PROFILING OF URINARY METABOLITES IN HUMAN PREGNANCY

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in the

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by

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Saskatoon, Saskatchewan

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ABSTRACT

Maternal urinary metabolites (organic acids and steroids) in human pregnancy were profiled by gas-chromatographic methods. Urine specimens were collected from pregnant women at two time intervals in gestation (12½-15½ weeks and 24½-27½ weeks). A group of nonpregnant women was also investigated. The 24-hour urinary creatinine excretion was used as a check of the completeness of urine collections. Urinary organic acids were isolated by anion-exchange chromatography (DEAE-Sephadex A-25). Urinary steroids and steroid conjugates were isolated by adsorption onto a neutral resin (Amberlite XAD-2). Steroid conjugates were hydrolyzed by enzymes from the digestive juice of the snail, *Helix pomatia*. Purified urinary organic acid and steroid extracts were methoximated and trimethylsilylated for gas-chromatographic analysis. Urinary acid profiles contained 50 recognizable peaks, 10 of which were unequivocally identified by mass spectral analysis (lactic acid, glycolic acid, sulfate, phosphate, erythronic acid, threonic acid, hippuric acid, citric acid, glucuronic acid, and uric acid). Urinary steroid profiles contained 16 recognizable peaks, although only one peak was identified by mass spectral analysis (pregnanediol).

The metabolite profiles were assessed for effects of diet, drugs, and intestinal microflora. Twelve acidic metabolites were excreted in greater amounts by pregnant women compared to nonpregnant women. Four acidic metabolites were excreted in greatly increasing amounts as pregnancy progressed: lactic acid, glycolic acid, the 4-deoxytetronic acids (only tentatively identified), and erythronic acid. Whereas lactic acid excretion was highly variable, the excretion of glycolic acid, the 4-deoxytetronic

acids, and erythronic acid increased simultaneously. The possible significance of these alterations in the urinary acid profile in pregnancy was discussed. Organic acid excretion in several cases of high-risk pregnancy showed slight deviations from the normal profile.

Urinary steroid excretion increased conspicuously as pregnancy progressed. The steroid metabolites could not be accurately quantified due to a lack of resolution with the present gas-chromatographic system. The present method would prove useful when large deviations in steroid excretion were to be confirmed.

Urinary organic acid-steroid profiling may not find application in health assessment of the general population, but the technique holds exciting possibilities for the assessment of certain high-risk pregnancies. The present work provides reference profiles which form a basis for future comparisons.

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LIST OF ABBREVIATIONS

AFP	=	alphafetoprotein
BSA	=	body surface area
BSA	=	N,O-bis(trimethylsilyl)acetamide
BSTFA	=	N,O-bis(trimethylsilyl)trifluoroacetamide
BW	=	body weight
CV	=	coefficient of variation
DEAE	=	diethylaminoethyl
DHEA	=	dehydroepiandrosterone
DMDCS	=	dimethyldichlorosilane
FID	=	flame ionization detector
GC	=	gas chromatography
GC-MS	=	gas chromatography-mass spectrometry
h	=	hour
HCG	=	human chorionic gonadotropin
HPL	=	human placental lactogen
%I	=	normalized intensity
M	=	molecular ion
m/e	=	mass to charge ratio
MO	=	methoxime
MO-TMS	=	methoxime trimethylsilyl
MS	=	mass spectrometry
MSTFA	=	N-methyl-N-trimethylsilyltrifluoroacetamide
MU	=	methylene unit
MW	=	molecular weight
P	=	probability that an event is due to chance
pCO_2	=	carbon dioxide pressure
psi	=	pounds per square inch
r	=	correlation coefficient
RWR	=	relative weight response
SD	=	standard deviation
TMCS	=	trimethylchlorosilane
TMS	=	trimethylsilyl
TSIM	=	trimethylsilylimidazole

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1. INTRODUCTION

1.1 Human pregnancy

1.1.1 Maternal physiological adjustments

The physiological adaptations of pregnancy involve widespread, complex, and fundamental adjustments to virtually every maternal system. Hormones of the fetus and placenta (estrogens, progesterone, and human placental lactogen) are primarily responsible for the reorganization of the mother's homeostatic mechanisms (Page _et al._, 1976). Radical resetting of maternal hypothalamic control centers (Guyton, 1976) is reflected in the reduced plasma osmolality, increased body temperature, and lowering of plasma carbon dioxide pressure (pCO_2) which are sustained throughout pregnancy (Hytten and Leitch, 1971). Adjustments presumably occur in the interests of the growing fetus, but once a single alteration is achieved, the maternal body may react with several additional compensatory mechanisms. For example, a steadily increasing plasma progesterone concentration produces the lowering of maternal plasma pCO_2. This aids the elimination of CO_2 by the fetus, but demands compensation to maintain the pH of maternal blood (Pearson, 1976). A corresponding reduction in plasma bicarbonate and sodium is achieved through increased renal excretion. This interplay of physiological adaptations produces the chronic respiratory alkalosis and compensatory metabolic acidosis which characterize pregnancy (Lim _et al._, 1976; Gallery and Gyory, 1979). The decrease in sodium concentration leads to a drop in plasma osmolality, which in turn leads to polyuria and periods of increased thirst -- symptoms observed in most women during the first trimester (Page _et al._, 1976).

Hemodynamic alterations (including increases in blood volume, cardiac output, and renal blood flow) are established in the early weeks of pregnancy (Hytten and Leitch, 1971). Such changes are anticipatory, preparing the way for fetal demands at a later stage. Maternal fat stores begin to increase early in gestation and reach a maximum in the second trimester, providing an important caloric reserve for the tripling of fetal weight over the last 13 weeks (Milley and Simmons, 1979). Pregnancy is an overall anabolic state for both the mother and her fetus. Maternal ability to store depot fat in early gestation, followed by the mobilization of those stores in late gestation, has led to the concept of an early anabolic phase followed by a later catabolic phase with respect to maternal fuel disposition in pregnancy (Knopp et al., 1981). The impaired effectiveness of maternal glucose removal from the circulation in late pregnancy serves to maintain an adequate plasma glucose supply to meet the obligate requirements of the fetus.

A general quiescence of metabolism in maternal tissues and decreased muscle tonus are energy-saving adaptations of pregnancy (Hytten, 1972). This reduced tempo may reflect a reduction in the circulating level of free thyroid hormone due to changes in thyroxine binding (Hytten and Leitch, 1971; Burrow, 1977). Low vagal tone, hypomotility, and decreased gastric secretion delay stomach emptying in pregnancy and lead to a high incidence of nausea and heartburn (Hytten and Leitch, 1971).

An increased plasma concentration for several components of the blood coagulation system produces the hypercoagulability of pregnancy which protects against excessive blood loss (Cavanagh et al., 1978). The

plasma concentration of other substances (hemoglobin, iron, amino acids) may decrease in pregnancy due to the increased plasma volume, but the total circulating quantity of that substance may be increased (Lind, 1980). The lowering of nutrient levels in the blood may favor transfer to the fetal rather than maternal tissues (Hytten, 1972).

The pregnant body must preserve a new homeostasis which would simulate pathology in the nonpregnant body. Clearly, new reference standards of normality must be applied in the assessment of pregnancy (Young _et al_., 1975). Failure to achieve the normal adaptive changes in pregnancy may deny the fetus of an optimum environment during its 40-week course of development.

1.1.2 Altered renal function

The kidneys of normal pregnant women probably enlarge during gestation, and dilatation of the renal calyces, pelves, and ureters is evident during the first trimester (Lindheimer and Katz, 1977). In the human, approximately one-fifth of the total cardiac output traverses the kidney (Lindheimer and Katz, 1977). One-third of the increased cardiac output in pregnancy is distributed to the kidneys, raising renal blood flow from a nonpregnant rate of 800 ml/min to a rate of 1200 ml/min by the 9th week of gestation (Hytten and Leitch, 1971). Glomerular filtration rate, a reflection of the renal plasma flow, is increased during the first trimester of pregnancy and remains elevated until delivery (Davison, 1974; Davison and Hytten, 1974; Dunlop, 1980). Since the glomerular filtration rate increases without substantial alterations in the production of creatinine, urea, and uric acid, renal clearance of these solutes is increased (Lindheimer and Katz, 1977).

In the normal nonpregnant state, nutrients passed by the renal glomeruli are reabsorbed with great efficiency by the proximal tubules (Hytten, 1973). In pregnancy, an upset in glomerulo-tubular balance occurs, resulting in a tendency for substances which are reabsorbed in the tubules to be excreted in greater amounts (Davison and Hytten, 1974). Whether each glomerulus is delivering more filtrate to an unchanged tubular system, or whether nephrons of low reabsorptive capacity are now being perfused, the result is a variable partial failure of tubular reabsorption. Almost all amino acids are excreted in greater amounts during pregnancy (Hytten and Cheyne, 1972). During early pregnancy the excretion of glycine, histidine, threonine, serine, and alanine doubles, and by the end of pregnancy the losses increase to fivefold (more than 1 mmol per 24 h). A second group of amino acids (lysine, tyrosine, cystine, taurine, leucine, phenylalanine, and valine) are excreted in greater amounts during the first trimester, but the levels fall thereafter. The remaining amino acids show little or no increase in excretion. The pattern of aminoaciduria is not related to the chemical structure or biological function of the amino acids (Hytten and Cheyne, 1972). Glucose excretion is highly variable in pregnancy and may exceed 1 g in 24 h (Hytten, 1973). The excretion of xylose, ribose, and fucose is significantly elevated in the first trimester (Date, 1964a). Lactose excretion is increased tenfold by the third trimester (Date, 1964a) Oligosaccharide excretion is also increased in pregnancy (Date, 1964b, 1964c), several being pregnancy-specific and detectable by the 13th week of pregnancy (Hallgren *et al.*, 1977; Hallgren and Lundblad, 1977a, 1977b). Water-soluble vitamins are excreted in excess in pregnancy, the excretion of

nicotinic acid and ascorbic acid being approximately doubled (Hytten, 1973). Folate excretion increases two- to fourfold (Fleming, 1972; Landon and Hytten, 1971). The excretion of vitamin B_{12} is also likely to increase (Hytten and Leitch, 1971). Urinary excretion of polyamines is increased in pregnancy (Russell and Durie, 1978; Russell et al., 1978). Putrescine and spermidine excretion is doubled, whereas spermine excretion may be increased 75-fold (up to 5 mg per 24 hours).

The mechanisms of altered renal function may be hormonal. Hytten and Cheyne (1972) discussed a pattern of aminoaciduria which was induced in men by giving 100 mg of cortisol daily. These workers noted that the similar aminoaciduria of pregnant women is apparent within the first two months of pregnancy although there is no convincing evidence of increased corticosteroid activity. Katz and Lindheimer (1977) reviewed the actions of hormones on the kidney and noted the parallel increase of progesterone levels and renal plasma flow during human gestation. They also cited evidence that estrogens decrease the tubular reabsorption of ascorbic acid in man. It is also possible that compression of large vessels in the abdomen by the pregnant uterus, combined with urinary tract dilatation, may alter renal function in pregnancy (Davison and Hytten, 1974).

The clinical consequences of altered renal function are substantial. Urinary tract dilatation may lead to retention of large volumes of urine, introducing errors in the collection of timed urine samples (Lindheimer and Katz, 1977). The bizarre wastage of nutrients in the urine may be deleterious in areas of the world where vitamin and protein intake is

marginal (Hytten, 1973). It is not known if the kidneys economize in these circumstances of dietary inadequacy. The high nutrient content of urine, combined with the tendency for stasis, increases the liability of urinary tract infection in pregnancy (Hytten, 1976). The increased renal clearance in pregnancy results in a more rapid elimination of drugs (Dickason *et al*., 1978). This is a protective mechanism, since nearly all drugs enter the fetal circulation following maternal administration (Green *et al*., 1979) and may exert different effects upon the fetus (Schardein, 1976).

1.1.3 Biochemical methods for monitoring risk pregnancies

Fetal growth in humans is affected by a variety of factors (Miller and Merritt, 1979). Some of these factors, including maternal parity and race, play a role in all pregnancies. Most of the abnormal factors affecting pregnancy are of the growth-retarding type. These include fetal factors (chromosomal aberrations, congenital malformations, inborn errors of metabolism), medical complications during pregnancy (acute or chronic hypertension; severe chronic disease involving heart, liver, lungs, kidneys, gastrointestinal tract, thyroid, or adrenal glands; severe chronic infections; continuous medication with corticoids, immunosuppressives, or growth-retarding drugs; abnormalities of the uterus, placenta, or umbilical cord), maternal behavioral conditions (lack of prenatal care, dietary inadequacy, low prepregnancy weight for height, age less than seventeen or more than thirty-five years, cigarette smoking or use of addicting drugs or alcohol), and environmental factors (high altitude, exposure to toxic substances, irradiation). A pregnancy associated with these detrimental factors is described as high-risk due

to the increased chance of problems developing for the mother or her fetus (Hobel, 1976). Complications may lead to miscarriage, premature birth, neonatal mortality, or neonatal morbidity (long-term physical, neurological, mental, or immunological handicap). Thus, there is a great need to monitor pregnancies for establishing risk.

Direct or indirect visualization of the fetus is possible through roentgenography, amniography, fetography, ultrasonic scanning, or fetoscopy (Burton _et al._, 1974). These methods can be used to detect anatomic defects in utero. Sampling of fetal tissues can be accomplished either directly or through aminocentesis followed by cultivation of amniotic fluid cells. Cytogenetic study of fetal cells will confirm a chromosomal abnormality, while appropriate enzyme assays on the amniotic fluid or cultured fetal amniocytes may confirm a metabolic disease (Burton _et al._, 1974). Various organic constituents of amniotic fluid may be assayed to evaluate fetal maturity: creatinine, lecithin/sphingomyelin ratio, total protein (Dito, 1980; Seeds, 1980), or cortisol (Bjorkhem _et al._, 1978; Dawood, 1977). Elevated amounts of certain metabolites in amniotic fluid are associated with specific fetal conditions: alphafetoprotein in neural-tube defects (Seppala and Ruoslahti, 1976); methylmalonic acid in methylmalonic aciduria (Morrow _et al._, 1970; Nakamura _et al._, 1976); methylcitric acid in methylmalonic aciduria (Naylor _et al._, 1980) or propionic aciduria (Sweetman _et al._, 1979b); glutaric acid in glutaric aciduria (Goodman _et al._, 1980; Kvittingen _et al._, 1981); pyroglutamic acid in pyroglutamic aciduria (Larsson _et al._, 1974); argininosuccinic acid in argininosuccinic aciduria (Goodman _et al._, 1973; Fleisher _et al._, 1979); mucopolysaccharides in Hurler's syndrome (Matalon _et al._, 1970);

17α-hydroxyprogesterone and pregnanetriol in congenital adrenal hyperplasia due to 21-hydroxylase deficiency (Jeffcoate et al., 1965; Frasier et al., 1975; Milunsky and Tulchinsky, 1977; Nagamani et al., 1978); and tetrahydro-11-deoxycortisol (tetrahydrosubstance S) in congenital adrenal hyperplasia due to 11β-hydroxylase deficiency (Rosler et al., 1979). Amniotic fluid dehydroepiandrosterone sulfate is elevated four- to fivefold in placental sulfatase deficiency (Hahnel et al., 1982).

The foregoing techniques of visualization or amniocentesis must be assessed in terms of the risks to the mother and fetus -- radiation exposure, infection, or other unidentified consequences (Chard, 1974; Scrimgeour, 1978). Amniocentesis should not be done before 13 weeks after the first day of the last menstruation due to technical aspects and the risks involved (Lindsten et al., 1976). In contrast, the collection of blood or urine specimens from the mother is virtually without risk. A revolution in prenatal diagnosis may be forthcoming with the recent isolation of fetal cells from maternal blood samples as early as 15 weeks gestation (Iverson et al., 1981). With a fluorescence-activated cell sorter these workers used antibodies to a paternal cell surface antigen to select fetal cells from the lymphocyte fractions of maternal blood samples. While cultivation of amniotic fluid cells requires up to three weeks, only three days of culture are required with the sorted fetal cells (Iverson et al., 1981).

In addition to cytological investigations, biochemical parameters in maternal body fluids can be monitored by serial determinations. Evaluation of a pregnancy is based on a combination of these parameters and parallel physical examination. Hormones of the fetoplacental unit

are the prevalent choice, although there is presently little definitive knowledge regarding control of fetoplacental hormone production or the precise role of these hormones. The status of an early complicated pregnancy is best assessed by monitoring a placental hormone. The placental proteins human chorionic gonadotropin (HCG) and human placental lactogen (HPL) give prognostic validity until the 13th to 14th week of gestation (Goebel, 1978). HCG is detectable in maternal serum and urine 9 to 10 days after conception, maximal values being reached between the 9th and 11th week of pregnancy (Keller, 1976b). HCG is important for the maintenance of early pregnancy because of its luteotrophic effect on the corpus luteum. The corpus luteum has a functional life of about 10 weeks, its primary role being progesterone secretion to maintain the early implanted blastocyst (Yoshimi _et al_., 1969). HPL, always measured in blood due to its short half-life, is first detectable around the 5th week of pregnancy. Levels rise to a peak at 36 weeks, thereafter showing a plateau or slight decline (Letchworth, 1976). A multitude of actions, including lactogenic, luteotrophic, and metabolic effects have been attributed to HPL. Its levels are interpreted to reflect placental function. If the production rates of HCG or HPL are low or decreasing, the likelihood of an unavoidable abortion is extremely great, whereas rising values are a good prognosis (Goebel, 1978). The same is true for levels of ovarian and placental steroid hormones (estrone, 17β-estradiol, and progesterone) which are induced by HCG and HPL. The plasma level of estrone and 17β-estradiol increases continuously during the first 9 weeks of pregnancy, followed by a more significant rise which is maintained until term (Kunzig and Geiger, 1976). Serum progesterone

(along with its excretory counterpart, urinary pregnanediol) rises steadily from conception until the end of pregnancy (Keller, 1976a). Highly specific and sensitive radioimmunological methods have been most widely used for routine assays of HCG, HPL, plasma estrogens, and plasma progesterone (Abraham, 1974).

The status of a late high-risk pregnancy is most often assessed by monitoring the fetoplacental steroid estriol. Estriol is produced by the placenta from the fetal adrenal precursor dehydroepiandrosterone sulfate, and thus serves as an indicator of disorders in the fetal compartment. The physiological function of estrogens is not well understood but they appear to promote growth by influencing cell proliferation and division, protein synthesis, and membrane stability (Kunzig and Geiger, 1976; Beling, 1977). Estriol production shows a pronounced increase only after the 12th week of gestation, due to the secretory differentiation of the fetal adrenal (Kunzig and Geiger, 1976). During pregnancy the concentration of estriol in 24-hour urine shows a 1000-fold increase compared to the luteal phase of the menstrual cycle, whereas the concentration of estrone and 17β-estradiol rises about 100-fold. Thus, the glucuronide conjugates of estriol comprise 90 to 95% of the classical estrogens excreted in late pregnancy urine, often as much as 50 mg per 24 h (Beling, 1977). For this reason the total urinary estrogen fraction is often equated with estriol and rapidly assayed by colorimetric methods, avoiding complex procedures for conjugate hydrolysis and resolution of components (Wolfrum, 1978). Reduced concentrations of urinary total estrogens almost always reflect risk situations in pregnancy and at birth (Goebel, 1978; Carlstrom and

Lindberg, 1980). Roughly 90% of women with a growth-retarded baby have estriol excretion below the normal range (Kunzig and Geiger, 1976).

Various other biochemical parameters have been studied for surveillance of high-risk pregnancies. The major plasma protein of the early human fetus, alphafetoprotein (AFP), has been used for the antenatal detection of neural-tube defects (anencephaly, spina bifida, and hydrocephaly), disorders in the digestive system, and congenital nephrosis (Goebel, 1978). Raised maternal serum AFP levels are usually associated with these severe fetal disorders, although amniotic fluid levels may be more reliable (Seppala and Ruoslahti, 1976). Assays for maternal serum enzymes of placental origin (oxytocinase, heat-stable alkaline phosphatase) have failed to meet the criteria for a clinical placental function test (Curzen, 1976). Recently it was proposed that metabolic activities of maternal leukocytes may serve as markers of metabolic changes in fetal cells (Mameesh _et al._, 1976; Crosby _et al._, 1977). Leukocyte pyruvate kinase activity was reduced and some kinetic properties of the enzyme were affected in mothers delivering fetally malnourished babies (Mameesh _et al._, 1976). The authors suggested that the altered response of this enzyme in fetal malnutrition could be due to an imbalance of nutrients and metabolites in the mother without overt nutrient deficiency. This imbalance could affect susceptible enzymes in critical pathways of energy metabolism, such as glycolysis, which are necessary for the rapidly replicating fetal and placental cells or maternal leukocytes. This metabolic approach to routine diagnosis has yet to be applied. Other workers have focussed on steroid hormones such as estetrol (15α-hydroxyestriol) which may be largely fetal in origin, but

further investigations are needed to determine the reliability of this parameter (Kunzig and Geiger, 1976; Klopper, 1977; Kundu _et al_., 1978).

Prenatal diagnosis of inborn errors of fetal metabolism through biochemical monitoring of maternal urine is emerging as a reality in specialized laboratories. The first such case, described by Morrow _et al_. (1970), involved the detection of elevated methylmalonic acid in the urine of a mother carrying a fetus affected with methylmalonic aciduria. Maternal urinary methylmalonate, quantitated by a colorimetric method, was elevated after 25 weeks of gestation. More recently, other investigators have detected the same inborn error of metabolism in utero by measuring an elevation in maternal urinary methylmalonate as early as 16 weeks (Mahoney _et al_., 1975), 22 weeks (Bakker _et al_., 1978), and 23 weeks (Nakamura _et al_., 1976). Congenital adrenal hyperplasia can be diagnosed prenatally on the basis of elevated levels of tetrahydrosubstance S (Rosler _et al_., 1979) and estriol (Cathro _et al_., 1969) in maternal urine. Placental sulfatase deficiency is reflected by the increased maternal excretion of steroid monosulfate conjugates of 16α-hydroxypregnenolone, 16α-hydroxydehydroepiandrosterone, and androstenetriol (Taylor and Shackleton, 1979; Hahnel _et al_., 1982). Not surprisingly, placental sulfatase deficiency and congenital adrenal hypoplasia are accompanied by reduced maternal urinary estriol excretion (Fliegner _et al_., 1972).

1.2 Urinary organic acids and steroids

The urine provides a unique reflection of body chemistry. Since the kidney tubules do not effectively reabsorb some metabolites (for

example, organic acids), the urine may contain higher concentrations of those metabolites than does the plasma (Goodman, 1980). The placental circulation may be capable of clearing any accumulation of metabolite that occurs during fetal life, but the maternal urinary excretion of that substance might provide a prenatal diagnosis (Milunsky _et al_., 1970). Thus, urine may afford a means of examining the fetus through the screen of the mother. Collection of urine is simple, causing a minimum of inconvenience to the patient.

While the excretion of other classes of compounds in pregnancy has been documented, little is known of organic acid excretion. The urinary organic acids comprise a category of compounds which reflect many important metabolic routes involving carbohydrates, amino acids, lipids, amines, nucleic acids, and vitamins. The organic acids form a heterogeneous class with many different functional groups (carboxyl, hydroxyl, carbonyl, sulfhydryl, amino, imino) and a wide range of structures (aliphatic, aromatic, imidazolic, indolic),(Nordmann and Nordmann, 1961).

Analysis of organic acids in urine can provide an easy, rapid diagnosis for about 50 of the 180 known inborn errors of metabolism (Tanaka and Hine, 1982; Watts, 1978; Jellum, 1977). In addition, organic acidurias have been reported which are characteristic of riboflavin deficiency (Goodman, 1981), biotin deficiency (Sweetman _et al_., 1979a), vitamin B_{12} deficiency (Higginbottom _et al_., 1978), vitamin B_6 deficiency (Rose, 1972), as well as generalized insufficiency due to the dietary neglect of alcoholism (Mamer _et al_., 1978). Dicarboxylic acid excretion is a recognized response in patients with

ketosis (Niwa _et al._, 1982b; Truscott _et al._, 1979b; Pettersen and Stokke, 1973; Pettersen _et al._, 1972). Certain malignancies are accompanied by an increased excretion of vanilmandelic acid and homovanillic acid (LaBrosse _et al._, 1980; Muskiet _et al._, 1977; Addanki _et al._,1976) or 5-hydroxyindole-3-acetic acid (Haverback et al., 1956).

Much more is known of urinary steroid excretion than of organic acid excretion. The urinary steroids form a more homogeneous group. All steroids have the basic cyclopentanoperhydrophenanthrene nucleus and differ only by their functional groups (hydroxyl, carbonyl), double bonds, or isomeric configurations. Steroids are excreted in various conjugated forms (glucuronides, sulfates, double conjugates) which add diversity to the metabolites seen in the urine (Bernstein _et al._, 1970).

Urinary steroids can provide an index of adrenal, gonadal, and fetoplacental function (Van der Molen _et al._, 1971) and thus are of obvious importance in determining pregnancy status. In addition, urinary steroids may reflect heritable defects in steroid metabolism (Finkelstein and Shaefer, 1979).

1.3 Benefits, limitations, and objectives of the present study

Any antenatal screening procedure contributing to the prevention of perinatal mortality or morbidity would prove highly cost-effective in both humane and economic terms. Complications recognized prenatally sometimes allow alterations to be made to the apparent course of pregnancy. Adverse conditions recognized late in pregnancy may be

curtailed by artificial induction of labor. Dietary therapy *in utero* is advantageous in specific instances. For example, prenatal therapy with vitamin B_{12} in cases of vitamin-B_{12}-responsive methylmalonic aciduria is possible since vitamin B_{12} crosses the placenta to accumulate in the fetus (Ampola *et al.*, 1975). The course of this prenatal therapy has been monitored by analysis of multiple maternal urine specimens which showed decreasing levels of methylmalonic acid as therapy continued to term (Ampola *et al.*, 1975; Nakamura *et al.*, 1976). In fetal disorders diagnosed prenatally but not responsive to dietary therapy *in utero*, treatment can be started immediately after birth. The rapid postnatal deterioration associated with such conditions is thereby averted. The application of new and existing biochemical methods will increase the number of disorders detectable in utero, as well as the potential for treatment of those disorders.

Investigations of human metabolism pose special problems because genetic and environmental factors cannot be controlled. In addition, the drastic changes in every maternal physiological system during pregnancy would add to this heterogeneity. Current forms of treatment (such as prophylactic use of iron, folic acid, and vitamins) may modify the "natural" response to pregnancy (Hytten and Leitch, 1971). Fetal outcome is often difficult to correlate with the prenatal biochemical assessment. Subsequent complications in pregnancy or birth-related trauma may disguise the original condition. In addition, diverse complications are often grouped under one vague term. For example, intrauterine growth retardation (Rosso and Winick, 1974; Jones

and Battaglia, 1977) may have a variety of causes including maternal malnutrition, abnormal placental function, genetic disposition, or multiple combinations of these contributing factors. While retrospective evaluation of pregnancy relies mostly on evaluation of the newborn, more attention could be paid to the physiology of the placenta (Klopper, 1977).

Changes in maternal urinary metabolite excretion may reflect the adaptations associated with normal human pregnancy (altered renal function; emergence of sequential enzymatic pathways as the fetus matures; alterations in metabolism due to the placenta). The study of changes occurring in normal pregnancy may help to explain the etiology of unsuccessful pregnancies.

The objectives of the present study were:

1) the development of suitable profiling methods for organic acids and steroids in biological fluids,
2) the application of those methods to 24-hour urine specimens from reference groups of pregnant and nonpregnant women,
3) the identification of any alterations in metabolite excretion in the course of normal pregnancy,
4) the comparison of metabolite excretion in high-risk pregnancies to metabolite excretion in normal pregnancy.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment and column materials

Equipment	Supplier
Gas chromatograph (5730A)	Hewlett-Packard
Automatic sampler (7672A)	Hewlett-Packard
Integrator (3380A)	Hewlett-Packard
Vortex-evaporator	Buchler
Temp-Blok module heater	Pierce

Gas chromatography materials	Supplier
Pyrex glass columns (coiled; 6 ft x 2 mm inside diameter)	Supelco
3% OV-101 on 100/120 Gas Chrom Q	Applied Science
3% SE-30 on 100/120 Gas Chrom Q	Applied Science
Molecular sieve 5A (60/80 mesh)	Hewlett-Packard
Low-bleed septa (Teflon-lined)	Supelco

Column materials	Supplier
Glass Econo-columns (20 cm x 0.7 cm)	Bio-Rad
DEAE-Sephadex A-25	Pharmacia or Sigma
Amberlite XAD-2	Sigma
Amberlyst A-26	Sigma

2.1.2 Reagents, chemicals, and enzymes

Silylating reagents	Supplier
N,O-Bis(trimethylsilyl)acetamide	Pierce
N,O-Bis(trimethylsilyl)trifluoroacetamide	Pierce
Dimethyldichlorosilane	Pierce
N-Methyl-N-trimethylsilyltrifluoroacetamide	Pierce
Sylon-CT	Pierce
Trimethylchlorosilane	Pierce
Trimethylsilylimidazole	Pierce
Tri-sil "TBT"	Pierce

Specialty solvents	Supplier
Glass-distilled methanol	BDH
Glass-distilled hexane	BDH
Silylation grade pyridine	Pierce

Chemicals and enzymes	Supplier
Alkane standard kit	Applied science
Ethoxyamine hydrochloride	Eastman-Kodak
Ethylmalonic acid	Aldrich
β-Glucuronidase (*Helix pomatia)*	Sigma
15α-Hydroxyestradiol	Squibb
2-Hydroxyestriol	Squibb
Methoxyamine hydrochloride	Eastman-Kodak
Methylsuccinic acid	Aldrich

All other biochemicals were obtained from Sigma.

Name and address of suppliers

Aldrich	Aldrich Chemical Co. (Canada) Ltd., Montreal, Quebec
Applied Science	Applied Science Labs., Inc., State College, Pennsylvania
BDH	BDH Chemicals, Terochem Laboratories Ltd., Edmonton, Alberta
Bio-Rad	Bio-Rad Laboratories, Mississauga, Ontario
Buchler	Buchler Instruments, Fort Lee, New Jersey
Eastman-Kodak	Eastman-Kodak Co., Rochester, New York
Hewlett-Packard	Hewlett-Packard (Canada) Ltd., Edmonton, Alberta
Pharmacia	Pharmacia (Canada), Dorval, Quebec
Pierce	Pierce Chemical Co., Rockford, Illinois
Sigma	Sigma Chemical Co., St. Louis, Missouri
Squibb	E. R. Squibb and Sons, Inc., Princeton, New Jersey
Supelco	Supelco, Inc., Bellefonte, Pennsylvania

2.2 Sample collection

The project entitled "Early Diagnosis of Human Growth and Development Disorders" was approved by the Principal's Advisory Committee on Ethics in Human Experimentation (Appendix A). Informed consent forms (Appendix B) were distributed to physicians in Saskatoon, who introduced the prenatal study to obstetrical patients. Information on the completed consent forms enabled us to contact the women regarding collection of samples. Samples were collected during two time intervals in pregnancy according to menstrual age (the number of weeks since the last menses began). The first collection interval (12½ weeks to 15½ weeks gestation) was the earliest time possible due to the delay dictated by the physician-patient interaction. The second collection interval (24½ weeks to 27½ weeks gestation) was followed by term and postpartum collections. In addition, 24-hour urine samples were collected from healthy nonpregnant women between the 7th and 9th day of the menstrual cycle, with the requirement that they were not using oral contraceptive agents.

Written instructions (Appendix C), which explained the protocol for sample collection, were distributed to the participants. These instructions were verbally reinforced at the time of collection, emphasizing the need for a complete 24-hour urine collection and the necessity of refrigerating or freezing the sample. The 24-hour urine collection began in the morning, after rejection of the overnight specimen, and was completed at exactly the same time the following morning. The urine collection was made directly into a 2-litre polyethylene bottle using a funnel. The time and quantity of food intake during the preceding 24 hours was recorded by each patient as a diet recall (Appendix D). Food intake

for the duration of the 24-hour collection period was recorded as a diet diary (Appendix E). Other data recorded were height, present weight, and drug status.

The total 24-hour urine volume was recorded. Aliquots in glass screw-cap vials were frozen immediately since the urine was collected without preservatives. Urine samples were collected from April 1979 to January 1981 and stored at -30°C until required for analysis.

Medical information was extracted from prenatal records (Appendix F and Appendix G) when the pregnancy terminated. These records contained details of previous obstetrical history and the assessment of the present pregnancy. The physicians categorized each pregnancy as either low-risk, moderate-risk, or high-risk according to the calculated fetal risk score. The infant discharge form (Appendix H) contained information on the status of the newborn.

Patients selected to comprise the category of normal pregnancy fulfilled certain criteria. These pregnancies were described as uneventful and resulted in the delivery of a healthy newborn. Only those patients with 24-hour urine collections taken in both time intervals during gestation were considered. Several additional patients were selected to comprise the category of high-risk pregnancy. Some of these pregnancies resulted in the death of the fetus or neonate.

2.3 Creatinine determination

Urine was diluted 10-fold with distilled water and assayed in duplicate by the method of Yatzidis (1974, 1975). The modified Jaffé method of Yatzidis measures the difference in absorbance at 500 nm of

two alkaline picrate reagents. The color produced with pH 9.65 reagent is due to protein only, whereas that with pH 11.50 reagent is due to protein as well as creatinine. Other noncreatinine chromogens give a measurable absorbance only with a more alkaline reagent (pH 12.00).

2.4 Extraction methods

2.4.1 Organic acid extraction

The DEAE-Sephadex A-25 anion exchange resin (new from the supplier or previously used for organic acid extraction) was swollen in distilled water for 24 hours. The swollen resin was washed on a medium porosity fritted glass filter funnel successively with the following: 2.0 M pyridinium acetate, 0.5 M hydrochloric acid, distilled water to neutrality, 0.5 M sodium hydroxide, and distilled water to neutrality (McQuade _et al._, 1981). The washed resin was stored in distilled water at 4°C until poured as a slurry into glass columns (Bio-Rad Laboratories, Econo-columns; 0.7 cm inside diameter) to a height of 8 cm. The polyethylene bed support in these columns allowed a reproducible flow rate (about 1 ml/min) which facilitated the simultaneous operation of 20 columns. Each column (bed volume, 3 ml; 0.5 g of dry Sephadex resin) was washed with 10 ml of distilled water and 20 ml of 0.5 M pyridinium acetate immediately prior to use (Chalmers and Watts, 1972c).

Each urine specimen or aqueous acid standard, previously stored at -30°C in 10-ml glass vials, was thawed at room temperature and stirred to homogeneity with a small magnetic stirring bar. An aliquot of urine calculated to contain approximately 1 mg of creatinine (0.50 to 2.00 ml) was combined with 1.00 ml of aqueous glutaric acid or β-phenylpyruvic

acid standard (200 μg/ml) in a 10 mm x 100 mm test tube. This mixture was adjusted to pH 7.5 with the addition of 0.25 ml of 1.0 M sodium bicarbonate (Horrocks _et al_., 1976). This slightly basic sample was applied to the head of a prepared DEAE-Sephadex column and allowed to drain in. Cations and neutral substances were eluted with 15 ml of distilled water and discarded. The acidic metabolites were eluted with two 10-ml volumes of 1.5 M pyridinium acetate into a 50-ml Erlenmeyer flask. Occasionally bubbles slowed the flow rate to less than 1 ml/min but these were conveniently removed by gentle stirring with a glass rod. The samples were frozen immediately at -80°C and later freeze-dried in 20-sample batches for precisely 14 hours (freeze-drying chamber, room temperature; condenser, -50°C; vacuum, 0.3 Torr; vacuum pump, 150 l/min), (Chalmers and Watts, 1972a). The dried residue in each flask was transferred to a 10 mm x 100 mm screw-top test tube with three 1.0-ml volumes of glass-distilled methanol (Dalgliesh _et al_., 1966). The sample was then evaporated to dryness in a vortex-evaporator after 70 min (Buchler Instruments vortex-evaporator; heating block, 20°C; vacuum pump, 25 l/min). The purified acidic extract thus prepared was derivatized for gas chromatography as described in the Methods, Section 2.5. Figure 2.4.1 outlines the organic acid extraction method and indicates the time required for each procedure. Twenty samples can easily be processed in one day if the lengthy lyophilization step is allowed to proceed overnight.

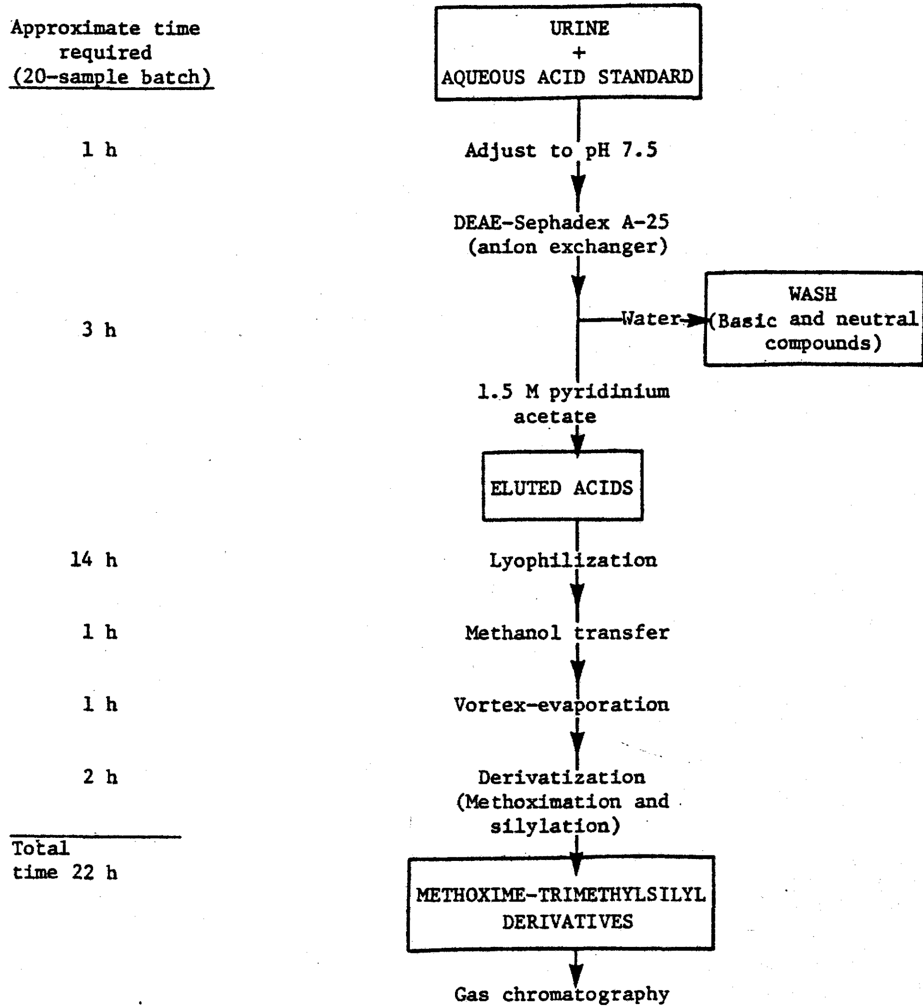


Figure 2.4.1 Outline of the organic acid extraction method, indicating the time required for each procedure.

2.4.2 Steroid extraction

The neutral resin Amberlite XAD-2 (60 g) was washed in succession with 600 ml each of methanol, acetone, and water prior to being used for the first time (Shackleton _et al._, 1970). Thereafter, the resin can be used repeatedly for many years with only methanol and water washes between each use (Bradlow, 1977; Leunissen and Thijssen, 1978). Resin fines were removed by a process of stirring and aspiration of the supernatant. The Amberlite XAD-2 resin (2 g) was transferred to glass columns (Bio-Rad Laboratories, Econo-columns with a polyethylene bed support; 0.7 cm inside diameter) which were used exclusively for steroid extractions. These glass columns, other glassware, and glass pipettes were previously silanized with 1% dimethyldichlorosilane (DMDCS) in toluene to prevent loss of steroids due to creepage (Kushinsky and Anderson, 1974). Resin aggregates and air bubbles were dispersed by inverting the column several times. Particles clinging to the glass were washed down with water. Each column (0.7 cm x 8 cm) was washed with 30 ml of distilled water immediately before use.

Each urine specimen or aqueous steroid conjugate standard, previously stored at -30°C in glass vials, was thawed at room temperature and stirred to homogeneity with a small magnetic stirring bar. An aliquot of urine calculated to contain approximately 10 mg of creatinine (5.0 to 20.0 ml) was combined with 2.00 ml of aqueous androsterone sulfate or 17β-estradiol-3β-D-glucuronide (200 μg/ml) in a test tube. This sample was applied to the head of a prepared XAD-2 column and allowed to drain completely. Because of the low specific gravity of the resin (1.03), a small amount of resin may float but this does not significantly

impair conjugate adsorption (Bradlow, 1968). The flow rate was kept constant (0.5 to 1.0 ml/min) throughout the entire extraction procedure. Faster flow rates affect the results only slightly (Osawa and Slaunwhite, 1970; Yapo et al., 1978). The columns were washed by pipetting two 10-ml volumes of distilled water onto the column, allowing the water to drain completely between applications. Hydrophilic substances such as glucose, urea, inorganic salts, amino acids, and low molecular weight organic acids were eluted in these washes (Osawa and Slaunwhite, 1970). The steroids were eluted with 12 ml of glass-distilled methanol into 12.5 mm x 125 mm screw-top test tubes. Methanol and ethanol are equally satisfactory eluents (Bradlow, 1968) but absolute alcohol tends to precipitate pigmented urinary constituents and impair conjugate recovery (Moore, 1972). The samples were evaporated to dryness in a vortex-evaporator after 4 hours (Buchler Instruments vortex-evaporator; heating block, 20°C; vacuum pump, 25 l/min). These dried residues were dissolved in 2 ml of acetate buffer (0.5 M acetic acid - 0.5 M sodium acetate, pH 4.5). The digestive juice of *Helix pomatia* was then added (0.2 ml of Sigma Type H-2 crude solution from *Helix pomatia*: β-glucuronidase activity, 100,000 units/ml; also contains sulfatase activity). The test tubes, sealed with teflon-lined screw caps, were incubated for 20 hours at 37°C in a covered shaking water bath. The liberated steroids were extracted by Amberlite XAD-2 as described above. The methanol eluates from the XAD-2 columns were passed through Amberlyst A-26 anion exchange columns to remove acidic impurities (Shackleton et al., 1970) only when steroid extracts were to be combined with the DEAE-Sephadex organic acid extracts. The methanol solutions were evaporated to dryness

in the vortex-evaporator. The purified steroid extracts thus prepared were derivatized for gas chromatography as described in the Methods, Section 2.5. Figure 2.4.2 outlines the steroid extraction method and indicates the time required for each procedure. Twenty samples can be processed in two days if the lengthy enzymatic hydrolysis step is allowed to proceed overnight.

2.5 Derivatization methods

2.5.1 Oximation

The keto groups present in keto acids and keto steroids were converted to their oxime derivatives (Chalmers and Watts, 1972b). Each oximating agent (methoxyamine hydrochloride, ethoxyamine hydrochloride, or hydroxylamine hydrochloride) was dissolved in silylation grade pyridine to form a saturated solution (1 g/50 ml), (Brooks and Harvey, 1970; Sternowsky et al., 1973; Axelson, 1978). These oximating reagents were stored at room temperature and remained chromatographically pure for many months.

All organic acids, steroids, or steroid conjugates were of the highest purity available from commercial sources and were used without further purification. The compounds were dissolved in silylation grade pyridine (1 mg/ml), (Sarkar and Malhotra, 1979), and stored at -30°C in 2-ml glass vials which were tightly capped with aluminum crimp-on seals containing teflon-lined silicone septa. All steroids and steroid conjugates were soluble in pyridine. Most organic acids were pyridine-soluble in their free acid form while most salts of acids (sodium, calcium, barium, lithium, and potassium salts) were not pyridine-soluble. These

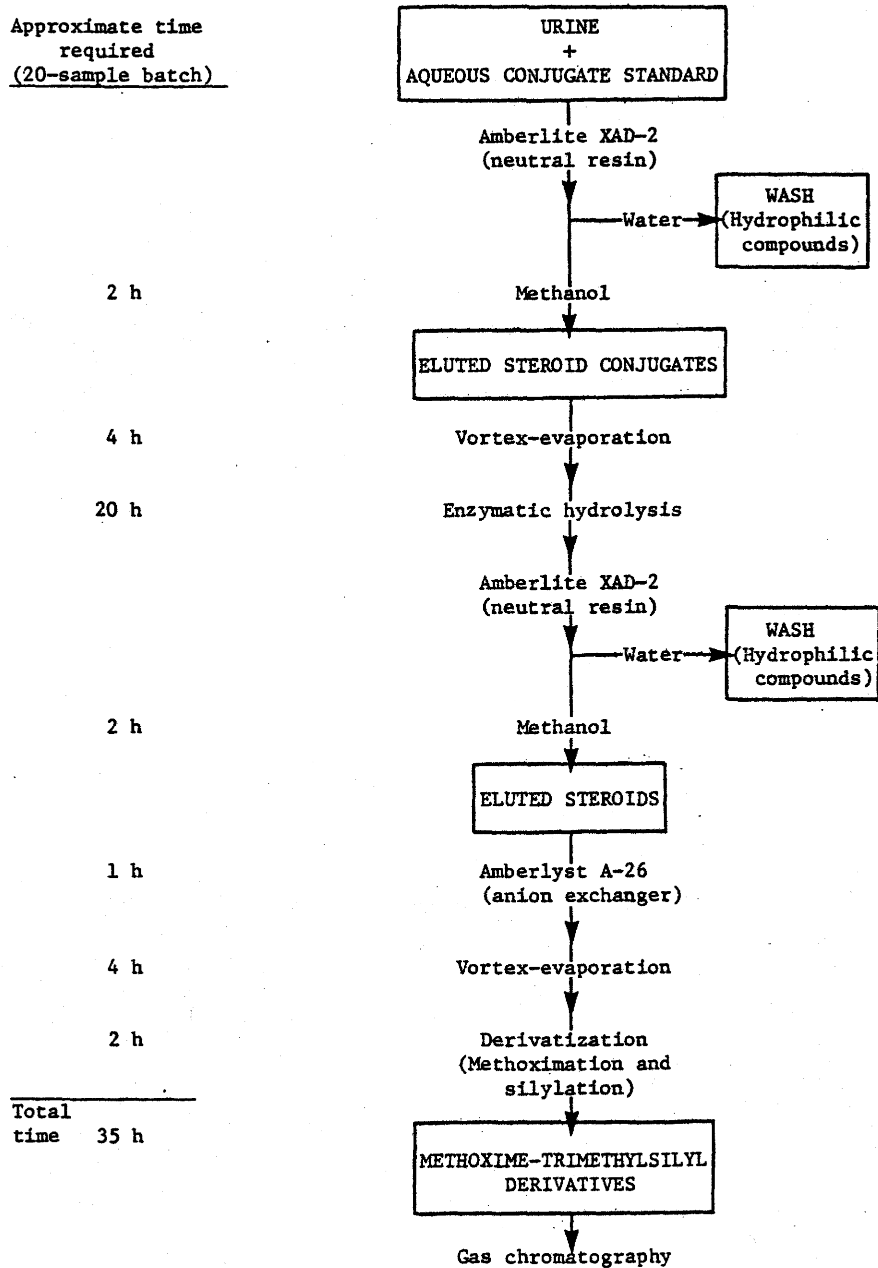


Figure 2.4.2 Outline of the steroid extraction method, indicating the time required for each procedure.

organic acid salts were converted to their free acid form and recovered by DEAE-Sephadex anion-exchange extraction or ethyl acetate-diethyl ether (solvent) extraction (Tanaka _et al._, 1980a, 1980b).

A suitable aliquot of a pyridine standard solution (containing organic acids, steroids, or steroid conjugates - singly, and later in suitable combinations selected according to retention times so as to produce a series of peaks with no overlap) was transferred to a 10 mm x 100 mm screw-top test tube. An equal aliquot of one of the oximating reagents was added to this pyridine solution. Likewise, an aliquot (250 μl) of an oximating reagent (usually methoxyamine hydrochloride in pyridine) was added to the dried methanol residues obtained from urinary organic acid or steroid extraction. The test tubes were tightly sealed with teflon-lined screw caps and placed upright into a controlled-temperature aluminum Temp-Blok module heater (Pierce Chemical Co.). The oximation reaction was allowed to proceed for 75 min at 60°C (as determined in the Results, Section 3.1.1).

2.5.2 Silylation

The reactive hydrogen atoms in the organic acids and steroids (hydrogen atoms of hydroxyl, carboxyl, sulfhydryl, amino, and imino groups) were replaced with silicon atoms to which were attached methyl groups. These trimethylsilyl derivatives can be produced by a variety of reagents with different silylating abilities (Chambaz and Horning, 1969; Gleispach, 1974; Nicholson, 1978). A mixture of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) was chosen for the silylation reaction for reasons outlined in the Results, Section 3.1.1.

The BSTFA/TMCS reagent (5:1,v/v) was prepared immediately prior to use from stock reagents previously stored at 4°C. An aliquot of the prepared silylating reagent was added to the sample test tubes after completion of the oximation reaction. A white precipitate of ammonium chloride was often produced with this addition, but that did not affect the reaction or the chromatography (Dalgliesh _et al_., 1966; Nicholson, 1978). Subsequent heating of the silylation mixture at 60°C for 60 min dissolved the ammonium chloride precipitate and resulted in a clear solution. The volume of silylating reagent used always equalled the volume of pyridine solution present for the oximation reaction (250 μl for urinary organic acid or steroid extracts). The test tubes were always tightly capped during the silylation reaction to prevent evaporation.

The oximated-trimethylsilylated samples were cooled to room temperature. An aliquot (100 μl) of each sample was transferred with a 100-μl glass syringe to a 200-μl cone-shaped glass vial containing 50 μg of tricosane. (A 50-μl portion of a 1.0 mg/ml solution of tricosane in hexane was previously evaporated undisturbed at room temperature over a 1-hour period.) The vials were tightly capped with aluminum crimp-on seals containing teflon-lined silicone septa. A 2-μl aliquot of each sample was withdrawn for injection into the chromatograph. The sample was injected into the gas chromatograph with the oven at an initial temperature of 70°C (followed by a 4-min initial isothermal delay) and the temperature was programmed to increase at 4°C/min to a final temperature of 300°C.

Straight-chain hydrocarbons were coinjected with the derivatized samples for the determination of methylene unit (MU) values (Dalgliesh _et al_.,

1966). An aliquot of hexane containing 1 mg/ml of even-numbered (C_{10} to C_{34}) or odd-numbered (C_9 to C_{27}) *n*-alkanes was added to the sample vial after all quantitative chromatographic runs were completed.

2.6 Gas-liquid chromatography

2.6.1 Chromatographic separation

2.6.1.1 Choice of column components

The wide range of column components available imparts versatility to the technique of gas chromatography (GC). Conventional packed columns were used exclusively, although capillary columns would have offered improved separation of sample components (Cramers et al., 1971; German and Horning, 1973). The chromatographic support chosen was Gas-Chrom Q (Applied Science Laboratories, Technical Bulletin No. 5: Gas-Chrom Q consists of siliceous earth treated with sodium carbonate, hydrochloric acid, potassium hydroxide, DMDCS in toluene, and methanol). The 100/120 mesh range (125 μm to 149 μm) was chosen for highest column efficiency (Ottenstein, 1973). From a wide variety of stationary phases (Horning et al., 1963; Husek and Macek, 1975), the nonpolar methylsilicones SE-30 and OV-101 were chosen for their chemical and thermal stability (Novotny and Zlatkis, 1971; Mueller et al., 1972; Coleman, 1973). The stationary phase routinely used was OV-101 (Ohio Valley Specialty Chemical Co.), because of its fluid state which results in increased efficiency at low temperatures (Trash, 1973). The usual 3% loading of the stationary phase was used (Ottenstein, 1973).

The carrier gas, certified nitrogen (Canadian Liquid Air Ltd.), was maintained at a flow rate of 25 ml/min through the columns (at 70°C).

Molecular sieve type 5A traps (calcium aluminosilicate; crystal lattice with pores of 5 ångströms in diameter) were installed in the carrier gas lines to remove moisture and other impurities. The molecular sieve was outgassed at a temperature of at least 200°C (for periods up to 8 hours) before installing the traps in the nitrogen lines. This action of heat was necessary to drive off the water of crystallization of the sieve to obtain a network of remaining empty holes (Bhatnagar, 1967). Failure to properly outgas the molecular sieve had little effect on the chromatography of standard solutions or the urinary steroid fraction, but completely destroyed the ability to chromatograph the urinary organic acid fraction. Urinary organic acid profiles obtained in this situation were identical to chromatograms with severe peak tailing which were published by Thompson (1977), and described by Chalmers et al. (1977) as an "occasional problem on one type of GC." These workers attributed the problem to column deterioration caused by the deposition of incompletely derivatized urinary sulfate and phosphate at the injector end of the column. Many hours were spent troubleshooting this problem. The impurities driven off of the molecular sieve (probably water, possibly calcium ions) irreversibly contaminated the column packing. It is possible that these impurities interacted with the urinary acid fraction to produce the smeared chromatograms. The problem was not further investigated once the source was identified and the molecular sieve properly outgassed. The seriousness of this problem emphasized the sensitivity of the GC column to chemical contamination.

2.6.1.2 Column packing and conditioning

Signs of failing column performance (peak tailing, shortened peak retention times, poor resolution of components, a rise in the base line, extraneous peaks) indicated that the columns should be repacked. As the columns aged, more active sites were exposed, rendering the stationary phase more polar. A column could normally be used for some 250 urinary samples before signs of deterioration were observed.

Pyrex glass coiled columns (6 ft x 2 mm inside diameter, 0.25 inch outside diameter) were base-washed and silanized according to the method of Leibrand and Dunham (1973). The glass was treated successively with 1% aqueous potassium hydroxide, methanol, toluene, Sylon-CT, toluene, methanol, and finally dried thoroughly in a stream of nitrogen. Silanization reduced the hydrophilic nature of the glass and helped to prevent adsorption of derivatives (Axelson, 1977; Rutten and Luyten 1972).

A glass wool plug (silanized glass wool) was inserted 1 cm into the detector end of the column. The empty column was weighed and then connected at the detector end to the suction of a water vacuum with intervening water trap. The column packing (3% OV-101 on 100/120 Gas-Chrom Q) was poured evenly into the injector end of the column through an attached funnel. Gentle tapping with a pencil was maintained until no further settling of the packing was noted. The column was again weighed and finally plugged at the injector end with silanized glass wool. (The glass wool plug was inserted 3 cm into the injection end of the column, allowing the injection syringe to deposit the sample just above the column packing.) The 6 ft x 2 mm columns held 1.9 - 2.2 g of packing.

The packed columns were returned to the GC oven (Hewlett-Packard, Model 5730A) and connected at their inlet end only. Nitrogen flow was restored to 25 ml/min and the outlet end of each column was capped to prevent backflow of air. The columns were conditioned at 50°C for 1 hour, and then heated at a rate of 2°C/min to an overnight temperature of 320°C (Walker *et al.*, 1977). The upper temperature limit of OV-101 is 350°C.

Once conditioned, the injection port and detector ends of the column were installed using graphite ferrules. The entire system was carefully leak tested to ensure freedom from carrier gas leakage and possible sample loss. Only teflon-coated silicone rubber septa (Microsep F-174, Supelco) were used in the injection port (temperature, 250°C). The use of these low-bleed septa minimized the occurrence of unexpected peaks which frequently arise from other septa. With less inert septa the bleeding of siloxane polymers will occur at high temperatures and appear as families of peaks resembling members of a homologous series of compounds (Smith and Sorrells, 1971).

Samples were injected onto the column with an automatic sampler (Hewlett-Packard, Model 7672A). The 10-μl glass syringe was automatically washed with hexane between samples. A thorough manual cleaning of syringes with potassium hydroxide, water, acetone, and chloroform was a daily routine (Walker *et al.*, 1977).

2.6.2 Chromatographic detection

2.6.2.1 Flame ionization detection

Routine analyses were performed using a Hewlett-Packard, Model 5730A gas chromatograph equipped with dual glass columns and dual flame

ionization detectors (FID). The detectors were operated in the differential mode (base line compensation), resulting in negligible base line rise with increase in temperature. The FID response was recorded by a Hewlett-Packard, Model 3380A reporting integrator. This instrument plots a chromatogram with the retention time printed at each peak apex (Healy et al., 1973). At the end of the run a report is printed which tabulates retention times and peak areas for a maximum of 54 peaks in one analysis. The integration cycle was initiated by the automatic sampler on injection of the sample and was stopped by the sampler after a preset time interval (usually 60 min). Peaks due to solvents and other reagents occurring before 4.0 min were traced out but not integrated. This was accomplished by use of the integrate "delay" control. Likewise, the use of the "area reject" control automatically rejected small peaks of a selected area (less than 1,000 area counts for urinary samples). The 3380A has a variety of integration capabilities: dropline allocation separates merged peaks; peaks on the tail of a solvent peak or other severely tailing peak are tangent-skimmed for more accurate area measurement (Cattran, 1975). The integrator was operated at an amplification of 10 x 16 (electrometer range x integrator attenuation), 0.5 μg of a straight-chain hydrocarbon giving a full-scale peak (approximately 200,000 area counts). Chart speed was 0.5 cm/min.

The individual FID is relatively insensitive to operating variables such as gas flow rates near the optimum values (Giuffrida, 1971) or detector temperature (Gill and Hartmann, 1967). In all analyses the detector was maintained at 300°C. Hydrogen flow rate was 30 ml/min (15 psi at the regulator) while the air flow rate was 250 ml/min

(25 psi at the regulator).

Bleed from the silicone gum rubber columns (Poulson and Jensen, 1971) and excess reagents used to make silyl derivatives (Giuffrida, 1971) are combusted to produce deposits of silicon dioxide on the FID collector electrodes (Lakeland and McDermott, 1968). These deposits were removed by manually cleaning the flame jets and collector electrodes (Walker et al., 1977) with 50% methanol/50% acetone after every 48 hours of FID operation. The reassembled detectors were purged for 1 hour at 400°C with nitrogen and air flow. The FID temperature was returned to 300°C and equilibrated before resumption of quantitative gas chromatography.

2.6.2.2 Mass spectrometry

The mass spectral analyses were performed by Dr. D. A. Durden of the Psychiatric Research Division, University Hospital, Saskatoon. The gas chromatographic separation used a conventional packed column as before (glass coil, 6 ft x 2 mm inside diameter; 3% OV-101 on 100/120 mesh Gas-Chrom Q) with helium as the carrier gas (30 ml/min). The 2-μl sample was injected into the GC with the oven at an initial temperature of 100°C (followed by an 8-min initial isothermal delay) and the temperature was programmed to increase at 4°C/min to a final temperature of 300°C. A jet separator (De Brauw, 1979) allowed the GC effluent to enter the mass spectrometer (Vacuum Generators Micromass, Model 7070F). The acquisition of mass spectral data was delayed for 4 min so as to vent off the solvent peak and avoid contamination of the ion source. The mass spectrometer was operated in the electron impact ionization mode. The mass spectrometry conditions were: ionizing voltage, 70 eV; source

block temperature, 250°C; source pressure, 10^{-5} Torr; accelerating voltage, 3 kV.

The mass spectrometer response was recorded by a Vacuum Generators Multi Spec data system. Automatic repetitive scanning of the mass range m/e 20-700 was completed in 3.0 sec. Complete spectra of all component peaks were accumulated on file. The computer system assigned mass values to each ion peak in the spectra, subtracted background where necessary, and presented normalized spectra graphically. The spectra were inspected individually and those of special interest were printed to form a permanent record.

3. RESULTS

3.1 Gas-liquid chromatography

3.1.1 Oximating agents and silyl donors

Three oximating agents (hydrochlorides of methoxyamine, ethoxyamine, and hydroxylamine) were chromatographed with a silylating reagent as shown in Figure 3.1.1.1, B to D. The chromatographic profiles of methoxyamine and ethoxyamine showed few peaks in addition to the solvent peak. Hydroxylamine was silylated to form the oxime-trimethylsilyl derivative which eluted at 10 minutes (Jakobs _et al._, 1977; Mamer _et al._, 1980). Additional silyl donors were chromatographed as shown in Figure 3.1.1.1, E to H. The name, abbreviation, and structure of each silyl donor is given in Table 3.1.1. BSTFA, alone or with the added catalyst TMCS, produced a narrow solvent peak. BSA and its by-products were less volatile than the fluorinated BSTFA as was seen by the wider solvent front. The BSA contained several additional peaks which increased in size after prolonged storage and exposure to moisture (Margosis, 1974; Munro _et al._, 1979). MSTFA showed several extra peaks despite the manufacturer's claim that it is the most volatile trimethylsilyl-amide available. TSIM obscured a large portion of the chromatogram. While Tri-sil "TBT" is a silylating mixture specially formulated for steroids, the large peak due to TSIM would hinder chromatographic analysis of organic acids.

Thus, the combination of reagents producing the minimum of peaks was seen to be methoxyamine or ethoxyamine hydrochloride in pyridine with BSTFA and TMCS. This combination of reagents has several advantages.

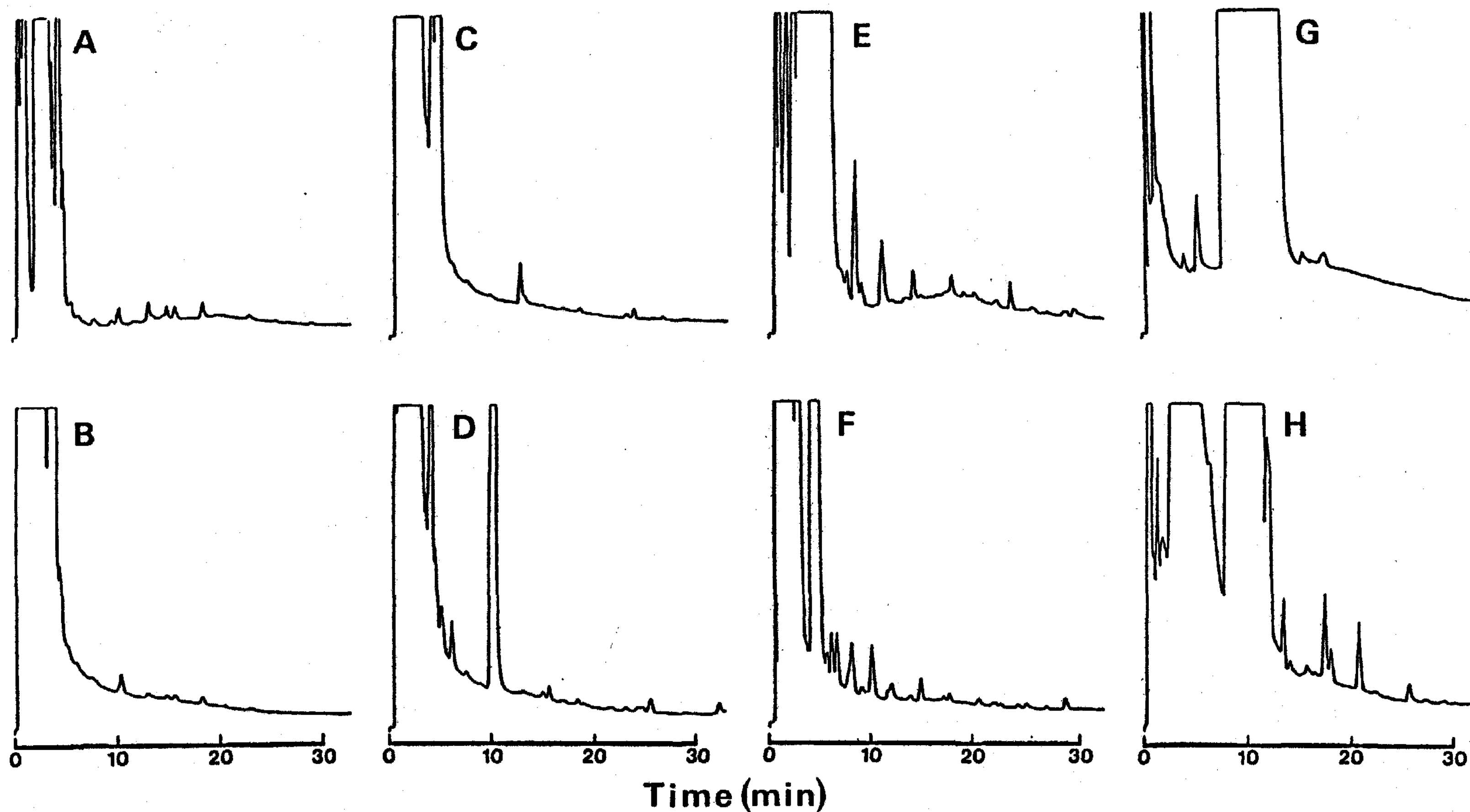


Figure 3.1.1.1 Gas-chromatographic profiles of oximating agents and silyl donors. All profiles represent 2-μl injections and temperature-programmed operation (70° to 300°C at 4°C/min with a 4-min initial isothermal delay) on 3% OV-101 stationary phase. The systematic name, abbreviation, and structure of each silyl donor is given in Table 3.1.1.1. The reagents are: (A) BSTFA and TMCS at 5:1 by volume, (B) 2% methoxyamine hydrochloride in pyridine with an equal volume of A, (C) 2% ethoxyamine hydrochloride in pyridine with an equal volume of A, (D) 2% hydroxylamine hydrochloride in pyridine with an equal volume of A, (E) BSA, (F) MSTFA, (G) TSIM, (H) Tri-sil "TBT" (TSIM, BSA, TMCS at 3:3:2 by volume).

Table 3.1.1 Systematic name, abbreviation, and structure of the silyl donors.

Systematic name	Abbreviation	Structure
N,O-Bis(trimethylsilyl)acetamide	BSA	$H_3C-C(-O-Si(CH_3)_3)=N-Si(CH_3)_3$
N,O-Bis(trimethylsilyl)trifluoroacetamide	BSTFA	$F_3C-C(-O-Si(CH_3)_3)=N-Si(CH_3)_3$
N-Methyl-N-trimethylsilyltrifluoroacetamide	MSTFA	$F_3C-C(=O)-N(CH_3)-Si(CH_3)_3$
Trimethylchlorosilane	TMCS	$H_3C-Si(CH_3)_2-Cl$
Trimethylsilylimidazole	TSIM	$Si(CH_3)_3$ attached to the N of an imidazole ring

Pyridine is a silylation catalyst, acting as an acid acceptor in the TMCS reaction (Pierce, 1968) as well as having excellent solvent properties. TMCS, water, and acids are all catalysts for the silylation reaction (Chambaz and Horning, 1969). Thus, amine hydrochlorides increase the rapidity of silylation (Van de Calseyde et al., 1972) and also enable the silylation of sodium salts of certain organic acids (Poole et al., 1976). The methoxime-trimethylsilyl (MO-TMS) system was chosen for routine analyses because mass spectral data is plentiful for this derivative.

The methoximation of dehydroepiandrosterone (DHEA) at three different temperatures was followed as shown in Figure 3.1.1.2. The disappearance of the DHEA-TMS peak paralleled the appearance of the MO-DHEA-TMS peak. The reaction was complete after 30 min at 60°C. To ensure complete methoximation of all keto compounds in urinary samples, methoximation was allowed to proceed for 75 min at 60°C. Labile keto acids (especially glyoxylic acid and oxalacetic acid) may decompose at elevated temperatures (Lohr and Warren, 1962; Andersson et al., 1973) and should be investigated individually if they are of special interest.

3.1.2 Retention data

Each compound was analyzed separately as its methoxime-trimethylsilyl (MO-TMS) derivative to check for chromatographic purity. (It was soon observed that all glassware must be carefully washed in chromic acid and thoroughly rinsed in distilled water to avoid spurious peaks.) Maleic acid chromatographed as two peaks, corresponding to maleic acid and fumaric acid (approximately 1:1), possibly indicating an interconversion of

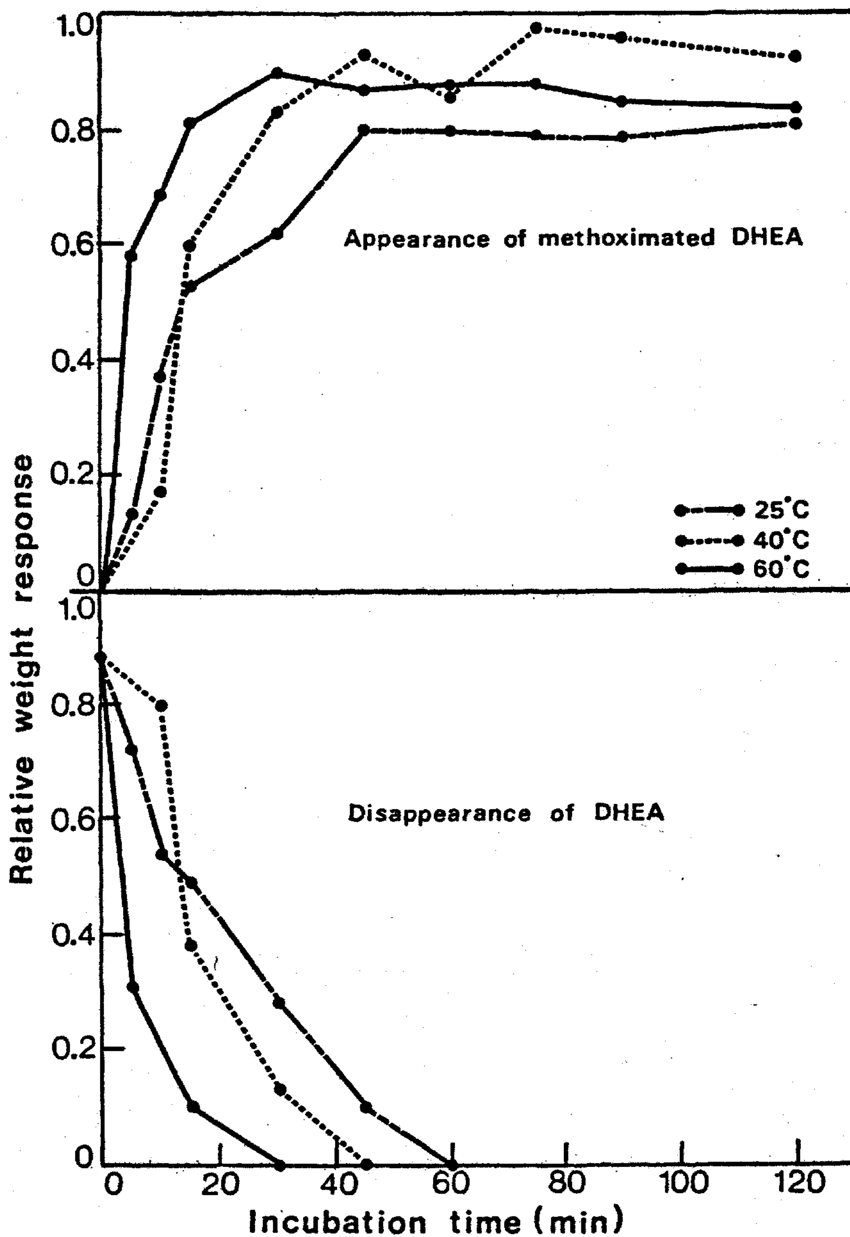


Figure 3.1.1.2 Relative weight response of dehydroepiandrosterone (DHEA) trimethylsilyl derivatives as a function of methoximation time and methoximation temperature. Addition of silylating reagents at the indicated times halted the methoximation reaction. The weight response is relative to the internal standard tricosane. Each point represents an average of two determinations.

the *cis*- and *trans*- isomeric forms. β-Glycerophosphate and α-glycerophosphate had slight cross-contamination (about 5%) confirming the supplier's claim of 95-99% purity. β-Hydroxybutyric acid in the free acid form (as a liquid) had several contaminants. Pyruvic acid (Von Korff, 1969) and lactic acid (Lockwood et al., 1965) have a reported tendency to form dimers but were always chromatographed as single peaks. Oxalacetic acid chromatographed as pyruvic acid but due to its unstable nature (Bachelard, 1965) spontaneous decarboxylation would not be surprising. The acylglycines hippuric acid and o-hydroxyhippuric acid (salicyluric acid) chromatographed as their mono- and di-TMS derivatives respectively. This formation of single derivatives for acylglycines agrees with results by O'Neill-Rowley and Gerritsen (1975) but is in contrast with the two derivatives formed by Ramsdell and Tanaka (1980). The aldonic acids (gluconic, galactonic, and ribonic acid) each formed two peaks corresponding to the free acid and its lactone (Kringstad and Bakke, 1977). Uric acid (as the free acid) and salts of several acids (glucaric acid calcium salt, glyceric acid hemi-calcium salt, isocitric acid trisodium salt, phthalic acid potassium salt, indoxyl sulfate potassium salt) did not give any peaks by the direct derivatization process. This was probably due to poor solubility and difficult silylation associated with certain salts of organic acids (Poole et al., 1976). All of these acids were detected after the anion-exchange isolation from aqueous solution which produced their pyridinium salts. These weakly ionic salts dissociate easily, allowing silylation to proceed more readily (Chalmers and Watts, 1972a).

All steroids investigated eluted as single peaks when chromatographed as their MO-TMS derivatives. In contrast, direct derivatization of steroid

glucuronide conjugates did not produce any response on the chromatogram. Methyl ester-trimethylsilyl ethers (Horning et al., 1967; Jaakonmaki et al., 1967b; VandenHeuvel, 1967) and propyl ester-trimethylsilyl ethers (Miyazaki et al., 1976) of steroid glucuronides have been reported as chromatographed and may be more stable than the present per(trimethylsilyl) derivatives (Billets et al., 1973). Steroid sulfate conjugates investigated (dehydroepiandrosterone sulfate and estrone 3-sulfate) chromatographed as the MO-TMS derivatives of the free steroids (dehydroepiandrosterone and estrone, respectively). Apparently an acid-catalyzed hydrolysis of the steroid sulfates resulted in the expulsion of SO_3 and consequent derivatization of the unconjugated steroid (Murray and Baillie, 1979).

For inter- and intralaboratory comparisons, the GC retention data were expressed as methylene unit (MU) values. The MU value is equal to the retention index of Kovats (1958) divided by 100. The MU value of a derivative describes its elution characteristics relative to the appropriate pair of flanking *n*-alkanes which differ by not more than two carbon atoms (Dalgliesh et al., 1966). The MU value for a given compound rarely varied more than 0.02. Slight deviations in temperature at the start of the GC run occasionally produced variations of up to 0.04 for compounds with MU values smaller than 13.00. The reproducibility of the temperature program was generally good, absolute retention times usually being within ± 20 sec of the average retention time over a prolonged period of analysis. When mixtures of the compounds were chromatographed, it was observed that a difference in MU value of 0.10 or more was needed for complete resolution of components. Differences of 0.05 to 0.10 produced twin peaks or shoulders, while compounds differing by less than 0.05

emerged as a single peak. When a large amount of a compound (more than 2 μg) was injected, the MU value was slightly increased. The excess amount caused a delay in peak retention time since that parameter was reported at the peak apex.

The MU values for MO-TMS derivatives of organic acids and steroids are given in Tables 3.1.2.1 and 3.1.2.2 respectively. The nonpolar stationary phase OV-101 has an affinity for nonpolar compounds and tends to separate components on the basis of their molecular size and shape. The acids of low molecular weight eluted with MU values between 10.04 (tiglic acid - monoTMS, MW 172) and 18.00 (hippuric acid - monoTMS, MW 251). The acids of higher molecular weight were retained longer, having MU values as high as 24.54 (biotin - monoTMS, MW 316). The steroids formed a more homogeneous group, eluting between MU 25.28 (androsterone-MO-monoTMS, MW 391) and MU 32.72 (cortisol-MO-triTMS, MW 636). Predictable relationships were evident for the MU values of members of a homologous series, for example the dicarboxylic acid - diTMS derivatives: oxalic acid, 11.26; malonic acid, 12.02; succinic acid, 13.12; glutaric acid, 14.00; adipic acid, 15.03; pimelic acid, 16.00; suberic acid, 16.93; azelaic acid, 17.99; sebacic acid, 18.89; undecanedioic acid, 19.88. Other authors have compiled comparable lists of MU values for TMS derivatives of organic acids (Butts, 1972; Petersson, 1977; Tanaka and Hine, 1982), inorganic anions (Butts and Rainey, 1971), and steroids (Luyten and Rutten, 1974; Pfaffenberger and Horning, 1977; Tomsova _et al_., 1980).

A more extensive analysis of the keto groups was made to aid in identification of keto compounds. The MU values for oxime trimethylsilyl derivatives of keto acids are listed in Table 3.1.2.3. When chromatographed

Table 3.1.2.1 Methylene unit values for methoxime trimethylsilyl derivatives of organic acids.

Gas-chromatographic analyses were performed on a 6-ft packed column containing 3% OV-101 on 100/120 mesh Gas Chrom Q.

* Indicates an acid with a carbonyl group which forms the methoxime derivative.
§ *cis* - Oxalacetic acid is decarboxylated to yield pyruvic acid.

MU value	Organic acid
10.04	Tiglic acid
10.36	Pyruvic acid*
10.36	*cis*-Oxalacetic acid*§
10.63	Lactic acid
10.70	α-Hydroxyisobutyric acid
10.74	Glycolic acid
10.93	α-Ketobutyric acid*
11.16	α-Furoic acid
11.26	Oxalic acid
11.34	α-Hydroxybutyric acid
11.48	Sulfate
11.67	β-Hydroxybutyric acid
11.70	α-Ketovaleric acid*
11.74	α-Hydroxyisovaleric acid
12.02	Malonic acid
12.12	α-Ketoisocaproic acid*
12.15	Methylmalonic acid
12.27	Benzoic acid
12.35	Levulinic acid*
12.47	α-Hydroxyisocaproic acid
12.54	α-Hydroxy-β-methylvaleric acid
12.63	Caprylic acid
12.69	Nicotinic acid
12.76	Phenylacetic acid
12.84	Ethylmalonic acid
12.90	Phosphate
12.99	Maleic acid
13.00	Picolinic acid

.....continued

Table 3.1.2.1 (continued)

MU value	Organic acid
13.12	Succinic acid
13.29	Methylsuccinic acid
13.48	Glyceric acid
13.53	Pyrrole-2-carboxylic acid
13.54	Fumaric acid
13.65	Pipecolic acid
14.00	Glutaric acid
14.34	α-Keto-γ-methiolbutyric acid*
14.37	3,3-Dimethylglutaric acid
14.55	Capric acid
14.72	Mandelic acid
14.89	Citramalic acid
15.01	Salicylic acid
15.03	Adipic acid
15.07	Acetopyruvic acid*
15.07	Malic acid
15.07	Pyroglutamic acid
15.18	*trans*-Cinnamic acid
15.47	3-Methyladipic acid
15.57	o-Hydroxyphenylacetic acid
15.58	m-Hydroxybenzoic acid
15.63	β-Phenylpyruvic acid*
15.76	α-Ketoglutaric acid*
15.78	β-Phenyllactic acid
15.85	Tropic acid
15.94	α-Hydroxyglutaric acid
15.96	m-Hydroxyphenylacetic acid
16.00	Pimelic acid
16.12	*meso*-Tartaric acid
16.14	Anthranilic acid
16.18	p-Hydroxybenzoic acid
16.25	β-Hydroxy-β-methylglutaric acid

.....continued

Table 3.1.2.1 (continued)

MU value	Organic acid
16.26	p-Hydroxyphenylacetic acid
16.35	α-Aminoadipic acid
16.43	Imidazoleacetic acid
16.49	Lauric acid
16.58	α-Ketoadipic acid*
16.69	Tartaric acid
16.75	Phthalic acid
16.82	Veratric acid
16.84	Homoveratric acid
16.93	3-Indoxylsulfuric acid
16.93	Suberic acid
16.95	Ribonic acid γ-lactone
17.17	Quinolinic acid
17.51	Vanillic acid
17.52	Orotic acid
17.56	Homovanillic acid
17.56	β-Glycerophosphoric acid
17.62	*cis*-Aconitic acid
17.82	Gentisic acid
17.87	p-Hydroxymandelic acid
17.92	α-Glycerophosphoric acid
17.99	Azelaic acid
18.00	Hippuric acid
18.20	Ribonic acid
18.23	3,4-Dihydroxybenzoic acid
18.29	3,4-Dihydroxyphenylacetic acid
18.40	Homogentisic acid
18.44	Shikimic acid
18.48	Myristic acid
18.50	Citric acid
18.50	Isocitric acid
18.51	Nicotinuric acid
18.78	3-Hydroxyanthranilic acid
18.87	Vanilmandelic acid

....continued

Table 3.1.2.1 (continued)

MU value	Organic acid
18.89	Sebacic acid
18.93	p-Hydroxyphenylpyruvic acid*
19.08	p-Hydroxyphenyllactic acid
19.14	Glucuronic acid γ-lactone*
19.20	Galactonic acid δ-lactone
19.20	Gluconic acid δ-lactone
19.29	Indole-3-acetic acid
19.43	4-Pyridoxic acid
19.69	Urocanic acid
19.76	Ascorbic acid-2-sulfate
19.82	Ascorbic acid
19.85	Glucuronic acid*
19.88	Undecanedioic acid
20.16	Pantothenic acid
20.40	Salicyluric acid
20.46	Palmitic acid
20.50	Kynurenic acid
20.66	Galactonic acid
20.66	Glucaric acid
20.66	Gluconic acid
20.84	Ferulic acid
21.20	Uric acid
21.39	Caffeic acid
21.65	Indole-3-pyruvic acid*
21.69	Indole-3-lactic acid
21.97	5-Hydroxyindole-3-acetic acid
22.13	Oleic acid
22.43	Stearic acid
22.63	Xanthurenic acid
24.54	Biotin

Table 3.1.2.2 Methylene unit values for methoxime trimethylsilyl derivatives of steroids.

M.U. value	Steroid
25.28	Androsterone
25.42	Etiocholanolone
25.93	Dehydroepiandrosterone
26.24	11-Ketoandrosterone
26.27	Estrone
26.29	5α-Androstan-3β,17β-diol
26.31	11-Ketoetiocholanolone
26.38	Androstenedione
26.60	β-Estradiol
26.72	Testosterone
27.10	11β-Hydroxyandrosterone
27.22	Equilenin
27.25	11β-Hydroxyetiocholanolone
27.85	Pregnanediol
28.00	5α-Cholestane
28.32	15α-Hydroxyestradiol
28.49	Progesterone
28.74	Tetrahydrosubstance S
28.94	Estriol
29.26	Pregnanetriol
29.38	17α-Hydroxyprogesterone
29.75	Tetrahydrocortisone
30.32	Tetrahydrocortisol
30.40	2-Hydroxyestriol
31.25	Cholesterol
31.89	Cholesterol acetate
32.06	Cortisone
32.43	Stigmasterol
32.72	Cortisol

Table 3.1.2.3 Methylene unit values for oxime trimethylsilyl derivatives of keto acids.

Keto acid	Not oximated	Methoxime	Ethoxime	ΔMU_1	TMS-oxime	ΔMU_2
Pyruvic acid	10.85	10.36	11.01	0.65	11.59	0.58
cis-Oxalacetic acid	10.88, 13.00, 13.50	10.36	11.00	0.64	11.57	0.57
α-Ketobutyric acid	11.79	10.93	11.54	0.61	11.98	0.44
β-Phenylpyruvic acid	17.01	15.63	16.14	0.51	16.34	0.20
α-Ketoglutaric acid	14.77, 16.21, 18.65	15.76	16.22	0.46	16.43	0.21
α-Ketoadipic acid	15.73, 17.10	16.58	17.04	0.46	17.27	0.23
p-Hydroxyphenylpyruvic acid	20.58	18.93	19.40	0.47	19.47	0.07
Glucuronic acid γ-lactone	18.26, 18.44	19.14	19.72	0.58	20.29	0.57
Glucuronic acid	19.59, 20.17, 20.85	19.85	20.35	0.50	20.89	0.54
Indole-3-pyruvic acid	24.28	21.65	22.12	0.47	22.15	0.03

ΔMU_1 = MU(Ethoxime) - MU(Methoxime)

ΔMU_2 = MU(TMS-oxime) - MU(Ethoxime)

Table 3.1.2.4 Methylene unit values for oxime trimethylsilyl derivatives of keto steroids.

Keto steroid	Not oximated	Methoxime	ΔMU_1	Ethoxime	ΔMU_2	TMS-oxime	ΔMU_3
Mono-keto steroid:							
Androsterone	24.51	25.28	0.77	25.88	0.60	26.36	0.48
Etiocholanolone	24.70	25.42	0.72	25.98	0.56	26.36	0.38
Dehydroepiandrosterone	25.25	25.93	0.68	26.55	0.62	27.11	0.56
Estrone	25.61	26.27	0.66	26.87	0.60	27.42	0.55
Testosterone	26.29	26.72	0.43	27.36	0.64	27.86	0.50
Di-keto steroid:							
Androstenedione	25.37	26.38	1.01	27.70	1.32	28.75	1.05
Progesterone	27.26	28.49	1.23	29.77	1.28	30.90	1.13

ΔMU_1 = MU(Methoxime) - MU(Not oximated)

ΔMU_2 = MU(Ethoxime) - MU(Methoxime)

ΔMU_3 = MU(TMS-oxime) - MU(Ethoxime)

without prior oximation, several keto acids produced multiple derivatives (*cis*-oxalacetic acid, α-ketoglutaric acid, α-ketoadipic acid, glucuronic acid γ-lactone, glucuronic acid). Identical values were obtained for pyruvic acid and *cis*-oxalacetic acid, probably indicating decarboxylation of the latter acid. The oximes eluted according to their order of increasing nonpolarity: methoxime, ethoxime, TMS-oxime. The difference in MU value (ΔMU_1, ΔMU_2) between various oxime derivatives was characteristic for each type of keto acid. Striking similarities were seen for α-ketoglutaric acid and α-ketoadipic acid, p-hydroxyphenylpyruvic acid and indole-3-pyruvic acid, glucuronic acid γ-lactone and glucuronic acid. Analogous results are listed in Table 3.1.2.4 for the keto steroids. The steroids analyzed without prior oximation produced single derivatives eluting prior to the methoximes as would be expected from the increased polarity of the free keto group. The difference in MU value (ΔMU_1, ΔMU_2, ΔMU_3) between oxime derivatives was uniform for mono-keto steroids, 0.58 ± 0.11 (mean $\pm$ SD, n = 15). The corresponding difference in di-keto steroid oximes was exactly double, 1.17 ± 0.13 (mean $\pm$ SD, n = 6). Thus, the mono-keto versus di-keto shift seen with steroid oximes would be helpful in identification of unknown keto steroids.

3.1.3 Flame ionization detection

Few aspects of the flame ionization detector's operation can be regarded as well understood (Cram et al., 1980). Response in the flame ionization detector (FID) is believed to arise from conductivity imparted by ions generated from combustion of organic eluates in the flame (Blades, 1973). There is a wide variation in detector geometry and

sample handling capacity (McWilliam, 1961; Ravey, 1978). The FID is reported to have a wide linear dynamic range (Keller, 1973), often cited as 10^7, while other detectors are usually 10^4 or less (Gill and Hartmann, 1967). To investigate the FID linearity, periodic response checks were implemented. Figure 3.1.3.1 illustrates the increase in the FID linear dynamic range following the removal of silicon dioxide deposits from the FID collector electrodes. These deposits insulated the electrodes and caused the FID response to fall off at high sample rates. Alexander and Garbutt (1965) similarly found peak areas were 30% greater when measurement was made directly after cleaning the detector. The linear range was narrowed to a maximum upper level of 1.0 μg of tetracosane after 72 hours of FID operation. Thus, the practical linear dynamic range extended from the minimum detectable level of 2.5 ng (approximately 1,000 integrator units) to the maximum upper linear level of 1.0 μg (approximately 400,000 integrator units) or over a range of 4×10^2.

To limit silicon dioxide deposits, the injection of freon (Walker et al., 1977) or the use of fluorinated silylating reagents was suggested (Stalling et al., 1968). The combustion of these reagents was reported to produce hydrofluoric acid which would react with silicon dioxide, converting it to the volatile silicon tetrafluoride. Use of these reagents did not noticeably lessen detector contamination. The rapidity of detector electrode contamination probably varies in magnitude with the design of the detector (Margosis, 1974). While most authors do not discuss FID contamination, others report that FID cleaning was not required even after four years of uninterrupted use (VandenHeuvel and Court, 1968). Routine cleaning of the FID, although time-consuming, was

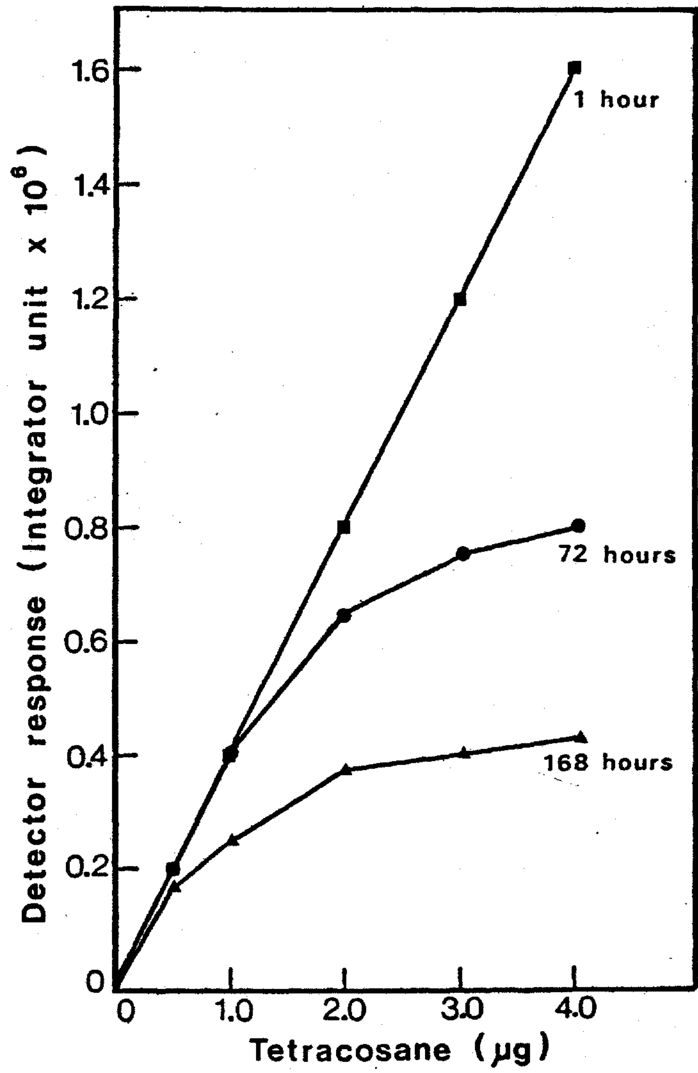


Figure 3.1.3.1 Linear dynamic range and time-dependent silicon dioxide suppression of the flame ionization detector response. Times indicate the hours of continuous FID operation since manual cleaning of the detector to remove SiO_2 deposits.

a welcome solution to the insidious problem of detector nonlinearity.

The relationship between the amount of compound present and the FID response was determined experimentally. Aliquots of pyridine standard solutions were methoximated, silylated, and chromatographed as shown in Figure 3.1.3.2. The relative weight response (RWR) was determined from:

$$\text{RWR} = \frac{\dfrac{\text{Area counts for compound}}{\text{Weight of compound}}}{\dfrac{\text{Area counts for tricosane}}{\text{Weight of tricosane}}}$$

The weight of tricosane present was 1.00 μg so,

$$\text{RWR} = \frac{\text{Area counts for compound}}{(\text{Weight of compound})(\text{Area counts for tricosane})}.$$

Table 3.1.3 lists the RWR for 60 organic acids and steroids arranged in order of increasing RWR. The small standard deviations indicate the reproducibility of the method and the stability of derivatives. The coefficient of variation ranged from 11% (oxalic acid, 0.34 $\pm$ 0.04, α-hydroxyisocaproic acid, 1.43 $\pm$ 0.16; α-furoic acid, 1.18 $\pm$ 0.13) to less than 1% for many compounds. The lower weight responses were characteristic of dicarboxylic acids (oxalic acid, 0.34; malonic acid, 0.52; methylmalonic acid, 0.79; glutaric acid, 0.86; succinic acid, 0.88), a tricarboxylic acid (citric acid, 0.95), keto compounds (β-phenylpyruvic acid, 0.54; 17α-hydroxyprogesterone, 0.67; pyruvic acid, 0.69; dehydroepiandrosterone, 0.88; cholesterol acetate, 0.88; androsterone, 0.93; estrone, 0.93). nonoximated carbonyls possessing nitrogen (pyroglutamic acid, 0.77; orotic acid, 0.90), and methoxy compounds (homoveratric acid, 0.65; veratric acid, 0.76; vanillic acid, 0.85;

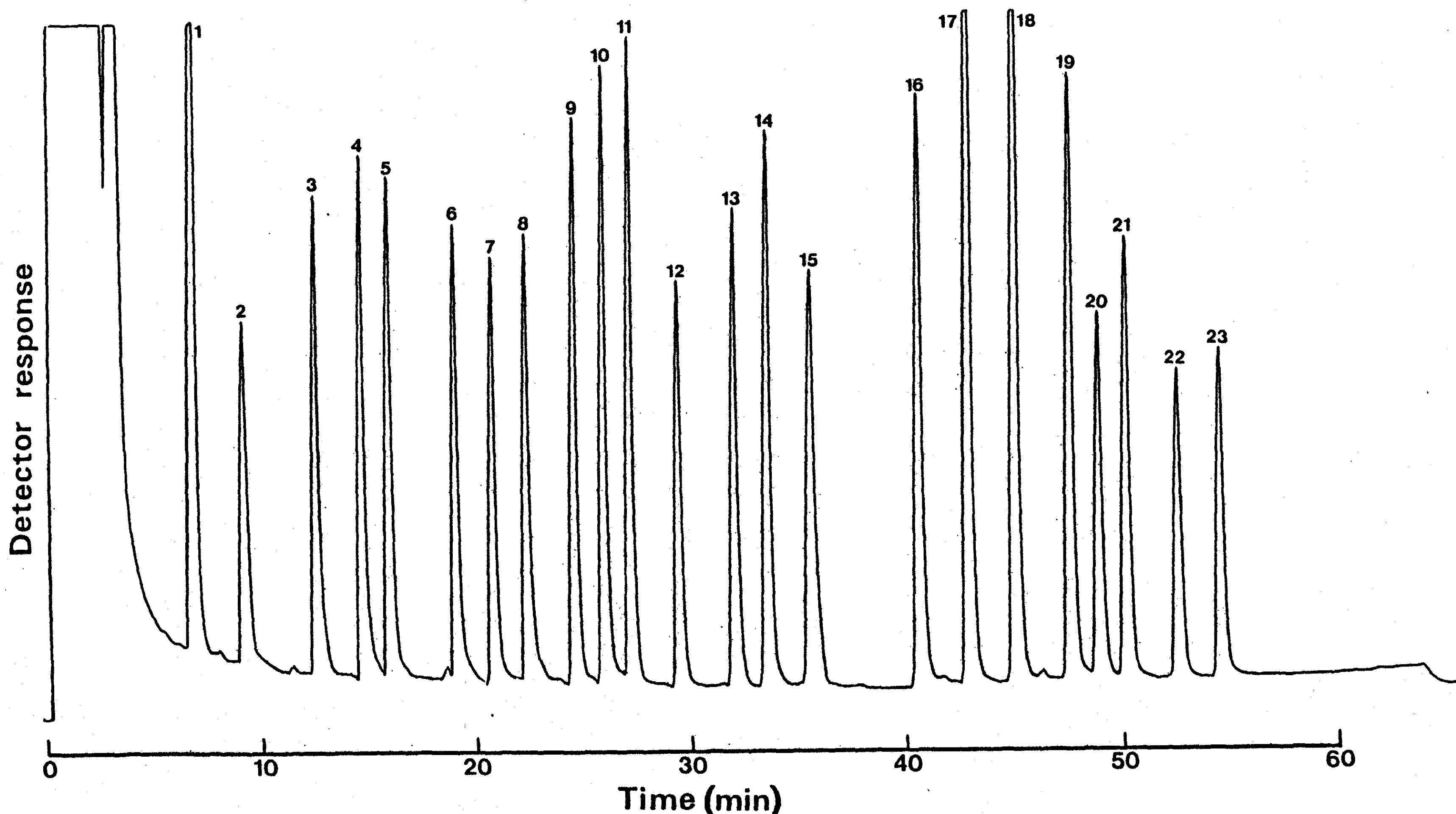


Figure 3.1.3.2 Gas-liquid chromatographic separation (flame ionization detection) of the methoxime trimethylsilyl derivatives of some organic acids and steroids. Analysis was on a 6-ft 3% OV-101 column programmed from 70° to 300°C at 4°/min with a 4-min initial isothermal delay. The peaks represent 0.5-1.0 μg of the following compounds: (1) Lactic acid, (2) Oxalic acid, (3) Methylmalonic acid, (4) Phenylacetic acid, (5) Succinic acid, (6) Glutaric acid, (7) Capric acid, (8) Pyroglutamic acid, (9) Phenyllactic acid, (10) p-Hydroxyphenylacetic acid, (11) Tartaric acid, (12) Vanillic acid, (13) Citric acid, (14) p-Hydroxyphenyllactic acid, (15) Glucuronic acid, (16) 5-Hydroxyindole-3-acetic acid, (17) Tricosane, (18) Tetracosane, (19) Androsterone, (20) Dehydroepiandrosterone, (21) β-Estradiol, (22) Pregnanediol, (23) Estriol.

Table 3.1.3 Weight responses and stability studies of methoxime trimethylsilyl derivatives.

The weight response is relative to the internal standard tricosane ($C_{23}H_{48}$). Twenty identical aliquots of each pyridine standard solution were methoximated, silylated, and chromatographed over a 25-h period.

Methoxime trimethylsilyl derivative	Relative weight response	
	Mean (n = 20)	Standard deviation
Oxalic acid	0.34	0.04
Malonic acid	0.52	0.05
β-Phenylpyruvic acid	0.54	0.04
Homoveratric acid	0.65	0.01
17α-Hydroxyprogesterone	0.67	0.03
Pyruvic acid	0.69	0.03
Veratric acid	0.76	0.03
Pyroglutamic acid	0.77	0.03
Methylmalonic acid	0.79	0.02
Vanillic acid	0.85	0.03
Homovanillic acid	0.86	0.01
Glutaric acid	0.86	0.02
Succinic acid	0.88	0.03
Dehydroepiandrosterone	0.88	0.02
Cholesterol acetate	0.88	0.03
Orotic acid	0.90	0.03
Capric acid	0.92	0.02
Androsterone	0.93	0.02
Estrone	0.93	0.02
Citric acid	0.95	0.05
Phenylacetic acid	0.96	0.02
Homogentisic acid	0.96	0.03
Tricosane	1.00	--
5α-Cholestane	1.00	0.01
Lauric acid	1.01	0.03
Suberic acid	1.01	0.03
5-Hydroxyindole-3-acetic acid	1.04	0.03

.....continued

Table 3.1.3 (continued)

Methoxime trimethylsilyl derivative	Relative weight response	
	Mean (n = 20)	Standard deviation
Cholesterol	1.05	0.04
p-Hydroxyphenylacetic acid	1.06	0.03
p-Hydroxyphenyllactic acid	1.08	0.04
Tartaric acid	1.10	0.05
Adipic acid	1.10	0.04
Lactic acid	1.11	0.03
Benzoic acid	1.11	0.02
Phenyllactic acid	1.11	0.03
Vanilmandelic acid	1.11	0.03
Tetracosane	1.11	0.01
Pregnanediol	1.11	0.04
m-Hydroxybenzoic acid	1.12	0.03
Glucuronic acid	1.13	0.03
α-Hydroxyisobutyric acid	1.15	0.02
o-Hydroxybenzoic acid	1.18	0.03
α-Furoic acid	1.18	0.13
β-Estradiol	1.18	0.02
Estriol	1.18	0.03
p-Hydroxymandelic acid	1.20	0.03
Indole-3-acetic acid	1.20	0.03
α-Hydroxyisovaleric acid	1.21	0.01
Malic acid	1.22	0.03
p-Hydroxybenzoic acid	1.26	0.02
Mandelic acid	1.27	0.04
Caffeic acid	1.28	0.03
Gentisic acid	1.30	0.03
3,4-Dihydroxybenzoic acid	1.32	0.02
o-Hydroxyphenylacetic acid	1.33	0.03
Tropic acid	1.34	0.04
m-Hydroxyphenylacetic acid	1.43	0.04
α-Hydroxyisocaproic acid	1.43	0.16
3,4-Dihydroxyphenylacetic acid	1.46	0.03
meso-Tartaric acid	1.55	0.04

homovanillic acid, 0.86). Polyhydroxy compounds, which would be enlarged by additional silyl groups, gave higher weight responses (pregnanediol, 1.11; glucuronic acid, 1.13; β-estradiol, 1.18; estriol, 1.18; 3,4-dihydroxybenzoic acid, 1.32; 3,4-dihydroxyphenylacetic acid, 1.46). Other FID response data for TMS derivatives of organic acids (Sarkar and Malhotra, 1979) and steroids (Shackleton and Honour, 1976) show similar trends. Analysis of these variations in RWR with the chemical nature of the compound showed a reduced response in the presence of oxygen and nitrogen atoms. Since authentic compounds are not always available, these trends can be used to estimate response factors (Edwards, 1978).

3.1.4 Mass spectrometry

The repetitively acquired mass spectra were accumulated on file and later inspected as a group, the computer-plotted chromatogram of the total ion current (Figure 3.1.4.1). The total ion chromatogram showed excellent agreement with the flame ionization chromatogram (Figure 3.1.3.2) with the expected differences in response due to the different methods of ionization. Isolated scans of special interest were inspected individually, such as the spectrum of vanillic acid - diTMS shown in Figure 3.1.4.2. Such histograms of normalized intensity (% I) versus mass to charge ratio (m/e) were analyzed manually for compound identification. Fragmentations related to the molecular ion (parent compound minus one electron) and fragments associated with the trimethylsilyl group have been tabulated by Markey *et al*. (1972). Indices arranged according to the most intense peak within windows of specified m/e (Markey *et al*., 1974) simplified the identification process.

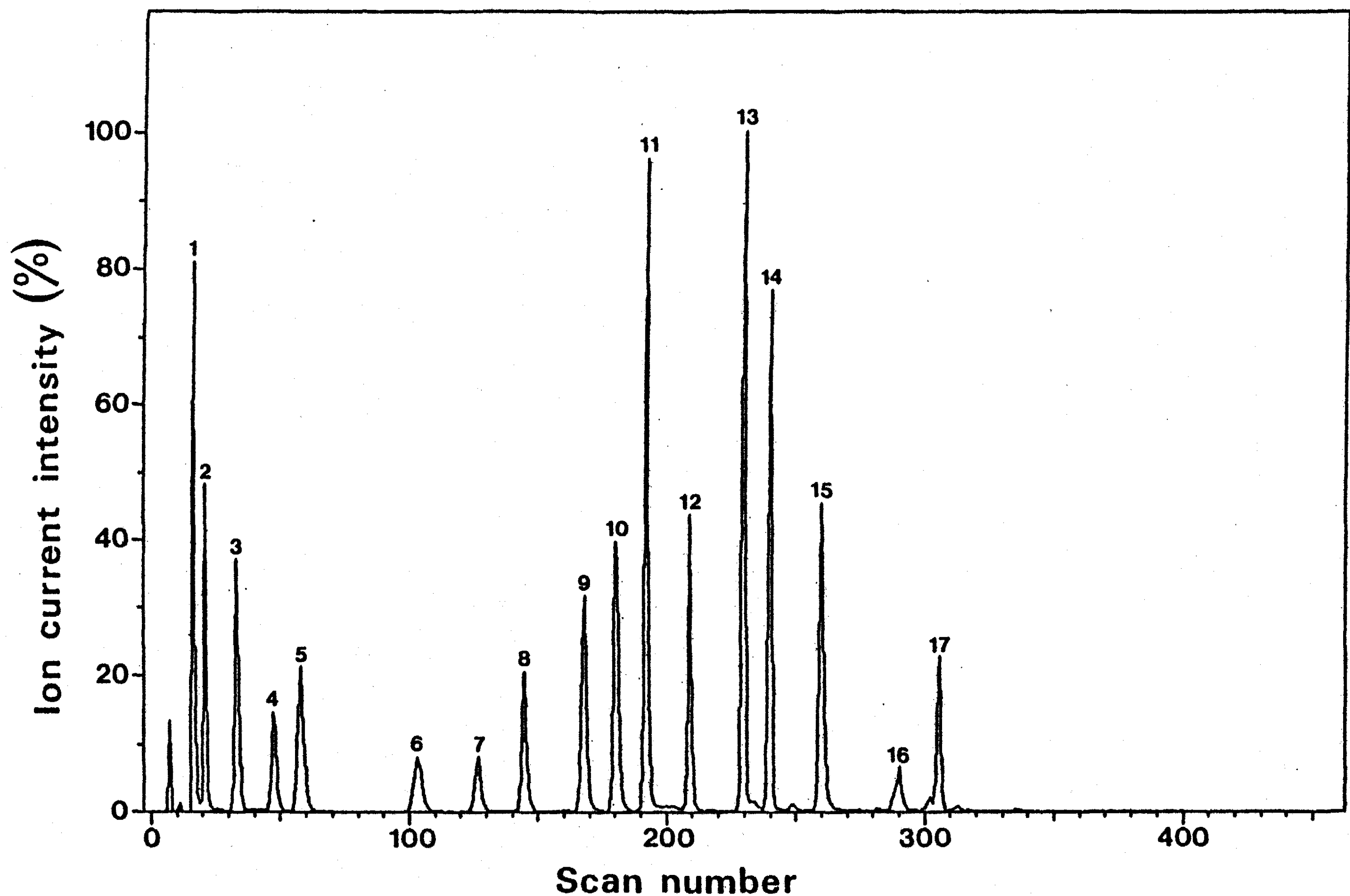


Figure 3.1.4.1 Total ion chromatogram from the mass spectrometric detection of the methoxime trimethylsilyl derivatives of some organic acids. GC separation was on a 6-ft 3% OV-101 column programmed from 100° to 300°C at 4°C/min with an 8-min initial isothermal delay. The peaks represent 0.5-1.0 μg of the following compounds: (1) Lactic acid, (2) Oxalic acid, (3) Methylmalonic acid, (4) Phenylacetic acid, (5) Succinic acid, (6) Glutaric acid, (7) Capric acid, (8) Pyroglutamic acid, (9) Phenyllactic acid, (10) ρ-Hydroxyphenylacetic acid, (11) Tartaric acid, (12) Vanillic acid, (13) Citric acid, (14) ρ-Hydroxyphenyllactic acid, (15) Glucuronic acid, (16) 5-Hydroxyindole-3-acetic acid, (17) Tricosane.

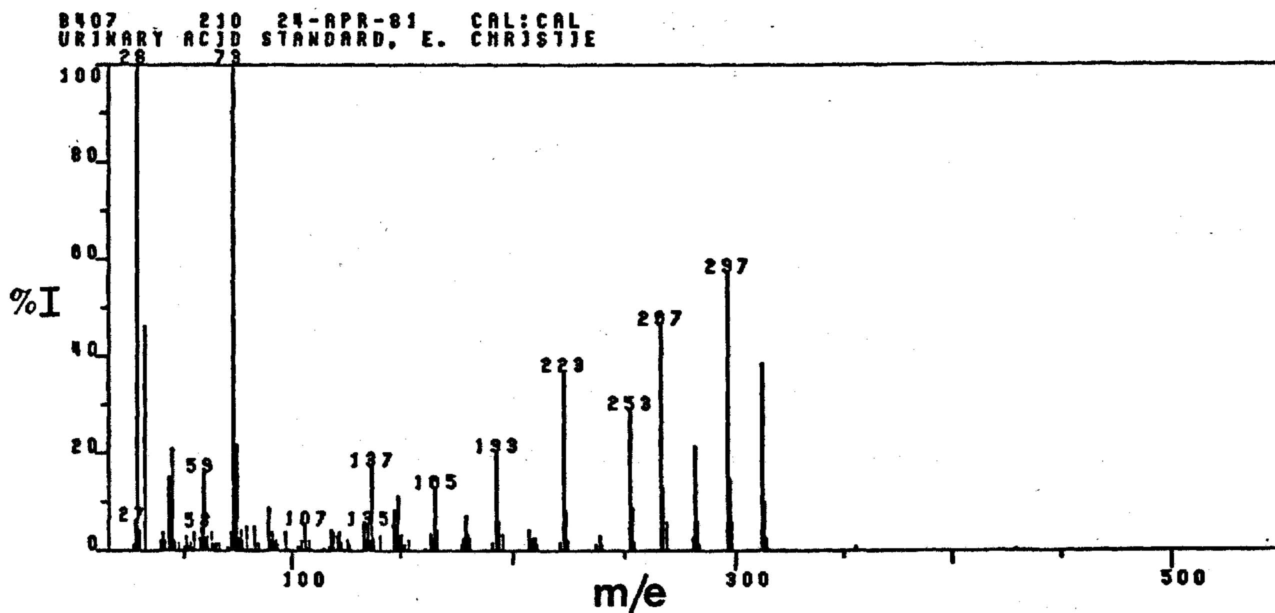
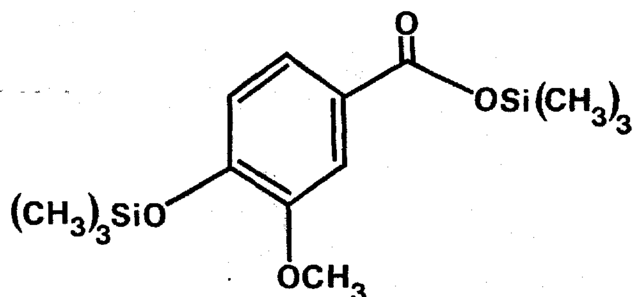


Figure 3.1.4.2 Electron ionization spectrum of vanillic acid-diTMS. This histogram of normalized intensity (%I) versus mass to charge ratio (m/e) corresponds to scan number 210 of the total ion chromatogram shown in Figure 3.1.4.1. The structure of the parent compound, vanillic acid-diTMS (MW 312), and the identities of major peaks are as follows:



m/e 312, molecular ion, M (frequently seen with aromatic compounds)

m/e 297, (M - 15) loss of a TMS methyl

m/e 223, (M - 89) loss of $OSi(CH_3)_3$

m/e 73, trimethylsiliconium ion (base peak to which all other peaks were normalized)

The intensities of the ions associated with a peak varied between scans obtained on the ascending or descending portion of the peak (Mamer et al., 1971).

3.2 Organic acid extraction

The recoveries of fifteen organic acids from aqueous solution by DEAE-Sephadex extraction are listed in Table 3.2. The extraction efficiencies were calculated according to the following equation:

$$\%\ \text{Recovery} = \frac{(\text{Area counts for acid})}{\begin{pmatrix}\text{Area counts}\\ \text{for tricosane}\end{pmatrix}\begin{pmatrix}\text{RWR for}\\ \text{acid}\end{pmatrix}\begin{pmatrix}\text{Weight of acid if}\\ \text{100\% recovery}\end{pmatrix}} \times 100.$$

The relative weight response (RWR) was obtained from Table 3.1.3. The recoveries ranged from 57.7% for pyroglutamic acid to 100% recovery for several acids. The coefficient of variation was less than 10% for all acids except citric acid (10.6%), oxalic acid (15.6%), and pyroglutamic acid (18.2%). These recoveries were considerably higher and more consistent than recoveries obtained by Chalmers and Watts (1972c), who experienced 50% recovery and often 50% coefficient of variation. The recovery experiments, performed over a 2-year period on aqueous acid standards stored at -30°C, showed no volatile artifacts. Figure 3.2 illustrates the gas-liquid chromatographic profile for extraction of glutaric acid by this procedure.

Acids of special interest were subjected to the DEAE-Sephadex procedure to check for chromatographic purity. Extraction of the aldonic acids or their lactones (gluconic acid and gluconic acid δ-lactone; galactonic acid and galactonic acid δ-lactone; ribonic acid and ribonic acid γ-lactone) gave the same equilibrium mixtures of approximately 50%

Table 3.2 Recovery of organic acids from aqueous solution by DEAE-Sephadex extraction. The recoveries were calculated from 15 replicate determinations.

Organic acid	Mean recovery %	Standard deviation %	Coefficient of variation %
Pyroglutamic acid	57.7	10.5	18.2
Lactic acid	79.3	5.5	6.9
Phenylacetic acid	84.4	3.4	4.0
Methylmalonic acid	88.7	3.9	4.4
5-Hydroxyindole-3-acetic acid	89.7	8.8	9.8
Glucuronic acid	90.6	3.6	4.0
Glutaric acid	92.0	7.0	7.6
Oxalic acid	93.4	14.6	15.6
Citric acid	93.7	9.9	10.6
Succinic acid	94.9	4.9	5.2
Phenyllactic acid	97.5	3.6	3.7
Vanillic acid	100.1	2.7	2.7
p-Hydroxyphenylacetic acid	102.2	2.8	2.7
Tartaric acid	102.9	5.5	5.3
p-Hydroxyphenyllactic acid	103.9	2.0	1.9

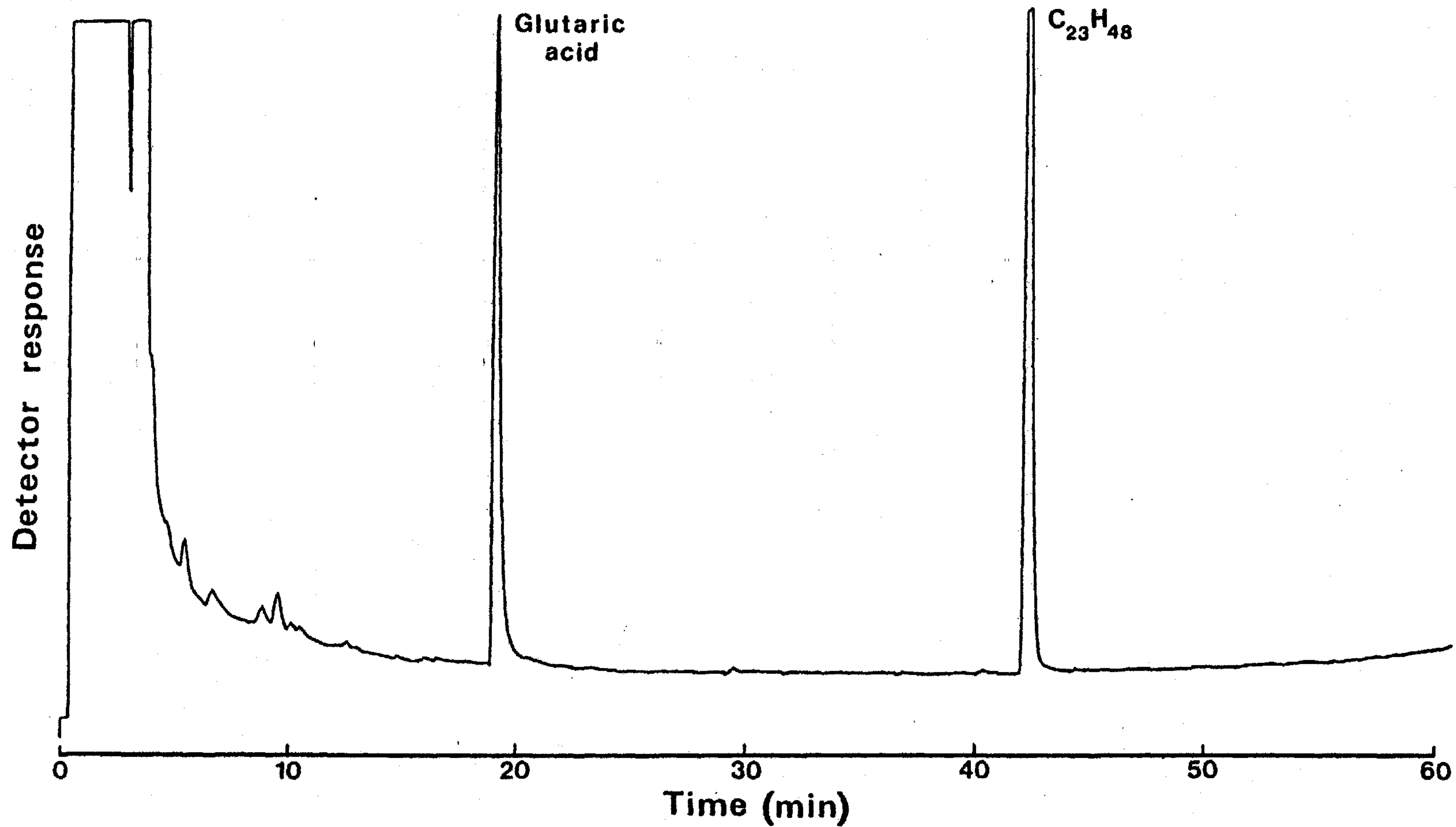


Figure 3.2 Gas-liquid chromatographic profile of glutaric acid extracted from an aqueous solution by DEAE-Sephadex. Glutaric acid was chromatographed as its diTMS derivative. Tricosane ($C_{23}H_{48}$) was the internal standard. Analysis was on a 6-ft 3% OV-101 column programmed from 70° to 300°C at 4°C/min with a 4-min initial isothermal delay.

acid, 50% lactone. Other workers noticed the same effect with deoxypentonic acids (Truscott et al., 1979a) and tetronic or deoxytetronic acids (Thompson et al., 1975). This effect was also observed with direct derivatization of the compounds (see Results, p.43) so it was unclear when the equilibration between forms occurred. Conversely, extraction of glucuronic acid γ-lactone produced more than 90% free glucuronic acid. Ascorbic acid extraction produced several peaks as would be expected from its tendency to undergo oxidation accelerated by alkalies (Windholz, 1976). Conversely, ascorbic acid-2-sulfate was extracted intact.

3.3 Steroid extraction

Overall recovery of the steroid extraction procedure was determined by carrying out the XAD-2 extraction, conjugate hydrolysis, and re-extraction without establishing the recoveries of individual steps. Buffer pH for deconjugation was varied between pH 4.0 and 6.0 as illustrated in Figure 3.3.1. For routine analyses the buffer used was pH 4.5 since pregnanediol recovery decreased with more alkaline buffers. Leon et al. (1960) identified the general pH optimum of 4.0 - 5.0 for enzymes from *Helix pomatia*. The entire procedure was tested against distilled water as shown in Figure 3.3.2 which illustrates the purity of the steroid extraction method. Artifacts due to *Helix pomatia* were not evident. Decomposition products and artifacts are reported to be minimal for *Helix pomatia* compared to other enzyme deconjugation methods (Curtius and Muller, 1967; Roy and Slaunwhite, 1970; Manson et al., 1972). The recoveries of steroids from an aqueous solution of steroid conjugates are listed in Table 3.3. The recoveries ranged from 64% for pregnanediol released from its 3α-glucuronide, to 100% recovery of estradiol and estriol

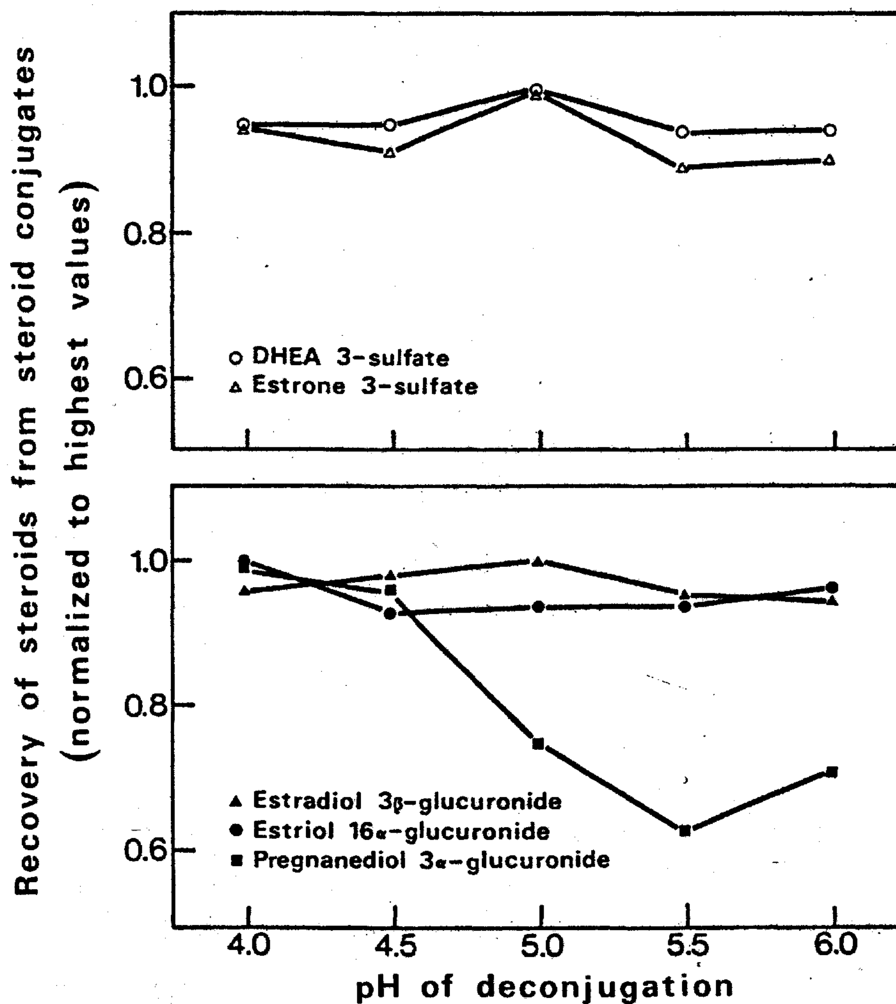


Figure 3.3.1 pH activity profiles of β-glucuronidase and sulfatase (*Helix pomatia*) on different substrates. Deconjugation was with *Helix pomatia* crude solution in 0.5 M sodium acetate buffer; 20 h at 37°C. Each point represents an average of three determinations.

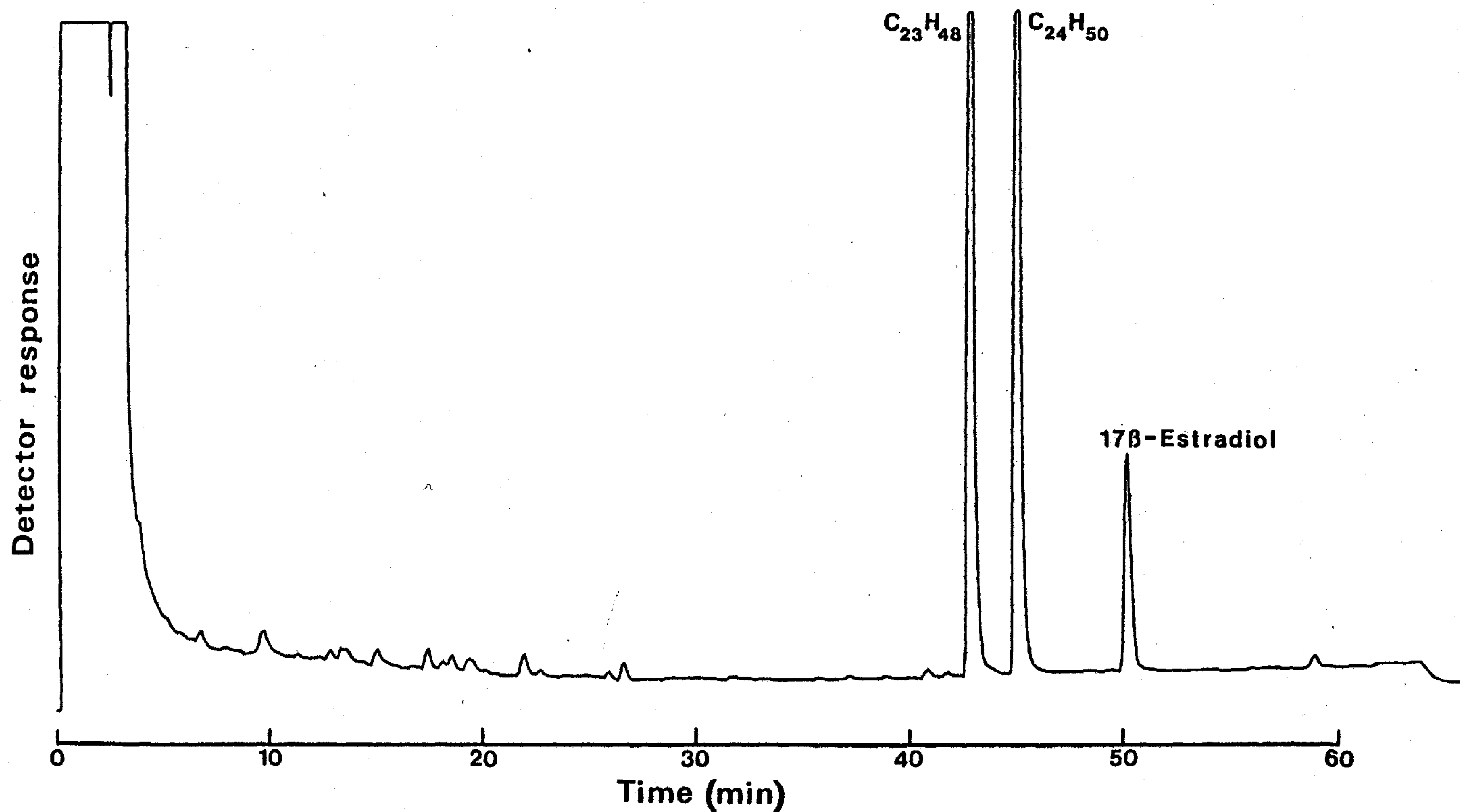


Figure 3.3.2 Gas-liquid chromatographic profile of 17β-estradiol extracted from an aqueous solution of 17β-estradiol 3β-glucuronide. Deconjugation was with *Helix pomatia* crude solution. Free 17β-estradiol was recovered by XAD-2 extraction and chromatographed as its diTMS derivative. Tricosane ($C_{23}H_{48}$) and tetracosane ($C_{24}H_{50}$) were the internal standards. Analysis was on a 6-ft 3% OV-101 column programmed from 70° to 300°C at 4°/min.

Table 3.3 Recovery of steroids from an aqueous solution of steroid conjugates by enzymatic deconjugation and subsequent XAD-2 extraction. The recoveries were calculated from triplicate determinations.

Deconjugation was with *Helix pomatia* crude solution containing β-glucuronidase and sulfatase activity (0.5 M sodium acetate buffer, pH 4.5; 20 h at 37°C.)

Steroid conjugate	Mean recovery %	Standard deviation %	Coefficient of variation %
Pregnanediol 3α-glucuronide	64.2	5.1	7.9
Estrone 3-sulfate	76.3	2.8	3.7
Dehydroepiandrosterone 3-sulfate	77.4	4.5	5.8
Estradiol 3β-glucuronide	97.0	3.1	3.2
Estriol 16α-glucuronide	107.8	3.5	3.2

from their glucuronide conjugates. The coefficient of variation was less than 10% for all steroids investigated. A major cause of loss is almost certainly inefficient conjugate hydrolysis, especially of the steroid sulfates. Sulfatases have a high degree of specificity toward the steroid part of the molecule (Roy, 1970) with the result that certain sulfates are probably not hydrolyzed (Leon et al., 1960). Other workers have routinely experienced 90% recoveries for most steroids by XAD-2 extraction procedures (Bradlow, 1968; Shackleton et al., 1970; Matsui et al., 1975; Leunissen and Thijssen, 1978).

3.4 Profiles of normal pregnant and nonpregnant women

3.4.1 Dietary information

Each participant completed the diet recall and diet diary forms (Appendices D and E). The prenatal record (Appendices F and G) also contained information concerning medications. These sources were reviewed for unusual or extreme dietary habits. One nonpregnant woman consumed 12 cups of coffee during the 24-hour collection period. Table 3.4.1 summarizes the drug status of the participants. Sixty percent of the pregnant women took a vitamin-mineral supplement while forty percent ingested iron salts. Only one woman did not take any dietary supplements throughout her pregnancy. Several women ingested iron salts in addition to vitamin-mineral supplements, leading to an intake of more than 100 mg of elemental iron per day. Antibacterial and antifungal agents were prescribed for three pregnant women. Occasional use of antihistaminics and analgesics was noted, use being more frequent in nonpregnant women. One nonpregnant woman consumed five ounces of alcohol.

Table 3.4.1 Drug status of the normal pregnant and nonpregnant women during the 24-hour collection.

The table indicates the number of subjects in each therapeutic category. The vitamin-mineral supplements contained the following: vitamin A (1,600 - 10,000 I.U.), vitamin D (200 - 400 I.U.), vitamin C (50 - 150 mg), iron (4 - 60 mg), calcium (0 - 134 mg), folic acid (0 - 5 mg), thiamine (0 - 3 mg), riboflavin (0 - 5 mg), niacinamide (0 - 25 mg), pyridoxine (0 - 1 mg), cyanocobalamin (0 - 3 mg).

Therapeutic category (Brand name or compound name)	Normal pregnancy (n = 25)		Normal nonpregnant (n = 15)
	12½ - 15½ wk	24½ - 27½ wk	
Vitamin-mineral supplement (Prenavite, Orifer-F, Paramettes)	15	16	1
Iron salts (ferrous gluconate or sulfate)	10	11	-
Antibacterial (Penicillin V)	-	2	-
Antifungal (Monistat)	1	1	-
Antiseptic-antifungal (domiphen bromide lozenges)	-	1	-
Antihistaminic (Chlor-Tripolon, Dimetapp, Contac-C)	1	1	2
Analgesic, antipyretic (acetaminophen, acetylsalicylic acid)	-	1	2
Alcohol (more than 2 oz.)	-	-	1

3.4.2 Urinary creatinine excretion

Urinary creatinine was determined by the color reaction between creatinine and picrate in a strong alkaline medium. The mechanism for this reaction probably involves the carbonyl group of the creatinine molecule (Kammeraat, 1978). The presence of several interfering substances possessing a carbonyl group (glucose, fructose, protein, keto acids) could cause spuriously elevated values in urinary creatinine determinations. The method of Yatzidis (1974, 1975) measures the difference in absorbance at 500 nm of two alkaline picrate reagents, thus subtracting the contribution of noncreatinine chromogens to give a true creatinine concentration.

Absorbance at 500 nm was linear with creatinine concentration from 0.5 to 20 mg/dl. Results of 25 urine analyses by the Yatzidis method showed an excellent correlation ($r = 0.993$) with results obtained by an automated sequential multiple analyzer (SMA-6). The SMA-6 continuous-flow method used dialysis to remove interfering noncreatinine chromatogens (Kammeraat, 1978).

Creatinine is a waste product of creatine metabolism. Ninety-eight percent of the total body creatine is in skeletal muscle and 2% of this is converted each day to creatinine (Borsook and Dubnoff, 1947). Thus, creatinine excretion is a function of muscle mass and is roughly proportional to body weight. Creatinine excretion (mg/24 h) did not change significantly with pregnancy (Table 3.4.2). A significant decrease ($P < 0.05$) was observed as pregnancy progressed when the creatinine excretion was expressed as the creatinine coefficient (mg/kg body weight/24 h). The average weight gain between collection periods for the 25 pregnant

Table 3.4.2 Urinary creatinine excretion in normal pregnant and nonpregnant women.

Group	N	Age range (years)	Mean age (years)	24-hour sample collection: time since last menses began (weeks)	Creatinine excretion/24 hour (Mean ± SD)			
					mg	mg/kg BW	mg/m^2 BSA	mg/prepregnancy m^2 BSA
Normal nonpregnant	15	19-39	27	1	1120 ± 260	18.9 ± 1.9	690 ± 100	690 ± 100
Normal pregnant	25	23-32	27	i) 12½ - 15½	1160 ± 190	19.7 ± 3.0*	710 ± 100	710 ± 100
				ii) 24½ - 27½	1210 ± 230	18.6 ± 3.5*	710 ± 130	750 ± 130

N = Number of participants
BW = Body weight
BSA = Body surface area (Du Bois and Du Bois, 1916)
*Significant difference by paired *t*-test ($P < 0.05$)

women was 6.7 $\pm$ 1.7 kg (mean $\pm$ SD). The components of this weight gain (conceptus, maternal fat, maternal blood volume) do not contribute to creatinine production (Clark *et al.*, 1951). Since calculations incorporating changes of body weight in pregnancy are illogical, Lindheimer and Katz (1977) suggested that excretion data in pregnancy should be expressed relative to the prepregnancy body surface area. Body surface area increased in the 25 pregnant women from a prepregnancy value of 1.62 $\pm$ 0.10 m^2 (mean $\pm$ SD) to values of 1.63 $\pm$ 0.10 m^2 and 1.71 $\pm$ 0.11 m^2 at the first and second collection intervals respectively. Body surface area for the 15 nonpregnant women was 1.61 $\pm$ 0.17 m^2. Creatinine excretion expressed relative to the present or prepregnancy body surface area was not significantly different in the three groups investigated (Table 3.4.2). Since the subjects were reasonably homogeneous in terms of body size (comparable prepregnancy body surface areas) and age (mean $\pm$ SD : 27 $\pm$ 3y for pregnant women, 27 $\pm$ 6y for nonpregnant women), it was decided that further evaluation of creatinine excretion would be appropriate using the units of g/24 h.

Creatinine excretion in the nonpregnant women was 1.12 $\pm$ 0.26 g/24 h (mean $\pm$ SD, n = 15) and in the pregnant women was 1.18 $\pm$ 0.21 g/24 h (mean $\pm$ SD, n = 50). Investigators generally agree that creatinine excretion does not change with pregnancy (Clark *et al.*, 1951), although few investigators have evaluated pregnant and nonpregnant groups simultaneously. Reported values for creatinine excretion include (g/24 h, mean $\pm$ SD) : 1.03 $\pm$ 0.39, n = 14, nonpregnant (Peters *et al.*, 1972); 1.17 $\pm$ 0.08, n = 10, nonpregnant (Vestergaard and Leverett, 1958); 1.25 $\pm$ 0.21, n = 19, nonpregnant (Clark *et al.*, 1951); 1.21 $\pm$ 0.18, n = 242, 12 - 40 weeks gestation (Clark *et al.*, 1951); 1.21 $\pm$ 0.20, n = 19, 20 - 40

weeks gestation (Chattaway et al., 1969).

Some individuals show great constancy of 24-hour urinary creatinine excretion, while for others the daily excretion must be rated as inconsistent (Vestergaard and Leverett, 1958; Scott and Hurley, 1968; Edwards et al., 1969). Generally, creatinine excretion in an individual rarely varies more than 10 to 15% and when it does, a collection error is usually identified (Lindheimer and Katz, 1977). Of the 25 pregnant women investigated, there were four whose daily creatinine excretion was greater at the second collection by more than 15% (by 21%, 26%, 28%, and 31%), as shown in Figure 3.4.2.1. Daily creatinine output does not depend on urinary volume (Cramer et al., 1967). The 65 samples had volumes of between 0.380 and 2.435 l (1.32 ± 0.43 l, mean ± SD). A highly significant inverse correlation ($r = -0.790$, $p < 0.001$) was observed between 24-hour urine volume and creatinine concentration (Figure 3.4.2.2). Metcalf (1977) observed an identical correlation ($r = -0.780$) for 81 samples of nonpregnancy urine. An incomplete 24-h sample would be indicated by a value in the lower left segment of Figure 3.4.2.2 (low urine volume and low creatinine concentration). The two points farthest below the correlation line (0.585 l, 1.03 g/l and 0.820 l, 0.74 g/l) represent creatinine excretion in the same woman at 13½ weeks and 27½ weeks of pregnancy. This would tend to indicate that the woman is a low excretor of creatinine (consistently 0.60 g/24 h, also see Figure 3.4.2.1), but one cannot dismiss the possibility of uniform losses of a portion of the total 24-hour sample. Incomplete collection would be implicated if the 24-hour excretion of other urinary metabolites was also below the average value expected. Some pregnancies may be

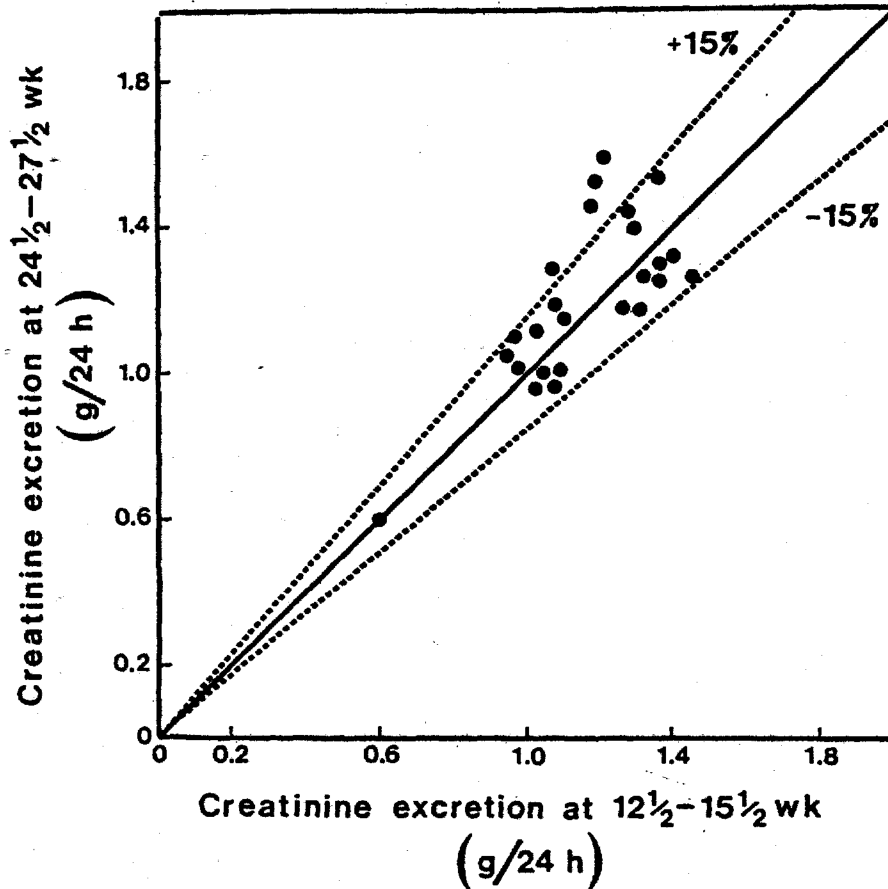


Figure 3.4.2.1 Comparison of 24-h creatinine excretion at two time intervals in pregnancy for 25 women. Points falling on the solid line would represent a perfect correlation. Dotted lines enclose the excretion values differing by less than ±15%, the approximate normal intra-individual variation in daily creatinine excretion.

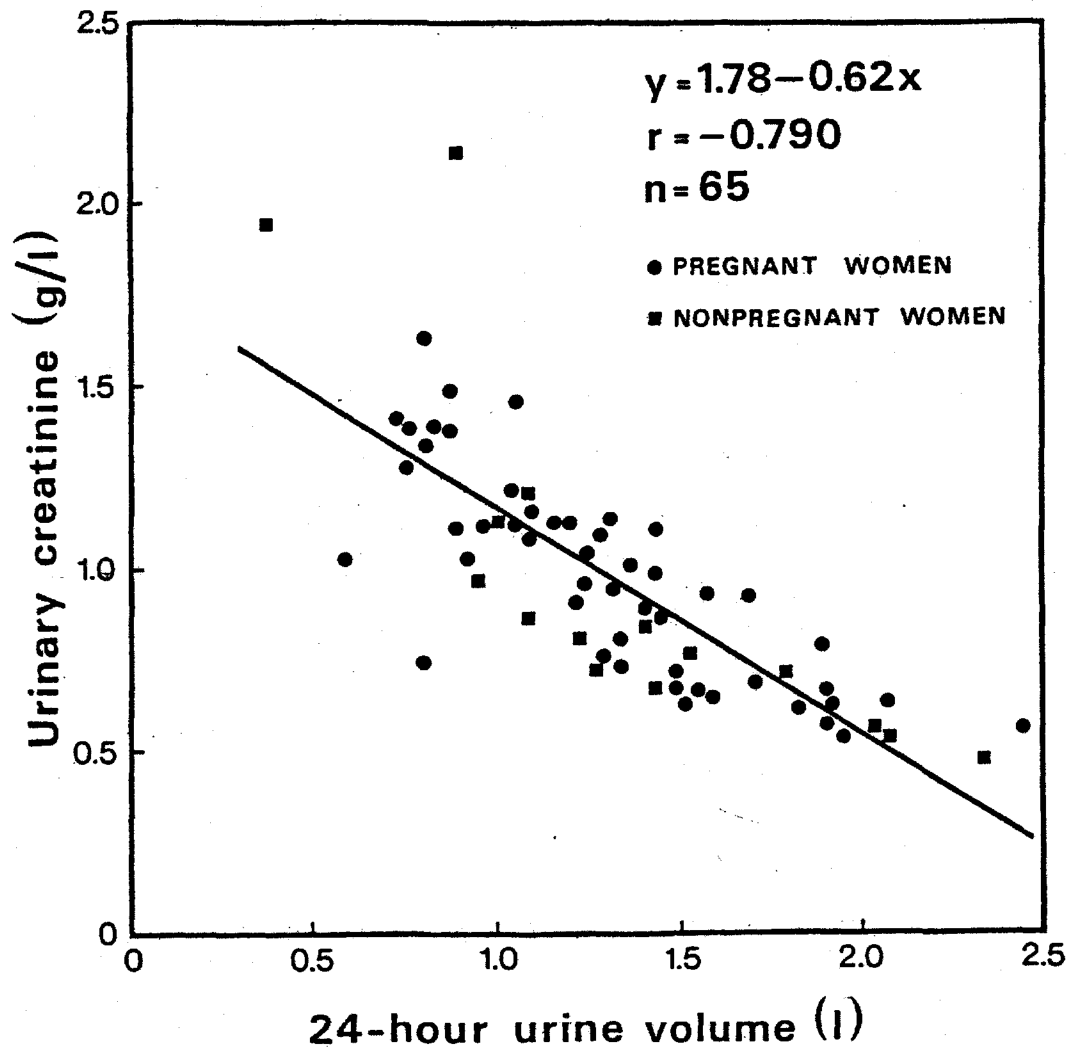


Figure 3.4.2.2 Comparison between the volume and creatinine concentration of 24-h urine specimens collected from normal pregnant and nonpregnant women. The data represent two collections from each of 25 pregnant women and single collections from 15 nonpregnant women.

associated with diminished creatinine excretion since creatine excretion may be increased in pregnancy (Clark et al., 1951). Renal changes associated with hypertension during pregnancy may also decrease the 24-h urinary creatinine excretion (Legge, 1980). Chattaway et al. (1969) investigated 10 pregnant women with pre-eclamptic toxemia at 32 - 40 weeks gestation and reported creatinine excretion of 0.79 ± 0.49 g/24 h (mean ± SD). Failure to empty the bladder completely due to urinary tract dilatation and increased urinary tract dead space may cause errors in the collection of timed urine samples in pregnancy (Lindheimer and Katz, 1977).

Samples deviating above the correlation line in Figure 3.4.2.2 (for example, 0.89 l with 2.14 g creatinine/l) may represent more than a 24-h collection. A high-creatine meat diet (Barrett and Addis, 1947) and intense physical exercise (Doolan et al., 1962) may each increase urinary creatinine excretion by 10%. Some authors report an increase in creatinine excretion during a 24-h fast (Tocci et al., 1972) while others have reported a decrease (Peters et al., 1972). Large elevations of urinary creatinine have been observed in schizophrenic patients during a transient psychotic episode (Pscheidt et al., 1966), indicating that emotional state may influence creatinine metabolism. Increased urinary 17-ketosteroid excretion (principally dehydroepiandrosterone, etiocholanolone, and androsterone) has been associated with elevated urinary creatinine excretion, suggesting hormonal control of muscle mass and thus creatinine output (Clark et al., 1951).

Despite the many factors influencing creatinine metabolism, the results above indicate that creatinine excretion did not change with

pregnancy. The daily excretion of creatinine was fairly constant as judged by intra-individual and inter-individual comparisons. Considering this constancy and the fact that instructions for specimen collection were stressed by written and verbal directions, we can be reasonably confident that the samples represent complete 24-h collections. For future reference, Figures 3.4.2.1 and 3.4.2.2 could be used to estimate the completeness of 24-h collections. Samples with a low creatinine content and below average content of other urinary metabolites probably represent incomplete collections. Excretion values in those cases could be corrected to give an equivalent of 1.0 g of creatinine per 24 hours. Knuppel _et al._ (1979) examined 489 urine collections in pregnancy and suggested that 24-h urine volumes less than 500 ml be corrected to give 1.08 g of creatinine per 24 hours.

3.4.3 Urinary organic acid excretion

Each urinary specimen was assayed in duplicate by the organic acid extraction method. An aliquot of aqueous glutaric acid was added to one sample, while aqueous β-phenylpyruvic acid was added to the other sample. These acids were chosen as recovery standards because they eluted in uncrowded portions of the urinary acid chromatogram. Any urinary peaks coeluting with these recovery standards were expressed relative to another urinary peak and subtracted to give the corrected area for each recovery standard. The recovery of glutaric acid (a dicarboxylic acid) was 95.1 ± 8.3% (mean ± SD, n = 76). The recovery of β-phenylpyruvic acid (a keto, monocarboxylic acid) was 95.6 ± 7.6% (mean ± SD, n = 76).

The urinary acid chromatograms contained several distinct peaks,

as well as many peaks which were poorly resolved from the major constituents. The complex chromatograms were interpreted by a series of calculations. The preliminary visual comparison of chromatograms was followed by the tabulation of peak areas into 50 recognizable peaks. Peaks which were not consistently resolved were combined into one peak report, which of course represented more than one recognizable component. All component areas were corrected to give 100% recovery by the glutaric acid or β-phenylpyruvic acid recovery data. The component areas were then expressed relative to the internal standard (*n*-tricosane) and corrected for total 24-h urine volume. Final results were expressed in terms of relative amount of component/24 h. The relative amount/24 h was transformed to mg/24 h by a conversion factor which could be applied when peak identity was confirmed. The conversion factor considered the extraction efficiency as well as the FID response factor unique for each compound. Figure 3.4.3.1 summarizes the series of calculations involved in the quantitation of identified metabolites. Table 3.4.3.1 lists the conversion factors for several organic acids. Not surprisingly, acids of similar structure had similar conversion factors, reflecting comparable extraction efficiencies and chromatographic characteristics.

The 2-μl injection produced a chromatogram of the constituents in an aliquot of urine containing 4 μg of creatinine (the equivalent of 1 mg of creatinine was extracted, while only 1/250 of the derivatized extract was injected into the gas chromatograph). A chromatogram of the acidic metabolites extracted from the urine of a normal pregnant woman at the 27th week of gestation is shown in Figure 3.4.3.2. The corresponding total ion chromatogram from mass spectrometric analysis

The quantitation of identified metabolites was calculated according to the following formula:

$$\text{mg/24 h} = \text{Relative amount/24 h} \times \text{Conversion factor}$$

$$\text{Relative amount/24 h} = \frac{\text{Area counts for compound}}{\left(\begin{array}{c}\text{Area counts}\\ \text{for tricosane}\end{array}\right)\left(\begin{array}{c}\text{Recovery of glutaric}\\ \text{or } \beta\text{-phenylpyruvic acid}\end{array}\right)} \times \frac{\begin{array}{c}\text{24-h urine}\\ \text{volume (ml)}\end{array}}{\begin{array}{c}\text{Urine volume}\\ \text{extracted (ml)}\end{array}}$$

$$\text{Conversion factor} = \frac{1}{\left(\begin{array}{c}\text{Extraction}\\ \text{efficiency}\\ \text{of compound}\end{array}\right)} \times \frac{1}{\left(\begin{array}{c}\text{Relative weight}\\ \text{response}\\ \text{of compound}\end{array}\right)} \times \frac{\text{Derivatization volume } (\mu l)}{\text{Injection volume } (\mu l)} \times \frac{1 \text{ mg}}{1000\ \mu g}$$

Extraction efficiency of compound = see Table 3.2, page 64

Relative weight response of compound = RWR = see Table 3.1.3, page 58

Derivatization volume = 500 μl

Injection volume = 2 μl

Where the extraction efficiency or relative weight response of the compound was not known, an estimate was made by consideration of structural similarities with other compounds.

Figure 3.4.3.1 Quantitation of identified metabolites.

Table 3.4.3.1 Conversion factors for organic acids. (See Figure 3.4.3.1 for an explanation of the conversion factor.)

Organic acid	Type of acid	Conversion factor
Oxalic	dicarboxylic	0.787
Pyroglutamic	amido, monocarboxylic	0.563
Methylmalonic	dicarboxylic	0.357
Glutaric	dicarboxylic	0.316
Phenylacetic	phenyl, monocarboxylic	0.309
Succinic	dicarboxylic	0.299
Vanillic	methoxy, hydroxy, phenyl, monocarboxylic	0.294
Lactic	hydroxy, monocarboxylic	0.284
Citric	hydroxy, tricarboxylic	0.281
5-Hydroxyindole-3-acetic	hydroxy, indole, monocarboxylic	0.268
Glucuronic	aldehydo, hydroxy, monocarboxylic	0.244
Phenyllactic	hydroxy, phenyl, monocarboxylic	0.231
p-Hydroxyphenylacetic	hydroxy, phenyl, monocarboxylic	0.231
p-Hydroxyphenyllactic	hydroxy, phenyl, monocarboxylic	0.223
Tartaric	hydroxy, dicarboxylic	0.221

is shown in Figure 3.4.3.3. Ten urinary acidic metabolites were identified by their mass spectra which matched previously published spectra (Markey et al., 1972; Markey et al., 1974; Thompson et al., 1975). While approximately 50 spectra were deemed to be of special interest, only the prominent peaks in the chromatogram were unequivocally identified. These prominent peaks were more easily identified due to their single component nature. In contrast, multicomponent peaks were difficult to interpret due to superimposed spectra (Mamer et al., 1971). The 4-carbon hydroxy-acid isomers erythronic acid and threonic acid gave identical spectra (Thompson et al., 1975). Ion intensities also varied according to the portion of the peak scanned. Background spectra due to the stationary phase and derivatizing reagents also probably complicated the mass spectral data (Brooks and Middleditch, 1971). The linear dynamic range of the mass spectrometer was narrower than desired. As a result, there was a tendency for major urinary components to overload the mass spectrometry system, while minor components were not identified. The introductory analyses performed assured us that the system was working correctly. Further analyses to identify minor urinary acidic metabolites should be performed on concentrated samples after removal of excess reagents.

Tables 3.4.3.2, 3.4.3.3, and 3.4.3.4 contain a complete list of the urinary acidic metabolites as well as 24-h excretion data for the pregnant and nonpregnant women. Columns in the tables indicate the possible identity of a peak (determined by coinjection of authentic acids), the confirmed identity of a peak (determined by mass spectrometry), and the identity of a peak according to other workers (Lawson et al. 1976;

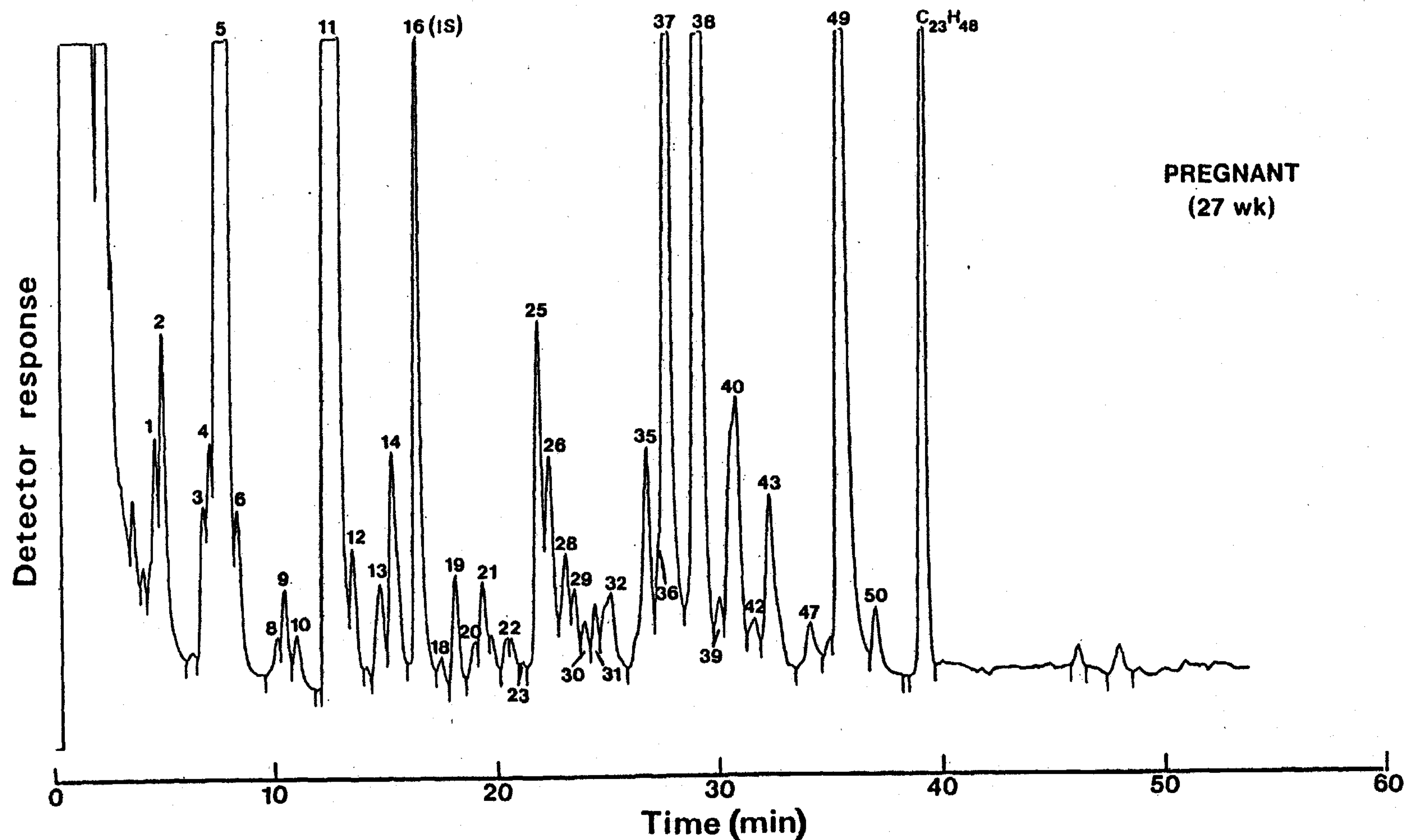


Figure 3.4.3.2 Gas-liquid chromatogram of acidic metabolites extracted from the urine of a normal pregnant woman at the 27th week of gestation. The methoxime trimethylsilyl derivatives were analyzed on a 6-ft 3% OV-101 column programmed from 70° to 300°C at 4°C/min with a 4-min initial isothermal delay. Injection volume was 2 µl. Amplification was 10 x 16 (via electronic integrator). The chromatogram represents an aliquot of urine equivalent to 4 µg of creatinine. Peak numbers correspond to peak identification given in Tables 3.4.3.2, 3.4.3.3, and 3.4.3.4. Peak 16 was the internal standard (IS) glutaric acid, used for sample recovery calculations. Excretion data were expressed relative to tricosane ($C_{23}H_{48}$).

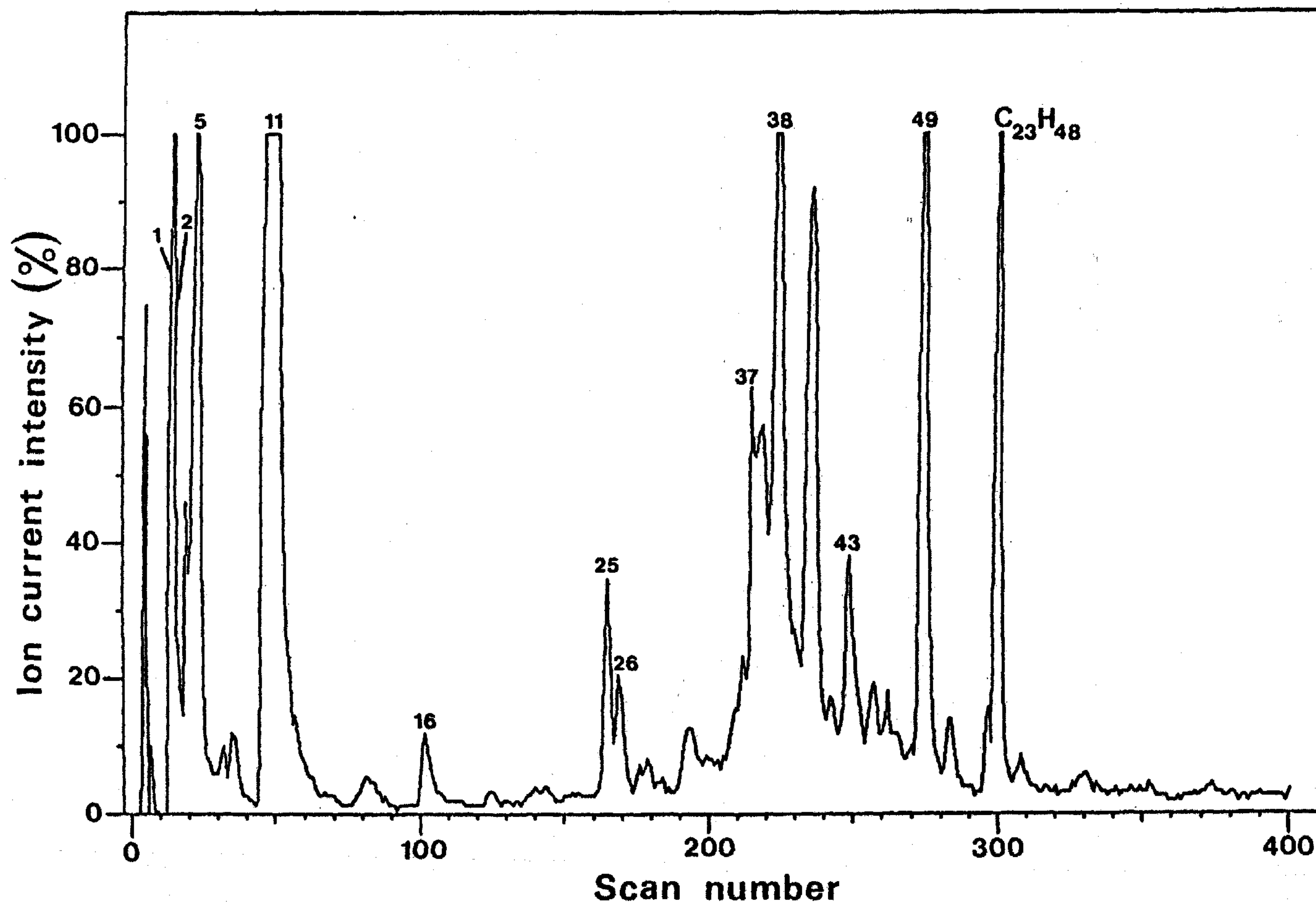


Figure 3.4.3.3 Total ion chromatogram from the mass spectrometric detection of the methoxime trimethylsilyl derivatives of the acidic metabolites extracted from the urine of a normal pregnant woman at the 27th week of gestation. GC separation was on a 6-ft 3% OV-101 column programmed from 100° to 300°C at 4°C/min with an 8-min initial isothermal delay. Peak numbers correspond with the gas-liquid chromatogram of the same sample shown in Figure 3.4.3.2. Peak numbers represent metabolites identified by their mass spectra as the following: (1) Lactic acid, (2) Glycolic acid, (5) Sulfate, (11) Phosphate, (16) Glutaric acid recovery standard, (25) and (26) Erythronic or Threonic acid, (37) Hippuric acid, (38) Citric acid, (43) Glucuronic acid, (49) Uric acid.

Table 3.4.3.2 Acidic metabolite excretion in normal pregnant and nonpregnant women. Group 1: Metabolites detected in all samples and fully quantitated.

The possible identities of peaks were determined by coinjection of derivatized acid standards. Unequivocal confirmation of peak identity was achieved only by gas chromatography-mass spectrometry (GC-MS).

The 24-h excretion data were calculated relative to the internal standard tricosane. An unpaired *t*-test was used to compare excretion in nonpregnant women to excretion in pregnant women at each stage of pregnancy. The significance levels are indicated in the columns of pregnancy data. A paired *t*-test compared the excretion data of pregnancy.

*P < 0.05
**P < 0.01
***P < 0.001
§ Compounds identified by other workers (Lawson et al., 1976; Petersson, 1977; Cohn et al., 1978).

Peak number	MU value	Possible peak identity (authentic acids coinjected)	Confirmed identity by GC-MS	Compounds identified by other workers§	Relative amount/24 h (mean ± SD) Nonpregnant (n = 15)	Pregnant (n = 25) 12½-15½ wk	24½-27½ wk	Paired *t*-test
1	10.63	Lactic acid	Lactic acid	Lactic acid	77 ± 76	227 ± 277*	416 ± 324***	*
2	10.74	Glycolic acid	Glycolic acid	Glycolic acid	154 ± 94	326 ± 99***	380 ± 122***	*
3	11.26	Oxalic acid	-	Oxalic acid	149 ± 75	151 ± 89	164 ± 79	
8	12.15	Methylmalonic acid	-	Methylmalonic acid β-Hydroxyisovaleric acid	73 ± 26	103 ± 40*	100 ± 32**	
10	12.41	Levulinic acid	-	-	70 ± 35	59 ± 31	78 ± 42	
12	13.15	Succinic acid	-	Succinic acid Pyrotartaric acid	208 ± 63	257 ± 71*	259 ± 61*	
13	13.51	Glyceric acid Pyrrole-2-carboxylic acid Fumaric acid	-	Glyceric acid	89 ± 52	131 ± 70	124 ± 53*	
14	13.67 and 13.74	Pipecolic acid	-	4-Deoxyerythronic acid 4-Deoxythreonic acid	223 ± 96	328 ± 89**	490 ± 122***	***
17	14.12	-	-	-	39 ± 30	71 ± 44*	61 ± 36	
19	14.56	Capric acid	-	2-Deoxytetronic acid	74 ± 19	112 ± 48**	124 ± 49***	

....continued

Table 3.4.3.2 (continued)

Peak number	MU value	Possible peak identity (authentic acids coinjected)	Confirmed identity by GC-MS	Compounds identified by other workers§	Relative amount/24 h (mean ± SD) Nonpregnant (n = 15)	Pregnant (n = 25) 12½–15½ wk	24½–27½ wk	Paired t-test
21	15.02 to 15.11	Salicylic acid Adipic acid Acetopyruvic acid Malic acid Pyroglutamic acid	-	Adipic acid Pyroglutamic acid	107 ± 84	105 ± 52	123 ± 56	
22	15.34 to 15.49	3-Methyladipic acid	-	5-Hydroxymethyl- 2-furoic acid	54 ± 31	80 ± 57	98 ± 55**	
25	15.82	α-Ketoglutaric acid β-Phenyllactic acid	Erythronic or threonic acid	Erythronic acid α-Ketoglutaric acid	373 ± 84	427 ± 107	496 ± 115***	**
26	15.99	m-Hydroxyphenylacetic acid Pimelic acid	Erythronic or Threonic acid	Threonic acid m-Hydroxyphenylacetic acid	278 ± 174	307 ± 135	310 ± 154	
28	16.27	β-Hydroxy-β-methylglutaric acid ρ-Hydroxyphenylacetic acid	-	β-Hydroxy-β-methyl- glutaric acid p-Hydroxyphenylacetic acid	114 ± 55	136 ± 80	151 ± 73	
32	16.92 to 17.03	3-Indoxylsulfuric acid Suberic acid Ribonic acid γ-lactone	-	Suberic acid Deoxypentonic acids	149 ± 38	205 ± 96*	218 ± 85**	
35	17.62	Homovanillic acid β-Glycerophosphoric acid *cis*-Aconitic acid	-	Homovanillic acid *cis*-Aconitic acid	268 ± 72	310 ± 84	386 ± 179*	
39	18.91	Vanilmandelic acid Sebacic acid ρ-Hydroxyphenylpyruvic acid	-	Vanilmandelic acid m-Hydroxyphenyl- hydracrylic acid	112 ± 19	107 ± 36	108 ± 38	
40	19.11 to 19.32	ρ-Hydroxyphenyllactic acid Glucuronic acid γ-lactone Galactonic acid δ-lactone Gluconic acid δ-lactone Indole-3-acetic acid	-	Gluconic acid δ-lactone Deoxyhexonic acids	557 ± 281	557 ± 177	592 ± 221	
43	19.89	Ascorbic acid-2-sulfate Ascorbic acid Glucuronic acid Undecanedioic acid	Glucuronic acid	Glucuronic acid	327 ± 121	389 ± 145	411 ± 141	

.....continued

Table 3.4.3.2 (continued)

Peak number	MU value	Possible peak identity (authentic acids coinjected)	Confirmed identity by GC-MS	Compounds identified by other workers§	Relative amount/ 24 h (mean ± SD)			Paired *t*-test
					Nonpregnant (n = 15)	Pregnant (n = 25)		
						12½-15½ wk	24½-27½ wk	
47	20.59	Galactonic acid Glucaric acid Gluconic acid	-	Glucaric acid Gluconic acid	126 ± 74	135 ± 67	115 ± 74	
50	22.01	5-Hydroxyindole-3-acetic acid	-	5-Hydroxyindole-3-acetic acid	57 ± 58	72 ± 76	68 ± 45	

Table 3.4.3.3 Acidic metabolite excretion in normal pregnant and nonpregnant women. Group 2: Metabolites detected in all samples but not fully quantitated. (See Table 3.4.3.2 for details.)

Category	Peak number	MU value	Possible peak identity (authentic acids coinjected)	Confirmed identity by GC-MS	Compounds identified by other workers§
A. Off-scale peaks	5	11.48	Sulfate	Sulfate	Sulfate
	11	12.90	Phosphate	Phosphate	Phosphate
	37	18.00	Hippuric acid	Hippuric acid	Hippuric acid
	38	18.50	Citric acid	Citric acid	Citric acid
	49	21.30	Uric acid	Uric acid	Uric acid
			Caffeic acid		
B. Partially obscured peaks	4	11.38	α-Hydroxybutyric acid	-	-
	6	11.67	β-Hydroxybutyric acid	-	β-Hydroxybutyric acid
					β-Hydroxyisobutyric acid
	7	12.02	Malonic acid	-	-
	27	16.18	ρ-Hydroxybenzoic acid	-	-
	36	17.86	ρ-Hydroxymandelic acid	-	ρ-Hydroxymandelic acid
			α-Glycerophosphoric acid		
	41	19.45	4-Pyridoxic acid	-	-

Table 3.4.3.4 Acidic metabolite excretion in normal pregnant and nonpregnant women. Group 3: Metabolites not detected in all samples. (See Table 3.4.3.2 for details.)

Peak number	MU value	Possible peak identity (authentic acids coinjected)	Confirmed identity by GC-MS	Compounds identified by other workers§	Number of subjects excreting detectable amounts (Range of excretion in relative amount/24 h) Nonpregnant (n = 15)	Pregnant (n = 25) 12½-15½ wk	Pregnant (n = 25) 24½-27½ wk
9	12.27	Benzoic acid	-	Benzoic acid	0	9 (21 - 670)	9 (24 - 331)
15	13.93	-	-	-	1 (16)	8 (trace - 97)	10 (6 - 214)
16	14.00	Glutaric acid	-	Glutaric acid	12 (6 - 74)	20 (trace - 174)	15 (trace - 197)
18	14.33	α-Keto-γ-methiolbutyric acid 3,3-Dimethylglutaric acid	-	3-Deoxytetronic acid 3-Methylglutaconic acid	11 (trace - 93)	23 (20 - 116)	25 (trace - 110)
20	14.83 to 14.90	Citramalic acid	-	-	15 (13 - 65)	23 (15 - 94)	23 (21 - 94)
23	15.58	o-Hydroxyphenylacetic acid m-Hydroxybenzoic acid	-	-	1 (19)	5 (12 - 63)	4 (24 - 105)
24	15.63	β-Phenylpyruvic acid	-	-	0	1 (43)	0
29	16.43	Imidazoleacetic acid	-	-	15 (trace - 87)	23 (trace - 171)	21 (trace - 185)
30	16.60	α-Ketoadipic acid	-	-	5 (trace - 91)	17 (trace - 70)	14 (trace - 145)
31	16.70	Tartaric acid	-	Tartaric acid	10 (37 - 504)	19 (40 - 1210)	20 (7 - 598)

.....continued

Table 3.4.3.4 (continued)

Peak number	MU value	Possible peak identity (authentic acids coinjected)	Confirmed identity by GC-MS	Compounds identified by other workers§	Number of subjects excreting detectable amounts (Range of excretion in relative amount/24 h)		
					Nonpregnant (n = 15)	Pregnant (n = 25)	
						12½-15½ wk	24½-27½ wk
33	17.16	Quinolinic acid	-	-	2 (33 - 64)	3 (trace - 71)	1 (35)
34	17.30	-	-	-	4 (4 - 274)	3 (16 - 89)	3 (10 - 49)
42	19.53	-	-	-	9 (42 - 77)	11 (30 - 143)	18 (23 - 211)
44	20.03	-	-	-	0	0	1 (386)
45	20.30	-	-	-	3 (44 - 115)	0	3 (60 - 99)
46	20.43	Salicyluric acid	-	Salicyluric acid	3 (420 - 3714)	2 (12 - 21)	2 (14 - 727)
48	20.82	Ferulic acid	-	-	4 (120 - 136)	18 (trace - 183)	10 (trace - 170)

Petersson, 1977; Cohn et al., 1978). The metabolites were grouped according to prevalence of excretion of detectable amounts, and listed in order of chromatographic elution. The minimum detectable amount was 1-2 mg excreted/24 h. In crowded regions of the chromatogram, 5 mg excreted/24 h was needed to identify a metabolite as a distinct peak. The three acidic metabolite groupings consisted of: group 1, metabolites detected in all samples and fully quantitated (Table 3.4.3.2); group 2, metabolites detected in all samples but not fully quantitated (Table 3.4.3.3); and group 3, metabolites not detected in all samples (Table 3.4.3.4).

Table 3.4.3.2 shows the means and standard deviations for acidic metabolites detected in all samples analyzed. The wide range of standard deviations (some $\pm$ 100% of the means) indicated the variability in organic acid excretion among normal subjects. An unpaired Student's *t* test was used to compare excretion in nonpregnant women to excretion in pregnant women at each stage of pregnancy. A paired Student's *t* test compared the excretion data of pregnancy. Of the 22 peaks in group 1, 10 peaks showed no significant difference between the nonpregnant and pregnant groups. No decreases were observed in 24-h excretion for the pregnant women compared to the nonpregnant women. Conversely, 12 acidic metabolites were excreted in greater amounts in pregnancy. Three metabolites, peak 13 (probably glyceric acid), peak 22 (probably 5-hydroxymethyl-2-furoic acid), and peak 35 (probably homovanillic acid and *cis*-aconitic acid) were significantly increased at the second trimester collection only. Five metabolites, peak 8 (probably methylmalonic acid and β-hydroxyisovaleric acid), peak 12 (probably succinic acid and pyrotartaric acid), peak 17 (unknown), peak 19

(probably 2-deoxytetronic acid), and peak 32 (probably suberic acid and deoxypentonic acids) were significantly increased at the first trimester collection with only slight changes as pregnancy progressed. Four metabolites, peak 1 (lactic acid), peak 2 (glycolic acid), peak 14 (probably the 4-deoxytetronic acids), and peak 25 (erythronic acid) were excreted in greatly increasing amounts as pregnancy progressed. These last four metabolites were major urinary peaks and thus unequivocally identified (except the 4-deoxytetronic acids). Since all were aldonic acids (hydroxy, monocarboxylic acids) the relative amount/24 h excretion data were converted to μmol/24 h using the lactic acid conversion factor of Table 3.4.3.1. The resultant excretion data are listed in Table 3.4.3.5 which also gives the range of values for each group of women. Since excretion of these four acidic metabolites showed such a dramatic increase in pregnancy, pairs of metabolites were further investigated to identify simultaneous increases in excretion. Table 3.4.3.6 gives the correlation coefficients for the paired urinary acidic metabolites. Lactic acid excretion correlated poorly with the excretion of the other acids. The excretion patterns for the remaining acidic metabolites (glycolic acid, erythronic acid, and the 4-deoxytetronic acids) correlated very well, possibly indicating a common origin. Figure 3.4.3.4 is a graphic illustration of the correlation between glycolic acid excretion and 4-deoxytetronic acid excretion. The excretion values for urinary acidic metabolites in four 24-h urine samples from one nonpregnant woman were remarkably consistent (see Figure 3.4.3.4). The excretion data for that individual remained constant over the year in which the samples were collected, regardless of dietary or seasonal conditions. Such

Table 3.4.3.5 Urinary excretion of some organic acids in normal pregnant and nonpregnant women.

Organic acid	24-h excretion, μmol/24 h ± SD (range): Nonpregnant (n = 15)	Pregnant (n = 25): 12½ - 15½ wk	Pregnant (n = 25): 24½ - 27½ wk	Paired *t*-test
Lactic acid	240 ± 240	710 ± 870*	1310 ± 1020***	*
	(50 - 840)	(90 -4060)	(210 - 4200)	
Glycolic acid	570 ± 350	1220 ± 370***	1420 ± 460***	*
	(160 -1490)	(640 -2000)	(790 - 2530)	
4-Deoxytetronic acids	530 ± 230	780 ± 210**	1160 ± 290***	***
	(210 - 990)	(460 -1240)	(740 - 1700)	
Erythronic acid	780 ± 180	890 ± 220	1040 ± 240***	**
	(420 -1090)	(520 -1290)	(530 - 1410)	

*P < 0.05 (Statistical significance was calculated as in Table 3.4.3.2)
**P < 0.01
***P < 0.001

Table 3.4.3.6 Correlation between paired urinary acidic metabolites in pregnant and nonpregnant women.

Lactic acid			
0.386**	Glycolic acid		
0.360**	0.667***	4-Deoxytetronic acids	
0.406***	0.584***	0.621***	Erythronic acid

**Correlation significant at $P < 0.01$

***Correlation significant at $P < 0.001$

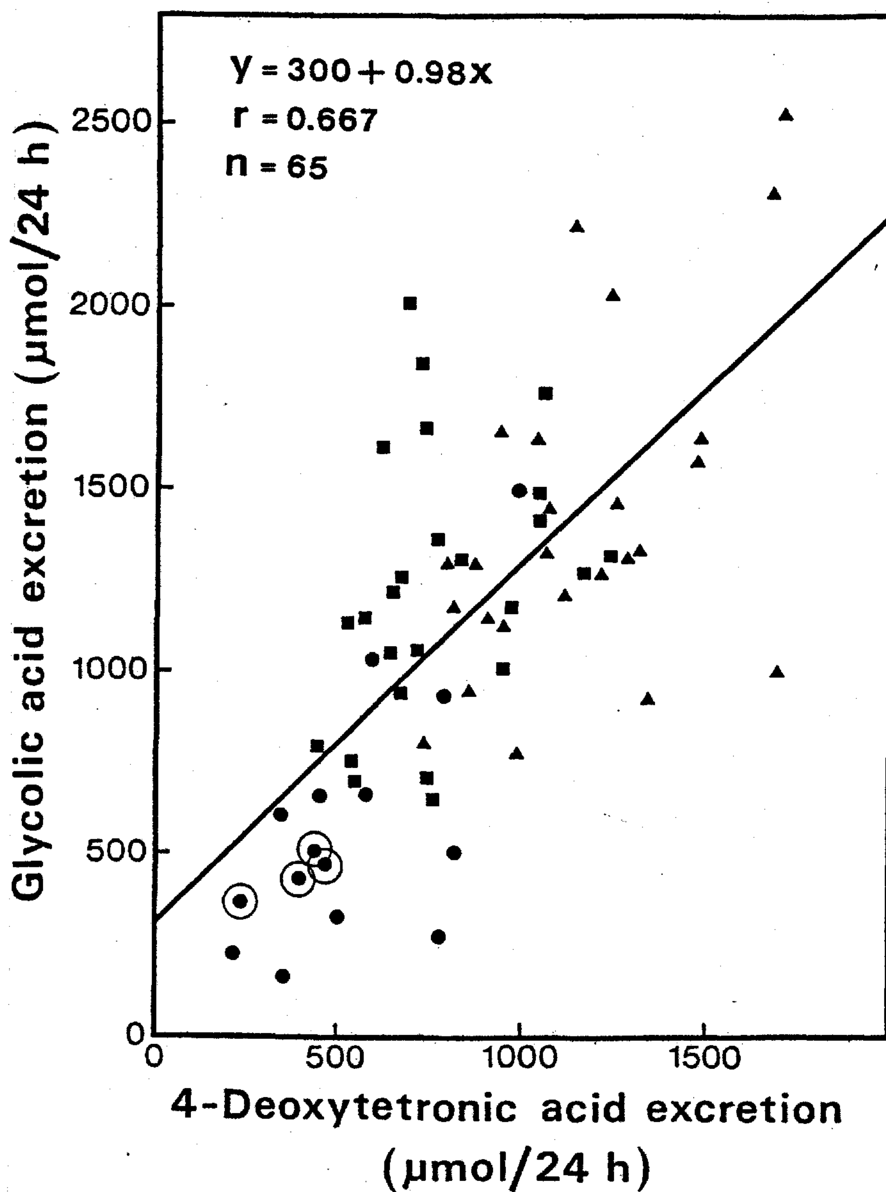


Figure 3.4.3.4 Correlation between glycolic acid excretion and 4-deoxytetronic acid excretion for pregnant and nonpregnant women. (● nonpregnant women, n = 15; ■ pregnant women, 12½-15½ weeks gestation, n = 25; ▲ pregnant women, 24½-27½ weeks gestation, n = 25) The four points circled in the lower left segment of the graph represent excretion values for one nonpregnant woman who collected a 24-h urine sample at four separate intervals over a 1-year period.

uniform intra-individual data would support the concept of biochemical individuality (Williams, 1956).

Table 3.4.3.3 lists the metabolites detected in all samples but not quantitated. Metabolites present in large amounts produced off-scale peaks, overloading the flame ionization detection system and resulting in nonlinear detection. These off-scale peaks (sulfate, phosphate, hippuric acid, citric acid, uric acid) usually had a wide methylene unit range. Such peaks partially obscured several smaller peaks which were seen as inconsistently resolved shoulders on the large peaks. The large sulfate and phosphate peaks especially dominated the early portion of the chromatogram. It would have been possible to quantitate the large peaks by injecting less of the derivatized sample into the gas chromatograph. Uric acid showed a tendency to precipitate out of solution and disappear from the chromatogram.

While metabolites detected in all samples are probably endogenous, metabolites detected in only a few samples are likely of exogenous origin. Table 3.4.3.4 lists 17 peaks whose occurrence was irregular, probably influenced by diet and drugs (Chalmers _et al._, 1976a) or other factors such as intestinal microorganisms (Scheline, 1968). Figure 3.4.3.5 shows the gas-liquid chromatogram of a normal pregnancy at 27 weeks of gestation which has a large amount of peak 31 (probably tartaric acid) and peak 50 (probably 5-hydroxyindole-3-acetic acid). Compounds such as 5-hydroxymethyl-2-furoic acid and tartaric acid are mostly of dietary origin (Lawson _et al._, 1976; Pettersen and Jellum, 1972). The ingestion of foods containing serotonin (for example, bananas) can increase the 5-hydroxyindole-3-acetic acid excretion markedly

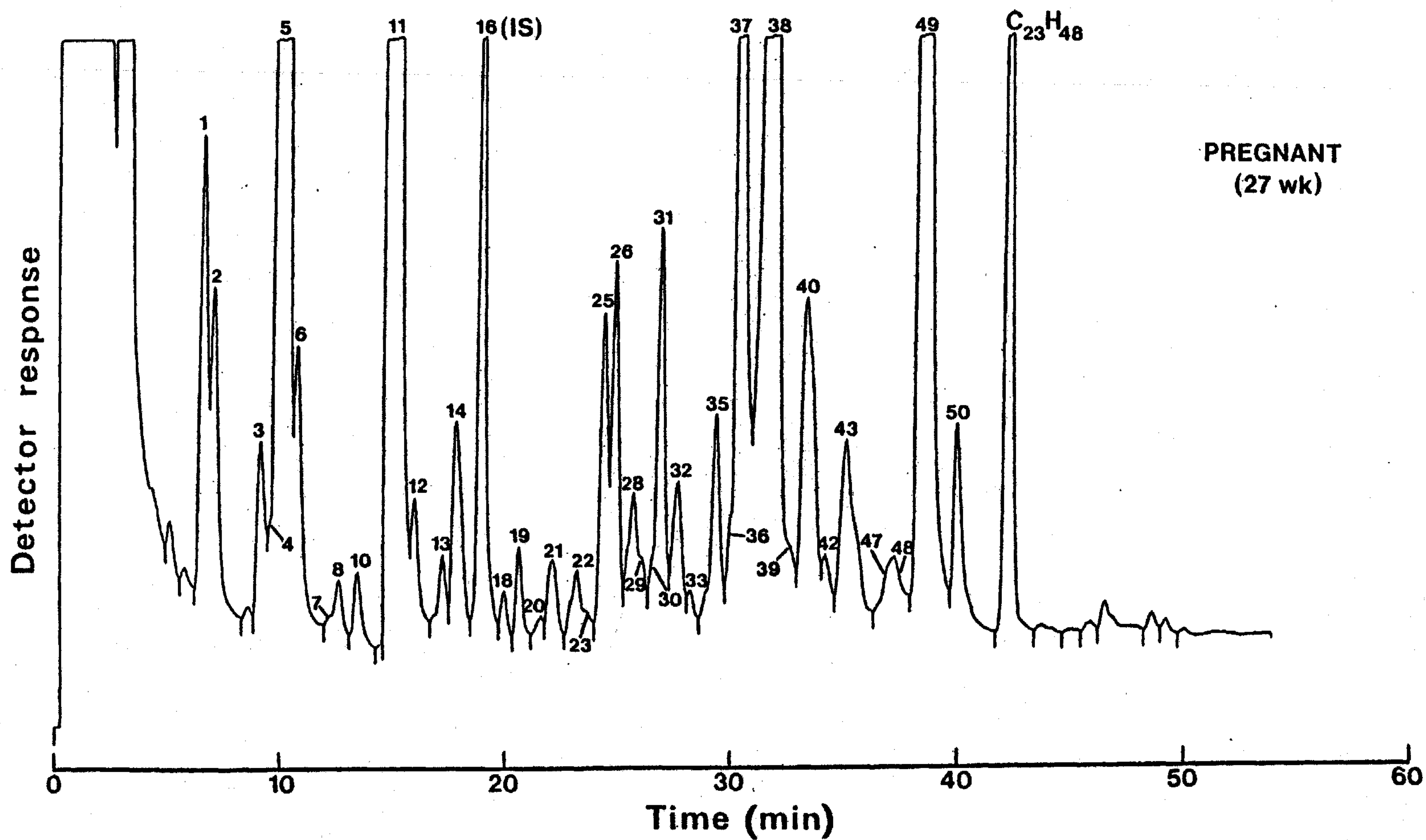


Figure 3.4.3.5 Gas-liquid chromatogram of acidic metabolites extracted from the urine of a normal pregnant woman at the 27th week of gestation. Note the increased amount of peak 31 (probably tartaric acid) and peak 50 (probably 5-hydroxyindole-3-acetic acid). See Figure 3.4.3.2 for an explanation of chromatographic conditions.

(Crout and Sjoerdsma, 1959). Pharmaceutical preparations may also produce unusual urinary acidic metabolites (Chalmers *et al.*, 1976a). A peak corresponding to salicyluric acid (peak 46) was observed in women who ingested acetylsalicylic acid. Salicyluric acid formation (salicylic acid conjugation with glycine) is the main route of salicylic acid elimination (Amsel and Levy, 1969), although alterations in intestinal flora may cause patients with gastrointestinal dysfunction to excrete salicyluric acid even though they do not receive salicylates (Finnie *et al.*, 1976). Peak 44 was observed in only one woman who also ingested an antihistaminic (Dimetapp: contained brompheniramine, phenylephrine, and phenylpropanolamine). Peak 34 was greatly elevated in one nonpregnant woman who ingested acetaminophen. No additional urinary peaks were observed for the nonpregnant woman who consumed 5 ounces of alcohol although ethyl β-D-glucosiduronic acid has previously been identified as a metabolite of ethanol in man when alcoholic volumes were consumed (Jaakonmaki *et al.*, 1967a). None of the nonpregnant women had detectable amounts of benzoic acid in their urine, while several pregnant women had considerable quantities present. Benzoic acid may be produced as the result of hippuric acid hydrolysis by hippuricase-forming bacteria in the urine (Hansen *et al.*, 1972). At least one pregnant woman had greatly increased amounts of benzoic acid in the urine due to the use of an antifungal agent (Monistat) which contained benzoic acid. Such overt alterations to the urinary acid chromatogram stressed the importance of considering drug metabolites or drug-induced metabolites when an unusual peak was encountered.

Further characterization of the urinary acid chromatogram was

carried out by a variety of procedures. Different oximating agents were employed to aid in the identification of keto acids. Glucuronic acid was the only keto acid clearly observed, while occasionally the peaks corresponding to α-ketoglutaric acid were seen. Careful consideration was given to an extensive list of 300 substances (antioxidants, putrefactive compounds, food additives, plasticizers, etc.) commonly encountered in biological fluids (Ramsey et al., 1980), although no such compounds were identified. The effect of specimen storage on the urinary acid profile was also investigated. Figures 3.4.3.6 and 3.4.3.7 show chromatograms of a urinary sample stored at -30°C for 5 months and 13 months, respectively. The chromatograms were identical, any minor differences being solely due to GC column performance. Indeed, the resolution of peak 1 (lactic acid) from peak 2 (glycolic acid) served as a good indicator of GC column performance. Retention times for the two peaks differed by 0.40 min with a newly packed GC column while a difference of less than 0.35 min indicated that the column needed to be repacked. Several urinary acid peaks were not always resolved. Peak 14 eluted as a single peak (MU 13.68) and as a doublet (MU 13.67 and MU 13.74). In addition, peaks 22 and 32 each encompassed at least two different compounds but inconsistent resolution necessitated the tabulation of peak areas as single peak reports.

The precision of the method was assessed using duplicate analyses as shown in Table 3.4.3.7. The coefficient of variation for the four peaks investigated (peak 1, lactic acid; peak 2, glycolic acid; peak 14, probably the 4-deoxytetronic acids; peak 25, erythronic acid) was usually less than 10%. The coefficient of variation varied only slightly

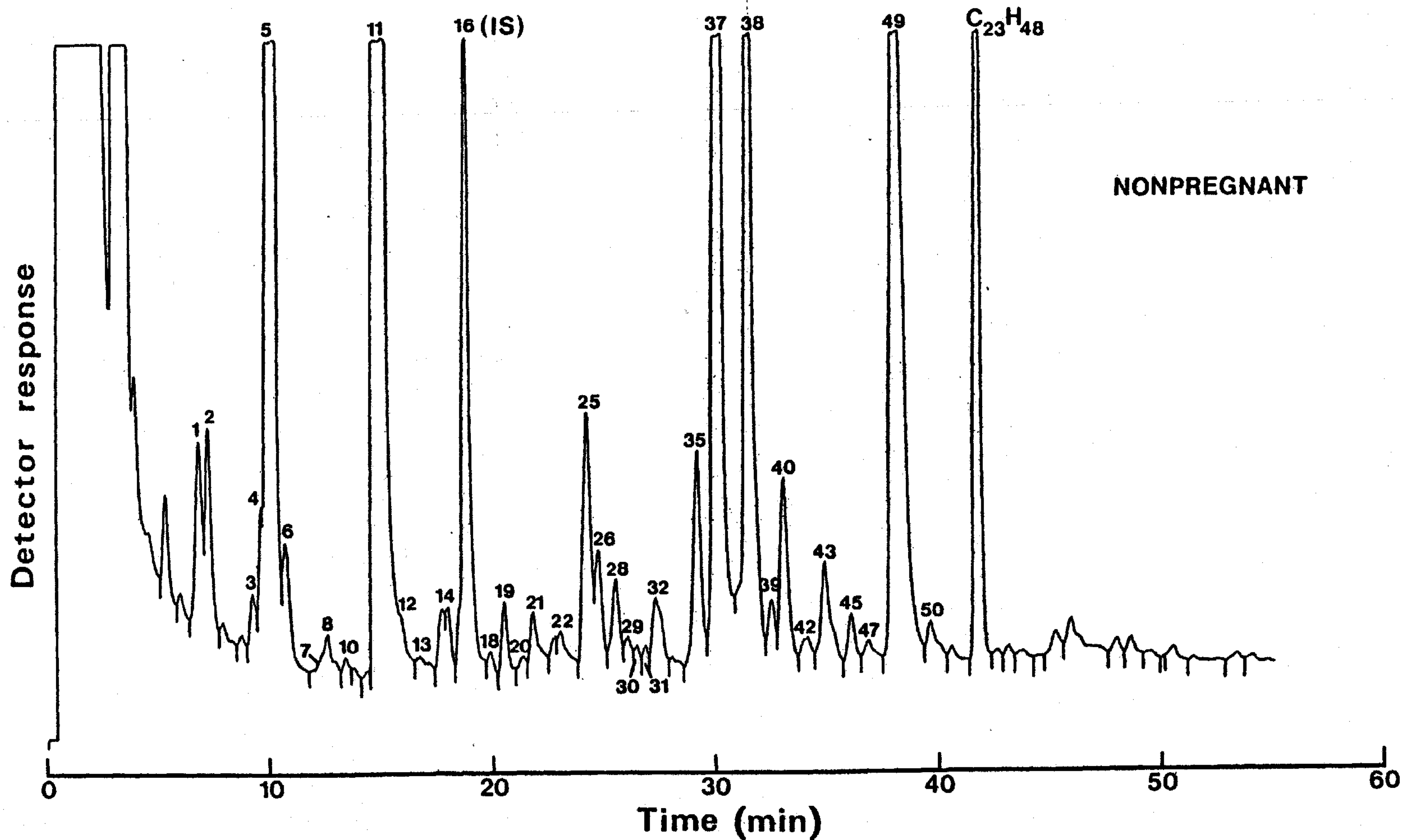


Figure 3.4.3.6 Effect of sample storage on the gas-liquid chromatogram of acidic metabolites extracted from the urine of a nonpregnant woman. The urine sample was stored 5 months at -30°C. Compare to Figure 3.4.3.7. See Figure 3.4.3.2 for an explanation of chromatographic conditions.

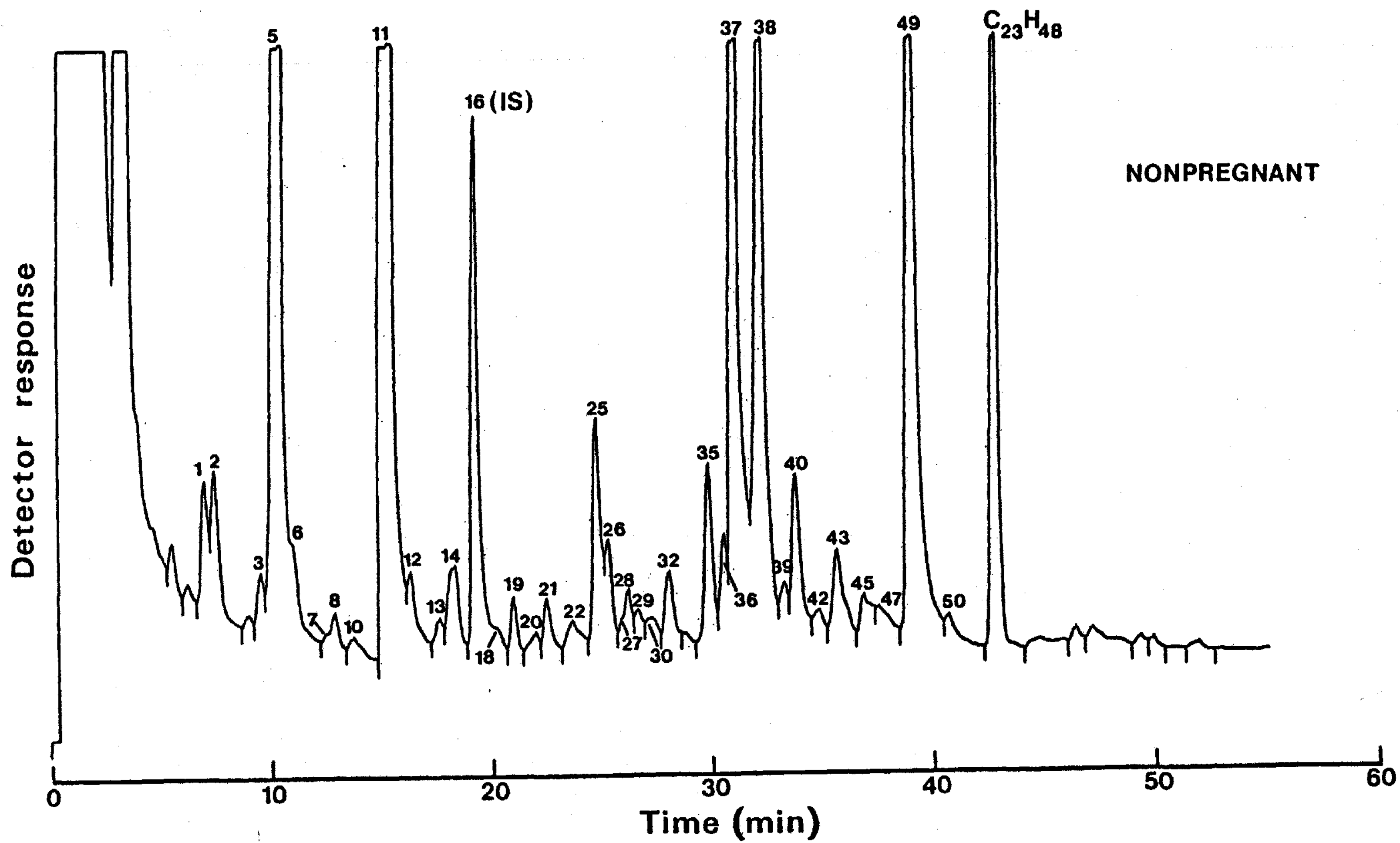


Figure 3.4.3.7 Effect of sample storage on the gas-liquid chromatogram of acidic metabolites extracted from the urine of a nonpregnant woman. The urine sample was stored 13 months at -30°C. Compare to Figure 3.4.3.6. See Figure 3.4.3.2 for an explanation of chromatographic conditions.

Table 3.4.3.7 Assessment of precision of the method using duplicate analyses.

The coefficient of variation (CV) of the results of duplicate determinations from their means was determined by the following (Snedecor, 1952; Abraham et al., 1971):

$$CV = \sqrt{\frac{\Sigma d^2}{2n}} \quad \text{where } d = \left[\frac{\text{highest value of each duplicate}}{\text{lowest value of each duplicate}} - 1\right] \times 100$$

and n = number of duplicate determinations.

The four prominent urinary acidic metabolies were used for the precision calculations. Ten duplicate determinations were performed by the same assay method on seven different days.

Peak no.	Organic acid	Coefficient of variation (CV) of the results of duplicate determinations from their mean (%) Day 1 n = 10	Day 2 n = 10	Day 3 n = 10	Day 4 n = 10	Day 5 n = 10	Day 6 n = 10	Day 7 n = 10	Overall CV (%) n = 70
1	Lactic	6.3	6.5	13.8	7.9	6.4	11.8	3.8	8.7
2	Glycolic	12.3	4.6	8.3	5.9	5.1	3.6	11.2	8.1
14	4-Deoxytetronic	7.9	9.2	7.3	14.9	6.2	11.4	13.2	10.4
25	Erythronic	9.8	5.3	3.7	14.6	9.7	7.7	7.9	9.1

Table 3.4.3.8 Assessment of precision of the method according to the analytical range of determinations.

The coefficient of variation was determined as in Table 3.4.3.7. Data for the four prominent urinary acidic metabolites (lactic acid, glycolic acid, 4-deoxytetronic acids, erythronic acid) were grouped according to analytical range.

Analytical range of determinations (Relative amount/24 h)	Number of duplicate determinations	Coefficient of variation (%)
< 49	10	11.8
50- 99	19	9.4
100-149	24	7.8
150-199	22	13.1
200-299	46	9.2
300-399	62	8.0
400-499	48	8.3
500-599	30	10.6
600-699	10	4.7
> 700	9	5.1
All values	280	9.1

according to the analytical range of determinations (Table 3.4.3.8). Thus, the organic acid extraction method had good analytical precision for the urinary acids investigated.

3.4.4 Urinary steroid excretion

The urinary steroid chromatograms contained several poorly resolved peaks. A chromatogram of the steroid metabolites extracted from the urine of a normal pregnant woman at the 27th week of gestation is shown in Figure 3.4.4.1. The corresponding total ion chromatogram from mass spectrometric analysis is shown in Figure 3.4.4.2. Pregnanediol (peak 8) was the only steroid metabolite identified from its mass spectrum. Table 3.4.4 contains a list of the possible peak identities. The steroid chromatogram was similar to published steroid profiles of pregnancy urine with pregnanediol and estriol as the predominant peaks (Horning _et al._, 1971; Fotsis _et al._, 1980). Capillary columns would, however, produce a profile where individual metabolites could be quantitated (Pfaffenberger and Horning, 1975). Indeed, the doublet of peaks corresponding to androsterone and etiocholanolone (peaks 1 and 2) provide an indication of the lack of resolution with the 6-ft packed columns.

Analysis of urine from nonpregnant women revealed small peaks corresponding to androsterone, etiocholanolone, 11β-hydroxyandrosterone, 11β-hydroxyetiocholanolone, and pregnanediol as would be expected for the follicular phase of the menstrual cycle (Ros and Sommerville, 1971; Viinikka and Janne, 1973; Fantl and Gray, 1977).

An aliquot of urine equivalent to 40 μg of creatinine was needed to produce a good urinary steroid profile. Since the Amberlite XAD-2

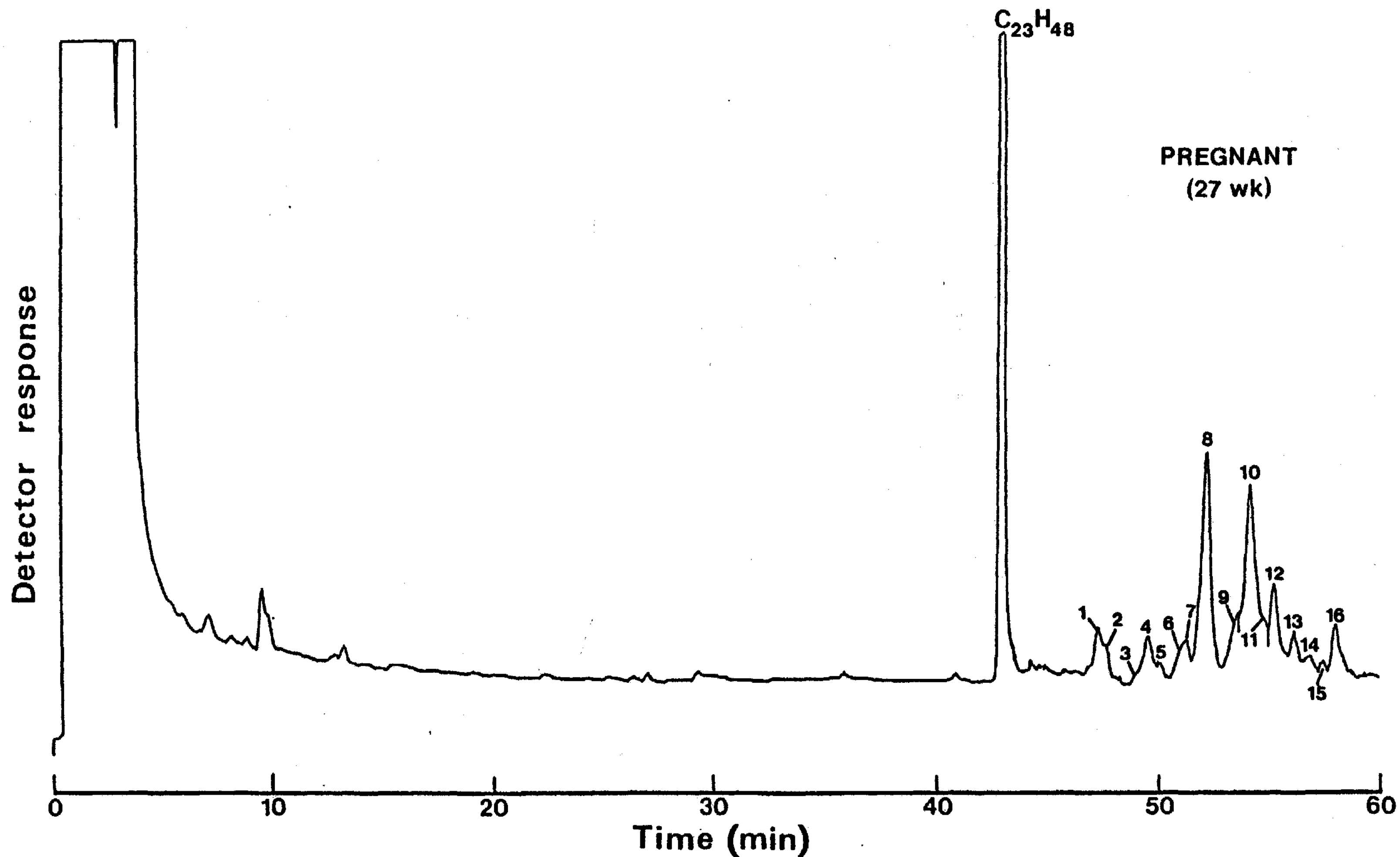


Figure 3.4.4.1 Gas-liquid chromatogram of steroid metabolites extracted from the urine of a normal pregnant woman at the 27th week of gestation. The methoxime trimethylsilyl derivatives were analyzed on a 6-ft 3% OV-101 column programmed from 70° to 300°C at 4°C/min with a 4-min initial isothermal delay. Injection volume was 2 µl. Amplification was 10 x 16 (via electronic integration). The chromatogram represents an aliquot of urine equivalent to 40 µg of creatinine. Peak numbers correspond to possible peak identifications given in Table 3.4.4.

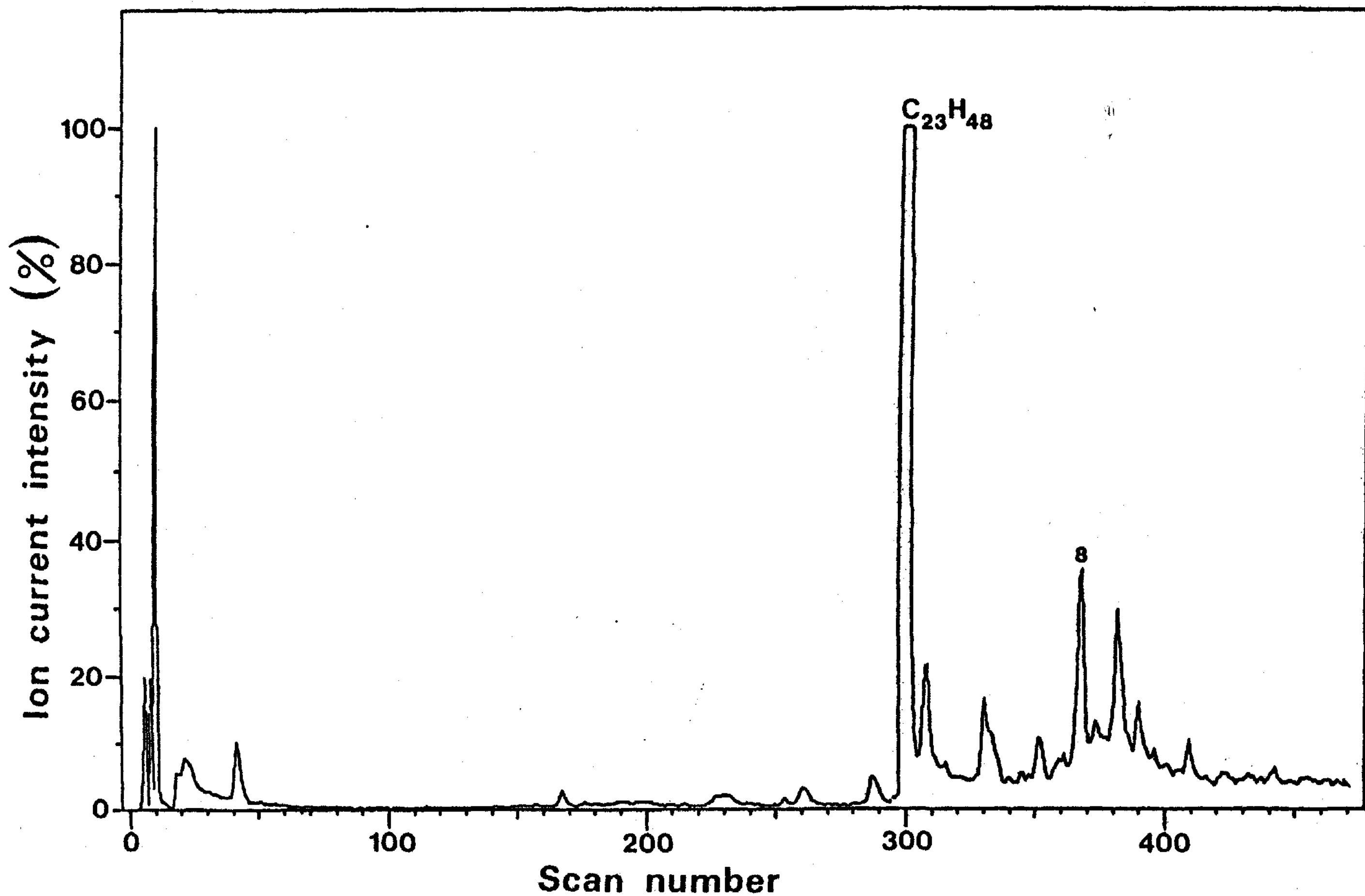


Figure 3.4.4.2 Total ion chromatogram from the mass spectrometric detection of the methoxime trimethylsilyl derivatives of the steroids extracted from the urine of a normal pregnant woman at the 27th week of gestation. GC separation was on a 6-ft 3% OV-101 column programmed from 100° to 300°C at 4°C/min with an 8-min initial isothermal delay. Peak numbers correspond with the gas-liquid chromatogram of the same sample shown in Figure 3.4.4.1. Peak 8 was identified by the mass spectrum as pregnanediol-diTMS.

Table 3.4.4 Steroid metabolite excretion in a normal pregnant woman at the 27th week of gestation.

The possible identities of peaks were determined by coinjection of derivatized steroid standards.

*Peak 8 was identified by the mass spectrum as pregnanediol-diTMS.

Peak Number	MU value	Possible peak identity (authentic steroids coinjected)
1	25.24	Androsterone
2	25.37	Etiocholanolone
3	25.90	Dehydroepiandrosterone
4	26.34	11-Ketoandrosterone, Estrone, 5α-Androstan-3β,17β-diol, 11-Ketoetiocholanolone, Androstenedione
5	26.57	β-Estradiol
6	27.14	11β-Hydroxyandrosterone
7	27.26	11β-Hydroxyetiocholanolone
8	27.77	Pregnanediol*
9	28.61	Tetrahydrosubstance S
10	28.88	Estriol
11	29.33	Pregnanetriol
12	29.50	Tetrahydrocortisone
13	30.20	Tetrahydrocortisol
14	30.50	2-Hydroxyestriol
15	30.75	-----
16	31.25	Cholesterol

resin adsorbs steroid conjugates quantitatively from as much as 40 times its volume of urine (Osawa and Slaunwhite, 1970), the aliquot of urine extracted can be adjusted to produce suitable chromatograms. The inefficiency of enzyme deconjugation would probably be the greatest drawback of the present method. Certain steroid sulfates are not hydrolyzed by enzymes from *Helix pomatia* (Leon et al., 1960). While urinary steroids are predominantly excreted as glucuronide conjugates, approximately 10% of the total steroids are mono- or disulfate conjugates (Eriksson and Gustafsson, 1970, 1972).

3.5 Profiles of some risk pregnancies

Acidic metabolite excretion was investigated in several cases of high-risk pregnancy. Some of these pregnancies resulted in the death of the fetus or neonate. Table 3.5 lists only those excretion values outside the range encompassed by two standard deviations (SD) above and below the means encountered in the group of reference pregnancies. The accepted range of normality for pregnant women would inevitably be wider than most reference populations due to the extensive physiological adaptations associated with pregnancy. Also, for every metabolite there was one or two of the reference pregnancies with an excretion value outside the designated mean $\pm$ 2SD. Indeed, the concept of a "normal range of values" (Sunderman, 1975) is probably too simplistic since absolute health does not exist and every human being deviates from normality in some respects (Williams, 1956). Such arguments point out the advantages of repeated observations on the same individual to define intraindividual variation (Harris, 1974). Nevertheless, the single 24-h urine collections from

Table 3.5 Acidic metabolite excretion in several cases of high-risk pregnancy.

Outcome of pregnancy	Time of sample collection (weeks)	Description of pregnancy	Acidic metabolite excretion compared to normal pregnancy: Outside 2 SD above mean (E) Outside 2 SD below mean (D)			
			M.U. value	Relative amount/24 h	E or D	Possible peak identity
Survival of child	26	Maternal hypothyroidism, compensated by Eltroxin 0.1 mg; low serum vitamin B_{12}	12.15	178	E	Methylmalonic acid β-Hydroxyisovaleric acid
			13.68	232	D	4-Deoxytetronic acids
	24	Maternal Crohn's disease	13.68	221	D	4-Deoxytetronic acids
	27	Maternal diabetes mellitus since 10 years of age, compensated by Toronto and NPH insulin	12.27	179	E	Benzoic acid
			12.41	182	E	Levulinic acid
Death of child	12	Normal pregnancy; sudden infant death syndrome at 6 months of age	13.68	140	D	4-Deoxytetronic acids
Death of fetus	17	Miscarriage (unexplained) at 20 weeks	16.27	925	E	β-Hydroxy-β-methyl-glutaric acid or p-Hydroxyphenylacetic acid
	26	Sudden intrauterine death (polyhydramnios) at 36 weeks	14.00	207	E	Glutaric acid

risk pregnancies were compared to the categories of normal pregnancy.

Three successful pregnancies were complicated by maternal disease (hypothyroidism, Crohn's disease, and diabetes mellitus). The woman with hypothyroidism compensated by dietary thyroid powder also had low serum vitamin B_{12}. Concentrations of vitamin B_{12} are decreased in maternal serum in normal pregnancy, not necessarily indicative of B_{12} deficiency (*Dietary Standard for Canada*, 1976, p.36; published by Health and Welfare Canada). The feeding of thyroid powder may stimulate food consumption causing an increase in protein intake. This accentuates vitamin B_{12} deficiency since metabolism of the branched-chain amino acids (valine and isoleucine) requires vitamin B_{12} (Batra et al., 1979). Vitamin B_{12} deficiency is characterized by increased excretion of methylmalonic acid, a metabolite of the branched-chain amino acids (Gompertz et al., 1967). Excretion of methylmalonic acid may be the first indicator of functional deficiency of vitamin B_{12}, a more sensitive indicator than serum vitamin B_{12} (Higginbottom et al., 1978). The acidic metabolite peak (MU 12.15) elevated in the pregnancy complicated by maternal hypothyroidism was not identified by mass spectrometry, but it would not be surprising if the metabolite was methylmalonic acid.

Three pregnancies (maternal hypothyroidism, maternal Crohn's disease, and the apparently normal pregnancy followed by death of the child at 6 months of age due to sudden infant death syndrome) were characterized by lower than normal levels of the acidic metabolites corresponding to the 4-deoxytetronic acids. These decreases in excretion were not attributable to incomplete 24-h collections. (The woman with

Crohn's disease excreted only 500 mg of creatinine/24 h, but the 24-h excretion data were corrected to give the equivalent of 1 g of creatinine/ 24 h.) Since neither the function nor the metabolism of deoxyaldonic acids is known (Haraguchi *et al.*, 1982a, 1982b), the significance of the decreased 4-deoxytetronic acid excretion was obscure. Likewise, slight elevations in acidic metabolites in the maternal diabetic and in the pregnancy terminated at 36 weeks due to polyhydramnios may or may not be of significance.

Peak 28 (MU 16.27) was greatly elevated in a pregnancy at 17 weeks which was later terminated by an unexplained miscarriage at 20 weeks (see Figure 3.5). If peak 28 was exclusively p-hydroxyphenylacetic acid, the 24-h excretion for normal pregnant and nonpregnant women was 31 $\pm$ 17 mg/24 h (n=65), while excretion in the risk pregnancy was 210 mg/24 h. Urinary p-hydroxyphenylacetic acid is derived from p-tyramine which can arise from dietary tyrosine due to the action of intestinal bacteria (Chalmers *et al.*, 1979). Normal p-hydroxyphenylacetic acid excretion has been reported as 11.6 $\pm$ 11.4 mg/24 h (Tocci *et al.*, 1972), 22.4 $\pm$ 1.5 mg/24 h (Davis and Boulton, 1981), and 31.3 mg/24 h (Horning *et al.*, 1966). In small-bowel disease, dietary tyrosine is degraded by intestinal bacteria which are present due to bacterial overgrowth syndromes (Chalmers *et al.*, 1979). Elevated urinary p-hydroxyphenylacetic acid (100-1000 mg/24 h) is rare in the absence of small-bowel disease (Chalmers *et al.*, 1979). The significance of the increased amount of peak 28 in the case of unexplained miscarriage was unknown.

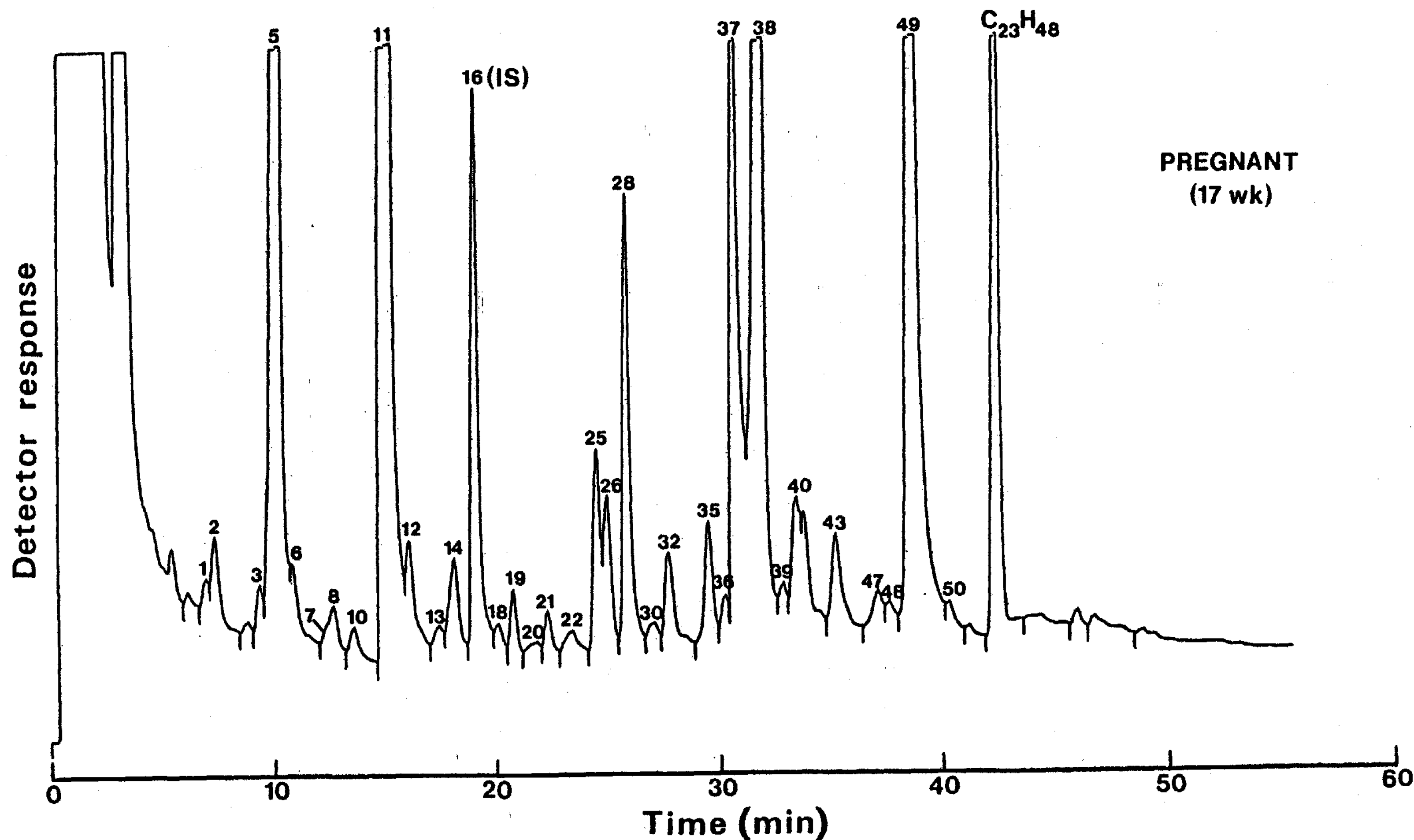


Figure 3.5 Gas-liquid chromatogram of acidic metabolites extracted from the urine of an abnormal pregnancy at the 17th week of gestation. The pregnancy was terminated at 20 weeks by an unexplained miscarriage. Note the increased amount of peak 28 (possibly ρ-hydroxyphenylacetic acid or β-hydroxy-β-methylglutaric acid). See Figure 3.4.3.2 for an explanation of chromatographic conditions.

4. DISCUSSION

4.1 Urinary metabolites in human pregnancy

The urine contains intermediates and end-products from the myriad metabolic processes occurring in the body. Compounds present in the urine may reflect the metabolism of the entire organism, as well as renal metabolism. There are an estimated 500 acidic compounds in the urine, 200 of which have been characterized (Spiteller and Spiteller, 1979). Thus, the urinary acid profile is complex, containing several recognizable components as well as many more components which are present in smaller quantities.

There was an amazing qualitative uniformity among urine samples despite the complexity of urine. All urinary acid profiles exhibited large peaks due to sulfate, phosphate, hippuric acid, citric acid, and uric acid. These compounds were recognizable landmarks in the chromatograms, normal adult excretion rates ranging from 0.5 g/24 h (citric acid, uric acid) to 1.0 g/24 h (sulfate, phosphate, hippuric acid), (Diem and Lentner, 1970). Several well-known organic acids (for example, β-hydroxybutyric acid and phenylacetic acid) could not be quantified because of their proximity to these large peaks. Peaks corresponding to several low-molecular-weight acids (lactic acid, glycolic acid, oxalic acid, succinic acid, erythronic acid, threonic acid) were also detected in all urine samples. The 24-hour excretion of these acids rarely exceeded 300 mg. Several acids (benzoic acid, tartaric acid, salicyluric acid) were encountered less frequently due to their dietary origin.

The quantitative variability of organic acid excretion among individuals was reflected by the wide range of excretion values encountered for some metabolites. This heterogeneity could be due to dietary influences and bacterial flora, as well as individual metabolic differences. Large intraindividual variations can occasionally be attributed to diet, especially for substances with a known exogenous origin. Several investigators examined organic acid excretion in individuals under extreme dietary conditions (low-carbohydrate, high-fat diet versus high-glucose diet) and observed only minimal changes in the excretion profiles (Chalmers *et al.*, 1976b). Other investigators studied excretion patterns of subjects on a uniform diet and observed highly individualized profiles (Young *et al.*, 1971; Witten *et al.*, 1973). Thus, undoubtedly the major factor affecting urinary acidic metabolite excretion is individual metabolic variation. Genetic constitution affects metabolic reactions and organ function, leading to an individualized biochemical profile. Genetic differences may be important determinants of kidney function (Williams, 1956). The range of excretion values for a given individual would be narrower than the population range. This argument encourages longitudinal health surveillance where the subject provides his own base line data for comparison of biochemical profiles. The present study compared urinary organic acid excretion by individual pregnant women at two time periods in gestation, thereby allowing a more controlled investigation of the effects of pregnancy on organic acid excretion.

The urinary acid profiles obtained for the normal nonpregnant women agreed well qualitatively with previously published patterns

(Lawson _et al._, 1976; Cohn _et al._, 1978). Unfortunately, quantitative comparison of the present results with previously published data was not possible. Other workers analyzed untimed morning urine specimens and did not calculate GC detector response factors. Thus, the majority of workers report excretion data as percentages of an internal standard per mg of urinary creatinine. In contrast, I have chosen to analyze 24-hour urine specimens and present data in a format which can be readily converted to mg/24 h or μmol/24 h.

This thesis contains the first description of urinary organic acid profiles in pregnancy. Previous investigators have focussed only on single metabolites which were often quantified by methods less reliable than the present gas-chromatographic methods. Other workers have determined maternal urinary methylmalonate levels (most recently Bakker _et al._, 1978) in pregnancies at risk for methylmalonic acidemia. Herzberg _et al._ (1977) claimed that glucaric acid excretion increases in pregnancy (from 2 mg/g creatinine in nonpregnant women to 5 mg/g creatinine by the second trimester), supposedly reflecting the induction of hepatic enzymes. In contrast, the present data showed no increase in glucaric acid excretion in pregnancy. Herzberg _et al._ (1977) quantified glucaric acid by measuring the inhibition of β-glucuronidase, an enzyme which is powerfully inhibited by the lactone of glucaric acid (Fishman, 1967). This indirect method of measuring glucaric acid output may be influenced by the presence of other β-glucuronidase inhibitors in urine, for example, sulfate ions (Graef _et al._, 1977) as well as citric acid and gluconic acid (Roy, 1970). Additional compounds excreted in increased amounts in pregnancy urine may add to

the complexity of this enzyme inhibition assay. For example, other polyhydroxycarboxylic acids (the aldonic and deoxyaldonic acids) could conceivably be additional β-glucuronidase inhibitors.

The obvious multicomponent nature of unsymmetric peaks in the urinary acid chromatograms stresses the need for caution when interpreting the data. Thus, until further mass spectrometric analyses are performed, discussion must be restricted to those peaks of interest which have been unequivocally identified (lactic acid, glycolic acid, erythronic acid).

Urinary lactic acid levels tended to increase with pregnancy, but excretion was rather sporadic. The wide range of excretion values emphasized the inadequacy of expressing the data as a mean $\pm$ SD. Lactate excretion in the nonpregnant women was somewhat lower than values reported by other workers (Judge and Van Eys, 1962; Elliot and Ribeiro, 1972), as shown in Table 4.1. These workers used the colorimetric method of Barker and Summerson (1941) which involves the conversion of lactic acid to acetaldehyde followed by color production with p-hydroxyphenyl. Lower values would be expected with the present gas-chromatographic method which is a more specific and direct measure of lactic acid. While lactate excretion never exceeded 100 mg/24 h in the nonpregnant women, values over 350 mg/24 h were encountered in pregnancy. Lactic acid excretion is increased with severe exercise (Miller and Miller, 1949). The lethargy and reduced energy expenditure characteristic of pregnancy (Hytten, 1972) argue against the increased lactate excretion of pregnancy being due to increased physical activity. Normally, lactate is almost completely reabsorbed by the kidney tubules

Table 4.1 Urinary lactic acid excretion in normal subjects.

Subjects	n	Lactic acid excretion (mg/24 h) Mean ± SD	Range	Method (Reference)
Male and female	20	41 ± 14	22 - 82	Colorimetric (Judge and Van Eys, 1962)
Male	30	46 ± 26	15 - 153	Colorimetric (Elliot and Ribeiro, 1972)
Female	17	46 ± 17	22 - 96	
Female	15	22 ± 22	5 - 75	Gas chromatography (present work)
Pregnant, 12½ - 15½ weeks	25	65 ± 79	8 - 366	
Pregnant, 24½ - 27½ weeks	25	118 ± 92	18 - 378	

when blood lactate concentrations are below the renal threshold of 60 mg/dl (Miller and Miller, 1949). Healthy subjects with renal glycosuria have normal blood lactate concentrations but they excrete lactate in their urine at levels about three times greater than that of normal subjects (Anderson and Mazza, 1963). Evidence such as this has supported the idea that lactate and glucose excretion are related. Glucose, bicarbonate, acetoacetate, phosphate, and several amino acids (alanine, valine, glycine, and tryptophan) are known to inhibit each other in renal transport studies (Drummond and Michael, 1964). Recent evidence suggests that such substances do not share a common renal transport carrier, but instead may compete for a common energy source (Short and Rosenberg, 1979). Glycosuria occurs in about 50% of all pregnant women and is very episodic in nature (Lind, 1975). As well, several amino acids (glycine, histidine, threonine, serine, and alanine) are excreted in excessive amounts during pregnancy (Hytten and Cheyne, 1972). Thus, the renal excretion of lactate in pregnancy may be governed by the same unknown factors which lead to the glycosuria and aminoaciduria of pregnancy.

Lactic acid is by far the dominant acidic constituent in amniotic fluid, present at 50 - 100 mg/dl (Hagenfeldt and Hagenfeldt, 1972; Nicholls _et al._, 1976; Wilkinson _et al._, 1979). The fetus is a consumer rather than a producer of lactate, although the precise fate of lactate in the fetus is unknown (Battaglia and Meschia, 1978). Lactate is supplied by the placenta to the fetus in large amounts (Burd _et al._, 1975). The placenta deposits lactate into both the fetal and maternal circulations (Milley and Simmons, 1979; Char and Creasy,

1976). The etiology of placental lactate production is unknown. Other tissues with high rates of lactate production (tumor, intestinal mucosa, kidney tubule) are also actively involved in transport (Milley and Simmons, 1979). While increased maternal lactate excretion may reflect normal placental function, the wide range of excretion values encountered would probably preclude the use of this urinary organic acid as a reliable indicator of pregnancy status.

Although lactic acid excretion was sporadic, the excretion of glycolic acid, the tentatively identified 4-deoxytetronic acids, and erythronic acid increased simultaneously (see Table 3.4.3.6, page 95). These three acidic metabolite peaks were consistently increased in the pregnant women, as seen by the small standard deviations of mean excretion values (see Table 3.4.3.5, page 94). They were excreted in approximately equimolar quantities within each group of women studied. The consistent, simultaneous increase in excretion of these metabolites points to their possible common origin.

There was excellent analytical precision for the determination of the four noteworthy urinary acidic metabolites (lactic acid, glycolic acid, the 4-deoxytetronic acids, and erythronic acid) as shown in Table 3.4.3.7, page 103. Thus, it is unlikely that these peaks would be formed due to random chemical degradation of compounds during the organic acid extraction procedure. The most extensive chemical and chromatographic studies of the aldonic and deoxyaldonic acids have been done by Petersson (1970, 1974, 1977). These acids may be formed by the action of alkali on various aldoses and deoxyaldoses (Morrison and Perry, 1966; Verhaar and De Wilt, 1969). Roesel _et al._ (1979) reported the production

of lactic acid (otherwise named 3-deoxyglyceric acid) from glucose by alkaline hydrolysis. The oxidation of maltose with 0.05 M sodium hydroxide gives 2-deoxytetronic acid (Fell et al., 1975). Thus, alkaline conditions should probably be avoided if artifactual aldonic acid formation is not to occur. This has, however, been disputed by Thompson et al.(1975) who used a barium hydroxide sample pretreatment and observed no artifacts. Glucose can apparently be leached from the dextran-based DEAE-Sephadex resin (Brewer et al., 1974). The Sephadex resin was initially washed with 2.0 M pyridinium acetate in the present method, while the buffer used for elution of the organic acid fraction was 1.5 M pyridinium acetate. Also, no extraneous peaks were observed with extractions of distilled water. The present organic acid extraction procedure did not involve extreme alkaline conditions. Conversely, no lactones of hydroxy acids were observed, such as may be formed under acidic conditions (Szafranek et al., 1974).

Neither the function nor the metabolism of aldonic and deoxy-aldonic acids is known (Haraguchi et al., 1982a, 1982b; Niwa et al., 1981). These acids were only a few years ago identified as regular constituents of urine. The urine of adults contains large amounts of erythronic acid, threonic acid, and 4-deoxythreonic acid. Moderate amounts of 4-deoxyerythronic acid and 2-deoxytetronic acid are found, while only trace amounts of 3-deoxytetronic acid are normally observed (Thompson et al., 1975). Since the appearance of these compounds is little affected by large dietary alterations, they are probably of endogenous origin. The oxidation of erythrose, a metabolite of glucose, probably gives rise to erythronic acid (Lawson et al., 1976). The

excretion of 2-deoxytetronic acid appears to be greatly increased by a high-glucose diet (Chalmers et al., 1976b; Tracey et al., 1981). The metabolism of 4-hydroxybutyric acid may produce 2-deoxytetronic acid as an intermediate (Niwa et al., 1982a; Walkenstein et al., 1964). Other 2-deoxyaldonic acids may be derived from 2-deoxyribose of deoxyribonucleic acid (Haraguchi et al., 1982a; Truscott et al., 1979a).

While the mechanisms are not yet known, alterations in glucose metabolism appear to influence the aldonic and deoxyaldonic acids. Glycolic acid increases simultaneously with several deoxyaldonic acids in rat heart muscle after a reduction in coronary blood flow (Haraguchi et al., 1982b). The glycolic acid is presumably formed from glyoxylic acid (Haraguchi et al., 1982b). It may be no coincidence that cleavage of the glucose molecule between the second and third carbon atoms would produce a 2-carbon fragment resembling glyoxylic acid and a 4-carbon fragment resembling erythritol. The 2-carbon fragment could lead to glycolic acid, while the 4-carbon fragment could lead to erythronic acid. Erythritol, a compound used as a coronary vasodilator in humans (Windholz, 1976), is also the normal 4-carbon sugar alcohol found in the urine of newborn infants (Horning and Horning, 1970). Cleavage of aldoses such as glucose would be analogous to the metabolism of ascorbic acid via threonic acid and oxalic acid (Tolbert et al., 1967; Hellman and Burns, 1958). Placenta, lens, and seminal vesicles are the only tissues known to contain aldose reductase (Van Heyningen, 1959). Thus, it is not known whether the presently observed increases in urinary excretion of aldonic and deoxyaldonic acids reflect fetal, placental, or maternal metabolism. This account of their unmistakable increase

in excretion with normal pregnancy poses exciting questions as to their reliability as indicators of pregnancy status.

4.2 Acceptability of the present methods

The present organic acid and steroid extraction methods were chosen because they offered the simplest, most reproducible, and most general extraction of each group of compounds. These multicomponent extractions did not optimize the recovery of all components such as could be achieved with methods developed for the analysis of single compounds, but the overall recoveries were generally good.

The majority of workers use the Amberlite-XAD adsorption procedure of Bradlow (1968) for urinary steroid extractions (Vrbanac *et al.*, 1982; Shackleton and Whitney, 1980). The present method performed well in the extraction of steroid conjugates from standard solutions. Due to the complexity of the urinary steroid fraction, however, the gas-chromatographic profiles of urine samples from pregnant women were difficult to interpret. High-resolution capillary columns would be needed in the gas-chromatographic analysis to accurately quantify the individual steroid metabolites. The present method, however, could be considered semi-quantitative since the trend of increasing steroid output in pregnancy was clearly visible (personal observation, data not presented). Indeed, when large deviations from the normal pattern have to be confirmed, no quantitative method is required.

A minority of workers use DEAE-Sephadex anion-exchange extraction of organic acids because the quicker solvent extraction methods have gained wide acceptance. The majority of workers use a diethyl ether-ethyl acetate extraction procedure (Knights *et al.*, 1975; Fitch *et al.*,

1979; Tanaka et al., 1980b) which discriminates against highly water-soluble acids. Other workers have used DEAE-Sephadex extraction procedures preceded by a barium hydroxide precipitation of the inorganic sulfate and phosphate (Thompson and Markey, 1975; Thompson et al., 1977; Gates et al., 1978) which also precipitates many organic acids. The present straightforward DEAE-Sephadex method allowed the quantitative extraction of many organic acids, including the noteworthy polyhydroxy-carboxylic acids (the aldonic and deoxyaldonic acids) which form 30-35% of total organic acid excretion (Chalmers et al., 1976a). These acids would be poorly recovered by solvent extraction techniques, and partially coprecipitated by barium hydroxide sample pretreatment. Thus, the present method allows a truly quantitative extraction of many urinary organic acids. In addition, the lyophilization, methanol transfer, and vortex-evaporation steps in the present procedure lead to more reproducible recoveries (Chalmers and Watts, 1972c) as well as convenience in performing the extractions. Recent publications have emphasized the superiority of anion-exchange extraction techniques compared to other organic acid extraction methods (Boujet et al., 1982; Rehman et al., 1982). High-resolution capillary columns would also increase the accuracy of quantitative organic acid analysis, but with hundreds of acidic metabolites potentially present in urine (Spiteller and Spiteller, 1979), even capillary columns could not be expected to resolve all components.

The present gas-chromatographic methods allow simultaneous organic acid-steroid profiling. The simple derivatization procedures involve oximation and silylation with no sample transfers or sample clean-up before gas chromatography. The low-molecular-weight organic

acids elute before the tricosane internal standard, while the high-molecular-weight steroids elute after tricosane. Thus, quantitative data pertaining to gas-chromatographic behavior of compounds (retention data; detector response factors) can be acquired simultaneously for organic acids and steroids. When examining biological fluids it may not be advisable to directly combine the organic acid and steroid fractions since unexpected cross-contamination of peaks could possibly occur. If the two fractions are extracted and derivatized in parallel, however, they can be analyzed sequentially on the same gas chromatograph under the same instrumental conditions. This is feasible where an automatic sample injector can operate 24 hours a day. Thus, the actual sample extraction procedures would be the limiting factors regarding speed of sample analysis.

The present methods were highly reliable (Buttner _et al._, 1975, 1976). The sample purification methods produced no artifacts. Neither did these methods cause destruction or chemical modification of compounds. Derivative formation was quantitatively reproducible. As little as 1-2 mg of compound in a 24-hour urine sample could be quantified, depending on the extraction efficiency and detector response characteristics of the compound. The recovery of compounds from standard solutions was highly accurate, approximating 100% in several instances. There was good analytical precision for the determination of organic acids in urine, as evidenced by the small coefficient of variation of duplicate determinations from their means. The present methods were also practical. If samples were extracted in 20-sample batches, approximately 1 day was needed for organic acid analysis, while 1½ days

were needed for steroid analysis. The methods can thus be used for a large number of assays where urgency of analysis is a secondary consideration. The resins employed for extraction (DEAE-Sephadex and Amberlite XAD-2) are reusable after appropriate washing procedures. Operation of the gas chromatograph was dependable once the problems of detector linearity and molecular sieve reactivation were corrected.

Various means were employed to identify unknown peaks when they occurred in the chromatograms. The methylene unit value was first determined, thus ruling out possible compound identities. Careful scrutiny was given to extractions of distilled water, thereby excluding the possibility of artifacts. Various oximating agents were employed, thus identifying keto compounds. The history of the urine specimen was investigated by reviewing previous dietary and drug intake. Tentative peak identifications were made on the basis of literature values published by other workers for compounds which were not commercially available. Finally, unequivocal confirmation of peak identity was attained by mass spectral analysis.

Thus, the application of these highly acceptable methods produced reference profiles of urinary metabolites in human pregnancy. These methods could be applied directly to other protein-free fluids, such as amniotic fluid (Ng _et al_., 1982; Nicholls _et al_., 1978), newborn urine (Bjorkman _et al_., 1976), and cerebrospinal fluid (Malcolm and Leonards, 1976). Recent improvements in ultrafiltration procedures (Issachar and Sweeley, 1981) would allow the present methods to be applied to plasma (Issachar _et al_., 1982) and other protein-containing body fluids.

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PROFILING OF URINARY METABOLITES IN HUMAN PREGNANCY

A Thesis

Submitted to the Faculty of Graduate Studies and Research

in Partial Fulfilment of the Requirements

For the Degree of

Master of Science

in the

Department of Biochemistry

by

Elva Jean Christie

Saskatoon, Saskatchewan

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Appendix A

UNIVERSITY OF SASKATCHEWAN

PRINCIPAL'S ADVISORY COMMITTEE ON ETHICS IN HUMAN EXPERIMENTATION

Dr. I.S. Mendelson (EC#78-3)

Your Project entitled "Early Diagnosis of Human Growth and Development Disorders"

has been approved by the Committee.

1. Therefore you are free to proceed with the following conditions:

2. Any significant changes of protocol should be reported to me for the Committee's consideration in advance of its implementation.

3. If you are applying for funds for this project to any of the following agencies, the required form is being completed by this office and forwarded to the agency with a copy enclosed herewith for your record:

- National Institute of Health
- Medical Research Council
- Canadian Heart Foundation

Sincerely,

Dr. T.W. Wilson, Chairman
Principal's Advisory Committee
on Ethics in Human Experimentation

TWW:paw

Appendix B

Informed Consent Form

A Prenatal Study into the Prevention of Disorders in Child Growth and Development

The Alvin Buckwold Centre Department of Pediatrics, University Hospital, Saskatoon would like to collect specimens from you during your pregnancy for the expressed purpose of research into prevention of disorders in growth and development. We would like to establish a biochemical profile of trace elements or minerals (zinc, copper, iron, manganese, chromium), urinary metabolites, hormones and vitamins. This research project may be of value to future generations but if abnormal biochemical values are found in your specimen, your doctor will promptly be informed. The specimens will be collected in the morning by our staff at your home with as little inconvenience to the doctor and yourself as possible.

The following samples are required if you agree to participate in the project:

a) End of 3rd month of pregnancy - fasting blood samples
- a 24 hr. urine collection

b) End of 6th month of pregnancy - fasting blood samples
- a 24 hr. urine collection

c) At the time of birth - a tube of maternal blood
- a tube of cord blood

d) Approximately 6 weeks post partum - a tube of fasting blood & a 24 hr. urine collection

We also request your permission to correlate the biochemical profile with the clinical assessment made by your doctors of your pregnancy and your newborn baby. Strict confidentiality will be maintained.

You are quite free to withdraw at any time from this study.

If you do not agree to participate in the study, this will not effect in any way the quality of medical care from your doctors.

If more information is required or you wish to visit the Alvin Buckwold Centre and its Laboratory, we will be glad to arrange a visit at a suitable time (343-5901). We thank you for considering our proposal and whatever is your decision, we wish you the best of success.

Respectfully yours,

I.S. Mendelson, Ph.D,
Assistant Professor of Pediatrics
Lecturer in Physiology

W.A. Zaleski, M.D., M.R.C.Psych., F.R.C.P.(C)
Professor, Department of Pediatrics

I agree to participate in the Alvin Buckwold Centre's prenatal study into the prevention of disorders in child growth and development.

Doctor's name________________ Doctor's Signature________________

Patient's name________________ Signature________________

Address________________

Telephone Number: Work________________ Home________________ SHSP#________________

Return to:

Alvin Buckwold Centre, Department of Pediatrics,
Room 45, Ellis Hall
University Hospital, Saskatoon, Saskatchewan,
S7N 0W8

To Be Filled in by Doctor:

Last Menstral Period ________________

Expected Date of
Confinement________________

Appendix C

Dear Participant,

Thank you for participating in our study into the prevention of disorders of child growth and development.

A member of our laboratory staff will be contacting you shortly regarding your preparation for the collection of specimens as follows:

(1) The night before the first morning visit, you must not eat or drink, except for water, after 12:00 midnight until our technologist arrives early in the morning.

(2) When our technologist arrives, she would like to collect a morning urine specimen, at which time the 24-hour urine collection will begin. The 24-hour collection will be completed when you void at exactly the same time the next day.

(3) A venous blood sample will also be taken.

(4) Arrangements will be made to pick up the 24-hour urine specimen the following day. If you are passing by the University Hospital, it can be deposited at Room 45, ground floor, Ellis Hall, Alvin Buckwold Centre.

Please retain this letter for our next visit in approximately 3 months.

If at any time you wish to withdraw, please feel free to inform us. If you have any questions or suggestions, we would like to hear them. We thank you again for your participation.

Our best wishes,

I.S. Mendelson, Ph.D.
Assistant Professor
Department of Pediatrics

W.A. Zaleski, M.D., M.R.C.Psych., F.R.C.P.(C)
Professor, Department of Pediatrics

Appendix D

DIET RECALL

Name:____Date:_____________________

From memory, please list as accurately as possible all food and beverages (and the approximate quantity of each) consumed in the previous 24 hours.

Breakfast

Lunch

Supper

Snacks

Medications

Appendix E

24-HOUR DIET DIARY

Name:____Date:____________________

Your present weight:___________________________________Height:__________________

In the following chart, please record all substances eaten for the duration of the 24-hour urine collection. This includes all food and beverages and the approximate quantity of each.

Time of meal (or snack)	Food or beverage
Example: 8:00 a.m.	8 oz. milk
9:30 a.m.	1 banana

Appendix F

Saskatchewan Health

Hospital Services Plan

Prenatal Record

Health 19 42 5 79

Name	Age	Date of Birth
Address		Telephone No.
Father of Child		Date of First Visit

Family Physician	Obstetrician	Pediatrician

Obstetrical History Including Abortions:

Date	Place of Confinement	Dur. Preg. Wks	Hours of Labour	Type of Delivery	Complications Mother and or Infant	Child Sex	Birth Wt	Pres. Health

Past illnesses:	No	Yes	Specify
Renal			
Cardiac			
Infections			
Rubella			
Venereal			
Allergies			
Operations			
Transfusions			
Other			

Family history:	No	Yes	Specify
Diabetes			
Cardiac			
Hypertension			
Tuberculosis			
Twins			
Malformations			
Mental Retardation			
Other			

Menstrual history:

Cycle ________ Duration __________

L.M.P. ____ Normal Abnormal

Method of contraception:

Date of Discontinuance

Pre-Preg. Weight | Height

History of present pregnancy: Specify

Nausea, Vomiting	No	Yes	
Bleeding	No	Yes	Date:
Fever	No	Yes	Date:
Radiation	No	Yes	Date:
Smoking	No	Yes	Amount:
Drug Use	No	Yes	Identify:
Nutrition	Poor	Good	

Current medications

Examination Date ____________________

General Condition:	Breasts:
Head & Neck:	Abdomen:
Lymph Nodes:	Spine:
Heart:	Limbs:
Lungs:	Pelvic Examination:

Fetal Risk Scoring "A" Baseline Data (Maximum Score is 3)

Age 35 +	1
40 +	2
Para 0	1
6 +	2
Interval less than 2 years	1
Obesity 200 lb. +	1
Diabetes - Mild, Moderate	2
Severe	3
Chronic Renal Disease	1
with Diminished Renal Function	3
Pre-Existing Hypertension	
140 + 90 +	1
160 + 110 +	2
Rh iso - Immunized Mother	2
Pos. History of Erythroblastosis	3

Total Score Part "A"

Previous Obstetrical History

Abortion	
Stillbirth	
Neonatal Death	
Surviving Premature Infant	
Antepartum Hemorrhage	
Toxemia	
Mid-Forceps Delivery	
Cesarian Section	
Major Congenital Anomaly	
Baby 10 lbs. +	
One Instance of Above	1
Two or More Instances of Above	2

Doctor's Chart

Appendix F

Full Term Deliveries	Prematures	Abortions 500 grams or less	Live Births: Surviving 7 days	Live Births: Dying before 7 days	Grav.	E.D.C.	Date of Quickening

V.D.R.L.	ABO Group	RH	Father of Child RH Genotype	Rubella Titre	Pap Smear	Notations for Delivery

Fetal Risk Scoring "B" Present Pregnancy (Maximum Score is 3)

e.g. Hydramnios 3

Bleeding, before 20 weeks
- Alone ... 1
- With Pain ... 2

Bleeding, after 20 weeks
- Ceased ... 1
- Continues ... 2
- With Pain ... 3
- With Hypotension ... 3

Spontaneous Premature
- Rupture of Membranes ... 1
- Latent Period 24 hr. + ... 2

Anemia 8 to 10 gms. ... 1
- Less than 8 gms. ... 2

No Prenatal Care ... 2
- 1 to 3 Prenatal Visits ... 1

Toxemia Mild - Moderate ... 1
- Severe ... 3

Hydramnios (Single Fetus) ... 3

Multiple Pregnancy ... 2

Abnormal Glucose Tolerance ... 1

Decreasing Insulin Requirement ... 3

Maternal Diabetic Acidosis ... 3

Maternal Pyrexia ... 1

Pyrexia + FHR Greater Than 160 ... 2

RH Negative
- With Antibody Titre ... 2
- With Amniotic Fluid Liley Zone III ... 3

C Gestational Age (At time of scoring)
- 28 Wks. or Under ... 4
- 29 - 32 Wks. ... 3
- 33 - 35 Wks. ... 2
- 36-37 Wks. ... 1
- 38 - 41 Wks. ... 0
- 42 Wks. ... 1
- 43 Wks. or More ... 2

Other Investigations

Date	Type	Findings

Prenatal Classes Yes No

Father For Case Room Yes No

Fetal Risk Score (Maximum is 10)

A. Baseline Data [ ] + B + C = Total Score

Evaluation Summary
- Score 0 to 2 Low Risk
- Score 3 to 6 Moderate Risk
- Score >6 High Risk

Date	Dur. of Preg. in Weeks	Fundus in Weeks	Present # and Position	F H Rate	Engage-ment	B P	WT	Edema	Urine Alb	Urine Sug	H B	Risk Score	Medications and Remarks	Return On

Appendix G

University Hospital, Saskatoon
INFANT DISCHARGE FORM

ANTEPARTUM:

Maternal Age ______ Antibodies: Yes ☐ No ☐ Specify: LMNP ☐ Grav. ______

Blood Type ______ Due ☐ Para. ______

Last HGB ______ Gestn. At Del. ☐ Abort. ______

Antepartum Status

ABNORMALITIES OF PREVIOUS PREGNANCIES: Yes ☐ No ☐
Specify:

ABNORMALITIES OF PREVIOUS INFANTS: Yes ☐ No ☐
Specify:

COMPLICATIONS OF THIS PREGNANCY: Specify:
None ☐ Toxemia ☐ Heart ☐
Bleeding ☐ Diabetes ☐ Other ☐

LABOR & DELIVERY: Fetal Distress: Yes ☐ No ☐
Spont. ☐ Specify Indications:
Operative ☐
COMPLICATIONS: Specify:
(Include Forceps)

CONDITION 1 & 5 Min. AFTER BIRTH (Grade as 0,1,or 2)

Tone ______ ______
Color ______ ______
Respirations ______ ______
Response ______ ______
Heart Rate ______ ______
Apgar ______ ______

First Breath ______ Secs.
First Cry ______ Secs.
Sustained Respirations ______ Secs.
ASPHYXIA: None ☐ Mod. ☐ Severe ☐

RESUSCITATION: None ☐
Oxygen Only ☐ I.P.P.B. & Mask ☐
I.P.P.B. & E.N.T.
Duration: ______ to ______ Mins.

Boy ☐ Alive ☐ Antiseptic Eye Drops ☐
Girl ☐ Stillborn ☐ Vit.K_1 ☐

Weight ______ G
Length ______ Cm
Head ______ Cm

Preterm ☐ Full Term ☐ Post Term ☐
Specify Gest. Age ______ Wks.
S.G.A. ☐ A.G.A. ☐ L.G.A. ☐
H_gB ______ G
Blood Type ______

ON EXAMINATION: Specify Abnormalities:

Scalp & Skull ☐ Mouth ☐ Kidneys ☐ Hips (Abduction) ☐
Fontanelles ☐ Heart ☐ Umbilicus ☐ Skin ☐
Sutures ☐ Femoral Pulses ☐ Inguinal Orfices ☐ Sucking ☐
Eyes ☐ Lungs ☐ Genitalia ☐ Rooting ☐
Ears ☐ Liver ☐ Rectum ☐ Moro ☐
Nose ☐ Spleen ☐ Extremities ☐

COMMENTS:

Signature: ______________________

DISCHARGE: Alive ☐ Died ☐ Weight ______ G. H_gB ☐ Feedings &/Or Medications:
Autopsy: Yes ☐ No ☐ Length ______ Cm.
Head ______ Cm.