentration. Most peaks were approximately the same in the experimental and control groups, illustrated by peak numbers 3,4,6,7 and 9. However, certain compounds were very different. For example, data for peaks at retention index 3017, 3126 and 3331 are tabulated to show that some peaks in the steroid profiles were apparently different in the two groups. Mass spectral interpretation indicated these compounds were metabolites of corticosterone.

Based on these preliminary results, the study was expanded to include a large enough number of subjects to make statistical comparisons between the PBBs exposed and control groups. The treatment protocol of Dent et al. (1976) was chosen because the physiological and biochemical effects of PBBs are well characterized with this regimen. Twenty one day-old female Sprague-Dawley rats were treated with a single 150 mg/kg I.P. dose of PBBs at day 21 of age.

Urines were collected 8 days later over a 24-hour period. Four animals were housed per metabolic cage with four cages in each experimental group. Urinary steroids were isolated and derivatized by method I. Samples were analyzed both by capillary column GC and GC/MS/DS.

Most GC peaks, determined by capillary column GC or GC/MS/DS, were not different between the control and PBBs groups. Although no qualitative differences were noted, certain peaks were quantitatively different. Data for those peaks where mass spectral interpretation was informative are presented below. The quantitative differences occurred in the "corticosteroid" region of the chromatogram and are given in Table 4 and Figures 18, 19 and 20. Values shown in Table 4 were calculated by dividing the peak area of a characteristic mass chromatogram for the compound in question by a characteristic mass chromatogram for the internal standard, cholesteryl butyrate, times the amount of internal standard added, divided by the mls of urine extracted and the concentration of creatinine. The group means ± S.D. are shown on the right. Control and PBBs data were compared by Student's t-test and found to be significantly different at $p<0.01$.

Figure 18 shows the mass spectrum for the first peak listed in Table 4. This is a typical mass spectrum for the MO-TMS derivative of a tetrahydroxy-pregnane-one. The molecular ion (m/z=683) and M^+-31 (m/z=652) are usually observed with these derivatives. Also seen are characteristic losses of M^+-90-31, M^+-2X90-31 and M^+-3X90-31.

Table 4. Changes in urinary excretion of some corticosterone metabolites in female rats following acute exposure to PBBs.

			Amount[b]				X ± S.D.
			CAGE NUMBER				
			1	2	3	4	
1)	$C_{21}O_5$[c]	Control	4.4	3.5	4.0	0.9	3.2 ± 1.6
		PBBs	15.9	8.0	7.6	10.6	10.5 ± 3.8*
2)	$C_{21}O_5$[c]	Control	1.0	2.4	2.8	2.2	2.1 ± 0.8
		PBBs	0.64	0.41	0.26	0.25	0.39 ± 0.18*
3)	$C_{21}O_5$[c]	Control	2.3	1.4	1.6	0.3	1.4 ± 0.83
		PBBs	6.7	3.6	7.0	3.7	5.3 ± 1.85*
4)	$C_{21}O_6$[d]	Control	1.7	1.8	3.8	3.2	2.6 ± 1.0
		PBBs	0.88	0.79	0.48	0.50	0.66 ± 0.2*

[a] 150 mg/kg i.p. to 21 day old female Sprague-Dawley rats. Urines were collected 8 days later. 16 animals per group. Urine samples pooled from 4 animals (n=4).

[b] For numbers 1,2 and 3, the value is the peak area of m/z 472 for the $C_{21}O_5$ steroid divided by the peak area of m/z 255 of the internal standard, cholesteryl butyrate, times the amount of internal standard added, divided by the mls of urine extracted and the concentration of creatinine.

For number 4 the value is the peak area determined by capillary column GC divided by the peak area of the internal standard, cholesteryl butyrate, times the amount of internal standard added, divided by the mls of urine extracted and the concentration of creatinine.

[c] A tetrahydroxy-pregnane-one.

[d] A pentahydroxy-pregnane-one.

*Significantly different from the control by Student's t-test, $p<0.01$.

Figure 18. Mass spectrum of the MO-TMS derivative of a tetrahydroxy-pregnane-one that increased in the urine of PBBs exposed female rats. The molecular ion (m/z=683) and M^+-31 (m/z=652) are observed, as are characteristic losses of M^+-90-31, M^+-2X90-31 and M^+-3X90-31. Likely structures for this steroid are 3α,11β,15α(or 16α),21-tetrahydroxy-5α-pregnane-20-one or 3α,6β,11β,15α(or 16α)-tetrahydroxy-5α-pregnane-20-one.

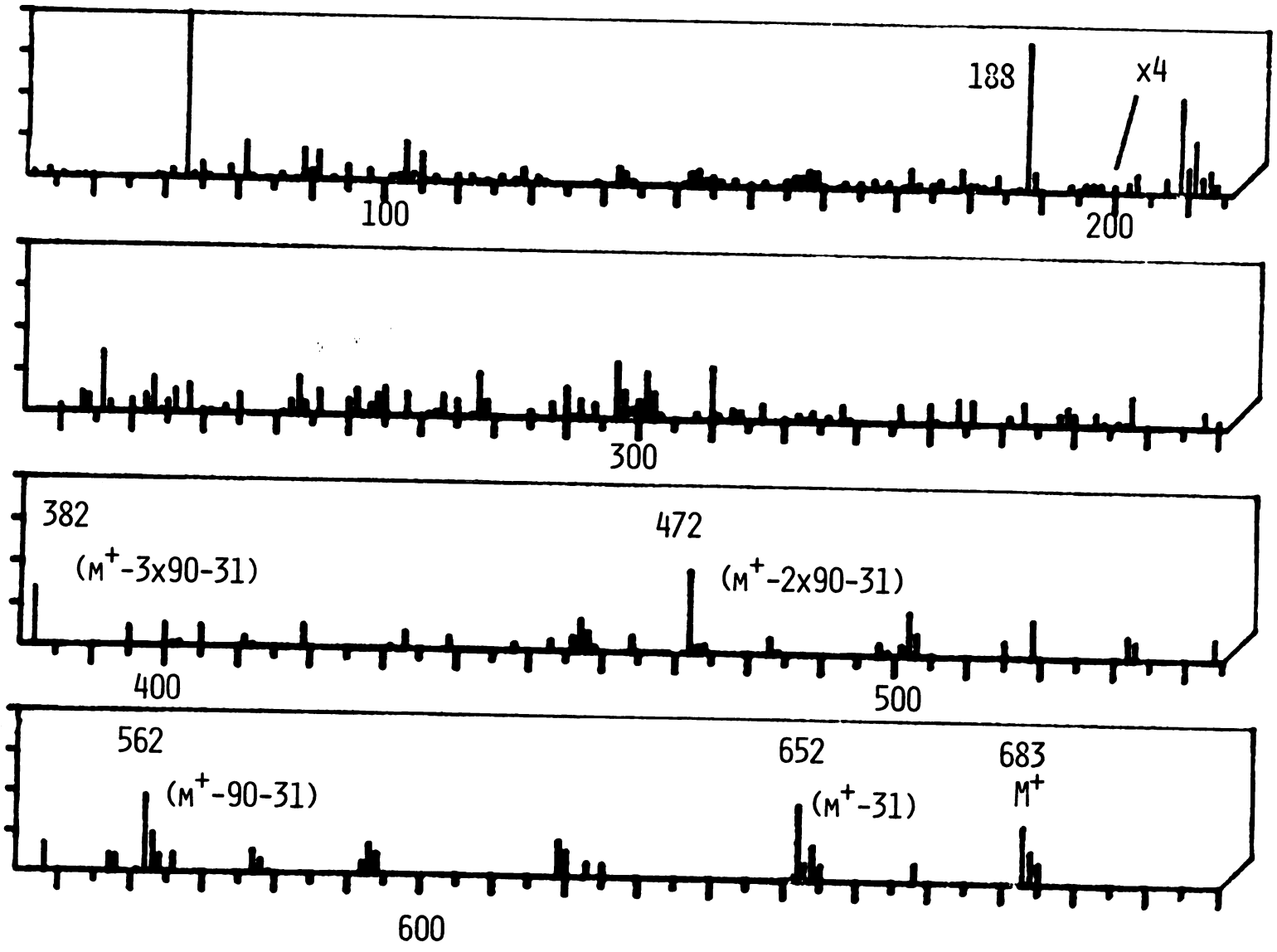

188
x4
100
200
300
382
(M+-3x90-31)
472
(M+-2x90-31)
400
500
562
(M+-90-31)
652
(M+-31)
683
M+
600

Figure 19. Mass spectrum of the MO-TMS derivative of a tetrahydroxy-pregnane-one that decreased in the urine of PBBs exposed female rats. The molecular ion (m/z=683) and M^+-31 (m/z=652) are observed, as are characteristic losses of M^+-90-31, M^+-2X90-31 and M^+-2X90-31. The ion at m/z=170 is characteristic of MO-TMS derivatives of C-15 hydroxy, C-20 keto pregnanes.

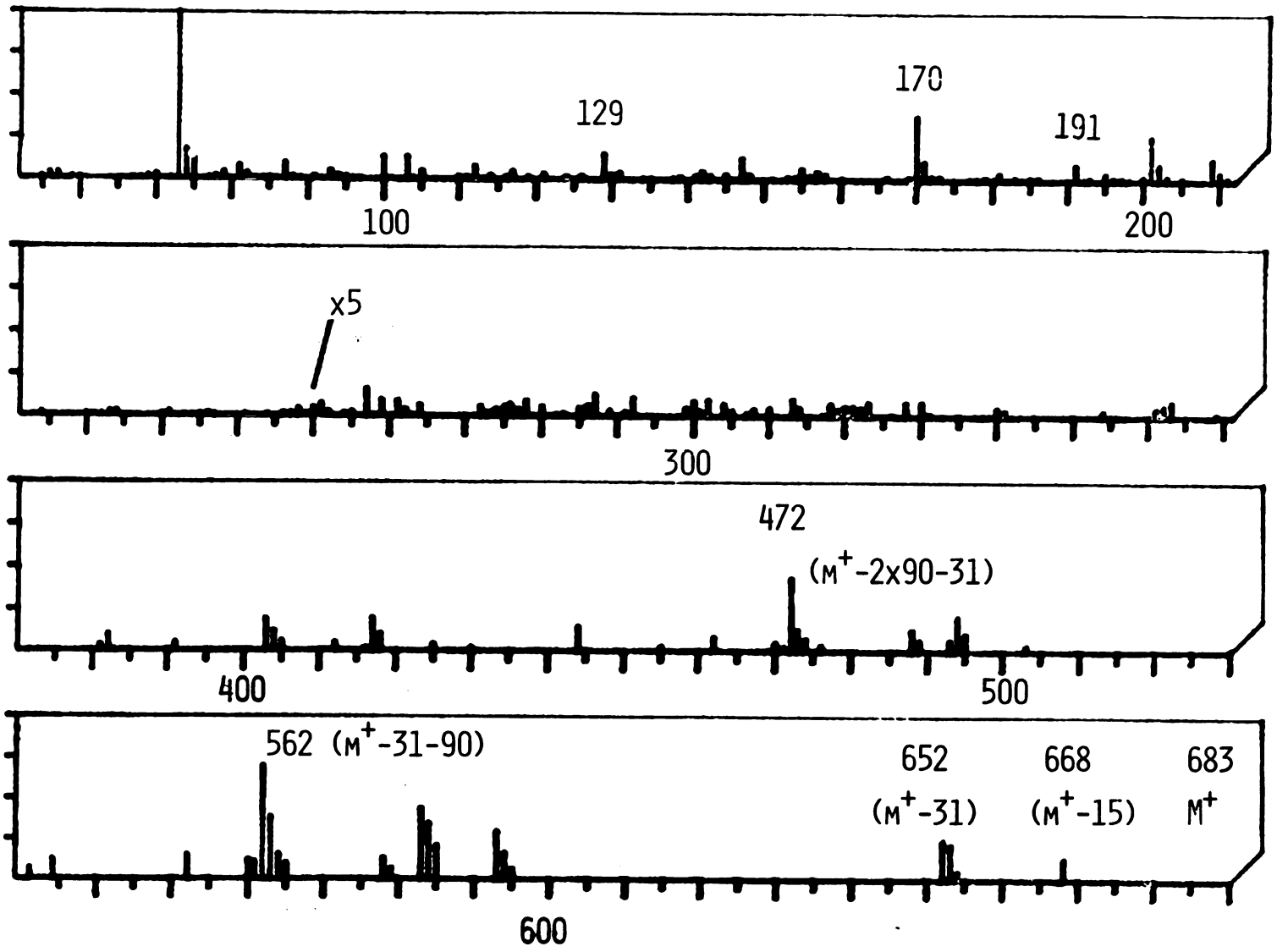
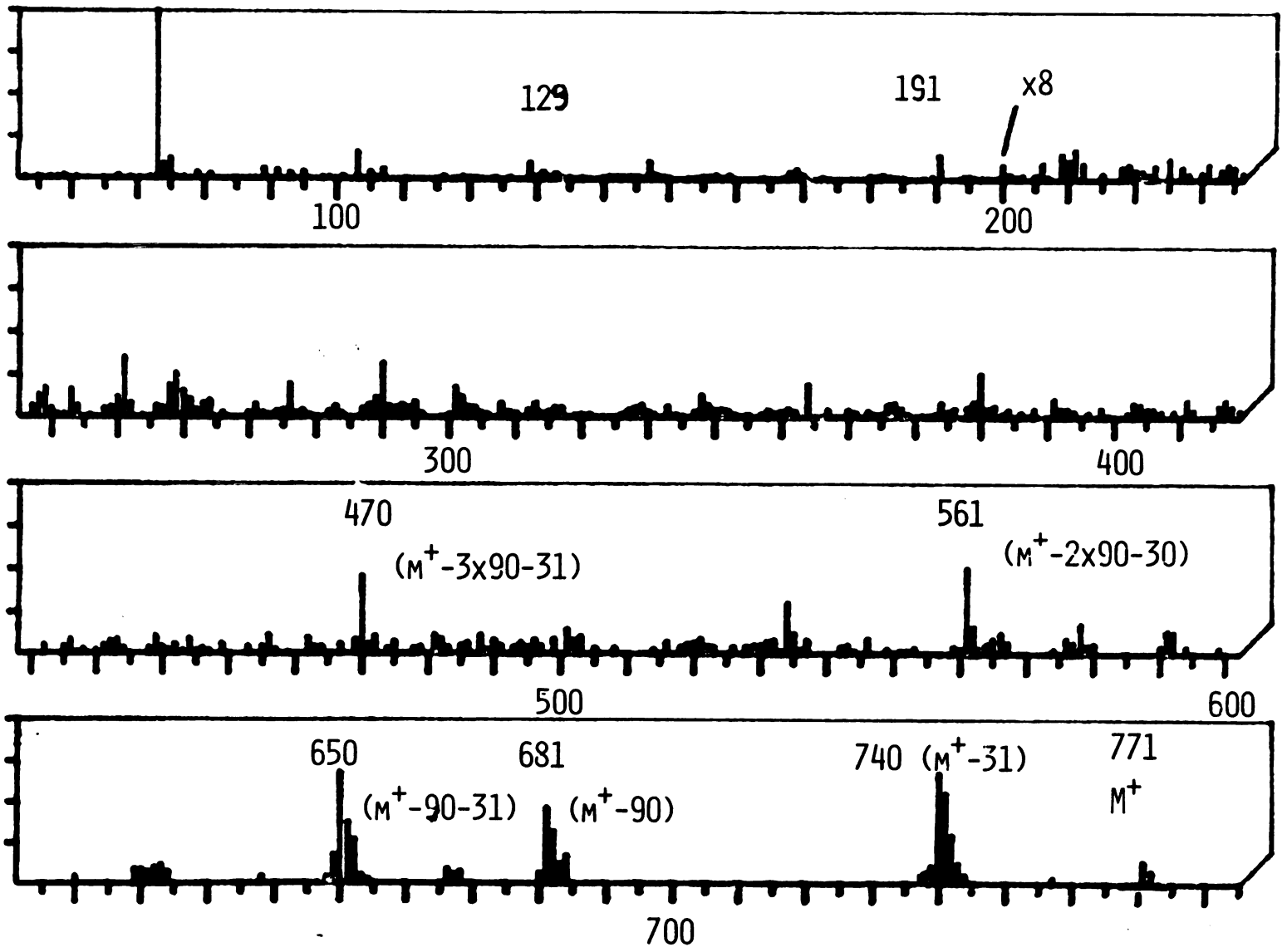

129
170
191
100
200
x5
300
472
(M+-2x90-31)
400
500
562 (M+-31-90)
652
(M+-31)
668
(M+-15)
683
M+
600

Figure 20. Mass spectrum of the MO-TMS derivative of a pentahydroxy-pregnane-one that decreased in the urine of PBBs exposed female rats. The molecular ion (m/z=771) and M^+-31 (m/z=740) are observed, as are characteristic losses of M^+-90-31, M^+-2X90-31 and M^+-3X90-31.


129
191
x8
100
200
300
400
470
(M+-3x90-31)
561
(M+-2x90-30)
500
600
650
681
740 (M+-31)
771
M+
(M+-90-31)
(M+-90)
700

The lower end of the mass spectrum contains an intense fragment at m/z=188. This is a characteristic ion for a C-21 hydroxyl, C-20 methoxime configuration or a C-16 (or 15) hydroxyl, C-20 methoxime configuration with the C-21 position reduced. This second configuration can occur by reduction of the C-21 hydroxyl by gut microbes (Eriksson et al., 1969). This particular steroid was observed to increase in the urine of PBBs-exposed subjects. This effect is most likely due to increased 6β,7α,15α(or 16α)-hydroxylation of corticosterone in the PBBs-exposed subjects. For example, likely structures for this steroid are 3α,11β,15α(or 16α), 21-tetrahydroxy-5α-pregnane-20-one or 3α,6β,11β,15α(or 16α)-tetrahydroxy-5α-pregnane-20-one. The third steroid listed in Table 4 is also a tetrahydroxy-pregnene-one with a similar mass spectrum to peak 1 and may represent the 3β-isomer of the first steroid. This steroid also increased in the urine of PBBs exposed subjects. The mass spectrum for peak 2 is shown in Figure 19. The high end of the mass spectrum shows the same characteristic ions (M^+, M^+-31, M^+-31-90, etc.). The low end shows an intense fragment ion at m/z=170, which is a characteristic fragment of MO-TMS derivatives of C-15 hydroxy, C-20 keto steroids. Ions at m/z=129 and 191 also are characteristic fragments. The ion at m/z=129 is derived from an A-ring with either a 2,3-diTMS or a 5-ene-3-TMS configuration. The ion at m/z=191 is characteristic of polyhydroxylated steroids with TMS-hydroxyls on adjacent carbons. For example, a 2,3-diTMS

configuration in the A-ring will give a m/z=191 fragment. This steroid was decreased in the urine of the PBBs-exposed subjects. PBBs have been shown to inhibit 5α-reduction of testosterone *in vitro* (Newton *et al.*, 1980, 1982). Thus, inhibition of 5α-reduction of corticosterone is one plausible mechanism by which PBBs could decrease the excretion of a urinary metabolite of corticosterone.

Figure 20 shows the mass spectrum of a pentahydroxy-pregnane-one that was decreased in the urine of PBBs-exposed subjects. Characteristic losses of 31, 90+31, 2X90+31, 3X90+31 from the molecular ion are seen. The low end of the mass spectrum shows ions at m/z=191 and 129. As stated above, m/z=129 is derived from the A-ring (2,3-diTMS or a 5-ene-3-TMS configuration) and 191 is characteristic of polyhydroxylated steroids with TMS-hydroxyls on adjacent carbons. Reduction of this steroid in the urine of PBBs-exposed rats could have occurred by the same mechanism that resulted in a decreased excretion of the second steroid listed in Table 4 (i.e., inhibition of 5α-reduction).

Because of the complicating aspects of the estrus cycle on urinary steroid excretion in female rats, it was decided at this point to use male rats exclusively as the experimental model. Attempts were also made to simplify the chromatogram by fractionating the hydrolyzed (free) steroid milieu with Sephadex LH-20 column chromatography by the method of Shackelton and Whitney (1980). The first experiment involved making multiple cuts (9 total) of the total

250 ml of eluant (data not shown). This resulted in greatly simplified capillary column gas chromatograms, but with extensive overlay of the elution of most components of the mixture. A second experiment was performed in which fractions were collected from 0-37 ml, 37-67 ml and 67-150 ml and analyzed by capillary column GC. These data are displayed in Figure 21. The top trace shows the chromatogram obtained for the 0-37 ml fraction, the middle trace the 37-67 ml fraction, and the bottom trace the 67-150 ml fraction. Since all three traces seen in Figure 21 are relatively uncomplicated (i.e., when compared to the non-fractionated GC trace), and there was still overlapping of a large number of peaks, the experiment was repeated collecting fractions from 0-55 and 55-150 ml. These data are displayed in Figure 22. The top GC trace is for the 0-55 ml fraction and the bottom trace is for the 55-150 ml fraction. These chromatograms were still far simpler than chromatograms from non-fractionated urine. However, since there was still some degree of overlap, most apparent in the middle region of the chromatograms, it was decided to use the LH-20 fractions to obtain "clean" mass spectra for the steroids occurring in male rat urine and not for quantitative purposes at this time.

Figure 21. Capillary column GC traces obtained for three different fractions from Sephadex LH-20 column chromatography. The method of Shackelton and Whitney (1980) was used to chromatograph the free steroid fraction. The top trace shows the chromatogram obtained with the 0-37 ml fraction, the middle trace is for the 37-67 ml fraction, and the bottom trace is for the 67-150 ml fraction. Chromatograms were obtained using a 60 m DB-1 fused silica capillary column (0.242 mm I.D., 0.1 μm film thickness).

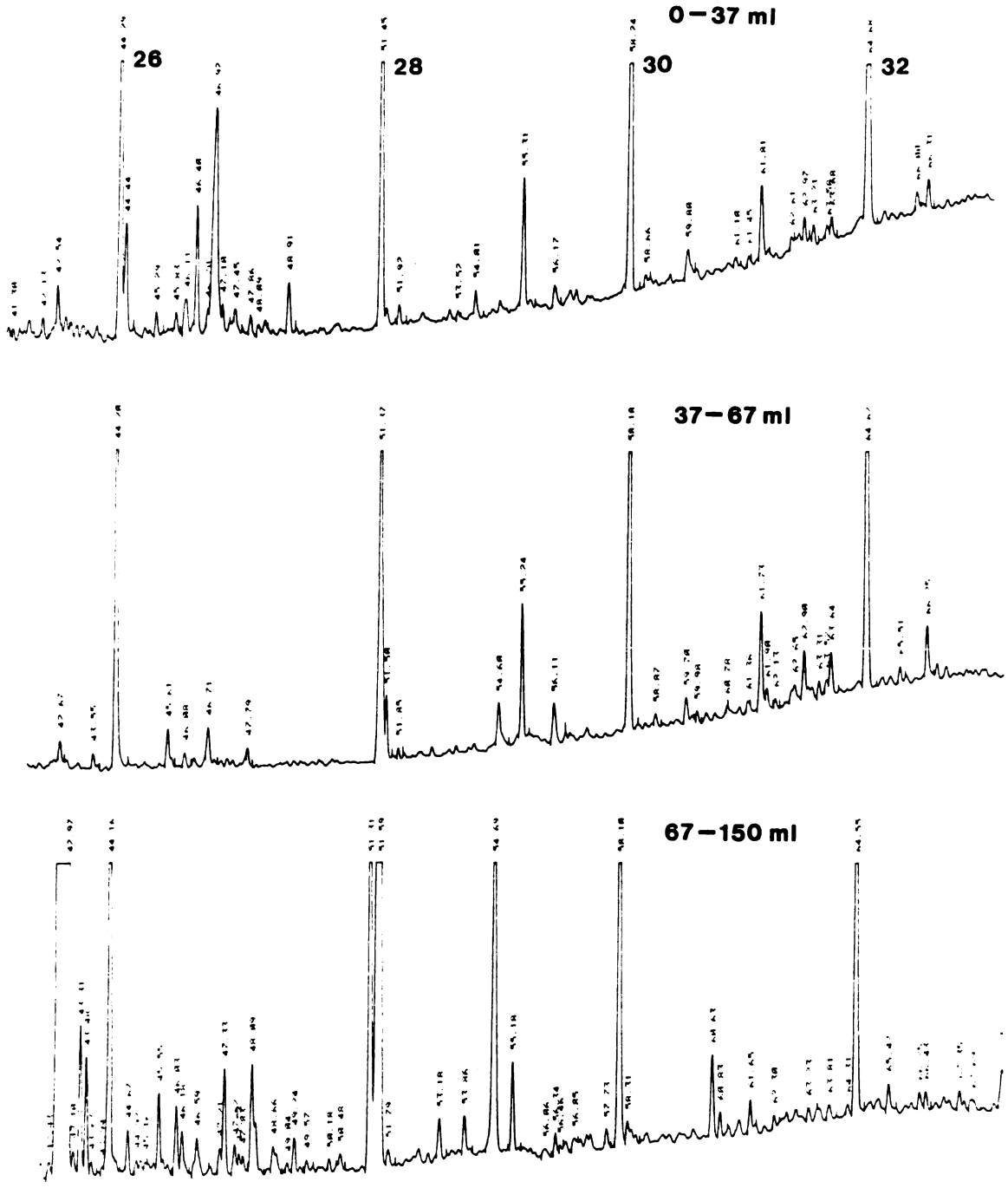
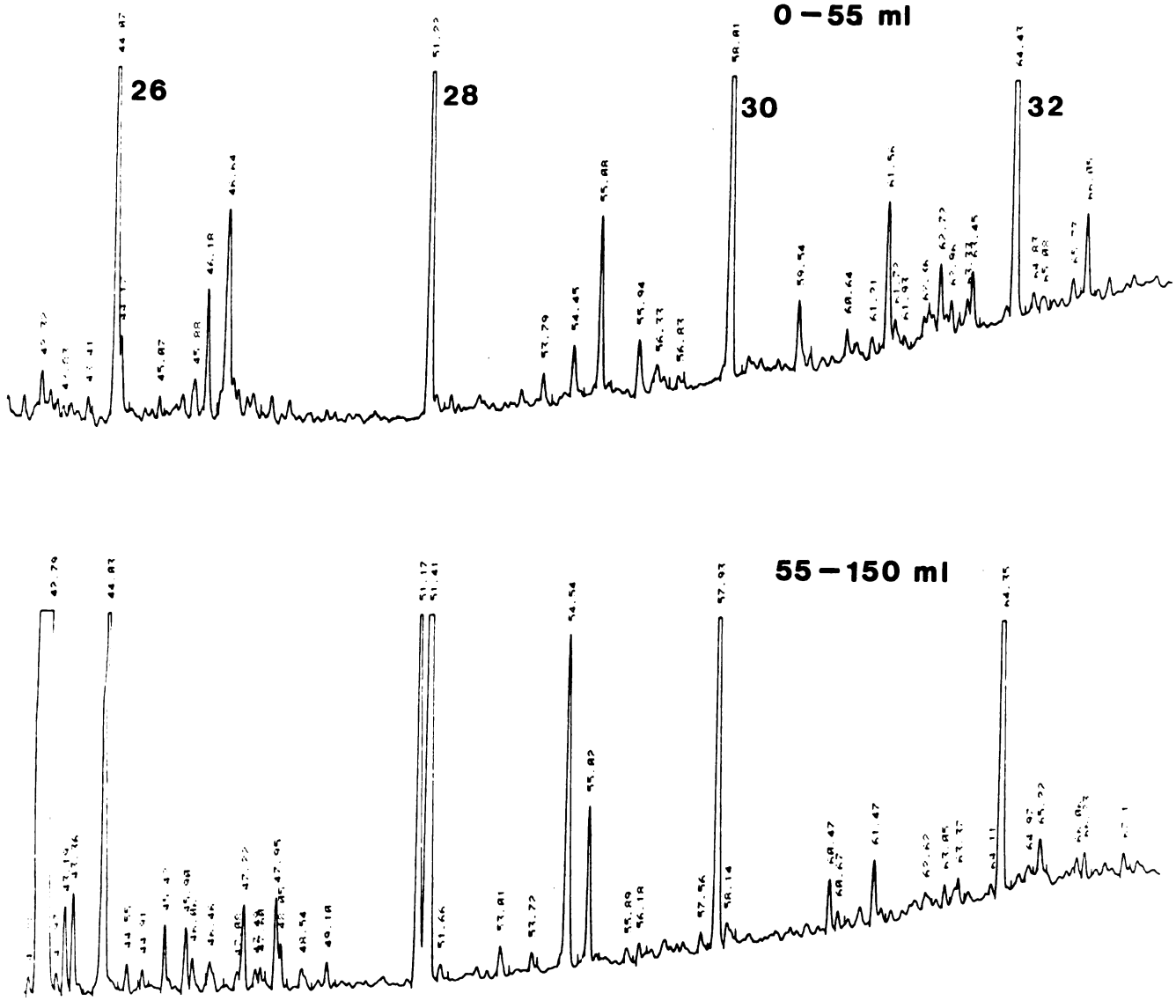

0–37 ml
26
28
30
32
37–67 ml
67–150 ml

Figure 22. Capillary column GC traces obtained for two different fractions from Sephadex LH-20 column chromatography. The method of Shackelton and Whitney (1980) was used to chromatograph the free steroid fraction. The top trace shows the chromatogram obtained with the 0-55 ml fraction, and the bottom trace is for the 55-150 ml fraction. Chromatograms were obtained using a 60 m DB-1 fused silica capillary column (0.242 mm I.D., 0.1 μm film thickness).


0 – 55 ml
26
28
30
32
55 – 150 ml

In the following experiment, the Dent et al. (1976) protocol was used with male rats (150 mg/kg PBBs administered to 21-day-old rats). Urines were collected at 28 days (pre-puberal) and 60 days (post-puberal). The concentration of total steroids in the pre-puberal samples was not sufficient for accurate mass spectral analysis, especially in the corticosteroid region where differences were expected to occur based on the previous results. Since approximately 4 to 5 times as much urine was collected from the post-puberal animals, these samples were chosen for detailed characterization.

Samples were prepared by Method II (see Section II) and analyzed by both capillary GC and GC/MS/DS. As with the previous experiment, most peaks were not quantitatively different between the control and PBBs groups, and no obvious qualitative differences were noted. Therefore, only data for those peaks where mass spectral interpretation was informative are presented below (Table 5). Mass spectral analysis (see below) characterized the top three steroids listed in Table 5 as tetrahydroxy-pregnane-ones and the bottom four steroids as 5α-pregnane-3,11,15,20,21-pentols. Of these compounds, two of the tetrahydroxy-pregnane-ones increased statistically (t-test, <0.05) and one was unchanged, while two of the four pregane pentols decreased.

Mass spectral characteristics of the tetrahydroxy-pregnane-ones are similar to what has previously been described, with M^+, M^+-31, M^+-31-90, M^+-31-2X90 and M^+-31-3X90

Table 5. Urinary metabolites of corticosterone in control and PBBs exposed adult male rats.

R. I.	Amount/mg creatinine[1] Average (S.D.) Control (n=4)	PBBs (n=4)
Tetrahydroxy-pregnane-ones		
3015	5.08 (1.65)	5.02 (2.62)
3106	0.20 (0.06)	1.19 (0.19)*
3164	1.70 (0.29)	16.0 (0.95)*
5-Pregnane-3,11,16,20,21-pentols		
3251	1.08 (0.41)	1.70 (0.62)
3270	3.17 (1.18)	0.84 (0.65)*
3307	4.18 (0.65)	6.46 (3.61)
3306	1.75 (0.66)	0.44 (0.04)*

[1] Values are average of four samples and are normalized to the internal standard and urinary creatinine. Standard deviation is in parentheses.
* Significantly different ($p<.05$).

occurring in the high end of the mass spectrum. Although mass spectral interpretations of the peaks at retention index 3015 and 3106 established that these compounds were tetrahydroxy-pregnane-one metabolites of corticosterone, the sterochemistry could not be determined. However, the corticosterone metabolite at 3164 had a very prominant ion at m/z = 188, a characteristic fragment for a C-21, TMS-hydroxyl, C-20 methoxime configuration or a C-16 (or 15) TMS-hydroxyl, C-20 methoxime configuration with the C-21 position reduced. Likely structures for this steroid are 3α,11β,15α(or 16α), 21-tetrahydroxy-5α-pregnane-20-one or 3α,6β,11β,15α(or 16α)-tetrahydroxy-5α-pregnane-20-one.

The bottom four steroids in Table 5 are 5α-pregnane-3,11,16,20,21-pentols. These steroids have previously been reported to occur in the urine of conventional male rats (see Introduction, Section III). The mass spectrum of a 5α-pregnane-3,11,16,20,21-pentol is shown in Figure 23. Characteristic ions at m/z = 625 (M^+-103), m/z = 535 (M^+-103-90), m/z = 445 (M^+-103-2X90), m/z = 355 (M^+-103-3X90) and m/z = 265 (M^+-103-4X90) occur for this series of steroid stereoisomers. The absolute configurations of the steroids listed in Table 5 could not be determined. However, the order of elution of the α- and β-enatiomers of 3,16 and 20-hydroxy-substituted steroids indicates that the two steroids which decreased in the PBBs subjects must have the same configuration in the 20 position, but different configurations at the 16 position.

Figure 23. Mass spectrum of a 5α-pregnane-3,11, 16,20,21-pentol. Characteristic ions at m/z = 625 (M^+-103), m/z = 535 (M^+-103-90), m/z = 445 (M^+-103-2X90), m/z = 355 (M^+-103-3X90) and m/z = 265 (M^+-103-4X90) occur for this series of steroid epimers and enatiomers. The fragment at m/z = 191 is very characteristic of polyhydroxylated steroids with TMS-hydroxyls on adjacent carbons.

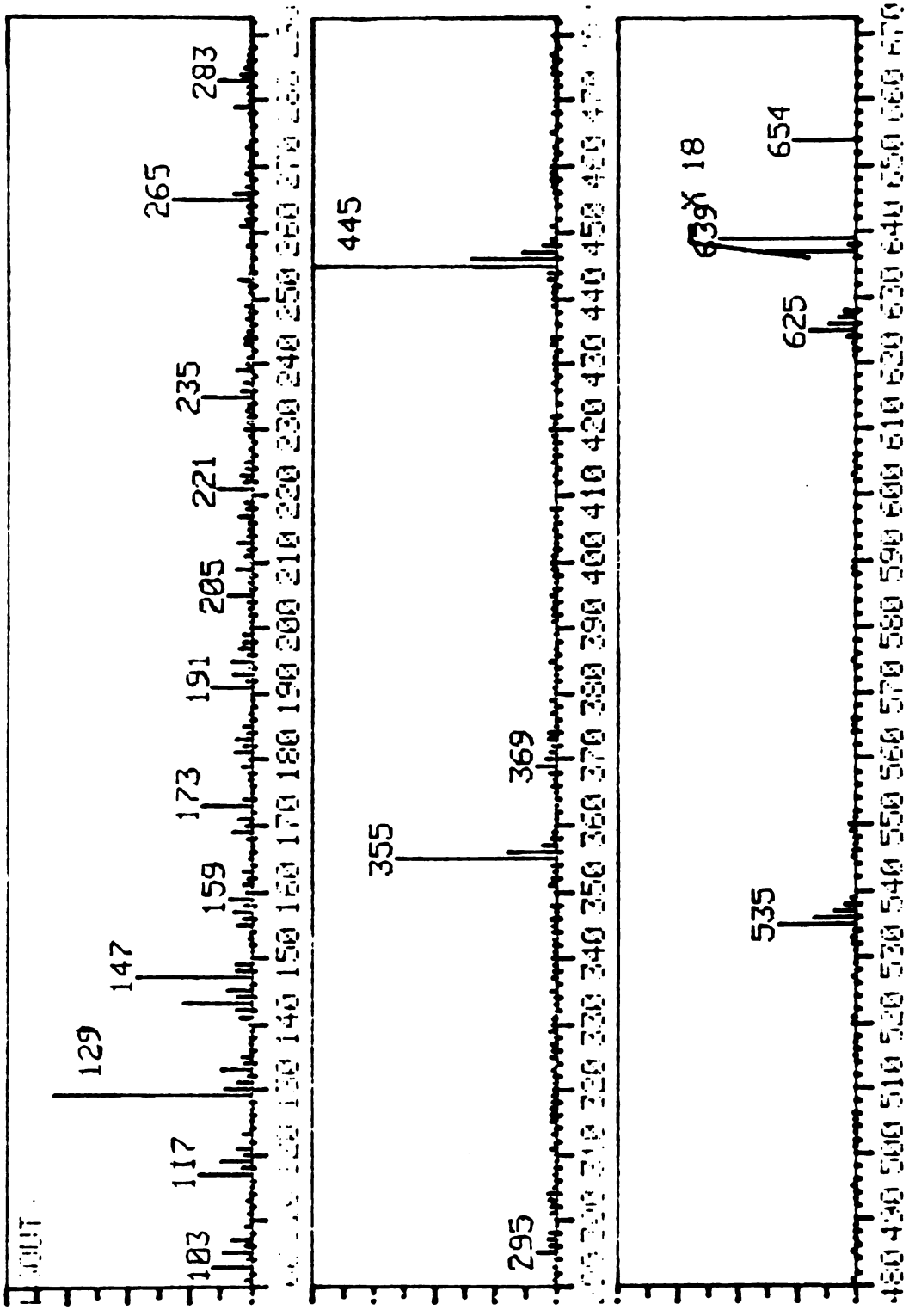

129
147
117
159
173
191
205
221
235
265
283
103
355
369
445
295
535
625
639
654
18

Figure 24. Capillary GC trace of urinary steroids obtained for a group of four male rats before (control) and after ("germ-free") antibiotic treatment. Trace was obtained using a 60 m DB-1 fused silica capillary column. MO-TMS derivatives of urinary steroids were prepared using Method II (Section II). Arrows show where peaks have been attenuated or are absent in the "germ-free" urine.

CONTROL
"GERM-FREE"
RR

α-Stereoisomers elute before β-stereoisomers at these positions (Eriksson, 1971b). Also, the four possible 3α-isomers will elute before any of the 3β-isomers (Eriksson, 1971b). The order of elution for the 16 and 20 position isomers is 16α,20α-, 16α,20β-, 16β,20α- and 16β,20β-. Therefore, the two pregnane pentols that decreased in the PBBs group must differ at the 16 position and must have the same steroichemistry at the 20 position. Of course, these steroids could either be 3α- or 3β-hydroxy-pregnanes.

Attempts were made to simplify the urinary steroid metabolic profile by making the subjects germ-free. This was atempted using a published method (Rowland et al., 1980) and involved addition of bacitracin, neomycin sulfate and streptomycin sulfate at a concentration of 2 mg/ml to the drinking water. Figure 24 shows the capillary GC trace of urinary steroids obtained for a group of four male rats before antibiotic treatment (control) and after antibiotic treatment ("germ-free"). A number of peaks were either absent or greatly attenuated in the "germ-free" chromatograms (none of these peaks could be identified by GC/MS). However, there are two problems with this procedure. First, the subjects would not drink the antibiotic contaminated water until they were very thirsty (2-3 days) and then only sparingly. Second, the animals were not completely germ-free since bacterial cultures of fecal material from all but a few of the subjects were positive.

DISCUSSION

A number of steroids reported to occur in male and female rat urine were not detected by the methods employed in these studies. These steroids include several C-19, O-2,3 metabolites of testosterone and C-21, O-2,3,4 metabolites of corticosterone and progesterone. It is possible that strain differences or differences in the intestinal flora could influence the urinary steroid metabolic profile. However, the most likely explanation for our inability to detect many of the steroids reported to be excreted in the urine of male and female rats is related to the initial volume of urine. The earlier studies were all qualitative in nature and large volumes of urine were used (100 ml or more for each sample preparation). In some of the GC/MS analyses in the present study, there were occurrences of weak ion currents that suggested the presence of some of these compounds. For example, in a few analyses, the presence of weak ion currents at m/z = 436 (M^+), 421 (M^+-15), 406 (M^+-30) and 326 (M^+-90) suggested the presence of a TMS derivative of an androstanediol. There was also some indication of the presence of androstanetetrol-TMS compounds (M^+=612). Larger volumes of urine need to be pooled in order to detect and identify these compounds.

Although certain differences were noted between the PBBs exposed and the control subjects in the urinary concentrations of some metabolites of corticosterone, none of these steroids could be fully characterized. Thus, it was impossible to describe a specific effect of PBBs exposure as the result of an increased or decreased activity of a specific metabolic step (i.e., increased 6β-,7α,15α or 16α- hydroxylation of corticosterone for example).

The large number of non-steroid compounds and the relatively low concentrations of steroids (i.e., compared to humans) in rat urine make studies of this type difficult and time consuming. The extensive enterohepatic circulation and metabolism of steroid hormones by gut microbes in the rat further complicated the interpretation of steroid profiles in this species.

In summary, PBBs administration was noted to alter the urinary steroid metabolic profile in male and female rats. Due to the complexity of the rat urinary steroid profile, the presence of a large number of non-steroidal substances, the extensive enterohepatic circulation of steroids and metabolism by gut microbes and the lack of reference compounds, it was impossible to accurately characterize the exact nature of this phenomenon. However, it is most likely that at least some of the increased urinary excretion of steroids seen in the PBBs group is related to stimulation of steroid hydroxylases. It is also likely that decreased urinary excretion of steroids in the PBBs group is related to inhibition of steroid reductases.

SECTION IV

Human Studies

CHAPTER IV

INTRODUCTION

This section describes the experiments using human subjects. The first series of experiments were designed to determine daily variations in the excreted concentrations of urinary steroids using "spot" urine collections and normalizing to urinary creatinine concentration. Also determined were daily variations in the excreted concentrations of urinary steroids using 24 hour urine collections and normalizing to total amount per 24 hours. It was necessary to determine the relative variability of using a "spot" urine collection protocol compared to that obtained for a 24 hour urine collection protocol to ascertain if "spot" urine collections were suitable for subsequent experiments. After establishment of the expected variability in normals, the urinary steroid metabolic profiles of a group of humans accidentally exposed to polybrominated biphenyls were compared to the steroid profiles obtained for an appropriate control group.

Catabolism of steroid hormones in man. The following overview of some aspects of steroid metabolism in man is presented as background for the discussion of results on human steroid profiles to follow. Because of the complexity

of steroid hormone metabolism and urinary excretion, it is necessary to present this material for the reader to understand and appreciate these results. More detailed accounts of this subject have been presented by Makin (1975) and Schulster et al. (1976).

Although the urine contains a surprisingly large number of steroid metabolites, these compounds are formed by relatively few enzymatic conversions. One of the common structural features of physiologically active steroid hormones, with the notable exception of the estrogens, is a 4-ene-3-one A-ring configuration. Reduction of this configuration will generally result in a loss of biological activity although there are exceptions to this generalization (i.e., 5α-reduction of testosterone enhances its activity in several target tissues). Reduction of the 4-ene-3-one system to a 3-hydroxy-5-dihydro configuration results in one of four stereoisomers: 3α-ol-5β; 3β-ol-5β; 3α-ol-5α; 3β-ol-5α. While all of these transformations do occur, the relative activities of the liver enzymes involved varies from species to species and also with sex. In lower animals the 3β-hydroxy configuration predominates while in man 3α-hydroxy steroids predominate.

In man, the first step in reduction of the A ring involves a 4-ene-5β-reductase or a 4-ene-5α-reductase and is an NADH or NADPH requiring reaction. In the second step either a 3α-hydroxy steroid dehydrogenase or a 3β-hydroxy-steroid dehydrogenase acts on the partially reduced steroid.

The 4-ene-5α-reductase is found in the microsomal fraction of the liver, adrenal, testes and ovaries while the 4-ene-5β-reductase is found in the soluble fraction of the same tissues. The 3α-hydroxysteroid dehydrogenase is found in the soluble and microsomal fractions of the liver and kidney and the microsomal fraction of the testes, ovaries and adrenal, while the 3β-hydroxysteroid dehydrogenase is found in the microsomal fraction of the liver, testes, ovaries and adrenal.

A second general pathway for reductive inactivation of steroids involves reduction of the 20-keto functional group of C-21 steroids to a 20α- or a 20β-hydroxy group. Both enzymes involved in these reactions, 20α- and 20β-hydroxysteroid dehydrogenase, require NADPH or NADH. The 20α-hydroxysteroid dehydrogenase is found in the soluble fraction of liver, kidney, muscle, adrenal, testes, ovaries and placenta, whereas the 20β-hydroxysteroid dehydrogenase is found in the soluble fraction of liver, muscle, testes and ovaries.

A third general pathway for inactivation of steroid hormones occurs through formation of a 17-keto derivative of 17-hydroxy C-19 steroids or 17-hydroxy C-21 steroids, the latter involving the cleavage of the C-20,21 side chain. For example, a 17β-hydroxysteroid dehydrogenase converts testosterone to 4-androstene-3,17-dione which can then undergo A-ring reduction to form either androsterone (3α-hydroxy-5α-androstane-17-one) or etiocholanolone (3α-hydroxy-5β-androstane-17-one).

Catabolism of the C-19 steroid hormones. The most important androgen produced by the testes is testosterone. Testosterone is derived from androstenedione, which also has weak androgenic activity. Another important source of androgens is the adrenal cortex where large quantities of dehydroepiandrosterone (DHEA) are produced. Androstenedione and 11β-hydroxyandrostenedione are also produced in the adrenal cortex. As was previously mentioned, one pathway for the catabolism of testosterone involves conversion first to androstenedione with subsequent reduction of the A-ring forming either androsterone or etiocholanolone. A second pathway involves the reduction of the A-ring of testosterone to yield either 5α-androstanediol (5α-androstane-3α,17β-diol) or 5β-androstanediol, both of which can be excreted as the glucuronide conjugate. DHEA can be hydroxylated at either the 7 or 16 positions (both α-hydroxylations) or the 17-keto group can be reduced to a 17β-hydroxy. These metabolites are conjugated with glucuronic acid before excretion in the urine.

Catabolism of progesterone. Progesterone and 17α-hydroxyprogesterone primarily undergo reduction to 3α-hydroxy-5β-pregnanes. Progesterone undergoes A-ring reduction to yield pregnanolone (3α-hydroxy-5β-pregnane-20-one) and subsequent reduction of the 20-keto group to a 20α-hydroxy yields pregnanediol, the major metabolite of progesterone. Both the pregnanediol and pregnanolone are

excreted as glucuronides. 17α-Hydroxyprogesterone undergoes A-ring reduction to yield 3α,17α-dihydroxy-5β-pregnane-20-one which can be reduced to pregnanetriol (5β-pregnane-3α,17α,20α-triol). Both of these metabolites are excreted as glucuronides. 17α-Hydroxyprogesterone can also undergo A-ring reduction and cleavage of the C-17,20 bond to yield etiocholanolone.

Catabolism of the adrenal steroids. Aldosterone also undergoes A-ring reduction with the major metabolite being 3α,5β-tetrahydroaldosterone which occurs in urine as the glucuronide conjugate. Other metabolites occur but only in small amounts. Glucocorticoids also undergo A-ring reduction. In man the major glucocorticoids secreted by the adrenal are cortisol and cortisone while corticosterone predominates in rats, rabbits and mice. The 11-deoxy derivatives of cortisol and corticosterone are also produced by the adrenal. Both the 4-ene-5α-reductase (microsomal fraction) and the 4-ene-5β-reductase (soluble fraction) will reduce these steroids. Cortisol is primarily converted to the 5β-isomer and corticosterone to the 5α-isomer. Further reductions occur at the 3, 11 and 20 positions. Cortisol and cortisone are interconvertible by means of an 11β-hydroxysteroid dehydrogenase (microsomal fraction of liver and adrenal).

The principal metabolites of cortisone are 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione (tetrahydro E; THE) and the 20α-hydroxy or 20β-hydroxy derivatives of THE

(α-cortolone and β-cortolone). The 5α-derivatives of these metabolites are also formed but to a lesser extent. Cortisol is also primarily converted to a 5β-derivative and its major metabolites are 3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one (tetrahydro F; THF) and the 20α-hydroxy and 20β-hydroxy derivatives of THF (α-cortol and β-cortol). The major metabolite of corticosterone is its 3α-hydroxy-5α-reduced derivative (3α,11β,21-trihydroxy-5α-pregnane-20-one; αTHB) although some of the 5β-derivative (THB) is produced. Both metabolites are excreted in the urine as the glucuronide conjugate. Some free cortisol and corticosterone are also usually present in the urine.

Catabolism of the C-18 steroids. The most physiologically active estrogen is estradiol-17β which is interconvertible with the somewhat less active estrone via action of a 17β-hydroxysteroid dehydrogenase. Estrogens can undergo hydroxylation at C-2,4,6,15,16 and 18 and many different metabolites appear in the urine. However, the quantitatively most important estrogen metabolite is estriol (1,3,5[10]-estratriene-3,16α,17β-triol). Estriol appears in the urine as a glucuronide. Estrone sulfate is also seen in the urine, but during pregnancy its major conjugate is the glucuronide.

Formation of steroid conjugates. The enzyme responsible for formation of steroid sulfates is a sulfokinase requiring ATP and Mg^{2+} which is found in the soluble fraction of the liver, testes, adrenal zona fasciculata and reticularis.

Glucuronides are formed by means of a glucuronyl transferase found in liver microsomes. This reaction requires UDP-glucuronic acid.

EXPERIMENTAL

Materials. All materials were obtained from the sources previously described in Section II.

Isolation of steroids. Steroids were isolated by Method II described in Section II.

Gas chromatography. Urinary steroid metabolic profiles were generated using a 60 m DB-1 bonded phase fused silica capillary column as previously described in Section II.

Gas chromatography/mass spectrography. Urinary steroid metabolic profiles were generated by GC/MS/DS as previously described in Section II.

Collection of urine. In the first series of experiments 24-hour urine samples were collected for six days, starting after the first void morning urine and ending with the first void morning urine the following day. Spot urine samples consisted of the first void morning urine. For the PBBs studies, spot urine samples were taken at various times of the day.

Selection of subjects for PBBs study. Samples were obtained by the Michigan Department of Public Health from patients enrolled in the "Long-Term PBB Study". A randomly selected high PBB subgroup (PBB $\geq$ 50 ppb) and an age- and sex-matched low PBBs subgroup (PBBs $\leq$ 2 ppb) were compared. A complete medical history was taken for all individuals enrolled in the study at the time of urine collection (a copy of the questionnaire used is displayed in Appendix I).

For the present study these questionnaires were used to select the most appropriate samples from those made available. Appendix II lists information for those factors deemed most important in sample selection for individuals used in the present study. Age, diabetes, kidney disease, liver disease, smoking and medications were the most important consideration. Individuals with diabetes, kidney disease, liver disease, or individuals who smoked were excluded from the study. Individuals over the age of 70 were excluded, as were individuals on medications known to affect microsomal mixed function oxidase activity (i.e., anticonvulsants). The ages of the low PBBs group were 21,21,33,33,39,42,47,48,49,50,54,60 and 69. The ages of the high PBBs group were 20,21,23,28,36,42,48,52,58,58,64,65 and 65. Other medical factors (such as recent surgery, high blood pressure) were also taken into consideration. Because of the complicating aspects of the menstrual cycle on excretion of urinary steroids in females, it was decided that male subjects would be used exclusively in the present study.

RESULTS

The first phase of the human experiments involved qualitative and quantitative determination of daily variations in the excreted levels of urinary steroids when normalized to 24-hour excretion rates and when normalized to urinary creatinine concentration. This was necessary to ascertain the relative variability for each mode of normalization.

Although 24-hour urine collections have been described by other investigators as being more reproducible than spot collection, they have one major disadvantage in that one is never sure that the total urine output for the entire 24-hour period was collected unless the collections are strictly supervised. It was decided that if the daily variation seen when data are normalized to urinary creatinine concentration is similar to the variation seen when normalizing to total 24-hour excretion rates, then this mode of normalization would be preferable in the current studies.

The 24-hour urinary steroid excretion pattern of a normal adult male over a 6 day period is shown in Table 6. Values were calculated by the MSSMET program in the following manner. The integrated area of the profiles of 'designate' ions (ions chosen for quantification) were divided by the integrated area of the designate ion of the internal

Table 6. Urinary steroid metabolic profiles of a normal adult male: 24 hour excretion patterns over a 6 day period[1].

Name	Retention Index	Amount/24 hours[2] Day 1	2	3	4	5	6
Androsterone	2532	2660	2420	2770	3310	2130	1780
Etiocholanolone	2547	2530	2420	2610	3770	2340	1820
DHEA[2]	2597	484	381	623	1890	1580	1010
11-keto-E[2]	2634	225	188	240	266	210	170
11β-hydroxy-A	2715	786	684	587	563	688	659
11β-hydroxy-E[2]	2731	448	347	444	437	309	334
16α-hydroxy-DHEA	2774	1780	1890	1520	2610	1450	975
Pregnanediol	2784	1930	1890	2060	2710	1820	1210
Pregnanetriol	2815	3570	3100	3320	3130	2190	2130
THS[2]	2845	--	--	15.7	40	--	--
THE[2]	2978	2060	1510	1610	1680	1330	1600
THA[2]	2979	31.7	93.8	93.3	--	101	101
THB	3000	154	23.9	114	157	96.7	100
allo-THB[2]	3016	269	29.6	240	186	101	61.8
allo-THE	3030	182	109	139	149	68.6	88.3
THF[2]	3030	1130	982	1000	1100	682	940
allo-THF	3040	698	456	554	486	360	428
Cortolone[2]	3059	485	451	461	459	299	437
β-Cortol	3088	428	376	271	268	158	294
β-Cortolone[2]	3090	759	573	454	357	274	486
Cortol[2]	3130	220	196	197	195	136	182

[1] Urine was collected starting after the first void morning urine and ended with the first void morning the following day.

[2] Values were calculated by the MSSMET program as follows: the integrated area of the GC peak of the designate ion for each steroid was divided by the integrated area of the GC peak of the designate ion for the internal standard. This ratio was divided by the number of ml of urine used, multiplied by the number of μg of internal standard added, and multiplied by the total volume of urine collected over the 24 hour period (ml). This value was converted to actual μg of sample for those steroids where a relative response factor had been determined (designated by a superscript[2]).

[3] Abbreviations: Androsterone, 3α-hydroxy-5α-androstane-17-one; Etiocholanolone, 3α-hydroxy-5β-androstane-17-one; DHEA, dehydroepiandrosterone, 3β-hydroxy-5-androstene-17-one; 11-keto-E,11-keto-etiocholanolone, 3α-hydroxy-5β-androstane-11,17-dione; 11β-hydroxy-A, 11β-hydroxy-androsterone, 3α,11β-dihydroxy-5α-androstane-17-one; 11β-hydroxy-E, 11β-hydroxy-etiocholanolone, 3α,11β-dihydroxy-5β-androstane-17-one; 16α-hydroxy-DHEA, 3β,16α-dihydroxy-5-androstene-17-one;

Table 6 (continued)

pregnanediol, 5β-pregnane-3α,20α-diol; pregnanetriol, 5β-pregnane-3α,17α,20α-triol; THS, 3α,17α,21-trihydroxy-5β-pregnane-20-one; THE, 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione; THA, 3α,21-dihydroxy-5β-pregnane-11,20-dione; THB, 3α,11β,21-trihydroxy-5β-pregnane-20-one; allo-THB, 3α,11β,21-trihydroxy-5α-pregnane-20-one; THF, 3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one; allo-THE, 3α,17α,21-trihydroxy-5α-pregnane-11,20-dione; allo-THF, 3α,11β,17α,21-tetrahydroxy-5β-pregnane-11-one; cortolone, 3α,17α,20α,21-tetrahydroxy-5β-pregnane-11-one; β-cortolone, 3α,17α,20β,21-tetrahydroxy-5β-pregnane-11-one; β-cortol, 5β-pregnane-3α,11β,17α,20β,21-pentol; cortol, 5β-pregnane-3α,11β,17α,20α,21-pentol.

standard. These ratios were divided by the volume (ml) of urine used (20), multiplied by the amount of internal standard added (10 μg), and multiplied by the total volume of urine collected over the 24 hour period (in ml). These relative concentrations were converted to actual μg of steroid per 24 hour whenever relative response factors had been determined.

The morning spot urinary steroid excretion patterns of a normal adult male over a 6 day period are shown in Table 7 (not the same subject as in Table 6). Values were calculated by the MSSMET program in the following manner. The ratios of integrated areas of the designate ions to the integrated area of the designate ion of the internal standard were divided by the volume (ml) of urine used (20) and the concentration of creatinine (mg/ml) and multiplied by the amount of internal standard added (10,000 ng). These values were converted to actual ng of sample per ng creatinine whenever relative response factors had been determined.

Excretion patterns of steroids were noted to be fairly consistent from day to day for major components (i.e., androsterone, etiocholanolone, pregnanediol, pregnanetriol, THE, THF, the cortolones and cortols). The large variation in the excretion pattern of DHEA was expected, based upon work by others (Van de Calseyde et al., 1972) which indicated that DHEA excretion is related to stress. Variations in the excretion patterns of some of the minor metabolites of adrenal corticosteroids were probably due to difficulties

Table 7. Urinary steroid metabolic profiles of a normal adult male: Morning spot urine excretion patterns over a 6 day period.[1]

Name[3]	Amount/mg Creatinine[2] Day 1	2	3	4	5[4]	6
Androsterone	1460	2050	1950	1410	838	1832
Etiocholanolone	1020	1340	1340	953	456	1240
DHEA[2]	950	250	830	1080	180	643
11-keto-E[2]	133	117	152	148	54.1	190
11β-hydroxy-A	519	359	637	452	256	573
11β-hydroxy-E[2]	86	100	81.9	91.6	33.9	59.8
16α-hydroxy-DHEA	104	125	179	215	83.4	--
Pregnanediol	1340	1420	1460	819	268	745
Pregnanetriol	958	1370	1010	931	381	1090
THS[2]	19.2	31.3	12.5	16.1	7.32	58.8
THE[2]	545	503	787	528	303	626
THA[2]	30.9	48.3	90.5	59.7	47.7	210
TBH[2]	43.8	44.8	79.6	47.9	36.1	58.6
allo-THB[2]	130	69.9	154	72.5	74.9	112
allo-THE	40.4	46.0	67.2	39.1	20.6	57.0
THF[2]	335	227	406	285	168	357
allo-THF	204	193	237	172	127	209
Cortolone[2]	123	143	202	140	79.3	165
β-Cortol	46.0	47.2	60.3	45.1	32.4	38.3
β-Cortolone[2]	114	143	169	126	81.9	130
Cortol[2]	57.6	50.3	67.5	42.9	34.1	47.5

[1] Urine was collected with the first void morning urine.

[2] Values were calculated by the MSSMET program as follows: the integrated area of the GC peak of the designate ion for each steroid was divided by the integrated area of the GC peak of the designate ion for the internal standard. This ratio was divided by the number of ml of urine used and the concentration of creatinine (mg/ml) and multiplied by the amount of internal standard added (10,000 ng). This value was converted to actual ng of sample for those steroids where a relative reponse factor had been determined (designated by a superscript[2]).

[3] Abbreviations are defined in Table 6.

[4] Values are consistently low and thus supect.

in detecting designate ions of these substances, and by the sharp gas chromatographic peaks of those that were present. As discussed in Section II, the capillary column was deliberately overloaded somewhat, such that larger peaks exhibited a degree of 'fronting' with a slight overlapping of isomers, to detect and quantify these minor metabolites. These analyses represented a reasonable balance of the need for good gas chromatographic separation with the sample size required for adequate quantification by MSSMET. Another problem which may contribute to errors in the quantification of minor metabolites arose when contributions to designate ion currents resulted from much larger co-eluting or adjacent peaks. This problem could be avoided by selection of ions that were unique, whenever possible. For example, intense ions in the THA mass spectrum all have a contribution from the much larger THE peak, except m/z 431, which is unique to THA and therefore the best ion for quantification of THA.

Table 8 shows the average urinary steroid excretion patterns over a 6-day period in absolute amounts for a 24 hr collection and relative to creatinine for a spot urine collection. Also shown are the designate ions used for quantification and the calculated relative response factors. Although the values listed in Tables 6 and 7 for those steroids for which a relative response factor was not available are not in absolute amounts, most of these values are within a factor of 2 or 3 of the actual amount since

Table 8. Average urinary excretion patterns over a 6 day period in absolute amounts for a 24 hour urine collection and relative to creatinine for a morning spot urine collection[1].

Name[2]	Designate ion m/z[3]	Relative Response[4]	24 Hour Collection µg/24 hours	Morning Spot Sample ng/mg creatinine
DHEA	260	1.47	950 ± 680	660 ± 410
11-keto-E	300	1.30	220 ± 38	130 ± 50
11β-hydroxy-E	448	0.98	390 ± 70	75 ± 27
THS	474	2.66	- -	24 ± 21
THE	488	1.13	1600 ± 270	550 ± 170
THA	431	1.91	70 ± 48	81 ± 73
THB	474	1.08	110 ± 53	52 ± 17
allo-THB	474	1.13	140 ± 110	100 ± 39
THF	472	1.67	970 ± 180	310 ± 90
Cortolone	449	0.24	430 ± 73	140 ± 45
β-Cortolone	449	0.45	480 ± 190	130 ± 29
Cortol	343	0.46	190 ± 31	50 ± 13

[1] Values are the average ± standard deviation over a 6 day collection period. Values for the 24 hour urine collection were calculated as described in Table 6 and values for the morning spot urine collection were calculated as described in Table 7.

[2] Abbreviations are defined in Table 6.

[3] Ion used in quantitation (designate ion). See Sections I and II for more detail.

[4] See Section II for calculation of relative response factor. Values were determined using an LKB-2091 GC/MS.

relative response factor values usually fall in the range of 0.33-3.0. Assuming that the relative response factors for these steroids are in fact within this range, the values in Tables 6 and 7 fell well within the range of reported urinary steroid excretion patterns for adult males (i.e. 17-keto-steroids, 7-20 mg day^{-1}; 17-hydroxy-steroids, 2-12 mg day^{-1}; pregnanetriol, 0.5-3.0 mg day^{-1}). The one exception was the apparently high excretion rate of pregnanediol assumed from the relative value in Tables 6 and 7. These values are very different from absolute levels because the designate ion (m/z 117) of the trimethylsilyl derivative of pregnanediol is a particularly major proportion of total ionization and the relative response factor is therefore unusually low.

Previous investigations have demonstrated large inter-individual variations in the excretion rates of urinary steroids (Pfaffenberger and Horning, 1977; Jurigskay and Kecskès, 1978; Moyer et al., 1978; Van de Calseyde et al., 1972; Fantl and Gray, 1977; Bailey et al., 1974; Völlmin, 1971; Setchell et al., 1976). Of course, certain variations in excretion in females are related to pregnancy and the estrus cycle, but even with males, a wide range of values have been reported. For example, some of the average values that have appeared in the literature for 24 hour excretion rates of androsterone in normal adult males (in mg per 24 hour) are: 2.8±0.4, n=12 (Jurigskay and Kescskès, 1978); 2.2±5.0 (±2 SD), n=23 (Moyer et al., 1978); 3.8±1.1, n=15

(Van de Calseyde et al., 1972); 2.7 with a range of 1.9-4.2, n=8 (Fantl and Gray, 1977); 2.03 with a range of 1.06-3.22, n=12 (Bailey et al., 1974); and 2.5 with a range of 1.7-2.6, n=2 (Völlmin, 1971). It is interesting that such a discrepancy exists in the literature for average excretion rates, a discrepancy that is apparently related to differences in methodology. Etiocholanolone also exhibits wide variations in excretion rates, with reported average values ranging from 1.2-4.8 mg per 24 hour (Jurigskay and Kecskès, 1978; Moyer et al., 1978; Van de Calseyde et al., 1972; Fantl and Gray, 1977; Bailey et al., 1974; Völlmin, 1971). DHEA exhibits the widest range in excretion rates (0.0-7.9 mg per 24 hour). Increases in the excretion of this steroid have been correlated with stressful situations (Spiteller, 1978). Pfaffenberger and Horning (1977) reported a minimum of 0.01, a maximum of 3.6 and an average of 0.7 mg DHEA/g creatinine in 21 male subjects. In the two individuals examined in the current study the urinary excretion rates of DHEA ranged from 0.38-1.9 mg per 24 hour for the 24 hour collection to 0.18-1.1 $\mu g\ mg^{-1}$ creatinine for the morning spot collection. Values given in Tables 6-8 for the other 17- keto urinary steroids also agree well with the values reported by other investigators (Pfaffenberger and Horning, 1977; Jurigskay and Kecskès, 1978; Moyer et al., 1978; Van de Calseyde et al., 1972; Fantl and Gray, 1977; Bailey et al., 1974; Völlmin, 1971; Setchell et al., 1976).

It has been reported that urinary excretion rates of the major metabolites of the corticosteroids also exhibit large inter-individual variations. Values ranging from 2.9-7.2 mg per 24 h and 1.1-8.1 mg/g creatinine have been reported for THE (Pfaffenberger and Horning, 1977; Van de Calseyde et al., 1972; Fantl and Gray, 1977; Vollmin, 1971). Values of 1.1-3.0 mg per 24 h and 0.44-2.9 mg/g creatinine have been reported for THF (Pfaffenberger and Horning, 1977; Fantl and Gray, 1977). Our values for excretion rates of the minor corticosteroid metabolites (i.e. THS, THA, THB, allo-THB, allo-THE) also agree with values reported by Setchell et al. (1976) and Pfaffenberger and Horning (1977).

In summary, the day to day variation in the urinary excretion of steroids when calculated per mg creatinine was comparable to that seen when the excretion rate was expressed as amount per 24 hours and was within the limits arbitrarily set at the beginning of these investigations (15-20% for the major urinary steroids). Also, the data for urinary steroid concentrations obtained by automated reverse library search of selected mass chromatograms were similar to those reported by other investigators using different methods of analysis.

Effect of accidental exposure to PBBS on urinary excretion of 6β-hydroxycortisol. A number of drugs are reported to be inducers of microsomal mixed function oxidases in man as well as other species. These include such agents as phenobarbital, antipyrine, rifampicin,

phenytoin, and carbamazepine (Ohnhaus and Park, 1979; Roots et al., 1979). 6β-Hydroxycortisol, a polar metabolite of cortisol excreted in the urine unconjugated (Frantz et al., 1961; Thrasher et al., 1969; Chamberlain, 1971; Werk et al., 1964; Yanaji et al., 1969; Berman and Green, 1971), has been reported to be a reliable indicator of hepatic microsomal MFO enzyme activity (Saenger et al., 1981; Berman and Green, 1971; Ohnhaus and Park, 1979; Stevenson et al., 1972; Poland et al., 1970; Roots et al., 1979). Saenger et al. (1981) reported that 6β-hydroxycortisol increased 4- to 7-fold in the urine of children on anticonvulsant therapy. The ratio of 6β-hydroxycortisol to total urinary 17-hydroxycortisol and free cortisol also increased. Phenytoin and carbamazepine have been reported to increase urinary 6β-hydroxycortisol (Roots et al., 1979) as have antipyrine, phenobarbital and rifampicin (Ohnhaus and Park, 1979). Other factors are also known to effect urinary excretion of 6β-hydroxycortisol. This urinary metabolite of cortisol is relatively high in pregnancy (Frantz et al., 1960; Katz et al., 1962), in certain cancer patients (Werk et al., 1964) and in the newborn (Daniilescu-Goldinberg et al., 1974). Estrogen therapy was reported to increase 6β-hydroxycortisol excretion (Katz et al., 1962). Thyroid dysfunction has also been reported to increase 6β-hydroxycortisol excretion relative to total urinary 17-hydroxycorticosteroids (Yamaji et al., 1969). The ratio of 6β-hydroxycortisol to 17-hydroxycorticosteroids is reported to be slightly higher in females than males (Thrasher et al., 1969).

The ability of certain drugs to increase urinary excretion of 6β-hydroxycortisol is classified as a "phenobarbital-like" effect on hepatic microsomal MFO enzyme systems. This is an important consideration since one of the effects of certain PBBs congeners in laboratory animals is a "phenobarbital-like" effect on hepatic MFO enzymes. In particular, those congeners with 2 or more ortho bromines have a "phenobarbital-like" effect in that they induce liver microsomal enzymes usually induced by phenobarbital and cause a proliferation of hepatic endoplasmic reticulum. Congeners without bromines in the ortho position induce microsomal enzymes usually associated with 3-methylcholanthrene exposure. Those congeners with one ortho bromine show moderate toxicity and are "mixed" inducers of liver microsomal enzymes (having both 3-methylcholanthrene and phenobarbital-like effects).

Individual components of the commercial mixture of PBBs which contaminated Michigan all contained at least one ortho bromine and 89% of the total mixture contained two ortho bromines (Moore and Aust, 1978; Moore et al., 1978, 1980). It would be reasonable to expect that one of the possible effects of this particular mixture of PBBs would be a "phenobarbital-like" effect in the exposed human population. Therefore, it follows that the exposed human population might have increased levels of urinary 6β-hydroxycortisol.

The electron impact mass spectrum of the methoxime-trimethylsilyl derivative of 6β-hydroxycortisol is given in

Figure 25. The molecular ion is seen as are characteristic losses of 15 (CH_3^+), 31 (OCH_3^+), 90 + 31 ($TMSiOH^+ + OCH_3^+$), 2X90 + 31 and 3X90 + 31. This derivative had a retention index of 3313 on the 50 m DB-1 capillary column and 3315 on the 3% OV-101 packed column. Table 9 shows the data obtained for 6β-hydroxycortisol by the MSSMET program. Also shown are data for a peak which occurred at retention index 3290. This peak exhibited the same ion currents as the major peak at R.I = 3313, was found in both the standard and the urine samples, and most likely is 6α-hydroxycortisol. These data were obtained from the packed column runs and were normalized to the internal standard and urinary creatinine. 6β-Hydroxycortisol was not present in high enough concentration to be observed in the capillary column GC/MS analyses. These data are included to demonstrate the sensitivity of the automated reverse library search. Although 6β-hydroxycortisol is a minor component of the urinary steroid mixture, this compound was found in approximately half of the samples analyzed and the averages obtained from these analyses were consistent with data obtained from the capillary column GC with FID analyses. Since this metabolite of cortisol is a minor component of the steroid mixture, the procedure utilizing capillary column GC with FID detection proved to be a more quantitative method of analysis, since this method had the required sensitivity (see Section II).

Figure 25. Electron impact mass spectrum of the methoxime-trimethylsilyl derivative of 6β-hydroxycortisol. The molecular ion is m/z=724. The m^+-31 ion (m/z=693) and subsequent losses of 90, 2X90 and 3X90 are very characteristic of methoxime-trimethylsilyl derivatives of steroids.

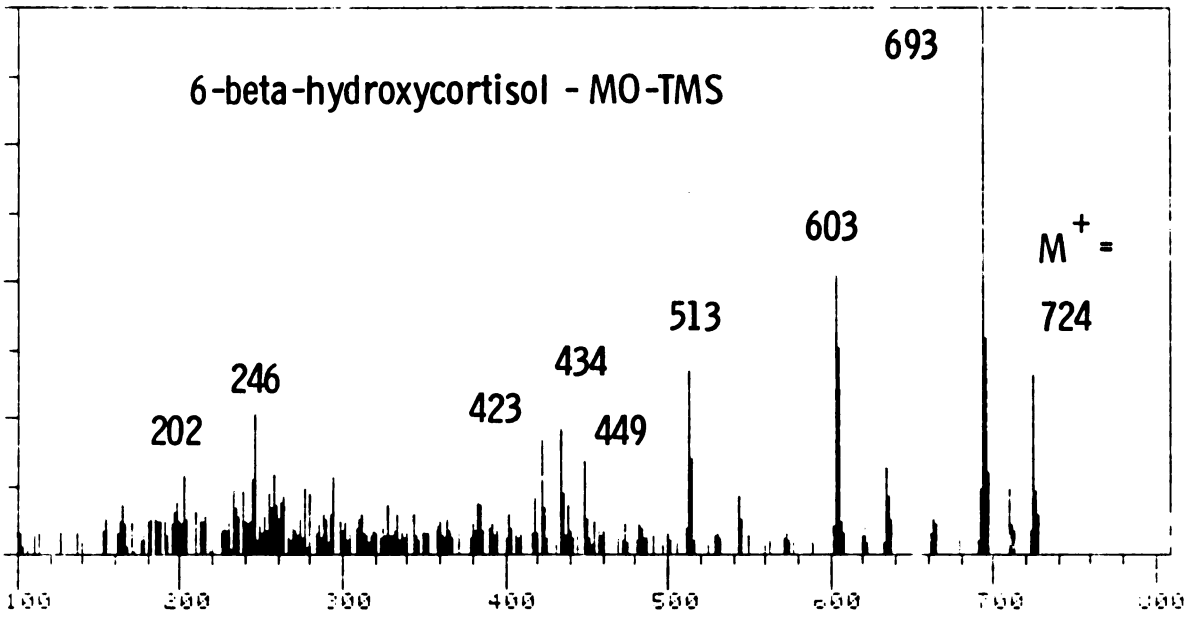

6-beta-hydroxycortisol - MO-TMS
693
603
M+ =
724
513
434
246
423
449
202
100
200
300
400
500
600
700
800

Table 9. Determination of 6β-hydroxycortisol by automated reverse library search of selected mass chromatograms[1].

	Retention Index[2]	
Group	3313	3290
Controls (n=13)	12.4 ± 14.2	1.2 ± 3.3
PBBs (n=14)	23.7 ± 24.0	1.8 ± 3.4
Phenobarbital (n=6)	118 ± 233	17.6 ± 45.8

[1] Data normalized to the internal standard (in ng) and urinary creatinine. Data were obtained from packed column runs since 6β-hydroxycortisol was not in high enough concentration to be observed in the capillary column GC/MS analyses.

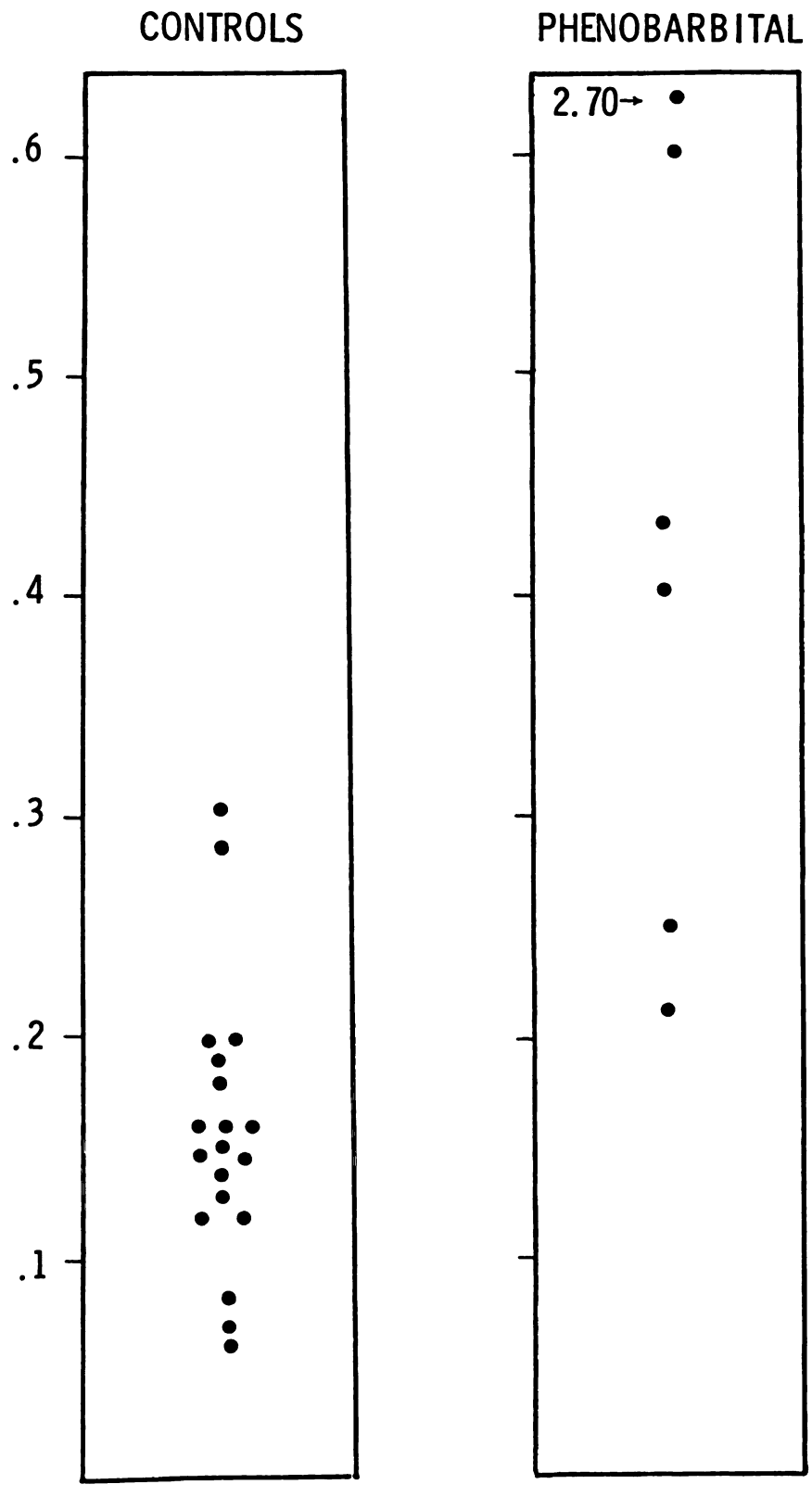
[2] The peak at retention index 3313 was the major peak obtained for the 6β-hydroxycortisol standard. The minor peak at 3290 had an almost identical mass spectrum and was probably 6α-hydroxycortisol.

The urinary concentrations of 6β-hydroxycortisol in the control and phenobarbital groups are plotted in Figure 26. All subjects were non-smoking adult males. Data are expressed as μg of 6β-hydroxycortisol/mg creatinine (a relative response factor for 6β-hydroxycortisol, and cholesteryl butyrate had previously been determined allowing conversion to actual amounts of compound). The levels of urinary 6β-hydroxycortisol were greatly elevated in some, but not all, of the phenobarbital exposed individuals. It should be noted that the standard deviation of the mean was nearly as great as, or greater than the mean in this group, which was the same pattern observed by other investigators (Saenger et al., 1981; Roots et al., 1979; Ohnhaus and Park, 1979).

Figure 27 shows the urinary 6β-hydroxycortisol concentrations in the PBBs and control groups (again, non-smoking adult males). The concentrations of urinary 6β-hydroxycortisol were significantly elevated in the PBBs group; t-test, $p<0.05$. This observation was not surprising in view of the literature described above. However, the effect is best described as "mild" since no dramatically increased concentrations were noted.

Effects of PBBs exposure on the urinary steroid metabolic profile in humans. Urinary concentrations of the major steroid components were most easily and acurately determned by capillary GC with FID. Data for the PBBs and control groups for the major urinary steroids have been

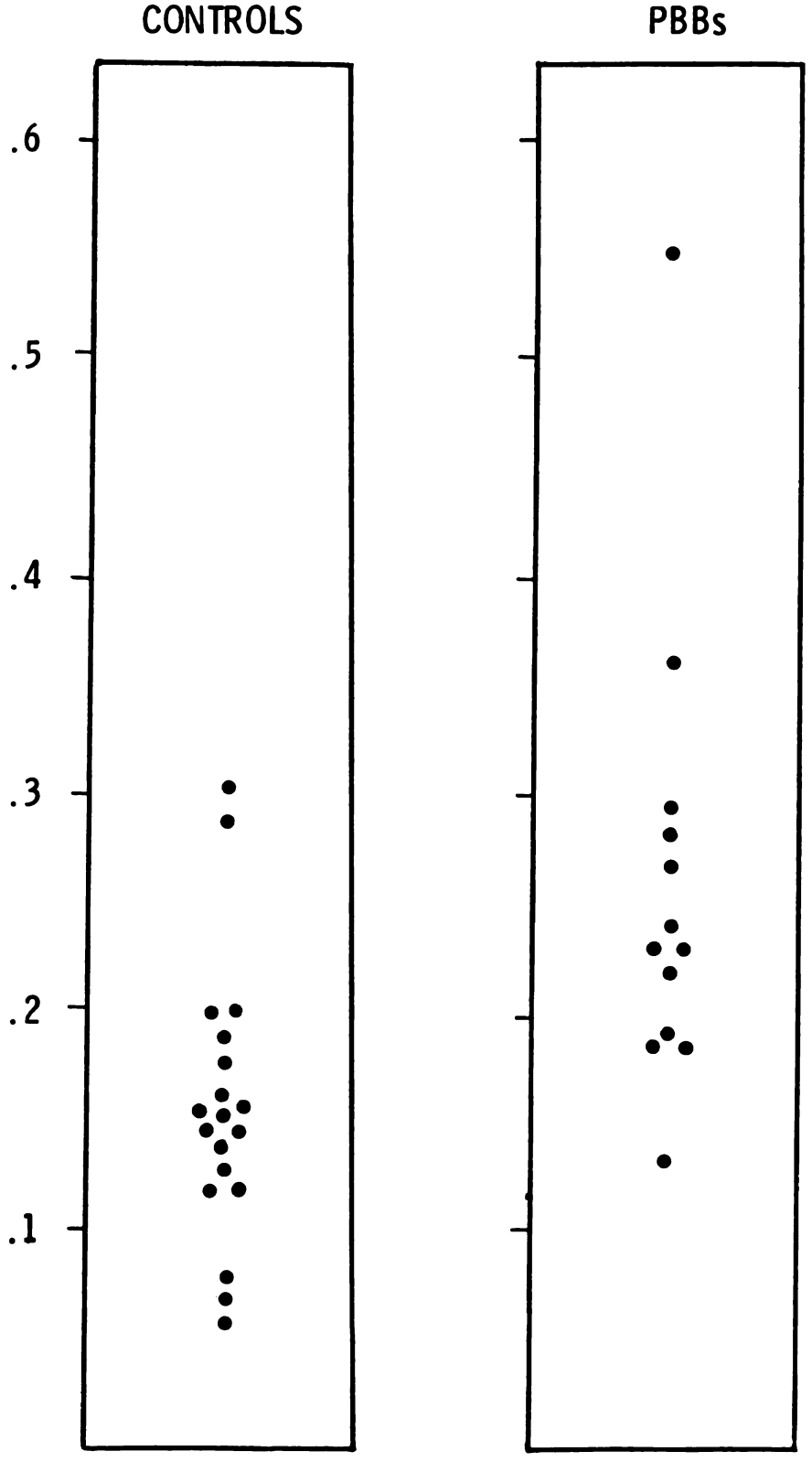
Figure 26. Dot plots of urinary concentrations of 6β-hydroxycortisol in phenobarbital and control groups. Data are for non-smoking healthy adult male subjects and are expressed as μg of 6β-hydroxycortisol/mg creatinine. Data were obtained using a 60 m DB-1 fused silica capillary (narrow bore, thin film).



ug of 6-beta-hydroxycortisol / mg creatinine

non-smoking adult male subjects

Figure 27. Dot plots of urinary concentrations of 6β-hydroxycortisol in PBBs exposed and control groups. Data are for non-smoking healthy adult male subjects and are expressed as μg of 6β-hydroxycortisol/mg creatinine. Data were obtained using a 60 m DB-1 fused silica capillary column (narrow bore, thin film).



ug of 6-beta-hydroxycortisol / mg creatinine

non-smoking adult male subjects

tabulated in Table 10. The data were generated using a 60 m DB-1 fused silica capillary column. Groups were compared for statistically significant differences by t-test with the level of significance at $p<0.05$. No differences were noted in the excretion rate of the major metabolites of testosterone (androsterone, etiocholanolone, DHEA, androstenedione). 11β-Hydroxyandrosterone, 11β-hydroxyetiocholanolone, 11-keto-androsterone and 11-keto-etiocholanolone are the major urinary steroids formed from 17α-hydroxy C-21 steroids by oxidative cleavage of the C-20,21 side chain. Of these particular steroids, 11-keto-etiocholanolone was increased in the PBBs group. The major urinary metabolite of progesterone, pregnanediol, was increased almost four-fold in the PBBs group. Certain of the major urinary metabolites of cortisol and corticosterone were also increased in the PBBs group. These steroids were THE, THF and α-cortolone. In general, the minor C-21 urinary steroids showed no tendency to be different. However, THB and THDOC were elevated in the PBBs group. Three unknown steroids, characterized by methylene unit retention indices, were also increased in the PBBs group. These data are tabulated in Table 11. The peak at RI = 3365 was most interesting in that the peak occurred in all but two of the PBBs subjects but was found in only two of the 13 control subjects.

Although the urinary levels of the major steroids and well separated minor steroids were more accurately

Table 10. Human urinary steroid metabolic profiles of non-smoking male subjects: Average plus standard deviation, normalized to the internal standard and urinary creatinine.

STEROID[1]	amount/mg creatinine[2] Control (n=13)	PBBs (n=14)
Androsterone	1.40 ± 0.70	1.60 ± 0.83
Etiocholanolone	1.30 ± 0.82	2.00 ± 2.00
11β-hydroxy-A	0.90 ± 0.50	1.30 ± 0.60
11β-hydroxy-E	0.80 ± 0.70	0.80 ± 1.00
DHEA	0.50 ± 0.80	0.70 ± 1.00
11-keto-andro.	0.20 ± 0.10	0.20 ± 0.10
11-keto-etio.	0.30 ± 0.30	0.50 ± 0.40*
Androstenedione	0.30 ± 0.20	0.40 ± 0.30
Pregnanediol	0.14 ± 0.04	0.50 ± 0.70*
Pregnanetriol	0.50 ± 0.20	0.60 ± 0.30
THE	3.00 ± 2.00	3.00 ± 2.00*
THF	1.90 ± 0.80	3.00 ± 1.00*
allo-THF	1.60 ± 0.90	2.00 ± 1.00
α-cortolone	0.90 ± 0.30	1.20 ± 0.40*
β-cortolone + β-cortol	0.70 ± 0.30	0.90 ± 0.30
α-cortol	0.40 ± 0.20	0.50 ± 0.20
THB	0.21 ± 0.07	0.40 ± 0.40*
allo-THB and allo-THA	0.50 ± 0.30	0.60 ± 0.30
allo-THE	0.30 ± 0.10	0.40 ± 0.10
THDOC	0.06 ± 0.05	0.12 ± 0.09*
THS	0.20 ± 0.10	0.30 ± 0.20
allo-THS	0.06 ± 0.09	0.10 ± 0.09

[1] Abbreviations are listed in Table 6.

[2] Relative response factors for MO-TMS derivatives of these steroids was not calculated. However, most of these values will be close to 1.0. Thus, values listed are very close to the actual amounts in μg/mg creatinine.

* Significantly different ($p<.05$)

Table 11. Unknowns elevated in the urine of PBBs exposed human subjects as determined by capillary GC with FID.

Average ± standard deviation[1]

R.I.[2]	amount/mg creatinine	
	Control (n=13)	PBBs (n=14)
2626	0.06 ± 0.03	0.18 ± 0.25*
3180	0.12 ± 0.05	0.20 ± 0.12*
3365	0.02 ± 0.05	0.10 ± 0.08*

[1] Normalized to the internal standard and urinary creatinine.

[2] Each compound was identified by these characteristic retention indices.

* Significantly different ($p<.05$).

determined by capillary GC with FID, there were a number of other steroids that were detected by GC/MS/DS. These included compounds in the MSSMET library where retention and spectral information were obtained from standards, compounds where retention and spectral information were obtained from other investigators and compounds where retention and spectral information were obtained directly from GC/MS analysis of the urinary milieu (i.e., entered in the MSSMET library as unknowns). These data are displayed in Table 12. Values are the integrated area of the designate ion for each steroid divided by the integrated area of the designate ion of cholesteryl butyrate (m/z = 368) times the amount of cholesteryl butyrate added (in mg) divided by the mls of urine used and the concentration of creatinine (in mg/ml). Data shown are the average ± S.D. for controls (n=13) and PBBs (n=14) subjects. Also shown are the averages ± S.D. for the phenobarbital treated subjects (same subjects for which 6β-hydroxycortisol concentrations are shown in Figure 26). Since these are for the most part minor urinary steroids, the data shown are for the packed column GC/MS/DS analyses. Also shown are values for THE and THF, which are included to demonstrate that the GC/MS/DS gave comparable results for the major urinary steroids as GC/FID (Table 10). Most of the steroids and unknowns listed in Table 12 are in very low concentration in urine. For this reason, these compounds were often not detected by automated reverse library search of selected mass chromatograms. As an

Table 12. Human urinary steroids in controls, PBBs exposed and phenobarbital exposed individuals as determined by GC/MS/DS[1].

Compound Number	N A M E	Controls (n=13)	PBBs (n=14)	Phenobarbital (n=6)
52	THE	1800 ± 900	3000 ± 2000	2000 ± 3000
58	THF	2000 ± 2000	3000 ± 4000	3000 ± 4000
7	17α-hydroxypregnenolone (3β,17α-dihydroxy-5β-pregnane-20-one)	50 ± 40	50 ± 60	200 ± 300
9	16β-hydroxy-DHEA (3β,16α-dihydroxy-5-androstene-17-one)	20 ± 20	20 ± 20	20 ± 30
11	16α-hydroxy-DHEA(3β,16β-dihydroxy-5-androstene-17-one)	600 ± 800	1000 ± 2000	2000 ± 3000
13	3β,20α-dihydroxy-5-pregnene	10 ± 20	20 ± 40	10 ± 20
14	15-keto-androstenediol (3β,17β-dihydroxy-5-androstene-16-one)	30 ± 30	60 ± 70	100 ± 100
15	15-β, 16α-dihydroxy-DHEA (3β,15β,16α-trihydroxy-5-androstene-17-one)	1.0 ± 3.0	2 ± 5	10 ± 20
16	Androstenetriol (5-androstene-3β,16α, 17β-triol)	30 ± 80	100 ± 200	10 ± 20
17	3α,16α-dihydroxy-5β-pregnane-20-one	10 ± 20	10 ± 10	30 ± 40
18	3α,6α-dihydroxy-5β-pregnane-20-one	10 ± 20	10 ± 10	30 ± 80
19	16α,18-dihydroxy-DHEA, peak 1 (3β,16, 18-trihydroxy-5-androstene-17-one)	2.0 ± 5.0	20 ± 50	1.0 ± 4.0

Table 12 (continued)

Compound Number	N A M E	Controls (n=13)	PBBs (n=14)	Phenobarbital (n=6)
20	16α,18-dihydroxy-DHEA, peak 2 (3β,16, 18-trihydroxy-5-androstene-17-one)	3.0 ± 5.0	20 ± 50	2.0 ± 4.0
21	Estriol (3,16α,17β-trihydroxy-1,3,5(10)-estratriene)	1.0 ± 1.0	10 ± 11	4.0 ± 9.0
22	3β,15α-dihydroxy-5α-pregnane-20-one	60 ± 50	80 ± 100	200 ± 300
23	3α,20α-dihydroxy-5α-pregnane-11-one	6.0 ± 8.0	8.0 ± 10	6.0 ± 14
24	3α-20α-dihydroxy-5α-pregnane-11-one	8 ± 15	20 ± 30	6.0 ± 14
25	5β-pregnane-3α,16α,20α-triol	70 ± 40	90 ± 60	500 ± 600
29	11-keto-pregnanetriol (3α,17α,20α-trihydroxy-5β-pregane-11-one)	20 ± 20	10 ± 10	15 ± 20
30	3α,11β,20α-trihydroxy-5β-pregnane	12 ± 10	10 ± 20	10 ± 20
42	3α,11β,20α,21-tetrahydroxy-5β-pregnane	70 ± 50	30 ± 40	20 ± 20
43	3α,11β,20α,21-tetrahydroxy-5α-pregnane	60 ± 50	40 ± 50	80 ± 100
44	3β,16α,20α-trihydroxy-5-pregnene	1.0 ± 2.0	2.0 ± 6.0	5.0 ± 6.0
45	16α-hydroxypregnenolone (3β,16α-dihydroxy-5β-pregnane-20-one)	10 ± 10	20 ± 30	6.0 ± 9.0
46	3β,16α-dihydroxy-5α-pregnane-20-one	10 ± 10	40 ± 60	7.0 ± 14
47	5-pregnene-3β,17α,20α-triol	30 ± 30	50 ± 60	30 ± 50

Table 12 (continued)

Compound Number	N A M E	Controls (n=13)	PBBs (n=14)	Phenobarbital (n=6)
50	5-androstene-3α,15β,16α,17β-tetrol	20 ± 40	100 ± 300	30 ± 60
60	1,3,16α-trihydroxypregnane-20-one	10 ± 30	40 ± 100	40 ± 90
119	$UN24_2$	10 ± 20	60 ± 200	3.0 ± 8.0
120	$UN25_2$	200 ± 200	300 ± 300	100 ± 90
121	$UN26_2$	90 ± 100	500 ± 2000	10 ± 20
122	$UN27_2$	0.0 ± 0.0	4.0 ± 20	0.0 ± 0.0
123	$UN28_2$	30 ± 20	30 ± 30	30 ± 40
124	$UN29_2$	400 ± 400	600 ± 400	400 ± 500
125	$UN30_2$	0.3 ± 1.0	2.0 ± 3.0	2.0 ± 4.0
126	$UN31_2$	0.4 ± 1.0	2.0 ± 8.0	0.0 ± 0.0
206	$UN110_2$	14 ± 16	100 ± 400	100 ± 200
209	$UN111_2$	4.0 ± 10.0	7.0 ± 22	4.0 ± 10
211	$UN111_2$	20 ± 20	50 ± 70	100 ± 200
212	$UN112_2$	30 ± 30	60 ± 70	200 ± 300
213	$UN113_2$	10 ± 10	3.0 ± 4.0	4.0 ± 6.0
214	$UN114_2$	10 ± 10	10 ± 20	6.0 ± 9.0

Table 12 (continued)

[1] Values are the integrated area of the designate ion for each steroid (or unknown) divided by the integrated area of the designate ion of cholesteryl butyrate (m/z = 368), times the amount of cholesteryl butyrate added (in ng), divided by the mls of urine used and the concentration of creatinine (in mg/ml). Data shown are the mean ± S.D. for low PBBs (n=13), and high PBBs (n=14) groups and for the phenobarbital treated subjects.

[2] Unknowns whose retention and spectral information were obtained directly from GC/MS analysis of the urinary milieu.

example, estriol (3,16α,17β-trihydroxy-1,3,5(10)-estratriene one of the major urinary metabolites of 17β-estradiol in humans) was found in 8 out of 14 of the high PBBs subjects and in only 4 out of 13 of the low PBBs groups. Estriol was observed to increase in the PBBs ($\bar{x}$ = 0.8 ± 1.4 in the low PBBs group and $\bar{x}$ = 5.9 ± 11.0 in the high PBBs group). Also, all estriol values in the high PBB group were higher than the highest value recorded in the low PBBs group. The data for estriol is typical of most of the data in Table 12 because this compound was either below or near the detection limits of the analytical system. For these analyses, the values determining whether a compound will be printed in the found file were set purposely low. This, of course, increased the possibility of obtaining "false positives". However, for this particular analysis of the GC/MS data, the object was to detect possible differences only, since most of these compounds were at or below the level of detection. Obviously, more sensitive methods of analysis would have to be employed to confirm or refute any differences noted when using an automated reverse library search for quantitative analysis of minor components of the urinary steroid milieu. Inspection of the data in Table 12 revealed that a number of compounds were apparently increased in the PBBs exposed individuals. Most interestingly, many of these compounds were 16α-hydroxy steroids. For example, large increases in the excretion

of androstenetriol (5-androstene-3β,16α,17β-triol) 16α,18-dihydroxy-DHEA, estriol, 3β,16α-dihydroxy-5α-pregnane-20-one and 5-androstene-3β,15β,16α,17β-tetrol were seen in the high PBBs group when compared to the control group. Other apparent differences were seen for UN24 and UN110. UN110 had the same retention index as the first compound listed in Table 11 (2626) and these may, in fact, be the same compounds. Mass spectral interpretation of UN110 indicated this compound might be a hydroxylated metabolite of progesterone.

DISCUSSION

The subjects. The data presented above indicate that PBBs exposed individuals may have altered excretion rates of certain urinary steroids compared to an appropriate non-exposed (or low level of exposure) group. These urinary samples were obtained through the Michigan Department of Public Health (MDPH), Division of Environmental Epidemology from individuals enrolled in a study examining possible long-term health effects of accidental PBBs exposure. Factors that were considered in the selection of subjects have been described above. What emerged from this selection process was two groups of apparently healthy male subjects who did not smoke and had no known, or reported, medical consideration that would obviously necessitate the removal of them from this study. Of course, one must trust the honesty of the respondents and this is not always a correct assumption. For example, the questionnaires were filled out by employees of the MDPH who interviewed each participant in the study. Under the interview conditions, certain subjects might have felt inhibited in providing a totally accurate medical history. For example, an individual who professed to be a non-smoker might in truth have an occasional cigarette but be reluctant to reveal this information.

However, the relatively large number of subjects selected for each group will tend to overcome the few instances of inaccuracy which most likely occurred in the interviewing process. For the most part, it is this author's belief that the high and low PBBs groups were closely matched with respect to those factors known to effect steroid hormone excretion.

6β-Hydroxycortisol. Data comparing the urinary levels of 6β-hydroxycortisol in the high and low PBBs groups were one of the more interesting aspects of this study. As previously stated, a number of drugs known to be classical inducers of hepatic P-450 microsomal mixed function oxidase system have been shown to increase the urinary levels of 6β-hydroxycortisol in a dose-dependent fashion, with the highest doses of various drugs causing a many-fold increase in the average for the treated group. With increased exposure (or dose), there was also a concomitant increase in the standard deviation of the group mean. If hepatic microsomal mixed function oxidase activity in individuals with "high" plasma levels of PBBs was induced by PBBs to the extent seen following administration of a large or maximum dose of one of the classical P-450 type inducers, then large increases in the excretion of 6β-hydroxycortisol, at least in some of the experimental subjects, should have occurred. Although the mean of the high PBBs group was significantly elevated over the low PBBs group, the increase was less than two-fold and no individual had a greatly elevated

concentration of 6β-hydroxycortisol. Therefore, it follows that the PBBs-exposed individuals were certainly not experiencing the same degree of stimulation of hepatic P-450 microsomal MFO enzyme stimulation that was seen following large or maximal doses of the drugs previously mentioned. What can be said is there was an apparently "mild" stimulation of these enzyme systems in the high PBBs groups similar to what has been reported to occur following "moderate" doses of the above-mentioned drugs.

This "mild" induction of hepatic microsomal mixed function oxidase enzyme systems was not surprising in view of the literature. First, the inductive affect of PBBs on hepatic P-450 enzyme systems is well established in experimental animals (Dent et al., 1976a, 1976b; McCormack et al., 1978; Newton et al., 1980, 1981) and would be expected to occur in man. Second, reports on workers exposed to DDT and endrin have also indicated a mild stimulation of 6β-hydroxycortisol excretion. Poland et al. (1970) studied the effect of intensive occupational exposure to DDT on drug and steroid metabolism. DDT (1,1,1-trichloro-2,2 bis(p-chlorophenyl)ethane) is similar to PBBs with respect to effects on hepatic microsomal MFO enzyme systems and storage in body fat tissues. In Poland's study, the concentration of DDT and DDT-related compounds was 20 to 30 times that of the control population. They reported that the serum half life of phenylbutazone (also a test for hepatic P-450 microsomal mixed function oxidase induction) was 19% lower in the

factory workers exposed to DDT and that urinary excretion of 6β-hydroxycortisol was 57% higher in this group. Both of these differences were significant at the $p<0.01$ level. Increased excretion of 6β-hydroxycortisol (2-3 fold; n=8) has also been reported to occur in workers exposed to endrin (Chamberlain, 1971). Both of these studies, involving exposure to fat soluble polyhalogenated organics similar to PBBs, report an effect on 6β-hydroxycortisol very much in line with that seen in the present investigation.

Table 13 summarizes some of the more important metabolic consequences of induction of hepatic microsomal mixed function enzyme systems. Although a number of consequences are harmful (or toxic) under certain situations, there are also potential beneficial consequences. Thus, it could be misleading to describe an apparent stimulation of hepatic cytochrome P-450 mixed function oxidase systems by PBBs in the exposed population as a toxic affect.

Urinary Steroid Metabolic Profiles. The urinary steroid metabolic profile of the high PBBs group showed some potentially important differences. With the exception of pregnanediol (the major urinary metabolite of progesterone) and possibly estriol, all of the steroids elevated in the high PBBs group are of adrenal origin. To reiterate, these steroids are 11-keto-etiocholanolone (formed by oxidative cleavage of the sidechain of a 17α-hydroxy-20-one C-21 steroid), THE, THF, α-cortolone, THB and THDOC. If there are, in fact, elevated concentrations of urinary steroids of

Table 13. Possible consequences of induction and/or inhibition of liver microsomal enzyme systems.

1) Increased metabolism of endogenous substrates

2) Decreased metabolism of endogenous substrates

3) Increased levels of minor metabolites of endogenous substrates or appearance of a new metabolite

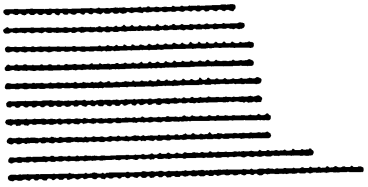
4) Shift in dose-reponse curve to drug therapy
   a) Attenuated therapeutic and/or toxic response to drug therapy
   b) Increased therapeutic and/or toxic reponse to drug therapy

5) Shift in dose-reponse curve to toxin exposure
   a) Attenuated response to accidental exposure to a toxin
   b) Increased response to accidental exposure to a toxin

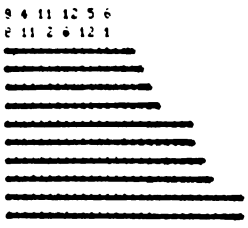
adrenal origin in the high PBBs group, this may indicate an effect of PBBs that is unrelated to stimulation of hepatic cytochrome P-450 mixed function oxidase induction since total urinary 17-hydroxycorticosteroids in humans does not change following administration of phenobarbital or rifampicin (Saenger et al., 1981; Ohnhaus and Park, 1979). Thus if PBBs do effect adrenal function in humans, an alternate explanation of the toxicodynamics of this effect must be found. Indeed, PCBs, compounds similar in chemical and toxicologic properties to PBBS, have been reported to elevate corticosterone and cortisol levels and increase adrenal size in rodents and rhesus monkeys (Sanders et al., 1974; Wasserman et al., 1973; Barsotti and Allen, 1975). If PBBs do in fact stimulate adrenal function in exposed humans, the effect may be of toxicological significance.

Statistical considerations. There are certain statistical problems associated with analysis of data of the type displayed in Tables 10-12. Although there were statistically significant differences noted in seven out of the 22 steroids listed in Table 10, the chance of finding differences between any two groups does increase with the number of comparisons made. For example, if 20 separate measurements are compared and the level of significance is arbitrarily set at $p<0.05$, one would expect to find at least one significantly different group of values by chance alone, assuming the measurements are stastically independent. Thus, a simple t-test, or a similar test, are not the most

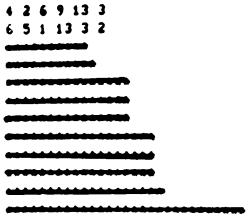
Figure 28. Plots of Student's t-statistic when urinary steroid data are randomly reassigned. Compounds are ordered by value of t-statistic (top plot). Six values were randomly picked from each group and reassigned to the other experimental group. The statistical comparisons were then repeated and the 10 largest values plotted. Data shown are for 18 replications of this process.



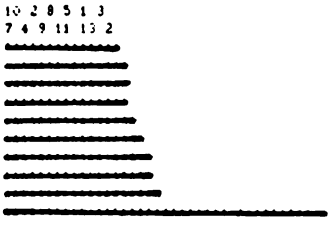
9 4 11 12 5 6
8 11 2 6 12 1



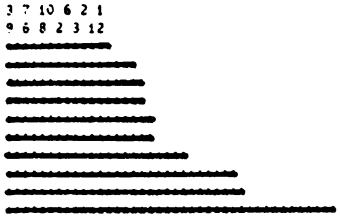
4 2 6 9 13 3
6 5 1 13 3 2



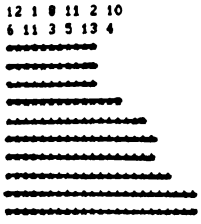
10 2 8 5 1 3
7 4 9 11 13 2



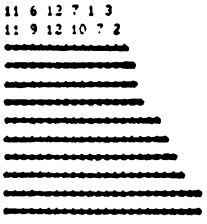
3 7 10 6 2 1
9 6 8 2 3 12



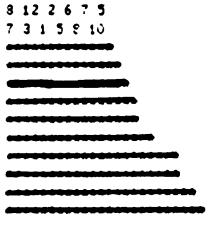
12 1 8 11 2 10
6 11 3 5 13 4



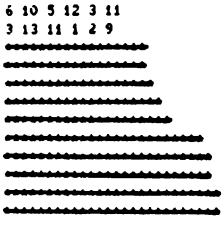
11 6 12 7 1 3
11 9 12 10 7 2



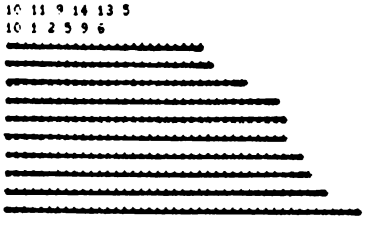
8 12 2 6 7 5
7 3 1 5 9 10



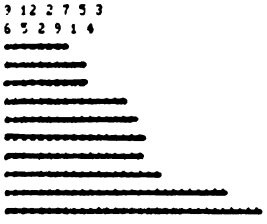
6 10 5 12 3 11
3 13 11 1 2 9



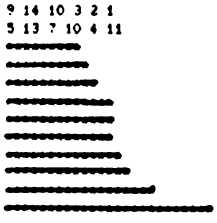
10 11 9 14 13 5
10 1 2 5 9 6



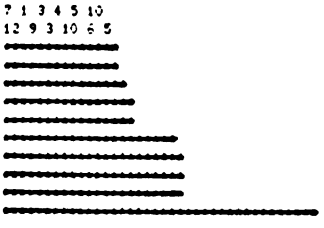
9 12 2 7 5 3
6 5 2 9 1 4



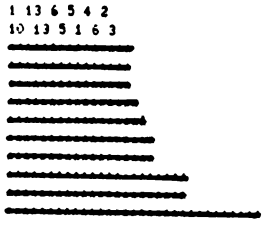
9 14 10 3 2 1
5 13 7 10 4 11



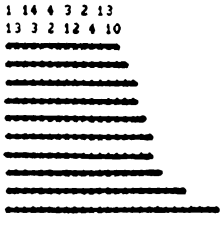
7 1 3 4 5 10
12 9 3 10 6 5



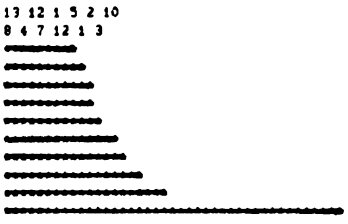
1 13 6 5 4 2
10 13 5 1 6 3



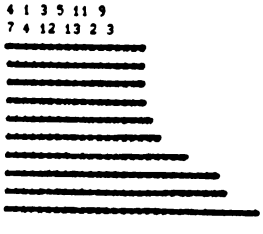
1 14 4 3 2 13
13 3 2 12 4 10



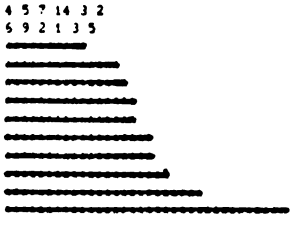
13 12 1 5 2 10
8 4 7 12 1 3



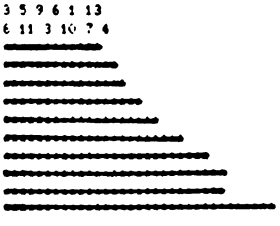
4 1 3 5 11 9
7 4 12 13 2 3



4 5 7 14 3 2
6 9 2 1 3 5



3 5 9 6 1 13
6 11 3 10 7 4



appropriate methods to use when comparing multiple variables between groups, as is the situation in metabolic profiling. However, there are tests to ascertain the level of significance that can be placed on these data as a whole. One test applied to the current data involved ranking each compound by its level of significance using the Student's t-statistic (LePage, 1983). This procedure involved first calculating the level of significance for each compound using the t-statistic, which is simply the difference between sample means divided by the standard error of difference of sample means and is calculated as follows:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{s^2_{\bar{X}_1} + s^2_{\bar{X}_2}}}$$

where $\bar{X}_1$ and $\bar{X}_2$ are means for groups 1 and 2 and $S^2_{\bar{X}_1}$ and $S^2_{\bar{X}_2}$ are standard errors of the means for groups 1 and 2. The absolute values of the ten most significant values (10 largest values) were tabulated in order of increasing significance. Bar graph plots of these data are shown at the top of Figure 28. Data are plotted with increasing t-statistic from top to bottom. Following this calculation, six subjects were randomly picked from the control group and reassigned to the high PBBs group and six subjects were randomly picked from the high PBBs group and reassigned to the control group. Following random reassignment of subjects, t-statistic values were again calculated for each compound. The absolute values of the ten most significant

values were tabulated in order of increasing significance. This process (randomization of the data and calculation of a new set of t-statistics) was then repeated a total of 24 times. Randomization of the data will have the tendency to attenuate differences occurring in the original data set although some greater differences will occur by chance alone with enough replications of the process. Thus, the relative number of times the sum of the t-statistics is greater than the original data set is a measure of the probability that the original distribution of means could have occurred by chance alone.

From this type of analysis of the data, the level of significance for the group as a whole was placed at $p<0.13$ (for 24 replications, the sum of the resultant t-statistics were greater than the original sum in three instances). It should be noted that the above consideration does not apply to statistical analysis of the 6β-hydroxycortisol data because the effect of PBBs on 6β-hydroxycortisol was proposed as a separate question. Thus, the level of significance for the effect on 6β-hydroxycortisol could correctly be placed at $p<0.05$.

SECTION V

Conclusions and Directions

CONCLUSIONS AND DIRECTIONS

The studies described in this dissertation can be classified as either analytical or as toxicological in nature. The analytical objectives of this dissertation were to:

1) Develop quantitative procedures for the isolation, purification and derivatization of steroids from urine and plasma.

2) Develop capillary column GC and GC/MS/DS techniques for the quantitative analysis of a mixture of steroids isolated from urine or plasma.

Satisfactory procedures have been developed for isolation, purification and derivatization of urinary and plasma steroids. Data were not shown for studies generating plasma steroid GC profiles. However, the methods developed for urine were found to be applicable to plasma samples. The reproducibility of these methods (demonstrated using human urine) has been demonstrated to be well within the original goal of ± 10%.

The capillary column GC techniques improved as the study progressed due to improvements in the efficiency of available columns and to further optimization of GC oven temperature programming rate and carrier gas linear velocity. This was apparent upon comparison of the quality and

information content of the capillary column urinary steroid metabolic profile shown in Figure 7 (page 51) which was obtained approximately one year after the start of the study on a 25 m OV-101 column, with the profile shown in Figure 8 (page 55), which was obtained in the later stages of the study on a 60 m DB-1 bonded phase column. The automated GC/MS/DS procedures described in Section II are capable of reproducibly identifying and quantitating urinary steroids by reverse library search analysis of selected mass chromatograms from full range repetitive scanning GC/MS data sets. The advantages and disadvantages of using packed and capillary columns in this type of sophisticated analysis have been exhaustively studied and compared to the advantages and disadvantages of using capillary columns with FID. Capillary GC with FID was adequate for quantitative analysis of most urinary steroids isolated from human urine. The major drawback to using capillary GC with FID was the time involved in analysis of the GC traces. Generation of human urinary steroid metabolic profiles by GC/MS/DS was not nearly as time consuming. The major drawback to using GC/MS/DS was the relative insensitivity of this method compared to capillary column GC with FID. Knowledge of these advantages and disadvantages proved invaluable in subsequent applications of these methods to the toxicological questions addressed in Sections III and IV.

The toxicological objectives of this dissertation were to:

3) Use the rat as a model to examine the effects of PBBs on the urinary steroid metabolic profile and correlate these changes with known specific effects these chemicals have on steroid metabolism.

4) Extend the study to a human population by examining the steroid metabolic profiles of a group of subjects accidentally exposed to PBBs.

The rat model was chosen primarily because many of the previous investigations into effects of PBBs on steroid hormone metabolism have used this species. Many of the effects reported were of such a magntude that they would be expected to be reflected by changes in the urinary steroid metabolic profile. In these experiments, PBBs did alter the urinary steroid metabolic profile in rats, although the data did not permit accurate characterization of the exact mechanism of action of any one observed effect. Attempts were made to characterize the observed effects as either phenobarbital-like or 3-MC-like in nature. However, the concentrations of corticosteroid metabolites in these particular samples were not sufficient for such a characterization.

In retrospect, it is apparent that the rat was an inadequate experimental animal model for several reasons. The extensive enterohepatic circulation and metabolism by gut microbes of steroid hormones in the rat complicated the urinary profile. The urinary steroids fraction isolated

using the procedures described in Section II contained many non-steroidal substances, many of which were in high concentration. The rat urinary steroid profile was also complicated by a large number of steroid stereoisomers and steroids with similar general structures (i.e., tetrahydroxy-pregnane-ones, for example). It was also difficult to obtain reference standards for most of these compounds. Finally, there is the problem of the relatively low concentration of steroid hormones in rat urine (relative to human urine).

Future research using the rat as a model must develop along three lines. First, much larger volumes of urine (100-200 mls) must be used to better characterize the rat urinary steroid metabolic profile (both male and female, conventional and germ-free). Second, better methods must be developed to obtain a germ-free condition in the experimental subjects. Third, the LH-20 fractionation (or possibly another column procedure) must be further investigated to obtain a "no-overlap" situation for those steroids deemed most important to the investigations.

More definitive statements can be made as to the effects of PBBs on humans. That any differences in the urinary steroid metabolic profile could be detected years following accidental exposure of humans to PBBs is, in itself, a major accomplishment. The exposure occurred approximately 8 years before the urine samples were collected and tolerance to

many of the effects of PBBs could have occurred during this time. The methods developed appear to be sensitive in detecting xenobiotic-induced alterations in steroid hormone metabolism.

Based upon the data presented in Section IV, the following statements can be made:

1) Exposure to PBBs has resulted in some degree of stimulation of 6β-hydroxycortisol excretion, assuming another factor(s) is not involved in the increased excretion of 6β-hydroxycortisol in the high PBBs group (Figure 27).

2) Exposure to PBBs has not resulted in the same degree of stimulation of 6β-hydroxycortisol excretion as that which usually would occur following chronic administration of a "large", or "maxmimal" dose of phenobarbital.

It is this author's opinion that a mild degree of phenobarbital-like stimulation of liver microsomal enzymes should not be classified as a toxic effect, but only as an effect that in some situations can be detrimental, and in other situations beneficial. The data presented in Table 10 which suggest an increased excretion of adrenal steroids, are also of interest from a toxicological point of view. If the increases in urinary steroids seen in the PBBs group are real and are, in fact, due solely to accidental exposure to PBBs, the most likely cause of this effect would be adrenal cortex hyperactivity. Furthermore, the enzymes involved in production of these urinary metabolites are not mixed function oxidases and are not inducible by phenobarbital.

If exposure to PBBs has resulted in adrenal cortex hyperactivity, this effect may be detrimental to the health of exposed individuals. Therefore, future investigations in this area are needed to confirm the effect of PBBs exposure on urinary excretion of steroids of adrenal origin. If this hypothesis is confirmed then experiments should be initiated to evaluate the degree of adrenal cortex activity of the PBBs exposed and non-exposed (or low-level exposure) human populations.

APPENDIX I

Michigan Department of Public Health,
Division of Environmental Epidemiology,
Health Questionnaire

APPENDIX I

Michigan Department of Public Health,

Division of Environmental Epidemiology Health Questionnaire

Date _____

SECTION B:

HEALTH HISTORY

1. Have you ever had any of the following health problems?

A. Diabetes 1. Yes __ 2. No __

IF YES TO A:

Are you presently taking insulin or any other medication for diabetes? 1. Yes __ 2. No __ 8. Does not apply __

B. Rheumatic fever or heart murmur? 1. Yes __ 2. No __

C. Heart attack, heart failure or heart surgery? 1. Yes __ 2. No __

D. High blood pressure? 1. Yes __ 2. No __

E. Stroke? 1. Yes __ 2. No __

F. Kidney Disease? 1. Yes __ 2. No __

G. Cancer (malignancy)? 1. Yes __ 2. No __

IF YES TO G:

Year of onset Year _____

Type or site of cancer _____

H. Liver Disease? 1. Yes __ 2. No __

IF YES TO H:

Year of onset Year _____

Type of liver disease _____

I. Any other diagnosed disease? 1. Yes __ 2. No __

IF YES TO I:

Year of onset Year _____

Please specify _____

2. Have you been hospitalized in the last year? 1. Yes __ 2. No __

IF YES:

Reason Hospitalized	Hospital	Date Entered	Date Released

3. Have you had surgery in the last year? 1. Yes __ 2. No __

IF YES:

Type of Surgery	Date of Surgery	Surgeon	Hospital

4. Are you presently on a:

A. Low salt diet? 1. Yes __ 2. No __

B. Low cholesterol diet? 1. Yes __ 2. No __

C. Diet to lose weight? 1. Yes __ 2. No __

D. Any other special diet? 1. Yes __ 2. No __

IF YES TO D:

What kind of special diet are you on? _____

5. Have you gained or lost at least 20 pounds in the past six months? 1. Yes, gained __ 2. Yes, lost __ 3. No __

MDPH Environmental Epidemiology

Health Questionnaire

SECTION C:

SMOKING, DRINKING AND OCCUPATIONAL EXPOSURE

Drinking

1. If one drink is defined as one bottle of beer, one glass of wine, or one mixed drink or shot of liquor, do you presently drink one or more drinks per week?
   1. Yes ___
   2. Used to, no longer __
   3. No, never drank that frequently __

———IF YES TO 1:———
On the average, how many drinks do you have in a week?
Number of drinks ____
Does not apply ___

Cigarette Smoking

2. Have you ever smoked cigarettes? (No means less than 20 packs of cigarettes or 12 oz. of tobacco in a lifetime, or less than 1 cigarette a day for 1 year.)
   1. Yes __ 2. No __

———IF YES TO 2:———

A. Do you smoke cigarettes? (as of 1 month ago)
   1. Yes __ 2. No __

B. How old were you when you first started regular cigarette smoking?
   Age in years ___
   Does not apply ___

C. If you have stopped smoking cigarette, completely, how old were you when you stopped?
   Age in years ___
   Does not apply ___

D. How many cigarette do you smoke per day now?
   Cigarettes per day ___

E. On the average of the entire time you smoked, how many cigarettes did you smoke per day?
   Cigarettes per day ___
   Does not apply ___

F. Do you or did you inhale the cigarette smoke?
   1. Does not apply ___
   2. Not at all __ 3. Some __
   4. Moderately __ 5. Deeply__

Pipe Smoking

3.	Have you ever smoked a pipe regularly? (Yes means more than 12 oz. of tobacco in a lifetime)	1. Yes __ 2. No __

IF YES TO 3:

For persons who have ever smoked a pipe:

A1.	How old were you when you started to smoke a pipe regularly?	Age ___
2.	If you stopped smoking a pipe completely, how old were you when you stopped?	Age stopped ___ Still smoking pipe ___ Does not apply ___
B.	On the average over the entire time you smoked a pipe, how much pipe tobacco did you smoke per week?	Oz./wk ___ (a standard pouch of tobacco contains 1.5 oz.) Does not apply __
C.	How much pipe tobacco are you smoking now?	Oz./wk ____ Not smoking currently __
D.	Do you or did you inhale the pipe smoke?	1. Never smoked __ 2. Not at all __ 3. Slightly __ 4. Moderately __ 5. Deeply __

Cigar Smoking

4.	Have you ever smoked cigars regularly? (Yes means more than 1 cigar a week for a year)	1. Yes __ 2. No __

IF YES TO 4:

For persons who have ever smoked cigars:

A1.	How old were you when you started smoking cigars regularly?	Age ___
2.	If you have stopped smoking cigars completely, how old were you when you stopped?	Age stopped ___ Still smoking cigars ___ Does not apply ___
B.	On the average, over the entire time you smoked cigars, how many cigars did you smoke per week?	Cigars per week ___ Does not apply __
C.	How many cigars are you smoking per week now?	Cigars per week ___ Not smoking currently __
D.	Do you or did you inhale the cigar smoke?	1. Never smoked __ 2. Not at all __ 3. Slightly 4. Moderately __ 5. Deeply __

Occupational Exposure

5. If occupational exposure is defined as the equivalent of daily exposure at your job for 3 or more months during your lifetime, have you been occupationally exposed to:

A. Silica, talc or sandblasting?	1. Yes, presently exposed ___ 2. Yes, past exposure ___ 3. No ____
B. Asbestos?	1. Yes, presently exposed ___ 2. Yes, past exposure ___ 3. No ____
C. Dust such as feed, grain, coal dust or sawdust?	1. Yes, presently exposed ___ 2. Yes, past exposure ___ 3. No ____
D. Smelting, foundry or welding fumes?	1. Yes, presently exposed ___ 2. Yes, past exposure ___ 3. No ____
E. Smoke, freon, auto exhaust or other fumes?	1. Yes, presently exposed ___ 2. Yes, past exposure ___ 3. No ____
F. Paints or solvents?	1. Yes, presently exposed ___ 2. Yes, past exposure ___ 3. No ____
G. Insecticides or herbicides?	1. Yes, presently exposed ___ 2. Yes, past exposure ___ 3. No ____
H. Capacitor, transformer or hydraulic fluids, or heat transfer chemicals?	1. Yes, presently exposed ___ 2. Yes, past exposure ___ 3. No ____

MDPH Environmental Epidemiology

Clinic Information Form

SOME OF THE TESTS WE WILL BE CONDUCTING TODAY MAY BE AFFECTED BY SUCH THINGS AS DRUGS AND MEDICINES YOU MAY BE TAKING, ILLNESSES OR INFECTIONS YOU MAY HAVE OR ALCOHOL CONSUMPTION. FOR THIS REASON WE ASK THAT YOU COMPLETE THE FOLLOWING QUESTIONS.

1. Do you have any infections such as a cold, boils or the flu? 1. Yes _ 2. No _ 3. DK _

IF YES:

A. What is the infection or illness? _____

B. When did it start? _____

2. Have you had any other illnesses in the past week? 1. Yes _ 2. No _ 3. DK _

IF YES:

What was the illness? _____

3. Are you currently having any trouble with an allergy, such as hay fever, asthma or hives? 1. Yes _ 2. No _ 3. DK _

IF YES:

What allergy is bothering you? _____

4. Have you consumed any alcohol in the last seven days? 1. Yes _ 2. No _ 3. DK _

IF YES:

In the last 7 days, how many drinks of each of the following types of liquor did you drink?

	NUMBER
a) 12 oz. glasses of beer	1. ___ 2. NA __ 3. DK __
b) 4 oz. glasses of wine	1. ___ 2. NA __ 3. DK __
c) mixed drinks or shots containing 1 1/2 ozs. of liquor	1. ___ 2. NA __ 3. DK __

5. Have you taken any prescription medicines for more than 2 weeks during the past year? 1. Yes _ 2. No _ 3. DK _

IF YES:

Medication Taken	Amount Per Day	Last Date Taken
_____	_____	_____
_____	_____	_____
_____	_____	_____

_____

6. Have you taken any over-the-counter drugs or home remedies for more than two weeks during the past year? 1. Yes _ 2. No _ 3. DK _

IF YES:

Drug or Remedy	Amount Per Day	Last Date Taken

7. Have you taken any medicines or drugs in the last two days for:

A. **Headache?** 1. Yes _ Drug _____ 2. No __ 3. DK _

B. **Sleep?** 1. Yes _ Drug _____ 2. No __ 3. DK _

C. **Birth Control?** 1. Yes _ Drug _____ 2. No __ 3. DK _

D. **Infection?** 1. Yes _ Drug _____ 2. No __ 3. DK _

E. **Blood Condition?** 1. Yes _ Drug _____ 2. No __ 3. DK _

F. **Nerves?** 1. Yes _ Drug _____ 2. No __ 3. DK _

8. Have you taken any other medicines or drugs in the past few days? 1. Yes _ 2. No _ 3. DK _

IF YES:

What medicine or drugs have you taken? _____

9. Do you take vitamins? 1. Yes _ 2. No _ 3. DK _

IF YES:

Type of Vitamin	Amount Per Day	Last Date Taken

THE REMAINING QUESTIONS ARE FOR WOMEN ONLY:

10. When did your last menstrual period begin? _____ month/day/year
Not Applicable _

11. Do you use birth control pills? 1. Yes __ 2. No __ 3. N/A __ 4. DK __

IF YES:

Brand of Birth Control Pill	Amount Per Day	Last Date Taken

12. Do you use female sex hormones other than birth control pills? 1. Yes ___ 2. No ___ 3. NA ___ 4. DK ___

IF YES:

Brand of Sex Hormone Pill	Amount Per Day	Last Date Taken
_____	_____	_____
_____	_____	_____
_____	_____	_____

When did you last have anything to eat or drink besides water.

NUMBER OF HOURS: 12 or more ____
8 - 11 ____
7 or less ____

APPENDIX II

Individual Human Subject Health Information

APPENDIX II
Individual Human Subject Health Information

Male - Low PBB Level

MSU#	CDC #	Birthdate	Age	Kidney Disease	Liver Disease	Diet Restrictions	Drinking Frequency
60	32039	4/14/34	47	no	no	no	no-3
04	32053	8/25/30	51	no	no	no	no-3
41	32099	5/09/17	64	no	no	yes	yes-2
19	32101	2/15/34	48	no	no	no	no-3
40	32104	11/18/21	60	no	hepatitis, 1944	no	no-3
21	32106	12/24/42	39	no	no	no	no-3
48	32109	5/15/38	42	no	no	no	no-3
07	32131	12/12/58	23	no	no	hypoglycemia	yes-1
46	32132	12/25/60	21	no	no	no	yes-1
05	32133	1/12/52	30	no	no	no	no-3
66	32135	7/06/48	33	no	no	no	yes-3
44	32137	2/23/32	50	no	no	no	no-3
20	32140	2/05/22	60	no	no	no	no-3
55	32155	5/10/32	49	no	no	no	no-3
56	32230	6/19/28	54	no	no	lose weight	no-3
22	32231	3/29/49	33	no	no	no	no-3
67	32233	6/15/12	69	no	no	no	no-2
57	32244	3/04/61	21	no	no	no	no-3
25	32229	3/21/48	33	no	no	lose weight	no-3

Male - Low PBB Level (Cont.)

MSU#	Smoking	Medications	PBB Level Last two Determinations		PCB Level Last two Determinations	
60	quit at 47	none	.001	.002	.007	.021
04	smokes pipe	Contact	.003	.003	.006	.005
41	yes	see chart	.002	.001	.006	.008
19	quit at 23	Tagamet	.001	.001	.007	.009
40	no	metahydrian	.002	.002	.007	.019
21	no	Tacaryl	.002	.004	,003	.006
48	cigars, quit at 30, never inhaled	Keflex aspirin	.002	.003	.005	.008
07	yes	none	.002	.003	.007	.006
46	no	aspirin	.001	.002	.003	.005
05	yes	none	.002	.003	.005	.005
66	yes	none	.003	.004	.005	.005
44	quit at 48	Flexeril, can't read some, aspirin	.002	.002	.005	.006
20	yes	none	.003	.004	.005	.011
55	no	none, vitamins	.003	.003	.006	--
56	no	none, vitamins	.005	.004	.005	--
22	no	aspirin	.001	.002	.006	--
67	quit at 55	Inderal, Halotestin, Dyazide, aspirin, vitamins	.003	.003	.006	--
57	no	Chlor-Trimeton	.003	.004	.005	--
25	no	can't read	.001	.002	.005	.011

Male - High PBB Level

MSU#	CDC #	Birthdate	Age	Kidney Disease	Liver Disease	Diet Restrictions	Drinking Frequency
62	32005	5/22/33	48	no	no	no	no-3
70	32007	9/26/61	20	no	no	no	no-3
72	32010	5/18/47	34	no	no	no	yes 10/wk
18	32021	12/06/29	52	no	no	no	yes 2/wk
33	32022	10/30/28	52	no	no	low salt	no
17	32043	3/24/16	65	no	no	no	no
35	32044	12/20/12	69	no	no	low sugar	yes-2
02	32046	7/04/60	21	no	no	no	yes
32	32084	8/09/62	20	no	no	no	no-2
31	32086	6/05/14	67	no	no	no	no-3
42	32088	2/09/54	28	no	no	no	no-3
14	32094	11/21/20	61	no	no	no	no-3
59	32095	3/02/18	64	no	no	no	no-3
50	32097	8/26/23	58	no	no	no	no-3
01	32177	2/07/20	62	no	no	no	yes-1
47	32178	3/26/24	58	no	no	no	no-3
63	32031M (32175)	4/20/45	36	no	no	no	no-3
26	32253	1/22/33	49	no	no	no	yes-1
24	32265	2/11/17	65	no	no	no	no-3
39	32071	5/11/31	50	no	no	no	yes-1
53	32243	10/21/39	42	no	no	no	yes-1

Male - High PBB Level (Cont.)

MSU#	Smoking	Medications	PBB Level Last two Determinations		PCB Level Last two Determinations	
62	no	Bufferin	1.21	1.93	.007	.01
70	no	tetracycline	.54	.97	.003	.015
72	cigars, does not inhale	none	.14	.15	.001	.008
18	quit at 21	vitamins	.037	.05	.008	.002
33	no	Thiuretic	.18	.18	.006	.007
17	no	can't read vitamins	.21	.28	.008	.006
35	quit at 56	Insulin, unable to read others Tylenol	.057	.051	.006	.007
02	no	none	.18	.23	.007	.009
32	no	none	.016	.053	.002	--
31	no	thyroid preparat'n Bufferin daily	.10	.19	.008	.005
42	quit at 21	can't read	.017	.087	.008	.007
14	yes	Lanoxin	.12	.11	.008	.009
59	no	antibiotics	.028	.053	.005	.012
50	quit at 45	none	.198	.21	.01	.01
01	yes	aspirin	.061	.065	.009	.012
47	no	none; vitamins, protein supplement	.12	.14	.01	.01
63	no	none	1.24	1.4	.007	.009
26	smokes pipe	aspirin	.24	.22	.015	.015
24	no	insulin	.18	.16	.12	.12
39	yes	Tuberculin	.051	.140	.006	.014
53	no	antibiotics	.23	.20	.011	.01

APPENDIX III

Glossary of Terms

Appendix III

Glossary of Terms

FID	Flame ionization detector
GC	Gas chromatography
GC/MS/DS	Gas chromatograph/mass spectrometer/data system
mass chromatogram (reconstructed mass chromatogram)	A plot of ion intensity vs. scan number generated from GC/MS/DS data base consisting of consecutively recorded mass spectra
3-MC	3-methylcholanthrene
MFO	Mixed function oxidase
PB	Phenobarbital
PBBs	Polybrominated biphenyls
PCBs	Polychlorinated biphenyls
Repetitive scanning mode	Mode of operation of a mass spectrometer whereby complete mass spectras are recorded sequentially
SIM	Selected ion monitoring. Mode of operation of a mass spectrometer whereby instrument is dedicated to recording (monitoring) ion current at only one or a few m/z values

LITERATURE CITED

LITERATURE CITED

AHOTUPA, M., HARTIALA, K. AND AITIO, A.: Effects of tetraethyl lead on the activities of drug metabolizing enzymes in different tissues of the rat. Acta Pharmacol. et Toxicol. 44: 359-363, 1979.

AITIO, A., AHOTUPA, M. AND PARKKI, M.G.: Inhibition of drug metabolizing enzymes by heavy metals *in vitro*. Biochem. Biophys. Res. Comm. 83: 850- 856, 1978.

ALDERCRUETZ, H., MARTIN, F. AND LINDSTROM, B.: Gas chromatographic and mass spectrometric studies on oestrogens in bile - 2. Man and non-pregnant women. J. Steroid Biochem. 9: 1197-1205, 1978.

ALLEN, J.R. AND BARSOTTI, D.A.: The effects of transplacental and mammary movement of TCBs on infant Rhesus monkeys. Toxicology 6: 331-340, 1976.

ALLEN, J.R., LAMBRECHT, L.K. AND BARSOTTI, D.A.: Effects of polybrominated biphenyls in nonhuman primates. J. Amer. Vet. Med. Assn. 173: 1485-1489, 1978.

ARNERIC, S.P., McCORMACK, K.M., BRASELTON, W.E. Jr. AND HOOK, J.B.: Altered metabolism of progesterone by hepatic microsomes from rats following dietary exposure to polybrominated biphenyls. Toxicol. Appl. Pharmacol. 54: 187-196, 1980.

AXELSON, M. AND SJOVALL, J.: Analysis of unconjugated steroids in plasma by liquid-gel chromatography and glass capillary mass spectrometry. J. Steroid Biochem. 8: 683-692, 1977.

BAILEY, E., FANOUGHTY, M. AND CHAPMAN, J.R.: Evaluation of a gas-liquid chromatographic method for determination of urinary steroids using high-resolution open-tubular glass capillary columns. J. Chromatogr. 96: 33-46, 1974.

BARSOTTI, D.A. AND ALLEN, J.R.: Effects of polychlorinated biphenyls on reproduction in the primate. Fed. Proc. 34: 338, 1975.

BATY, J.D. AND WADE, A.P.: Analysis of steroids in biological fluids by computer-aided gas-liquid chromatography-mass spectrometry. Anal. Biochem. 57: 27-37, 1974.

BEGUE, R.J., MORINIERE, M. AND PADIEU, P.: Urinary excretion of 5β-pregnane-3α,6α,21α-triol in human gestation. J. Steroid Biochem. 9: 779-784, 1978.

BERMAN, M.I. AND GREEN, O.C.: Acute stimulation of cotrisol metabolism by pentobarbital in man. Anesthesiology 34: 365-369, 1971.

BICKERS, D.R., KAPPAS, A. AND ALVARES, A.P.: Difference in inducibility of cutaneous and hepatic drug metabolizing enzymes an ctychrome P450 by polychlorinated biphenyls and 1,1',1'-tricholoro-2-bis(p-chlorphenyl) ethane (DDT). J. Pharmacol. Exp. Ther. 188: 300-309, 1974.

BIRNBAUM, L.S., BAIRD, M.B. AND MASSIE, H.R.: Pregnenolone-16α-carbonitrille-inducible cytochrome P450 in rat liver. Red. Comm. Chem. Pathol. Pharmacol. 15: 553-562, 1976.

BITMAN, J. AND CECIL, G.C.: Estrogenic activity of DDT analogs and polychlorinated biophenyls. J. Agr. Food Chem. 18: 1108-1112, 1970.

BLUMBERG, W.E.: Enzymic modification of environmental intoxicants: the role of cytochrome P-450. Quarterly Reviews of Biophysics 2: 452-482, 1978.

BONHAUS, D.W., McCORMACK, K.M., BRASELTON, W.E. Jr. AND HOOK, J.B.: Effect of dietary exposure to polybrominated biphenyls on the microsomal metabolism of estrogens and the uterotropic action of administered estrogens in rats. J. Toxicol. Environ. Hlth,

BRADLOW, H.L.: Extraction of steroid conjugates with a neutral resin. Steroids 11: 265-272, 1968.

BURTON, G.V. AND MEIKLE, A.W.: Partial adrenal insufficiency following chronic methylmercury poisoning. Clin. Res. 25: 145a, 1977.

CHAMBERLAIN, J.: The determination of urinary 6-oxygenaged cortisol in evaluating liver function. Clin. Chem. Acta 34: 269-271, 1971.

CHANDLER, J.A., TIMMS, G.B., NORTON, M.S. AND GROOM, G.V.: Effects of cadmium administration in vivo on plasma testosterone and the ultrastructure of accessory sex organs of the rat. J. Endocrinol. 69: 21p, 1976.

COHEN, S.L., HO, P., SUZUKI, Y. AND ALSPECTOR, F.E.: The preparation of pregnancy urine for an estrogen profile. Steroids 32: 279-295, 1978.

CONNEY, A.H.: Environmental factors influencing drug metabolism. In: "Fundamental of Drug Metabolism and Distribution." LaDu, B.N., Mandel, H.G. and Way, E.L. Eds., Williams and Wilkins Company, Baltimore, pp. 253-278, 1971.

CONNEY, A.H., LEVIN, W., JACOBSON, M. AND KUNTZMAN, R.: Effects of drugs and environmental chemicals on steroid metabolism. Clin. Pharm. Ther. 14: 727-741, 1973.

CONNEY, A.H.: Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev. 19: 317-366, 1976.

CRONHOLM, T., ERIKSSON, H., AND GUSTAFSSON, J.-A.: Excretion of endogenous steroids and metabolites of [4-C14]pregnenolone in bile of female rats. Eur. J. Biochem. 19: 424-432, 1971.

DANIILESCU-GOLDINBERG, D., AND GIROUD, C.J.P.: Metabolism of cortisol by the human newborn infant. J. Clin. Endocrinol. Metab. 38: 64-70, 1974.

DANNAN, G.A., AND AUST, S.D.: "Structural requirements for the microsomal metabolism of polybrominated biphenyl congenres", manuscript in preparation.

DANNAN, G.A., MOORE, R.W., AND AUST, S.D.: Studies on the microsomal metabolism and binding of polybrominated biphenyls (PBBs). Environ. Health Perspect. 23: 51-61, 1978.

DANNAN, G.A., MOORE, R.W., BESAW, L.C., AND AUST, S.D.: 2,4,5,3',4',5'-Hexabromobiphenyl is both a 3-methylcholanthrene- and a phenobarbital-type inducer of microsomal drug metabolizing enzymes. Biochem. Biophys. Res. Commun. 85: 450-458, 1978.

DENT, J.G., NETTER, K.J., AND GIBSON, J.E.: Effects of chronic administration of polybrominated biphenyls on parameters associated with hepatic drug metabolism. Res. Comm. Chem. Pathol. Pharmacol. 13: 75-82, 1976a

DENT, J.G., NETTER, K.J. AND GIBSON, J.E.: The induction of hepatic microsomal metabolism in rats following acute administration of a mixture of polybrominated biphenyls. Toxicol. Appl. Pharmacol. 38: 237-249, 1976b.

DERR, S.K.: In vivo metabolism of exogenous progesterone by PCB-treated female rats. Bull. Contam. Toxicol. 19: 729-732, 1978.

DE WEERDT, G.A., BEKE, R., VERDIEVEL, H. AND BARBIER, F: Quantitative determination of fecal bile acids as their methyl esters by the repetitive scan technique. Biomed. Mass Spectrom. 7: 515-520, 1980.

DUNCKEL, A.E.: An updating of the polybrominated biphenyl disaster in Michigan. J. Amer. Vet. Med. Assoc. 167: 838-841, 1975.

ERIKSSON, H.: Steroids in germ-free and conventional rats. Unconjugated metabolites of [4-C14]pregnenolone and [4-C14]corticosterone in faeces from female rats. Eur. J. Biochem. 16: 261-267, 1970.

ERIKSSON, H.: Absorption and enterohepatic circulation of neutral steroids in the rat. Eur. J. Biochem. 19: 416-423, 1971a.

ERIKSSON, H.: Steroids in germ-free and conventional rats. Metabolites of [4-C14]pregnenolone and [4-C14]corticosterone in urine and faeces from male rats. Eur. J. Biochem. 18: 86-93, 1971b.

ERIKSSON, H. AND GUSTAFSSON, J.-A.: Excretion of steroids in adults. Steroids in urine from pregnant women. Eur. J. Biochem. 16: 268-277, 1970.

ERIKSSON, H. AND GUSTAFSSON, J.-A.: Metabolism of corticosterone in the isolated perfused rat liver. Eur. J. Biochem. 20: 231-236, 1971.

ERIKSSON, H. AND GUSTAFSSON, J.-A.: Steroids in germ-free and conventional rats. Distribution and excretion of labeled pregnenolone and corticosterone in male and female rats. Eur. J. Biochem. 15: 132-139, 1970a.

ERIKSSON, H. AND GUSTAFSSON, J.-A.: Steroids in germ-free and conventional rats. Steroids in the mono- and disulfate fraction of faeces from female rats. Eur. J. Biochem. 16: 252-260, 1970b.

ERIKSSON, H. AND GUSTAFSSON, J.-A.: Steroids in germ-free and conventional rats. Sulpho- and glucuronohydrolase activities of caecal contents from conventional rats. Eur. J. Biochem. 13: 198-202, 1970c.

ERIKSSON, H., GUSTAFSSON, J.-A., AND POUSETTE, A.: Metabolism of androstenedione and progesterone in the isolated perfused rat liver. Eur. J. Biochem. 27: 327-334, 1972.

ERIKSSON, H., GUSTAFSSON, J.-A., AND SJOVALL, J.: Steroids in germ-free and conventional rats. 21-Dehydroxylation by intestinal microorganisms. Eur. J. Biochem. 9: 550-554, 1969.

ERIKSSON, H., GUSTAFSSON, J.-A., AND SJOVALL, J.: Studies on the structure, biosynthesis and bacterial metabolism of 15-hydroxylated steroids in the female rat. Eur. J. Biochem. 19: 433-441, 1971.

FANTL, V. AND GRAY, C.H.: Automated urinary steroid profiles by capillary column gas-liquid chromatography and a computer integrator. Clin. Chem. Acta 79: 237-253, 1977.

FRANTZ, A.G., KATZ, F.H., AND JAILER, J.W.: 6-Beta-hydroxy-cortisol high levels in human urine in pregnancy and toxemia. Proc. Soc. Exp. Biol. Med. 105: 41-43, 1960.

FRANTZ, A.G., KATZ, F.H., AND JAILER, J.W.: 6β-hydroxy-cortisol and other polar corticosteroids: Measurement and significance in human urine. J. Clin. Endocrinol. 21: 1290-1303, 1961.

FRIES, G.E. AND MARROW, G.S.: Excretion of polybrominated biphenyls into milk of cows. J. Dairy Sci. 58: 947-951, 1975.

GARDINER, W.L. AND HORNING, E.C.: Gas-liquid chromatographic separation of C-19 and C-21 human urinary steroids by a new procedure. Biochem. Biophys. Acta 115: 524, 1966.

GATES, S.C., YOUNG, N.D., HOLLAND, J.F., AND SWEELEY, C.C.: Computeraided qualitative analysis of complex biological fluids by combined gas chromatography-mass spectrometry. In Advances in Mass Spectrometry in Biochemistry and Medicine. A. Frigerio and N. Gastagnoli, Eds., Spectrum Publications, New York, 1976, pp. 483-495.

GATES, S.C., YOUNG, N.D., HOLLAND, J.F., AND SWEELEY, C.C.: Aumated multicomponent analysis of biological mixtures by gas chromatography-mass spectrometry. Ibid., vol. 2, pp. 171-181.

GATES, S.C., SMISKO, M.J., ASHENDEL, C.L., YOUNG, N.D., HOLLAND, J.F., AND SWEELEY, C.C.: Automated simultaneous qualitative and quantitative analysis of complex organic mixtures with a gas chromatography-mass spectrometry-computer system. Anal. Chem. 50: 433-441, 1978a.

GATES, S.C., DENDRAMIR, N., AND SWEELEY, C.C.: Automated metabolic profiling of organic acids in Human Urine I. Description of Methods. Clin. Chem. 24: 1674-1679, 1978b.

GATES, S.C., SWEELEY, C.C., KRIVIT, W., DeWITT, D., AND BLAISDELL, B.E.: Automated metabolic profiling of organic acids in human urine. II. Analysis of urine samples from "healthy" adults, sick children, and children with neuroblastoma. Clin. Chem. 24: 1680-1698, 1978c.

GATES, S.C. AND SWEELEY, C.C.: Quantitative metabolic profiling based upon gas chromatography. Clin. Chem. 24: 1663-1673, 1978d.

GELLERT, R.J.: Uterotrophic activity of polychlorinated biphenyls (PCB) and induction of precocious reproductive aging in neonatally treated female rats. Environ. Res. 16: 123-130, 1978.

GERMAN, A.L. AND HORNING, E.L.: Thermostable open tube capillary columns for the high resolution gas chromatography of human urinary steroids. J. Chromatogr. Sci. 11: 76-82, 1973.

GRADY, R.R., KITAY, J.I., SPYKER, J.M. AND AVERY, D.L.: Postnatal endocrine dysfunction induced by prenatal methylmercury or cadmium exposure in mice. J. Environ. Pathol. Toxicol. 1: 187-197, 1978.

GUNSALUS, I.C., PEDERSON, T.C., AND SLIGAR, S.G.: Oxygenase-catalyzed biological hydroxylations. Ann. Rev. Biochem. 44: 377-407, 1975.

GUSTAFSSON, B.E., GUSTAFSSON, J.-A., AND SJOVALL, J.: Steroids in germ-free and conventional rats. 2. Identification of 3α-,16α-dihydroxy-5α-pregnan-20-one and related compounds in faeces from germ-free rats. Eur. J. Biochem. 4: 568-573, 1968a.

GUSTAFSSON, B.E., GUSTAFSSON, J.-A., AND SJOVALL, J.: Steroids in germ-free and conventional rats. 3. Solvolyzable sterol conjugates in germ-free and conventional rat faeces. Eur. J. Biochem. 4: 574-577, 1968b.

GUSTAFSSON, J.-A.: Steroids in germ-free and conventional rats. 7. Identification of C-19 and C-21 steroids in faeces from conventional rats. Eur. J. Biochem. 6: 248-255, 1968.

GUSTAFSSON, J.-A.: Steroids in germ-free and conventional rats. Steroid monosulphates in urine from female rats. Eur. J. Biochem. 14: 560-566, 1970.

GUSTAFSSON, J.-A. AND LISBOA, B.P. Studies on the metabolism of C-21 steroids in rat liver. Hydroxylation of progesterone in rat liver microsomes. Eur. J. Biochem. 15: 525-530, 1970.

GUSTAFSSON, J.-A. AND LISBOA, B.P.: Studies on the metabolism of C-19 steroids in rat liver. 6α-Hydroxylation of 3-oxo-Δ^4-steroids in rat liver microsomes. Eur. J. Biochem. 12: 369-374, 1970a.

GUSTAFSSON, J.-A. AND LISBOA, B.P.: Studies on the metabolism of C-19 steroids in rat liver. Biosynthesis of saturated 17-oxo-C-19,O-3 steroids in rat liver microsomes. Eur. J. Biochem. 16: 475-480, 1970b.

GUSTAFSSON, J.-A. AND LISBOA, B.P.: Studies on the metabolism of C-19 steroids in rat testis. 6α- and 6β-hydroxylation of testosterone in rat testis preparations. Eur. J. Biochem. 13: 554-557, 1970c.

GUSTAFSSON, J.-A. AND SJOVALL, J.: Steroids in germ-free and conventional rats. 5. Identification of C-19 steroids in faeces from germ-free rats. Eur. J. Biochem. 6: 227-235, 1968a.

GUSTAFSSON, J.-A. AND SJOVALL, J.: Steroids in germ-free and conventional rats. 6. Identifiaction of 15α- and 21-hydroxylated C-21 steroids in faeces from germ-free rats. Eur. J. Biochem. 6: 236-247, 1968b.

GUSTAFSSON, J.-A., LISBOA, B.P., AND SJOVALL, J.: Studies on the metabolism of C-19 steroids in rat liver. Eur. J. Biochem. 5: 437-443, 1968a.

GUSTAFSSON, J.-A., LISBOA, B.P., AND SJOVALL, J.: Studies on the metbolism of C-19 steroids in rat liver. Eur. J. Biochem. 6: 317-324, 1968b.

HADLEY, W.M., MIYA, T.S. AND BOUSQUET, W.F.: Cadmium inhibition of hepatic drug metabolism in the rat. Toxicol. Pharmacol. 28: 284-291, 1974.

HAEGELE, M.A. AND TUCKER, R.K.: Effects of 15 common environmental pollutants on eggshell thickness in mallards and coturnix. Bull. Environ. Contam. Toxicol. 11: 98-102, 1974.

HARBISON, R.D. AND DIXON, R.L.: (Conference Organizers), Proceedings of symposium on target organ toxicity: Gonads (reproductive and genetic toxicity). Environ. Hlth. Perspect. 24: 1-128, 1978.

HARRIS, S.J., CECIL, H.C. AND BITMAN, J.: Embryotoxic effects of polybrominated biphenyls (PBBs) in rats. Environ. Hlth. Perspect. 23: 295-300, 1978.

HITES, R.A. AND BIEMANN, K.: Computer evaluation of continuously scanned mass spectra of gas chromatographic effluents. Anal. Chem. 42: 885-860, 1970.

HONOUR, J.W., TOURNIAIRE, J., BIGLIERT, E.G. AND SHACKLETON, C.H.L.: Urinary steroid excretion in 17α-hydroxylase deficiency. J. Steroid Biochem. 9: 495-505, 1978.

HORNING, E.C. AND HORNING, M.G.: Metabolic profiles: Gas-phase methods for analysis of metabolites. Clin. Chem. 17: 802-809, 1971a.

HORNING, E.C. AND HORNING, M.G.: Human metabolic profiles obtained by GC and GC/MS. J. Chromatogr. Sci. 9: 129-140, 1971b.

HORNING, E.C., HORNING, M.G., SZAFRANEK, J., VAN HOUT, P., GERMAN, A.L., THENOT, J.P. AND PFAFFENBERGER, C.D.: Gas phase analytical methods for the study of human metabolites. Metabolic profiles obtained by open tubular capillary chromatography. J. Chromatogr. 91: 367-378, 1974.

HORNING, J.W., TOURNIAIRE, J., BIGLIERT, E.G., AND SHACKLETON, C.H.L.: Urinary steroid excretion in 17α-hydroxylase deficiency. J. Steroid Biochem. 9: 495-505, 1978.

JACKSON, T.F. AND HALBERT, F.S.: A toxic syndrome associated with the feeding of polybrominated biphenyls contaminated protein concentrate to dairy cattle. J. Am. Vet. Med. Assoc. 65: 437-439, 1974.

JOHNSTON, C.A., DEMAREST, K.T., McCORMACK, K.M., HOOK, J.B., AND MOORE, K.E.: Endocrinological, neurochemical and anaboic effects of PBBs in male and female rats. Toxicol. Appl. Pharmacol., 1980.

JURIGSKAY, Z. AND KECSKES, L.: Steroid spectrum in human urine as revealed by gas chromatography. I. Quantitative analysis of C -C O steroids. Acta Biochim. et Biophys. Acad. Sci. Hung. 13: 279-291, 1978.

KATZ, F.H., LIPMAN, M.M., FRANTZ, A.G., AND JAILER, J.W.: The physiologic significance of 6β-hydroxycortisol in human corticoid metabolism. J. Clin. Endocrino. Metab. 22: 71-77, 1962.

KLINGENBERG, M.: Pigments of rat liver microsomes. Arch. Biochem. Biophys. 75: 376-386, 1958.

KOHLI, J., AND SAFE, S.: The metabolism of brominated aromatic compounds. Chemosphere 5: 433-437, 1976.

KOHLI, J., WYNDHAM, C., SMYLIE, M., AND SAFE, S.: Metabolism of bromobiphenyls. Biochem. Pharmacol. 27: 1245-1249, 1978.

KRASNY, H.C. AND HOLBROOT, D.J.: Effects of cadmium on microsomal hemoproteins and heme oxygenase in rat liver. Mol. Pharmacol. 13: 759-765, 1977.

KURATSUNE, T., YOSHIMURA, T., MATSUGAKA, J. AND YAMAGUCHI, A. Epidemiologic study on Tusho, a poisoning caused by ingestion of rice oil contaminated with a commercial bi and polychlorinated biphenyl. Environ. Hlth. Perspect. 1: 119-128, 1972.

LAMBRECHT, L.K., BARSOTTI, D.A. AND ALLEN, J.R.: Responses of nonhuman primates to a polybrominated biphenyl mixture. Environ. Hlth. Perspect. 23: 139-146, 1978.

LANCRANJAN, J.: Reproductive ability of workmen occupationally exposed to lead. Arch. Environ. Hlth. 30: 396-401, 1975.

LEPAGE, R.: Michigan State Univ., personal communication.

LEUNISSEN, W.J.J. AND THIJSSEN, J.H.H.: Quantitative analysis of steroid profiles from urine by capillary gas chromatography. I. Accuracy and reproducibility of sample preparation. J. Chromatogr. 146: 365-380, 1978.

LISBOA, B.P., GUSTAFSSON, J.-A., AND SJOVALL, J.: Studies on the metabolism of C-19 steroids in rat liver. 1. Hydroxylation of testosterone in rat liver microsomes. Eur. J. Biochem. 4: 496-505, 1968.

LU, A.Y.H., SOMOGYI, A., WEST, S., KUNTZMAN, R., AND CONNEY, A.H.: Pregnenolone-16α-carbonitrile: A new type of inducer of drug-metabolizing enzymes. Arch. Biochem. Biophys. 152: 457-462, 1972.

LUCIER, G.W., LEE, I.P., AND DIXON, R.L.: Effects of environmental agents on male reproduction. In Johnson, A.D. and Gomes, W.R.: The Testis. Advances in Physiology, Biochemistry, and Function, Vol. IV, Academic Press, Inc., New York, 1977, pp. 578-598.

LUDWIG, H., SPITELLER, M., EGGER, H.J., AND SPITELLER, G.: Correlation of emotional stress and physical exertion with urinary metabolic profiles. Israel J. Chem. 16: 7-11, 1977.

LUYTEN, J.A. AND RUTTEN, G.A.F.M.: Analysis of steroids by high resolution gas liquid chromatography. Application to urinary samples. J. Chromatogr. 91: 393-406, 1974.

MAKIN, H.L.J.: "Biochemistry of Steroid Hormones." Blackwell Scientific Publications, Osney Mead, Oxford, 1975.

MARSH, P.G. AND FENNESSEY, P.V.: Detection and quantitation of steroid levels. Proc. West. Pharmacol. Soc. 22: 91-96, 1979.

MARTIN, F., JOHNSON, S., McPHERSON, M. AND HOLLAND, J.F.: Dual processor foreground/background GC/MS data system. 28th Annual Conference on Mass Spectrometry and Allied Topics, MPMP22, New York, May 1980.

MASON, H.S.: Mechanisms of oxygen metabolism. Adv. Enzymol. 19: 79-233, 1957.

MASON, J.I., ESTABROOK, R.W., AND PURVIS, J.L.: Testicular cytochrome P450 and iron-sulfur protein as related to steroid metabolism. Ann. N.Y. Acad, Sci. 212: 406-419, 1973.

MATTHEWS, H.B., KATO, S., MORALES, N.M., AND TUEY, D.B.: Distribution and excretion of 2,4,5,2',4',5' hexabromobiphenyl, the major component of Firemaster BP-6. J. Toxicol. Environ. Health 3: 599-605, 1977.

MATTHEWS, H., FRIES, G., GARDNER, A., GARTHOFF, L., GOLDSTEIN, J., KU, Y., AND MOORE, J.: Metabolism and biochemical toxicity of PLBs and PBBs. Environ. Health Perspect. 24: 147-155, 1978.

MAUME, B.F., MILLOT, C., MESNIER, D., PATOURAUX, D., DOUMAS, J., AND TOMORI, E.: Quantitative analysis of corticosteroids in adrenal cell cultures by capillary column gas chromatography combined with mass spectrometry. J. Chromatogr. 186: 581-594, 1979.

McCORMACK, K.M., KLUWE, W.M., RICKERT, D.E., SANGER, V.L., AND HOOK, J.B.: Renal and hepatic microsomal enzyme stimulation and renal function following three months exposure to polybrominated biphenyls. Toxicol. Appl. Pharmacol. 44: 539-553, 1978.

McCORMACK, K.M., ARNERIC, S.P., AND HOOK, J.B.: Action of exogenously administered steroid hormones following perinatal exposure to polybrominated biphenyls. J. Toxicol. Environ. Hlth. 5: 1085-1094, 1979.

McCORMACK, K.M., BRASELTON, W.E. Jr., SANGER, V.L., AND HOOK, J.B.: Residual effects of polybrominated biphenyls following perinatal exposure in rats. Toxicol. Appl. Pharmacol. 53: 108-115, 1980a.

McCORMACK, K.M., LEPPER, L.F., WILSON, D.M., AND HOOK, J.B.: Biochemical and physiological sequelae to perinatal exposure to polybrominated biphenyls: A multigeneration study in rats. Toxicol. Appl. Pharmacol.

McINTOSH, E.N., UZGIRIS, V.I., ALONSO, C.A., AND SALHANICK, H.A.: Spectral properties, respiratory activity and enzyme systems of bovine corpus luteum mitochondria. Biochemistry 10: 2909-2916, 1971.

McINTOSH, E.N., MITANI, F., UZGIRIS, V.I., ALONSO, C.A., AND SALHANICK, H.A.: Comparative studies on mitochondrial and partially purified bovine corpus luteum cytochrome P450. Ann. N.Y. Acad. Sci. 212: 392-403, 1973.

MEANS, J.R. AND SCHNELL, R.C.: Cadmium-induced alteration of the microsomal monooxygenase system in male rat liver: Effects on hemoprotein turnover and phospholipid content. Toxicology Letters 3: 177-184, 1979.

MEANS, J.R. AND SCHNELL, R.C.: Interaction between cadmium and phenobarbital on the activity of the hepatic microsomal monooxygenase system in the male rat. Res. Comm. Chem. Path. Pharm. 27: 187-190, 1980.

MEIGS, R.A. AND RYAN, K.J.: Cytochrome P450 and seroid biosynthesis in the human placenta. Biochim. Biophys. Acta 165: 476-482, 1968.

MEREDITH, P.A., CAMPBELL, B.C., MOORE, M.R. AND GOLDBERG, A.: The effects of industrial lead poisoning on cytochrome P450 mediated phenazone (antipyrine) hydroxylation. Europ. J. Clin. Pharmacol. 12: 235-239, 1977.

MOORE, R.W. AND AUST, S.D.: Purification and structural characterization of polybrominated biphenyl congeners. Biochem. Biophys. Res. Commun. 84: 936-942, 1978.

MOORE, R.W., DANNAN, G.A., AND AUST, S.D.: Structure-function relationships for the pharmacological and toxicological effects and metabolism of polybrominated biphenyl congeners. In: Molecular Basis of Environmental Toxicity (R.S. Bhatnager, ed.). Ann Arbor Science Publishers, Inc., Ann Arbor, Michigan, pp. 173-212, 1980.

MOORE, R.W., O'CONNOR, J.V., AND AUST, S.D.: Identification of a major component of polybrominated biphenyls as 2,2',3,4,4',5,5'-heptabromobiphenyl. Bull. Environ. Contam. Toxicol. 20: 478-483, 1978.

MOYER, T.P., CARPENTER, P.C., JIANG, N.-S., AND MACHACEK, D.: Series on clinical testing. 2. Fractionation of urinary ketosteroids: Procedures and clinical significance. Mayo Clin. Proc. 53: 601-606, 1978.

MURPHY, B.E.P. AND D'AUX, R.C.D.: The use of Sephadex LH-20 column chromatography to separate unconjugated steroids. J. Steroid Biochem. 6: 233-237, 1975.

NELSON, J.A.: Effects of dichlorodiphenyltrichloroethane (DTT) analogs and polychlorinated biphenyls (PCB) mixtures on 17β-estradiol-^{3}H binding to rat uterine receptor. Biochem. Pharmacol. 23: 447-451, 1974.

NEWTON, J.F., BRASELTON, W.E. Jr., LEPPER, L.F., McCORMACK, K.M. AND HOOK, J.B.: Effects of polybrominated biphenyls (PBBs) on the microsomal metabolism of testosterone. The Pharmacologist 22: 173, 1980.

NEWTON, J.F., BRASELTON, W.E. Jr., LEPPER, L.F., McCORMACK, K.M. AND HOOK, J.B.: Effect of polybrominated biphenyls on the reductive metabolism of testosterone by hepatic microsomes. Toxicol. Appl Pharmacol. 63: 142-149, 1982.

NOVOTNY, M. AND ZLATKIS, A.: High resolution chromatographic separation of steroids with open tubular glass columns. J. Chromatogr. Sci. 8: 347-350, 1970.

NOVOTNY, M., MASKARINEC, M.P., STEVERINK, A.T.G. AND FARLOW, R.: High resolution gas chromatography of plasma steroidal hormones and their metabolites. Anal. Chem. 48: 468-472, 1976.

NOWICKI, H.G. AND NORMAN, A.W.: Enchanced hepatic metabolism of testosterone, 4-androstene-3,17-dione, and estradiol-17β in chickens pretreated with DTT or PCB. Steroids 19: 85-99, 1972.

OHNHAUS, E.E. AND PARK, B.K.: Measurement of urinary 6β-hydroxycortisol excretion as an *in vivo* parameter in the clinical assessment of the microsomal enzyme-inducing capacity of antipyrine, phenobarbitone and rifampicin. Eur. J. Clin. Pharmacol. 15: 139-145, 1979.

OMURA, T., AND SATO, R.: A new cytochrome in liver microsomes. J. Biol. Chem. 237: 1375-1376, 1962.

OMURA, T. AND SATO, R.: The carbon monoxide binding pigment of liver microsomes. J. Biol. Chem. 239: 2370-2385, 1964.

ORBERG, J.: A comparison between some effects of two pure chlorobiphenyls (2,4',5-trichlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl) on the hepatic drug metabolizing system in the female mouse. Acta Pharmacol. et Toxicol. 39: 145-151, 1976.

ORBERG, J. AND INGVAST, C.: Effects of pure chlorobiphenyls (2,4',5-TCB and 2,2',4,4',5,5'-HCB) on the disappearance of ^{14}C from the blood plasma after intravenous injection of 4-^{14}C progesterone and on the hepatic drug metabolizing system in the female rat. Acta Pharmacol. et Toxicol. 41: 11-17, 1977.

ORBERG, J., JOHANSSON, N., KILHSTROM, J.E., AND LUNDBERG, C.: Administration of DTT and PCB prolongs oestrous cycle in mice. Ambio. 1: 148-149, 1972.

ORBERG, J. AND KIHLSTROM, J.E.: Effects of long-term feeding of polychlorinated biphenyls (PCB, Clophen A60) on the length of the oestrous cycle and on the frequency of implanted ova in the mouse. Environ. Res. 6: 176-179, 1973.

ORBERG, J. AND LUNDBERG, C.: Some effects of DDT and PCB on the hormonal system in the male mouse. Environ. Physiol. Biochem. 4: 116-120, 1974.

PFAFFENBERGER, C.D. AND HORNING, E.C.: High resolution biomedical gas chromatography. Determination of human urinary steroid metabolites using glass open tubular capillary columns. J. Chromatogr. 112: 581-594, 1975.

PFAFFENBERGER, C.D. AND HORNING, E.C.: Sex differences in human urinary steroid metabolic profiles determined by gas chromatography. Anal. Biochem. 80: 329-343, 1977.

PLANTONOW, W.S., LIPTRAP, R.M., AND GEISSINGER, H.D.: The distribution and excretion of polychlorinated biphenyls (Aroclor 1254) and their effect on urinary gonadal steroid levels in the boar. Bull. Environ. Contam. Toxicol. 7: 358-365, 1972.

POLAND, A., GREENLEE, W.F., AND KENDE, A.S.: Studies on the mechanism of action of the chlorinated dibenzo-p-dioxins and related compounds. Ann. N.Y. Acad. Sci. 320: 214-230, 1979.

POLAND, A., SMITH, D., KUNTZMAN, R., JACOBSON, M., AND CONNEY, A.H.: Effect of intensive occupational exposure to DDT on phenylbutazone and cortisol metabolism in human subjects. Clin. Pharmacol. Ther. 11: 724-732, 1970.

REIMENDAL, R. AND SJOVALL, J.: Analysis of steroids by off-line computerized gas chromatography-spectrometry. Anal. Chem. 44: 21-29, 1972.

REIMENDAL, R. AND SJOVALL, J.: Computer evaluation of gas chromatographic-mass spectrometric analysis of steroids from biological materials. Anal. Chem. 45: 1083-1089, 1973.

RICKERT, D.E., DENT, J.G., CAGEN, S.Z., McCORMACK, K.M., MELROSE, P., AND GIBSON, J.E.: Distribution of polybrominated biphenyls after dietary exposure in pregnant and lactating rats and their offspring. Environ. Hlth. Perspec. 23: 63-66, 1978.

RINGER, R.K. AND POLIN, D.: The biological effects of polybrominated biphenyls in avian species. Fed. Proc. 36: 1894, 1977.

ROOTS, I.: Effect of rifampicin on urinary excretion of 6β-hydroxycortisol and glucaric acid in man and animal. Naunyn-Schmiedeberg's Arch. Pharmacol. 307: R 72, 1979.

ROOTS, I., HOLBE, R., HOVERMANN, W., NIGAM, S., HEINEMEYER, G., AND HILDEBRANDT, A.G.: Quantitative determination of HPLC of urinary 6β-hydroxycortisol, an indicator of enzyme induction by rifampicin and antiepiteptic drugs. Eur. J. Clin. Pharmacol. 16: 63-71, 1979.

SAENGER, P., FORSTER, E., AND KREAM, J.: 6β-Hydroxycortisol: A noninvasive indicator of enzyme induction. J. Endocrinol. Met. 52: 381-384, 1981.

SAFE, S., JONES, D., AND HUTZINGER, O.: Metabolism of 4,4'-dihalogenobiphenyls. J. Chem. Soc. Perkin I, 357-359, 1976.

SANDERS, O.T., ZEPP, R.L., AND KIRKPATRICK, R.L.: Effect of PCB ingestion on sleeping times, organ weights, food consumption, serum corticosterone and survival of albino mice. Bull. Environ. Contam. Toxicol. 12: 394-399, 1974.

SANDERS, O.T. AND KIRKPATRICK, R.L.: Effects of a polychlorinated biphenyl (PCB) on sleeping times, plasma corticosteroids, and testicular activity of white-footed mice. Environ. Physiol. Biochem. 5, 308-313, 1975.

SCHROEDER, H.A. AND MITCHENER, M.: Toxic effects of trace elements on the reproduction of mice and rats. Arch. Environ. Hlth. 23: 102-106, 1971.

SCHULSTER, D., BURSTEIN, S., AND COOKE, B.A.: "Molecular Endocrinology of the Steroid Hormones." John Wiley and Sons, New York, 1976.

SETCHELL, K.D.R., ALME, B., AXELSON, M. AND SJOVALL, J.: The multicomponent analysis of conjugates of neutral steroids in urine by lipophilic ion exchange chromatography and computerized gas chromatography-mass spectrometry. J. Steroid Biochem. 7: 615-629, 1976.

SHACKELTON, C.H.L. AND MITCHELL, F.L.: The measurement of 3β-hydroxy-Δ5-steroids in human fetal blood, amniotic fluid, infant urine and adult urine. Steroids 10: 359-385, 1967.

SHACKELTON, C.H.L. AND WHITNEY, J.O.: Use of Sep-pak cartridges for urinary steroid extraction: Evaluation of the method for use prior to gas chromatographic analysis. Clin. Chem. Acta 107: 231-243, 1980.

SHACKELTON, C.H.L., SJOVALL, J., AND WISEN, O.: A simple method for the extraction of steroids from urine. Clin. Chem. Acta 27: 354-356, 1970.

SHACKELTON, C.H.L., GUSTAFSSON, J.-A., AND SJOVALL, J.: Steroids in newborns and infants. Identification of steroids in urine from newborn infants. Steroids 17: 265-280, 1971.

SHACKELTON, C.H.L. AND TAYLOR, N.F.: Identification of the androstene- triolones and androstenetetrols present in the urine of infants. J. Steroid Biochem. 6: 1393-1399, 1975.

SHACKLETON, C.H.L. AND HONOUR, J.W.: Simultaneous estimation of urinary steroids by semi-automated gas chromatography. Investigation of neonatal infants and children with abnormal synthesis. Clin. Chem. Acta 69: 267-283, 1976.

SJOVALL, J.: Analysis of steroids by liquid-gel chromatography and computerized gas chromatography-mass spectrometry. J. Steroid Biochem. 6: 227-232, 1975.

SJOVALL, J. AND AXELSON, M.: General and selective procedures for GC/MS analysis of steroids in tissues and body fluids. J. Steroid Biochem. 11: 129-134, 1979.

SLADEK, N.E. AND MANNERING, G.J.: Evidence for a new P-450 hemoprotein in hepatic microsomes from methylcholanthrene treated rats. Biochem. Biophys. Res. Comm. 24: 668-674, 1966.

SLADEK, N.E. AND MANNERING, G.J.: Induction of drug metabolism. I. Differences in the mechanism by which polycyclic hydrocarbons and phenobarbital produce their inductive effects on microsomal N-demethylating systems. Mol. Pharmacol. 5: 174-185, 1969a.

SLADEK, N.E. AND MANNERING, G.J.: Induction of drug metabolism. II. Qualitative differences in the microsomal N-demethylating systems stimulated by polycyclic hydrocarbons and by phenobarbital. Mol. Pharmacol. 5: 186-199, 1969b.

SPITELLER, G.: The language of biological fluids. Pure Appl. Chem. 50: 205-217, 1978.

STEVENSON, I.H., BROWNING, M., CROOKS, J., AND O'MALLEY, K.: Changes in human drug metabolism after long-term exposure to hypnotics. Br. Med. J. 4: 322-324, 1972.

SWEELEY, C.C., GATES, S.C., THOMPSON, H.R., et al.: Techniques for quantitative measurements by mass spectrometry. In: Quantitative Mass Spectrometry in Life Sciences, A.P. DeLeenheer and R.R. Roncucci, Eds., Elsevier, Amsterdam, 1977, pp. 29-48.

SWEELEY, C.C.: Quantitative metabolic profiling analysis with a gas chromatography-mass spectrometry computer system. Proc. Japanese Soc. Med. Mass Spectrom. 4: 15, 1979.

TAKAGI, Y., OTAKE, T., KATAOKA, M., MURATA, Y., ABURADA, S., AKASAK, S., HIMOTO, K., UDA, H., AND KITAWA, T.: Studies on the transfer and distribution of C-polychlorinated biphenyls from maternal to fetal and sucking rats. Toxicol. Appl. Pharmacol. 38: 549-558, 1976.

THENOT, J.P. AND HORNING, E.C.: Methoxime-trimethylsilyl ether derivatives of human urinary steroids for gas chromatography and gas chromatography-mass spectrometry studies. Anal. Letters 5: 21-33, 1972.

THRASHER, K., WERK, E.E., CHOI, Y., SHOLITON, L.J., MEYER, W., AND OLINGER, C.: The measurement, excretion and source of urinary 6-hydroxycortisol in humans. Steroids 14: 455-468, 1969.

VAN DE CALSEYDE, J.F., SCHOLTIS, R.J.H., SCHMIDT, N.A., AND LEIJTEN, C.J.J.A.: Clin. Chem. Acta 38: 103-111, 1972.

VILLENEUVE, D.C., GRANT, D.L., PHILLIPS, W.E.J., CLARK, M.L., AND CLEGG, D.J.: Effects of PCB administration on microsomal enzyme activity in pregnant rabbits. Bull. Environ. Contam. Toxicol. 6: 120-128, 1971a.

VILLENEUVE, D.C., GRANT, D.C., KHERA, K., CLEGG, D.J., BAER, H., AND PHILLIPS, W.E.J.: The fetotoxicity of a polychlorinated mixture (Aroclor 1254) in the rabbit and in the rat. Environ. Physiol. 1: 67-71, 1971b.

VOLLMIN, J.A.: High resolution gas chromatography of urinary steroids on glass capillary columns. Clin Chem. Acta 34: 207-214, 1971.

VRBANAC, J.J., BRASELTON, W.E. Jr., HOLLAND, J.F., AND SWEELEY, C.C.: Automated qualitative and quantitative metabolic profiling analysis of urinary steroids by a gas chromatography-mass spectrometry-data system. J. Chromatogr. 239: 265-276, 1982.

VRBANAC, J.J., PINKSTON, J.D., AND SWEELEY, C.C.: Automated metabolic profiling analysis of human urinary steroids by a gas chromatography-mass spectrometry-data system. Biomed. Mass Spectrom. 10: 155-161, 1983.

WASSERMAN, D., WASSERMAN, M., CUCOS, S., AND DJAVAHERIAN, M.: Function of adrenal gland-zona fasciculata in rats receiving polychlorinated biphenyls. Environ. Res. 6: 334-338, 1973.

WERK, E.E., Jr., MacGEE, J., AND SHOLITON, L.J.: Altered cortisol metabolism in advanced cancer and other terminal illnesses: Excretion of 6-hydroxycortisol. Metabolism 13: 1425-1438, 1964.

WIEKRAMASINGHE, R.H.: Biological aspects of cytochrome P450 and associated hydroxylation reactions. Enzyme 19: 348-376, 1975.

WILLIAMS, R.J.: "Individual Metabolic Patterns and Human Disease: An Exploratory Study Utilizing Predominantly Paper Chromatographic Methods", Univ. Texas Publication No. 5109, p. 7, 1951.

YAMAJI, T., MOTOHASHI, K., MURAKAWA, S., AND IBAYASHI, H.: Urinary excretion of 6β-hydroxycortisol in states of altered thyroid function. J. Clin. Endocrinol. Metab. 29: 801-806, 1969.