IMPROVEMENT OF THE ORAL BIOAVAILABILITY OF 17β-ESTRADIOL BY A PRODRUG APPROACH. STABILITY KINETICS, PHARMACODYNAMIC AND BIOAVAILABILITY STUDIES

BY

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1993
To my brother
Bharat
and my parents
Sita and Umed Patel
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<tr>
<td>K</td>
<td>equilibrium constant</td>
</tr>
<tr>
<td>k</td>
<td>rate constant</td>
</tr>
<tr>
<td>k&lt;sub&gt;obs&lt;/sub&gt;</td>
<td>observed rate constant</td>
</tr>
<tr>
<td>(\gamma)</td>
<td>gamma</td>
</tr>
<tr>
<td>(\lambda)</td>
<td>wavelength</td>
</tr>
<tr>
<td>(\mu)</td>
<td>ionic strength</td>
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<td>nm</td>
<td>nanometers</td>
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<td>psi</td>
<td>pounds per square inch</td>
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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

IMPROVEMENT OF THE ORAL BIOAVAILABILITY OF 17β-ESTRADIOL BY A PRODRUG APPROACH: STABILITY KINETICS, PHARMACODYNAMIC AND BIOAVAILABILITY STUDIES

By

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An imidomethyl prodrug of 17β-estradiol was synthesized to improve its oral delivery by preventing a hepatic first pass effect. The compound synthesized was 2'-saccharinylmethyl estradiol. It was designed to undergo chemical hydrolysis under physiological conditions by pseudo-first-order kinetics.

The prodrug was found to undergo specific base catalysis in buffer media. It was also observed that the rate of hydrolysis was dependent on the concentration of the non-aqueous solvent (methanol or dioxane) used during the experiments. Under physiological conditions at 37°C the prodrug was relatively more stable in rat liver homogenate than in rat plasma. The prodrug did not exhibit significant
differences in its half-lives in biological media in the presence and absence of the enzyme poison, tetraethyl pyrophosphate.

Pharmacodynamic studies were conducted in ovariectomized rats to compare the potencies of 17β-estradiol with that of the prodrug. An eightfold increase in potency was observed (an increase in uterine weight) for the prodrug compared to estradiol when the prodrug and estradiol were administered orally. A significant lowering of the hormone levels (follicular stimulating hormone and luteinizing hormone) compared to controls was observed when the prodrug was administered. No such lowering was observed when an equimolar dose of estradiol was administered. Intravenous administration of equimolar doses of 17β-estradiol and its prodrug demonstrated no significant difference in the measured activity (increase in uterine weight). This indicates complete in vivo cleavage of the prodrug to the drug.

Bioavailability studies conducted on the prodrug and estradiol in ovariectomized rats demonstrated approximately a fivefold increase in bioavailability by the prodrug compared to 17β-estradiol. However, further experiments are required to establish this ratio.

The 2'-saccharinylmethyl derivative of 17β-estradiol serves as a model compound for improving oral bioavailability of phenolic drugs by protecting them against premature metabolism. However, the compound to which the promoiety is
to be attached also plays a significant role. The effectiveness of the model compound tested here (estradiol) may have been more promising if a more water soluble prodrug had been made which exhibited a better balance of hydrophilicity and lipophilicity. The saccharinylmethyl promoiety is a promising candidate for protecting drugs containing phenolic groups susceptible to enzymatically mediated conjugation reactions.
CHAPTER 1
INTRODUCTION

Oral ingestion of drugs is the most common and convenient route of administration, especially when frequent dosing is necessary. It is estimated that 9 million women in the United states and 55 million women worldwide are currently taking estrogens which have proven beneficial in either hormonal replacement therapy or as oral contraceptives.

Research on estrogens began in 1900 when Halban and Knauer (1) independently transplanted rabbit ovaries and concluded that these organs stimulate uterine growth by secretion of a biologically active substance into the blood. Of an even greater significance was the discovery by Loewe and Lange in 1926 (2) of a female sex hormone and the observation that the concentration of the hormone varied with the phase of the menstrual cycle. In 1928, Zondek reported large amounts of the hormone, estrogen, in the urine during pregnancy. This finding proved beneficial to the chemists, who soon isolated an active substance in crystalline form from that source. A few years later its chemical structure was elucidated as estra-1,3,5 (10)-triene-3-ol-17-one or estrone.
Since then it has been demonstrated that another estrogen, 17β-estradiol (Fig. 1.1), is the most potent, and the major secretory product of the ovary. It has the greatest potency to bind to the estrogen receptor in the target tissues and is regarded as the principal active natural estrogen. It is readily oxidized to estrone, which in turn can be hydroxylated and reduced to estriol. These transformations take place in the liver, where there is free interconversion between estrone and estradiol. All these three estrogens are excreted into the urine as glucuronide and sulfate conjugates.

Figure 1.1: 17β-Estradiol
During pregnancy, estrogens are synthesized in large quantities and a pregnant mare excretes about 100 mg daily.

The formation of estrogens occurs primarily in (but is not limited to) the gonads, placenta and the adrenals, but peripheral tissues such as liver, fat, skeletal muscles, and hair follicles can also form significant quantities of the hormone.

Role Of Estrogen In The Ovarian Cycle

The principal physiological effects of the estrogens are exerted on the organs of the female reproductive tract. Estrogen is responsible for the growth and development of the fallopian tubes, uterus, vagina and external genitalia that characteristically occur in the transition from sexual immaturity to the sexually mature state following puberty. Once maturation has occurred, however, these accessory organs of reproduction do not remain static; deprivation of ovarian hormones by ovariectomy results in severe involution and atrophy of these organs. Although a gland of origin is seldom regarded as a target for its own hormone, estrogen appears to play a critical role in ovarian function. Ovarian function is controlled by two gonadotropic hormones secreted by the anterior pituitary gland: follicle stimulating hormone (FSH) and luteinizing hormone (LH). These in turn are released by
gonadotropin releasing hormone (GnRH). FSH plays a key role in the proliferation and the differentiation of the granulosa cells of the ovary responsible for estrogen synthesis and also of the thecal cells. LH synergizes with FSH in promoting follicular development and is required for the production and secretion of the follicular hormones. Estrogen production and maturation of the follicle depend on complex interactions between the two gonadotropins and between the thecal and granulosa cells. A unique feature of this system is that estrogen plays a pivotal role in the entire maturation process, which includes increased capacity to produce estrogen. Estrogen causes proliferation of the granulosa cells, and along with FSH, enhances their ability to respond to stimulation by increasing their capacity to bind LH and FSH. Estrogen thus stimulates its own production.

The foregoing pattern of ovarian and pituitary hormones in the blood results from intricate interactions between the pituitary, ovaries and the CNS. The pattern of the hormones in the blood during the ovarian cycle are well established (Fig. 1.2). In a normal ovarian cycle there exists three phases. First is a follicular phase, with respect to the ovary, where several follicles may begin to develop though ultimately only one reaches maturity. This lasts for about two weeks. This is followed by ovulation, the mid-point of the cycle, followed by a luteal phase (lasting for two weeks). If pregnancy has not resulted during ovulation, there is a sharp decline of the hormone levels, marked by
menses. The central event of each ovarian cycle, ovulation, is marked by a characteristic dramatic LH surge that precedes it by 1 or 2 days. A corresponding peak in FSH is also seen at this time, but is considerably less pronounced. FSH begins to rise 1 or 2 days before the onset of menstruation. Administration of ovarian hormones, either estrogen or progesterone or both, produces ovarian atrophy and failure of follicular development. In fact, this observation led to the development of the oral contraceptives. Ingestion of estrogen prevents the appearance of the bursts of secretion and gradually brings LH concentration down as seen during the follicular phase. In fact, in sufficient doses, estrogens can suppress both phases by feedback action on the hypothalamus.

**Estrogens In Substitution Therapy**

Estrogens are used in substitution therapy when menopausal symptoms occur: (a) after cessation of ovarian function, (b) following ovariectomy or X-ray or radium therapy, or (c) in the natural menopause (climacteric). Therapy ameliorates the symptoms such as vasomotor instability (hot flushes), prevents or reverses urogenital atrophy, and slows or prevents osteoporosis. However, the beneficial effect of estrogen therapy on mood swings is unpredictable.
Figure 1.2: Plasma concentrations of ovarian hormones and gonadotropins in women during normal menstrual cycles (3).
Estrogens are used in young women in whom there is failure of steroidogenesis; treatment brings about acceleration of delayed development of the primary and secondary sexual characteristics. A number of menstrual irregularities may be treated by estrogens such as amenorrhea, dysmenorrhea, endometriosis and in dysfunctional uterine bleeding. They can be used as hypocholesteremic drugs, but the side effects are usually unacceptable to the male recipient. Estrogens are also used in the treatment of acne vulgaris and hirsutism, to inhibit the growth of prostatic cancer in men, and to inhibit carcinoma of the breast or carcinoma elsewhere in the reproductive tract of women who are more than four years beyond the menopause.

The side effects induced by estrogens are numerous and vary in severity. Nausea and vomiting (3), and also headaches are frequent side effects of estrogen treatment. Estrogens decrease folate absorption, an effect that could cause neurological impairment and megaloblastic anemia in elderly users; however, solid data on this effect is still lacking. Estrogens cause some changes in blood clotting factors, and there is considerable evidence that they increase the incidence of thromboembolism in both superficial and deep veins. They are also known to alter hepatic function and decrease glucose tolerance. Changes in the blood include increase in blood proteins such as thyroxine- and glucocorticoid-binding proteins, which may alter endocrine relationships. It has long been held that estrogens increase
the incidence of breast cancer in women and also increase the risk of cervical and uterine carcinoma.

Naturally occurring estrogens are not orally effective because they are destroyed almost totally in a single pass through the liver. The half-life of estradiol is 40–50 minutes in man, but other estrogens persist much longer. Estrogens circulate both in free and conjugated forms. They are bound in varying amounts to albumin and to a specific sex hormone binding globulin, to transcortin and to glycoproteins. Estrogens are excreted primarily in the conjugated form in the urine while some free estrogens are secreted into bile and so later excreted in feces. However, most return to the systemic circulation by the enterohepatic route. They are rapidly absorbed from intramuscular sites, mucous membranes, skin and other sites of therapeutic application.

**Dosage Forms**

Among the prevalent dosage forms are conjugated estrogens, micronized estradiol and other synthetic estrogen derivatives. Orally administered estradiol (Fig. 1.1) is well absorbed but undergoes considerable first pass metabolism in the liver, leading to a systemic bioavailability of only 11% (4). This poor bioavailability is attributed to the presence of the 17β-OH groups which can be readily oxidized to give
estrone and to the phenolic -OH group at the C-3 position in
the molecule which makes it susceptible to conjugation as the
sulfate and glucuronide metabolites (4) shown in Fig.1.3. Conjugation of estrone also occurs. Transmucosal and vaginal
preparations of these agents are well absorbed but lack
patient compliance as they tend to be inconvenient to use.

Transdermal patches recently have gained attention, but
intersubject variability and fluctuation in blood levels
limit their usage. At present, oral agents are the most
widely used, of which ethinyl estradiol (present in most
preparations) has demonstrated a bioavailability of about 40%
in female volunteers (5). Ethinyl estradiol was designed as
an orally active agent by Inhoffen and Howley in 1938 (6). The increased oral effect of ethinyl estradiol is related to
its slower loss from circulation when compared to the natural
hormone, estradiol (7). Mestranol, the 3-methyl ether of
ethinyl estradiol, is demethylated in the liver to the
active compound ethinyl estradiol; only about 54% of the dose
is demethylated, therefore more is required in order to
generate the same therapeutic effect.

Conjugated estrogens, though known to be metabolites,
are effective in delivering estradiol. The plasma
pharmacokinetics of different conjugates after oral
administration of estradiol and estrone are well
characterized (8). Conjugated estrogen preparations are
comprised mainly of estrone-3-sulfate, which is enzymatically
hydrolysed in the liver to yield estrone.
Figure 1.3: Conjugation of 17β-estradiol to form the sulfate and glucuronide metabolites.

Estrone has a longer biological half-life, compared to estradiol, because of its enterohepatic recirculation and interconversions between estrone and estradiol. Oral administration of the conjugate, compared to free estrogen, results in a relatively protracted rise of the conjugated estrone in plasma which is desirable. One possible explanation of the differences observed in the plasma levels could be the difference in the velocity of the intestinal
absorption between the free and the sulfoconjugated estrogens (9). Another possible explanation for the protracted influx of the conjugated estrogens could be the steps involved in the transhepatic transport of the free and the conjugated forms. Since both the free and conjugated estrogens are recovered to similar extents in the bile, one could suggest that differences in distribution and protein binding play a minor role in the hepatic availability of these compounds. However, uptake of free estrogen is considerably different from the uptake of estrone sulfate in isolated rat liver cells. While the former is lipophilic and is taken up in less than 15 seconds, the latter is water soluble and the uptake proceeds over 5 minutes (10). Isolated liver cells were used in this case in order to characterize the kinetics of the steps involved in biliary elimination. The results suggested that the uptake of estrone sulfate is active and can proceed against a concentration gradient; the transmembrane transport of the charged molecule is slow in both directions. In contrast, uptake of unconjugated estrogens, as well as release, should proceed rapidly via passive diffusion through the membrane. Hence, in vivo the major driving force for the uptake of estrone sulfate from the blood into the liver cells may be active transport, while in the case of free estrogens a concentration gradient due to extensive metabolism of the estrogens within the liver cells may be of major importance. Because uptake of estrone sulfate has been shown to follow saturation kinetics (10), it may be the rate limiting step.
Transdermal dosage forms of estradiol have proven equally effective as conjugated estrogens and, in contrast, well tolerated. Studies which compared the pharmacokinetics of transdermal estradiol with orally administered micronised estradiol and conjugated estrogens (11) in postmenopausal women have shown higher estradiol/estrone ratios with the Estraderm patch than with Estrace (micronized estradiol) or Premarin (conjugated estrogens). Conjugated estrogens preparation contain not less than 50% and not more than 65% of sodium estrone sulfate, and not less than 20% and not more than 35% of sodium equilin sulfate, calculated on the basis of the total conjugated estrogens content. Among the other preparations for oral administration are esterified estrogens which contain 75-85% of sodium estrone sulfate and 6-15% of sodium equilin sulfate. However, the dosage required varies, for example: following parenteral administration at the given doses, the following estrogens are approximately equipotent; estradiol, 50ug; ethinyl estradiol, 50ug; mestranol, 80ug. The daily requirement of estrogen is about 60 ug of estradiol but its poor oral bioavailability requires a higher dose to be administered, e.g. some of the recommended doses of the following preparations indicated for replacement therapy are: estradiol, 1-2 mg; conjugated estrogens, 1.25 mg; estrone, 2 mg.

Non-steroidal synthetic substances which display estrogenic activity include stilbene compounds and those compounds which may be considered to be more or less
structurally related to stilbenes, e.g. diethylstilbestrol. Metabolism at the stilbene double bond occurs which possibly leads to reactive intermediates, such as epoxides.

Thus, it appears that the high potency exhibited by estrogens comes with a price, i.e., they have toxic side effects and so the lower the administered dose, the better. What would be most ideal in this case is the administration of the hormone itself in a dose as small as possible to attain a maximum therapeutic effect. Estradiol would in this case be recognized by the human body as inherently native and in acceptable concentrations. However, this approach seems unfeasible due to the low bioavailability of 17β-estradiol attributed to rapid glucuronidation and sulfation at the phenolic-OH. The available data show that a prerequisite for estrogenic action of steroidal compounds is a free phenolic group. Steroids without the free 3-hydroxyl function must be converted to phenols to gain estrogenic activity (12). Pharmaceutical modifications affecting the physical characteristics of the steroid would be ineffective in this case, as the problem lies in its chemical structure, making it susceptible to hepatic first pass effect. The alternative is to temporarily modify the structure at the C-3 site, so that it could bypass the liver. This approach is termed the prodrug approach. Ideally, a prodrug is considered pharmacologically inert and should serve only as a carrier until required to release the parent compound. In this case a prodrug is required which could be ingested orally, bypass
conjugation in the liver and then release the hormone when it enters the systemic circulation. This presumes that there exists a relationship between the pharmacological response of the hormone and the concentration it exhibits in the blood.

**Background - Literature Review**

The term "prodrug" was first used by Albert (13) to describe compounds which undergo biotransformation prior to exhibiting their pharmacological effects. Although terms such as drug latency, bioreversible derivatives, etc have been used synonymously with the term 'prodrug', Sinkula and Yalkowsky (14), Higuchi and Stella (15) and Roche (16) have standardized the term 'prodrug' to describe compounds which undergo enzymatic or nonenzymatic chemical transformations within the body prior to exhibiting their intended pharmacological action(s).

The prodrug approach to preventing oral first pass metabolism of 17β-estradiol has been explored in the past by Hussain et al. (17), where three ester prodrugs (17β-estradiol-3-acetylsalicylate, 17β-estradiol-3-salicylate, 17β-estradiol-3-anthranilate) (Fig.1.4) were synthesized. The reported bioavailability in male beagle dogs for the acetylsalicylate and the anthranilate esters were 17-fold and 5-fold higher, respectively, compared to 17β-estradiol. In
this case, the 3-OH blocking substituents were enzymatically cleaved to release the drug. Enzymatic cleavage by esterases and amidases is relied upon heavily, rather than chemical cleavage, due to the prevalence of these enzymes in the mammalian body. A disadvantage to this approach could be premature cleavage of the prodrug before it passes the liver, or there could be interindividual variation in the enzymatic activity exhibited per se.

Naltrexone, an opioid antagonist is currently used for the treatment of opioid addiction. Due to extensive oral first pass effect (5-6% bioavailability), prodrugs have been synthesized by Hussain et al. (18) as shown in Fig. 1.5. A number of prodrug esters of the 3-OH group were prepared, the: (a) anthranilate; (b) acetylsalicylate; (c) benzoate; and (d) pivalate. The oral bioavailability of these prodrugs was determined in dogs. Compounds (a) and (b) exhibited the greatest enhancement of naltrexone bioavailability (an absolute bioavailability of 45% and 28%, respectively), whereas the rest exhibited an absolute bioavailability of less than 10%. Nalbuphine, a narcotic agonist/antagonist which has a reported oral bioavailability of less than 10% of the dose (19) has also been subjected to the prodrug approach (20), where again the acetylsalicylate and anthranilate esters as shown in Fig. 1.6 have improved the oral bioavailability 4 and 9 fold, respectively.
Figure 1.4: Structures of ester prodrugs of 17β-Estradiol
Figure 1.5: Structures of ester prodrugs of naltrexone
General approaches with a model phenolic compound (acetaminophen) have been studied (21). Various derivatives of the compound were synthesized and tested for both in vitro and in vivo behavior. The comparative behavior of the pivalyl ester and the methyl, ethyl, methoxymethyl and pivaloxymethyl ether prodrugs of acetaminophen were studied. The 4-pivaloxymethyl ether of acetaminophen was found to be more chemically stable than the 4-pivalyl ester of the drug. Rapid in vitro conversion rates observed in intestine and liver homogenates and lack of detectable levels of intact prodrugs following intraduodenal administration strongly supports the possibility of conversion of the two prodrugs to acetaminophen during their absorption. This resulted in poor bioavailability of the drug. These results were consistent (premature metabolism) with the findings reported for bacampicillin (22), talampicillin (23) and pivampicillin (24).

Therefore, one can conclude that prodrugs which undergo conversion to the parent drug during their absorption, or which depend for conversion on the enzymes present in the gut lumen, gut wall and/or liver, are not good candidates for protecting the drug against presystemic metabolism. Thus, it was decided to avoid this approach altogether and resort to a protective mechanism based on chemical cleavage.
Figure 1.6: Structures of ester prodrugs of nalbuphine
Bundgaard et al. (25) studied amino acid phenylcarbamates as prodrugs of phenols which were designed to undergo non-enzymatic hydrolysis. The stability of the derivatives was studied in aqueous buffer solutions and in various physiological media. The carbamates were found to be rather stable in weakly acidic solutions but were hydrolysed more easily at physiological pH. The rates were found to increase with decreasing pKa value of the phenol. A t1/2 of 1.8 min was found for a compound having a −NO2 group para to the phenolic moiety versus a t1/2 of 208 h for a compound having a −OCH3 group at the same position. Similar trends were observed by Getz (26) where the rate of hydrolysis of the imidomethyl prodrugs of phenols was relatively more affected by the acidity of the phenol than by that of the imide. Bundgaard et al. (25) noticed that the hydrolyses of the amino acid phenylcarbamates were not catalysed significantly by liver and intestinal wall enzymes, but that human plasma showed a marked catalytic effect. This effect was ascribed to a catalysis by serum albumin.

The half-lives of most of the compounds studied by Bundgaard were several hours both in buffer and physiological media. This would ensure the stability of the prodrugs following oral administration. However, on reaching the systemic circulation, a relatively faster rate of hydrolysis would be needed to avoid the clearance of the prodrug from circulation before its cleavage to yield the parent compound.
The Choice of a Protecting Group for the 3-OH of Estradiol.

When an NH-acidic compound is allowed to react with formaldehyde, reversible N-hydroxymethylation occurs.

\[
RCONH_2 + CH_2O \rightleftharpoons R - CONH - CH_2OH
\]

\[
RCONHR' + CH_2O \rightleftharpoons R - CON(R') - CH_2OH
\]

The kinetics of decomposition of a large number of N-hydroxymethylated amides, imides, carbamates and hydantoins has been studied in aqueous solution (27). It was found that: (a) the decomposition exhibited a first order dependence on the hydroxide ion concentration up to a pH of at least 12; and (b) the rates of hydrolysis increased sharply with increasing acidity of the parent N-H compound. A relationship between \( \log k \) (where \( k \) is the apparent second order hydroxide ion catalytic rate constant) and the pKa of the parent nitrogen acids was established (28). From this relationship it could be predicted that the requirement for a decomposition reaction half-life of less than 1 minute at pH 7.4 and 37°C is that the parent NH-acidic compound possess a pKa value of less than 10.8. It has been established that the reaction mechanism for the decomposition of the N-hydroxymethylated derivatives of amides and imides involves a stepwise pathway, with an N-hydroxymethyl anion as an intermediate undergoing a rate determining N-C bond cleavage.
As can be expected on the basis of this reaction mechanism, the rate of decomposition of N-hydroxymethyl derivatives was not catalysed by enzymes (29).

The structural effects on the decomposition rate of N-Mannich bases derived from amides and imides involve steric effects, basicity of the amine component and also the acidity of the amide-type component (30, 31). As in the case of the hydroxymethyl derivatives, one of the ways to greatly enhance the decomposition rate of these compounds is to increase the acidity of the parent amide-type function, i.e., the ability to withdraw electrons from the amide-like nitrogen anions and stabilize them. This leads to an increased rate of N-C bond cleavage. This concept was used to choose a group that would serve to protect the phenolic moiety of the compound in question. Imidomethyl promoieties were chosen because imides (pKa 2-9) were more acidic in nature than amides (pKa 10 - 15) and therefore would serve as better leaving groups. This would make imidomethyl prodrugs of phenols more labile than the corresponding amidomethyl prodrugs if the same mechanism of hydrolysis was operating in both cases.

Getz et al. (26) have observed that the rate of hydrolysis of various imidomethyl and amidomethyl prodrugs of 4-nitrophenol was proportional to the acidity of the parent nitrogen acid. Fig. 1.7 shows a plot of log kobs vs pKa of the imides that were studied. From this observation it appeared that incorporation of o-benzoic sulfimide (saccharin, pKa 1.6) as the imide part of an imidomethyl
Figure 1.7: Pseudo-first-order rate constants (min$^{-1}$) versus the acidic pKa of the parent imide. Compound I (saccharinylmethyl prodrug of 4-nitrophenol), compound II (phthalimidomethyl prodrug of 4-nitrophenol), compound III (succimidomethyl prodrug of 4-nitrophenol), (pH 9.0, 0.1M, $\mu = 0.15$, 25°C) (26)
promoiety would make it a good candidate for temporarily protecting the phenolic moiety on 17β-estradiol. The saccharinylmethyl prodrug was expected to have a relatively fast rate of hydrolysis due to the high acidity of saccharin. It was also expected to have a first order dependence on the hydroxide ion concentration. The decomposition products resulting from the hydrolysis of the prodrug would in this case be saccharin, formaldehyde and 17β-estradiol. The toxicity of saccharin has been extensively studied and found to be sufficiently safe to be approved as a food additive. Moreover, the dose of the prodrug that would be administered would be low so as to yield only microgram quantities of saccharin. A variety of biochemical reactions in the human body yields a normal blood concentration of formaldehyde of 2x10⁻⁵ M (32), therefore, toxicity resulting from formaldehyde formation should also be minimal.

The rate determining step involved in the decomposition of a prodrug comprised of an imide linked to a phenol through a methylene linkage will not be hydrolysis of the N- or O-hydroxymethylated derivative since their hydrolysis will be very fast (20, 27). Instead, it will be the nucleophilic attack by hydroxide ion on the methylene group to yield either the N-hydroxymethyl or O-hydroxymethyl intermediates as discussed by Getz (26). The first alternative is rate limiting specific base attack on the methylene group, facilitated by electron withdrawal by the imide. This seems feasible due to the presence of the two strongly electron
withdrawing groups (C=O and SO₂) on the saccharin molecule. The withdrawal of electron density by the imide nitrogen causes a partial positive charge to develop on the methylene carbon and leads to breaking of the O-C bond, formation of 17β-estradiol anion, and hydroxymethylsaccharin which is hydrolyzed to yield saccharin and formaldehyde. This would be expected to occur only if the phenol was more acidic than the imide and presumably better able to delocalize a formal negative charge. The second alternative is rate limiting specific base attack on the methylene group facilitated by withdrawal of electrons by the phenolic portion. The withdrawal of electron density by the phenol causes a partial positive charge to develop on the methylene carbon and leads to breaking of the N-C bond, formation of saccharin anion and 3-hydroxymethyl estradiol which is rapidly hydrolyzed to yield 17β-estradiol and formadehyde. Getz (26) found that the former alternative was apparently the mechanism of hydrolysis of imidomethyl prodrugs of phenols even for very acidic imides such as saccharin.

Figure 1.8: 2'-Saccharinylmethyl ether of 17β-Estradiol
Figure 1.9: $S_n2$ mechanism of cleavage of the 2'-saccharinyl methyl ether 17β-estradiol after the proposal of Getz (26).
CHAPTER 2
SYNTHESIS OF THE 2'-SACCHARINYLMETHYL ETHER OF 17β-ESTRADIOL

Introduction

A difficulty that frequently arises in the alkylation of 1,3-dicarbonyl compounds is the concurrent formation of both C-alkylated and O-alkylated products (33). A more common example of usually predominant O-alkylation is the alkylation of phenols. In general, O-alkylation competes significantly with C-alkylation only when the active methylene compounds involved are acidic. In such cases the equilibrium concentration of the enol tautomer is often relatively high. Alkylation at the more electronegative atom of the ambident anion (at oxygen rather than at carbon for an enolate anion) is usually favored by the use of polar, aprotic solvents especially hexamethylphosphoramide, anhydrous acetone, or dimethylformamide. The presence of large (R₄N⁺ >, K⁺ >, Na⁺ >, Li⁺) cations, which have a tendency to dissociate from the anion, will favor O-alkylation. C-Alkylation of phenols is favored by the use of heterogeneous reaction conditions or by the use of protic solvent which can hydrogen-bond with the oxygen atom of the anion.

A reaction in a solution of a particular enolate anion
with a particular alkylation agent will give the greatest proportion of O-alkylation when reaction conditions (solvent, cation, temperature) are chosen which allow the maximum amount of free enolate to be present. Presumably, the greatest fraction of the negative charge is located on oxygen, the most electronegative atom present, and the maximum opportunity for O-alkylation exists when the oxygen atom is not shielded by association with a metal cation or a hydrogen-bonding solvent.

The choice of the alkylation agent may also exert a significant influence on the proportions of C- and O-alkylated products. The proportion C- and O-alkylated products has been correlated with the principle of hard and soft acids and bases which states that hard Lewis acids prefer to coordinate to hard Lewis bases and vice versa. Soft Lewis acids and bases are characterized as being highly polarizable; usually the donor atoms of soft bases are large and in a low oxidation state with a relatively low electronegativity. Hard Lewis acids and bases exhibit low polarizability; the donor atoms of hard bases are usually small and highly electronegative. Thus, the common leaving groups of alkylation agents, listed in order of increasing hardness, would be \(-\text{I}<-\text{Br}<-\text{Cl}<-\text{O-SO}_2\text{R}\). The relatively small, electronegative oxygen atom of enolate anions and other ambident anions is harder than the larger, less electronegative, and more polarizable carbon atom. The result is that O-alkylation is more favored when the leaving group
is $-\text{OSO}_2\text{R}$ and C-alkylation is more favored when the leaving group is $-\text{I}$.

The alkylation agent frequently employed for reaction with an enolate anion is an alkyl halide. Both primary and secondary halides may be used successfully, however, tertiary alkyl halides having at least one beta hydrogen atom are usually of little value as alkylation agents because the major reaction that occurs when these materials are treated with enolate anions is a bimolecular elimination, leading to formation of an olefin.

Studies of alkylation in dipolar, aprotic solvents such as hexamethylphosphoramide, dimethylformamide, dimethyl sulfoxide and 1,2-dimethoxyethane have demonstrated that very substantial increases in the rates of reaction of enolate anions with alkylation agents result when they are used. Their advantage over protic solvents, such as alcohols, lies in the fact that they presumably do not solvate the enolate anion and, consequently, do not diminish its reactivity as a nucleophile. On the other hand, these aprotic solvents do have the ability to solvate the cation, separating it from the cation-enolate anion pair and leaving a relatively free anion in the reaction mixture.

The title compound was synthesized according to the steps illustrated in Fig 10. Saccharin was hydroxymethylated with formaldehyde (37%) (34), halogenated with phosphorus pentachloride (35) and the product was then used to alkylate the phenoxide anion of 17β-estradiol. The reactivity of the
alkyl halide was enhanced by in situ Finkelstein displacement of the chloride by iodide (36).

**Materials**

**Chemicals**

Figure 2.1: Steps involved in the synthesis of the 
2'- saccharinyl methyl ether of 17β-estradiol
**Miscellaneous**

**Instrumental**

IR spectra were recorded for solids in KBr discs on a Perkin-Elmer 1420 Ratio Recording Infrared Spectrophotometer. 90 MHz $^1$H NMR spectra were recorded in deuterochloroform (CDCl$_3$) and hexadeuteriodimethyl sulfoxide (DMSO-d$_6$) using tetramethyilsilane (TMS) as the internal standard on a Varian EM-390 NMR spectrometer. Signal positions are reported in parts per million downfield from TMS ($\delta$ ppm) and coupling constants (J) are reported in hertz (Hz). Melting point ranges were determined in capillary tubes, with a Thomas Hoover melting apparatus (Arthur J. Thomas Co., Philadelphia, PA) and are corrected. Elemental microanalysis was carried out on the final product by Atlantic Microlab, Inc., Norcross, Georgia. Thin layer chromatography with diethyl ether as the mobile phase was used to identify fractions containing the drug and/or prodrug. The fluorescent plates (0.25mm silica gel with fluorescent indicator UV254) were viewed under ultraviolet light (Chromato-vue, Ultraviolet Products, San Gabriella, CA.). The compounds were dried under vacuum in an oven (National Appliance Co., Model 585). The final product (prodrug) was characterized by a single peak on an HPLC system: $\lambda$anal = 220nm, Analyst series 7800, equipped with a Constametric II LDC pump and Spectromonitor III UV detector.
Methods

N-hydroxymethylsaccharin

To 10g (0.055 moles) saccharin in a 250 ml Erylenmeyer flask was added 100 ml (1.2 mole) of 37% w/w solution of formaldehyde. This was stoppered loosely and stirred on a warm hot plate until all the saccharin had dissolved. The solution was then left at room temperature until cool, and then placed in a refrigerator for two days. A white precipitate formed. The liquid was decanted and the precipitate was filtered. The residue was then placed in a beaker and covered with perforated aluminum foil. The beaker was placed in a vacuum oven at 30°C for two days. The melting point of the dried solid was: 125-131°C; yield: 10g (0.047 mole, 86%); NMR (DMSO-d6) \( \delta 5.13 \) (s, 2H, N-CH\(_2\)-OH), \( 4.52 \) (s, 1H, -OH), \( 7.93-8.29 \) (multiplet, 4H, Ar-H).

N-chloromethylsaccharin

In a 250 ml Erylenmeyer flask was placed 8 g (0.038 mole) of N-hydroxymethylsaccharin and 7.8 g (0.038 mole) of phosphorus pentachloride. These were suspended in 50 ml of anhydrous diethyl ether. The mixture was stirred for 3 hours
at room temperature. A white precipitate formed which was filtered, and then dried overnight in a vacuum desiccator at room temperature: M.P.: 139-145°C; yield: 7.55 g (0.033 mole, 86%); NMR (CDCl₃) δ 5.75 (s, 2H, N-CH₂-Cl), 7.95 - 8.38 (multiplet, 4H, unresolved, ArH).

2'-Saccharinylmethyl ether of 17β-estradiol

In a 50 ml pear-shaped flask was placed 1 g (0.0037 mole) of 17β-estradiol, 0.85 g (0.0037 mole) of N-chloromethyl saccharin, 0.506 g (0.0037 mole) of anhydrous potassium carbonate and 0.53 g (0.0037 mole) of anhydrous sodium iodide. The mixture was suspended in 25 ml of anhydrous acetone. The flask was loosely stoppered, and the mixture was stirred at room temperature for two days. The reaction mixture at the end of two days appeared to be frothy white. It was poured into a separatory funnel. The residual contents were rinsed from the flask into the funnel with dichloromethane. The organic mixture was extracted once with 20 ml of 0.1N NaOH solution. Then it was dried with about 2 g of anhydrous sodium sulfate. The mixture was stirred for 10 minutes, then filtered. The dichloromethane solution was concentrated in a round-bottom flask with a Rotavap to give a gummy residue. About 15 g of silica was added to the flask with 100 ml of diethyl ether. The flask was swirled until the
gum dissolved. The solvent was then removed using the Rotavap.

A chromatography column was set up by first partly filling a 5 cm diameter column with anhydrous diethyl ether. About 400 g of silica was added maintaining the solvent layer about 5" above the silica layer at all times. To this was added the mixture of silica and adsorbed reaction product. The solvent was eluted dropwise at a steady rate of about 7 ml/min. Fractions were collected and spotted on TLC plates (mobile phase, diethyl ether) and observed under UV after development. Fractions containing the desired compound, Rf = 0.38, were combined in a round bottom flask and the ether was evaporated with a Rotavap. A viscous residue was obtained, which was dissolved in methanol (15 ml). The solution was then poured into a smaller flask and concentrated under vacuum. When the volume was reduced to about 7 ml, the liquid was decanted into a 10 ml flask. The flask was covered with perforated aluminum foil and set aside for three days. A white suspension formed. The mother liquor was decanted and the solid was filtered and dried in a desiccator: M.P.: 112-118°C; yield: 1.19 g (0.025 mole, 70%); NMR (CDCl3) δ 0.73 (s, 3H, CH3), 1.0 - 2.9 (multiplet, 15H, methylene envelop), 3.66 (multiplet, 1H, 17-CH), 5.69 (s, 2H, N-CH2-O-), 6.79 (s,1H, ArH), 6.82 (d,1H, ArH, J=9 Hz), 7.13 (d, 1H, ArH, J=8.9 Hz), 7.66 - 8.1 (multiplet, 4H, ArH). 17β-Estradiol: IR (KBr disc) 3450 (OH), 3200 (OH) cm⁻¹; prodrug: IR (KBr disc) 3410 (OH), 1740 (C=O), 1330 (SO2), and 1196 cm⁻¹ (SO2).
Elemental analysis for C_{26}H_{29}N_{1}O_{5}S_{1}: found (theoretical), C: 66.78 (66.80), H: 6.26 (6.21), N: 3.03 (2.99). Differential scanning calorimetry for prodrug, scanning rate: 5.0°C/min, onset temperature: 98°C, heat of fusion: 36160 J/mole, estimated purity: 94.09%.

Discussion

The percentage yields of N-hydroxymethylsaccharin and N-chloromethylsaccharin were comparable (~86%) and reproducible based on 5 g of starting material. When a synthesis with larger amounts of saccharin was attempted (12-20 g), more unreacted material was found and the percentage yield of the N-hydroxymethylsaccharin dropped to 45%. Therefore, 5 g or less of saccharin was optimal for synthesis. Another factor that improved the yield of N-hydroxymethylsaccharin was the use of an unopened or relatively new bottle of formaldehyde solution (37% w/w). The difference in percentage yield between using an unopened bottle and an opened one was greater than 20%. This probably resulted due to the evaporation of the formaldehyde from the solution or the formation of paraformaldehyde. The reaction between the N-hydroxymethylsaccharin and phosphorus pentachloride did not encounter any difficulties. The reaction involving N-chloromethylsaccharin and 17β-estradiol required strictly
anhydrous conditions due to the susceptibility of the product (prodrug) to hydrolysis. The yield of the prodrug was found to decrease when more than 1 g of estradiol was used. Thus, the yield obtained starting with 2 g of 17β-estradiol was only about 37%. Moreover, the column chromatography conditions mentioned above hold valid only for a reaction where 1 g or less of 17β-estradiol was used. The extraction procedure involving the removal of the unreacted 17β-estradiol by salt formation with sodium hydroxide (0.1N) should be done only once and very rapidly, as the product (prodrug) is highly susceptible to hydrolysis under alkaline conditions. The precipitation of the final product led to formation of an amorphous solid substance in almost all cases which made it difficult to determine its purity by differential scanning calorimetry. Attempts made to run DSC on the amorphous product resulted in no apparent endotherm. When a crystalline form was obtained, it revealed an endothermic peak at 103°C and an estimated percentage purity of 94.09%. This purity estimate is not regarded as accurate, as the heating rate (5°C/min) was higher than that usually thought to be optimal (<2°C/min) (37).
CHAPTER 3
STABILITY STUDIES OF THE 2'-SACCHARINYL METHYL ETHER OF 17β-ESTRADIOL IN BUFFER SYSTEMS AND PHYSIOLOGICAL MEDIA

Introduction

As discussed previously in Chapter 1, the kinetics of decomposition of N-hydroxymethylamides in aqueous solution have been shown to exhibit first order dependence, up to pH 12, on the hydroxide ion concentration. Reaction rates also increased sharply with increasing acidity of the parent amide. Based on this discussion, and the observations of Getz (26), the hydrolytic stability of a prodrug such as the 2'-saccharinylmethyl ether of 17β-estradiol was expected to be higher at lower pH values than at higher pH values.

Kinetic studies of drug substances are preferably performed in aqueous buffers. However, stability studies of the prodrug were conducted in mixed aqueous-organic solvent systems consisting of 30% or 50% v/v aqueous methanol, as the prodrug was poorly soluble in aqueous media alone. Studies were also performed in 30% and 50% v/v aqueous dioxane.
Stability Studies In Mixed Solvent Systems

Until recently (38) the choice of a reaction solvent was made rather empirically, and often with little consideration of its effect on the rate or course of the reaction. However, the influence of the solvent can be profound. By the proper choice of the solvent, the products from a given set of reactants can be completely changed, and in some cases, the direction of the reaction can be reversed. In the case of the $S_{N2}$ mechanism, if the substrate is neutral, as is the case with the prodrug in this study, and the nucleophile is charged (OH$^-$ anion in this case), then the resulting charge dispersion in the transition state of the $S_{N2}$ mechanism is better stabilized in less polar solvents than in solvents with higher polarity (39). Therefore, in a water-methanol mixture, the overall solvent polarity is decreased and the reaction is favored compared to that with water alone as the solvent. Thus, one would expect to see higher reaction rates with increasing methanol concentrations (40). This type of reaction is illustrated in Figure 3.1:

\[
Y^- + \overset{\delta^+}{C-X} \rightarrow [\overset{\delta^-}{Y} \overset{\delta^+}{C} \overset{\delta^-}{X}]
\]

Figure 3.1: $S_{N2}$ reaction: a charged nucleophile and a neutral substrate
where $Y^-$ is the OH$^-$ anion, and C-X is the neutral species (prodrug). This has a highly dispersed charge in the transition state as compared to the initial (reactant) state. In terms of solvation phenomena, one must consider the difference between the free energies of solvation of the reactants and of the transition state. This difference in solvation free energy is conveniently expressed in terms of a medium effect or transfer activity coefficient (41). Transfer activity coefficients are particularly useful for examining reaction rates involving bimolecular nucleophilic attack by anions. These reaction rates are particularly sensitive to solvent change.

The thermodynamics of the solvent effect can be analyzed in terms of the free energies of transfer of substrate, nucleophile, and transition state from a reference solvent to the solvent of interest. In an $S_N2$ reaction of the charge type as shown in Fig. 3.1, a point charge on $Y^-$ is dispersed in the transition state. The $\Delta G_{tr} (Y^-)$ (the free energy of transfer of OH$^-$ from methanol to a dipolar aprotic solvent) is large and positive, whereas, $\Delta G_{tr} (R-X)$ (substrate) is zero or slightly negative. Furthermore, the free energy of transfer of the transition state is slightly positive (39). Small ions with localized charge such as OH$^-$, F$^-$ and Cl$^-$, which are good hydrogen bond acceptors, are much better solvated by protic solvents than by aprotic solvents. Therefore, the free energy of activation of the reaction will be smaller in dipolar aprotic solvents (and the rate faster),
largely as a result of poor solvation of the nucleophile. Water-methanol systems are amphiprotic in nature, in which case the reaction rates will be faster than those observed in water alone, as a result of lower solvent polarity, but slower than the rates observed in dipolar aprotic solvents (32). On the other hand, investigations of the alkaline hydrolysis of ethyl benzoates and ethyl acetate have been studied in dimethyl sulfoxide, DMSO (42), a dipolar aprotic solvent. The increased reaction velocity in DMSO has been attributed to the increased activity of the hydroxide ion caused by its decreased solvation in DMSO.

**Solvent - Structure**

Solvent structure-making and -breaking are also relevant. This effect is related to the ability of the solvent molecules to accept and donate hydrogen bonds. As studied by Butler et al. (43) and Schulman (44), the structural integrity of water is maintained in water-methanol mixtures up to 0.7 mole fraction for methanol, whereas, in the case of water-dioxane mixtures (45), the structural integrity is lost at 0.05 mole fraction of dioxane. Structured water is described as highly directional, cooperative, covalent hydrogen bonded water clusters (46).
The pH Scale in Non-Aqueous Media

Numerical values of activities and other free-energy related functions cannot be directly compared for different solvent media (47). To express activities in two solvents on the same scale, we must refer them to a single standard state. Consider, for example, the meaning of \( \text{paH} \) (\( \text{paH} = -\log a_H \)), where \( a_H \) is the activity of the hydrogen ions. An identical numerical value in methanol and in water will not represent the same free energy for the hydrogen ion in the two media because the solvation free energies for methanol and for water are not likely to be equal. The transfer activity coefficient, or the medium effect of transferring a solute 'i' from water to a given nonaqueous solvent is denoted by \( m_{yi} \) (47). The value of \( m_{yi} \) is a property of two ideal solutions, and is determined from the nature of the solute and the two solvents. At a given temperature and pressure it is constant and independent of the concentration of other substances present in solution. The analogous term \( s\gamma_H \) can be defined as the activity coefficient of the hydrogen ions due to a salt effect. When referred to the aqueous standard state, the activity coefficient of the hydrogen ion in methanol is a product of the salt effect (\( s\gamma_H \)), and the medium effect (\( m\gamma_H \)). Non-aqueous activity is designated by an asterisk. For the case where \( i = \) the hydrogen ion, the activity and molality are related as follows (47):
\[ aH^* = mH \gamma H \]

where:  
- \( aH^* \) is the activity of the hydrogen ions in the non-aqueous state  
- \( mH \) is the molality of the hydrogen ions  
- \( \gamma H \) is the activity coefficient (\( \gamma \)) of the hydrogen ions of salt 's', which becomes unity at infinite dilution in the non-aqueous solvent.

The medium effect can be used as a conversion factor from a non-aqueous to an aqueous activity scale. Thus, in the case of hydrogen ion activity, we have:

\[ aH = aH^* \gamma H \]

and

\[ paH = paH^* - \log \gamma H \]

to correlate \( paH \) scales in water and methanol (41). The transfer activity coefficient for the proton in methanol at 25°C is +1.68, which means that a solution of a given conventional \( paH^* \) in methanol would have a lower \( paH \) (would be more acidic) on the aqueous \( paH \) scale by 1.68 units. On the other hand, Bates, Paabo, and Robinson (48) interpreted nonaqueous pH data without using 'myi' in methanol-water mixtures. In their study, relationships were derived between pH and \( paH^* \), and the practical applications were demonstrated...
on buffer solutions in alcohol-water media. They estimated a salt-effect coefficient for the chloride ion ($\gamma_{\text{Cl}}$) from the Debye-Hückel equation (Eqn. 3.1). The Debye-Hückel equation 3.1 gives an activity coefficient for the hydrogen ion in dilute solutions.

$$\log \gamma = -\frac{A|Z_+Z_-|^{1/2}}{1 + B \Delta I}$$  \hspace{1cm} \text{Eqn. 3.1}

In the above equation, $I$ is the ionic strength, $Z_+$ and $Z_-$ are the ionic charges, $\Delta$ is the ion-size parameter in Ångstroms, $\Delta = 0.509$, and $B = 0.329$ for aqueous solutions at 25°C.

Using Eqn 3.1, Bates, Paabo, and Robinson calculated the constant $\delta$, where $\delta = [(\Delta E_j/0.05916) - \log m\gamma_H]$, and $\Delta E_j$ is the difference in the liquid-junction potential arising at (a) the boundary between the KCl salt bridge and the standard solution the pH of which is known, and (b) the same boundary between the KCl salt bridge and a mixed aqueous organic solution. The calculation of $\log m\gamma_H$ makes use of the following:

$$\log m\gamma_H = \log m\gamma_{\text{HCl}} - \log m\gamma_{\text{Cl}}$$  \hspace{1cm} \text{Eqn. 3.2}

The values of $\log m\gamma_{\text{HCl}}$ for alcohol-water systems are obtained from data compiled by Strehlow (49).
The term log \( mYH \) relates to the transfer activity coefficient of the hydrogen ion in that medium. The value for \( \delta \) was related to the \( \text{pa}H^* \) scale as in Eqn. 3.3.

\[
\text{pa}H^* = \text{pH} - \delta
\]

Eqn. 3.3

The \( \delta \) value in pH units at 25°C in 50% (wt%) or 0.4 mole fraction methanol in water is 0.13. For each alcohol-water medium studied, \( \delta \) values were fairly constant and independent of pH, so that interpretation of operational pH numbers via Eqn. 3.3 became possible.

**Stability Studies of the Prodrug of 17\( \beta \)-estradiol**

Hydrolysis of the prodrug was intended to occur at desired rates in the absence of enzyme catalytic effects. Hence, reactions were performed in physiological media which had been treated with enzyme inhibitors or denaturants. These were tetraethylpyrophosphate (TEPP) (35 \( \mu \)g/ml and 100 \( \mu \)g/ml) (50) which is effective against a wide spectrum of esterases, and sodium fluoride (20 mg/ml and 40 mg/ml) which denatures proteins (51). An observation of no difference in the rate constants between systems with and without the enzyme inhibitor or denaturant would suggest that these enzymes have no catalytic effect on the hydrolysis of the prodrug. In another set of experiments the hydrolytic behavior of the
prodrug in the presence of heat treated rat plasma and rat liver homogenate was studied. A standard compound, 4-nitrophenyl acetate (52), was used to demonstrate enzymatic activity in the enzyme preparation used.

In the present investigation, particular interest was focussed on whether the prodrug was stable during its passage through the GIT, the liver and into the systemic circulation. As the prodrug was to be evaluated in vivo, its stability was studied in solutions containing 50% methanol and 50% v/v aqueous buffers with nominal pH values of 5 to 8. Stability was also studied in human plasma, rat plasma and 20% rat liver homogenate containing 3.38% v/v methanol. All the stability studies were carried out in triplicate.

Materials

Chemicals

Co., Inc., Hauppauge, N.Y.); sodium phosphate, HPLC grade, M.W.: 136.08, purity: 99.6%; sodium phosphate, dibasic heptahydrate, M.W.: 268.07, purity: 99.7%; potassium phosphate, monobasic, M.W.: 136.09, purity: 99.5%; sodium fluoride, M.W.: 41.99; sodium chloride, M.W.: 58.45, (Sigma, St. Louis, MO); potassium chloride, M.W.: 74.55; sodium acetate, HPLC grade, M.W.: 136.08, purity: 99.6%; sodium hydroxide, 1N solution; sodium hydroxide, M.W.: 40.00, purity: 97.9%; hydrochloric acid, 1N solution; standard buffer solutions, pH: 2.0, 4.0, 7.0, 10; glacial acetic acid, M.W.: 60.05, purity: 99.7%; 1,1,1,3,3,3-hexamethyldisilazane, M.W.: 161.4 (Sigma); 17β-estradiol, M.W.: 272.4, (Sigma); 17α-ethinylestradiol-3-methyl ether (mestranol), M.W.: 310.4 (Sigma); 17α-ethinylestradiol, M.W.: 296.4 (Sigma); progesterone, M.W.: 314.5 (Sigma); 4-androstene-3,17-dione, M.W.: 286.42, purity: 98% (Aldrich, Milwaukee, WI).

Instruments and equipment

High performance liquid chromatograph: Analyst series 7800, equipped with a Constametric II LDC pump and Spectromonitor III UV detector (LDC, Milton Roy, Riviera Beach, FL); Chart Recorder, Omniscrobe Recorder, Houston Instruments; Water bath, (Haake, W.Germany, distributed by Fisher); pH meter, Orion Research digital pH/millivolt meter 611; HPLC microliter syringes, (Hamilton Company, Reno, NE);
Evaporator, N-Evap, Analytical Evaporator, (Organomations Associates, Inc., South Berlin, MA); centrifuge, Dynac II Centrifuge, Clay Adams, (Becton Dickinson & Co., Rutherford, NJ); Vortexer, (Vortex-Genie, Scientific Industries, Inc., Bohemia, NY); glass test tubes, (Kimble Borosilicate Glass), 13x100 mm, 16x100 mm; pasteur pipets, 146 mm. Very small volumes of liquids (<20 µl) were pipetted with a constant expulsion rate micrometer syringe (Hamilton Co., Reno, NE). Larger volumes (20 - 5000 µl) were transferred with Eppendorf pipettors (Fisher Sci.)

Methods

Buffer Solutions

All buffer solutions for kinetic studies were prepared from distilled deionized water. To weighed amounts of the buffer salts, sufficient water was added to yield a solution of the desired molarity (0.05M). An ionic strength of 0.145M was maintained using sodium chloride. All buffer solutions were prepared fresh and used within twenty-four hours of preparation. The pH of the buffer solution was measured using a pH meter calibrated at 37°C before use. The pH values of aqueous-organic reaction mixture were measured before and after some of the experiments. Only small changes (i.e., as ± 0.01) in the apparent pH values were observed in those
experiments, so apparent pH values were not routinely measured for experiments using these buffers and concentrations of substrate (i.e., $1 \times 10^{-4}$ M).

**Stock Solutions**

Stock solutions of drug, prodrug and the internal standard were prepared in methanol or 1,4-dioxane.

When appearance of the drug and disappearance of the prodrug were monitored, 4-androstene-3,17-dione was used as an internal standard. When only the disappearance of the prodrug was monitored, as for the studies in physiological media, mestranol was used as an internal standard.

**Physiological Media**

**Rat liver homogenate (20% v/v in 1.15% KCl-pH 7.4 phosphate buffer)** (53)

A female Sprague-Dawley rat was anesthetized with diethyl ether and sacrificed by decapitation. The liver was excised and placed in a beaker containing ice-cold 1.15% w/v KCl, buffered to pH 7.4 with potassium phosphate. It was rinsed twice with the buffer to remove as much blood as possible. It was then patted dry on a paper towel and weighed as quickly as possible. The liver was placed in a beaker containing four times its weight of buffer. It was then
sliced with a pair of scissors and transferred with the buffer to a glass homogenizing vessel (Potter-Elvejhem type). The liver was homogenized with a motor-driven Teflon pestle, using four complete up and down strokes. The homogenate was then centrifuged in a Sorvall polycarbonate centrifuge tube at 2,500 rpm for 10 minutes to separate nuclei and cell debris. The supernatant was then used for the stability studies. The liver homogenate used for the study was kept in an ice bucket until use.

**Rat plasma studies.** Blood was collected from female Sprague-Dawley rats after decapitation. It was centrifuged for 10 minutes at 3000 rpm. The plasma portion was used for the stability studies. The plasma was either kept in an ice bucket or stored frozen (-5°C) until use.

**Human plasma studies.** Fresh human plasma was obtained from Civitan Regional Blood Center, Gainesville, FL.

**Hplc Assay Validation**

Since several analytical procedures were necessary to study the stability of the prodrug in different media, corresponding calibration curves for 17β-estradiol and the prodrug were generated. These used different wavelengths for
detection, different mobile phases for HPLC analysis, and/or
different procedures for extraction from plasma.

Stability studies in buffered media were conducted under
the following chromatographic conditions; column: Zorbax
octadecylsilane, 4.6 mm ID x 15 cm; guard column: Zorbax
Reliance Cartridge 6 mm ID x 1.2 cm; mobile phase:
acetonitrile:acetate buffer (pH 3.8), 55:45, with tetrabutyl
ammonium phosphate (1 x 10^-4 M); UV detection at λ = 225 nm;
flow rate: 1.35 ml/min; pressure: 1450 psi; AUFS: 0.005;
chart speed: 0.5 cm/min; injection loop: 100 µl.

The HPLC conditions for stability studies in
physiological media are those mentioned above, except for the
mobile phase, acetonitrile: acetate buffer (pH 3.8), 70:30;
flow rate: 0.9 ml/min; pressure: 1100 psi; UV detection at λ =
280 nm. Only the disappearance of the prodrug could be
monitored when the mobile phase consisted of
acetonitrile:acetate buffer (pH 3.8), 70:30 because there was
interference from the plasma impurities. The choice of a
longer wavelength (λ = 280 nm) for detection helped alleviate
this problem when the stability of the prodrug in plasma was
determined.

Calibration curves for the prodrug after its extraction
from physiological media could not be achieved with
consistency, due to the instability of the prodrug in
physiological media. Therefore, the concentration of the
prodrug remaining at different sampling times could not be
determined. Peak heights obtained from chromatographic traces
of the prodrug and the internal standard were used to obtain peak height ratios. The natural logarithm of the peak height ratios, rather than the concentrations of prodrug, were plotted against time to determine the pseudo-first-order rate constants (k) for degradation of the prodrug in physiological media.

**Calibration Curves**

**Calibration curve for 17β-estradiol and the prodrug with UV detection at 225 nm**

Calibration curves were generated for the prodrug, drug and the internal standard, 4-androstene-3,17-dione. Stock solutions of the drug, prodrug and the internal standard were made in 100% methanol. Appropriate volumes of the stock solutions were pipetted into tubes, diluted with the mobile phase and immediately injected onto the HPLC system. The HPLC conditions are those given on p.51. The calibration range was 50 - 1000 ng/ml for the drug, 200 - 2400 ng/ml for the prodrug, with 600 ng/ml of the internal standard.

**Calibration curve for 17β-estradiol and the prodrug with UV detection at 280 nm**

The procedure was similar to that described for above. The HPLC conditions are those given on p.51 except that UV
detection was at $\lambda = 280$ nm. The calibration range was 50 - 2000 ng/ml for the drug, 150 - 4000 ng/ml for the prodrug, with 500 ng/ml of the internal standard 4-androstene-3,17-dione.

**Calibration curve for 17β-estradiol after its extraction from plasma**

A stock solution of the drug was made in 100% rat plasma. Appropriate volumes were pipetted into a mixture of blank plasma, internal standard (fixed volume added to all tubes) and normal saline such that the final volume on addition of the stock solution would be 1.0 ml. Normal saline was added to dilute the plasma and simplify the extraction procedure. Two milliliters of diethyl ether was then added to each tube. The mixture was vortexed for two minutes and then centrifuged for ten minutes at 3000 rpm. The supernatant was removed with a pasteur pipet, placed in a silylated glass tube, and evaporated under nitrogen. The extraction procedure was repeated three times. The residue obtained on drying was reconstituted with 1.0 ml of the mobile phase and injected onto the HPLC. The HPLC conditions are those given on p.51 except that the UV detection was at $\lambda = 280$ nm. The calibration range studied was between 50 - 3000 ng/ml of 17β-estradiol with 500 ng/ml of the internal standard 4-androstene-3,17-dione.
Calibration curve for the prodrug using mestranol as an internal standard

Stock solutions for the prodrug (0.2 mg/ml) and the internal standard (0.2 mg/ml) were made in 100% methanol. The concentration range studied was 100 - 2000 ng/ml of the prodrug.

Appropriate volumes of the prodrug solution were pipetted into tubes containing 5 μl of the internal standard solution. To these tubes was then added 1.0 ml of the mobile phase, the contents were mixed and the solutions were injected onto the HPLC. The HPLC conditions are those given on p.51, except for the mobile phase; acetonitrile:acetate buffer (pH 3.8), 70:30; flow rate: 0.9 ml/min; pressure: 1100 psi; UV detection at λ = 280 nm.

Calibration curve for the prodrug after extraction from rat plasma using mestranol as an internal standard

Stock solutions for the prodrug (0.2 mg/ml) and the internal standard (0.2 mg/ml) were made in 100% methanol. The concentration range studied was 100 - 2000 ng/ml of prodrug.

To 500 ml of ice-cold plasma was added 50 μl of the prodrug solution. This was mixed and appropriate aliquots were added to tubes containing 4.0 ml of ice-cold acetonitrile. To each tube was then added 5 μl of the internal standard solution. The tubes were vortexed for two
minutes each and then centrifuged at 3000 rpm for ten minutes. The supernatant was then evaporated under nitrogen. To these tubes was added 2.0 ml of diethyl ether. The mixture was vortexed for one minute, 1.0 ml of normal saline was added, and the mixture was again vortexed for one minute. The tubes were centrifuged for ten minutes at 3000 rpm and the supernatant pipetted into fresh tubes. The supernatant was then evaporated under nitrogen, and the residue was reconstituted with 1.0 ml of mobile phase. The reconstituted residue was injected onto the HPLC system. The HPLC conditions are those given on p.51, except for the mobile phase; acetonitrile: acetate buffer (pH 3.8), 70:30; flow rate: 0.9 ml/min; pressure: 1100 psi; UV detection at $\lambda = 280$ nm.

This double extraction procedure was necessary to remove plasma impurities.

**Experiment 1**

*Stability Studies of the Prodrug of 17β-estradiol in 50% v/v Aqueous Methanol*

**Hplc conditions**

The stability of the prodrug was studied in a mixed solvent system comprising 50% v/v methanol and buffer with pH 4.98, 5.88, 6.98 or 7.98. At pH 6.98, both the disappearance
of the prodrug and the appearance of the drug were monitored. The chromatographic conditions for this HPLC assay were as given on p. 51.

When only the disappearance of the prodrug was monitored (buffer pH 4.98, 5.88 and 7.98), the HPLC conditions were the same as above, except for the mobile phase; acetonitrile:acetate buffer (pH 3.8), 70:30; flow rate: 0.9 ml/min; pressure: 1100 psi.

Procedure

To each of three 15 ml test-tubes was added 5.0 ml of buffer solution, 60 µl of mestranol stock solution (0.5 mg/ml in methanol) and 4.7 ml of methanol. The contents of the tubes were mixed by gently vortexing, the test-tubes were then tightly capped and placed in a water bath at 37°C. Ten minutes later, 240 µl of a stock solution of the prodrug (0.5 mg/ml in methanol) was added to each tube (zero time), and the contents were vortexed. At appropriate timed intervals, 200 µl aliquots were pipetted into tubes containing 2 ml of ice-cold mobile phase. These samples were injected onto the HPLC with the described conditions (p.51). The peak heights of the drug, internal standard and the prodrug were recorded, converted to concentrations by reference to the calibration curve, and the natural logarithms of the concentrations were
plotted against time. Pseudo-first-order rate constants \((k)\) were obtained from the following equation (Eqn. 3.4):

\[
\ln C_t = \ln C_0 - kt \quad \text{Eqn. 3.4}
\]

where; \(C_t\) is the concentration at time \(t\), and 
\(C_0\) is the starting concentration at \(t=0\)

**Experiment 2**

*Stability Studies of the Prodrug of 17\(\beta\)-estradiol in 30% v/v Aqueous Dioxane, 50% v/v Aqueous Dioxane and 30% v/v Aqueous Methanol*

These experiments were conducted in order to determine whether there was a difference in the pseudo-first-order rate constants for degradation of the prodrug in the two solvent mixtures, i.e., methanol-water and dioxane-water. The buffers used in this study were pH 6.98 and pH 7.98. Degradation of the prodrug was relatively fast at those pH values. Only the disappearance of the prodrug was monitored.

**Procedure**

a) **Stability study in 30% v/v aqueous dioxane:** To 7.0 ml of the buffer solution in a 10 ml test-tube at 37°C, was added 60 \(\mu\)l of the internal standard (4-androstene-3,17-dione) (2
mg/ml of 1,4-dioxane) and 2.7 ml of 1,4-dioxane. The tube was vortexed and placed in the water bath for twenty minutes. To this was added 240 μl of the prodrug solution (2 mg/ml in 1,4-dioxane), then the tube was vortexed and placed in the water bath. Sampling was done periodically by pipetting 200 μl of solution into 2 ml of cold mobile phase. Samples of these mixtures were then injected onto the HPLC.

b) Stability study in 50% v/v aqueous dioxane: The procedure for this experiment was the same as that described for the 30% v/v dioxane study, except that 5.0 ml of the pH 7.98 buffer solution and 4.7 ml of 1,4-dioxane were added to the test-tube.

c) Stability study in 30% v/v aqueous methanol: The procedure for this experiment was the same as that described for 30% v/v dioxane, except that methanol was substituted for 1,4-dioxane.

HPLC conditions for a), b) and c): The chromatographic conditions were the same as described under Expt. 1 except for the following; mobile phase, acetonitrile:acetate buffer (pH 3.8), 70:30; flow rate: 0.9 ml/min; pressure: 1100 psi.
Experiment 3

Stability Studies of the Prodrug of 17β-estradiol in 30%, 50%, 60% and 70% v/v Aqueous Methanol

These experiments were carried out in order to determine the rate constants for the degradation of the prodrug with increasing methanol concentration in the reaction mixtures. Concentrations above 70% methanol were not studied due to precipitation of the buffer solutions.

Procedure

The procedure was similar for all concentrations except for the methanol content.

a) 30% v/v methanol: To 4.9 ml of pH 6.98 phosphate buffer in a 10 ml test-tube at 37°C was added 840 µl of the internal standard (4-androstene-3,17-dione) (0.05 mg/ml in methanol). The tube was placed in a water bath for twenty minutes. To this was added 1.26 ml of the prodrug solution (0.1 mg/ml in methanol) and the tube was vortexed. Samples (500 µl) were taken at appropriate intervals and added to tubes containing 2.0 ml of chilled mobile phase. The contents were vortexed and then the tubes were placed in an ice bucket until samples of the supernatant were injected onto the HPLC.
b) 50% v/v methanol: To 5.0 ml of pH 6.98 phosphate buffer in a 10 ml test-tube at 37°C was added 1.2 ml of the internal standard (4-androstene-3,17-dione) (0.05 mg/ml in methanol) and 0.2 ml of methanol. The tube was placed in a water bath for twenty minutes. To this was added 3.6 ml of the prodrug solution (0.05 mg/ml in methanol) and the tube was vortexed. Samples (200 µl) were taken at appropriate intervals and added to tubes containing 2.0 ml of chilled mobile phase.

c) 60% v/v methanol: The procedure was the same as that for the 50% v/v methanol mixture, except for the volume of buffer used (4.0 ml) and the methanol content (1.2 ml).

d) 70% v/v methanol: The procedure was the same as that for the 50% v/v methanol mixture, except for the volume of buffer used (3.0 ml) and the methanol content (2.2 ml).

HPLC conditions are as described on p.51.

**Experiment 4**

**Demonstration of One to One Conversion of the Prodrug to the Parent Drug**

The results obtained from the stability studies of the prodrug in buffer solutions with 50% v/v methanol in pH 6.98 phosphate buffer and 30% v/v dioxane in pH 8.0 phosphate buffer were used to determine if one to one conversion of the
prodrug to the drug had occurred. HPLC conditions are as described on p. 51.

**Experiment 5**

**In Vitro Stability Study of the Prodrug in Rat Liver Homogenate (20% in 1.15% w/v KCl-pH 7.4 Phosphate Buffer)**

**Procedure**

To 0.5 ml of the fresh liver homogenate (p. 49, less than two hours old) in a 1.0 ml vial containing 2.0 ml of pH 7.4 buffer was added 5 µl of mestranol (1 mg/ml in methanol). The mixture was gently vortexed and placed in the water bath at 37°C for 20 minutes. To the test-tube was added 15 µl of the prodrug solution (1 mg/ml in methanol). The tube was gently vortexed and placed back in the water bath. At appropriate intervals, 25 µl samples were pipetted into another tube containing 4 ml of ice-cold acetonitrile and the mixture was vortexed. These tubes were agitated in a shaker for 15 minutes, and centrifuged for 10 minutes at 3000 rpm. The acetonitrile layer was removed from the tubes with a pasteur pipet and placed in a third test-tube where the acetonitrile was evaporated under nitrogen. To these tubes was added 1 ml of mobile phase, the contents were vortexed, and a sample was injected onto the HPLC.

HPLC conditions: The chromatographic conditions were the same as described for Expt. 1 except for the following; mobile
phase, acetonitrile:acetate buffer (pH 3.8), 70:30; flow rate: 0.9 ml/min; pressure: 1100 psi.

**Experiment 6**

**In Vitro Stability Study of the Prodrug in Heat Denatured Rat Liver Homogenate (20% in 1.15% w/v KCl-pH 7.4 Phosphate Buffer)**

**Procedure**

The procedure for this experiment was the same as that mentioned for Expt.5, except that heat treated liver homogenate was used for the experiment. A test-tube containing the homogenate was placed in a boiling water bath for twenty minutes. The tube was then centrifuged for 15 minutes at 3000 rpm. The supernatant was used for the experiment.

HPLC conditions: As described under Expt. 5.

**Experiment 7**

**In Vitro Stability Study of the Prodrug in Rat Liver Homogenate (20% in 1.15% w/v KCl-pH 7.4 Phosphate Buffer) in the Presence of Tetraethylpyrophosphate (TEPP, 100 µg/ml)**

**Procedure**

The procedure for this experiment was the same as that mentioned for Expt.5, except that 25 µl of a fresh solution
of TEPP (2 mg/ml in water) was added to the reaction mixture 5 minutes prior to the addition of the prodrug.

HPLC conditions: As described for Expt. 5

**Experiment 8**

**In Vitro Stability Study of the Prodrug in Rat Plasma (3.84% v/v Methanol) at 37°C**

**Procedure**

The procedure and the HPLC conditions, were the same as those described for the rat liver homogenate study, for Expt. 5, except for the physiological medium (p. 50) and for the fact that the plasma was not diluted with buffer as the liver homogenate had been.

**Experiment 9**

**In Vitro Stability Study of the Prodrug in Heat Denatured Rat Plasma (80%) at 37°C**

**Procedure**

Heat treated plasma was obtained by mixing plasma with pH 7.4 phosphate buffer (80:20 v/v) and placing the tube in a boiling water bath for twenty minutes. The cooled mixture was filtered and then used for the study.

To each of three test-tubes was added 500 μl of heat treated rat plasma and 10 μl of mestranol solution (1 mg/ml
in methanol). These tubes were placed in a water bath at 37°C for twenty minutes. The hydrolysis was initiated by adding 15 μl of the prodrug solution (1 mg/ml in methanol) to the reaction mixture. Sampling was accomplished at the desired intervals by adding 25 μl of the mixture to test-tubes containing 4.0 ml of ice-cold acetonitrile. The tubes were vortexed and centrifuged at 3000 rpm. The supernatant from each tube was then added to a silylated tube and evaporated under nitrogen. The residues were reconstituted with 1.0 ml of mobile phase and samples were injected onto the HPLC.

HPLC conditions: The chromatographic conditions were the same as described for Expt. 1 except for the following; mobile phase, acetonitrile:acetate buffer (pH 3.8), 70:30; flow rate: 0.9 ml/min; pressure: 1100 psi.

**Experiment 10**

**Stability Study of the Prodrug in Rat Plasma in the Presence and Absence of Tetraethylpyrophosphate (TEPP) at Concentrations of 35 μg/ml at 25°C and 100 μg/ml at 37°C**

The stability of the prodrug was studied in the presence and absence of TEPP to determine if enzymes present in plasma catalyzed the hydrolysis of the prodrug. Two concentrations of the enzyme inhibitor were studied; 35 μg/ml was the suggested concentration (52) and 100 μg/ml (25 μl of 2 mg/ml solution) was arbitrarily chosen to determine if there was a concentration effect.
Procedure

The procedure was the same as described for the rat liver homogenate study, except for the physiological medium that was used.

HPLC conditions: The chromatographic conditions were the same as described for Expt. 1 except for the following; mobile phase, acetonitrile:acetate buffer (pH 3.8), 70:30; flow rate: 0.9 ml/min; pressure: 1100 psi.

Experiment II

Stability Study of the Prodrug in Rat Plasma in the Presence and Absence of Sodium Fluoride at Concentrations of 20 mg/ml and 40 mg/ml at 25°C

Procedure

To each of three test-tubes was added 500 μl of rat plasma and 17 μl of mestranol solution (1 mg/ml in methanol). To one of the tubes was added 130 μl of aqueous sodium fluoride (100 mg/ml) and to another was added 130 μl of aqueous sodium fluoride (200 mg/ml). To all three tubes was then added 10 μl of the prodrug solution in methanol (5 mg/ml) and the contents were vortexed. Sampling was done periodically by adding 30 μl of the mixture to a test-tube containing 4 ml of ice-cold acetonitrile. The contents were vortexed for one minute and then centrifuged (3000 rpm) for
ten minutes. The supernatant was transferred to another test-tube and evaporated to dryness. Two milliliters of diethyl ether and 1.0 ml of water were added to each tube. The tubes were vortexed for one minute and centrifuged again for ten minutes (3000 rpm). The supernatants were added to silylated tubes and evaporated under nitrogen. The residues were reconstituted with 1.0 of mobile mobile phase and a sample of each was injected onto the HPLC. HPLC conditions are those described under Expt. 10.

Experiment 12

Stability Study of p-Nitrophenyl Acetate in pH 7.4, 0.05 Molar Phosphate Buffer at 37°C by Spectrophotometry

Procedure

The following were the settings on the Cary UV spectrophotometer: Scan rate: 5 nm/sec; Period: 1.0; Range: 2.0; Balance: 4.0; Chart display: 20 nm/sec; Mode: double beam; Spectral band width: 1.0, Water bath temperature: 37°C.

A solution of p-nitrophenyl acetate (1 x 10^{-3} M) was prepared in pH 4.0 acetate buffer. Phosphate buffer (pH 7.4, 2.0 ml) was pipetted into two UV cuvettes. The cuvettes were covered with lids and placed into the sample and reference chambers. The cuvettes were maintained at 37°C with a water bath and a baseline was recorded from 520 nm to 260 nm. The cuvette in the sample compartment then was spiked with 200 μl
of the p-nitrophenyl acetate stock solution, inverted, and quickly returned to the sample chamber. Spectra were recorded of the solution every few minutes over a period of approximately nine hours.

Experiment 13

Stability Study of p-Nitrophenyl Acetate in Rat Plasma or 20\% Rat Liver Homogenate at 37°C

Procedure

Stock solutions of p-nitrophenyl acetate (0.4 mg/ml in methanol) and saccharin (internal standard, 0.4 mg/ml in water) were prepared. To 0.5 ml of the rat liver homogenate (p. 49) or rat plasma was added 40 μl of the saccharin solution. This mixture was placed in a water bath at 37°C. After ten minutes the hydrolysis was initiated by spiking the mixture with 20 μl of the p-nitrophenyl acetate solution. Sampling was done periodically by taking 30 μl of the mixture and adding it to test-tubes containing 4.0 ml of acetonitrile. The tubes containing the samples were vortexed and centrifuged for ten minutes at 3000 rpm. The supernatants were then added to silylated tubes and evaporated to dryness under nitrogen. The residues were reconstituted in 1.0 ml of acetonitrile: acetate buffer (50:50) and aliquots were injected onto the HPLC.
HPLC conditions: The chromatographic conditions are same as described for Expt. 1 except for the following; mobile phase, acetate buffer (pH 3.8): acetonitrile, 70:30; flow rate: 1.0 ml/min; pressure: 1995 psi.; \( \lambda_{\text{anal.}} = 280 \) nm.

**Experiment 14**

**In Vitro Stability Study of the Prodrug in Human Plasma (3.84% v/v Methanol) at 37°C**

**Procedure**

The procedure and the HPLC conditions, were the same as those described for the rat liver homogenate study, Expt.5, except for the physiological medium.

**Results**

**Hplc Assay Validations**

Means ± standard deviation (SD) for the slopes and student's t-tests were determined for all the intercepts that were obtained from the calibrations curves described above (pp. 51-55). These results are reported in Tables 3.1, 3.2, 3.3, 3.4, 3.5, 3.6 and 3.7. The t-test in this case determines if there is a significant difference between the value zero and the test value (\( \mu \)). The p-value signifies the
probability that the null hypothesis (\( \mu = 0 \)) is true at a specified confidence interval. A 95% confidence interval was chosen here.

Calibration curves for 17\( \beta \)-estradiol are plotted in Figures 3.2, 3.4, 3.6, while curves for the prodrug are plotted in Figures 3.3, 3.5, 3.7 and 3.8. The calibration curves of the prodrug on extraction from rat plasma could not be reliably reproduced since the prodrug is relatively unstable in physiological media.

Table 3.1: Mean ± SD for the slope and student's t-test on the intercepts obtained from calibration curves for 17\( \beta \)-estradiol using 4-androstene-3,17-dione as an internal standard: 225 nm wavelength of detection.

<table>
<thead>
<tr>
<th>Slope</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.27 x 10^{-3}</td>
<td>1.32 x 10^{-3}</td>
<td>0.01 x 10^{-2}</td>
</tr>
<tr>
<td>1.37 x 10^{-3}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.21 x 10^{-3}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.42 x 10^{-3}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intercept</th>
<th>Mean</th>
<th>SD</th>
<th>t-stat</th>
<th>p; ( \mu = 0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.16 x 10^{-1}</td>
<td>6.94 x 10^{-2}</td>
<td>0.70 x 10^{-1}</td>
<td>2.11</td>
<td>1.25 x 10^{-1}</td>
</tr>
<tr>
<td>1.57 x 10^{-2}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.01 x 10^{-2}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.36 x 10^{-1}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2: Mean ± SD for the slope and student's t-test on the intercepts obtained from calibration curves for the prodrug in mobile phase using 4-androstene-3,17-dione as an internal standard: 225 nm wavelength of detection.

<table>
<thead>
<tr>
<th>Slope</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$5.69 \times 10^{-4}$</td>
<td>$5.30 \times 10^{-4}$</td>
<td>$0.04 \times 10^{-3}$</td>
</tr>
<tr>
<td>$4.89 \times 10^{-4}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$5.10 \times 10^{-4}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$5.41 \times 10^{-4}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intercept</th>
<th>Mean</th>
<th>SD</th>
<th>t-stat.</th>
<th>p; $\mu = 0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$8.56 \times 10^{-3}$</td>
<td>$1.73 \times 10^{-2}$</td>
<td>$1.10 \times 10^{-2}$</td>
<td>3.22</td>
<td>$2.45 \times 10^{-1}$</td>
</tr>
<tr>
<td>$2.02 \times 10^{-2}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$9.14 \times 10^{-3}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$3.12 \times 10^{-2}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3: Mean ± SD for the slope and student's t-test on the intercepts obtained from calibration curves for 17β-estradiol in mobile phase using 4-androstene-3,17-dione as an internal standard: 280 nm wavelength of detection.

<table>
<thead>
<tr>
<th>Slope</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$8.45 \times 10^{-4}$</td>
<td>$8.20 \times 10^{-4}$</td>
<td>$0.04 \times 10^{-3}$</td>
</tr>
<tr>
<td>$7.67 \times 10^{-4}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$8.41 \times 10^{-4}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$8.28 \times 10^{-4}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3 -- continued

<table>
<thead>
<tr>
<th>Intercept</th>
<th>Mean</th>
<th>SD</th>
<th>t-stat.</th>
<th>p; \mu = 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1.63 \times 10^{-2}</td>
<td>-1.17 \times 10^{-2}</td>
<td>1.50 \times 10^{-2}</td>
<td>1.59</td>
<td>3.00 \times 10^{-1}</td>
</tr>
<tr>
<td>-3.04 \times 10^{-2}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.12 \times 10^{-5}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.29 \times 10^{-6}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4: Mean ± SD for the slope and student's t-test on the intercepts obtained from calibration curves for the prodrug in mobile phase using 4-androstene-3,17-dione as an internal standard: 280 nm wavelength of detection.

<table>
<thead>
<tr>
<th>Slope</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.17 \times 10^{-3}</td>
<td>1.16 \times 10^{-3}</td>
<td>0.03 \times 10^{-3}</td>
</tr>
<tr>
<td>1.19 \times 10^{-3}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.12 \times 10^{-3}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.16 \times 10^{-3}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intercept</th>
<th>Mean</th>
<th>SD</th>
<th>t-stat.</th>
<th>p; \mu = 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.93 \times 10^{-2}</td>
<td>2.87 \times 10^{-2}</td>
<td>1.00 \times 10^{-2}</td>
<td>5.98</td>
<td>1.15 \times 10^{-2}</td>
</tr>
<tr>
<td>4.16 \times 10^{-2}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.91 \times 10^{-2}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.46 \times 10^{-2}</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Table 3.5: Mean ± SD for the slope and student t-test on the intercepts obtained from calibration curves for 17β-estradiol on extraction from rat plasma using 4-androstene-3,17-dione as an internal standard: 280 nm wavelength of detection.

<table>
<thead>
<tr>
<th>Slope</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.44 x 10^-4</td>
<td>8.07 x 10^-4</td>
<td>0.04 x 10^-3</td>
</tr>
<tr>
<td>8.35 x 10^-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.37 x 10^-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.10 x 10^-4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intercept</th>
<th>Mean</th>
<th>SD</th>
<th>t-stat.</th>
<th>p; μ = 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.24 x 10^-4</td>
<td>1.13 x 10^-3</td>
<td>2.00 x 10^-3</td>
<td>1.26</td>
<td>2.98 x 10^-1</td>
</tr>
<tr>
<td>2.42 x 10^-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.27 x 10^-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.82 x 10^-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.6: Mean ± SD for the slope and student's t-test on the intercepts obtained from calibration curves for the prodrug in mobile phase using mestranol as an internal standard: 280 nm wavelength of detection.

<table>
<thead>
<tr>
<th>Slope</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.03 x 10^-4</td>
<td>8.14 x 10^-4</td>
<td>0.01 x 10^-3</td>
</tr>
<tr>
<td>8.30 x 10^-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.00 x 10^-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.24 x 10^-4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.6: Mean ± SD on the intercepts obtained from calibration curves for the prodrug on extraction from plasma using mestranol as an internal standard: 280 nm wavelength of detection.

<table>
<thead>
<tr>
<th>Intercept</th>
<th>Mean</th>
<th>SD</th>
<th>t-stat.</th>
<th>p; ( \mu = 0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1.27x10^{-2}</td>
<td>9.10 x 10^{-4}</td>
<td>1.00 x 10^{-2}</td>
<td>0.13</td>
<td>9.06 x 10^{-1}</td>
</tr>
<tr>
<td>-8.90x10^{-3}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.54x10^{-3}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.77x10^{-2}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.7: Mean ± SD on the slope and student's t-test on the intercepts obtained from calibration curves for the prodrug on extraction from plasma using mestranol as an internal standard: 280 nm wavelength of detection.

<table>
<thead>
<tr>
<th>Slope</th>
<th>Mean</th>
<th>± Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.21 x 10^{-4}</td>
<td>3.81 x 10^{-3}</td>
<td>3.09 x 10^{-3}</td>
</tr>
<tr>
<td>6.89 x 10^{-3}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intercept</th>
<th>Mean</th>
<th>± Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.85 x 10^{-4}</td>
<td>2.33 x 10^{-2}</td>
<td>2.31 x 10^{-2}</td>
</tr>
<tr>
<td>4.65 x 10^{-2}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3.2 Calibration curve in mobile phase for 17β-estradiol using 4-androstene-3,17-dione (600 ng/ml) as an internal standard: 225 nm wavelength of detection.

Fig. 3.3 Calibration curve in mobile phase for prodrug using 4-androstene-3,17-dione (600 ng/ml) as an internal standard: 225 nm wavelength of detection.
Fig. 3.4 Calibration curve in mobile phase for 17β-estradiol using 4-androstene-3,17-dione (600 ng/ml) as an internal standard: 280 nm wavelength of detection.

Fig. 3.5 Calibration curve in mobile phase for prodrug of 17β-estradiol at using 4-androstene-3,17-dione (600 ng/ml) as an internal standard: 280 nm wavelength of detection.
**Fig. 3.6** Calibration curve for 17β-estradiol on extraction from plasma using 4-androstene-3,17-dione (600 ng/ml) as an internal standard: 280 nm wavelength of detection.

**Fig. 3.7** Calibration curve in mobile phase for prodrug using mestranol (1000 ng/ml) as an internal standard: 280 nm wavelength of detection.
Fig. 3.8 Calibration curve for prodrug on extraction from plasma using mestranol (1000 ng/ml) as an internal standard: 280 nm wavelength of detection.

Results for Experiment 1

Stability of the Prodrug in 50% v/v Aqueous Methanol

The stability of the prodrug in the solvent system comprising 50:50 v/v methanol and buffers having pH values 4.98, 5.88, 6.98 and 7.98 at 37°C yielded pseudo-first-order rate constants calculated from Eqn. 3.4. The hydrolysis reaction was studied in triplicate for each pH value, which gave observed mean values of the rate constants (kobs) as listed in table 3.8.
Table 3.8: Pseudo-first-order rate constants and the respective half-lives at different pH values.

<table>
<thead>
<tr>
<th>pH measured at 37°C</th>
<th>$k_{obs}$ (min$^{-1}$)</th>
<th>Half-life (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td></td>
</tr>
<tr>
<td>4.98</td>
<td>3.87x10$^{-4}$ ± 0.03x10$^{-4}$</td>
<td>1790 (30 h)</td>
</tr>
<tr>
<td>5.88</td>
<td>4.98x10$^{-3}$ ± 0.02x10$^{-2}$</td>
<td>139</td>
</tr>
<tr>
<td>6.98</td>
<td>5.35x10$^{-2}$ ± 0.07x10$^{-2}$</td>
<td>13</td>
</tr>
<tr>
<td>7.98</td>
<td>6.47x10$^{-1}$ ± 3.00x10$^{-2}$</td>
<td>1.07</td>
</tr>
</tbody>
</table>

Figures 3.9 and 3.10 show the pseudo-first-order plots obtained by plotting the data obtained from one of three replicates against the time recorded.

Figure 3.9: Pseudo-first-order plots of degradation of the prodrug in buffer systems with pH values 4.98 and 5.88.
Figure 3.10: Pseudo-first-order plots of degradation of the prodrug in buffer systems with pH values 6.98 and 7.98.

Figure 3.11 on p.81 shows the partial pH rate profile obtained by plotting the logarithm of the mean values of the pseudo-first-order rate constants obtained in 50% v/v non-aqueous buffer mixture versus the measured pH values. The plot has a slope of 1.07 with SD ± 0.04.

Results for Experiment 2

Stability of the Prodrug of 17β-estradiol in 30% v/v Aqueous Dioxane, 50% v/v Aqueous Dioxane and 30% v/v Methanol

Tables 3.9 and 3.10 lists the mean values ± SD of the $k_{obs}$ values with the respective half lives for these experiments
studied in pH 6.98 and pH 7.98 phosphate buffer (0.05 M, μ = 0.145) at 37°C, respectively.

Table 3.9: Observed rate constants for the hydrolysis of the prodrug in pH 6.98 phosphate buffer with varying percentages of organic cosolvent.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>k_{obs} (min^{-1})</th>
<th>Mean ± SD</th>
<th>Half-life (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% v/v Dioxane</td>
<td>1.20x10^{-2} ± 0.08x10^{-2}</td>
<td>57.8</td>
<td></td>
</tr>
<tr>
<td>50% v/v Dioxane</td>
<td>4.63x10^{-3} ± 0.03x10^{-3}</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>30% v/v Methanol</td>
<td>3.36x10^{-2} ± 0.01x10^{-2}</td>
<td>20.6</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.10: Observed rate constants for the hydrolysis of the prodrug in pH 7.98 phosphate buffer with varying percentages of organic cosolvent.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>k_{obs} (min^{-1})</th>
<th>Mean ± SD</th>
<th>Half-life (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% v/v Dioxane</td>
<td>3.69x10^{-2} ± 0.1x10^{-2}</td>
<td>18.8</td>
<td></td>
</tr>
<tr>
<td>50% v/v Dioxane</td>
<td>1.33x10^{-2} ± 0.3x10^{-2}</td>
<td>52.1</td>
<td></td>
</tr>
<tr>
<td>30% v/v Methanol</td>
<td>3.48x10^{-1} ± 0.9x10^{-2}</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.11 exhibits the plots obtained from the results listed under tables 3.8, 3.9 and 3.10.
Results for Experiment 3

Stability of the Prodrug of 17β-estradiol in 30%, 50%, 60% and 70% v/v Aqueous Methanol

The use of varying percentages of methanol in the stability experiments in order to solubilize the prodrug was shown to give a significant difference in the pseudo-first-order rate constants. Therefore, additional experiments (besides those in 30% v/v and 50% v/v aqueous methanol) were carried out in 60% v/v and 70% v/v methanol. These used phosphate buffer (pH 6.98, 0.05 M, υ = 0.145) at 37°C. Attempts to study the hydrolysis of the prodrug in solution...
with >70% v/v methanol led to precipitation of buffer salts. Attempts to study rate constants below 30% v/v methanol were not made as it was not clear that the prodrug was soluble at lower methanol concentrations. Table 3.11 shows the pseudo-first-order rate constants obtained in 30%, 50%, 60% and 70% v/v aqueous methanol.

Table 3.11: Observed rate constants for the hydrolysis of the prodrug in pH 6.98 phosphate buffer with varying percentages of methanol.

<table>
<thead>
<tr>
<th>Solvent (with pH 6.98 buffer)</th>
<th>k_{obs} (min^{-1})</th>
<th>Half-life (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% v/v methanol</td>
<td>2.88 x 10^{-2} ± 0.06 x 10^{-2}</td>
<td>24.0</td>
</tr>
<tr>
<td>50% v/v methanol</td>
<td>6.19 x 10^{-2} ± 0.60 x 10^{-2}</td>
<td>11.2</td>
</tr>
<tr>
<td>60% v/v methanol</td>
<td>7.79 x 10^{-2} ± 0.80 x 10^{-2}</td>
<td>8.90</td>
</tr>
<tr>
<td>70% v/v methanol</td>
<td>1.02 x 10^{-1} ± 0.08 x 10^{-1}</td>
<td>6.79</td>
</tr>
</tbody>
</table>

Figure 3.12 shows a plot of the mean values of the pseudo first-order rate constants (three replicates) versus the mole fraction of methanol present in the media. The plot yields a slope ($R^2 = 0.998$) having a value $2.07 \times 10^{-1}$ and a negative intercept of $-4.30 \times 10^{-3}$. The standard error about the Y intercept is: $4.68 \times 10^{-3}$, at $\alpha = 0.05$, df = 2.
Figure 3.12: Plot of the pseudo-first-order rate constants versus mole fraction of methanol in mixtures with pH 6.98 phosphate buffer.
Results for Experiment 4

Demonstration of One to One Conversion of the Prodrug to the Parent Drug

The results for this experiment were computed from the 50% v/v aqueous methanol studies where both the disappearance of the prodrug and the appearance of the drug were quantitated. For one to one conversion, the rate of disappearance of the prodrug (k) would be expected to equal the rate of appearance of the drug (k1).

To compute these rates, the Sigma minus plot (54) was employed. This is a semilogarithmic plot of the difference between the amount of the drug that can be formed at infinite time (A∞) and the cumulative amount (At) formed at time t. Equation 3.5 represents such a plot.

\[ \ln(A_\infty - At) = \ln A_\infty - k_1t \]

Eqn. 3.5

where
- \( At \) = cumulative amount of drug at time t.
- \( A_\infty \) = total amount of drug formed at time \( \infty \).
- \( k_1 \) = rate of appearance of the drug.

Table 3.12 shows the pseudo-first-order rate constants obtained by using the above equation for the appearance of the drug in methanol: pH 6.98 buffer (50:50 v/v) and dioxane: pH 7.98 phosphate buffer (30:70 v/v) at 37°C.
Table 3.12: Pseudo-first-order rate constants observed for the appearance of the drug and the disappearance of the prodrug.

<table>
<thead>
<tr>
<th>pH of the buffer used and solvent</th>
<th>$k_1$ (min$^{-1}$)</th>
<th>$k$ (min$^{-1}$)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.98 (methanol)</td>
<td>$4.89 \times 10^{-2}$</td>
<td>$5.04 \times 10^{-2}$</td>
<td>$4.97 \times 10^{-2} ± 0.10 \times 10^{-2}$</td>
</tr>
<tr>
<td>7.98 (dioxane)</td>
<td>$3.47 \times 10^{-2}$</td>
<td>$3.67 \times 10^{-2}$</td>
<td>$3.57 \times 10^{-2} ± 0.10 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

Figure 3.13 shows plots of the time dependent concentration of the drug and of the prodrug. Figure 3.14 shows the rate of appearance of the drug as obtained by using Eqn. 3.5 and Fig. 3.15 shows the pseudo-first-order rate of disappearance of the prodrug obtained by using Eqn. 3.4.

Fig. 3.13: Appearance of the drug and disappearance of the prodrug in pH 6.98 buffer:methanol (50:50 v/v)
Figure 3.14: Sigma minus plot for the rate of hydrolysis of the prodrug based on measurement of the appearance of the drug in pH 6.98 phosphate buffer: methanol (50:50 v/v)

Figure 3.15: Rate of disappearance of the prodrug in pH 6.98 phosphate buffer: methanol (50:50 v/v)
Results for Experiments 5, 6 and 7.

**In Vitro Stability in Rat Liver Homogenate: pH 7.4, 1.15% KCL Phosphate Buffer (20:80 V/V) at 37°C**

These experiments were conducted in order to determine whether the prodrug was susceptible to catalytic effects of the enzymes present in rat liver. The prodrug was found to be relatively stable in heat treated rat liver homogenate and TEPP (100 μg/ml) treated homogenate; no degradation rates could be reliably established. Figure 3.16 shows the plot for these experiments. In rat liver homogenate, the $k_{obs} \pm SD$ was $1.40 \times 10^{-2} \pm 0.10 \times 10^{-2}$ with a $t_{1/2} = 49.5$ min.

![Graph showing stability study of the prodrug in rat liver homogenate (20%), in heat treated rat liver homogenate and in rat liver homogenate in the presence of TEPP.](image-url)
Results for Experiments 8 and 9.

**In Vitro Stability of the Prodrug in Rat plasma (96%) and Heat Treated Rat Plasma (80%) at 37°C**

These experiments were conducted in order to determine the stability of the prodrug in rat plasma in the presence and absence of enzymes. Heat treatment was carried out in order to destroy the plasma enzymes. Table 3.13 lists the pseudo-first-order rate constants obtained ± SD and the corresponding half-lives.

Results for Experiment 10

**Stability of the Prodrug in Rat Plasma in the Presence and Absence of TEPP (35 µg/ml at 25°C; 100 µg/ml at 37°C)**

These experiments were carried out to determine if the rate of degradation was affected by the presence or absence of rat plasma enzymes. The biological samples were treated with TEPP to destroy enzyme activity. Table 3.13 lists the rate constants ± SD and the corresponding half-lives.
Table 3.13: Pseudo-first-order rate constants and the respective half-lives observed in physiological media with varying conditions.

<table>
<thead>
<tr>
<th>Temp</th>
<th>Medium</th>
<th>$k_{obs} \pm SD$ (min$^{-1}$)</th>
<th>$t_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>Rat plasma (96%)</td>
<td>$3.28 \times 10^{-2} \pm 0.20 \times 10^{-2}$</td>
<td>21.11</td>
</tr>
<tr>
<td></td>
<td>+ TEPP (35 µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>Rat plasma (96%)</td>
<td>$3.48 \times 10^{-2} \pm 0.30 \times 10^{-2}$</td>
<td>19.91</td>
</tr>
<tr>
<td></td>
<td>Heat treated plasma (80%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>Rat plasma (96%) + TEPP (100 µg/ml)</td>
<td>$2.27 \times 10^{-1} \pm 0.30 \times 10^{-1}$</td>
<td>3.05</td>
</tr>
</tbody>
</table>

**Results for Experiment 11**

**Stability Study of the Prodrug in Rat Plasma in the Presence and Absence of Sodium Fluoride at Concentrations of 20 mg/ml and 40 mg/ml at 25°C**

Sodium fluoride was used as an alternative to TEPP to determine whether there was a significant difference in the rate constants obtained from the two experiments. Table 3.14 lists the rate constants ± SD and the corresponding half-lives.
Table 3.14: Pseudo-first-order rate constants and the respective half-lives observed in physiological media with varying sodium fluoride concentrations at 25°C.

<table>
<thead>
<tr>
<th>Medium</th>
<th>$k_{\text{obs}} \pm \text{SD (min}^{-1})$</th>
<th>$t_{1/2}$ (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat plasma (96%)</td>
<td>$3.28 \times 10^{-2} \pm 0.20 \times 10^{-2}$</td>
<td>21.11</td>
</tr>
<tr>
<td>Rat plasma (96%) + NaF (20 mg/ml)</td>
<td>$3.22 \times 10^{-2} \pm 0.10 \times 10^{-2}$</td>
<td>21.52</td>
</tr>
<tr>
<td>Rat plasma (96%) + NaF (40 mg/ml)</td>
<td>$3.08 \times 10^{-2} \pm 0.05 \times 10^{-2}$</td>
<td>22.50</td>
</tr>
</tbody>
</table>

Results for Experiment 12

Stability of p-Nitrophenyl Acetate in Phosphate Buffer (pH 7.4, 0.05 M) at 37°C Using UV Spectrophotometry.

The results for this experiment were computed from spectra obtained by periodically scanning the reaction medium (520 nm - 260 nm). The absorbance of the spectrum was measured at 400 nm for the formation of p-nitrophenol. Since p-nitrophenyl acetate is hydrolyzed to form p-nitrophenol, formation of the phenol can be used to estimate the rate of degradation of the ester. Therefore, the natural logarithm of the difference in the absorbance units of the phenol at time infinity and at time $t$ was plotted against time to obtain the first-order rate constant (Sigma minus plot): $k_{\text{obs}} = 1.56 \times 10^{-3}$ min$^{-1}$, range = $2.2 \times 10^{-4}$, $n = 2$, $t_{1/2} = 7.4$ h. The
pseudo-first-order rate constants were determined from linear least squares analysis of the data. Figure 3.17 shows the spectra obtained from this experiment and Figure 3.18 shows the sigma minus plot.

Figure 3.17: Spectra showing the formation of the p-nitrophenol and the degradation of p-nitrophenyl acetate in pH 7.4 phosphate buffer at 37°C.
Results for Experiment 13

Stability of p-Nitrophenyl Acetate in Rat Plasma and 20% Rat Liver Homogenate at 37°C

The rate of degradation of p-nitrophenyl acetate in these media was too fast to be monitored by HPLC, hence no rate constants could be established. Figure 3.19 (p.93) and Figure 3.20 (p.94) show the peaks obtained for chromatograms of reaction solutions after addition of p-nitrophenyl acetate in the presence and absence of the plasma or the homogenate respectively. The times given are those that elapsed between commencement of the reaction and injection onto the HPLC.
Figure 3.19: HPLC traces of p-nitrophenyl acetate in pH 7.4 phosphate buffer and in rat plasma.

Scale: 1 cm = 4.6 min
Figure 3.20: HPLC trace of the p-nitrophenyl acetate in pH 7.4 phosphate buffer and in rat liver homogenate (20%).
Results for Experiment 14.

In Vitro Stability of the Prodrug in Human Plasma (96%) at 37°C

These experiments were conducted to determine the stability of the prodrug in human plasma: \( k_{\text{obs}} = 4.37 \times 10^{-2} \pm 0.40 \times 10^{-2} \text{ min}^{-1} \), \( n = 3 \), \( t_{1/2} = 15.9 \text{ min} \).

Discussion

Calibration Curves

The high performance liquid chromatograph used to conduct the stability experiments exhibited significant background noise. Consequently the values obtained for the slopes and intercepts from the calibrations showed day-to-day variability. T-tests on the intercepts gave 'p' values which denoted a significant difference between the value zero and the intercept at a 95% confidence interval. However, the correlation coefficients obtained from these calibration curves were acceptable. Also, since almost all the injections of the samples obtained from any single study were carried out within 24 hours after calibration of the instrument, no day-to-day variability caused difficulty. Moreover, the kinetic studies exhibited first-order behavior. Therefore, it was possible to compare experiments, since absolute
concentrations of the compounds under study were not necessary to establish the pseudo-first-order rate constants. Analyses of samples from in vitro buffer and biological studies at longer wavelength $\lambda = 280$ nm were easier to perform as the instrument exhibited less baseline noise at the longer wavelength. However, higher concentrations were necessary to study the kinetics at this wavelength, as both the prodrug and 17β-estradiol exhibited lower molar absorbances at 280 nm compared to those at 225 nm.

**Stability of the Prodrug in Buffer Systems**

Since the solubility of the prodrug was very low, it became necessary to use aqueous-organic solvent mixtures to study its stability at various apparent pH values. Methanol was chosen as the solvent, as it is more 'water-like' in its behavior than other solvents (41). Thus a 50:50 v/v methanol: buffer mixture was used for the initial in vitro studies. The prodrug exhibited pseudo-first-order hydrolysis behavior at the pH values studied: 4.98, 5.88, 6.98 and 7.98; the plots obtained from these studies are shown in Figures 3.9 and 3.10. A plot (Fig. 3.11) of the logarithm of the pseudo-first-order rates obtained from these experiments versus the measured pH values at 37°C exhibited a nearly first order dependence on the hydroxide ion concentration. This was evident from the slope (1.07) obtained from the plot. This
observation was in agreement with the proposed mechanism of cleavage, based on the leaving abilities of phenols (26) as discussed in Chapter 1. The prodrug exhibited expected shorter half-lives at higher pH values, since imidomethyl prodrugs have been shown to be susceptible to specific base catalysis. For imidomethyl prodrugs of phenols using acidic imides, e.g. saccharin (26), specific base attack on the methylene group followed by loss of the phenol portion in an $S_N2$ reaction is the apparent mechanism of hydrolysis.

Methanol was initially used for the purpose of solubilizing the prodrug. It was later found to play a part in the reactivity of the prodrug. This was recognized when hydrolysis studies were performed in varying methanol concentrations. A significant decrease in the degradation rate constants with decreasing methanol concentrations was observed with one buffer system at a single pH value (Figure: 3.12). One explanation could be that $S_N2$ reactions are favored by less polar solvents than polar ones. Mixing of methanol with the aqueous buffer will result in lowering of the polarity of the medium. Systems with lower polarity allow stabilization of the transition state involved in Type 1 $S_N2$ mechanisms of cleavage (40). The transition state in this case comprises a neutral substrate (prodrug) and a charged nucleophile (OH$^-\$). As a result, faster rates should be observed in methanol-buffer systems than in buffer alone. Dioxane, on the other hand, has been shown in this research to decrease the rate of hydrolysis of the prodrug with
increasing dioxane concentration. This is evident from experiments conducted in the presence of 30% v/v and 50% v/v dioxane with pH 6.98 and 7.98 buffer. The half-lives in these media were 58 min and 150 min respectively at pH 6.98, and 19 min and 52 min at pH 7.98. Due to an increase in the hydroxide ion concentration from pH 6.98 to pH 7.98, there is an increase in the rate constants involved with the experiments at a higher pH value, as expected. But the remainder of the data suggests that dioxane is playing a role in decreasing the rate constants with increasing concentrations of dioxane. If a Type 1 $S_N2$ mechanism were to be favored by decreasing polarity, we should expect to observe faster rates of hydrolysis with dioxane also ($\varepsilon = 2.21$). This would have been the case if it was not also for the solvent structure required for these reactions. As discussed in the introduction to this Chapter, the structural integrity of water is necessary for the reaction to take place successfully. This includes hydrogen bonding of the water molecules which in turn favors the movement of the negative charge on the nucleophile towards the substrate for a subsequent attack. As studied by Schulman (44, 45), the structural integrity of water in a methanol-water system is maintained up to 0.7 mole fraction of methanol, whereas, for a dioxane-water system, it is maintained up to only 0.05 mole fraction of dioxane. Experiments conducted with increasing methanol concentrations have demonstrated increased rates with increasing methanol concentrations; a plot (Fig. 3.12)
of $k_{obs}$ versus the mole fraction of methanol resulted in a slope of approximately 0.2, and an intercept of approximately zero. If the solubility of the prodrug was not a problem, then it would have been possible to study the stability at lower methanol concentrations. In this case, a deviation from the linear plot in Fig. 3.12 would be expected, giving an intercept value significantly different from zero. The prodrug should be unstable at pH 6.98 regardless of the co-solvent.

The pH values of these mixed systems have not been studied here. However, as discussed earlier in the chapter, it does not change significantly in terms of the activity of the hydrogen ions.

**Stability of the Prodrug in Biological Systems**

**Rat liver homogenate studies**

The lability of the prodrug was studied in biological media in order to establish its stability en route to sites where the parent drug expresses its activity. As the liver is a major site for metabolism of drugs, it was necessary to determine the stability of the prodrug in the presence of the monooxygenase and other enzyme systems. Only the disappearance of the prodrug was monitored in the biological media studied, therefore the solvent system used for
chromatography did not allow the observance of any metabolite (more polar) peaks. Plots of prodrug concentration-time profiles are given in Fig. 3.16. In vitro stability studies in rat liver homogenate (20%) demonstrated an average half-life of 50 min at 37°C. The longer half-lives in this medium compared to buffer:methanol with a similar pH suggests that the prodrug is somewhat stabilized by the presence of rat liver enzymes. It may be adsorbed onto some of the homogenate components like the protein glutathione transferase absent in human plasma. Introduction of enzyme poisons such as tetraethylpyrophosphate and the use of heat treated liver homogenate demonstrated that the prodrug was even more stable under those conditions than in untreated media. This observation suggests that the prodrug may be somewhat susceptible to the monooxygenase system present in the rat liver. The prodrug was less stable in the presence of liver homogenate that had been treated with an enzyme poison than in the presence of heat treated homogenate. Precipitation of the proteins due to heat treatment might have precluded the prodrug from effectively being solubilized. However, no further experiments were conducted on this aspect so it is difficult to draw a valid conclusion on the difference between the results obtained from the two sets of experiments.
Rat plasma studies

Stability studies of the prodrug in rat plasma have demonstrated that the prodrug is less stable in rat plasma than in rat liver homogenate. The average half-life observed for these experiments at pH 7.4 was about 3.05 min \( (t_{1/2} = 13 \text{ min in 50:50, pH 6.98 buffer:methanol mixture}) \) which suggests rapid hydrolysis of the prodrug on reaching the systemic circulation. Plasma treated with TEPP at a concentration of 100 \( \mu \text{g/ml} \) at 37°C demonstrated no significant difference in the half-life (2.68 minutes) (Table 3.13). Stability studies were also conducted at a lower temperature (25°C) to slow the rates for comparison purposes (Table 3.13), and using 35 \( \mu \text{g/ml} \) of TEPP. Again it was evident that there was no significant difference between the half-lives in plasma, and in plasma treated with the enzyme inhibitor. It appears that the esterases present in plasma do not play a role in the degradation of the prodrug. When heat treated plasma was used (where the protein content was precipitated and filtered), a decrease in the rate constants was observed at 37°C \( (t_{1/2} = \sim 22 \text{ min}) \).

Bundgaard et al. (25) have shown that albumin plays a catalytic role in the hydrolysis of phenyl carbamates of amino acids. Evidence similar to the results of the present study were obtained where pretreatment of plasma at 65°C changed the half-life of the phenyl carbamate from 0.5 h (untreated plasma) to 1.7 h (heat treated plasma). The half-
life in aqueous buffer was 19 h. Relatively smaller changes were observed between untreated and heat treated plasma in the case of the phenyl carbamate prodrugs compared to the case of the 2'-saccharinylmethyl prodrug of 17β-estradiol. This could be attributed to the degree of precipitation of the plasma proteins in the two studies. A gentle heat treatment was used for the precipitation of the proteins for the plasma used for the phenylcarbamate prodrugs; whereas for the 2'-saccharinylmethyl prodrug, the plasma was placed in boiling water. Further studies on the phenylcarbamates in human plasma in the presence of an enzyme poison (physostigmine: 10\textsuperscript{-4} M), demonstrated that the rates of hydrolysis of the carbamate with/without the poison were similar. This observation is similar to what has been observed here, as shown in Table 3.13, for the prodrug of 17β-estradiol under similar conditions. However, the prodrugs studied by Bundgaard undergo a proposed ElcB mechanism while that proposed for this prodrug in SN\textsubscript{2}. Therefore, it can be suggested that albumin plays a catalytic role in both of these base-catalyzed hydrolysis mechanisms. It can be speculated that its role as a general base could be due to ionization of the carboxylates of aspartate and glutamate residues, or the imidazoles of histidine residues.

Stability studies of the prodrug in rat plasma in the presence of another protein denaturant, NaF, demonstrated similar results. No significant differences in half-lives were observed as shown in Table 3.14 for the prodrug in rat
plasma, and rat plasma containing 20 mg/ml and 40 mg/ml of sodium fluoride at 25°C. Also, no significant difference was observed in the half-lives obtained in rat plasma treated with sodium fluoride and rat plasma treated with TEPP at 25°C. This suggests that esterases present in the rat plasma do not significantly contribute to the hydrolysis of the prodrug.

In order to verify the presence of enzymatic activity in rat plasma and rat liver homogenate, a known substrate (p-nitrophenyl acetate) for these enzymes was studied under similar conditions to the prodrug studies.

Jencks (55) has reported a half-life of 37.3 h for the hydrolysis of p-nitrophenyl acetate in pH 6.15, 0.1 M phosphate buffer at 25°C. Assuming a slope of 1.0 for log $k_{obs}$ versus pH, as for specific base hydrolysis, one would obtain a half-life of 3.76 h at pH 6.98 under similar conditions.

The hydrolysis of the phenyl acetate in pH 7.4 buffer was much slower ($t_{1/2} = 7.40$ h), than in the presence of biological media. Figure 3.17 shows the spectra obtained from the buffer study, and Figure 3.18 shows the first order plot for the rate of appearance of the product, i.e. p-nitrophenol. The rate of hydrolysis of p-nitrophenyl acetate in both rat liver homogenate and plasma could not be established as the substrate degraded within seconds on exposure to the biological media. Figures 3.19 and 3.20 show HPLC traces of samples obtained within 20-40 seconds of
addition of p-nitrophenyl acetate in buffer to plasma or liver homogenate. These traces show complete hydrolysis of the ester within the stated times.

The stability of the prodrug was also studied in human plasma (~96%). The average half-life of the prodrug in this medium was longer (t½ = ~15 min) than in rat plasma (t½ = ~3 min) at 37°C. The differences in the rates could be explained on the basis of the albumin content (56) present in the rat and human plasma as discussed earlier. The albumin content in rat plasma is about 7% versus 4% in human plasma. If albumin plays a catalytic role in the hydrolysis of these compounds (25), a faster rate of hydrolysis of the prodrug would be expected in rat plasma than in human plasma.
CHAPTER 4
PHARMACODYNAMIC ACTIVITY OF A PRODRUG OF 17β-ESTRADIOL
IN COMPARISON TO 17β-ESTRADIOL

Introduction

It is a major goal of clinical pharmacology to understand the relationship between dosage of a drug and its resulting effect. The study of pharmacokinetics seeks to explain the time course of drug concentration in various body components, e.g. blood, urine or tissues. However, the time course of drug concentration cannot in itself predict the time course or magnitude of drug effect. When drug concentration at the effect site (e.g. a receptor) has reached equilibrium and the response is constant, the concentration-effect relationship represents the pharmacodynamics for that particular drug. The central concepts of pharmacodynamics are potency - reflecting the sensitivity of the organ or tissue to a drug, and efficacy - describing the maximum response. These concepts have been embodied in a simple mathematical expression, the Emax model (Eqn.4.1), which provides a practical tool for predicting drug response analogous to the compartmental model in pharmacokinetics for predicting drug concentration.

\[
E = \frac{\text{Emax} \times C}{\text{ED}_{50} + C}
\]

Eqn. 4.1
This is the simplest model which adequately describes drug effect over the whole range of concentrations, where "E" is the effect, "C" is the concentration or dose (mg/kg), "Emax" is the maximum effect attributable to the drug and "ED50" is the concentration or dose producing 50% of the Emax.

The concentration effect curve for some drugs is not represented simply by the hyperbolic form of the Emax model. This deviation was noted by Hill (57). He found empirically that his observations could be explained by the addition of an extra parameter which altered the simple hyperbolic form of Eqn. 4.1. This model is called the sigmoid Emax model and is defined in Eqn. 4.2, where N is a number influencing the slope of the curve represented by the equation. When N = 1, the usual hyperbolic Emax model results, but when N is greater than 1, the curve becomes sigmoid with a steeper slope in its central region. Thus, N quantifies the steepness of the curve.

\[
E = \frac{Emax \times C^N}{ED_{50} + C^N}
\]  

Eqn. 4.2

The model has been extensively used in other areas, for example in enzyme kinetics and protein binding (58). Its use for pharmacodynamic phenomena may be justified empirically
because it has two important properties: (a) it predicts a maximum effect for the drug to achieve; and (b) it predicts no effect when no drug is present.

One of the types of relevant concentration-response relationship occurs when the elicited response is not directly related to plasma drug concentration. The response elicited is delayed to some extent in this case and reaches its maximum when the plasma concentration of the drug is at its minimum. In the case of 17β-estradiol, the measured response is uterine ballooning in ovariectomized rats. Uterine ballooning has been defined as that condition where the uterus contains more than 100 mg of fluid (59). The responses to estrogen in the rat uterus can be classified into at least two independent groups which Tchernitchin (60) has suggested are mediated by independent mechanisms. Each of these groups can be selectively blocked, stimulated and inhibited without interfering with the other group of responses to estrogen.

The first group of responses to estrogen includes increase in uterine RNA and protein synthesis, increases in the content of some specific uterine enzymes, and the morphological and functional differentiation of target cells. These responses are considered to be genomic responses to estrogen, since they are blocked by inhibitors of RNA and protein synthesis.

The second group of responses to estrogen includes estrogen-induced uterine eosinophilia, edema, increased
vascular permeability, release of histamine and uterine luminal fluid accumulation. It has been suggested that these responses are mediated by the uterine eosinophil leukocytes which migrate from the blood to the uterus under estrogen stimulation (61). These responses are considered to be non-genomic responses to estrogen. This is based on the fact that estrogen-induced migration to the uterus, as well as the eosinophil-mediated responses are not blocked by inhibitors of RNA and protein synthesis. According to the mechanism proposed by Tchernitchin, the eosinophils that migrate to the uterine stroma under the effect of estrogen stimulation degranulate and release their peroxidasosomes (specific granules) and dense granules into the uterine intercellular substance. These granules contain collagenase, β-glucuronidase, cathepsin, arylsulphatase, peroxidase, the major basic protein, other cationic proteins and other agents. Some of these enzymes depolymerize the mucopolysaccharides and collagen from uterine intercellular substance, which in turn osmotically increase uterine water content (i.e., estrogen-induced edema).

Estradiol administration causes an increase in two specific binding components in uterine nuclei of mature ovariectomized rats. One of these sites (type I) represents the estrogen receptor which binds estradiol with high affinity (dissociation constant, ~1nM) and low capacity (~1 pmol/uterus) and is translocated from the cytoplasm to the nucleus. The second component (type II) binds estradiol with
a higher capacity than type I sites and displays a saturation curve which is sigmoidal. Hence, no accurate estimation of the dissociation can be made. Figure 4.1 (estrogen receptors) shows the events involved in protein and RNA synthesis. The differential stimulation of type II sites by estradiol suggests that this is a specific estrogenic response and highly correlated with uterine growth. A single injection of estradiol results in long term retention of type I sites (>6 h), rapid and sustained elevations of type II sites (1 - 72 h), and true uterine growth. Studies conducted by Markaverich et al. (62) suggested that estrogen stimulation of true uterine growth may require long term (6 - 24 h) nuclear retention of type I sites and sustained elevation of type II sites. These elevations in nuclear type II sites persist (1 - 72 h) long after the disappearance of type I sites (24 h) from the nucleus.

Figure: 4.1 Estrogen receptors: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; ECF, extracellular fluid; ERC, estrogen receptor complex. Estrogen bound to steroid binding globulin diffuses into the target cell and binds to the receptor to form an ERC, which in turn activates DNA leading to RNA and protein synthesis (63).

Use of the ovariectomized rat uterus as an end-point for
estrogen assay was suggested by Bülbring and Burn (64). The state of estrous can be induced in ovariectomized, sexually mature female rats by administration of estrogenic substances. The approximate onset and duration of events in the normal rat estrous cycle are shown in Fig. 4.2. A small quantity of luteinizing hormone (LH) is secreted during the late morning of day 3 which causes estrogen secretion. Luteolysis of the most recent crop of corpora lutea takes place at this time also, probably due to LH secretion. The estrogen is responsible for the uterine ballooning and vaginal changes, the mating behavior, and luteinizing hormone-follicle-stimulating hormone (LH-FSH) release on the following two days. The surge of LH necessary for triggering ovulation is released after 14 hours on day 4. This LH release is closely tied to the time of day as defined by the light-dark cycle. There is a daily facilitation of this mechanism, but release of LH only occurs on one day of the cycle, which is the day estrogen levels are high. Estrogen levels also promote FSH secretion and possibly prolactin. The LH surge, in addition to causing ovulation, also terminates the ongoing estrogen secretion and starts progesterone secretion by the ovary. As a result of the lowering of estrogen secretion and its negative feedback on LH and FSH, the LH and FSH secretion levels are allowed to begin increasing, thus initiating the next crop of follicles. Figure 4.3 depicts the cyclic control of ovarian function.
Figure 4.2: Rat estrous cycle. LH(t), tonic release of luteinizing hormone; LTX, luteolysis; Estrogen, estrogen secretion from the ovary; UTER.BALL., uterine accumulation of ovulatory intraluminal fluid; LH(s), ovulatory surge of LH; FSH, ovulatory surge of FSH; PROL., prolactin day of proestrous; PROG., progesterone secretion after critical period; M.B., mating behavior; VAG.CORN., vaginal cornification; 0, ovulation (65).
Figure 4.3: (left) Early follicular phase. Immature follicles secrete small amount of E\(_2\) (Estradiol) which inhibits LH and FSH secretion to tonic levels. The negative feedback mechanism of E\(_2\) is exerted at the level of the pituitary and the hypothalamus. (middle) Time of LH surge. The growing follicles and the dominant follicle secrete larger amounts of E\(_2\). When levels of E\(_2\) exceed a certain concentration, the negative feedback loop is interrupted, and positive feedback takes effect, inducing the preovulatory LH and FSH surges. (right) Luteal phase. High levels of P (progesterone) inhibit positive feedback of E\(_2\). P also inhibits follicular development. When luteolysis occurs and the corpus luteum regresses, the inhibitory action of P is removed and a new cohort of follicles begin to grow, and LH and FSH concentrations increase (66).

The 4 day cycle of the rat includes the stages: diestrous, proestrous, estrous and metestrus; whereas the 5
day cycle of the rat includes the stages: proestrous, estrous, metestrous, diestrous and second day of diestrous. The 5 day cycle is similar to the 4 day cycle, except that the LH and estrogen secretion both appear to be delayed by 24 hours.

Plasma LH levels in female rats are significantly elevated for 17 days following ovariectomy from basal levels of $8 \pm 1$ ng/ml to $246 \pm 56$ ng/ml; also, plasma FSH levels are ~10-fold higher than those observed in intact diestrous rats ($2148 \pm 235$ vs $201 \pm 7$ ng/ml) (67).

In ovariectomized rats, preovulatory LH-like surges occur when single (68) or multiple injections (69) of estrogen are administered. Similarly, ovariectomized rats that have received Silastic capsules containing $17\beta$-estradiol exhibit daily afternoon surges for as long as 10 days (70). Although these Silastic implants produce plasma estradiol levels which are within normal physiological ranges for proestrous rats (75 - 90 pg/ml), as little as 12 pg/ml of plasma $17\beta$-estradiol will exert a positive feedback action on LH release in gonadectomized female rats (71).

The title compound, i.e., the prodrug of $17\beta$-estradiol, was compared with $17\beta$-estradiol in terms of pharmacodynamic activity based on the above discussion. Ovariectomized Sprague-Dawley rats were employed in experiments conducted to evaluate the response elicited by oral doses of both compounds in terms of pharmacodynamic activity, i.e., increase in uterine weight, decrease in LH and FSH levels and
response elicited by both the compounds, on intravenous administration. One purpose of these experiments was to determine if cleavage of the prodrug to the drug had occurred in vivo.

**Materials**

**Chemicals**

17β-estradiol (1,3,5 (10)-estratriene-3, 17β-estradiol (Sigma), MW: 272.4; prodrug of 17β-estradiol as described in Chapter 2; 1,2-Propanediol (Aldrich Chemical Company, Inc., Milwaukee, WI), MW: 76.1, purity: 99%, LD$_{50}$ orally in rats: 30g/kg; Methoxyflurane (2,2-dichloro-1,1-difluoroethyl methyl ether) (Pitman-Moore Inc., Mundelein, IL), inhalation anesthetic for veterinary use only.

**Miscellaneous**

**Surgical**

All surgical instruments were obtained from Roboz Surgical Instrument company., Inc., 1000 Connecticut Avenue, N.W., Washington, D.C. 20036; Black braided silk non-absorbable suture, non-sterile, U.S.P. (Deknatel, Fall River,
MA, division of Pfizer Hospital Products Group, Inc.).

Animals

Female Sprague-Dawley rats obtained from Charles River, Wilmington, MA, weighing 250-300 g were used throughout this study. They were housed 2 per cage with free access to standard laboratory chow and drinking water under conditions of constant temperature (21 ± 2°C). Food was withheld for 12 hours prior to oral administration of the drug or the prodrug. The light and dark cycles at the animal housing facility were twelve hours each (light: 6.30 a.m.-6.30 p.m.).

Surgery

Animals were bilaterally ovariectomized under mild methoxyflurane anesthesia within 3 days upon arrival. They were housed under the above mentioned conditions for 15 days after ovariectomy.

Data Fitting

The dose-response data was fitted to the Hill equation (Eqn. 4.2) using non-linear curve fitting software; MINSQ
The Akaike Information Criterion (AIC) makes use of the Akaike Information Criterion (AIC) (72, 73). The AIC attempts to represent the "information content" of a given set of parameter estimates by relating the coefficient of determination to the number of parameters (or to the number of degrees of freedom) that were required to obtain the fit. The AIC is dependent on the magnitude of the data points as well as the number of observations. The AIC is defined by the formula (Eqn. 4.3):

\[
AIC = n \times \ln \left( \sum_{i=1}^{n} w_i \left( Y_{obs_i} - Y_{cal_i} \right)^2 \right) + 2p \quad \text{Eqn. 4.3}
\]

where:
- \( n \) = number of observations
- \( w = \) weight defined as \( 1/(\text{obs}(I)^{WF}) \), where WF is a weighting factor. A weighting factor of zero causes all points to be weighted equally, while a weighting factor of 2 results in weights inversely proportional to the squares of the observed values.
- \( p = \) number of parameters
- \( Y_{obs_i} = \) observed values (wts); \( Y_{cal_i} = \) fitted values.

MINSQ uses a modified AIC called the Model Selection Criterion (MSC), defined by the formula (Eqn. 4.4):
\[
\text{MSC} = \ln \left( \frac{\sum_{i=1}^{n} w_i (Yobs_i - \bar{Yobs})^2}{\sum_{i=1}^{n} w_i (Yobs_i - Ycal_i)^2} \right) - \frac{2p}{n} \quad \text{Eqn. 4.4}
\]

\[
\bar{Yobs} = \text{average of observed values}
\]

The MSC will give the same rankings between models as the AIC, but it has been normalized so that it is independent of the scaling of the data points. Furthermore, the most appropriate model will be that with the largest MSC (because we want to maximize "information content" of the model).

The statistical information provided by MINSQ includes:

a) the best fit estimate for each parameter;
b) the standard deviation of each estimate;
c) 95% confidence ranges for each parameter, based on the assumption of model linearity near the least squares minimum; and
d) the variance-covariance matrix and the correlation matrix. MINSQ also provides the coefficient of determination, which is a measure of the fraction of the total variance accounted for by the model.

**Experiment 1**

**Administration of parent drug and prodrug**

Oral doses of the drug and the prodrug were given in
100% 1,2-propanediol. The doses tested in this experiment were: 1, 3, 5, 7.5, 10, 15 and 30 mg/kg equivalent of 17β-estradiol for the prodrug and 1, 10, 50, 100, 200 and 400 mg/kg of 17β-estradiol.

The number of rats having similar body weights, was four, employed per dose. Not more than three doses were tested at a time. Each time, there was also a control group of rats where the number of rats ranged between 2-4. The results obtained from the control groups were later pooled.

Fifteen days after ovariectomy, oral doses were administered by gavage to overnight fasted rats. These rats were individually housed and kept from food for at least 3 hours after drug administration before they were returned to their previous conditions. The rats were sacrificed by decapitation 72 hours after dosing, the uterus from each rat was dissected, trimmed of the excess fat and weighed immediately. Results are expressed as organ weight (mg) per 100 g body weight.

**Experiment 2**

In order to determine that the responses elicited from the oral study above were not affected by incomplete cleavage of the prodrug, a study was conducted where equimolar doses of the drug and the prodrug were administered intravenously into the jugular vein of ovariectomized rats (n = 6). The
dose employed in this case was 8.0 mg/kg of 17β-estradiol and 17β-estradiol equivalent prodrug, since this was the value that elicited a 50% response (ED50) by the prodrug (Expt. 1) from a log-dose versus response plot. Seventy-two hours after dosing, each rat was sacrificed and the uterus was excised, trimmed of excess fat, and weighed immediately. Results are expressed as mg/100 g body weight.

Experiment 3

In order to determine the hormone levels, FSH and LH, blood samples from a seventy-two hour study were collected where 8 mg/kg of 17β-estradiol and 17β-estradiol equivalent prodrug were administered to ovariectomized rats (n = 5). These doses were made in propylene glycol. The blood samples were analysed for FSH and LH levels and no significant difference (p = 0.9346) was found between treatments with prodrug and drug. Therefore, a twenty-four hour study was carried out to establish FSH and LH levels in ovariectomized rats on oral administration of the drug (n = 5), the prodrug (n = 6) or the vehicle alone (propylene glycol n = 3); and to observe if there was a difference at that time interval. A dose of 8 mg/kg of 17β-estradiol and 17β-estradiol equivalent prodrug each in propylene glycol, and 0.5 ml of the vehicle as a control was administered to ovariectomized rats fifteen days after surgery. The rats were sacrificed by decapitation
twenty-four after dosing, their truncal blood collected, plasma was separated and stored at -20°C, and later analysed for the hormone levels by radioimmunoassay. For determination of LH concentrations, the LH antibody S-10 provided by the NIADDK Pituitary Hormone Distribution Program was used. The LH levels were quantitated using the LH RP-2 reference preparation. For determination of FSH concentrations, the FSH antibody S-11 provided by the NIADDK was used. The FSH levels were quantitated using the FSH RP-2 reference preparation.

Results

Experiment 1

The uterine weights obtained from this experiment were pooled together and expressed as mg/100 g body weight. The increase in uterine weight was calculated by subtracting the mean values obtained from the control rats from the mean values obtained from rats given the various doses of drug and prodrug. These differences were plotted against the log-dose values and were fitted to the sigmoid Emax model using MINSQ.

Figure 4.4 and 4.5 show the log-dose response plots obtained on oral administration of the prodrug and the drug respectively. The following are the statistical values (Table
4.1 and Table 4.2) obtained on fitting the data points.

Table 4.1 Parameters obtained for the Hill equation (Eqn. 4.2) on oral dosing of 17β-estradiol.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimated value</th>
<th>SD</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emax</td>
<td>319.48</td>
<td>38.06</td>
<td>198.34 - 440.63</td>
</tr>
<tr>
<td>N</td>
<td>2.39</td>
<td>1.21</td>
<td>-1.47 - 6.24</td>
</tr>
<tr>
<td>ED50</td>
<td>64.94</td>
<td>13.31</td>
<td>22.75 - 107.31</td>
</tr>
</tbody>
</table>

Table 4.2: Parameters obtained for the Hill equation (Eqn. 4.2) on oral dosing of the prodrug of 17β-estradiol.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimated value</th>
<th>SD</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emax</td>
<td>354.13</td>
<td>25.51</td>
<td>283.31 - 424.94</td>
</tr>
<tr>
<td>N</td>
<td>4.01</td>
<td>1.07</td>
<td>1.04 - 6.98</td>
</tr>
<tr>
<td>ED50</td>
<td>7.80</td>
<td>0.58</td>
<td>6.19 - 9.41</td>
</tr>
</tbody>
</table>
Figure 4.4: Log dose response curve obtained on oral dosing with the prodrug of 17β-estradiol (1 - 30 mg/kg estradiol equivalent prodrug). Filled circles represent the observed values; line indicates the fitted curve obtained with MINSQ.
Figure 4.5: Log dose response curve obtained on oral dosing with 17β-estradiol (1 - 400 mg/kg estradiol). Filled circles represent the observed values; line indicates the fitted curve obtained with MINSQ.
Experiment 2

The mean values of the uterine weights obtained on intravenous dosing of ovariectomized rats with equimolar amounts of the drug and the prodrug, i.e., 8.0 mg/kg, were expressed as mg/100 g body weight. Table 4.3 shows the numerical values obtained from this experiment and the average values are plotted as shown in Fig. 4.6. A one way analysis of variance (confidence level: 95%) was carried out on the values and the statistical output data are as shown in Table 4.4.

Table 4.3. Numerical values obtained on intravenous dosing of equimolar amounts of drug and prodrug.

<table>
<thead>
<tr>
<th>Dose: 8.0 (mg/kg)</th>
<th>Uterine weights (mg/100 g body weight)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equivalent prodruq</td>
<td>480.62</td>
<td>464.35 ± 74.03</td>
</tr>
<tr>
<td></td>
<td>534.81</td>
<td></td>
</tr>
<tr>
<td></td>
<td>531.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>372.67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>402.50</td>
<td></td>
</tr>
<tr>
<td>17β-estradiol</td>
<td>347.37</td>
<td>503 ± 92.61</td>
</tr>
<tr>
<td></td>
<td>612.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>509.41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>579.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>498.78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>475.96</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.3--continued

<table>
<thead>
<tr>
<th>Controls</th>
<th>161.13</th>
<th>159 ± 2.54</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>157.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>162.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>157.14</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4: A one way analysis of variance for the uterine weights obtained on intravenous dosing of 8.0 mg/kg of 17β-estradiol and estradiol equivalent prodrug.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>F-ratio</th>
<th>Sig. level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>4276.94</td>
<td>1</td>
<td>4276.94</td>
<td>0.594</td>
<td>0.4686</td>
</tr>
<tr>
<td>Error</td>
<td>64799.83</td>
<td>9</td>
<td>7199.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total corrected</td>
<td>69076.77</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Experiment 3

The FSH and LH levels obtained after twenty-four hours on oral dosing at the ED50 value of the prodrug, i.e., 8.0 mg/kg of estradiol and estradiol equivalent prodrug, were expressed as ng/ml and plotted as shown in Fig. 4.7. The
values obtained from this experiment were subjected to a one way analysis of variance to determine significant differences. Tables 4.5 and 4.6 show the statistical output obtained. The percentage binding for the LH and FSH assays were 46.69% and 44.86%, respectively; the coefficient of variation for the LH and FSH assays were 4.42% and 3.17%, respectively.

Figure 4.6: Mean increase in uterine weights obtained on intravenous administration of 8.0 mg/kg of 17β-estradiol and estradiol equivalent prodrug.
Figure 4.7  Follicular stimulating hormone (FSH) and luteinizing hormone (LH) levels obtained on oral administration of 8.0 mg/kg of 17β-estradiol and estradiol equivalent prodrug.
Table 4.5: A one way analysis of variance for the FSH values obtained on oral dosing of 8.0 mg/kg of estradiol equivalent prodrug.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>F-ratio</th>
<th>Sig. level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between drug and prodrug</td>
<td>9.27</td>
<td>1</td>
<td>9.27</td>
<td>8.26</td>
<td>0.184</td>
</tr>
<tr>
<td>Error</td>
<td>10.1</td>
<td>9</td>
<td>1.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total corrected</td>
<td>19.37</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between prodrug and controls</td>
<td>18.1</td>
<td>1</td>
<td>18.1</td>
<td>27.30</td>
<td>0.0012</td>
</tr>
<tr>
<td>Error</td>
<td>4.64</td>
<td>7</td>
<td>0.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total corrected</td>
<td>22.74</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between drug and controls</td>
<td>2.54</td>
<td>1</td>
<td>2.54</td>
<td>1.303</td>
<td>0.2971</td>
</tr>
<tr>
<td>Error</td>
<td>11.70</td>
<td>6</td>
<td>1.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total corrected</td>
<td>14.25</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.6: A one way analysis of variance for the LH values obtained on oral dosing of 8.0 mg/kg of estradiol equivalent prodrug.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>F-ratio</th>
<th>Sig. level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between drug and prodrug</td>
<td>1.53</td>
<td>1</td>
<td>1.53</td>
<td>11.54</td>
<td>0.0079</td>
</tr>
<tr>
<td>Error</td>
<td>1.19</td>
<td>9</td>
<td>0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total corrected</td>
<td>2.72</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between prodrug and controls</td>
<td>0.55</td>
<td>1</td>
<td>0.55</td>
<td>6.54</td>
<td>0.0377</td>
</tr>
<tr>
<td>Error</td>
<td>0.59</td>
<td>7</td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total corrected</td>
<td>1.144</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between drug and controls</td>
<td>0.09</td>
<td>1</td>
<td>0.09</td>
<td>0.399</td>
<td>0.5574</td>
</tr>
<tr>
<td>Error</td>
<td>1.42</td>
<td>6</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total corrected</td>
<td>1.51</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Discussion

The results of the present pharmacodynamic studies in rats indicate that the prodrug is approximately 8 times as potent as 17β-estradiol on oral administration to ovariectomized rats (Figs. 4.4 and 4.5). This is evident from the ED50 values estimated from the Hill equation. Tables 4.1 and 4.2 show the respective parameters obtained for drug and prodrug on fitting the data.

Since no previous reference is available on the responses observed after seventy-two hours from orally administered estradiol, the accuracy of the bioassay cannot be commented upon. As for the precision of the assay, the maximum percentage of coefficient of variation observed for the prodrug was 23% (10 mg/kg estradiol equivalent prodrug) and the minimum was 7% (30 mg/kg estradiol equivalent prodrug); for 17β-estradiol, the maximum coefficient of variation observed was 18% (50 mg/kg) and the minimum value observed was 9% (200 mg/kg). Lower coefficient of variation values are observed at higher doses probably due to saturation of the response; whereas, maximum variation is observed at the steeper part of the curve where the change in uterine weight is highly responsive to the change in the dose. Increasing the number of animals (n) in the experiment would probably improve the statistics of the observations. The standard error about the mean (for the increase in uterine weight) for the prodrug was relatively greater than
that for the drug. The amount of estradiol administered as parent drug was much greater than the amount administered as prodrug, but the responses achieved on administering estradiol via the prodrug were relatively higher for comparable doses, which suggests that the prodrug is more efficacious in delivering the drug. The log dose response plots show values which do not fall onto the fitted line. These are not outliers (±4 standard deviations), and have been taken into consideration during the fitting by MINSQ.

The increase in oral potency of the prodrug compared to the parent drug can be attributed to the prodrug being able to protect the drug from hepatic first pass effect, or presystemic metabolism. This would allow more 17β-estradiol to get into the circulation and lesser amounts converted to water-soluble glucuronide and sulfate conjugates which are readily excreted.

A statistically insignificant difference (p = 0.4686) in the pharmacodynamic activity was observed for intravenous administration of the drug and the prodrug in equimolar doses, suggesting virtually complete cleavage of the prodrug to drug in the systemic circulation.

A twenty-four hour study was carried out to establish FSH and LH levels in ovariectomized rats on oral administration of the drug and the prodrug. The samples from the seventy-two hour study had been analysed for FSH and LH levels, and showed no significant difference (p = 0.9346) between treatments with prodrug and parent drug. This led to
the decision to determine the levels after twenty-four hours as well as seventy-two hours.

From the twenty-four hour study, a significant decrease occurred in the follicular stimulating hormone levels \( (p = 0.0012) \) and luteinizing hormone levels \( (p = 0.0377) \) on oral administration of the prodrug \( (8.0 \text{ mg/kg estradiol equivalent prodrug}) \) compared to the controls (Fig. 4.7). No such difference was observed on oral administration of an equimolar dose of \( 17\beta\)-estradiol \( (8.0 \text{ mg/kg}) \) for FSH \( (p = 0.2971) \) or LH \( (p = 0.5574) \) levels. Since the FSH and LH levels are inversely related to the circulating estrogen concentrations, values which are comparable to the control values suggest that the oral administration of the parent drug at a dose of \( 8.0 \text{ mg/kg} \) was not enough to induce a negative feedback action on these hormones, whereas, an equivalent oral dose of the prodrug was sufficient to induce such a negative feedback action on these hormones. Another explanation for this observation could be that the prodrug was able to sustain an effect up to twenty-four hours by slowly releasing estradiol. If sampling for the FSH and LH levels had been carried out at time points earlier than twenty-four hours, probably no significant difference between the effects exerted by the drug and the prodrug on the hormone levels would have been observed. Further discussion of the bioavailability of \( 17\beta\)-estradiol from oral doses of the prodrug will be based on pharmacokinetic measurements (Chapter 5).
CHAPTER 5
ORAL BIOAVAILABILITY STUDIES OF A PRODRUG OF 17β-ESTRADIOL: A COMPARISON WITH 17β-ESTRADIOL

Introduction

Many pharmacokinetic studies are concerned with the bioavailability of the drug. Bioavailability, in simple terms, refers to the rate and extent of drug absorption. The rate at which a drug reaches the systemic circulation is an important consideration for drugs used to treat acute conditions, whereas, the extent of absorption is usually the more important factor for drugs that are administered repetitively for the treatment of subchronic or chronic conditions. The average drug concentration in plasma at steady-state during repetitive administration is directly proportional to the amount absorbed from each dose but is independent of the rate of absorption. Pharmacokinetic theory is well developed and generally well accepted for the determination of the extent, or relative extent, of absorption of a drug from a dosage form. The amount of drug reaching the systemic circulation after oral administration is often less than the administered dose. There are many reasons for this, one being presystemic metabolism. Drugs that
are subjected to chemical and enzymatic degradation before reaching the systemic circulation often display this phenomenon; this may occur in the gut wall, gut lumen, or in the liver during the first pass. Estimation of absolute availability after oral administration almost always requires comparison with data obtained after intravenous administration. Almost all bioavailability studies are concerned with the systemic availability or relative availability of a drug after oral administration. Availability is also a consideration after intravenous administration of a chemical derivative of a drug (a prodrug) that is intended to produce the drug itself in the body. If the prodrug is both converted to the drug and eliminated by other routes before its conversion, the availability of the drug is less than complete.

Bioavailability

Systemic availability may be estimated based on drug concentrations in plasma. The most commonly used method for estimating availability is the comparison of the total area under the drug concentration in plasma versus time curve, AUC, after (a) oral administration of the test formulation and after (b) intravenous administration of a standard.
Area under the curve

The area under the concentration-time curve (AUC) is defined as follows:

\[ \text{AUC} = \int_{0}^{\infty} C \, dt \quad \text{Eqn. 5.1} \]

where: \( C \) is the concentration

In the usual single-dose pharmacokinetic study, blood sampling is stopped at some time \( t' \) when drug concentration, \( C_p \), is measurable. Hence, estimation of the area under the blood level-time curve from zero time to infinity, \( \text{AUC}_\infty \), must be carried out in two steps. The area under the curve from zero time to \( t' \) is calculated by means of the trapezoidal rule (74). To this partial area one must add the area under the curve from \( t' \) to infinity, which is usually estimated as follows:

\[ \int_{t'}^{\infty} C \, dt = \frac{C_p}{\lambda_n} \quad \text{Eqn. 5.2} \]

where \( \lambda_n \) is 2.303 times the slope of the terminal exponential phase of a plot of log drug concentration versus time. The sum of the two partial areas is \( \text{AUC} \).

In referring to the availability of a drug after oral administration, the term systemic availability (F) is used.
Usually, the area under the curve obtained from oral administration of the drug is compared with that obtained on its intravenous administration; 100% availability is expected when the drug is directly introduced into the systemic circulation. Therefore, the term 'F' is defined as follows when equal doses are given orally and intravenously:

\[
F = \frac{\left( \int_0^\infty C \, dt \right)_{\text{oral}}}{(\int_0^\infty C \, dt)_{\text{i.v.}}} = \frac{\text{AUC}_{\text{oral}}}{\text{AUC}_{\text{i.v.}}}
\]  
Eqn. 5.3

When different doses (D) are administered orally and intravenously, then:

\[
F = \frac{D_{\text{i.v.}} \times \text{AUC}_{\text{oral}}}{D_{\text{oral}} \times \text{AUC}_{\text{i.v.}}}
\]  
Eqn. 5.4

Pharmacokinetics of estrogens in humans (ethinylestradiol)

Oral contraceptive steroids are known to be well absorbed in humans although bioavailability varies from drug to drug. For example, levonorgestrel is completely bioavailable, norethisterone has an average bioavailability of 70%, and ethinylestradiol, too, is subject to presystemic metabolism so that a mean bioavailability of 40-45% is observed. The absolute bioavailability of ethinylestradiol
was studied by Back et al. (75) and Hümple et al. (76). The two groups administered the drug by both oral and intravenous routes in 100 mg and 50 mg doses, respectively. The bioavailability of the oral preparations, relative to the intravenous one, was an average of 40% although there were considerable interindividual variations (between 20 and 65%). Ethinylestradiol clearly undergoes first pass elimination in humans, as well as in various animal species [e.g., dogs, Hirai et al. (77) and monkeys, Back et al. (78)]. The main site of presystemic metabolism is the gut wall, with the production of ethinylestradiol sulfate. Ethinylestradiol in the liver is mainly hydroxylated at the 2-position; but a wide variety of hydroxylated and methylated metabolites are formed and these are present both free and as glucuronide and sulfate conjugates. Ethinylestradiol can be conjugated directly at the 3-position and thus is liable to enterohepatic recirculation. Ethinylestradiol sulfate concentrations in plasma are many times higher than that of the unchanged drug. During long term treatment with oral contraceptives, steady-state plasma concentrations of ethinylestradiol (24 hours after administration) are between 100 and 200 pg/ml. These levels essentially exert a positive feedback on the production of the pituitary hormones - FSH and LH. The terminal half-life of ethinylestradiol in humans varies from 6-20 hours, and the apparent volume of distribution of these contraceptive steroids on intravenous administration varies between 1.5 - 4.3 l/kg. All
contraceptive steroids are known to bind to plasma albumin. Ethinylestradiol is bound to plasma albumin (97 - 98%) and is known to cause induction of the sex hormone binding globulin (SHBG).

Comparative pharmacokinetics of estradiol versus conjugated estrogens on oral administration in humans.

The main difference observed between administration of free estrone or estradiol and conjugated estrogens was a much more protracted influx of estrogens from the intestine into the plasma compartment, with a tendency to give more sustained plasma levels, if conjugated estrogens were administered (79). There was a discontinuity seen in plasma estrogen levels 10-12 hours after oral ingestion of all the preparations examined indicating enterohepatic circulation.

Conjugated estrogens contain not less than 50% and not more than 65% of sodium estrone sulfate, and not less than 20% and not more than 35% of sodium equilin sulfate, calculated on the basis of the total conjugated estrogens content (USP).

The plasma kinetics of different conjugates after oral administration of estradiol and estrone compared with those of Premarin (conjugated equine estrogens, Ayerst Labs. N.Y.) were analyzed by Schindler et al. (79). They showed that there was an increase in the metabolism of sulfoconjugated estrone to estradiol after oral administration of the
conjugate when compared to oral administration of estrone. In their study, four preparations were studied; namely estradiol, estrone, estrone sulfate and conjugated estrogens which consist primarily of estrone-3-sulfate. The study was conducted on twelve female subjects with normal menstrual cycles. The data showed a relatively protracted rise of the conjugated plasma estrone fraction occurred when estrone sulfate and conjugated estrogens were administered compared to when free estrone and the estradiol were administered. One of the possible explanations for the differences observed in the plasma levels could be the difference in the velocity of the intestinal absorption between the free and the sulfoconjugated estrogens. Another possible explanation for the protracted influx of conjugated estrogens compared to free estrogens could be the steps involved in the transhepatic transport of the free and the conjugated estrogens (80). Since both the estrogens are recovered to a similar extent in the bile, one could suggest that differences in protein binding and distribution play a minor role in the hepatic availability of these compounds. But the uptake of free estrogens is considerably different from the uptake of estrone sulfate. While the former is taken up in less than 15 seconds, the latter is water soluble and the uptake proceeds for over 5 minutes. Isolated liver cells were used in order to characterize the kinetics of the steps involved in the biliary elimination. If estrone sulfate was added to a suspension of isolated liver cells, it was taken
up by the cells over a period of about 5 minutes with intracellular accumulation of the sulfate, accompanied by a decrease of the solution sulfate fraction. On the other hand, if estrone was added to a suspension of isolated liver cells, some 40% of the compound was taken up within 15 seconds. The results suggest that uptake of estrone sulfate is active and can proceed against a concentration gradient; the transmembrane transport of the charged molecule is slow in both directions. In contrast, uptake of unconjugated estrogens, as well as release, should proceed rapidly via passive diffusion through the membrane. Hence, in vivo, the major driving force for the uptake of estrone sulfate from the blood into the liver cells may be active transport, while in the case of free estrogens a concentration gradient due to rapid metabolism within the liver cells may be of major importance. Because uptake of estrone sulfate has been shown to follow saturation kinetics (81), uptake may be the rate limiting step if high concentrations are in contact with the cells.

Pharmacokinetics of estrogens in other species

Non-oral routes of administration of estrogens are desirable due to the extensive first-pass elimination exhibited by estrogens. Nasal absorption enhancement of 17β-estradiol by dimethyl-β-cyclodextrin in rats and rabbits was
studied by Hermens et al. (82). The cyclodextrin was used as a solubilizer as well as an absorption enhancer. That formulation demonstrated a significantly higher estradiol absorption than an estradiol suspension, in both rabbits and rats. AUC values, bioavailabilities and $t_{\text{max}}$ (time of maximum concentration) values with estradiol after intravenous and intranasal administration were calculated. In rabbits, a cyclodextrin formulation of estradiol exhibited a bioavailability of $94 \pm 41\%$, whereas, an estradiol suspension exhibited a bioavailability of $25 \pm 16\%$ compared to its intravenous administration. In rats, the cyclodextrin formulation of estradiol exhibited a bioavailability of $67 \pm 16\%$, whereas, an estradiol suspension exhibited a bioavailability of $22 \pm 5\%$ compared to its intravenous administration. The absolute bioavailability in rats (67%) was less than that in rabbits (94%). This was attributed to interspecies differences and experimental animal conditions that can affect nasal drug absorption. Moreover, the differences in bioavailability was attributed to the presence of a very active cytochrome P450-dependent drug metabolizing system in rodent olfactory epithelia.

Ester prodrugs studied by Hussain et al. (17) for improved oral 17$\beta$-estradiol bioavailability in dogs have also demonstrated an improvement in the relative bioavailability. Equimolar doses (35 mg/kg) of estradiol and prodrugs were administered to three male beagle dogs. A 17-fold increase in bioavailability was observed for the salicylate derivative
and a 5-fold increase was observed for the anthranilate derivative as discussed in Chapter 1. The oral study was conducted for a period of 4 hours after administration of the compounds. On oral administration, estradiol was reported to have a mean half-life of 10 min, while the salicylate and anthranilate derivatives were reported to have half-lives of 36 min and 27 min, respectively.

An unusual family of long chain fatty acid C-17 esters of estradiol is known to be synthesized by various tissues of several species, including humans (83). These compounds are known to produce prolonged estrogenic responses in vivo. A single i.v. injection of a representative ester, estradiol stearate, to immature rats is known to produce a prolonged uterotropic effect as well as a significant advancement of puberty (84). When intact, these compounds do not bind to the estrogen receptor, thus, they are not inherently estrogenic. Their estrogenic potency is the result of a sustained release of free estradiol to the target tissues. A study was conducted by Larner et al. (85) to determine the underlying mechanisms involved in the extended action of these esters. Specifically, was the unusual property a result of decreased clearance from the blood or an increased resistance to metabolism? While both these conditions would lead to a sustained supply of estradiol, the latter would show that the hydrophobic steroid could be sequestered in tissues as its ester and could slowly release the parent steroid through the action of esterases. Radiolabelled estradiol and its stearic
and arachidonic acid 17-esters were employed in the study. The steroids were administered intravenously via the jugular vein and the data obtained from the study was fitted to a two-compartment body model. The marker for metabolism was the conversion of C-17-^3H to ^3H_2O on cleavage of the the ester residue, followed by oxidation of the C17-OH to C17=O. While the clearance of the long chain esters from blood was somewhat slower than that of estradiol (t1/2 = ~16 min versus 2 min, respectively), the rates of metabolism were dramatically different. The half-life of metabolism for the two representative esters, 17-stearate and 17-arachidonate, were found to be 580 min and 365 min respectively, while the t1/2 for estradiol was about as fast as its clearance from blood (~2 min). When the effect of chain length was studied, an inverse relationship between the size of the acyl group and the clearance from the blood was found, i.e., esters of longer chain carboxylic acids were cleared more slowly. Nevertheless, when the acyl group was lengthened from C12 to C14, the rate of clearance increased and was even faster with C18. With all the esters that were tested, the rate of metabolism decreased steadily with increasing chain length. The results obtained from this study suggested that the rate-limiting step in the rate of metabolism of estradiol esters is the hydrolysis the ester. However, another possibility that should be considered is that the various esters are partitioned to a different extent into hydrophobic compartments. Thus, the release of the esters from these
sites may control their availability to the esterases and, in this manner, explain the variable rates of hydrolysis of the esters according to the degree of unsaturation as well as chain length. Thus, the prolonged estrogenic action of the esters was attributed to the slow release of estradiol esters from a hydrophobic compartment and their subsequent rapid hydrolysis.

The pharmacokinetics of tritiated ethinylestradiol (EE) has been studied in rabbits by Back et al. (86). The study used (a) an intravenous dose of labelled EE and (b) oral dose of unlabelled EE given prior to an intravenous administration of labelled EE. The disappearance of ethinylestradiol from the blood following intravenous administration of the steroid was monitored for approximately six hours. The exponential disappearance was biphasic, the first phase having a half-life of 5 min and the second having a half-life of 69 min without a loading dose and 6 min and 190 min with a loading dose. The plasma clearance was found to be 150 ml/min without a loading dose (69 ml/min with loading dose) which approaches total hepatic blood flow and suggests that EE is a very highly cleared drug which is subject to a significant first pass effect. Data obtained from bile and plasma for unmetabolized EE and the metabolites of EE suggested that oral dosing of EE prior to intravenous administration of EE reduces the metabolism of the compound by saturating the enzymes in the liver. This was evidenced from high levels of
unmetabolized EE found in plasma and in bile after a loading dose. Moreover, the conjugate concentration was found to be higher in plasma than in bile, which suggested that the mechanism whereby conjugates are excreted into the bile appeared to be overloaded by the oral dose. It was concluded that the pharmacokinetics of EE was dose dependent and consideration of this phenomenon should be taken when multiple dosing is involved.

**Materials**

**Chemicals**

17β-estradiol (1,3,5 (10)-estratriene-3, 17β-diol) (Sigma), MW: 272.4; prodrug of 17β-estradiol as described in Chapter 2; 1,2-Propanediol (Aldrich Chemical Company, Inc., Milwaukee, WI), MW: 76.1, purity: 99%, LD50 orally in rats: 30g/kg; Heparin sodium injection (Elkins-Sinn, Inc., Cherry Hill, N. J.), : 1000 USP units/ml; Atropine sulfate injection (LyphoMed, Inc., Rosemont, IL), USP, 1 mg/ml; Pentobarbital sodium injection, USP, 20 mg/ml; Heparin Sodium 1000 units and 0.9% Sodium Chloride Injection (Baxter Healthcare Corp., Deerfield, IL); 5% Dextrose and 0.9% Sodium Chloride Injection, USP (Baxter Healthcare Corp., Deerfield, IL); Methoxyflurane (Pittman-Moore Inc., Mundelein, IL); 17β-estradiol radioimmunoassay kit: Coat-A-Count Estradiol,
(Diagnostic Product Division, Los Angeles, CA)

**Surgical**

All surgical instruments were obtained from Roboz Surgical Company., Inc., (1000 Connecticut Ave., N.W., Washington, D.C.); Black braided silk non-absorbable suture, non-sterile, USP (Denaktel, Fall River, MA); Silastic cannula (Fisher Scientific, Orlando, FL), i.d. 0.5 mm, o.d. 1.0 mm; Syringes (Monoject, Sherwood Medical, St. Louis, MO), 1.0 cc; Needles (Monoject, Sherwood Medical, St. Louis, MO), 0.9 mm x 40 mm; Microcentrifuge tubes (Fisher Scientific, Orlando, FL), 1.8 ml; Oral feeding needles (Popper and Sons, Inc., New Hyde Park, N.Y.); Micro-hematocrit capillary tubes, Hemato-Seal, tube sealing compound (Fisher Scientific, Orlando, FL).

**Instruments**

Microcentrifuge (Fisher Scientific, Orlando, FL), model 235 C; Micro-hematocrit centrifuge, Micro-hematocrit capillary reader, (Damon, IEC Division, Needham Hts, MA); Gamma Counter, model 28023 (ICN Micromedic Systems, subsidiary of ICN Biomedical Inc., Huntsville, AL 35805)
**Animals**

Female Sprague-Dawley rats obtained from Charles River, Wilmington, MA, weighing 250-300 g were used throughout this study. They were housed, 2 per cage, after ovariectomy with free access to standard laboratory chow and drinking water under conditions of constant temperature (21 ± 2°C). On the day of jugular cannulation they were housed 1 per cage. Food was withheld for 12 hours prior to oral administration of the drug or the prodrug.

**Methods**

**Surgery**

Animals were bilaterally ovariectomized within 3 days upon arrival under mild methoxyflurane anaesthesia. They were kept under the above mentioned conditions for 7 days after surgery. Then the rats were equipped with a Silastic cannula introduced into the entrance of the right atrium via the external jugular vein, according to the method described by Steffens (87). The surgery was performed under phenobarbital anaesthesia. Atropine sulfate (10 μl/100g body weight) was administered to these rats to avoid excessive secretions. During recovery for at least one week, the rats were housed individually and handled every day in order to habituate them
to the sampling procedure. The cannulae were flushed every other day with heparinized saline to prevent them from blocking before the experiment.

Administration of the steroids and blood sampling

Fifteen days after surgery and seven days after jugular cannulation, the rats were dosed with either 17β-estradiol or the prodrug. Rats were fasted overnight, however, water was accessible at all times. Oral and i.v. doses were prepared in 1,2-propanediol. The oral doses were administered with oral feeding needles, and the intravenous doses were administered into the tail vein.

Blood sampling was carried out by withdrawing 250 μl of blood via the cannula. The blood was stored in microcentrifuge tubes placed in an ice bucket. The tubes were centrifuged for two minutes at 12000 G in a microcentrifuge. The plasma contents were pipetted into microcentrifuge vials and stored at -5°C until analysis. Blood sampling was done more frequently initially than at later time points. The cannulae were flushed with heparinized saline after each sampling. The rats that were involved in the oral study were not given food for at least twelve hours after oral administration of the compound. This was done to avoid any interference of the food with the study as steroids are known to undergo enterohepatic recirculation. However, the rats
involved in the oral study were injected every two hours with 200 µl 5% dextrose and 0.9% sodium chloride USP. The hematocrit was measured after each sampling.

**Determination of 17β-Estradiol concentration**

Since the levels of the steroid present in the samples were in pg/ml, and not more than 100 µl of plasma was available for analysis, it was necessary to use a commercial radioimmunoassay (RIA) kit. The samples obtained from the prodrug study were subjected to hydrolysis before analysis. This was done by placing the samples in a water bath at 37°C for 30 minutes. Therefore, the concentration of 17β-estradiol that was determined at each time by radioimmunoassay included estradiol from prodrug that might not have hydrolysed in vivo at the time of sampling. However, in vitro rat plasma studies have demonstrated a half life for the prodrug of ~3 min which suggests that the amount of estradiol in the sample formed from the prodrug after sampling was probably minimal.

The analysis of the rat plasma samples was performed with a commercially available radioimmunoassay kit: Coat-a-Count Estradiol. The kit is highly specific for 17β-estradiol, with a percentage cross reactivity from 0.001% to 4.4 for different steroids, except for estrone which is ~10%. Red blood cells and heparin are reported not to interfere with the immunoassay. The RIA procedure is based on antibody
coated tubes. To the coated tube is added labeled estradiol and the sample whose concentration is to be determined. $^{125}I$-labeled estradiol competes with estradiol present in the samples during an incubation period (3 hours) during which binding takes place. After incubation, separation of bound labeled estradiol from free labeled estradiol is achieved by decanting the contents of the tube. The bound steroid remaining in the tube is then counted in a gamma counter for 1 minute. The counts are inversely related to the amount of estradiol present in the sample. The quantity of estradiol in the sample is determined from a standard curve generated with known concentrations of estradiol.

Data fitting

The gamma counter is equipped with a computer and data reduction software. The software (RIA Aid, Robert Marcel Associates, Inc., Arlington, MA 02174) is designed to compute a standard curve. The standard curve is generated by a plot of (net counts/maximum counts) X 100 versus log dose. The net counts are those obtained from the standards containing estradiol and the maximum counts are obtained from a blank standard where no estradiol is present. An example of the standard curve is shown in Figure 5.1. The curve is fitted to a Rodbard 4 parameter logistic model as described below in Eqn. 5.1. The model is an unweighted four parameter logistic.
\[ Y = \frac{(a - d)}{(1 + (x/c)^b)} + d \]  

Eqn. 5.1

where:

- a: Left asymptote
- b: Slope factor
- c: Inflection point
- d: Right asymptote

The Y-axis represents the ratio of counts obtained from the standard to the maximum counts obtained from 1.0 ml of the \(^{125}\text{I}\)-labeled estradiol tracer solution included in the kit. The X-axis represents the log-dose of the standards used in the calibration.

Both the standards and the samples were run in duplicate. The average number of counts/sample was used for calculation purposes. The standards that were used were those included in the commercial kit and ranged from 20 - 3600 pg/ml.

The parameters for the curve exhibited in Figure 5.1 are:

- a: \(2.29 \times 10^4\)
- b: \(8.15 \times 10^{-1}\)
- c: \(1.64 \times 10^2\)
- d: \(6.24 \times 10^2\)
- r: 0.999
Figure 5.1: Example of a standard curve generated for the RIA procedure.

**Determination of the stability of the prodrug of 17β-estradiol in rat plasma at different temperatures and in the presence and absence of enzyme poisons**

From the in vitro stability studies as discussed in Chapter 3, the prodrug was found to be unstable in rat plasma ($t_{1/2} = \sim 3 \text{ min}$). Since the bioavailability studies involved the administration of the prodrug, blood samples obtained from the rat during the study might consist of both the drug and the prodrug at that time. In order to establish the correct concentration of the estradiol at the time of sampling it was necessary to arrest the hydrolysis of the prodrug present in the samples as quickly as possible. As the
in vitro half-life of the prodrug was found to be relatively short, it was imperative to design a method which would rapidly inhibit its cleavage. Several different procedures were attempted which included the following temperatures: 0°C (ice bucket), 5°C, and 20°C (room temperature); enzyme poisons: NaF (20 mg/ml), tetraethylpyrophosphate (35 μg/ml). Only single runs were carried out for each condition studied.

Procedure

a) Stability study of the prodrug in rat plasma (96%) at 5°C and 20°C in the absence and presence of TEPP (35 μg/ml).

To two test-tubes was added 1.0 ml of plasma, 35 μl of TEPP solution (1 mg/ml in water) and 10 μl of 4-androstene-3,17-dione solution (1 mg/ml in methanol). One of the tubes was placed in the refrigerator which was at a temperature of 5°C, and the other in a water bath at 20°C. For another set of two tubes the same procedure was carried out except that TEPP was not added this time. All four of the test-tubes were spiked with 5μl of the prodrug solution (5 mg/ml in methanol), vortexed, and the time was noted. Sampling was done by pipeting 50 μl of the mixture into 2 ml of diethyl ether and vortexing the mixtures. Sampling was carried out for at least thirty minutes as that would be the critical time period during which the samples were to be left unattended during the pharmacokinetic study. Water (1 ml) was added to the test-tubes and the mixtures were vortexed. The tubes were
then centrifuged for 10 min at 3000 rpm, and the supernatants were evaporated to dryness under nitrogen. The residues were reconstituted with 1 ml of 70:30 acetonitrile: acetate buffer (pH 3.8, 0.001M) and sample were injected onto the HPLC using conditions described on p. 51 in Chapter 3.

b) Stability study of the prodrug in rat plasma (96%) at 0°C in the absence and presence of TEPP (35 μg/ml) and NaF (20 mg/ml)

To two test-tubes was added 0.5 ml of plasma and 17 μl of mestranol solution (1 mg/ml in methanol). To one of the tubes was added 17 μl of TEPP solution (1 mg/ml in water) and to the other was added 130 μl of NaF solution (100 mg/ml in water). The tubes were placed in an ice-bucket. Another set of two tubes were taken and the same procedure was carried out except that TEPP and NaF were not added this time. All four of the test-tubes were spiked with 10 μl of prodrug solution (5 mg/ml in methanol) and vortexed. The time was noted. Sampling was done periodically by withdrawing 30 μl of the mixture and adding it to tubes containing 4 ml of ice-cold acetonitrile. Sampling was carried out for a longer period of time (5 - 10 h) than in experiment a) so as to determine the stability of the prodrug if stored at 0°C. The tubes were centrifuged at 3000 rpm for 10 minutes, the supernatants were added to another set of silylated tubes and evaporated to dryness. To each of those residues was added 2
ml of diethylether and 1 ml of water. The tubes were vortexed and centrifuged at 3000 rpm for 10 minutes. The supernatants were added to another set of silylated tubes and evaporated to dryness. Those residues were reconstituted with 1 ml of 70:30 acetonitrile: acetate buffer (pH 3.8, 0.001M) and samples were injected onto the HPLC using conditions described on p. 51 in Chapter 3.

Experimental Design And Procedure: Bioavailability Studies

The bioavailability studies were carried out in ovariectomized female Sprague-Dawley rats weighing 250-300 g. Not more than three rats were involved in the study at any given time. The data were later pooled together for the determination of the oral bioavailability. Table 5.1 gives the experimental design.

The molar doses chosen for the oral study of 17β-estradiol were approximately ten times greater than those for the prodrug. The doses employed in this study were similar to those used for the pharmacodynamic study. This selection was based on the results obtained from the pharmacodynamic studies where an 8 - 10 times greater potency was demonstrated by the prodrug as compared to the drug. However, for the intravenous study, equivalent doses of drug and prodrug were used, i.e., 5 mg/kg and 10 mg/kg of 17β-estradiol or estradiol equivalent prodrug, respectively.
Table 5.1: Experimental design involving oral and intravenous administration of drug and prodrug.

<table>
<thead>
<tr>
<th>Oral dose</th>
<th>17β-estradiol mg/kg</th>
<th>Prodrug as equivalent estradiol mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td># of rats</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>i.v. dose</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td># of rats</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

The following numbering was assigned to the rats dosed under each study:

Intravenous 17β-estradiol (5 mg/kg): Rats #1, #2  
(10 mg/kg): Rats #3, #4

Oral 17β-estradiol (100 mg/kg): Rats #9, #10, #11  
(200 mg/kg): Rats #12, #13, #14

Intravenous prodrug as estradiol equivalent: (5 mg/kg): Rats #5, #6  
(10 mg/kg): Rats #7, #8

Oral prodrug as estradiol equivalent: (8 mg/kg): Rats #15, #16, #17, #18  
(15 mg/kg): Rats #19, #20, #21

Results from rats #: 2, 7, 9, 10, 17, 18, and 21 were rejected due to reasons explained under the discussion section of this chapter.
Results

Calibration curves for RIA

The analyses of the samples obtained from the studies were carried out using a radioimmunoassay procedure. Calibration curves were obtained each time an analysis was conducted. The standards that were used were those included in the commercial kit and ranged from 20 - 3600 pg/ml. Table 5.2 shows the slopes ± SD obtained from several calibration curves generated during the analysis of the samples.

Table 5.2: Slopes and mean ± SD of the slopes obtained during analysis of the estradiol samples.

<table>
<thead>
<tr>
<th>Slope values</th>
<th>Mean</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.22 x 10⁻¹</td>
<td>8.08 x 10⁻¹</td>
<td>1.6 x 10⁻²</td>
</tr>
<tr>
<td>8.04 x 10⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.81 x 10⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.18 x 10⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.16 x 10⁻¹</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results for the stability of the prodrug of 17β-estradiol in rat plasma at different temperatures and in the presence and absence of enzyme poisons

a) Stability study of the prodrug in rat plasma (96%) at 5°C and 20°C in the absence and presence of TEPP (35 μg/ml).

The prodrug was found to be relatively unstable under the above mentioned conditions. The degradation observed from the plots was found to be considerably slower at the temperatures studied (5°C and 20°C) than the rates observed at 37°C as described in chapter 3. However, no rates could be established with accuracy because only a few samples were analysed. But the experiment did establish the fact that the prodrug was not stable if stored at those temperatures.

The plots obtained from this study are shown in Figure 5.2. The sampling was not carried out for a longer period of time as was the sampling at 0°C (b). The Y-axis has been magnified in order to determine if significant hydrolysis of the prodrug was occurring during the time the study was conducted. The scatter of the data points is similar to that observed in the subsequent experiment (b), however, the plots under Figure 5.3 have a wider Y-axis range and thus the scatter of the data points is not as obvious.
Figure 5.2: Degradation of the prodrug in rat plasma under the following conditions: 1) TEPP (35 μg/ml) at 5°C; 2) TEPP (35 μg/ml) at 20°C; 3) at 5°C; 4) at 20°C
b) Stability study of the prodrug in rat plasma (96%) at 0°C in the absence and presence of TEPP (35 μg/ml) and NaF (20 mg/ml)

The stability of the prodrug at 0°C temperature was studied for a longer period of time than at 5°C and 20°C. Only single runs were carried out under any of the conditions studied. The following are the half-lives obtained at this temperature as shown in Table 5.3:

Table 5.3: Effect of TEPP and NaF on the half-life of the prodrug at 0°C.

<table>
<thead>
<tr>
<th>Medium</th>
<th>t1/2 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>220</td>
</tr>
<tr>
<td>Plasma + NaF (20 mg/ml)</td>
<td>189</td>
</tr>
<tr>
<td>Plasma + TEPP (35 μg/ml)</td>
<td>189</td>
</tr>
</tbody>
</table>

Figure 5.3 exhibits the pseudo-first-order plots obtained from this study.

From the above two experiments it was established that the degradation of the prodrug on sampling could not be arrested. Therefore, it was not possible to extract the drug and the prodrug and analyze them individually. Only the total concentration could be estimated by hydrolysing any prodrug present at 37°C before analysis.
Figure 5.3: Degradation of the prodrug in rat plasma under following conditions; 1) Plasma at 0°C; 2) NaF (20 mg/ml) at 0°C; 3) TEPP (35 μg/ml) at 0°C.

(1) \[ y = 1.15 - 3.14e^{-3x} \] 
\[ R^2 = 0.992 \] 
\[ t_{1/2} = 220 \text{ min} \]

(2) \[ y = 1.32 - 3.65e^{-3x} \] 
\[ R^2 = 0.970 \] 
\[ t_{1/2} = 189 \text{ min} \]

(3) \[ y = 0.966 - 3.66e^{-3x} \] 
\[ R^2 = 0.989 \] 
\[ t_{1/2} = 189 \text{ min} \]
Results for the Bioavailability Studies

Data Analysis of Concentrations Obtained After Intravenous Administration of 17β-estradiol and its Prodrug.

The concentration values determined on RIA analysis of the samples obtained on intravenous administration of the drug were evaluated to determine the time-course of the drug. The following data analysis was carried out:

Test of superimposability.

Two doses were employed in the study to determine whether or not a concentration dependency was involved. Figures 5.4 and 5.5 exhibit the dose normalized plots obtained on intravenous administration of the drug and the prodrug. The doses studied were 5 mg/kg and 10 mg/kg of the drug and an equimolar amount of the prodrug. The number of rats tested per dose was 2. Therefore, a total of 4 rats were involved per study of either the drug or the prodrug.

To test for linearity between doses, a plot of log (plasma concentration/dose) versus the time recorded should theoretically exhibit the 4 profiles superimposed onto each other. Such an exercise was carried out for both the drug and the prodrug and the plots are as shown in Figures 5.4 and 5.5. The x-axes have different time limits in order to magnify the time-course of the estradiol levels.
Figure 5.4: Test for superimposability for dose dependency on intravenous administration of 17β-estradiol to rats: #1, #2 (5 mg/kg), and rats: #3, #4 (10 mg/kg) plots (1) until end of study, (2) up to 500 min, (3) up to 100 min, (4) up to 20 min.
Figure 5.5: Test for superimposability for dose dependency on intravenous administration of prodrug as 17\(\beta\)-estradiol equivalent to rats: #5, #6 (5 mg/kg), and rats: #7, #8 (10 mg/kg), plots (1) until end of study, (2) up to 500 min, (3) up to 100 min, (4) up to 20 min.
The initial period in the plots, i.e., up to a 100 min and 500 min, show relatively a better superimposition than the later period for drug and prodrug, respectively. However, overall the prodrug exhibits relatively better superimposition than the drug.

**Area under the curve**

In order to determine the area under the zero moment curves for the concentration-time profiles by the trapezoidal rule (74) it was necessary to determine their terminal slopes. A regression was carried out on the last three data points to obtain the slopes. These values were then used to calculate the area under the curve remaining after $t'$ as described earlier (pp 135-136).

The terminal slopes that were determined for the intravenous studies were not determined from the same time frame since the regression obtained from some of the slopes was not acceptable. This was particularly true for the intravenous study involving estradiol. As a result, only a part of the profile was considered where such an observation was made.

Tables 5.4 and 5.5 exhibit the area under the curve and terminal slopes for drug and prodrug obtained on regression analysis with their respective standard error about the slope, means ± range.
Table 5.4

AUC\textsuperscript{∞} obtained by the trapezoidal rule from the concentration-time profiles on intravenous administration of 17β-estradiol.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Weight kg</th>
<th>Dose mg/kg</th>
<th>(k_{\text{terminal}}) (\text{min}^{-1})</th>
<th>S.E. about the slope</th>
<th>R(^2)</th>
<th>&quot;t'*&quot; for AUC(_t) min</th>
<th>AUC(_t) (\mu\text{gmin/ml})</th>
<th>AUC\textsuperscript{∞} (\mu\text{gmin/ml})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.275</td>
<td>5</td>
<td>1.05 \times 10^{-3}</td>
<td>2.05 \times 10^{-4}</td>
<td>0.963</td>
<td>1266</td>
<td>76</td>
<td>78</td>
</tr>
<tr>
<td>3</td>
<td>0.295</td>
<td>10</td>
<td>1.46 \times 10^{-3}</td>
<td>2.30 \times 10^{-4}</td>
<td>0.975</td>
<td>1561</td>
<td>308</td>
<td>309</td>
</tr>
<tr>
<td>4</td>
<td>0.315</td>
<td>10</td>
<td>3.49 \times 10^{-3}</td>
<td>7.38 \times 10^{-4}</td>
<td>0.957</td>
<td>1183</td>
<td>200</td>
<td>201</td>
</tr>
</tbody>
</table>

Slope: Mean ± SD; 2.00 \times 10^{-3} ± 1.31 \times 10^{-3}

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Weight kg</th>
<th>Dose mg/kg</th>
<th>(k_{\text{terminal}}) (\text{min}^{-1})</th>
<th>S.E. about the slope</th>
<th>R(^2)</th>
<th>&quot;t'*&quot; for AUC(_t) min</th>
<th>AUC(_t) (\mu\text{gmin/ml})</th>
<th>AUC\textsuperscript{∞} (\mu\text{gmin/ml})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.290</td>
<td>5</td>
<td>7.10 \times 10^{-4}</td>
<td>3.46 \times 10^{-4}</td>
<td>0.808</td>
<td>2935</td>
<td>65</td>
<td>66</td>
</tr>
<tr>
<td>3</td>
<td>0.295</td>
<td>10</td>
<td>6.80 \times 10^{-4}</td>
<td>2.19 \times 10^{-4}</td>
<td>0.905</td>
<td>4398</td>
<td>326</td>
<td>329</td>
</tr>
</tbody>
</table>

Slope: Mean ± Range; 6.95 \times 10^{-4} ± 1.50 \times 10^{-5}
Table 5.5

AUC∞ obtained by the trapezoidal rule from the concentration-time profiles on intravenous administration of the prodrug of 17β-estradiol.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Weight (kg)</th>
<th>Dose (mg/kg)</th>
<th>kterminal (min⁻¹)</th>
<th>S.E. about the slope</th>
<th>R²</th>
<th>&quot;t&quot; for AUCₜ min</th>
<th>AUCₜ (μg/min/ml)</th>
<th>AUC∞ (μg/min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.230</td>
<td>5</td>
<td>1.79 x 10⁻³</td>
<td>7.66 x 10⁻⁵</td>
<td>0.998</td>
<td>1272</td>
<td>67</td>
<td>69</td>
</tr>
<tr>
<td>6</td>
<td>0.265</td>
<td>5</td>
<td>1.06 x 10⁻³</td>
<td>1.27 x 10⁻⁴</td>
<td>0.985</td>
<td>1528</td>
<td>133</td>
<td>137</td>
</tr>
<tr>
<td>7</td>
<td>0.295</td>
<td>10</td>
<td>1.37 x 10⁻³</td>
<td>4.18 x 10⁻⁴</td>
<td>0.915</td>
<td>1098</td>
<td>160</td>
<td>182</td>
</tr>
<tr>
<td>8</td>
<td>0.265</td>
<td>10</td>
<td>1.42 x 10⁻³</td>
<td>9.92 x 10⁻⁵</td>
<td>0.995</td>
<td>1623</td>
<td>211</td>
<td>216</td>
</tr>
</tbody>
</table>

Slope: Mean ± SD; 1.41 x 10⁻³ ± 2.99 x 10⁻⁴
Data Analysis of Concentrations Obtained After Oral Administration of $17\beta$-estradiol.

The concentration values determined by RIA analysis of the samples obtained on oral administration of $17\beta$-estradiol were evaluated to determine the time-course of the drug. The terminal slopes were determined for the drug and used to calculate the area under the zero moment curve after time $t'$.

Relatively higher oral doses were administered compared to the intravenous doses and sampling was carried out for a similar length of time for both. The concentrations observed from oral administration of the drug were not comparable to those observed on intravenous administration at similar time points. Moreover, the oral study was not conducted for more than seventy-two hours so as to observe concentrations comparable to the intravenous study. The rates observed at similar time points for oral and intravenous doses, showed a difference of one order of magnitude. This can be seen under Tables 5.4 and 5.6, where the $t'$ value for AUC$_t$ is similar for both intravenous (rats #2 and #3) and oral doses (rats #11, #12, #13, #14), whereas the rates observed at these time points differ by one order of magnitude.

For rats #1 and #4 (intravenous $17\beta$-estradiol), considerable error was observed in the data beyond 1300 min, therefore, the data beyond this time point was rejected. In order to compare the terminal slope and the AUC$\infty$ values
Table 5.6

AUC∞ obtained by the trapezoidal rule from the concentration-time profiles on oral administration of 17β-estradiol.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Weight (kg)</th>
<th>Dose (mg/kg)</th>
<th>Kterminal (min⁻¹)</th>
<th>S.E. about the slope</th>
<th>R²</th>
<th>&quot;t&quot; for AUCₜ</th>
<th>AUCₜ (µg.min/ml)</th>
<th>AUC∞ (µg.min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>0.290</td>
<td>100</td>
<td>1.75 x 10⁻³</td>
<td>7.14 x 10⁻⁶</td>
<td>0.999</td>
<td>3042</td>
<td>148</td>
<td>149</td>
</tr>
<tr>
<td>12</td>
<td>0.275</td>
<td>200</td>
<td>1.00 x 10⁻³</td>
<td>1.75 x 10⁻⁴</td>
<td>0.999</td>
<td>2864</td>
<td>98</td>
<td>104</td>
</tr>
<tr>
<td>13</td>
<td>0.280</td>
<td>200</td>
<td>1.28 x 10⁻³</td>
<td>1.43 x 10⁻⁵</td>
<td>0.999</td>
<td>2991</td>
<td>154</td>
<td>157</td>
</tr>
<tr>
<td>14</td>
<td>0.260</td>
<td>200</td>
<td>1.84 x 10⁻³</td>
<td>3.41 x 10⁻⁵</td>
<td>0.999</td>
<td>2187</td>
<td>164</td>
<td>167</td>
</tr>
</tbody>
</table>

Slope: Mean ± SD; 1.47 x 10⁻³ ± 3.97 x 10⁻⁴
obtained from rats #1 and #4, to rat #3, data for rat #3 was considered until 1561 min as seen under Table 5.4. Rats #2 and #3 (intravenous 17β-estradiol) exhibited acceptable data until ~3000 min and ~4000 min respectively. Terminal slopes for the data from these rats (#1, #2, #3 and #4) were determined to calculate the area under the zero moment curve after time t' as listed in Table 5.4. Table 5.6 exhibits the area under the curve and terminal slopes for the drug obtained on regression with their respective standard error about the slope, means ± range.

Data Analysis of Concentrations Obtained After Oral Administration of the Prodrug of 17β-estradiol.

The concentrations determined on RIA analysis of the samples obtained on oral administration of the prodrug of 17β-estradiol were evaluated to determine the time-course of the drug.

In order to calculate the area under the zero moment curve after time t', the terminal rates obtained from the intravenous study of the prodrug were used. These rates were applied at concentrations values similar to those obtained on intravenous dosing of the prodrug. It was possible to do so in this case (when compared to the estradiol study) since similar doses for both the oral and intravenous studies were employed. This had to be done because slopes obtained from the oral study exhibited too much variability to be used to determine
the AUC∞ values.

Table 5.7 exhibits the AUC∞ values for estradiol obtained on oral dosing of the prodrug. Table 5.8 exhibits the time points where the terminal slopes were determined with the corresponding concentrations for rats #5, #6 and #8 (intravenous prodrug administered rats). Rat #7 (iv) exhibited higher concentrations at similar time points when compared to orally administered doses, therefore, its slope was not used for AUC∞ calculations.

Table 5.7: AUC∞ for estradiol obtained on oral administration of the prodrug employing kterminal values (Table 5.5) obtained on intravenous administration of the same.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Dose: mg/kg</th>
<th>'Cp' μg/ml</th>
<th>&quot;t'&quot; min</th>
<th>AUCt</th>
<th>AUC∞</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>8</td>
<td>0.0054</td>
<td>2216</td>
<td>31</td>
<td>37</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>0.0042</td>
<td>2722</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>19</td>
<td>15</td>
<td>0.0050</td>
<td>2522</td>
<td>29</td>
<td>34</td>
</tr>
<tr>
<td>20</td>
<td>15</td>
<td>0.0053</td>
<td>3287</td>
<td>24</td>
<td>29</td>
</tr>
</tbody>
</table>

Table 5.8: kterminal values obtained at concentrations (Cp) for estradiol at time (t') on intravenous administration of the prodrug.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Dose: mg/kg</th>
<th>'Cp' μg/ml</th>
<th>&quot;t'&quot; min</th>
<th>kterminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5</td>
<td>0.005</td>
<td>1272</td>
<td>1.79 x 10^{-3}</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>0.005</td>
<td>1528</td>
<td>1.06 x 10^{-3}</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>0.007</td>
<td>1623</td>
<td>1.42 x 10^{-3}</td>
</tr>
</tbody>
</table>
Oral bioavailability calculations

The oral bioavailability of the prodrug was calculated using equation 5.3 as stated earlier except for the consideration of weight of the animals. Equation 5.6 was used to calculate the absolute oral bioavailability of 17β-estradiol on oral administration of drug and prodrug.

\[
\% F = \frac{Dose_{i.v.} \times AUC_{oral} \times Rat Weight_{i.v.}}{Dose_{oral} \times AUC_{i.v.} \times Rat Weight_{oral}} \times 100
\]

Eqn. 5.6

Using the AUC∞ listed in Tables 5.7 and 5.8, the following values (Table 5.9) were obtained for the oral bioavailability of 17β-estradiol upon oral administration of the prodrug.

Table 5.9: Absolute percentage bioavailability of 17β-estradiol on oral administration of the prodrug when compared to intravenous administration of the same.

<table>
<thead>
<tr>
<th>% Bioavailability</th>
<th>Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat# (iv)</td>
<td>Rat #15</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>8</td>
<td>21</td>
</tr>
</tbody>
</table>
The objective of this research was to improve the oral bioavailability of 17\(\beta\)-estradiol by administration of the prodrug. Therefore, it was logical to calculate the oral bioavailability of the drug from the prodrug by comparing it to the data obtained from intravenous administration of 17\(\beta\)-estradiol.

In order to calculate the area under the zero moment curve time "t'", the terminal slopes obtained from the intravenous study of 17\(\beta\)-estradiol were considered. In order to use these slopes for AUC\(\infty\) calculations, the concentrations from both the data sets (oral and iv) have to be similar. For this reason the terminal slopes for rats #1, #2 and #4 (rats administered intravenous estradiol) were taken into consideration (Table 5.4: first three values) to calculate the AUC\(\infty\) for oral prodrug.

The AUC\(\infty\) values obtained upon oral administration of the prodrug of 17\(\beta\)-estradiol using slopes from data obtained on intravenous administration of 17\(\beta\)-estradiol are listed in Table 5.10.

Using the AUC\(\infty\) values listed under Table 5.4 for intravenous estradiol and 5.10 for oral prodrug, the following values (Table 5.11) were obtained for the oral bioavailability of 17\(\beta\)-estradiol from oral administration of the prodrug.
Table 5.10: AUC∞ of estradiol obtained on oral administration of the prodrug employing kterminal values obtained on intravenous administration of 17β-estradiol.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Dose: mg/kg</th>
<th>'Cp' μg/ml</th>
<th>&quot;t&quot;' min</th>
<th>AUCt</th>
<th>AUC∞</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>8</td>
<td>0.001</td>
<td>4491</td>
<td>35</td>
<td>36</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>0.003</td>
<td>4173</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>19</td>
<td>15</td>
<td>0.003</td>
<td>4296</td>
<td>29</td>
<td>31</td>
</tr>
<tr>
<td>20</td>
<td>15</td>
<td>0.005</td>
<td>4213</td>
<td>24</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 5.11: Absolute percentage bioavailability of 17β-estradiol on oral administration of the prodrug when compared to intravenous administration of 17β-estradiol.

<table>
<thead>
<tr>
<th>% Bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
</tr>
<tr>
<td>Rat# (iv)</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

The results in Tables 5.9 and 5.11 are comparable in terms of the oral bioavailability values obtained. This suggests that rapid in vivo hydrolysis of the prodrug must have occurred on intravenous administration of the prodrug which resulted in similar AUC∞ and hence comparable bioavailability values.

The oral bioavailability of 17β-estradiol obtained using
the AUC∞ values listed in Table 5.4 and 5.6 was calculated and is as listed in Table 5.12.

Table 5.12: Absolute percentage bioavailability of 17β-estradiol on oral administration of the drug when compared to intravenous administration of the same.

<table>
<thead>
<tr>
<th>% Bioavailability</th>
<th>Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat# (iv)</td>
<td>Rat #11</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

Discussion

The oral bioavailability studies conducted for the drug and the prodrug give further insight on disposition and activity of 17β-estradiol. It would have been of interest to monitor both the drug and the prodrug after oral and intravenous administration of the prodrug, but, due to lack of any practical approach to doing so, only the parent drug levels were monitored. The assumed instantaneous hydrolysis of the prodrug seemed reasonable based on its in vitro stability in rat plasma. This in effect precluded the possibility of determining if in vivo hydrolysis of the prodrug was the rate
limiting step in the observation of biological activity.

The intravenous study of the drug and the prodrug involved two rats per dose. However, due to scatter and indecipherable profiles obtained from rat #7 (10 mg/kg estradiol equivalent prodrug), the data from this rat was not considered for bioavailability calculations. For rats #2 and #3 it was feasible to determine the slopes for concentration versus time plots beyond 3000 min. Therefore the terminal slopes exhibited longer half-lives (~17 h) compared to the half-lives of approximately 6 h for the drug and 8 h for the prodrug as the data was only analyzed up to ~1500 min.

Intravenous administration of the drug and the prodrug in equimolar doses exhibited similar terminal slopes (k_{terminal}) as seen in Tables 5.4 and 5.5. However, data for the prodrug exhibited relatively better regression parameters and lower standard error about the slopes than did data for the drug. The AUC_{\infty} values obtained on intravenous administration of the drug and prodrug were comparable, within biological variability. The AUC_{\infty} ± SD was 95 ± 37 μgmin/ml for the 5 mg/kg dose and 227 ± 56 μgmin/ml for the 10 mg/kg dose.

Oral administration of 17β-estradiol exhibited comparable k_{terminal} values for rats #11, 12, 13 and 14. Rats #9 and #10 (100 mg/kg oral estradiol) were rejected as the profiles observed from these studies did not demonstrate a decline in the terminal phase, but rather an increase. It appeared that there was still an input of estradiol into the blood circulation even after 1500 min. This could be due to the
enterohepatic recirculation often observed with steroids which makes interpretation of the data difficult. AUC∞ values calculated for the oral estradiol (rats #11, #12, #13 and #14) time-concentration profiles, demonstrated acceptable regression of the terminal slopes. Therefore these slopes were considered for the AUC∞ calculations. The AUC∞ values obtained upon oral administration of estradiol are as shown in Table 5.6. The AUC∞ was 149 µgmin/ml for the 100 mg/kg dose and AUC∞ ± SD was 143 ± 34 µgmin/ml for the 200 mg/kg dose. A significant scatter in the terminal slopes was observed when the prodrug was given orally. Data from rats #17, #18 (8 mg/kg) and #21 (15 mg/kg) were rejected from oral bioavailability considerations as it was not feasible to decipher the concentration-time profiles. The profiles exhibited a sharp rise in concentration reaching a maximum within the first 10 min, followed by a decrease in the concentration and then a subsequent rise. The terminal phase of concentration decrease had still not been reached when the sampling was discontinued (seventy-two hours). Therefore, it was not practical to calculate the area under the curve from such profiles. The rest of the rats demonstrated reasonable profiles so their results were used to calculate the oral bioavailability. However, considerable scatter was observed in the terminal slopes. Therefore, the profiles obtained on intravenous administration of the prodrug were used to calculate the last part of the AUC∞. In order to do so, the concentrations from both intravenous and oral prodrug studies
were compared to establish similarity. Once established, \( C_p \) from the oral study was divided by the \( k_{\text{terminal}} \) obtained from the intravenous study. Tables 5.7 and 5.8 exhibit the concentrations for the oral and intravenous studies. The last '\( C_p \)' value from the oral study will in effect decline with the same rate as that for the last '\( C_p \)' value for intravenous study for which the \( k_{\text{terminal}} \) values are established. Table 5.7 exhibits the \( \text{AUC}_{\infty} \) values for oral administration of the prodrug obtained by this method.

Similarly, \( \text{AUC}_{\infty} \) values were calculated using the terminal slopes obtained on intravenous administration of the prodrug. However, no significant difference in \( \text{AUC}_{\infty} \) values was observed as seen from Table 5.7 and 5.10. This suggests that upon intravenous administration of the drug or the prodrug, hydrolysis of the prodrug is not the rate limiting step. If this had been the case, then we would have observed significant differences in the \( \text{AUC}_{\infty} \) values obtained from intravenous administration of the drug and the prodrug, which would then have been reflected in the bioavailability calculations.

Oral bioavailability calculations employing Eqn. 5.6 yielded values exhibited in Tables 5.9, 5.11 and 5.12. Individual rats involved in the intravenous study were compared to individual rats in the oral study to yield multiple values for '%F'. The mean %F was found to be ~4% and ~16% for estradiol when delivered as drug and prodrug, respectively. This suggests a 4-fold increase in
bioavailability of 17β-estradiol when the prodrug is administered orally. However, further experiments are required to establish this ratio accurately. A greater increase could have been observed if a more water-soluble prodrug was made. The highly lipophilic nature of the 2′-saccharinylmethyl prodrug would render it poorly soluble in gastro-intestinal fluids, leading to slow dissolution after precipitation from the 1,2-propanediol vehicle. This could be one explanation for the observation of an increase in blood levels on oral administration in some of the rats, suggesting a steady zero order input that lead to a plateau in the concentration versus time profile.
The oral route is the most common and convenient route for administration of drugs. The drugs chosen for therapy are normally cleared from the body after the desired therapeutic effect is achieved. However, some drugs are prematurely eliminated from the body, due to their rapid metabolism and therefore are unable exert their maximum therapeutic effect. Drugs possessing phenolic moieties are one such example. Chemical modifications of the structure of the drug molecule to protect it from rapid metabolism might compromise the activity of the drug. A better alternative would be to chemically protect the site of metabolism, but only transiently. Such a prodrug approach could temporarily protect the site of metabolism until the drug reached the desired site of action where it would ideally revert to the parent molecule. The promoiety used to protect the site of metabolism preferably should not exert any pharmacological effect itself.

The test compound chosen in this research was 17β-estradiol which is known to undergo rapid conjugation to form water soluble products and be rapidly eliminated from the body before exerting its desired therapeutic effect. Its
reported oral bioavailability in human female volunteers is about 11% (4). However, it is a compound that is produced by the body, which makes it a desirable molecule for therapy. Prodrugs of estradiol have been investigated in the past. The design of prodrugs requires the introduction of relative instability into the prodrug so that it releases the drug molecule in a desired time frame. One design approach takes advantage of enzymes to release the drug from the prodrug. Unfortunately, these enzymes, except for a very few, are ubiquitous. Therefore, a challenge to the design of prodrugs that are enzymatically labile is designing the structure of the prodrug molecule so as to make it a poor substrate for the enzymes until it reaches its desired site of action. A second design approach takes advantage of a chemical mechanism of hydrolysis to release the drug from the prodrug. This approach also eliminates the possibility of intersubject variability that may affect enzymatic regeneration of the drug from the prodrug.

The major site of metabolism of 17β-estradiol is the 3-OH group. In this research it has been protected by a saccharinyl pro moiety attached to the 3-OH through a methylene ether linkage - an imidomethyl type of prodrug. The prodrug was designed based on past research where it was observed that a large number of N-hydroxymethyl derivatives of imides demonstrated a first-order dependence on the hydroxide ion concentration and on the acidity of the imide for their cleavage. Although the mechanism for hydrolysis of
hydroxymethylamides is entirely different from what is possible for the imidomethyl type of prodrug of a phenol, the fact that the imide functioned as a leaving group in the final step of hydrolysis of the hydroxymethylamides suggested that, if an imide with sufficiently good leaving group potential was used for the imide portion, the imidomethyl prodrug might hydrolyse by an $S_N2$ (or $S_N1$) mechanism at a fairly rapid rate. Therefore, saccharin ($o$-benzoic sulfimide), having a $pK_a = 1.6$, was used as the imide portion of the promoiety.

However, Getz (26) has observed that the rate of hydrolysis of model phenolic prodrugs where the imide portion was varied from saccharin ($pK_a = 1.6$) to succinimide ($pK_a = 9.6$), was more dependent on the acidity of the phenol than on the acidity of the imide. The rate of hydrolysis of the prodrugs was first-order with respect to hydroxide ion concentration and trapping experiments with cyanide yielded only imidoacetonitriles (26). Thus the mechanism of hydrolysis of imidomethyl prodrugs of phenols has been proposed to be $S_N2$ with specific base attack on the methylene group, followed by loss of the phenol but not the imide portion (26).

In vitro stability studies of the estradiol prodrug in buffers at nominal pH values of 5, 6, 7 and 8 suggested a pseudo-first-order dependence on the hydroxide ion concentration. This was in agreement with the suggested mechanism of hydrolysis for these type of prodrugs (26). The
estradiol prodrug exhibited an in vitro half-life of ~3 min in rat plasma suggesting that once the prodrug reached the circulation it would rapidly cleave to release the estradiol. On the other hand, a half-life of ~50 min in rat liver homogenate suggested that the prodrug was relatively stable in an organ where the parent drug is known to undergo rapid glucuronidation under in vivo conditions. Hydrolysis of the prodrug in human plasma gave a half-life of ~15 min. This suggests that the prodrug would probably not be eliminated faster than it was cleaved. The possibility of enzymatic hydrolysis of the prodrug was ruled out by the fact that the prodrug exhibited similar half-lives in the presence and absence of an enzyme poison and a chemical denaturant. However, heat treatment of plasma and liver homogenate led to significantly longer half-lives of the prodrug in those media. This could be attributed to the catalytic nature of albumin as observed by other investigators during studies involving plasma (25). Further studies are needed in order to verify this observation.

It was necessary to use an organic cosolvent for the stability experiments due to the insolubility of the prodrug in aqueous systems. There was an increase in the rates of hydrolysis when increasing amounts of methanol were used. However, there was a decrease in rates when increasing amounts of dioxane were used. Getz (26) had observed a similar increase in rates of hydrolysis of model phenolic prodrugs using methanol as a cosolvent. However, dioxane was
not used as a cosolvent in any of his experiments (26). A Type 1 $S_N2$ reaction (Figure 3.1) where the negative charge on the nucleophile has been dispersed in the transition state should proceed faster in mixed solvent systems which are less polar than water, than in water based on the analysis of the $\Delta G_{tr}$ for the nucleophile, substrate and transition state (see Chapter 3). Thus, the hydrolysis of the prodrug should proceed faster both in water-methanol and water-dioxane mixtures than in water. However, this does not take into account the ability of the two solvent systems to facilitate the diffusion of the hydrogen ion. In water and in most water-methanol mixtures, the ability of the components of the mixtures to both donate and accept hydrogen bonds make it easy for hydroxide to approach the electrophilic site of the substrate by a proton transfer mechanism (Figure 6.1). The structure of water remains reasonably intact. On the other hand, in water-dioxane mixtures, the dioxane can only accept hydrogen bonds so that the transfer mechanism is broken by the insertion of the dioxane molecules. The structure of water is not left intact (Figure 6.2). The result is slower rates of hydrolysis with increasing dioxane concentration. This effect of dioxane as a cosolvent on water structure apparently more than compensates for the effect of dioxane on $\Delta G_{tr}$. 
Figure 6.1: Mechanism of proton transfer proposed for a water-methanol system.
Figure 6.2: Prevention of proton transfer through water molecules due to breaking of the structure of water in water-dioxane system.
Pharmacodynamic studies in ovariectomized rats involving comparable doses of drug and prodrug showed that an eight fold increase in the potency of the prodrug (increase in uterine weights) compared to estradiol could be achieved with the prodrug. The other parameter tested was decrease in hormone levels: FSH and LH. These are known to increase significantly during menopause and ovariectomy. Since they hold an inverse relationship with steroid concentration, a decrease in the hormone levels would signify estrogenic activity. A significant decrease in these levels compared to control rats was observed for the prodrug when it was administered orally to ovariectomized rats. No decrease was observed when an equimolar dose of estradiol was administered. Intravenous administration of equimolar doses of estradiol and its prodrug to rats demonstrated that there were no significant differences in the uterine weights of rats treated with either the prodrug or estradiol. This indicated that complete cleavage of the prodrug to estradiol had taken place.

Bioavailability studies conducted in ovariectomized rats demonstrated a four-fold increase in oral bioavailability of 17β-estradiol via the prodrug approach. Intravenous doses of drug and prodrug exhibited comparable AUC∞ values well within biological accuracy, indicating that the prodrug exhibited the desired in vivo instability. A significant scatter in the data obtained upon intravenous administration of the drug and
prodrug was observed beyond 1500 min, therefore, it was not feasible to assess the data up to seventy-two hours.

Oral administration of the drug and the prodrug resulted in an increase in concentrations of estradiol beyond 1500 min in some rats which suggests enterohepatic recirculation. However, the rest of the rats did not exhibit such behavior. Intravenous administration of the drug and prodrug exhibited no such behavior in any of the rats. This could be because the compound does not encounter the liver immediately on intravenous administration, but rather is distributed throughout the body. Whereas, on oral administration, the liver is the first organ encountered by the compound; this probably leads to the elimination of some of the compound in the bile, followed by its reabsorption into the circulation. The low solubility of the drug and the prodrug and the inherent nature of enterohepatic recirculation observed with steroids made the assessment of the oral bioavailability in terms of concentrations difficult. Further experiments are required with a larger number of rats to make a significant claim about increased bioavailability of estradiol using the prodrug approach.

In conclusion, imidomethyl prodrugs of phenols are promising in terms of protecting phenolic drugs from premature metabolism and expected to be reliable in terms of interindividual variability as their cleavage is not enzyme mediated.
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard J. Prankerd, Chair
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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