A SURVEY OF DETECTION METHODS FOR OVULATION AND A PROPOSED METHOD FOR ESTROGEN DETERMINATION

by

Maria Lanni

* * * * * * *

Submitted in partial fulfillment
of the requirements for
Honors in the Department of Chemistry

UNION COLLEGE

June, 1981

ABSTRACT

LANNI, MARIA A Survey of Detection Methods for Ovulation and a Proposed Method for Estrogen Determination. Department of Chemistry June 1981.

A rapid, accurate method of determining the exact phase of a menstrual cycle at a given time is extremely important. This information is vital to doctors studying menstrual abnormalities and more importantly as an aid in conceptive and contraceptive decisions. Many methods based on changes in the body have been used to detect ovulation. These nonquantitative methods depend on the cyclical variations in basal body temperature, physical and chemical properties of mucus and content of saliva. These changes are influenced by changing hormone levels. Specifically, estrogen levels rise several days prior to ovulation, therefore, methods for determining estrogen concentration in blood and urine are of interest. Most methods involve hydrolysis and purification followed by radioimmunoassay, colorimetry, fluorimetry or enzymatic determinations. None of the methods proved satisfactory due to accuracy limitations or time demands.

A method involving hydrolysis, ether extraction and separation and detection using high performance liquid chromatography is proposed. A 2,4 - dinitrophenylhydrazine derivative of estrone was investigated in order to increase its extinction coeficient. A similar 2,4DNP-cyclohexanone derivative was made and the limits of sensitivity of the

HPLC - UV instrument using the derivative were found to be $1x10^{-6}$ g/mL. The 2,4DNP - estrone derivatization failed to occur.

The molar extinction coefficients for estrone, estradiol and estriol were found to be 2207, 1902, and 2114 respectively. HPLC retention times were $4.55 \pm .10$ for estrone, $4.4 \pm .5$ for estradiol and $3.25 \pm .10$ min. for estriol in separate acetonitrile solutions. Difficulty was encountered in separating estrone and estradiol from a mixture of the three standards although various HPLC programs were tried. Areas of further investigation are discussed.

I would like to thank Professor Karen Quasl for giving me the opportunity to research an extremely interesting and vital subject and in addition, for working with me until the very end.

TABLE OF CONTENTS

LIST OF FIGURES

Introduction

An accurate method of determining the exact phase of a menstrual cycle at a given time is extremely important. This information is vital to doctors studying menstrual abnormalities and, more importantly, as an aid in conceptive and contraceptive decisions.

The average menstrual cycle lasts 28 days (1, 2). It can be divided into three phases. The first phase is an infertile phase. Its duration varies, usually lasting 15 days or longer (3, 1). This preovulatory phase starts on the first day of menstruation when the uterine lining is being shed from the previous cycle. In the preovulatory or follicular phase a new egg-containing follicle ripens in the ovary and the uterine lining begins to rebuild. When the egg is ready to be released the follicle bursts. This is ovulation, the second phase, which lasts a few hours. The ripe egg is carried from the ovary through the fallopian tubes where it can be fertilized by sperm cells for up to 24 hours (3, 2).

In the post-ovulatory or luteal stage the lining of the uterus is built up to accomodate a fertilized egg and eventual fetus. If fertilization does not occur the lining will degrade, causing menstruation and the beginning of a new cycle. The length of the luteal phase is 14 ± 1 day. The fertile period is then, very short. Since the egg can only be fertilized for 24 hours and sperm can only live for up to 5 days (3, 2) in the reproductive tract of the female, conception can only occur on five days of a cycle. Intercourse can not result in pregnancy on at least 80% of the days.

In cases where a conception is planned, it is necessary to know when the few fertile days are in order to increase the chances of a pregnancy. This is especially true when low sperm counts are involved.

On the other hand, many couples are trying to avoid conception. A method of contraception is needed which, unlike the pill, IUD and other devices, will not interfere with the body in any way, yet will be more effective than the rythmn or natural family planning methods.

The pill may cause thromboembolic disorders (blood clotting problems) which may lead to strokes. In addition, headaches, leg cramps, shortness of breath, chest pains, increased blood pressure, aggravation of epilepsy and asthma, precipitation of diabetes, and increased susceptibility to vaginitis due to altering the chemical balance in the vagina, and possible increased irritability and depression have been attributed to the pill (4).

Common side effects of the I.U.D. are cramping and abdominal pain, increased menstrual flow and irregular bleeding. Problems in insertion may cause infection in the uterus and fallopian tubes. The IUD can not be used by women with endometriosis, venereal disease, vaginal or uterine infection, small uterus, uterine deformations, cardiac disease, anemia, or heavy menstrual flow (4).

The two prestated methods interfere with the body during the entire cycle. Many women feel an entire month is sacrificed for the sake of five days. If a woman's fertile period could be predicted, conception could be prevented by abstaining or using a condom, diaphragm or other barrier method on the fertile days.

There are many cyclical changes occuring in a woman's body during the month. By pinpointing these changes and correlating them to the phases of the menstrual cycle, a method of detecting the phase a woman is in can be developed.

This paper is a survey of detection methods and an attempt to develop a simple, accurate, quantitative means by which a woman can predict her fertile period.

CHAPTER I

A wide variety of cyclical physical changes occur in the body throughout the menstrual cycle. These cyclic variations are influenced by changing hormonal levels. Various methods of predicting fertile periods have been developed based on these changes.

One popular method is based on the fact that the rising progesterone level during the luteal phase causes a thermogenic change in the body. At ovulation the woman's temperature rises an average of 0.3°C (3). The method consists of carefully recording the morning basal body temperature (BBT). Typical temperature cycles are shown in figures 1-3. The infertile period after ovulation occurs after the temperature rise (Figure 1).

One disadvantage of this method is that sometimes the graphs are hard to interpret because small temperature differences are subject to significant fluctuations (Figure 2). Another problem is that BBT is affected by non-hormonal influences such as illness, late night hours and alcohol consumption (Figure 3).

There are other physical changes which should be mentioned. Just before ovulation the cervix dialates and breasts may feel tender. When the follicle bursts there may be slight bleeding and a pain called mittleschmertz. These phenomena can not be used as detection methods due to their subjective nature.

Other prediction methods are based on changes occuring in the cervical mucus. Researchers have found cyclic behavior in quantity,

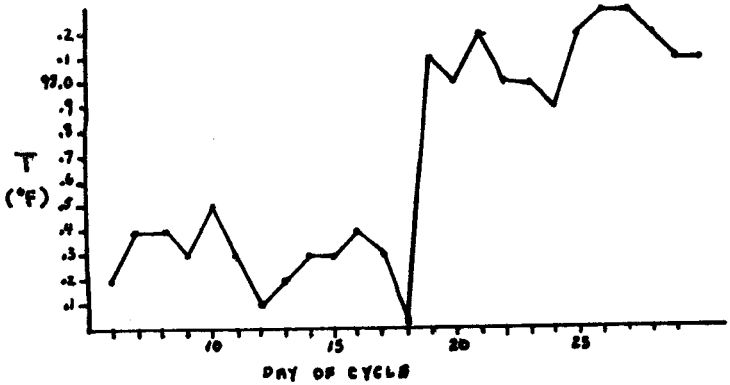


Figure 1. A normal BBT cycle.

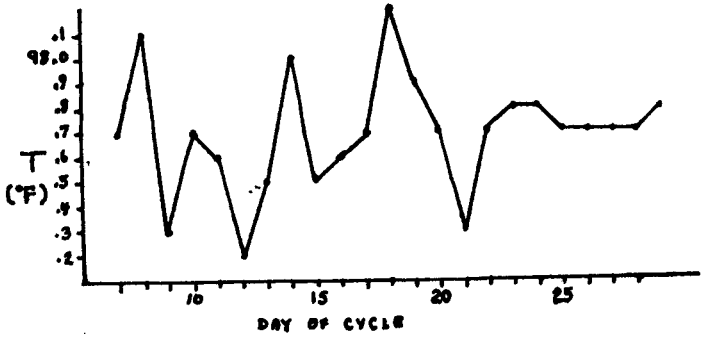


Figure 2. A monophasic BBT cycle.

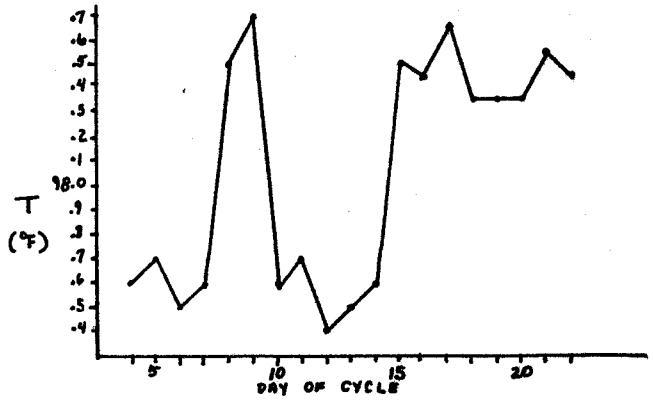


Figure 3. An abnormal BBT. The temperature variation was caused by a cold.

viscosity, cellularity and pH of mucus. Variations in spinnbarkeit, ferning and penetrability to sperm have also been investigated.

The consistency of mucus discharge varies from day to day. There is no discharge noted after menstruation. Several days later a sticky, cloudy, viscous discharge is apparent. Midcycle, just before he basal body temperature rise, the mucus becomes copious, thin, clear and lubricative. After ovulation there is a decrease in amount and an increase in viscosity (8, 9, 7).

Cervical mucus is a hydrogel consisting of 90-99% water, inorganic salts, sugars, glycogen, cholesterol and fifteen amino acids (10, 11). One and a half to eleven percent of the mucus is a suspension of proteins and gel-forming carbohydrate-rich glycoproteins called mucins (10, 11). Contributions to the cervical mucus come from the peritoneum, ovaries, fallopian tubes, cervix and vagina in the form of secretions and cellular material.

Changes in cervical mucus have been shown to correspond with changes in hormonal levels (10, 9). Under the preovulatory influence of increasing estrogen levels, viscosity decreases. This is due to a decrease in the ratio of proteins to carbohydrates and a corresponding increase in water content. The quantity of mucus also increases. After ovulation, when progesterone levels rise, the protein to carbohydrate ratio and viscosity increase while the quantity of mucus decreases noticeably (3, 10, 11).

Moghissi attempted to characterize the changes in quantity, viscosity, cellularity and pH using a rating scale of one to four (1). His results are found in Figures 4-7. Quantity and viscosity change as previously described. Cellularity, the estimated number of leucocytes, is at a minimum at ovulation (12). Moghissi determined the pH of cervical mucus to remain at an average of $8.3 \pm .3$ (1). Other studies have found that the pH varies from an acidic 6.8 (in non-fertile periods) to a basic 8.0 (at midcycle) suggesting that pH could be used for a test of fertility (13, 14). The apparent discrepancy can be explained by distinguishing between cervical mucus pH and vaginal pH. The pH of the vagina reportedly remains between 4 and 5 (15). Neutralization of the mucus by the acid vagina occuring most notably when there is scanty mucus, causes the mucus to appear acidic. Therefore, a pH test is actually only measuring mucus quantity.

Erbring used the term spinnbarkeit to discuss the elasticity or fibrosity of mucus. Spinnbarkeit can be measured by stretching the mucus between a slide and coverslip and noting how easily it breaks. Early in the cycle spinnbarkeit values are low. Several days before ovulation, the mucus becomes highly elastic and easily stretched. This is due to the existence of long macromolecular threads (11). After ovulation, elasticity decreases rapidly (9). There have been many attempts to quantify spinnbarkeit values, however, none have been standardized or consistently accurate in determining ovulation (9).

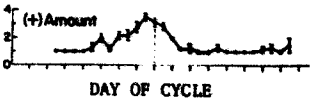


Figure 4. Variation in amount of cervical mucus (35).

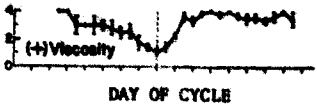


Figure 5. Variation in viscosity of cervical mucus (35).

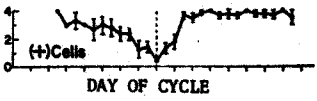


Figure 6. Variation in number of cells in cervical mucus (35).

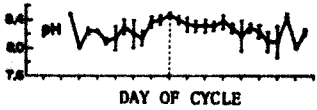


Figure 7. Variation in pH during the menstrual cycle (35).

Ferning is another property of mucus. In 1946 Papanicolaou discovered that mucus dries in a crystalline "fern" pattern (16). The ferning occurs due to interactions of sodium chloride and protein (9). For the first seven days of an average cycle ferning occurs in 10% of a smear of mucus. By the thirteenth day ferning has increased to 85%. Ferning peaks at ovulation and decreases afterward. Cyclic ferning has also been found in female nasal mucus (16) and saliva (17). Ferning more accurately pinpoints ovulation than spinnbarkeit yet it is still not easily quantified.

Another trait of mucus is sperm penetrability which is dependent on the structure of the mucus fibers (9). Near ovulation, the mucus is very receptive to sperm (11, 9, 10, 12). As mentioned earlier, there are not many leucocytes in the mucus at this time. Also, the copious mucus protects the sperm from the acidic conditions and phagocytes in the vagina. The sperm metabolize the abundant glycogen and other reducing substances present at this time (14). The orderly arrangement of mucoid molecules and low viscosity aid the movement of the sperm (11). In contrast, the mucus' high viscosity and randomly coiled mucoid molecules make it difficult for sperm to travel during infertile periods (11).

Quantitative determination of spermigration and other observed changes in the mucus are of little practical use as a conceptive or contraceptive aid. This is especially true since any infection in the vaginal tract will render these observations useless.

Methods which are more chemical in nature than those previously discussed have also been developed. Much work has been done on developing tests for alkaline phosphatase, glucose and chloride ion variations during the menstrual cycle.

The enzyme alkaline phosphatase is active in saliva. Its concentration reportedly peaks zero to ten days before ovulation (18). Lorincz developed a color test using filter paper saturated with alkaline phosphatase sensitive chemicals. In the presence of alkaline phosphatase the paper turns from white to blue. The "blue" days could be assumed fertile days. The enzyme appears to originate from cells lining the oral cavity. The noted increase in enzymatic activity is caused by an increased number of cells present in the saliva during ovulation. However, because samples would vary considerably in cell content, this method might be unreliable.

The glucose content of saliva has also been found to cycle. At ovulation, the follicular contents of hormones, carbohydrates and proteins empty into the abdominal cavity. They are absorbed thereby elevating the blood and urine glucose levels (19). Daves and Balin devised a color test using a stick coated with glucose oxidase, orthotoluidine and peroxidase. The "dextrostick" catalyzed the oxidation of glucose to gluconic acid and hydrogen peroxide. Hydrogen peroxide and orthotoluidine form a blue color of varying intensity in the presence of peroxidase. The test is inaccurate since the glucose content of blood is highly dependent on the amount and type of food injested (6).

Another color test was developed for changing chloride levels. It was thought that sodium chloride concentration might cycle since it is involved in ferning. McSweeney and Sbarra developed a spot test by precepitating brown silver chromate from a mixture of soluble silver and soluble chromate salts onto a test paper. Treating the test paper with varying chloride ion concentration would cause a white precipitate of silver chloride to form leaving a spot of varying intensity. McSweeney and Sbarra found the sodium chloride concentration high at ovulation and low before and after.

The actual sodium chloride content of mucus remains at 7% (11, 3). Therefore, the test measures the quantity of mucus present thus leaving it subject to many inaccuracies in sample collection.

Other studies have attempted to quantify changes in salivary phosphates (20), salivary calcium and sodium (21), proteins (14) and citric acid. These changes seemed to correlate with the basal body temperature shift.

To date none of the hormonally influenced changes discovered have lead to a good prediction test for ovulation.

CHAPTER II

The cyclic patterns of the menstrual cycle are caused by variations in hormone levels. None of these changes have proved accurately determinable. It seemed logical to look to the source of change: variations in hormone levels.

During the follicular phase, the pituitary released follicle stimulating hormone (FSH) which causes ovarian follicle growth. At the same time the follicles produce estrogen which initiates rebuilding of the uterine lining. When the estrogen level rises, the pituitary reduces FSH production and releases lutenizing hormone (LH). Estrogen production decreases while LH production peaks sharply, causing one follicle to rupture and release the ripe egg. The corpus luteum (remnant of follicle) begins to release progesterone. At the same time a second rise in estrogen levels occurs. Together, these two hormones halt the growth of other follicles and prepare the uterus for the ovum. Menstruation occurs when progesterone levels decline due to the degeneration of the corpus luteum (23). Figures 1-4 show typical hormone level cycles. Follicle stimulating hormone peaks 1 to 2 days before ovulation (Figure 1). Estrogen levels peak several days before (Figure 2), where the LH surge occurs at ovulation (Figure 3). Progesterone (Figure 4) and estrogen both increase after ovulation (1). The first and largest estrogen peak occurs prior to ovulation. A test for increasing estrogen levels would predict impending ovulation. A test for estrogen would also be useful during pregnancy because levels of estrogen excretion reflect fetoplacental

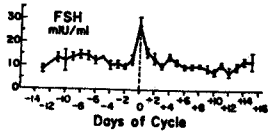


Figure 1. Follicle stimulating hormone levels throughout the menstrual cycle (35).

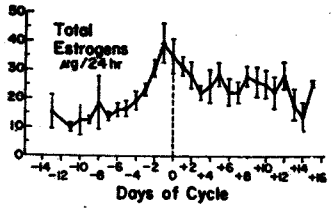


Figure 2. Estrogen levels throughout the menstrual cycle (35).

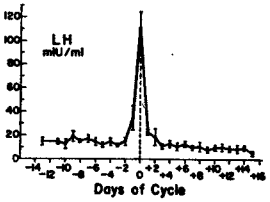


Figure 3. Lutenizing hormone levels throughout the menstrual cycle (35).

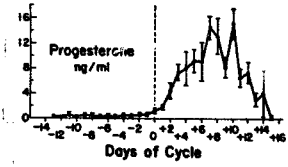
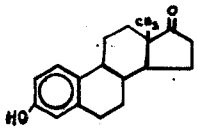


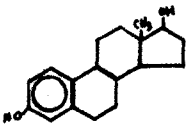
Figure 4. Progesterone levels throughout the menstrual cycle (35).

function (24). If estrogen levels fall below 5 mg per 24 hours there is a high chance of fetal death, premature labor, preeclampsia, or anencephalic pregnancy (25, 26).

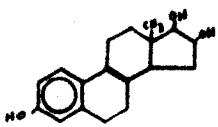
There are three basic forms of estrogen, estrone (E1), estradiol (E2) and estriol (E3). These are shown below.



estrone



estradiol



estriol

The estrogens are produced in the endocrine glands (27). Both estrone and estradiol have been found in high concentration in the follicular fluid during the follicular and luteal phases (28). Prior to ovulation estradiol is produced by the largest follicle, while after ovulation the corpus luteum produces it. Estrone is synthesized outside of the ovary (30).

Obtainable sources of estrogen include bile, blood and urine (29). In the urine, most estrogens are conjugated with glucuronic acid or sulfuric acid (29, 30). Conjugation makes the estrogens water soluble and facilitates urinary excretion.

Concentrations of each estrogen range from 5-30 mg/24 hr. urine sample in non-pregnant women (1, 31) with estrogen concentrations in pregnant women being 1000 times greater. Estrone is the most abundant of the three during the follicular phase (Figure 5) (31, 44). Due to

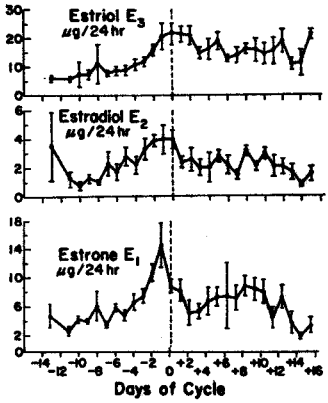


Figure 5. Variations in estrogens during the menstrual cycle.

its low concentration a sensitive method is needed for detection of urinary estrogens.

Many methods to quantify the concentration of estrogens in blood plasma and urine have been devised. Most are comprised of 3 parts: hydrolysis, purification and detection. Urine is collected for 24 hours in most cases but studies have found that 12 hour urine samples also give accurate results (33, 34).

Urine samples are usually hydrolyzed by acids or enzymes to free the steroids from conjugation (35). In acid hydrolysis, the urine is boiled with concentrated hydrochloric acid. Up to 20% of the estrogen may be lost by this method unless the urine is first diluted. Even when diluted, up to 9% losses have been reported (30, 36). Enzymatic hydrolysis consists of reacting the urine with β-glucuronidase and phenolsulfatase (30, 37, 38). Enzymatic hydrolysis of the three estrogens is more complete and occurs without large losses, however the incubation period may be as long as 96 hours (39).

Purification is accomplished either before or after hydrolysis. The samples are usually purified by extraction, ammonium sulfate precipitation, gel filtration or chromatographic methods. Two or more of these methods are often combined.

Hydrolyzed urine can be extracted into ethyl ether, petroleum ether, toluene or benzene. The lower aqueous phase is discarded (36). The estrogens remain in the organic solvent. Sodium chloride may be added to improve extraction of estriol and light petroleum ether may improve

extraction of estrone (36). The organic phase is often washed with a weak base (36, 40). Then a final organic extraction is made.

Another purification method uses ammonium sulfate precipitation which usually precedes hydrolysis. Addition of ammonium "effectively removes many substances that interfere with estrogen assays" including substances which may cause emulsions in an extraction step (41).

Gel filtration using sephadex columns is also used for purification. The estrogens are held back on the column until eluted with a buffer. The estrogens elute in two bands from the columns (39, 42).

Estrogens can also be separated and purified using thin layer chromatography (42, 44). The phenolic steroids are located on the silica gel plates by spraying with potassium ferricyanide reagent (43).

Other chromatographic methods use ion exchange resin (32), magnesium silicate (46) and alumina columns (47) to separate the estrogens.

After hydrolysis and purification the estrogens are detected and quantified. Radioimmunoassay, colorimetry, fluorimetry, gas liquid chromatography followed by mass spectroscopy or electron capture detection, are among the detection methods. For blood plasma, radioimmunoassay and enzymatic determinations are used. All of the above methods can be used for urinary analysis.

Radioimmunoassay (RIA) is often used as a check on other methods since it is more accurate (48, 49). Two major types of RIA are used. In the first, the double isotope derivative method, the estrogens are tagged

by forming derivatives with radioactive chemicals (50). The second involves competitive protein binding which utilizes an antibody-antigen reaction. An estrogen specific antibody is bound to radioactively labeled estrogen. When the sample of unknown estrogen concentration is added, reversible binding competition between the labeled and unlabeled estrogens occurs. The bound estrogens are separated from the free estrogens and the bound sample is counted using a scintillation counter. The amount of radioactivity detected is inversely related to the amount of estrogen in the sample (50, 51). Calibration curves are necessary for both RIA methods. Good purification techniques are required since the RIA antisera are susceptible to interference from other urinary compounds (45). Also, phytoestrogens, which are estradiol-like plant substances can combine with the protein to give false results (52). Another problem is the danger of radioactive contamination.

A colorimetric method for measuring estrogen levels in non-pregnancy urine has been developed by Brown (47). A solution prepared by dissolving quinol in sulfuric acid is known as the Kober reagent (35). This reagent is mixed with the hydrolyzed, purified estrogens to develop a colored complex that forms on warming. This colored complex can be measured spectrophotometrically. Quantitative color measurement is easy for pregnancy urines where the estrogen concentrations are high and interference is relatively low. For non-pregnancy urines, Brown's method consisted of acid hydrolysis, followed by methylation of the phenolic group and extraction into benzene or light petroleum ether. Further separation of the estrogens is accomplished on alumina columns (47).

To minimize interference by glucose Brown added sodium borohydride (47). The methylated estrogens show absorbance at 516nm(1 & 3) and 518nm (2) (47). Despite prior purification the Allen correction (1) for impurities must be used due to the large number of interferring impurities. This method does not lend itself to routine analysis due to the complicated purification steps.

$$\text{corrected reading} = 2D_{\substack{516 \\ \text{or} \\ 518}} - (D_{480} + D_{552}) \quad (1)$$

D is the optical density at the subscripted wavelength (47).

Ittrich also used the Kober reagent to form an estrogen complex. However, he developed a specific extraction method and measured the product fluorimetrically. Since fluorimeters are very sensitive (10 ng E2/mL) (50) the low estrogen concentrations could be determined with better accuracy. The urine was hydrolyzed, reacted with Kober reagent, extracted, then measured fluorimetrically. The Ittrich reagent for extraction of the estrogen complex consists of o-nitrophenol in chloroform (54) or in a solution of ethanol mixed with sym-tetrachloroethane (36). The product is then excited at 546 and 490 nm followed by fluorescence at 565 and 520 nm (36).

Impurities from glucose interference are removed with sodium borohydride but other organic impurities which cause quenching must be corrected for with equation(2)(54).

$$\text{Corrected Fluorescence} = F_{546/565}{}^{-2} \left(F_{490/520}\right) \quad (2)$$

F is fluorescence at the subscripted wavelengths.

The estrogen content of a sample is calculated by comparison to a standard calibration curve (36).

Many researchers have attempted to modify the Ittrich method by modifying hydrolysis and purification steps but impurities still interfere (41, 55-57). Purified urinary estrogens can also be determined using a gas chromatogram with either an electron capture detector or a mass spectrometer. Trimethylsilyl derivitives are often made to increase the volatility of the compound prior to the chromatographic step. This method is limited by the sensitivity of the gas chromatograph (51). Variations of this method have been investigated (32, 43, 44, 46, 58, 59).

Enzyme catalyzed conversion can be used to analyze estrogen concentration in both plasma and urine. Transhydrogenase causes interconversion of E2 and E1 with an accompanied conversion of NAD to NADH. NADH absorbs light at 340 nm. The spectrophotometrically determined NADH concentration is directly related to estrogen concentration (61).

The estrogen to creatinine ratio in pregnancy urine has been studied to avoid the necessity of 24 or 12 hour urine collection. The E/C ratio of first morning urine has been found to correlate with estrogen concentration determined in 24 hour urines (26). No literature on non-pregnancy E/C ratios was found.

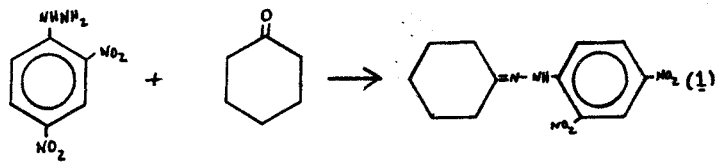
Additional methods have been developed for pregnancy estrogen values. One of the more interesting determinations involved an estrogen-latex agglutination inhibition reaction. Latex particles were coated with an antiestrogen antibody. Competitive interference by estrogen stopped the agglutination reaction of the latex and antibody. The amount of agglutination is inversely related to the estrogen concentration. The reported sensitivity is poor (.1 mg/l) (62).

In some laboratories estrogen determinations are still being performed by injecting animals with hormones and quantifying changes in the animals bodies. This method is not very chemical in nature and implies that a satisfactory test has not yet been developed (63).

CHAPTER III

The long range goal of the project entails developing a method of determining estrogen levels with minimum preparation and instrumentation. Due to various problems in previous methods I have proposed a less rigorous procedure whereby the urinary estrogens, after enzymatic hydrolysis and diethyl ether extraction, are analyzed with a high performance liquid chromatograph (HPLC) equipped with an ultraviolet spectrophotometer. A varian HPLC model 5000 with an attached Tracor U.V. spectrophotometer model 970 A was used. A Cary Model 118 U.V. spectrophotometer was also used in the experiments.

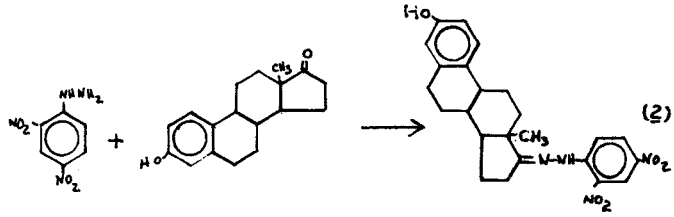
The preparation of a 2,4-dinitrophenylhydrazone of estrone was attempted in hope that it might increase the extinction coefficient substantially, thereby increasing the sensitivity of the U.V. spectrophotometer. Estrone is the largest contributor to total estrogens in the follicular phase (31, 33). Its properties have been modified by reaction with hydrazine (60). Based on this literature it was thought that a 2,4-dinitrophenylhydrazine (2,4DNP) derivative could be prepared. The U.V. behavior of the derivative should be similar to the 2,4DNP derivative of cyclohexanone (64). With this in mind, a systematic investigation of the cyclohexanone derivative was conducted. The limits of sensitivity of the U.V. spectrophotometer to the cyclohexyl-2,4-dinitrophenyl-hydrazone (III) were determined. The 2, 4 DNP was prepared by the Fieser Method (65). The 2,4-dinitrophenylhydrazine (I) reagent was reacted with cyclohexanone (II) to yield product (III) (1).



2,4-dinitrophenyl-hydrazine (I) | cyclohexanone (II) | 2,4-DNP-cyclohexanone derivative (III)

The observed melting point of the orange derivative was 158-161 C. The literature value is 162 C. The maximum absorbancy of (III) occurred at a wavelength of 380 nm. (max). A U.V. spectrum of (III) was run on the Tracor spectrophotometer (Figure 1). The sensitivity of the HPLC-UV system was determined to be $1x10^{-6}$ g/mL. Figure 2 shows the final chromatogram of (III) at $1x10^{-6}$ g/mL in diethyl ether. Figure 3 shows the chromatogram of diethyl ether. The peak due to the derivative can be clearly seen.

An estrone -2,4DNP derivative was attempted: estrone was dissolved in ether and 2,4DNP was added. If reaction (2) had occurred a shift in the max and a sharp increase in extinction coefficient for estrone would be expected.



2,4-dinitrophenylhydrazine (I) | estrone (IV) | 2,4DNP-estrone derivative (V)

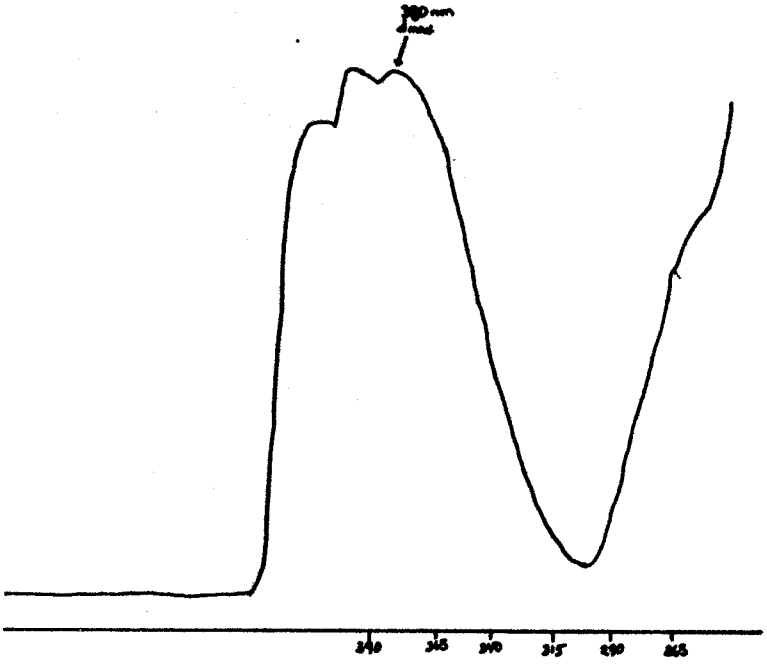


Figure 1. UV spectrum of 2,4DNP-cyclohexanone derivative (III) on Tracor spectrophotometer.

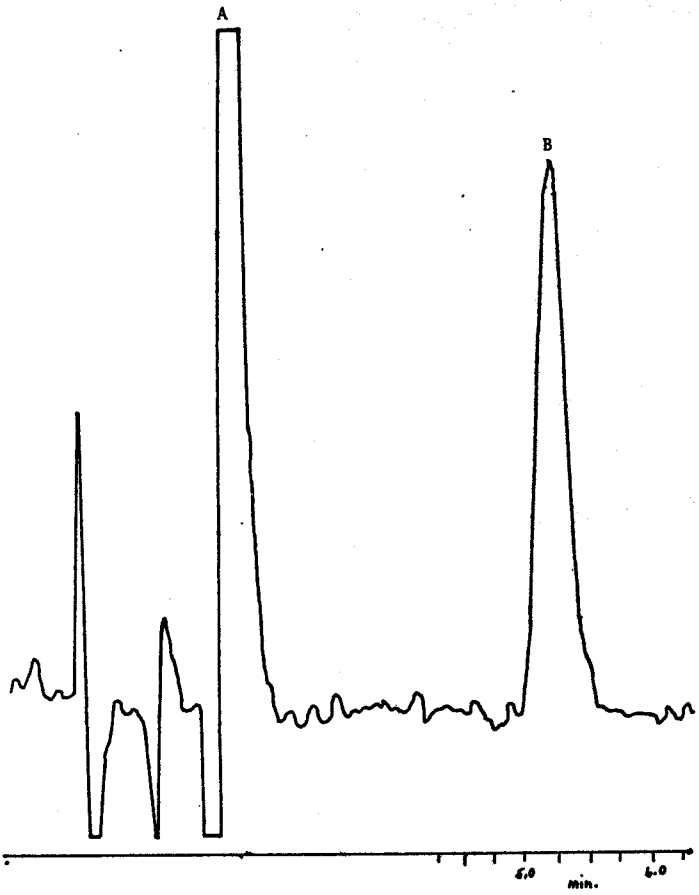


Figure 2. HPLC chromatogram of 2,4DNP-cyclohexanone (III) derivative at sensitivity limit. Concentration: $1x10^{-6}$g/mL. Flow rate: 1.5 ml/min. Detection wavelength: 380 nm. Sensitivity: 005 AU/mV. Peak A: diethylether. Peak B: (III).

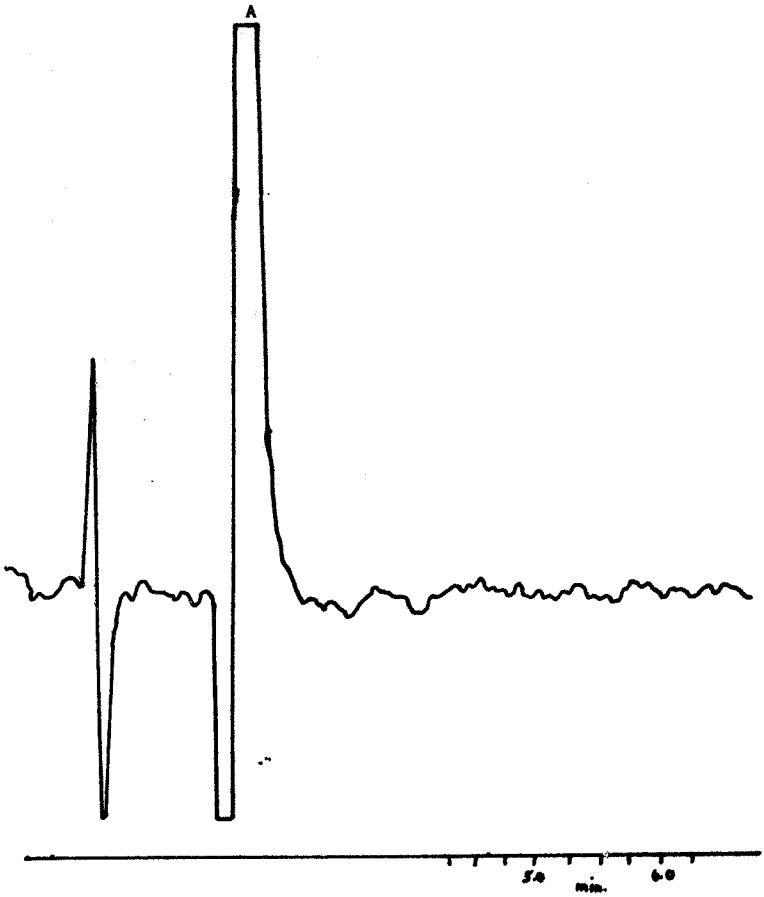


Figure 3. HPLC chromatogram of diethylether. Flow rate: 1.5 mL/min. Detection wavelength: 380 nm. Sensitivity: 005 AU/mV. Peak A: diethylether.

U.V. Spectra were obtained using the Cary 118 spectrophotometer. The spectra for estrone (IV), 2,4DNP (I) and the mixture of (I) and (IV) are shown in Figures 4 and 5. Peaks in the spectrum for (IV) occur at 290 and 278 nm while the λ max for (I) is 342 nm. No drastic spectral changes were detected in the mixture after 1, 3, 20 and 96 hours (Figure 6). The only change was an insignificant shift in the λ max of (I) from 342 nm to 345 nm. To determine if a product had formed but was undected in the spectra the mixture was analyzed on the HPLC. If the reaction had occurred the HPLC would separate the product from the reactants. As shown in Figures 7-10 no additional peak was observed at wavelength 340 nm. It appears that (V) does not form readily under these conditions.

A second aspect of this study was to examine the UV spectra and HPLC behavior of estrone (E1), estradiol (E2) and estriol (E3). In his work, Burce accomplished a similar separation with synthetic estrogens from oral contraceptives (66). Burce reported the following conditions: the estrogens were dissolved in acetonitrile; the mobile phase was 60% acetonitrile to 40% water; and, detection wavelengths were 210, 220 and 230 nm.

In this procedure I dissolved the estrogens in acetonitrile noting that the order of decreasing solubility was estradiol, estrone, estriol. Figures 11-13 show the U.V. spectra obtained for these solutions. Beer's Law (3) was used to calculate the molar absorptivity.

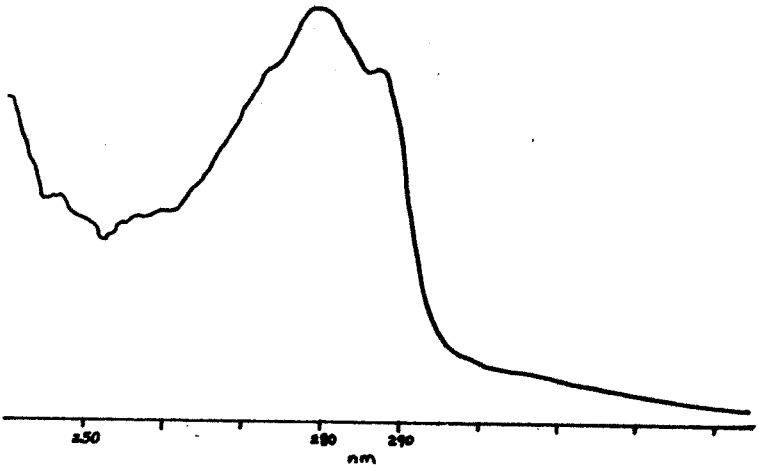


Figure 4. UV spectrum of estrone (IV). max: 278 nm. Prior to addition of 2,4-dinitrophenylhydrazine reagent (I).

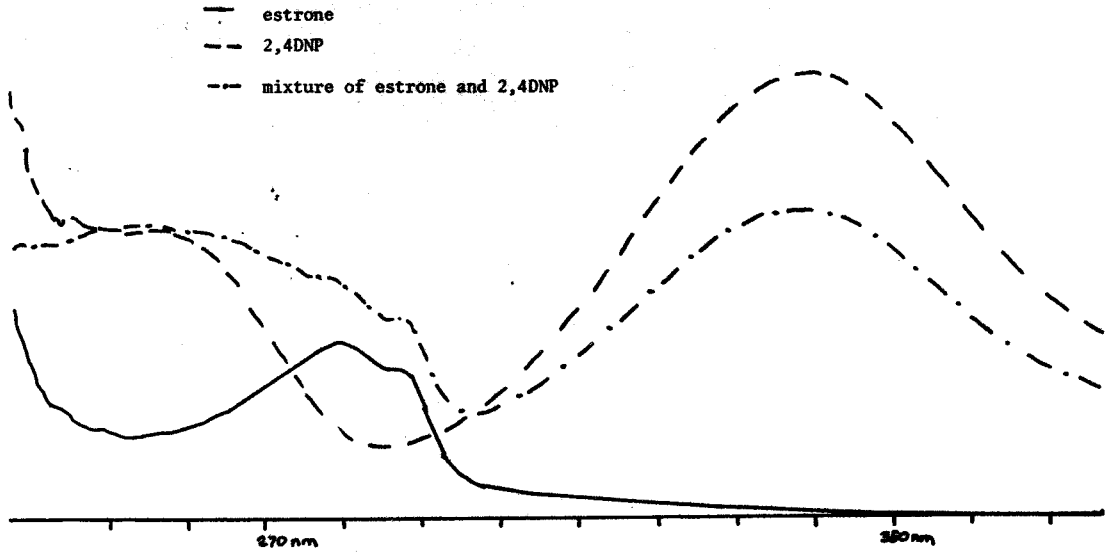


Figure 5. UV spectra of estrone (IV), 2,4-DNP (I) and mixture of (IV) and (I) at t=0 hours.

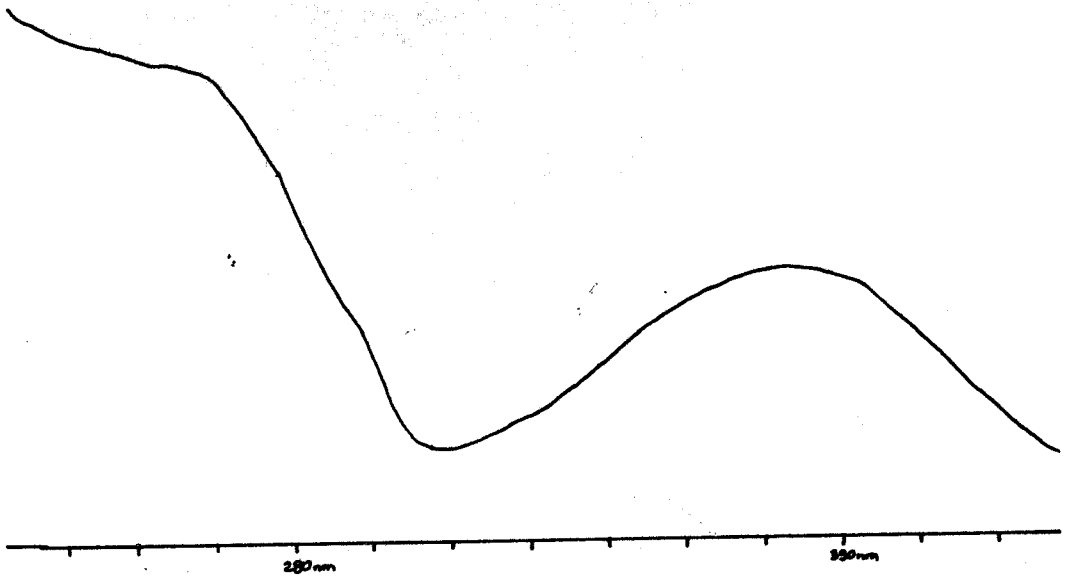


Figure 6. UV spectrum of mixture of estrone (IV) and 2,4DNP (I) at t=96 hours.

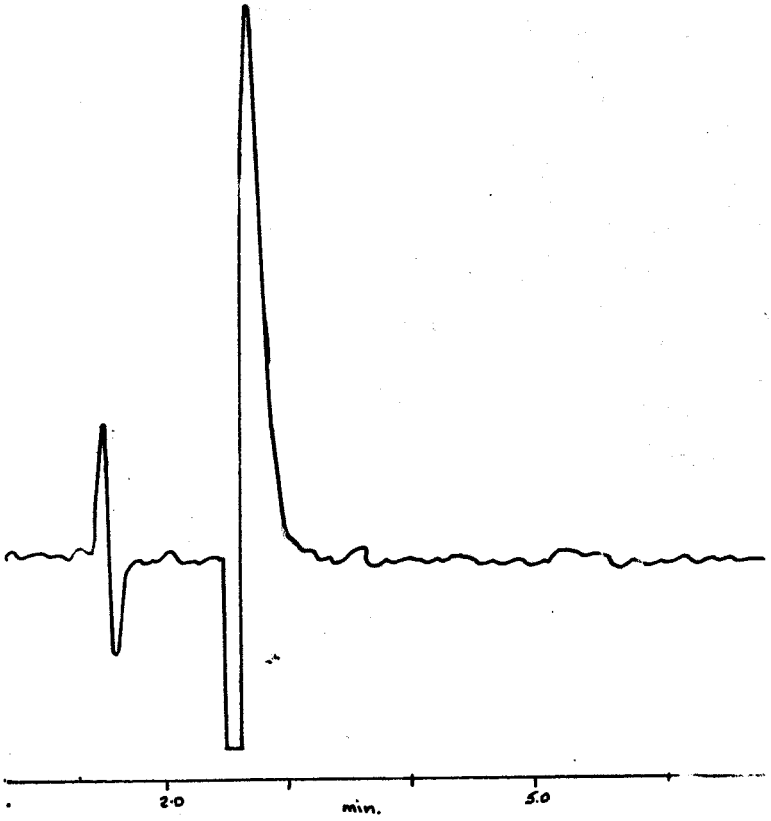


Figure 7. HPLC chromagram of diethylether. Detection wavelength: 340 nm.

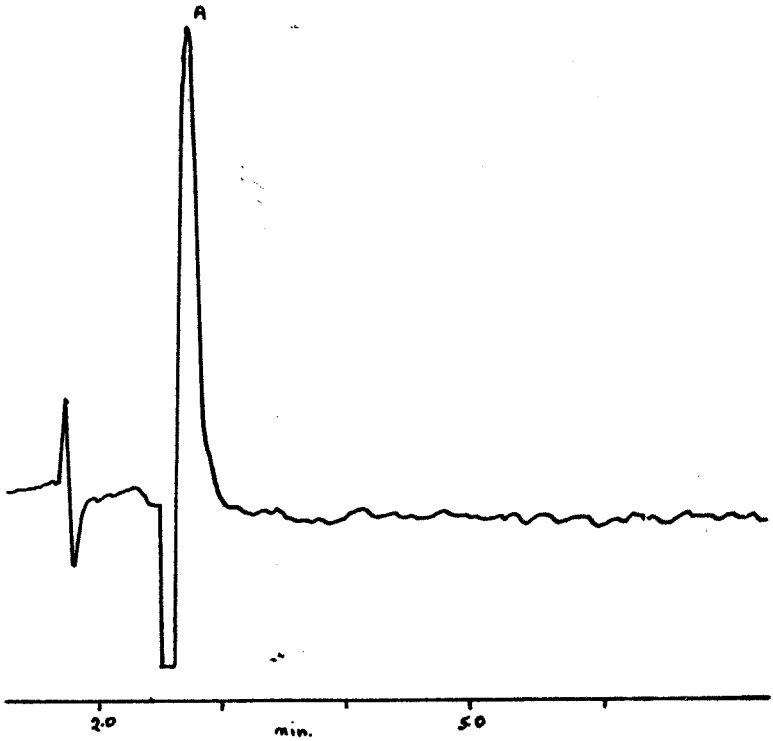


Figure 8. HPLC chromatogram of estrone (IV) at 340 nm. Estrone does not absorb light at this wavelength. Peak A: diethylether.

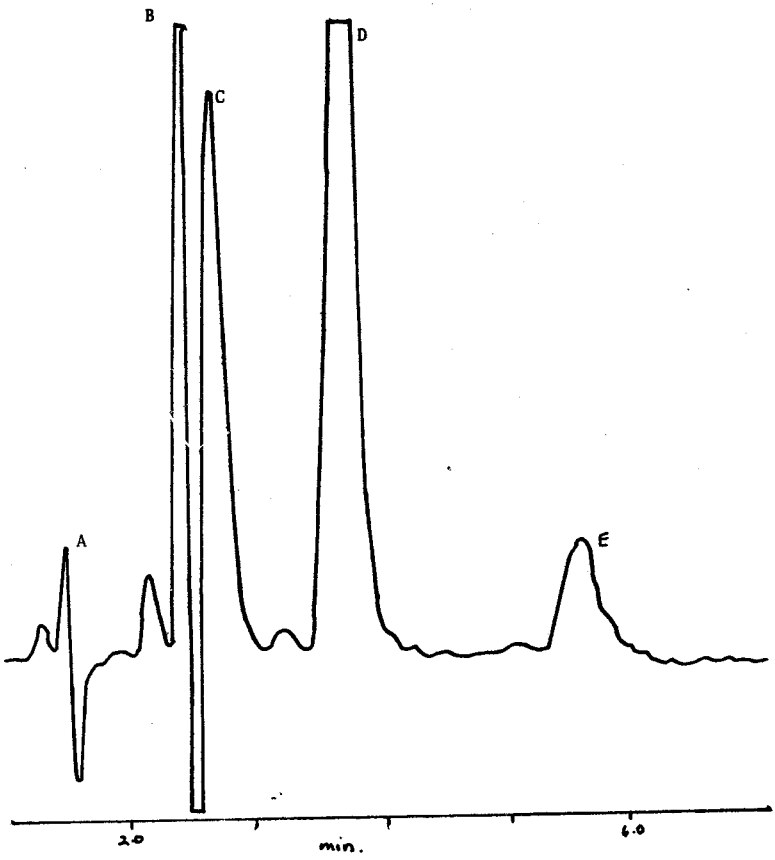


Figure 9. HPLC chromatogram of 2,4DNP (I) in diethylether. Detection wavelength: 340 nm. Peaks A, B, D and E: 2,4DNP reagent components. Peak B: diethylether.

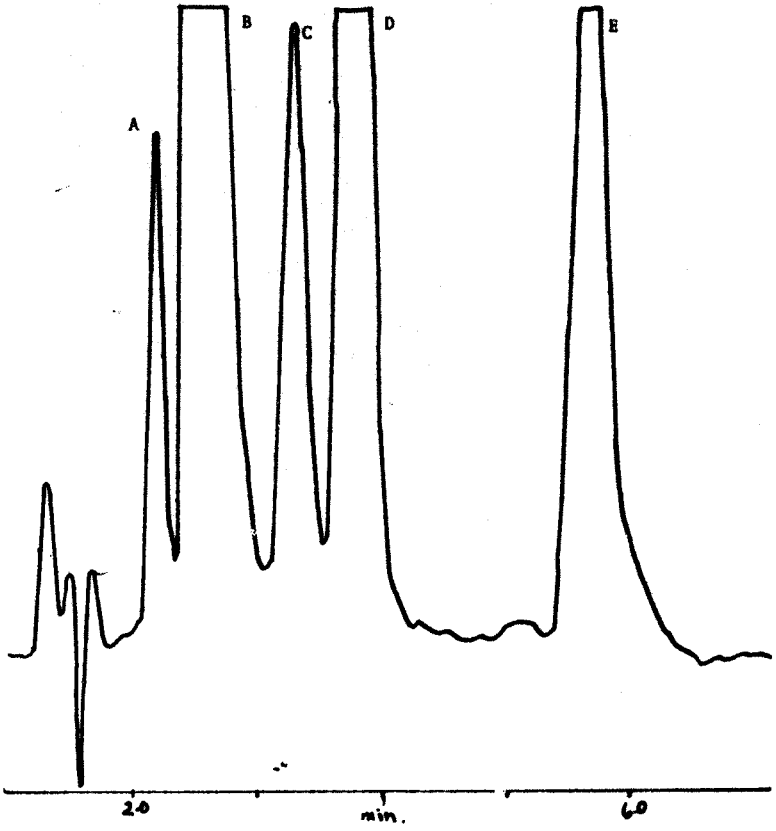


Figure 10. HPLC chromatogram of estrone (IV) and 2,4DNP (I) in diethylether. Detection wavelength: 340 nm. Peaks A, B. D and E are due to 2,4DNP (I). Peak C is diethylether. No 2,4DNP-estrone derivative (V) is detected.

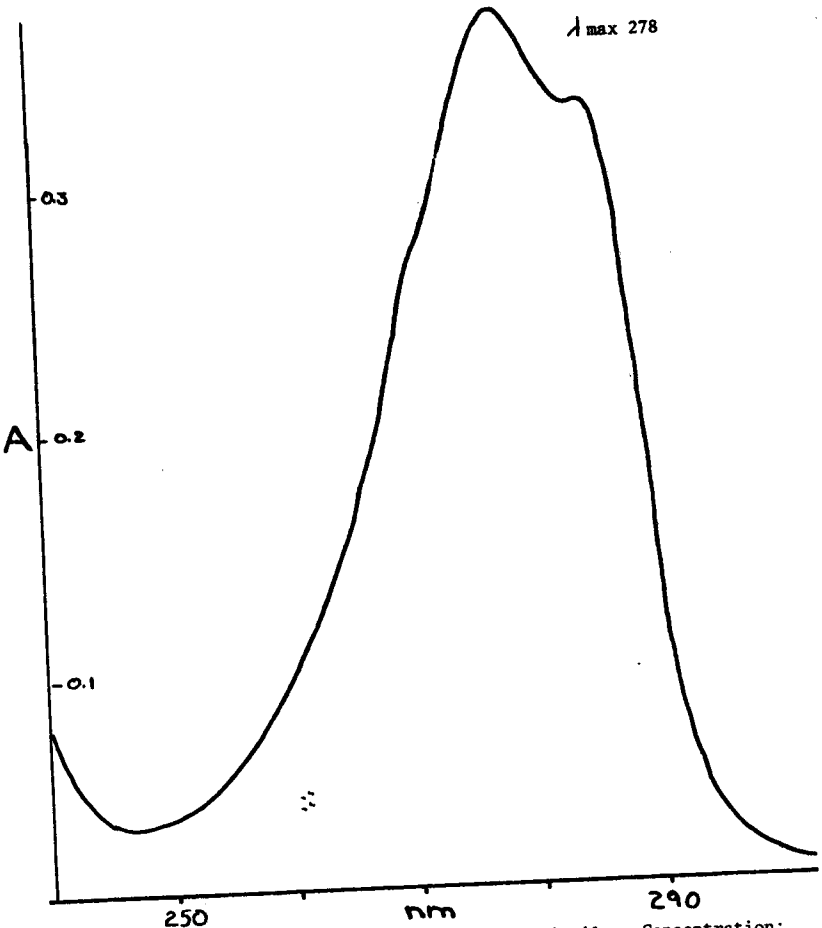


Figure 11. UV spectrum of estrone (E1) in acetonitrile. Concentration: 4.2×10^{-3} g/100 mL.

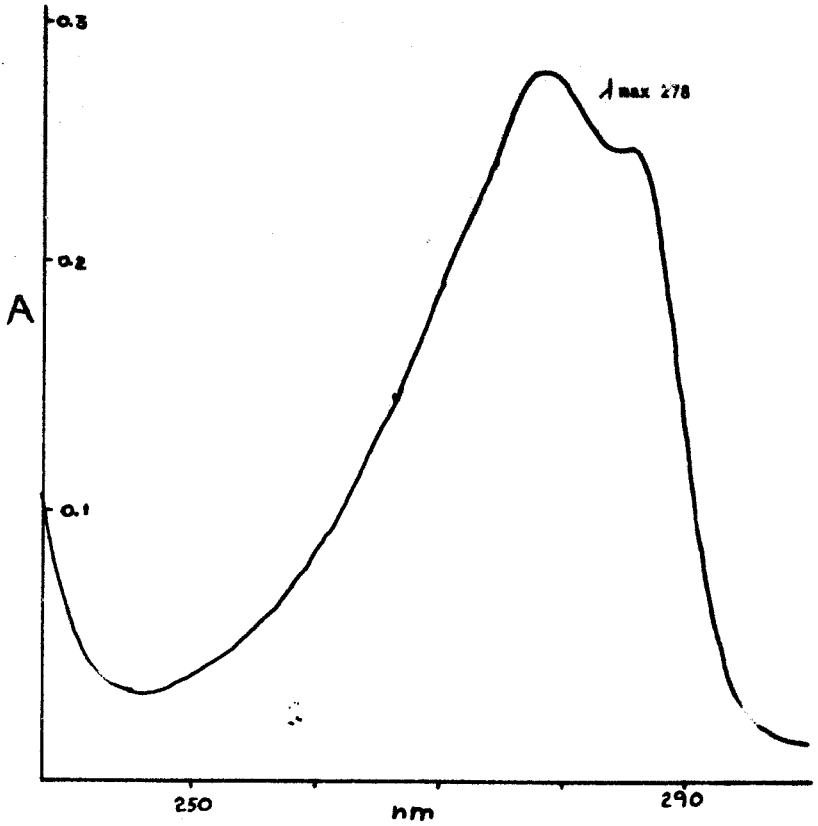


Figure 12. UV spectrum of estradiol (E2) in acetonitrile. Concentration: 4.0×10^{-3} g/100 mL.

$$A = \varepsilon b c \qquad (3)$$

A is measured absorbance,

ε is molar absorptivity,

b is the pathlength (1.0 cm), and c is concentration.

The concentrations used were 4.2×10^{-5} g/mL estrone, 4.0×10^{-5} g/mL estradiol and 4.2×10^{-5} g/mL estriol. The wavelength of maximum absorption was 278 nm for all three. The average absorbance at λmax was 0.342 for estrone, 0.282 for estradiol and 0.310 for estriol. The average calculated molar absorptivities for estrone, estradiol and estriol were 2207 ℓ/mole cm., 1902 ℓ/mole cm. and 2114 ℓ/mole cm respectively. A tabulation of these data appears below.

Table 1 ULTRAVIOLET ABSORBANCE OF ESTROGENS

Estrogen	Concentration	max	Absorbance	ε
	g/ℓ	nm		ℓ/mole cm
E1	0.042	278	0.342	2207
E2	0.041	278	0.282	1902
E3	0.042	278	0.310	2114

The observed molar absorptivities are consistent with the literature values for the $\pi \rightarrow \pi^*$ B band transition observed in phenols (67).

Optimum conditions were found to be 65%/35% acetonitrile/H_2O mobile phase, 1.0 mL/min flow rate and a 278 nm detection wavelength. The wavelengths used by Burce were not accessible in this experiment due to absorption of the solvent system in this region. The retention times for E1 and E3 were $4.55 \pm .10$ mins (Fig. 14) and $3.25 \pm .10$ mins. (Fig. 15) respectively. The retention time for E2 was $4.5 \pm .5$ mins. (Fig. 16). Its behavior was more uncertain than both E1 and E3 due to varying pressures in the HPLC.

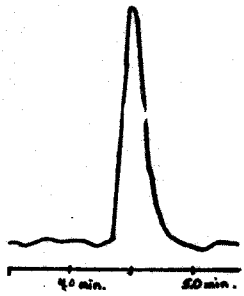


Figure 14. HPLC chromatogram of estrone. Retention time: 4.5 min.

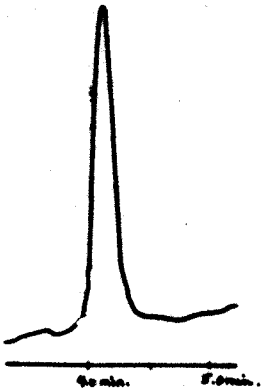


Figure 15. HPLC chromatogram of estradiol. Retention time: 4.15 min.

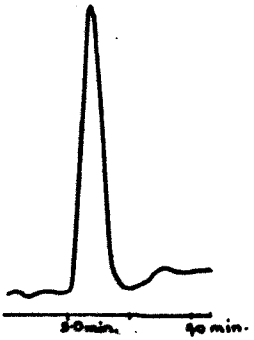
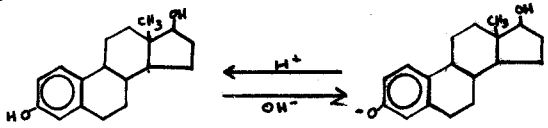


Figure 16. HPLC chromatogram of estriol. Retention time: 3.2 min.

A run of estrone, estradiol and estriol in equal concentrates at 2.0 mL/min. showed that estradiol and estrone could not be separated under the conditions used (Figure 17). The estrone-estradiol peak is twice as high as the estriol peak. The mobile phase was changed to 40%/60% acetonitrile/water but no separation occurred (Figure 18). Neither a flow program of 20%/80% acetonitrile/water changing to 65%/35% (Fig. 19) acetonitrile/water in three minutes nor a decreased flow rate (1.0 mL/min) (Figure 20) improved resolution.

A qualitative extraction of estradiol from alkaline solution was successful. Estradiol was dissolved in water containing sodium hydroxide (>> 1M). A UV spectrum show the absorbance peak for estradiol anion (VII) (Figure 21).



estradiol (VI) estradiol anion (VII)

The solution was mixed with ether, 12 molar hydrochloric acid was added and after extraction the aqueous layer was separated. No UV absorption of (VII) was observed in the water layer (Figure 22). A spectrum of the ether layer showed the presence of protonated estradiol (VI) (Figure 23).

The results of these preliminary experiments suggest many areas of further investigation. The ethyl ether extraction procedure should be extended to include estrone and estriol. In addition the method should

be quantified. The difficulty in separating estrone from estradiol indicates that different HPLC conditions particularly, a different mobile phase should be tried. Derivitization of the two estrogens might change their HPLC behaviors allowing separation. The HPLC detection limits for the estrogens must be determined. These limits may be improved by using shorter wavelengths (210-230 nm.). The transition of phenols at these wavelengths is stronger. To use the shorter wavelengths the solvent must be UV grade.

Other areas of study could involve determination of the conditions for β-glucuronidase hydrolysis and further investigation into the unique phenolic character of the estrogens.

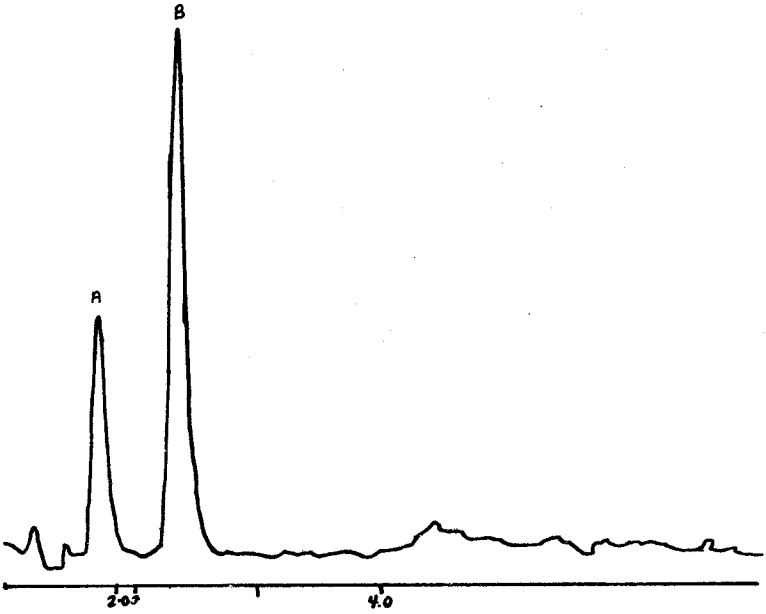


Figure 17. HPLC chromatogram of E1, E2 and E3 in acetonitrile. Flow rate: 2.0 mL/min. Mobile phase: 65%/35% CH_3CN/H_{20}. Peak A: E3. Peak B: E1 and E2.

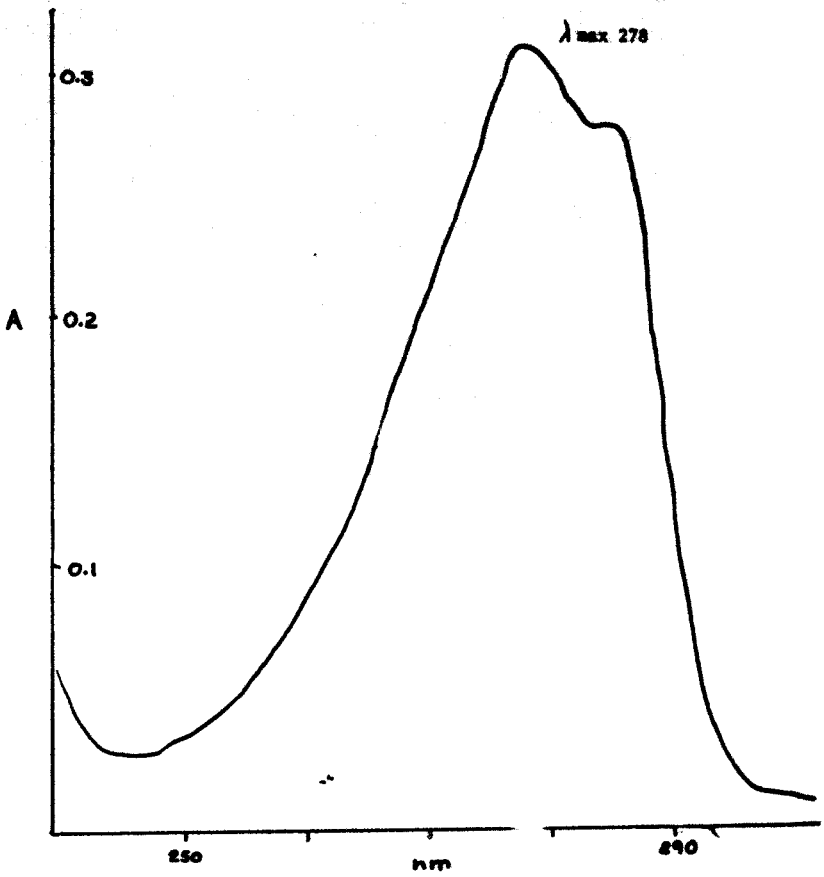


Figure 13. UV spectrum of estriol (E3) in acetonitrile. Concentration: 4.2×10^{-3} g/100 mL.

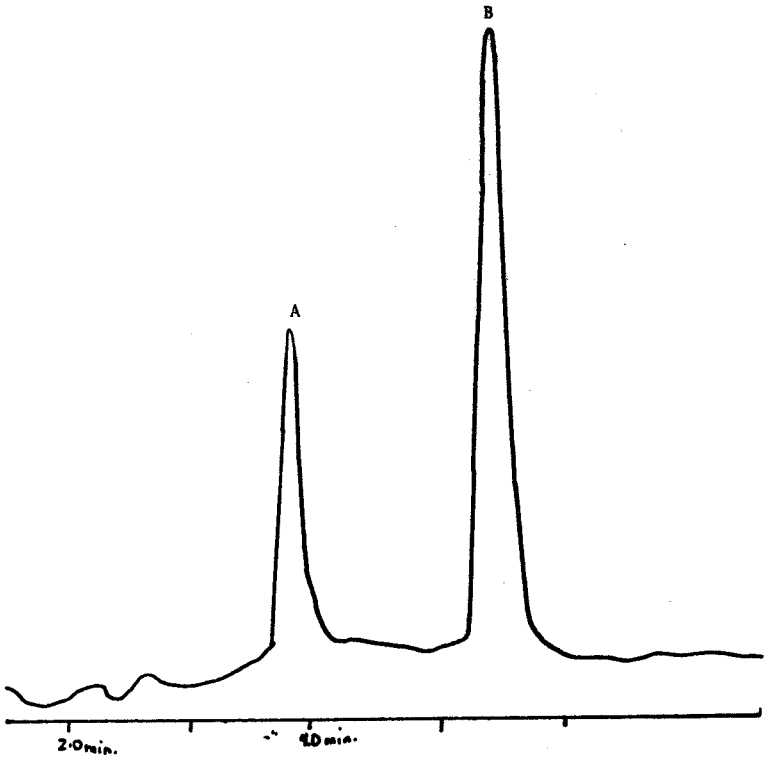


Figure 18. HPLC chromatogram of E1, E2 and E3 in acetonitrile. Mobile Phase: 40%/60% acetonitrile water. Flow rate: 1.0 mL/min. Peak A: E3. Peak B: E1 and E2.

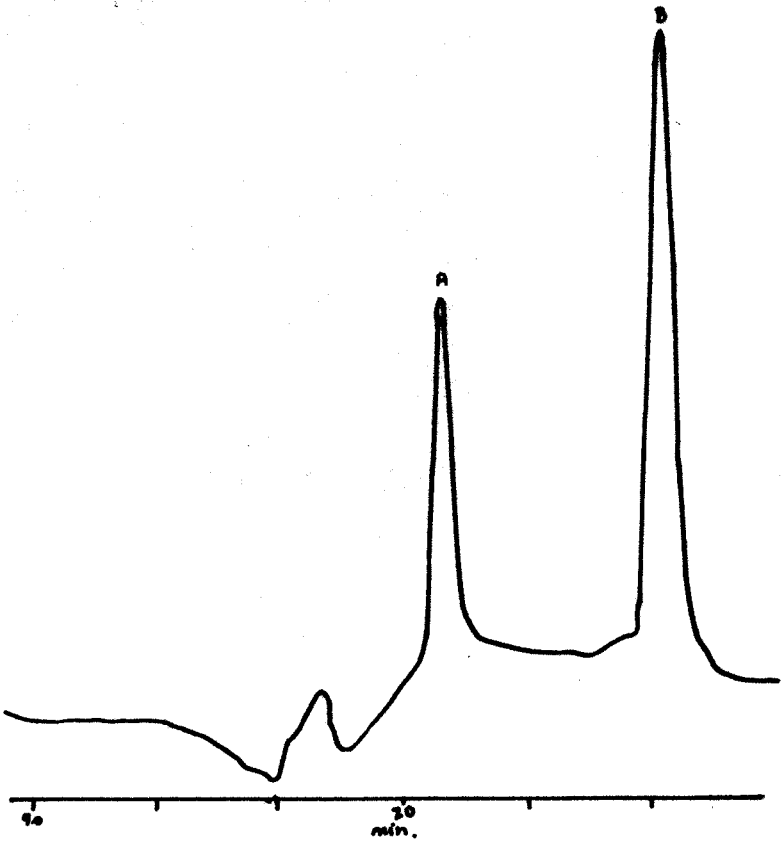


Figure 19. HPLC chromatogram of E1, E2 and E3 using flow program 20%/40% acetonitrile/water changing to 65%/35% in three minutes. Flow rate: 1.0 mL/min. Peak A: E3. Peak B: E1 and E2.

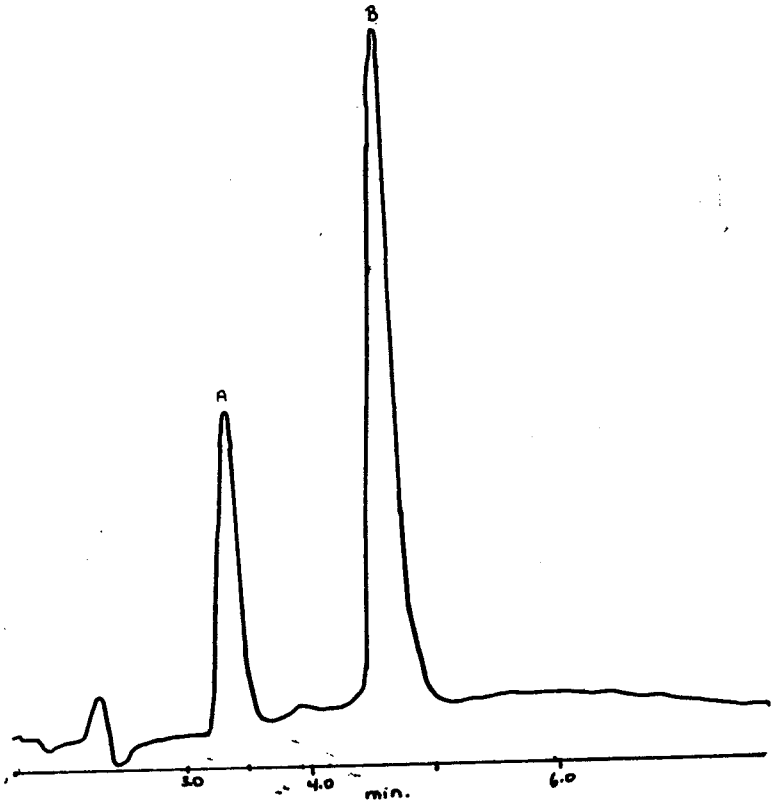


Figure 20. HPLC chromatogram of E1, E2 and E3. Flow rate: 1.0 mL/min. Mobile phase 65%/35% acetonitrile/water. Peak A: E3. Peak B: E1 and E2.

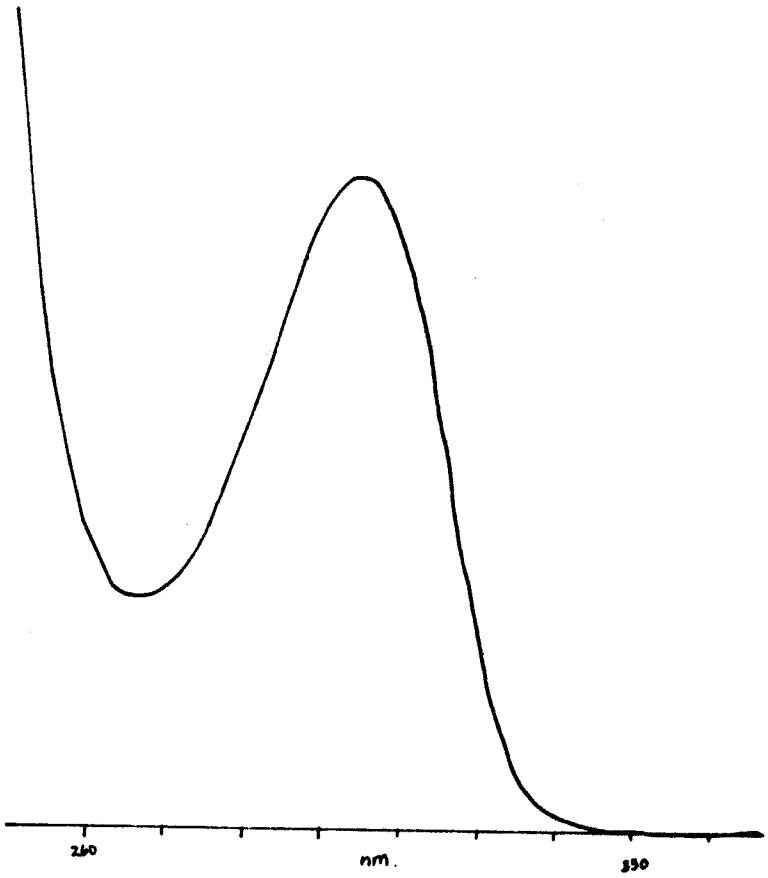


Figure 21. UV spectrum of estradiol anion (VIII).

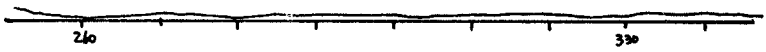


Figure 22. UV spectrum of aqueous layer.

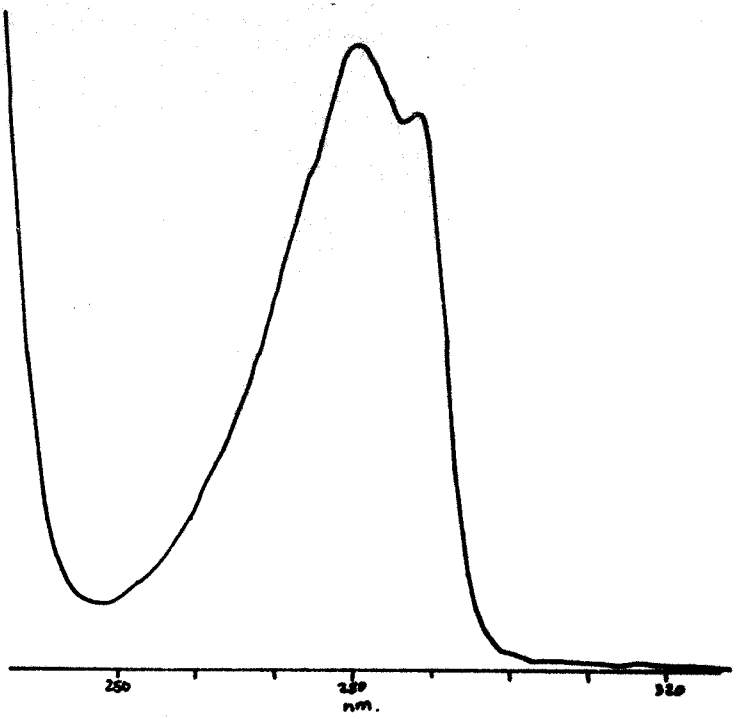


Figure 23. UV spectrum of organic layer.

References

1. Moghissi, K., Syner, F.M., and Evans, T.N. "A Composite Picture of the Menstrual Cycle." Am. J. Obstet. Gynecol., 114: 405-418 (1972).

2. Lane, C. "Deciphering Your Cycle Through Fertility Awareness." Mother's Manual, 14-15 (May-June 1980).

3. France, J.T., Boyer, K.G. "The Detection of Ovulation in Humans and it's Approach in Contraception." J. Reprod. Fertil., (Suppl) 22: 107-20 (April 1975).

4. Hyde, J.S. Understanding Human Sexuality. N.Y.: McGraw Hill, 1979.

5. Hilgers, T.W., Bailey, A.J. "Natural Family Planning II. Basal Body Temperature and Estimated Time of Ovulation." Obstet.-Gynecol., 53(3): 333-9 (March 1980).

6. McSweeney, D.J. and Skarra, A.J. "A New Cervical Test for Hormone Appraisal." Am. J. Obstet. Gynecol., 88: 705 (1964).

7. Shapiro, H.I. The Birth Control Book. N.Y.: St. Martin's Press, 1977.

8. Hilgers, T.W. et al., "Natural Family Planning I. The Peak Symptom and Estimated Time of Ovulation." Obstet. Gynecol., 52(5): 575-82 (November 1978).

9. Elstein, M., Moghissi, K.S. and Boeth R., Cervical Mucus in Human Reproduction. Copenhagen: Scriptor, 1973.

10. MacDonald, R.R., "Cervical Mucus: The Picture So Far." Post-Grad. Med. J., (Suppl) 28-33 (December 1967).

11. Odeblad, E., "The Functional Structure of Human Cervical Mucus." Acta Obstet. Gynecol. Scand., (Suppl. 1, 59): 47 (1968).

12. Grollman, A., Essentials of Endocrinology. Philadelphia, Pa.: J. B. Lippincott Co., 1941.

13. Chantler, E.N., Lo, K., and Elstein, M., "Carbonic Anhydrase Activity in Human Cervical Mucus and it's Response to Various Contraceptive Regimes." Br. J. Obstet. Gynaecol., 84(9): 705-7 (Sept. 1977).

14. Vickery, B.H., and Bennett, J.P., "The Cervix and its Secretion in Mammals." Physiol. Rev., 48: 135-154 (1968).

15. Lang, W.R., "Vaginal Acidity and pH. A Review." Obstet. Gynec. Survey, 10: 546-560 (April 1975).

16. Kullander, S. and Sohesson, B., "Studies on Saliva in Menstruating, Pregnant and Post Menopausal Women." Acta Endocr., 48:329-336 (1965.)

17. Oster, G. and Yang, S.L. "Cyclic Variation of Sialic Acid Content in Saliva." Am. J. Obstet. Gynecol., 114:190 (1972).

18. "Time of ovulation wasily predicted with paper 'testape'." OB-GYN Observer, (February 1972).

19. Davis, R.H. and Balin, H. "Saliva Glucose: A Useful Criterion for Determining the Time of Fertility in Women." Am. J. of Ob. Gyn., 115:287-288 (1973).

20. Ben-Aryeh, H., Filmar, S., Gutman, D., et al. "Salivary Phosphate as an Indicator of Ovulation." Am. J. of Ob. Gyn., 125: 871-874 (1976).

21. Puskulian, L., "Salivary Electrolyte Changes in the Normal Menstrual Cycle." J. Dent. Res., 51: 1212 (1972).

22. Hartman, C., "Literary Survey for Compounds in Urine and Saliva Suitable as a Basis for a Color Test of Ovulation." Fertil. Steril., 16: 77 (1965).

23. Maisel, A.Q., The Hormone Quest. N.Y.: Random House, 1965.

24. O'Herlihy, C. and Martin, R.H., "Screening for Fetal Risk with Urinary Estrogen-Creatinine Ratio." Br. J. Obstet. Gynaec., 87, 5: 388-92 (May 1980).

25. Miyakawa, I., et al. "Rapid Assays of Urinary Estriol in Pregnant Women." Int. J. Gynaecol. Obstet., 15 4: 291-5 (1978).

26. Joshi, U.M., et al. "Use of the Ratio of Estrogens to Creatinine in the Management of High Risk Pregnancy." Indian J. Med. Res., 68: 67-720 (July 1978).

27. Picnus, G., The Hormones. N.Y.: Academic Press, 1948.

28. Baird, D.T. and Fraser, I.B., "Concentration of Oestrone and Oestradiol in Follicular Fluid and Ovarian Venous Blood of Women." Clin. Endocrinol. (Oxf), 4, 3: 259-66 (May 1975).

29. Bjoro, K., "Oestriol Assay in Obstetric Practice" Acta Endocrinol., 69 Suppl 161: 5 (1972).

30. Brown, J.B. and Blair, H.A.F. "The Hydrolysis of Conjugated Oestrone, Oestradiol - 17β and Estriol in Human Urine." J. Endocrinol., 12: 411-424 (1958).

31. Brown, J.B. "A Chemical Method for Determination of Oestriol, Oestrone and Oestradiol." Bio. Chem. J. 60: 185-193 (1955).

32. Morreal, C.E., Dao, T.L. and Lonergan, P.A., "An Improved Method for the Detection of Estrone, Estradiol and Estriol in Low Titer Urine." Steroids, 20, 4: 383-397 (October 1972).

33. Hänhel, R. and Stenhouse, N.S., "Variation of Total Estrogen Excretion Within 24 Hours and From Day to Day." Europ. J. Steroids 2: 349 (1967).

34. Stenhouse, N.S. and Hänhel, R. "Total Estrogen Excretion Per Day Estimated From a 12 Hour Sample." Clinical Chemistry, 16, 5: 439 (1970).

35. Stimmel, B.F., "The Fractionation and Photometric Determination of Estrogen in Human Pregnancy Urine." J. Biol. Chem., 162: 99 (1946).

36. Brown, J.B., et al. "A Rapid Method for Estimating Oestrogens in Urine Using a Semi-Automatic Extractor." J. Endocr., 42: 5-15 (1968).

37. Adlercreutz, H., "Studies on the Hydrolysis of Gel Filtered Urinary Oestrogens." Acta Endocrinol., 57: 49-68 (1968).

38. Seddon, R.M. and Attwood, E.C. "Total Urine Oestriol by a Modification of a Commercial Plasma Oestriol Kit." Ann. Clin. Biochem., 15, 6: 338-9 (November 1978).

39. Beling, C.A. Acta Endocrinol., Suppl 79: 91 (1963).

40. Tenhaeff, D., Maruhn, D., and Elenore, A.H.L., "A Simple and Reliable Method for the Determination of Oestriol in Urine." Clin. Chim. Acta, 31: 37-43 (1971).

41. Pinkus, G.S. and Pinkus, J.L., "Sulfate Precipitation of Conjugated Estrogens in Pregnancy Urine" Clin. Chem., 16: 824 (1970).

42. Fisher, R.A., Anderson, D.C. and Burke, C.W., "Simultaneous Measurement of Unbound Testosterone and Estradiol Fractions in Undiluted Plasma." Steroids. 24, 6: 809-24 (December 1974).

43. Touchstone, J.C., and Muravec, T. "Urinary Estrogens I - Improved Method for Quantitation of Urinary Estrogens." Steroids, 17: 285-303 (1971).

44. Eberlein, R.W. "The Measurement of Low Levels of Estrone and Estradiol - 17β in Urine, Employing Ion-Exchange, Thin Layer and Gas-Liquid Chromatography." Steroids, 14: 553-573 (1969).

45. Kjeld, J.M. et al. Measurement of Unconjugated Testosterone and Oestradiol in Urine." Clin. Chim. Acta, 80, 2: 271-84 (Oct. 1977).

46. Ronco, G., Desinet, G., and Bezoc, J.P., "Use of Magnesium Silicate Before Gas-Liquid Chromatography for Determination of Total Estrogens in Urine During Pregnancy." Clin. Chim. Acta, 81, 2: 119-23 (December 1977).

47. Brown, J.B., "A Chemical Method for Determination of Oestriol, Oestrone and Oestradiol." Bio. Chem. J., 60: 185-193 (1955).

48. Purvis, K. et al., "Indices of Gonadal Function in the Human Male." Clin. Endocrinol. (Oxf) 4, 3: 247-58 (May 1975).

49. Kirkish, L.S., et al., "Plasma Estriol vs. Estrogen as an Index to Fetal Status." Clin. Chem., 24, 10: 1830-2 (October 1978).

50. Bischoff, F. and Bryson, G., "The Pharmacodynamics and Toxicology of Steroids and Related Compounds." Adv. Lipid Research, 15: 61-157 (1977).

51. Knox, B.S. and France, J.T., "Measurement of Unconjugated 17β - Estradiol in Plasma by Competitive Protein Binding." Clin. Chem., 18: 42 (1972).

52. Newsome, F.E. and Kitts, W.D., "A Method for Separating Oestrogens and Phytoestrogens on Sephadex LH-20 Columns." Steroids, 26, 2: 215-7 (August 1975).

53. Brown, J.B., et al., "Further Observations on the Kober Colour and Ittrich Fluorescence Reactions in the Measurement of Estriol, Estrone and Estradiol." J. Endocrinol. 40: 175 (1968).

54. Miklosi, S., et al., "A Rapid Method for the Estimation of Oestriol in Plasma During Pregnancy." Steroids, 26, 5: 671-81 (Nov. 1975).

55. Echaute, E. and Demeester, G., "Fluorimetric Determination of Total Estrogens After Gel Filtration of Enzymatically Hydrolyzed Urine." J. Clin. Endorinol. and Metabolism, 25: 480 (1965).

56. Corns, M. and James, V.H.T., "Determination of Total Oestrogens in Non-Pregnancy Urine by a Modification of Ittrich's Fluorimetric Technique." Clin. Chim. Acta, 28: 469 (1968).

57. Frye, R. et al., "A Semi Automated Fluorimetric Method for Total Estrogens in Pregnancy Urine." Clin. Chem., 23, 10: 1819-25 (October 1977).

58. Cohen, S.L., et al. "The Preparation of Pregnancy Urine for an Estrogen Profile." Steroids, 32, 2: 279-93 (September 1978).

59. Fels, J.P., et al., "Quantitative Estimation of Urinary Estrogens During the Menstrual Cycle by Gas-Liquid Chromatography with Glass Capillary Tubes." J. Steroid Biochem., 11, 3: 1303-8 (September 1979).

60. Nicholas, J.C., et al., "Enzymatic Determination of Estradiol and Estrone in Plasma and Urine." Clin. Chim. Acta, 89, 1: 59-70 (October 1978).

61. Walker, M.S. and McGlip, I., "Excretory Patterns of Urinary Free 11 - Hydroxy Corticosteroids and Total Oestrogens." Ann. Clin. Biochem., 15, 4: 201-2 (July 1978).

62. Sudo, N. and Takeuchi, S., "Evaluation of a 3 Minute Semi-Quantitative Test Kit for Urinary Estrogens of Pregnant Women." Obstet. Gynecol., 55, 1: 108-13 (January 1980).

63. Papanicolaou, A.D. and Adamopolos, D.A., Studies on the Urinary Excretion of Gonadotropins During the Normal Menstrual Cycle in Young Women." J. Obstet. Gynaecol. of the British Commonwealth. 77: 961-966 (November 1970).

64. Dyer, J.R., Applications of Absorption Spectroscopy of Organic Compounds. N.J.: Prentice-Hall, Inc., 1965.

65. Fieser, L.F., Organic Experiments. Lexington, Mass.: Ratheon Education Co., 1968.

66. Burce, G.L., "Determination of Synthetic Estrogens in Oral Contraceptive Dosage forms." Liquid Chromatography at Work, Varian Instrument Division, Palo Alto, CA.

67. Silverstein, R.M., Bassler, G.C. and Morrill, T.C., Spectrometric Identification of Organic Compounds. N.Y.: John Wiley and Sons, Inc., 1974.