The University of Manitoba

THE DISPOSITION OF ETHYLESTRENOL IN THE RAT

by

LESLIE JULES BOUX

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

Faculty of Pharmacy

Winnipeg, Manitoba, Canada

February, 1979

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ABSTRACT

The disposition of ethylestrenol in the rat and the *in vitro* metabolism by rat liver homogenate was studied utilizing ^{3}H-ethylestrenol as a radioactive tracer.

It was determined that 33% of an oral dose was absorbed from the alimentary canal and that a total of 17% of an oral dose was excreted in the urine and 83% in the faeces within ten days. Distribution experiments showed that oral doses of ^{3}H-ethylestrenol were circulated throughout the rat, but kidney tissue was found to contain 2 1/2 - 3 times and liver tissue 5 - 7 times the specific radioactivity of all other tissues. Samples of urine and faeces from orally dosed rats were examined for metabolites. Unchanged ethylestrenol was the only unconjugated drug-related compound detected in the urine and faeces, but two dihydroxylated dihydro-ethylestrenol metabolites and a trihydroxylated dihydro-ethylestrenol metabolite were detected from the glucuronide conjugates in the faeces.

Measurement of the excretion after intravenous doses showed that the drug excretory system could handle high concentrations of the drug. It was also found that 39.7% of an intravenous dose was excreted in the urine and 50.6% in the faeces in five days. Excretion after an intramuscular dose was found to be very slow. The average $t_{1/2}$ for the excretion of the radioactivity after an intramuscular dose of ^{3}H-ethylestrenol was 5.5 days. The major metabolite

produced by the *in vitro* metabolism of ethylestrenol by rat liver homogenate was identified as 17α-ethyl-17β-hydroxy-4-estren-3-one and 17α-ethyl-5ξ-estrane-3ξ,17β-diol was tentatively identified as a minor metabolite.

ACKNOWLEDGEMENT

I wish to express my gratitude for the considerable guidance given by Dr. J. W. Steele on performing this research and preparing this thesis, the technical criticisms and enlightenment given by Diane Smith and Ricky Araneda, and the extensive encouragement given by my wife, Heather.

Financial assistance from the Medical Research Council and the Faculty of Pharmacy is also gratefully acknowledged.

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INTRODUCTION

A. Anabolic Steroids

Since a report in 1935 (Kolchakian and Murlin, 1935), stating that an extract of male human urine, when injected into dogs, could cause weight gain and a marked drop in urinary nitrogen, the belief that certain male hormones possess an "anabolic" action has led to the development of a class of drugs known as anabolic steroids. They have been developed to "promote the synthesis and storage of cytoplasmic protein and stimulate the growth of tissues," i.e. the anabolic effect (Camerino and Sciaky, 1975). It has also been the aim to separate this effect from the action inherent in male sex hormones of promoting development of the secondary sex characteristics of the male, i.e. the androgenic effect. This class of drugs has been the topic of some controversy in the last few years, due to the lack of knowledge concerning their mode of action, coupled with the fact that they are subject to substantial abuse by the athletic community.

1. Evaluation and Classification

The main problem in anabolic steroid development has been to synthesize analogues of sex hormones, such that these compounds have enhanced anabolic activity and the least possible androgenic effect. This has necessitated the development of a variety of tests to differentiate between the two drug actions.

The assay for androgenic activity was the simplest to design, as any measurement of the growth of an accessory sex organ or sex characteristic would suffice. The most commonly used indicator is the weight of the ventral prostate and/or the seminal vesicles of the rat (Kolchakian, 1975).

The methods to measure anabolic activity were much more difficult to design and in recent years have come under some criticism. Two immediate possibilities, total body weight and nitrogen balance, have both had limited use due to the many technical problems inherent in these methods. Because the objective of anabolic drugs is to increase the amount of the body's lean tissue, other tests measuring the growth or mass of specific muscles have been developed. Most of the work done today is based on a method of this type. The method, developed in 1950 (Eisenberg and Gordon, 1950) and since modified (Hershberger et al., 1953; Desaulles and Krahenbuhl, 1962), utilizes the weight gain of the levator ani muscle of castrated rats, after seven days of drug treatment. For the sake of comparison, the weights of the ventral prostate and of the seminal vesicles are also taken at this time as an indicator of androgenic activity. This enables researchers to compare the two effects, and the resulting ratio of the gain in weight of the muscle to the gain in weight of the other organs is called the anabolic/androgenic ratio.

This method, however, has been severely criticized by several researchers. It was shown (Hayes, 1965) that the muscle used is the "dorsal bulbocavernosa" and is not the levator ani muscle, the former being definitely sex linked. Thus the method, although relatively simple and inexpensive, may yield somewhat misleading results and should now only be used for initial laboratory screening (Potts et al., 1977). Some researchers feel that all these drugs may not have the anabolic activity that is attributed to them, and that a few are on the market today only by virtue of the simplified test (Hervey et al., 1976). Thus, the need is obvious for more specific tests to accurately determine the anabolic activity of drugs.

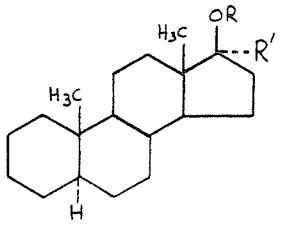
Anabolically active steroids can usually be classified into two large groups depending on their basic structure:
(a) Androstanes: These are C-19 steroids having a methyl group at position 10;
(b) Estranes: These are C-18 steroids having a hydrogen at position 10. These are sometimes called 19-nor-androstanes.

In each of these two main groups are two subgroups (Figure 1):
(i) 17β-hydroxysteroids: Members of this group often have the 17-hydroxyl group esterified, with the length of the ester chain determining the duration of anabolic action. Short chains of approximately two to three carbon atoms give rise to shorter-acting compounds, while those with chains of

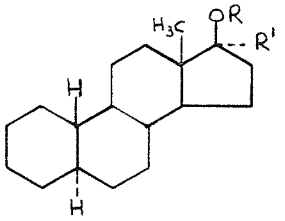
length seven to ten carbon atoms give longer-acting compounds. The products in this subgroup are not orally active and therefore see limited clinical use. More often the compound is converted to its analogue in the second subgroup, conferring oral activity.

(ii) 17α-alkyl-17β-hydroxysteroids: The alkyl group is usually a methyl or ethyl substituent. Members of this class are orally active and can be used for both oral and parenteral administration.

Figure 1. A classification system for anabolic steroids



androstane type



estrane type

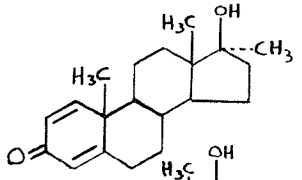
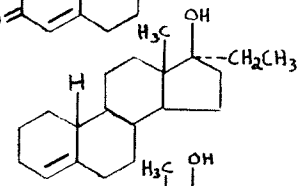
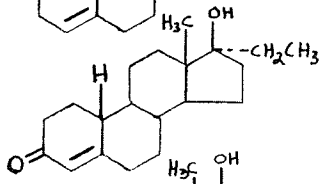
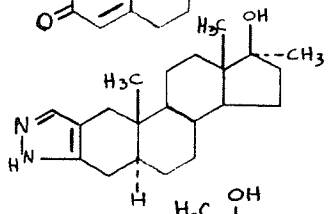
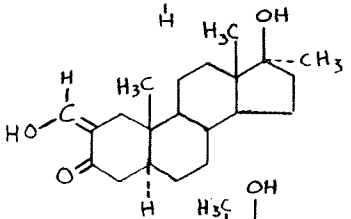
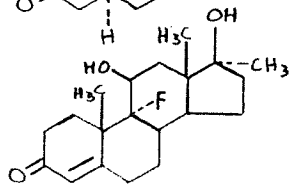
substituent	subgroup (i)	subgroup (ii)
R	-H or ester	-H
R'	-H	$-CH_3$ or $-CH_2CH_3$

Of the six steroids available in Canada for use as anabolic agents (Rotenburg, 1978), all have 17α-alkyl groups and are thus orally active (see Table 1). Four of these are androstane derivatives and the remaining two are estrane derivatives.

2. Uses and Abuses

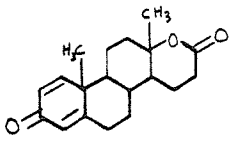
Although anabolic steroids have a wide influence on

Table 1. The anabolic steroids available in Canada

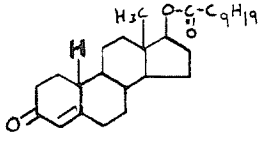
Common Name	Chemical Structure	I.U.P.A.C. Name	Brand Name/ Manufacturer
Methandrostenolone		17β-hydroxy-17α-methyl-1,4-androstadien-3-one	Danabol/ Ciba-Geigy
Ethylestrenol		17α-ethyl-4-estren-17β-ol	Maxibolin/ Organon
Norethandrolone		17α-ethyl-17β-hydroxy-4-estren-3-one	Nilevar/ Searle
Stanozolol		17α-methyl-2'H-5α-androst-2-eno[3,2-c] pyrazol-17β-ol	Winstrol/ Winthrop
Oxymetholone		17β-hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one	Adroyd/ Parke Davis and Company
Fluoxymesterone		11β,17β-dihydroxy-9α-fluoro-17α-methyl-4-androsten-3-one	Halotestin/ Upjohn

metabolic processes and have been suggested for treatment of a vast range of conditions, their clinical importance remains limited to the few areas in which there has been some evidence of success. These areas include the treatment of advanced metastatic breast cancer, aplastic anemia, and wasting diseases in the elderly. In addition, there is the controversial use by athletes to increase their muscle size and strength (Gribbin and Flavell Matts, 1976).

The use of anabolic steroids in the treatment of breast cancer developed from the use of more powerful androgens. These had originally been used to obtain regression of tumour growth, but had the side effect of serious masculinization in many patients (Segaloff et al., 1953). The shift to anabolic agents lowered the incidence of these side effects and still maintained a reasonable level of tumour regression. The use of the drugs, however, remains relatively empirical because the mode of action in producing regression is unclear and the methods for predicting which patient will respond to a given treatment not well developed (Gordan, 1977). The drugs which are now being used to treat this disease, such as Δ^1-testololactone and nandrolone decanoate are probably better classed as weak androgens than as anabolic steroids



Δ'-testololactone



nandrolone decanoate

(Gribbin and Flavell Matts, 1976). It still remains uncer-

tain whether a purely anabolic steroid would produce tumour regression if free of androgenic activity, as it is possible that it is this latter effect which accounts for the anti-cancer activity.

The treatment of aplastic anemia is another use of anabolic steroids in clinical practice (Young et al., 1977). Here, again, the influences of androgenic and anabolic steroids are extremely variable. Some researchers report remission rates as high as 50% (Sanches-Medal et al., 1969) while others have reported that steroid therapy had little influence in the outcome of the disease (Li et al., 1972). It is now generally thought that the treatment of aplastic anemia by anabolic therapy is likely only to influence the milder cases (Lancet, 1975), but that until more definite treatment is available for severe cases, anabolic treatment might as well continue.

The use of anabolics in geriatric practice resulted from a special problem in the treatment of the elderly. For a variety of reasons, many of them neglect their diet and allow themselves to fall into a state of apathy in which catabolism exceeds anabolism. It is these cases in which physicians see a benefit from anabolic steroids. It is suggested that anabolics be given along with other measures, such as a high protein diet and vitamin supplements, to effect improved muscle tone and bulk. In studies where this has been done, under conditions of weight loss, depression,

and muscular wasting, there has been some degree of success, producing general weight gain, increased activity, and overall clinical improvement (Lye and Ritch, 1977; Kopera, 1977).

Although anabolic steroids obviously have limited use in general clinical practice, much research is still being done due to the widespread and often unsupervised use by athletes. The belief that the use of steroids during training will increase both muscle mass and strength, coupled with the fact that the detection of these drugs can be (made) difficult, has led to increased misuse of the drugs. It has been estimated that as many as 75% of professional football players and 80-90% of all weightlifters in the world are taking steroids (Wade, 1972). Other "power" events such as the rowing and throwing events also have a high percentage of competitors who have repeatedly taken anabolic steroids (Freed et al., 1975). This extensive misuse of the drugs has concerned researchers, because of the reports of toxicity related to long-term use of anabolic steroids. The risk that athletes might do themselves serious injury by taking anabolics has led researchers to attempt to determine the actual toxic limits and mode of action of the drugs. It is hoped that this will shed some light on the causes and extent of any hazardous side-effects.

There has been much controversy recently in the scientific community as to whether or not there is an actual anabolic action of these drugs in healthy individuals, such as

athletes. A number of workers have tried to test the effect in athletes, some using "clinical" doses and others using doses more typical of actual use (sometimes as high as twenty to one hundred times the recommended therapeutic dose). Some have concluded that the drugs help to increase strength and body weight, while others report no effect (Tahmindis, 1976; Freed et al., 1975; Ryan, 1977). Even two recent papers, measuring hormone levels and endocrine functions in order to measure physiological effects, reported conflicting results for several indicators (Hervey et al., 1976; Holma and Aldercreutz, 1976). This situation in the literature, along with the consideration of ethics in sports (which led to sports administration bodies banning the use of these drugs by athletes), has resulted in a dilemma for the individual athlete. Although the general trend today is still to take anabolic steroids, it has been suggested that more athletes should approach researchers to supervise their drug-taking and monitor physiological parameters in order to avoid any permanent damage resulting from drug use (editorial, Med. S. Aust., 1976).

3. Mode of Action

The mode of action of the anabolic steroids which might produce increased lean weight is, for the most part, unknown. Although a fair body of knowledge concerning metabolic effects has been collected over the years, very little is yet clearly explained by events at the cellular level.

After the initial work by Kolchakian, it was found that paralleling the retention of nitrogen was a decrease in the excretion of calcium, potassium, phosphate, creatinine, and water (Camarino and Sciaky, 1975). More recent studies showed that during the administration of anabolic steroids plasma testosterone, aldosterone, and some pituitary hormone levels were lowered (Kilshaw et al., 1975; Holma and Aldercreutz, 1976). The retention of water could be explained by the increased binding capacities of newly formed proteins, although water retention has also been observed after high doses of other steroids. An explanation of the observed reduced hormone levels could be inhibition of synthesis related to high levels of pseudo-androgen in the system, which may be the cause of such side-effects as lowered sex libido. The retention of calcium, potassium, and phosphate ions may result from either increased protein production or mere water retention in the tissues. It has been suggested that an anabolic effect would involve one or more of the following factors:

(a) a higher rate of protein synthesis;

(b) a lower rate of protein catabolism;

(c) a lower rate of amino acid catabolism.

Bartlett (1953) showed that during increased nitrogen retention, both an elevated rate of protein synthesis and a lowered rate of amino acid breakdown to urea could be observed.

In a paper by Mayer and Rosen (1975), a theory was postulated that incorporates all three aspects, two of them being secondary to the initial effect. They proposed that the anabolic effects of all androgens is mediated through the interaction of these hormones with the glucocorticoid receptors in the muscle. Their proposal was based on three observations:

(a) that treatment with glucocorticoids produces a severe catabolic response in muscles, and androgens can reverse this;

(b) that specific binding proteins have been shown to exist in rat skeletal muscle for glucocorticoids, but have not been found for androgens; and

(c) that androgens can compete with the glucocorticoids for binding to the specific glucocorticoid receptors in the muscle tissue.

Thus the androgens (and anabolics) might interfere with and block the catabolic response of tissue to circulating glucocorticoids.

Another, similar hypothesis proposes that anabolic steroids interfere with glucocorticoids by inducing enzymes in the liver to increase the metabolism of glucocorticoids to inactive metabolites (Colby and Kitay, 1972). This proposal is supported by other work which indicates that there may be a mechanism by which anabolic steroids enhance the activity of drug metabolizing enzymes in the liver (Gillette, 1963;

Selye, 1970).

Although these theories begin to elucidate a mode of action for the effects of anabolic steroids, some researchers still regard the existence of a purely anabolic action to be an open question. This is based on publications which report finding no "permanent" weight gain in animals after drug treatment (Hervey and Huchinson, 1973).

4. Undesirable Effects

Most of the undesirable effects of taking anabolic steroids result either from the residual androgenic activity of the drugs or some type of liver toxicity. Whereas the effects of the first variety are relatively minor and usually reversible by termination of the drug administration, the effects of anabolics on the liver are viewed as potentially more serious.

Since it has been impossible to completely separate anabolic from androgenic activity, a number of side-effects resulting from residual androgenicity have been documented. Some of the effects that have been associated with anabolic steroids are acne, flushing, decreased libido, testicular atrophy, salt and water retention, and hypertension (Percy, 1977). Other effects such as lowered sperm count and temporary sterility have also been reported (Cooper and Craig, 1975). In women, side-effects resulting from an increase in androgenic activity in the body have also been seen. Such effects as virilization (skin coarsening, facial hair growth,

voice deepening), clitoral enlargement, and interference with the menstrual cycle are all effects to be expected when taking anabolic steroids. All of the effects caused by the androgenicity of the anabolic steroids in women also seem to be reversible on termination of the administration of the drugs.

With increasing frequency, anabolic steroids have been implicated in various forms of liver toxicity. Although it is only the 17α-alkylated steroids that show this characteristic, it is these drugs which are being most widely used, as they are orally active. Hospital surveys (Westaby et al., 1977), review articles (Johnson, 1975; Scheuer and Lehmann, 1977), and case studies (Young et al., 1977) all mention anabolic steroids as causes of cholestatic jaundice, peliosis hepatitis, leukemia, and hepatocellular carcinoma. Complications with anticoagulant therapy have also been reported (Howard et al., 1977). Liver toxicity is usually seen after prolonged use of anabolic steroids, so it is rarely reported in conjunction with athletes, since athletes usually stop taking steroids after noticing the milder, more reversible symptoms.

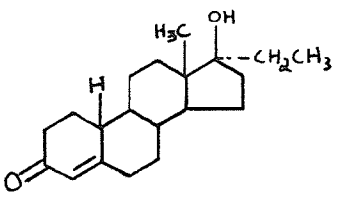
B. Ethylestrenol

Ethylestrenol (17α-ethyl-4-estren-17β-ol) was first synthesized in 1959 (DeWinter et al., 1959). A preliminary study (Overbeek et al., 1962), using the levator ani assay, stated that ethylestrenol possessed high myotropic (anabolic) and low androgenic properties that would make it useful

as a potent anabolic steroid. Another study (Ruchelman and Ford, 1963) demonstrated that there was a trend towards a positive nitrogen balance when ethylestrenol was given, and that ethylestrenol could reverse a negative nitrogen balance induced by dexamethasone (a glucocorticoid). Thus ethylestrenol came to be used medically for conditions characterized by wastage of protein and abused non-medically to improve muscle development and athletic performance.

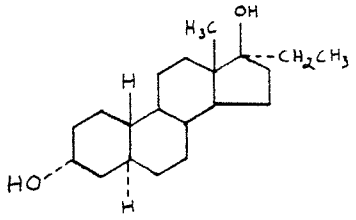
During the last ten years, very little research has been done with ethylestrenol. Only about forty-five research articles have been published in which ethylestrenol was studied. One-sixth of these deal with the use of ethylestrenol in the treatment of blood disorders, while three-quarters deal with the ability of ethylestrenol to induce hepatic microsomal enzymes. Most of this latter work was done at the University of Montreal, by a group of researchers headed by Selye and Tache. A recent review (Kourounakis *et al.*, 1977) summarized this work.

Only a few papers have been published that deal with the detection of ethylestrenol or its metabolites. Okada *et al.* (1969) reported the *in vitro* metabolism of ethylestrenol by rabbit liver slices to norethandrolone (17α-ethyl-17β-hydroxy-4-estren-3-one) and a more polar Δ^4-3 keto

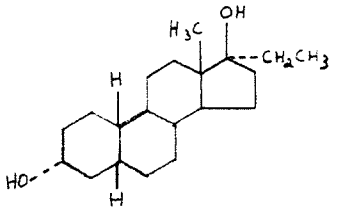


norethandrolone

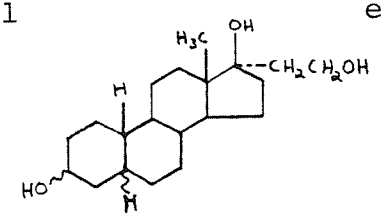
metabolite. Brooks and Middleditch (1971) reported on the suitability of gas-liquid chromatography/mass spectrometry for the detection of estrenols and their chloromethyl(dimethyl)silyl ethers. Hara and Mibe (1975) reported on the suitability of high pressure liquid chromatography in the detection of natural and synthetic steroid pharmaceuticals, including ethylestrenol. In vivo urinary metabolites from man and the marmoset monkey were reported by Ward et al. (1975, 1977) who found that ethylestrenol was metabolized to norethandrolone and its metabolites. These secondary metabolites were two tetrahydro metabolites (5α and 5β-17α-ethyl-estrane-3α, 17β-diol), a hydroxy-norethandrolone, a hydroxy-tetrahydro metabolite, and 17α-ethyl-5ξ-estrane-3ξ,17β,21-triol.



17α-ethyl-5α-estrane-3α,17β-diol



17α-ethyl-5β-estrane-3α,17β-diol



17α-ethyl-5ξ-estrane-3ξ,17β,21-triol

There is little available information concerning the disposition of ethylestrenol in human or any other animals. Aside from the small amount of metabolism work mentioned

above, there has been no research on the fate of ethylestrenol after it enters the body. It is not known what percentage is absorbed into the blood system, whether it is circulated throughout the body, and by which routes and how quickly it is excreted. Indeed, very little of this information is available for any of the anabolic steroids.

C. Aims of this Study

This study forms a part of a much larger research project, outlining the absorption, distribution, metabolism, and excretion of the anabolic steroids in the rat. Because the anabolic steroids are widely used and hepatotoxic, it is hoped that research into the disposition of the drugs will give some indications as to the causes of this toxicity. The aim of this study is to provide disposition data for ethylestrenol in the rat, utilizing tritium-labelled ethylestrenol as a radioactive tracer. This study is intended to provide information about:

(a) the percentage of drug absorbed after an oral dose;

(b) the distribution of the drug after an oral dose and any tissue localization;

(c) the time course and route of excretion of the drug and its metabolite(s) after oral, intramuscular, and intravenous dosing;

(d) the quantity of unchanged drug excreted and the quantities and identities of any metabolites;

(e) the identity of *in vitro* metabolites, and the differences

and/or similarities between *in vivo* and *in vitro* metabolism; (f) comparison of the above with other anabolic steroids.

METHODS

A. Chemicals

^{3}H-ethylestrenol [20,21-^{3}H-17α-ethyl-4-estren-17β-ol] and non-radioactive ethylestrenol were gifts of the Drug Metabolism and Development Department of Organon, N.V., Oss, Holland. Norethandrolone was a gift from G. D. Searle and Co., Chicago, Ill., U.S.A. All other chemicals were of reagent grade or better and most were purchased from Canadian Laboratory Supplies (Canlab), Fisher Scientific, or Sigma Chemical Co.

^{3}H-ethylestrenol (6.3 mCi/mg*) was stored as received in benzene solution. A working stock solution was prepared by evaporating approximately one-quarter of the solution to dryness and redissolving in 25 ml of 95% ethanol. This gave a solution of approximately 5.5$_{\mu}$Ci/100$_{\mu}$l) (12.2 x 10^{6} dpm/100$_{\mu}$l). Radiochemical purity of the working stock solution was determined by thin-layer chromatography followed by extraction of all spots and liquid scintillation counting of the extracts. Only one spot other than ^{3}H-ethylestrenol was found (at the origin) and accounted for 3% of the radioactivity. A gas-liquid chromatogram of this stock solution was also obtained and showed only one peak, corresponding to ^{3}H-ethylestrenol.

The chemical purity of the non-radioactive ethylestrenol was also determined by thin-layer and gas-liquid chromato-

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*for a list of the symbols and abbreviations used, see Appendix.

graphy and showed only one spot on the thin-layer plate and one peak on the gas-liquid chromatogram.

B. Instruments and Equipment

The following is a list of the brand names and models of the instruments and equipment used:

liquid scintillation counter - Beckman, LS-9000

freeze-dryer - Virtis, Model 10-100

tissue blender - Brinkmann, Willems Polytron

carbon-hydrogen analyzer - Coleman, Model 33

gas-liquid chromatograph - Varian, 1840

rotary evaporator - Buchi, Rotavap-R

mass spectrometer - Finnigan Quadrupole, Model 1015

refrigerated centrifuge - International Equipment Co. (IEC), Model B-20.

C. Animals

The rats used were male, Sprague-Dawley strain, obtained from Bio Laboratories, St. Paul, Minn., U.S.A. The rats were 50-53 days old and had been fasted 18-24 hours at the time of dosing. The average weight of the rats used was 170±30 g.

D. Dosing

1. Oral

Oral doses were prepared by dissolving 30-60 mg of ethylestrenol in an aliquot of the ^{3}H-ethylestrenol stock solution (100 or 200 μl) in a 4 ml test tube. Another aliquot of the stock solution was taken at the same time