THE UNIVERSITY OF CALGARY

Metabolism of Anabolic Steroids in Cynomolgus Monkey

by

Jasna Dmitrovic

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA

JUNE, 1998

© Jasna Dmitrovic 1998

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-34956-X

Canada

Abstract

The objective of this study was to determine if the cynomolgus monkey has similar urinary metabolite patterns of anabolic steroids as humans using ethylestrenol and norethandrolone as model compounds.

Monkey and human metabolic study urine samples collected after oral administration were analyzed by GC/MS for the presence of the various metabolites of the steroids. The monkey and human urinary metabolic patterns were then compared.

Major metabolites identified in human were also identified in the cynomolgus monkey: 17α-ethyl-5β-estran-3α,17β-diol, norethandrolone and two 17α-ethyl-5ξ-estran-3ξ,17β,21-triol compounds. In addition, some minor metabolites were also observed in both species: 17α-ethyl-5α-estran-3α,17β-diol, and 17α-ethyl-5ξ-estran-3ξ,17β,16ξ-triol.

The second objective was to develop a method for the isolation of an ethylestrenol metabolite from the urine of the cynomolgus monkeys. A successful method was developed for 17α-ethyl-5β-estran-3α,17β-diol.

Acknowledgements

I would like to thank my supervisor and mentor, Dr Siu Chan, for this master degree thesis. I am indebted to him for providing this opportunity for me. I would also like to extend my gratitude for his continuous immense support, assistance, patience and advice in seeing this project through to completion.

My special thanks to Dr Merle Olson, for his encouragement and assistance during my research.

I would also like to thank Dr Merle Olson, Dr Morley Hollenberg and Dr Raghev Yamdagni for agreeing to act as members of my thesis committee.

Finally, I would like to recognize the most important people in my pursuit of this degree. To my mom and my husband, had it not been for their support and help, this may never have happened.

Table of Contents

List of Tables

List of Figures

Abbreviations

ALS	Automatic sampler
CAL	Calibrator
EI	Electron impact
GC/MS	Gas chromatograph/ mass spectrometer
GC/MSD	Gas chromatograph/ mass selective detector
IS	Internal standard
M^+	Molecular ion
MeOH	Methanol
m/z	Mass to charge ratio
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
RT	Retention time
RRT	Relative retention time
SIM	Selected ion monitoring
TIC	Total ion chromatogram
TMS	Trimethylsilyl
TMSI	Trimethyliodosilane
TMSO	$OSi(CH_3)_3$ - group
TMSOH	$HOSi(CH_3)_3$ - group

1. INTRODUCTION

1.A.1. HISTORY

For many centuries it was believed that symptoms of aging in men were caused by testicular failure. This stimulated the search for an active principle of the testicles. When isolated, this active principle would restore sexual, mental and physical vigor in aging men.

In 1926 at the University of Chicago, Professor Fred C. Koch assisted by a young medical student, Lemuel C. McGee, succeeded in isolating twenty milligrams of a testicular substance out of 40 pounds of bull testicles [1]. They have proved that this substance had male sex hormone capacities by injecting it into capons as it prevented signs of castration.

Three years later Koch and Dr T.F. Gallagher continued to purify the male hormone and along with Dr A.T. Keynon performed the first human experiment on a eunuch to prove the efficacy of the male sex hormone in humans [1].

Now there was no more doubt that the male sex hormone existed, so the momentum was gathering in the scientific world to further isolate, identify and synthesize it. In 1931, A. Butenandt extracted a whisper of pure crystals that showed male hormone activity by filtering 25000 liters of male human urine [2]. However, his main contribution was not in isolating the male hormone from urine but in determining its exact chemical formula. In

the summer of 1935 pharmacologist E. Laqueur, working in Amsterdam, extracted a few milligrams of the pure male hormone using Koch's procedures, determined its precise chemical structure and named it testosterone [2]. The same year, two different scientists, Ruzicka, a Yugoslav chemist and Butenandt, a German chemist, synthesized testosterone from cholesterol [1,2]. Within a few years, a number of physicians and researchers began using testosterone in both clinical and research settings.

1.A.2. CLINICAL USES OF TESTOSTERONE AND SYNTHESIS OF ANABOLIC STEROIDS

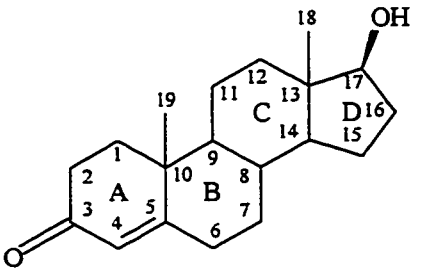
Experimental studies in both animals and humans showed that testosterone possess both anabolic and androgenic effects. The androgenic effects (masculinizing) are those involving development and maintenance of primary and secondary sexual characteristics, while the anabolic effects (tissue building) are stimulation of protein synthesis and muscle growth.

Testosterone therapy, during the period of early clinical experimentation, was considered beneficial in a number of medical conditions [1]. Some of the uses are: treatment of testosterone deficiency, some mental disorders, hypertension and heart disease, premature boy and girl infants, anemia, male impotence, contraception in men, nephrotic syndrome, female menopause, dysfunctional uterine bleeding, also for enhancement of muscle mass, strength, endurance and reflexes of normal older men and others.

Within a few years of the discovery of testosterone, attempts to alter the testosterone molecule to enhance the anabolic effects and reduce or alleviate the undesired androgenic effects occurred. As a result, many synthetic derivatives of testosterone, anabolic steroids, were developed. However, because the anabolic and androgenic actions of anabolic steroids appear to be mediated through the same receptor complex [3], it was not possible to produce an anabolic steroid that is free of testosterone's androgenic functions. Many of the clinical uses of anabolic steroids are highly controversial. However, from the beginning, they have had two widely accepted medical uses [2]. Anabolic steroids have been used for the treatment of certain types of anemia (because of their ability to stimulate erythropoiesis) and for stimulating sexual development in hypogonadal males (replacement therapy).

1.A.3. STRUCTURAL ANALOGS OF TESTOSTERONE: ANABOLIC STEROIDS

Anabolic steroids are synthetic analogs of testosterone (structure 1). Testosterone has a cyclopentanoperhydrophenantrene ring system (the basic structure of all steroids), which consists of four rings (labeled A, B, C and D) [4]. Furthermore, testosterone has a keto group in the C-3 position, a double bond at the C-4 position and a hydroxyl group in beta orientation at C-17 position. The spatial orientantation of the functional groups are alpha (α) when they are below the plane of the ring system, or beta (β) when above and if the orientation is unknown then it is denoted as xi (ξ).

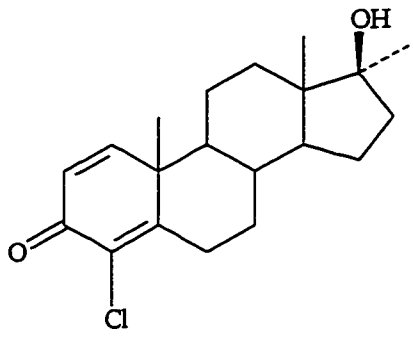


Structure 1. Testosterone, 4-androsten-17β-ol-3-one

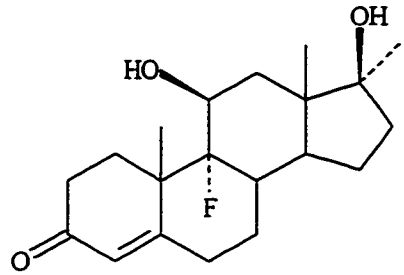
The presence of the 17β-hydroxyl group is common to all anabolic steroids, indicating this group is important for their biological activity. We can divide anabolic steroids in five groups, depending on their structural modifications:

1. steroids with 17α-alkylation,
2. steroids that have the 19-methyl group removed,
3. steroids with changes in the A-ring,
4. steroids with an extra ring fused to the A-ring,
5. and steroids with changes in other rings.

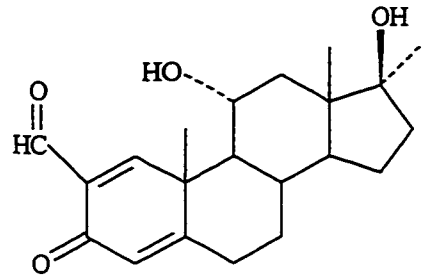
The chemical structures of some anabolic steroids are shown in Fig. 1.



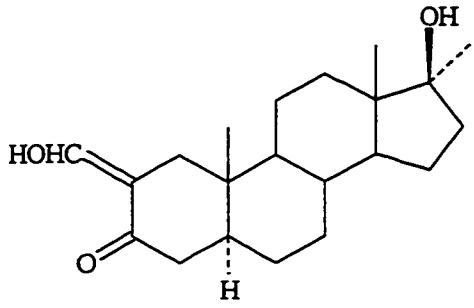
Chlorodehydromethyltestosterone



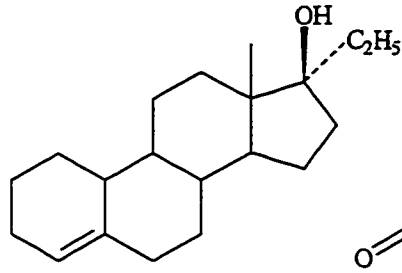
Fluoxymesterone



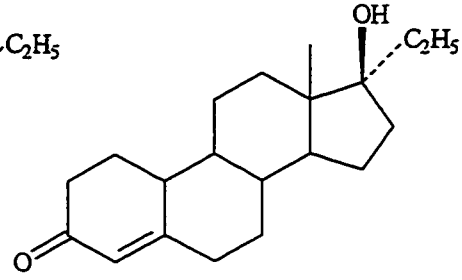
Formebolone



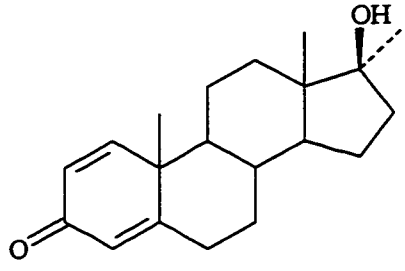
Oxymetholone



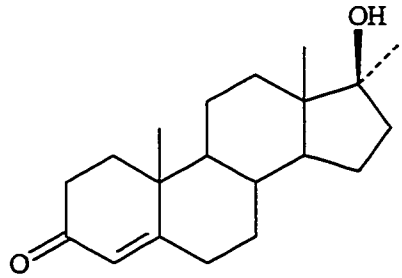
Ethylestrenol



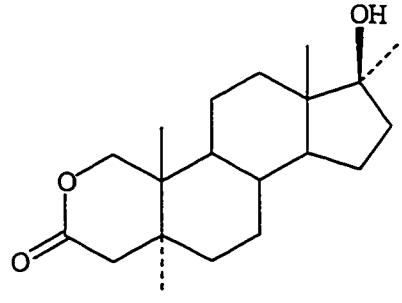
Norethandrolone



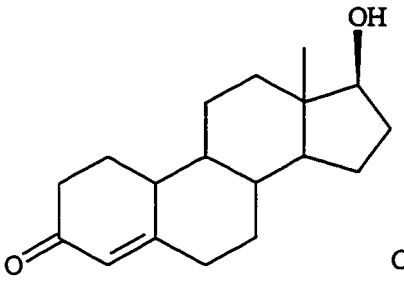
Metandienone



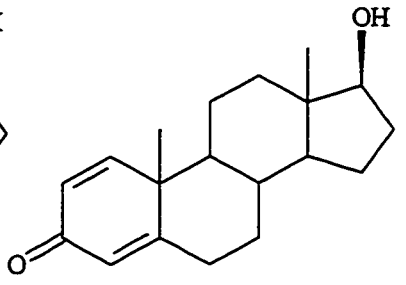
Methyltestosterone



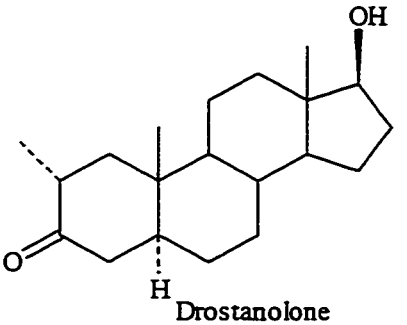
Oxandrolone



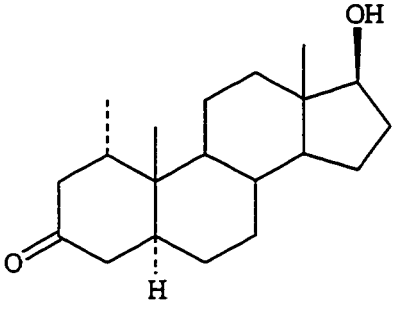
Nandrolone



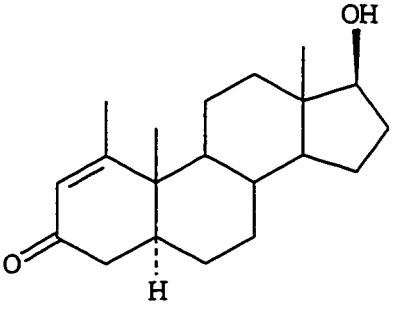
Boldenone



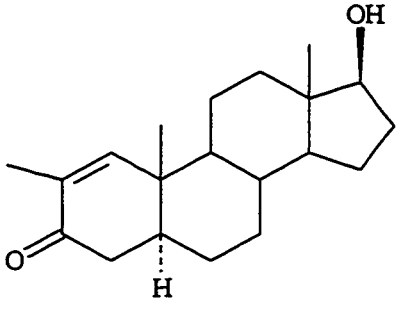
Drostanolone



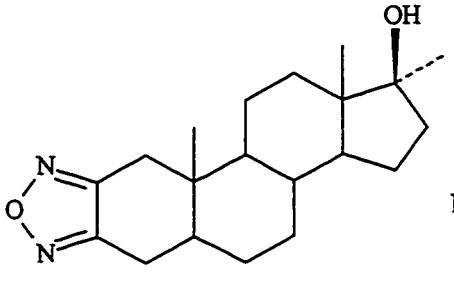
Mesterolone



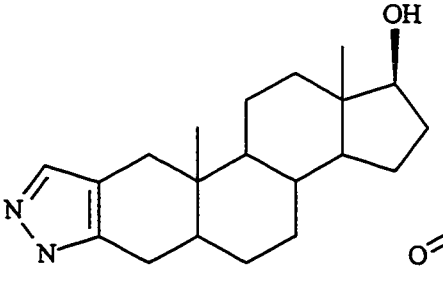
Metenolone



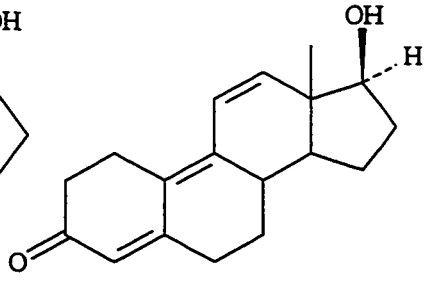
Stenbolone



Furazabol



Stanozolol



Trenbolone

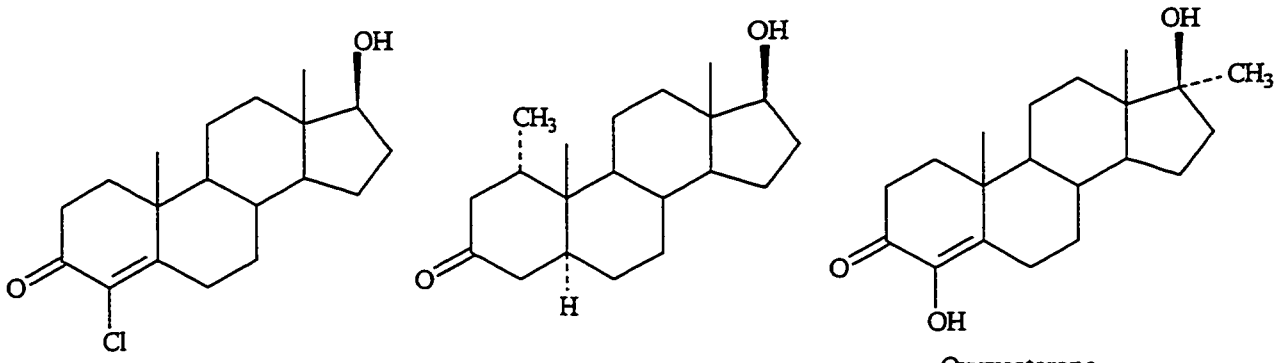


Figure 1. Structures of some anabolic steroids

1.A.4. ABUSE OF ANABOLIC STEROIDS

Anabolic steroids were reportedly first used, during World War II [1,3]. They were given to German troops to enhance their muscle strength and increase their aggressiveness. In 1954, the first reports appeared of athletes using anabolic steroids to increase weight and power. Over the following years, the use of anabolic steroids by normal young males especially those participating in athletic events has increased remarkably.

Today, the use of anabolic steroids has reached almost epidemic proportions, and it is not restricted to sports only, but is also a societal problem [1,5]. The misuse of anabolic steroid drugs has become so widespread that national and international sports organizations (including the International Olympic Committee in 1974 [9]) as well as governmental agencies (such as the Canadian and United States governments) have

imposed a ban upon their use for non-medical purposes. In 1990 anabolic steroids were reclassified in the United States as controlled substances (Schedule III substances under the Steroid Trafficking Act of 1990) [1,4].

Recent data suggests that there may be over 3,000,000 regular anabolic steroid users in the United States alone [1]. Most of these users buy steroids illegally in a estimated $300- to 400- million annual black market (according to a report by the US General Accounting Office) [5].

Among teenagers, i.e. at the high school level, the abuse of anabolic steroids has been rising to epidemic levels over the past 20 years [5]. Over two-thirds of the users initiated anabolic steroids when they were 16 years of age or younger (high risk for growth suppression), and improvement of "physical appearance" was the main reason for 26.7% of the users.

Surveys have shown that steroid use may afflict between 5% to 11% of teenaged boys and 0.5% to 2.5% of girls in grades 7 through 12 [5]. About 40 percent of the first time users progress to hard-core steroid use [1]. Although it is currently much less than that by boys, steroid use by high school girls is perhaps the fastest growing segment of total steroid use.

In 1993, it was reported by the Canadian Centre for Drug-free Sport that 3% of Canadian high school students surveyed (aged 16 and over) were using anabolic steroids and 28% of the same age group reported personally knowing someone who was using anabolic steroids [6]. Killip and Stennett, in 1989, found that the prevalence of steroid use among 146 classes of high school athletes in an Ontario school district was 4.5%.

The use of anabolic steroids for performance enhancement in sports began in the 1950s and has steadily increased to the present day [1,3]. These drugs are used by athletes to improve the development of muscle mass, strength and power, and in some cases muscle definition.

Anabolic steroids offer an advantage only for strength sports, as opposed to aerobic or skilled sports [5]. Football, wrestling, weight lifting, some track and field events, baseball and swimming are the primary sports for steroid users.

In the past, up to 100% of national and international competitors in the sports of weight lifting, shot-put, javelin throwing, and body-building have illegally used anabolic steroids [5]. Surveys done between 1970 and 1988 at the five major universities showed a 15% to 20 % use rate among college athletes.

However, steroid use is popular not only among young people for appearance-altering reasons or in sports among athletes to improve their physical performance. In a number of entertainment arenas muscular men and women have become superstars. Steroid use is also gaining popularity among police officers and armed forces personnel. The imposingly muscular body obtained by months and years of steroid use is transmitted by the press, television, and the cinema, and has a major impact on today's youth. In many cases, these overly muscular "heroes" portray very violent roles, which may translate to the true picture of a steroid user's personality. There are many adverse health effects of anabolic steroid use, and their non-medical use should be discouraged.

1.A.5. ADVERSE EFFECTS OF ANABOLIC STEROID USE

The abuse of anabolic steroids is associated with serious adverse effects to the liver and the cardiovascular, central nervous, musculoskeletal, and reproductive/endocrine systems [1,3,5]. Some adverse reactions occur rapidly (e.g., altered reproductive functions) but others may be delayed several years (e.g., cancer, heart disease).

Much of the data on side effects of anabolic steroids probably is understated because it derives from studies of users ingesting the drugs in manufacturer's recommended doses for medical reasons. Many of the adverse effects can be anticipated on the basis of hormonal physiology, and some are reversible after the drug is discontinued. Other adverse effects are less well understood, for example, structural changes in the liver and the development of various tumors.

In abuse the doses of anabolic steroids may be in excess of 1000 times the normally prescribed doses [3, 7]. Consequently it is reasonable to anticipate that the number and severity of adverse effects will significantly exceed those reported with therapeutic doses. However, even if the risk was well established, there are those users who will feel the potential benefits derived from anabolic steroid use are so compelling that they are willing to use the drug no matter what the consequences.

Tables 1-4 contain an extensive listing of the adverse physical and psychological effects that can be associated with anabolic steroid use and abuse [1]. Any user, depending on the number of anabolic steroids taken, the dosages, and the duration of the steroid use can experience any or all of these physical and psychological effects.

Table 1: **Minor Abnormalities Due to Anabolic Steroid Use in Men [1]**

Hypertension	Disturbances in sleep cycles
Acne	Increased appetite
Fluid Retention	Gynecomastia
Abnormal liver function tests	Deepening of the voice
Change in testicular size & function	Increased sebaceous gland secretion
Psychologic disturbances	Viral illness after cessation
Penile enlargement	Increased energy level
Increased libido	Cessation of depression
Changes in hair growth pattern and distribution	Rebound resetting of hormone balance
Epistaxis	Increased aggression
Enhancement of coping mechanisms	Withdrawal loss of libido
Withdrawal depression	

Table 2: **Minor Abnormalities Due to Anabolic Steroid Use in Women [1]**

Hypertension	Disturbances of sleep cycle
Acne	Increased appetite
Fluid Retention	Reduction of body fat
Abnormal liver function tests	Menstrual disturbances
Psychological disturbances	Increased energy level
Reduction of breast tissue	Deepening of the voice
Clitoral enlargement	Cessation of depression
Epistaxis	Increased aggression
Changes in hair-growth pattern and distribution	Increased sebaceous gland secretion
Viral illness after cessation	Withdrawal depression
Rebound estrogenization	Increased vascularity
Ruddiness of face	Withdrawal loss of libido

Table 3: **Major Conditions Associated with Anabolic Steroid Use in Humans [1]**

Liver
- hepatocellular carcinoma
- peliosis hepatis
- benign liver tumors
- clinical hepatitis

Cardiovascular
- myocardial infarction and death
- acceleration of vascular disease

Psychological
- increased hostility, mental intensity, anger, aggressiveness
- increased desire to excel
- increased libido
- probable enhancement of mental alertness
- psychological dependance and withdrawal symptoms
- decreased inhibition to use other medications
- tendency toward "one-track mindedness"
- tendency toward violence

Table 4: **Psychological Changes Induced by Anabolic Steroids [1]**

While on the Drugs	*After Drug Use*
Increases in self-esteem, sex-drive appetite, explosive hostility and violence, mental intensity, energy, tolerance to pain, desire to train	Increases in depression, listlessness, apathy, desire for the drug
Decreases in ability to accept failure or poor performance, general tolerance, inhibitions about other drug use	Decreases in self-esteem, sex drive, hostility and violence, desire to train intensely
Other changes, including sleeping disturbances and nightmares	Other changes, including a return to normal sleep patterns and ability to control violent behavior

1.A.6. THE FIGHT AGAINST DRUG USE

Misuse of anabolic steroids has resulted in a ban on anabolic steroid use for non-medical purposes for two reasons. First, there is a concern for the health of the user, particularly for prepubescent adolescent users, because of the very harmful side effects of anabolic steroid use. Second, fair competition among athletes, as anabolic steroids are performance-enhancing drugs and athlete using them have an added advantage.

One of the measures that are carried out to deter the abuse of anabolic steroids is drug testing. Surveys taken between 1970 and 1988 at five major universities, showed a 15-20% anabolic steroid use rate among college athletes [5]. Recent NCAA (National Collegiate Athletic Association) survey for self-reported drug use have detected a steroid usage rate of approximately 2.5% overall (5% among football players), suggesting that random drug testing initiated in 1989 has gradually deterred the abuse of anabolic steroids in the college ranks [5].

As very few anabolic steroids are excreted without being metabolized and the parent steroids are detected for only a short period after administration, an understanding of the metabolism is needed for drug testing [4,8]. Furthermore, structures of the metabolites need to be elucidated and, if possible, metabolites synthesized for use as reference materials.

Metabolic changes of the steroid molecule are generally grouped into two kinds of metabolism, phase I and phase II [9]. In phase I reactions steroids are usually converted into more polar compounds to inactivate the drug and to facilitate its elimination from the

body by enzymatically catalyzed oxidation, reduction or hydroxylation. The common metabolic pathways of anabolic steroids are [4,9]:

1) Oxidation of the 17β-hydroxyl group into a keto group,
2) Epimerization of the functional groups at the 17 position, for steroids with 17α-alkyl substitution,
3) Reduction of the 3-keto group to a hydroxy (which mostly gives 3α isomer) and reduction of the C-4,5 double bond when two isomers 5α and 5β can be formed,
4) Hydroxylation, most often, in position 6 and 16.

Phase II reactions are conjugation reactions, coupling of the anabolic steroid or its metabolite with glucuronic acid or sulfate, which help elimination of the steroid from the body [9]. However, not all anabolic steroids and their metabolites are excreted as conjugates in urine. Some may be excreted free, unconjugated, but most of them are excreted as conjugates, mainly as glucuronides and to a smaller extent as sulfates.

Pure substances of the metabolites, particularly conjugates, for most anabolics are not available from commercial sources. As a consequence of this there are no reference standards available for analysis. This lack of availability of suitable calibration standards makes quantitative analysis impossible.

Therefore, it is necessary to conduct metabolic studies, not only to determine the metabolic fate of these compounds, but also to produce "positive" urine that can be used as reference samples (assay quality control).

Currently in drug testing laboratories only human source urine is used as positive control samples for anabolic steroids. The positive samples are obtained via self-administration

by laboratory staff. This human production of positive urine could be an ethical and a legal problem.

An animal model that would metabolize anabolic steroids similar to man would be extremely valuable. Some attempts were made to use rats and pigs but with little success. Primates may offer the best or only option to obtain positive control urine.

The research animals used in our study were the cynomolgus monkeys. As they are nonhuman primates their physiology is very similar to human. At the time this project was started there were no metabolites of ethylestrenol or norethandrolone available as pure substances from commercial sources. This was the main reason for choosing these drugs as model compounds for the isolation of their major metabolites. Furthermore, there is limited information on the metabolism of ethylestrenol and norethandrolone, therefore we have chosen to study these compounds in detail.

2. OBJECTIVES

I To determine if the cynomolgus monkey has similar urinary metabolite patterns as human, by analyzing, by GC/MS, excretion study urine collected from the monkeys after administering orally ethylestrenol and norethandrolone, and comparing with human ethylestrenol and norethandrolone excretion study urine.

II Develop a method for isolation of a ethylestrenol metabolite from the urine of the cynomolgus monkey using column chromatography and extraction with organic solvents.

3. HYPOTHESIS

The cynomolgus monkey produces similar urinary metabolites of ethylestrenol/norethandrolone as human.

1.B. ETHYLESTRENOL/NORETHANDROLONE

Both ethylestrenol (17α-Ethyl-4-estren-17β-ol) and norethandrolone (17α-Ethyl-4-estren-17β-ol-3-one) are 17α-ethyl steroids Fig. 2. Norethandrolone was synthesized in 1957 by Colton et al. and ethylestrenol by De Winter et al. in 1959 [9]. The only structural difference between the two steroids is the presence of a keto group in 3-position in norethandrolone. The first step in the metabolic pathway of ethylestrenol is oxidation to norethandrolone, therefore after this step the metabolism of ethylestrenol is identical to that of norethandrolone [4,9].

In 1971, Brooks et al. [10] identified 17α-ethyl-5α-estran-3α,17β-diol and its 5β isomer (Fig. 2) as glucuronides and sulfates in urine of a male volunteer after oral administration of a single dose (50 mg) of norethandrolone. They have synthesized the two metabolites to confirm their identities. They also detected another metabolite they suggested to be a trihydroxy analog, hydroxylated at the 17α-ethyl group (Fig. 2).

Ward et al. [11] have investigated the metabolism of norethandrolone and ethylestrenol in man and marmoset monkey (Callithrix jacchus) in 1977. Using GC/MS, they analyzed the urine of a male volunteer given a 20 mg and a marmoset monkey given a 5 mg oral dose of the anabolic steroid. They observed, both in man and the marmoset monkey, that ethylestrenol is oxidized at C-3 to form norethandrolone prior to further metabolism. No ethylestrenol was detected in urine. They have also concluded that the urinary metabolite patterns of ethylestrenol and norethandrolone were the same.

In man the major metabolites detected were 17α-ethyl-(5β)-estran-3α,17β-diol,

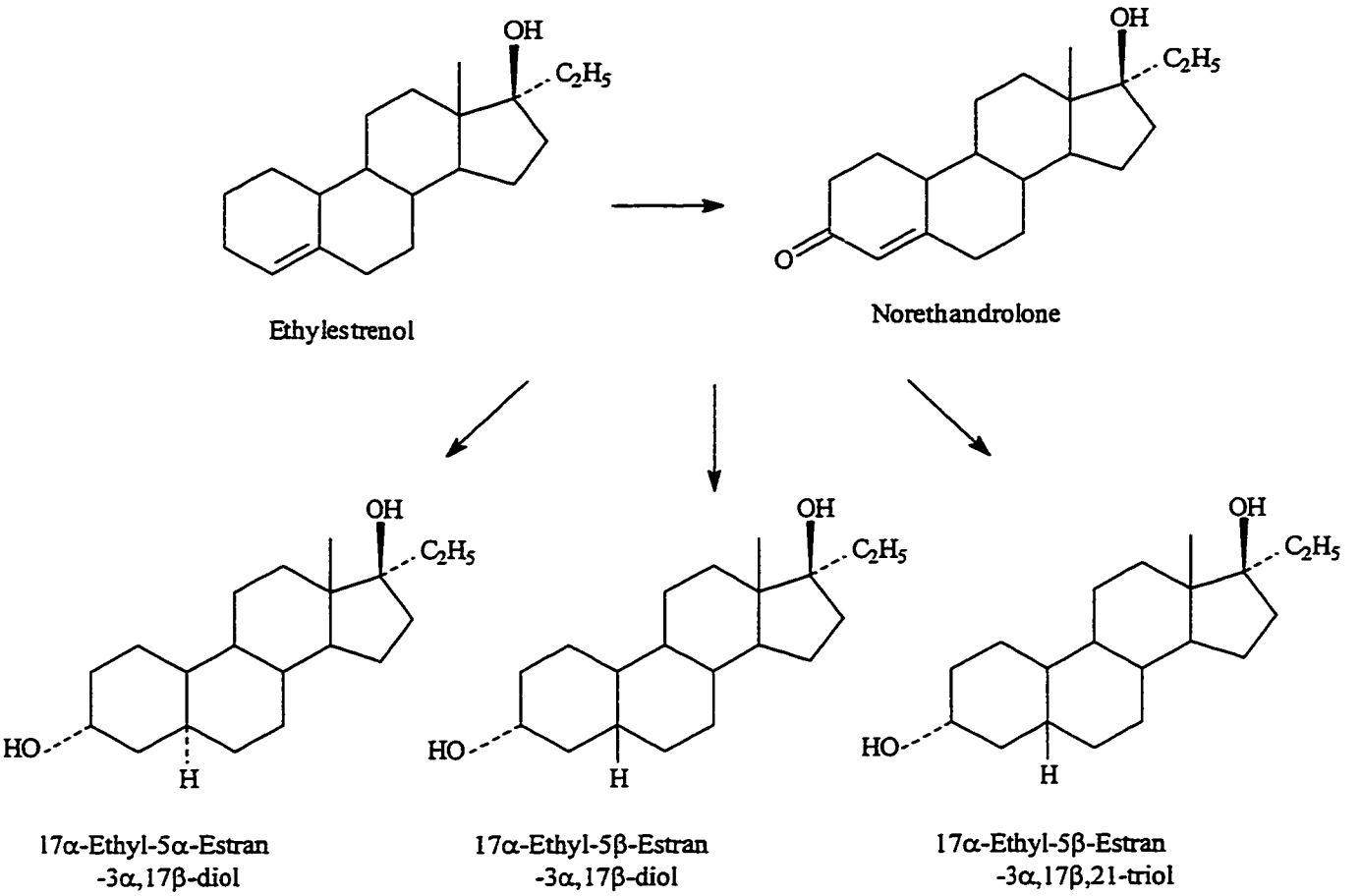


Figure 2. Metabolism of ethylestrenol/norethandrolone

norethandrolone and a trihydroxy analog believed to be 17α-ethyl-(5ξ)-estran-3ξ,17β,21-triol. However, marmoset monkey excretes norethandrolone unchanged or as hydroxylated norethandrolone. Also they detected in monkey urine both 3α,5α- and 3α,5β-tetrahydro metabolites but did not find metabolites with a hydroxyl group in the D-ring or side-chain.

Liu et al. [12] analyzed by GC/MS in 1991, a norethandrolone excretion study urine

collected for 35 hours after an oral administration. They have detected norethandrolone and nine of its metabolites (Fig. 3). Structures of seven metabolites were postulated. Variations of their concentrations in urine were determined by comparing the height of their base peaks (Fig. 4).

In 1992, Schänzer and Donike could identify only 17α-ethyl-5β-estran-3α,17β-diol in human norethandrolone excretion urine, no 5α-isomer was detected [13]. Both isomers were synthesized as reference substances for identification of the metabolites. A tri-hydroxy metabolite tentatively identified as 3α,5β-tetrahydro metabolite hydroxylated at the 17α-ethyl group, was also detected.

Schänzer reported in 1996 that it has been confirmed the main metabolites of ethylestrenol and norethandrolone are: 17α-ethyl-5α-estran-3α,17β-diol, 17α-ethyl-5β-estran-3α,17β-diol and 17α-ethyl-5ξ-estran-3α,17β,21-triol [9]. The 3α-hydroxy configuration of the metabolites is proposed because all metabolites are excreted as conjugates that can be hydrolyzed with β-glucuronidase [9]. The tetrahydro metabolite hydroxylated at the ethyl side chain, was proposed to be 17α-ethyl-5β-estran-3α,17β,21-triol. The EI mass spectrum of the TMS derivative of this metabolite shows abundant D-ring fragment ions at m/z 144 and 157, confirming the side chain hydroxylation [9]. Besides these metabolites, further hydroxy metabolites have been detected, but their structures remain unknown. Furthermore, recently it has been confirmed by Geyer, Donike, and Schänzer that 3α-hydroxy-5α-estran-17-one (norandrosterone) and 3α-hydroxy-5β-estran-17-one (noretiocholanolone), which are the main metabolites of nandrolone, are in low amounts excreted as metabolites of ethylestrenol.

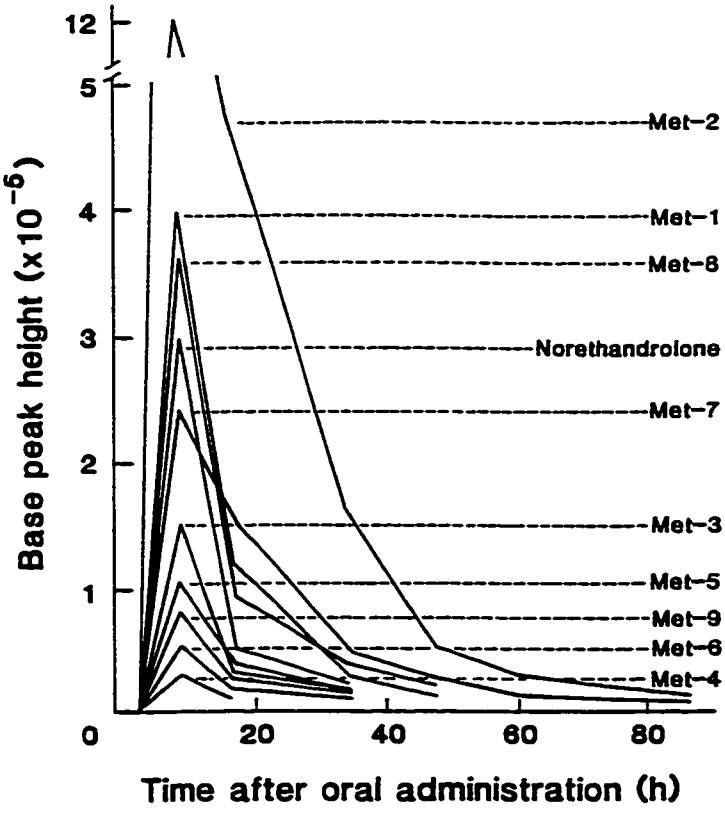


Fig. 4 Variation of norethandrolone and its metabolites' concentration in human urine.

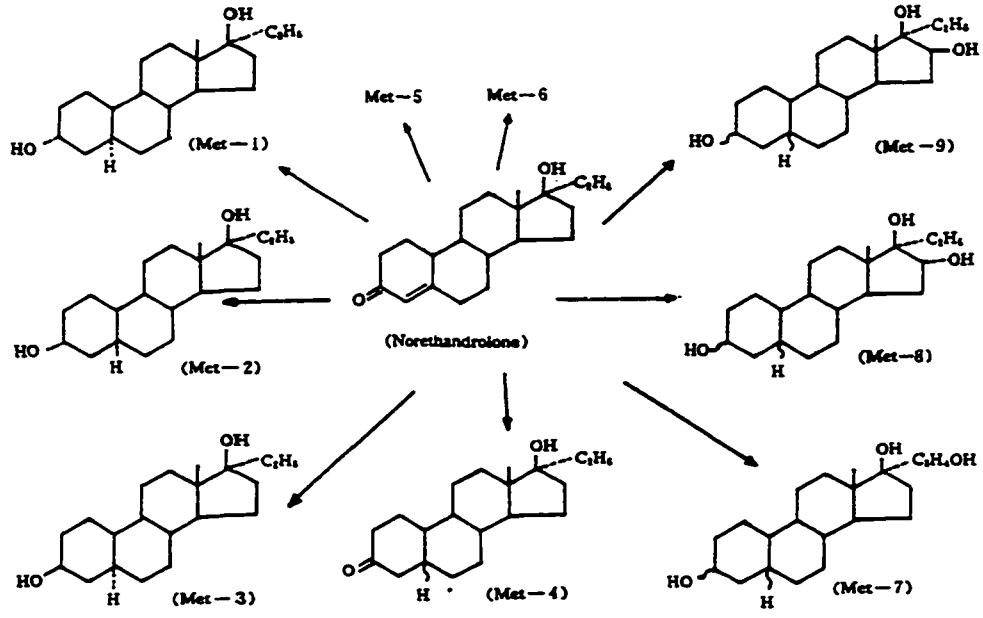


Fig 3. Metabolic pathways of norethandrolone in man.

4. EXPERIMENTAL

4. 1. RESEARCH ANIMALS: *CYNOMOLGUS MONKEYS*

The cynomolgus monkey is a nonhuman primate, and belongs to the division of anthropoids [14]. Nonhuman primates closely approximate human beings physiologically therefore they are the most desired research animals. They are also the most expensive animals that are found in research establishments. The cynomolgus, or crab-eating macaque is from the group of the Old World monkeys that originate from Africa and Asia. It has also been called M. *irus* in the past, but now M. *fascicularis* is the accepted name. They range in size from 10 to 20 kg, with a life span of 10 to 25 years under optimal conditions.

The two male cynomolgus monkeys are kept in individual metabolic cages in a separate room isolated from other animals. Under the cage there are pens where the urine is collected, but also feces and food. In order to reduce the contamination of the urine a screen is placed over the collection tray and the tray is sloped to allow the urine to be collected in a corner. As these animals are very messy they require a considerable amount of cleaning. High-pressure hoses are used to flush the cages and pens.

The research monkeys are fed on a complete monkey diet, which is made into large dry chunks, containing 15% protein [14]. They do not need nutritional supplements, but fruit, vegetables and other treats are given too. The daily intake of food is around 4% of the

body weight, but a lot of the food will be thrown around and wasted. Water bottles are attached to the cages. Fruit juices are given as a treat.

For this research project approval form the Faculty of Medicine Animal Care Committee was obtained under the title: Metabolism of anabolic steroids in primates. The approved protocol is under an ID: 91017 with a start date 91/06/01 and this document is reproduced on page 23 and 24.

PROTOCOL ID: 91017 Status: A Previous Id: Cross Reference:
Title: Metabolism of anabolic steroids in primates.

	(YY-MM-DD)		
Start Date:	91-06-01	Faculty:	Medicine
End Date:	99-06-01	Department:	MID
Approval Date:	91-10-08		
Review Date - Last:	96-12-01	Classification:	011502
Next:	97-12-01	Category:	B
		Acute/Chronic:	C
Funding approved: Y		Anaesthesia:	
Funding Agency:	American Col. Pathol	Analgesics:	
		Contraindicated:	

Replacement:
Reduction:
Refinement:

Infectious Agent:
Chemical Agent: Stanozolol
Biological Material:
Radioisotope:
Other Agent:

INVESTIGATORS:

Principal Investigator: Dr. M.E. Olson (OLSONM)
(403) 220-6835 Ext.
(403) 288-6151 Ext.

Authorized Investigators: Dr. S.C. Chan (CHANS)
(403) 670-1674 Ext.

Other Personnel: J. Fourneir (FOURNJ)
(519) 679-2111 Ext.

ANIMAL UTILIZATION:

Animal Description			No. Auth.	Used YTD	Total Used
Primates	Cynomolgus	Cynomolgus	4	2	2

PROTOCOL PROCEDURES:

Intact male cynomologus monkeys (approx. 20 kg) will be injected or fed an anabolic steroid for 3 days. Twenty-four hour urine samples will be collected. Urine will be collected in the pan below the animal.

In order to reduce contamination of urine, a screen will be placed over the collection tray and the tray will be sloped to allow urine to collect in a corner. Urine will be filtered and frozen immediately for analysis at a later date. Urine will also be used to separate and identify metabolites.

NOTE: These animals will be housed at the Health Protection Branch in Ottawa. Occasional animals may be brought to Calgary.

PROTOCOL OBJECTIVE:

Objective: To determine if macaque urine can be used as positive control urine for drug test laboratories.

Problem: Currently only human source urine can be used as a positive control sample for anabolic steroids in drug test laboratories. Many laboratories administer the drugs to staff members to obtain the samples. An animal model that would metabolize the anabolic steroid similar to man would be extremely valuable. We attempted to use rats and pigs but they do not metabolize stanozolol like humans. Primates may offer the best or only option to obtain positive control urine.

4.2. DRUG ADMINISTRATION

24 hours prior to drug administration pre-dose urine was collected. The cynomolgus monkey was administered orally 100 mg of ethylestrenol two times a week, on Mondays and Wednesdays, for four months. As the drug was in powder form it was well incorporated into peanut butter or a banana and given as a treat. The twenty-four hour urine samples that were collected in the pen under the cage were poured off into containers labeled with the date of collection, and these samples were stored at 4 °C until the whole week's collection is finished. The Monday to Friday samples were pooled together in a 4 L plastic urine container labeled as M-F. The Saturday and Sunday samples were pooled together and container labeled as S-S. The two pools were stored at 4 °C until 3 L of urine was collected, when the urine was frozen. 1 L of urine was collected for approximately ten days. Over the period of four months that ethylestrenol was administered, six pools were collected and they were labeled:

1. SP0001 M-F
2. SP0002 S-S
3. SP0003 M-F
4. SP0004 S-S
5. SP0005 M-F
6. SP0006 M-F

After the collection of ethylestrenol positive urine was finished the monkey was left to rest for approximately four weeks. After four weeks of no drug administration, a twenty-

four hour urine sample was collected and it was tested for the presence of ethylestrenol metabolites. When it was determined that ethylestrenol metabolites are not detectable in the urine, the monkey is "clean", the next drug was administered.

Norethandrolone was fed in doses of 100 mg every day for a period of a week. All urine samples collected were pooled.

4.3. GC/MS ANALYSIS

Metabolic study urine was analyzed for the presence of the anabolic steroid (parent compound) and/or various metabolites of the steroid by the method first developed by Masse, et al [15] and later modified by Chan, et al for detection of anabolic steroids [16,17]. The anabolic steroids are isolated from urine by C_{18} solid-phase extraction, conjugated metabolites are enzymaticly hydrolyzed, then all free steroids are recovered by liquid-liquid extraction with diethyl ether and converted to trimethylsilyl derivatives prior to instrumental analysis.

Before extracting, 500 ng of methyltestosterone is added as an internal standard to the 5 mL urine sample. Steroid metabolites may be excreted free (unconjugated), but most of them will be excreted as conjugates, mainly as glucuronides, and to a smaller extent, as sulfates [4]. In the first step of the extraction procedure, both, free and conjugated steroid metabolites are extracted from the urine by solid-phase extraction.

A C_{18} Sep-Pak cartridge (Sep-Pak Cartridges, Waters Co., Milford, MA) is first activated with methanol and water, and then the urine sample is passed through the cartridge. Some of the water-soluble impurities are washed by a water wash, and then the free and

conjugated steroids are eluted with methanol. The methanol is removed by evaporation. As conjugates of steroids are not easily extractable into an organic solvent, hydrolysis of the steroid conjugates is the next step in the procedure. Conjugates are hydrolyzed with a crude extract of Helix promatia, type H-2 (Sigma Chemical Co., St. Louis, MO) which consists primarily of β-glucuronidase and some sulfatase activity. Sigma has determined the β-glucuronidase activity to be approximately 100000 units/mL and 5000 sulfatase units/mL in this crude solution. They define that one unit of β-glucuronidase will liberate 1.0 μg/mL of phenolphthalein from phenolphthalein glucuronide per hour at 37 °C at pH 5.0 (30 min assay). Also, one unit of sulfatase will hydrolyze 1.0 μmole p-nitrocatechol sulfate per hour at pH 5.0 at 37 °C.

The hydrolysis is carried out in acetate buffer pH 5.2 at 60 °C for two hours. After the hydrolysis is completed, the pH is adjusted to basic with potassium carbonate. Free metabolites are extracted with diethyl ether, and the organic solvent is removed by evaporation under nitrogen at 40 °C.

To enhance gas chromatographic performance and detection, extracted metabolites are converted into trimethylsilyl (TMS) derivatives before they are subjected to instrumental analysis by GC/MS, with 100 μL of a MSTFA: TMSI: Dithioerytritol mixture at 70 °C. The derivatizing mixture is a mixture of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (Sigma Chemical Co., St. Louis, MO) (MSTFA) and trimethyliodosilane (Sigma Chemical Co., St. Louis, MO) (TMSI) (1000:3/v:v) containing 2 mg/mL of dithioerythritol (Sigma Chemical Co., St. Louis, MO). Steroid metabolites are converted to TMS-enol-ether and TMS-ether derivatives. Keto groups present in the steroid

molecule are first converted into their enol form so the hydroxy group is converted to a TMS-enol-ether derivative. Trimethyliodosilane in the derivatizing mixture is used as a potent catalyst to promote the formation of the enol form, and dithioerthyritol functions as a reducing agent to maintain the ketone in the enol form. Hydroxy groups present in the steroid molecule are converted to TMS-ether derivatives by N-methyl-N-(trimethylsilyl)-trifluoroacetamide.

The flow chart of this extraction procedure is shown on page 30.

The instrumental analysis was performed on a Hewlett Packard 5970 GC/MSD. Two μL of the derivatized extract was injected in splitless mode onto the GC/MSD operating in full scan mode. The gas chromatographic and mass spectrometric conditions for this analysis are listed in Table 5. The metabolic study urines analyzed were pre-dose monkey urine, ethylestrenol monkey urines (all of the pools collected in the four month period of administration), human norethandrolone urine and norethandrolone monkey urine.

Table 5. Gas Chromatographic and Mass Spectrometric Conditions for the Analysis of Anabolic Steroids

- Gas Chromatograph Conditions:

Column: DB-1 polymethyl siloxane, 20 m x 0.25 mm i.d., film thickness: 0.25 μM

Carrier gas: helium

Linear velocity: 40-45 cm/sec at 200 °C

Injection mode: splitless, purge on at 0.5 min

Oven temperatures:

Initial:	170 °C	Time: 0 min
Program rate:	15 °/min	
Intermediate (1):	200 °C	Time: 0 min
Program rate:	1 °/min	
Intermediate (2):	214 °C	Time: 0 min
Program rate:	4 °/min	
Intermediate (3):	250 °C	Time: 0 min
Program rate:	7 °/min	
Final:	280 °C	Time: 6.71 min

Run time: 36 minutes
Other temperatures:
Injection port: 280 °C
Transfer line : 300 °C
Equilibration time: 0.5min

- Mass Selective Detector Conditions:

Solvent delay: 4 min
Source temperature: 150-200 °C
Configuration file: *ATUNE.U
Scan mass range: 80-800 amu
Scans per second: 1.19

TOTAL FRACTION EXTRACTION FLOW CHART

5 mL urine
↓
add: 500 ng of methyltestosterone as internal standard
↓
apply on activated
C_{18} SEP-PAK cartridge
↓
wash with 5 mL water, then elute with 10 mL methanol
↓
evaporate to dryness under air at 52 °C
↓
add
↓
1 mL 0.2 mol/L sodium acetate buffer, pH 5.2
100 µL Helix pomatia crude extract
↓
vortex gently, then incubate at 58 °C for 2 hours
↓
cool, then add
↓
100 mg potassium carbonate
5 mL diethyl ether
↓
shake 10 minutes, then centrifuge 5 minutes
↓
ORGANIC PHASE
↓
transfer to 13 x 100 mm centrifuge tube
↓
dry with 0.1 g anhydrous Na_2SO_4
↓
transfer to 13 x 100 mm centrifuge tube
↓
evaporate to dryness under N_2 at 40 °C
↓
add: 100 µL MSTFA:TMSI (1000:3/v: v) with 0.2 mg dithioerythritol
↓
flush with N_2, cap and then vortex gently
↓
incubate at 70 °C for 30 minutes
↓
transfer to 200 µL flat glass insert in 2 mL ALS vial
↓
inject 2 µL onto GC/MSD

4.3.1. GC/MS ANALYSIS OF PURE STANDARDS

For norethandrolone, ethylestrenol and 17α-ethyl-5β-estran-3α,17β-diol, which are available as pure standards, full scan mass spectral data and retention times were obtained. A 1 mg/mL solution in methanol of norethandrolone and ethylestrenol was prepared. 17α-ethyl-5β-estran-3α,17β-diol was purchased as a 100 μg/mL solution in 1,2-dimethoxyethane. For each compound an aliquot containing 5 μg was transferred into a screw-cap tube, the organic solvent removed by evaporation and the compound derivatized into a TMS derivative. The sample was injected onto a GC/MSD operating in full scan mode, as described earlier.

4.4. ISOLATION OF ETHYLESTRENOL METABOLITE

After collecting a larger volume of ethylestrenol positive urine from a multiple dose study isolation of a metabolite was attempted using the ethylestrenol pool labeled SP0003 M-F.

4.4.A. Development of the isolation procedure

4.4.A.1. Initial clean-up of the urine

The isolation procedure was developed using a small sample (50 mL) of ethylestrenol urine. First the sample was centrifuged to remove precipitate.

The initial clean-up step of the raw urine sample is a solid phase extraction, which removes many polar substances present and also concentrates the sample. As mentioned earlier, in the standard procedure for GC/MS analysis of steroid metabolites in urine solid phase extraction with octadecylsilane cartridges or Amberlite XAD-2 columns, as an alternative, are used in this step [16,13]. As Amberlite XAD-2 resin is inexpensive a "scaled-up" version of the Amberlite XAD-2 column was prepared.

Amberlite XAD-2 is an insoluble cross-linked polystyrene copolymer, produced as white beads in a 20 to 60 mesh size range. About 80 g of the resin was activated by thoroughly washing with acetone, methanol and deionized water. Then the XAD-2 slurry was poured into the column. After the beads were placed in a column containing water, additional water was passed upflow through the column at a rate sufficient to expand the bed by about 50%. This would classify the beads according to size, thereby allowing more uniform flow through the bed during subsequent cycles. Backwash continued until complete classification was achieved (about 10 minutes). Following the backwash, the water was drained from the column, taking care to retain one inch of water above the top of the resin bed. Subsequently four bed volumes of water were passed downflow through the bed. The length of the column bed was 22.2 cm and the diameter 2.6 cm.

50 mL of ethylestrenol urine was passed through the column of Amberlite XAD-2 resin onto which the free steroids and steroid conjugates were absorbed. The column was washed with one bed volume of water (about 120 mL) and with the same volume of different mixtures of water and methanol (20%, 30%, 40% and 50% MeOH:H2O). All of these fractions (fr.1,2,3,4 and 5) were collected separately. The fractions were

concentrated by evaporation of the solvent, water and methanol. The metabolites retained on the column were recovered by elution with two times two bed volumes of methanol (fr.6 and 7). The methanol was removed by evaporation.

After they were hydrolyzed and derivatized (as described later), each of the wash fractions was tested for the presence of ethylestrenol metabolites by GC/MS (the results of the GC/MS analysis are discussed in section 5.B). It was determined that no metabolites were eluted by any of these washes, and decided to use only a 50%MeOH: 50%H_2O wash in the future.

4.4.A.2. Hydrolysis of steroid conjugates

As steroid metabolites are mostly excreted as conjugates, hydrolysis of the steroid conjugates is the next step in the isolation method. The concentrate of fr.1, 2,3,4,5,6 and 7 was reconstituted in 2 mL of 0.2 mol/L acetate buffer pH 5.2, and 200 μL of crude extract of Helix promatia was added. The hydrolysis was performed at 58 °C for four hours.

4.4.A.3. Extraction of the free steroids into an organic phase

After the hydrolysis free steroid metabolites were extracted into an organic phase. Diethyl ether was selected as the solvent for liquid-liquid extraction. The pH of the buffer solution was previously adjusted to alkaline pH 9-10 by adding potassium carbonate. The extraction was performed with three 5 mL volumes of diethyl ether. The diethyl ether

was pooled and evaporated. Fractions 1-7, except fraction 6, were derivatized and tested for the presence of ethylestrenol metabolites, by GC/MS.

4.4.A.4. Purification and separation of free metabolites

Further purification of the urine extract and separation of the free metabolites was accomplished with lipophilic Sephadex LH-20 (hydroxypropyl Sephadex) column chromatography. Sephadex LH-20 columns were used by many groups for fractionating unconjugated steroids and eluent solvent systems have usually been composed of chlorinated hydrocarbons with an alcohol (e.g. methylene chloride-methanol) or a hydrocarbon with an alcohol (e.g. cyclohexane-ethanol) [18-24].

A few different solvent systems were tried out for the Sephadex LH-20 column chrtomatography:

a. Heptane 50% : Methylene chloride 50% : Ethanol 1%
b. Methylene chloride 95% : Methanol 5%
c. Chloroform 50% : Heptane 50% : Ethanol 1% : Water to saturation
d. Chloroform 50% : Heptane 50% : Ethanol 0.25% : Water to saturation
e. Chloroform 50% : Heptane 50% : Ethanol 5% : Water to saturation
f. Chloroform 80% : Ethanol 20%
g. Chloroform 100%

Solvent systems containing methylene chloride (a and b) were very difficult to use due to the volatility of methylene chloride. As the solvent would evaporate, the column would

dry out and break up so these solvent systems were abandoned before applying the sample. Likewise solvent systems f and g were abandoned as in these solvents the gel was floating and it was not possible to achieve satisfactory upflow rate.

Application of solvent system c, chloroform 50% : heptane 50% : ethanol 1% : water to saturation, was very successful, not only because it was easy to use and had a good flow rate only under gravity feed, but also it provided good separation of the ethylestrenol metabolite (see 5.B.2.). However as only one metabolite was eluted with solvent system c, additional solvent systems d and e were applied, unsuccessfully, to try to elute other metabolites.

The column was prepared by using about 45 g of Sephadex LH-20 previously swollen in the eluent. The gel was allowed to settle and excess solvent was removed until a fairly thick slurry was formed. The slurry was carefully poured down a glass rod into a column designed for use in organic solvents. The column bed was 38 cm long and the diameter was 1.8 cm. After the column bed had settled it was washed with 3-4 bed volumes of the eluent.

Hydrolyzed fraction 6 from the XAD column chromatography was applied as a sample after reconstituting it in 1 mL of the eluent. This urine extract was a complex mixture of numerous compounds including many endogenous steroids with similar properties as the desired metabolites.

In the elution step, 45 fractions of about 10 mL each were collected (4,5 bed volumes) in approximately four hours. Fraction 1 was collected before the sample application as a blank. The steroid metabolites were recovered from each fraction by liquid-liquid

extraction with three times 3 mL of diethyl ether. The diethylether was pooled and evaporated and fractions derivatized and analyzed by GC/MS for the presence of ethylestrenol metabolites. The analysis of the GC/MS data and elution profile (graph 1) are discussed in section 5.B.

4.4.B. Isolation of ethylestrenol metabolite from 500 mL of urine

After the urine was centrifuged, a XAD column was prepared. 110 g of XAD powder was preconditioned and column packed as mentioned earlier. The height of the bed was 28.6 cm and the diameter 2.6 cm.

While the urine sample (500 mL) was pumped onto the XAD packed column the eluent coming off the column was collected as one fraction (fr. A)

After all the sample has passed through the column a water wash (one volume of the column bed) was applied onto the column, followed with a 50% MeOH : 50% H_2O wash (three bed volumes).

Both of the washes were collected as fr. B and fr. C.

Steroid metabolites were eluted with four bed volumes of methanol (fr. D).

Fractions A, B and C were tested for remaining ethylestrenol metabolites. It was determined that they had either trace quantities, or no remaining ethylestrenol metabolites. Fraction D was concentrated by rotary evaporation of methanol.

After removing the methanol, fraction D was reconstituted with 3 mL of 0.2 M acetate buffer pH 5.2 and hydrolyzed with 2 mL of Helix promatia for 5 hours at 58 °C.

After the hydrolysis was completed free steroids were extracted with three 5 mL portions of diethyl ether. The diethylether portions were pooled and the solvent removed by evaporation under a stream of nitrogen. The extract was reconstituted in 1 mL of methanol and a 50 μL portion was derivatized and analyzed by GC/MS (section 5.B.2). The remaining 950 μL of extract in methanol was divided in two parts: I - 100 μL and II – 850 μL.

Meanwhile, a Sephadex column was prepared using 45 g of Sephadex LH-20 powder, as described earlier. The dimensions of the column bed were: 27.5 cm height and 2.1 cm diameter.

The column was tested using part I of the methanol extract. The 100 μL sample was applied onto the column and four fractions were collected:

I.1. 150 mL
I.2. 200 mL
I.3. 100 mL
I.4. 200 mL

A portion (20 mL) of each fraction was tested for the presence of the desired ethylestrenol metabolite on the GC/MS, after removing the solvent and derivatization of the portion. It was concluded that almost all of the metabolite was eluted in fraction I.2 and no other ethylestrenol metabolite or endogenous steroid was present in fraction I.2 (section 5.B.2).

At this time, the purification of part II (850 μL) of the methanol extract was continued on an Sephadex LH-20 column (40.2 cm x 1.8 cm) and similar results were achieved. Fractions collected were:

II.1. 150 mL
II.2. 200 mL
II.3. 100 mL
II.4. 200 mL

The main fraction collected was 200 mL (II.2), the eluent was evaporated and the concentrate was reconstituted in 1 mL of methanol. 20 µL aliquot was derivatized and analyzed by GC/MS (section 5.B.2).

4.4.C. Yield of the isolated metabolite

In the summer of 1997, 17α-Ethyl-5β-estran-3α,17β-diol became available as a 100 µg/mL standard from RADIAN (cat. # E-905). This synthesized ethylestrenol metabolite was purchased, and it was used to determine the yield of the isolated ethylestrenol metabolite and also its structure.

First the concentration of the ethylestrenol metabolite in metabolic study urine SP0003 M-F, used for isolation, was determined. A calibration curve was prepared by spiking an appropriate amount of the standard into 5 mL of blank urine for each calibrator (CAL 100, 200 and 500 ng/mL). Also 5 mL of blank urine was aliquoted for calibrator 0 ng/mL. Dilutions of the ethylestrenol urine were prepared (x5, x10 and x20 dilution) in blank urine, and 5 mL of the dilutions aliquoted. The calibrators and samples were extracted and derivatized according to the total fraction extraction procedure (page 24) and analyzed by GC/MSD operating in selected ion monitoring mode (SIM).

This SIM analysis was created for the quantitation of the ethylestrenol metabolite. The gas chromatographic conditions of this analysis are the same as for the full scan analysis

listed in Table 5. However, the mass selective detector monitors only for chosen masses unique for the ethylestrenol metabolite and internal standard (methyltestosterone). The ions monitored for the ethylestrenol metabolite were m/z 421, 331 and 241 and for methyltestosterone m/z 446 and 301. Also, a computer macro program was created to automatically print: the total ion chromatogram (TIC), the ion chromatograms of selected ions within the specified retention time range for each compound, the mass spectrum on the apex of the peak of the target compounds and the calculation of the ion abundance ratios and the area ratio.

The area ratio of each calibrator was plotted in relation to the concentration and the slope was used to determine the concentration of the metabolite in the ethylestrenol urine.

To determine the concentration of the ethylestrenol metabolite in fractions I.2 and II.2 a set of calibrators was also prepared. First a 10 μg/mL solution of the standard was prepared in methanol. The calibrators (CAL 0, 100, 200, 500 and 1000 ng) were prepared by aliquoting 0, 10, 20, 50 and 100 μL of the 10 μg/mL standard solution into separate, labeled, tubes. A 20 μL aliquot of fraction I.2. was aliquoted into a screw-cap tube. For the quanitation of fraction II.2 first a x10 dilution was prepared by mixing 5 μL of the fraction and 45 μL of methanol and then 10 μL of this dilution was aliquoted into a screw-cap tube. To both samples and all calibrators 50 μL of a 10 μg/mL solution of methyltestosterone was added as internal standard. The methanol was removed by evaporation from each tube and the samples and calibrators derivatized with 100 μL of the MSTFA:TMSI: dithioerythritol mixture at 70 °C for 30 minutes.

Derivatized calibrators and samples were analyzed on the GC/MSD in the ethylestrenol SIM quantitation method. The results of the analysis were used to plot the calibration curve and determine the concentration in the samples.

The results of the two quantitations were used to calculate the yield of the isolation procedure.

5. RESULTS

5.A. METABOLISM OF ETHYLESTRENOL/NORETHANDROLONE IN THE CYNOMOLGUS MONKEY

After completing the full scan GC/MS analysis of the pure standards (norethandrolone, ethylestrenol and 17α-ethyl-5β-estran-3α,17β-diol) (see 4.3.1.), the pre-dose monkey urine, ethylestrenol metabolic monkey urine, human and monkey norethandrolone metabolic urine (described in 4.3), the GC/MS results were analyzed.

Total ion chromatograms (TIC) of the pre-dose and post-dose monkey urine were compared to determine all the additional peaks that are present in the post-dose urine (Figure 5), as they would be produced by the ethylestrenol metabolites. To compare the metabolic pattern of ethylestrenol/norethandrolone in monkey and human, a comparison of the TIC of the ethylestrenol monkey urine was done with a TIC of the human norethandrolone urine (Figure 6). The interpretation of each additional peak in these two comparisons as well as peaks of corresponding retention times, relative retention times and mass spectra in the second comparison, would be very complicated and a very time consuming procedure. These urine extract are complex mixtures of not only metabolites of the anabolic steroid but also endogenous steroids and many other compounds from the urine matrix. To simplify the search for the metabolites of interest published data on human urinary metabolites of ethyestrenol and norethandrolone was used.

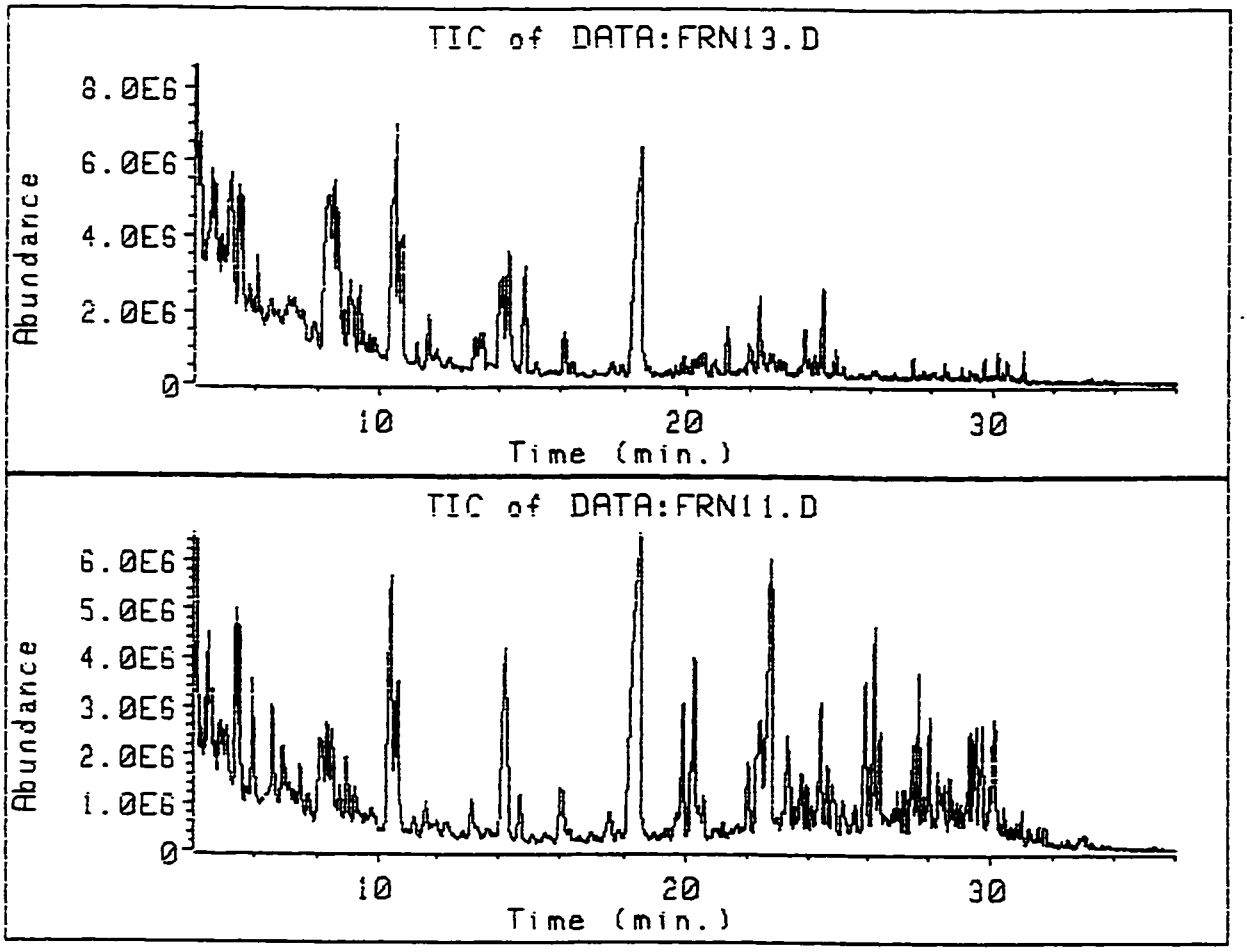


Figure 5. Full scan TIC of pre-dose (top) and post-dose (bottom) monkey urine

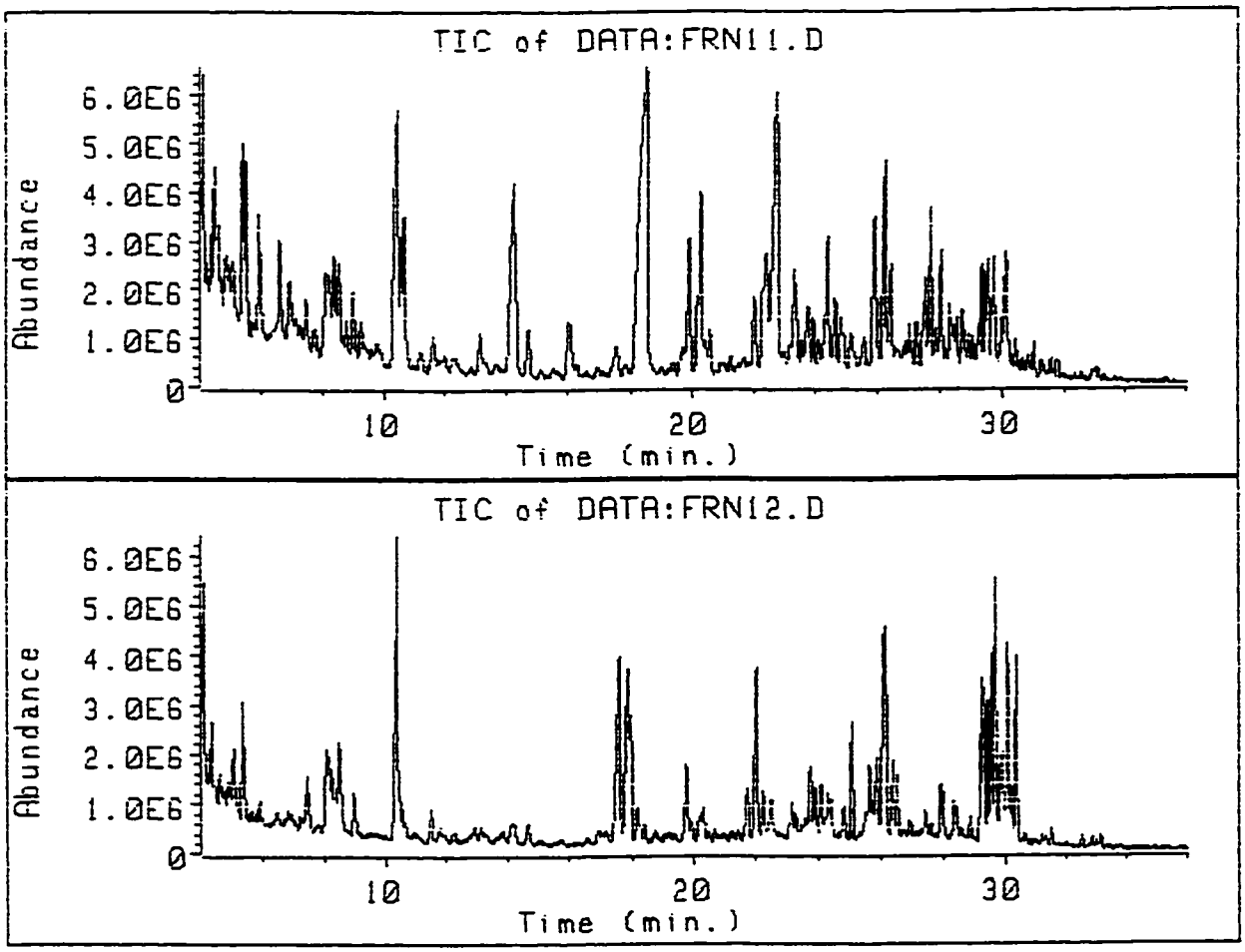


Figure 6. Full scan TIC of monkey ethylestrenol metabolic urine (top) and human norethandrolone metabolic urine (bottom)

As mentioned earlier, the first step of the metabolic pathway of ethylestrenol is oxidation to norethandrolone (Figure 2 – section 1.B.). After this step the metabolism of ethylestrenol is identical to that of norethandrolone, and the major metabolites that are found in human are di- and tri-hydroxy metabolites and norethandrolone. The reduction of the 3-keto group yields the di-hydroxy metabolites. Additional hydroxylation of the 17α-ethyl group or in position C-16 produces the tri-hydroxy metabolites.

In the derivatization procedure for GC/MS analysis these metabolites are converted into trimethylsilyl (TMS) derivatives. The literature survey has also provided us with EI (electron impact) mass spectral data of the TMS derivatives of the metabolites and its interpretation. Furthermore, for the pure standards that are available to purchase we have acquired information on the full scan mass spectral data and retention times of their TMS derivatives (norethandrolone, ethylestrenol and 17α-ethyl-5β-estran-3α,17β-diol).

The mass spectrum of a di-hydroxy metabolite (Figure 7 shows the EI mass spectrum of TMS derivative of 17α-ethyl-5β-estran-3α,17β-diol) has no molecular ion (M^+, m/z 450) present [13]. The highest ion at m/z 435 is M^+-15 fragment (loss of a methyl group) and m/z 421 is M^+-29 fragment (loss of the 17α-ethyl group). The most abundant fragments are the base ion at m/z 157 and a fragment ion at m/z 144, both D-ring fragments (Figure 8) [13]. Fragments at m/z 331 and 241 are m/z 421 –TMSOH and m/z 421 –2TMSOH respectively. (In the following figures TMSO represents $OSi(CH_3)_3$ group.)

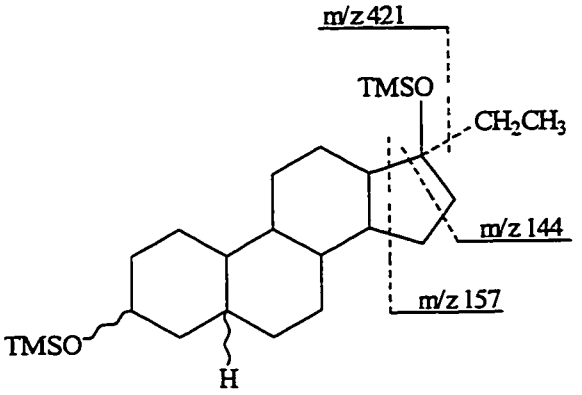


Figure 8. Fragmentation of two hydroxy metabolites

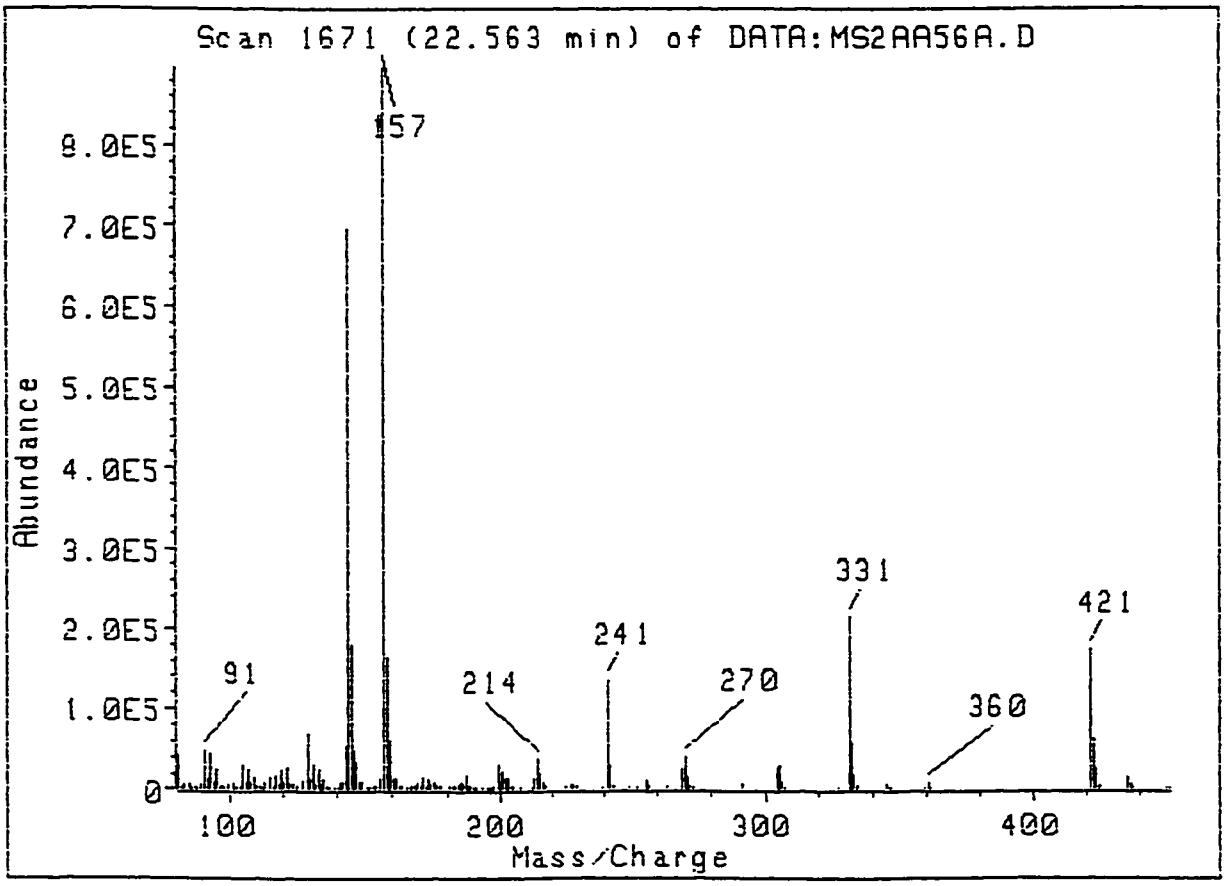


Figure 7. Full scan mass spectrum of 17α-ethyl-5β-estran-3α,17β-diol

Similarly, tri-hydroxy metabolites, hydroxylated at the 17α-ethyl group, would have characteristic fragment ion at m/z 421 (cleavage of the C-17 side chain – C_2H_5OTMS) and also fragment ions at m/z 232 and 245 produced by the D-ring fragmentation (Figure 9) [12]. It has also been proposed that the less abundant tri-hydroxy metabolites are hydroxylated in position C-16 [12]. These metabolites would have fragment ion m/z 232 and 245 from the D-ring cleavage but could also have m/z 509 fragment produced by the loss of the 17α-ethyl group (Figure 10). The molecular ion of the tri-hydroxy metabolites is at m/z 538. A fragmentation pattern has been proposed to explain fragment ion m/z 217 also seen in the mass spectrum of the tri-hydroxy metabolites (Figure 11) [12].

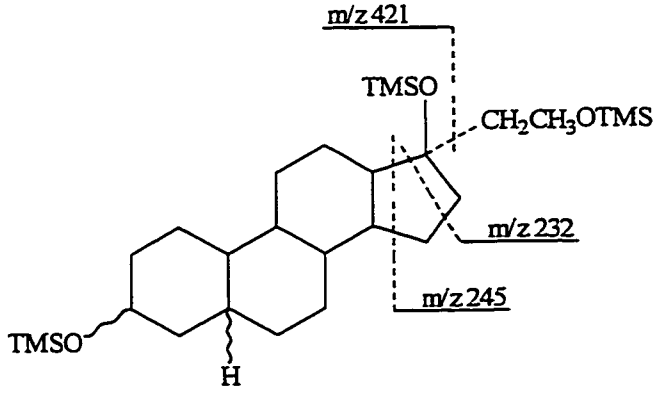


Figure 9. Fragmentation of 3,17,21-tri-hydroxy metabolites

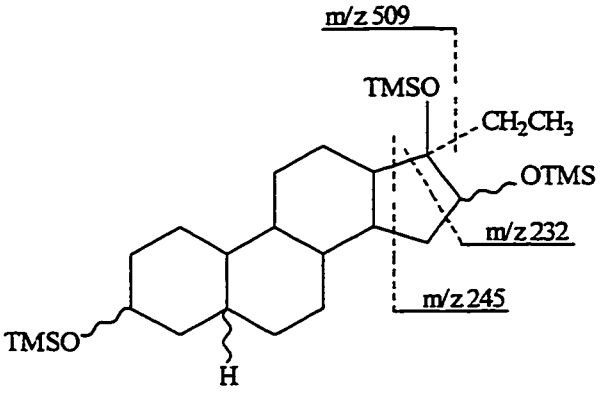


Figure 10. Fragmentation of 3,17,16-tri-hydroxy metabolites

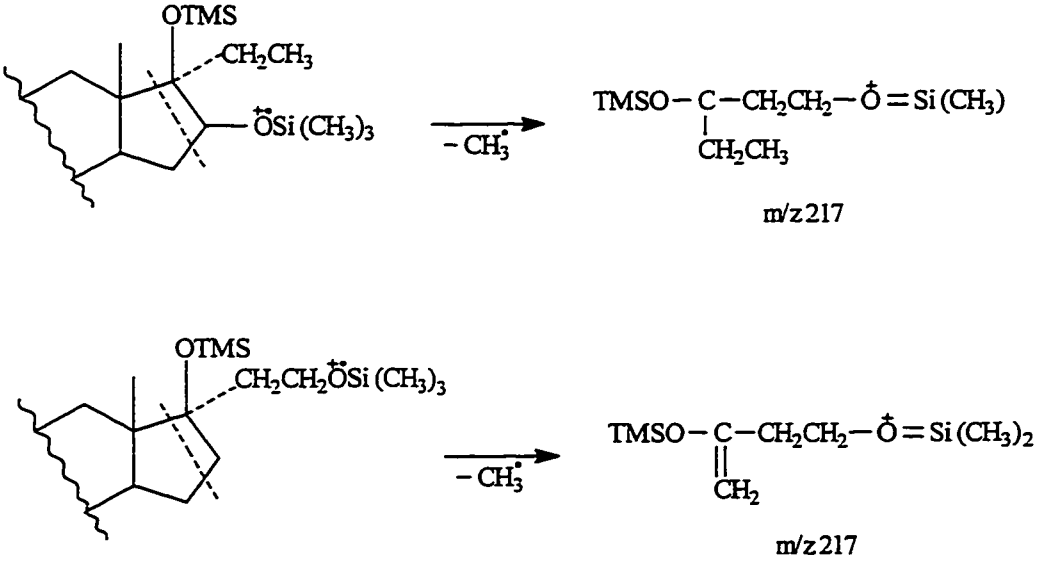


Figure 11. Fragmentation pattern of tri-hydroxy metabolites

The EI mass spectrum of norethandrolone (Figure 12) shows the molecular ion at m/z 446 and the base ion at m/z 287, which as well as fragment ion m/z 300, is a product of the D-ring fragmentation (Figure 13) [12]. Cleavage of the 17α-ethyl group produces fragment ion m/z 417.

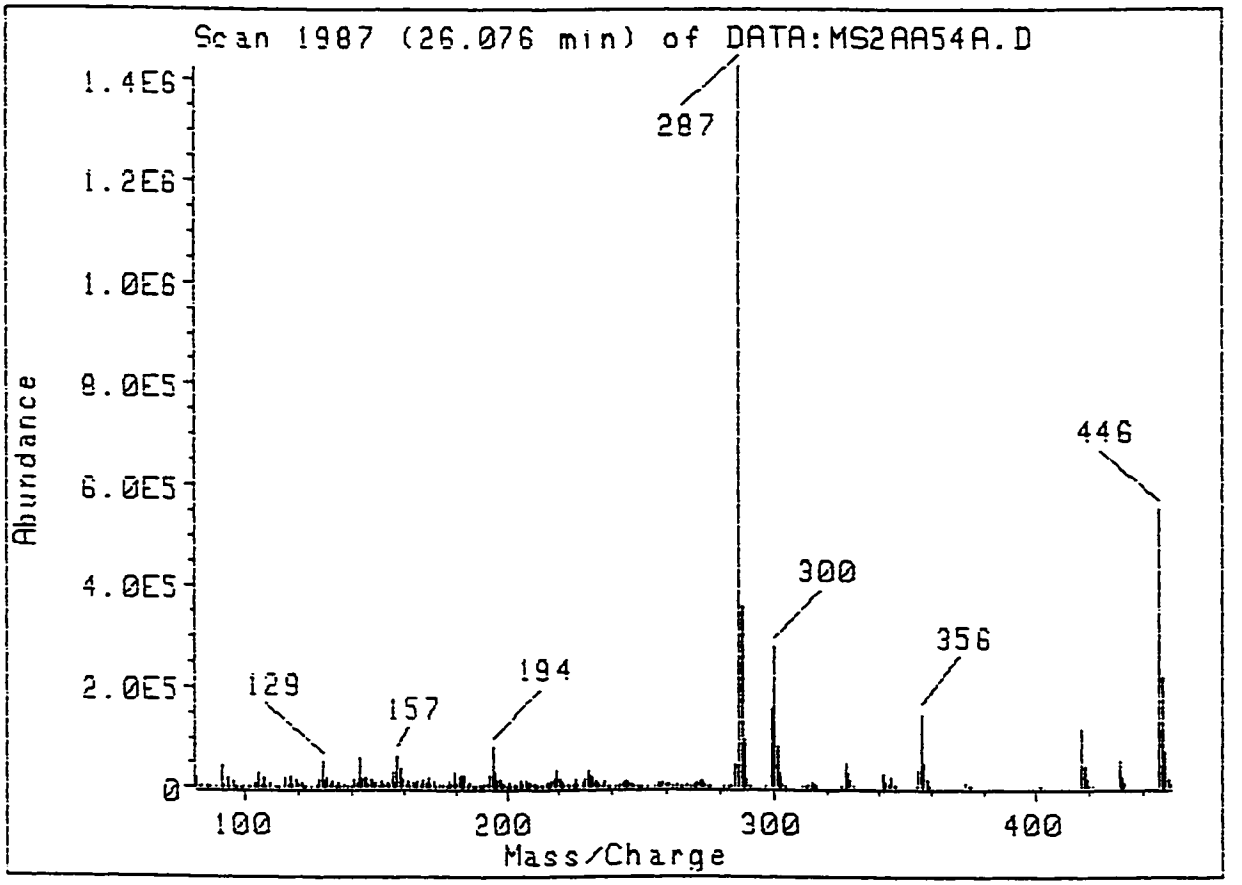


Figure 12. Full scan EI mass spectrum of norethandrolone -TMS derivative

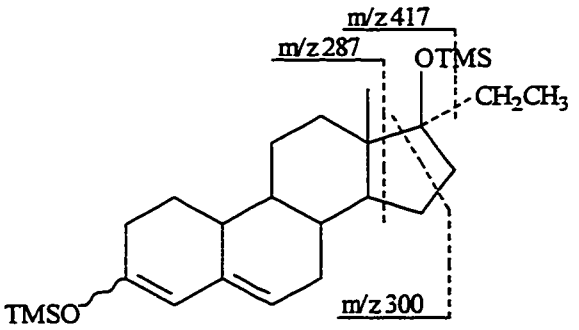


Figure 13. Fragmentation of norethandrolone

Based on the fragmentation patterns of the TMS derivatives of the metabolites a strategy was developed to analyze the full scan mass spectral data collected. Three characteristic fragment ions have been chosen for each different category of metabolites (number and position of hydroxy groups) and norethandrolone. By extracting the three ion chromatograms from the TIC and overlapping them it became easier to locate the peaks that have all representative ions present, therefore are the peaks of the metabolites. After searching the full range of the TIC a time window has been chosen for each category for the presentation of results. The ions chosen and time windows are shown in Table 6.

Table 6. Characteristic ions and time windows for ethylestrenol/norethandrolone metabolites

	ION (m/z)	ION (m/z)	ION (m/z)	Time window (min)
3, 17 – diol	144	157	421	21 – 25
3, 17, 21 – triol	217	245	421	25 – 29
3, 17, 16 –triol	217	245	509	25 – 29
Norethandrolone	287	300	446	24 – 28

5.A.1. Pre-dose monkey urine

Search for ethylestrenol/norethandrolone metabolites in the full scan mass spectral data of the pre-dose urine shows that no metabolites are present. When the characteristic ion chromatograms are extracted from the TIC we see only base line noise (Figure 14,15,16 and 17).

5.A.2. Ethylestrenol monkey metabolic urine (SP0003 M-F)

Extracted ion chromatograms of m/z 144, 157 and 421, Figure 18, show only one di-hydroxy metabolite present at retention time 22.726. The mass spectrum of this diol is shown in Figure19.

Peaks of the tri-hydroxy metabolites, hydroxylated in C-3,17 and 21 position, are located by extracting from the TIC ion profiles m/z 217, 245 and 421, shown in Figure 20. Two

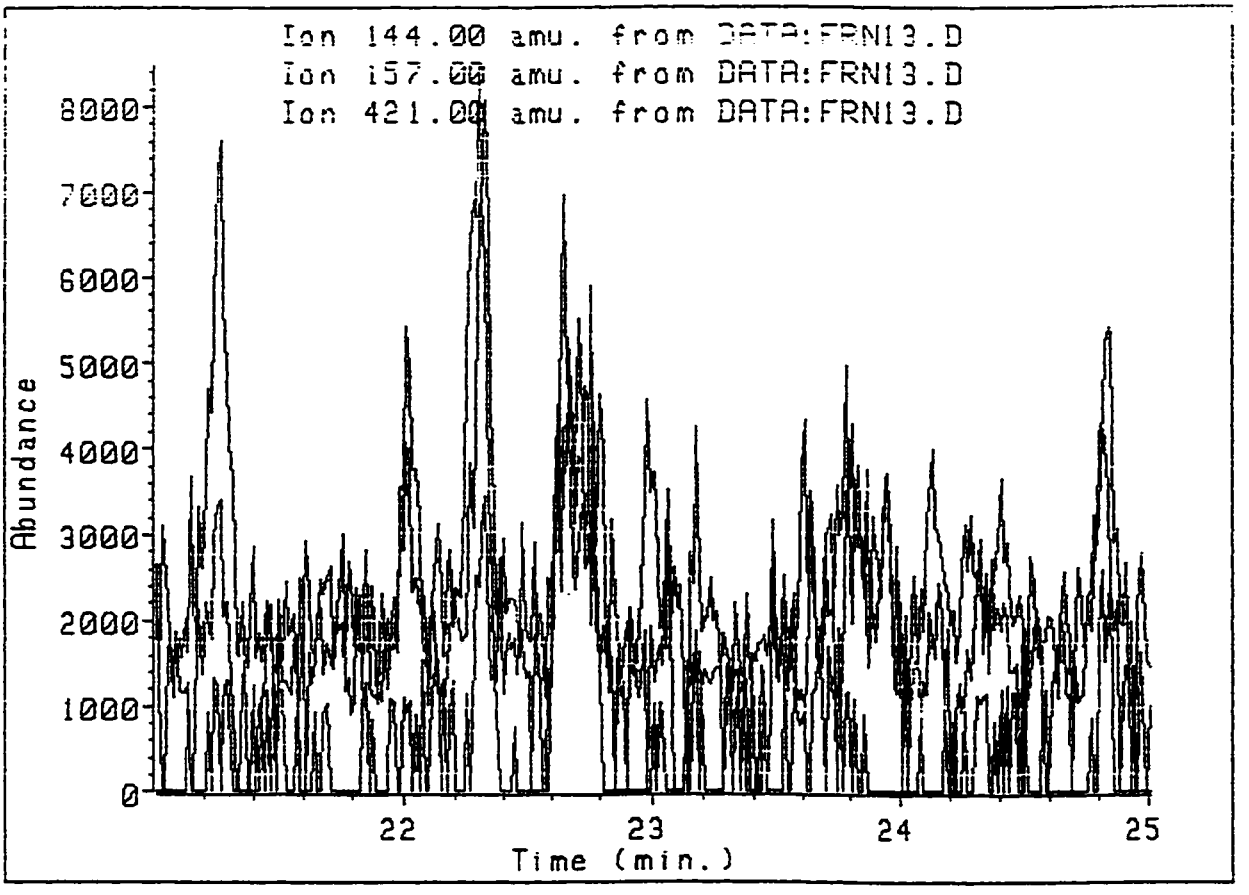


Figure 14. Extracted ion chromatograms m/z 144, 157 and 421 merged – pre-dose monkey urine full scan

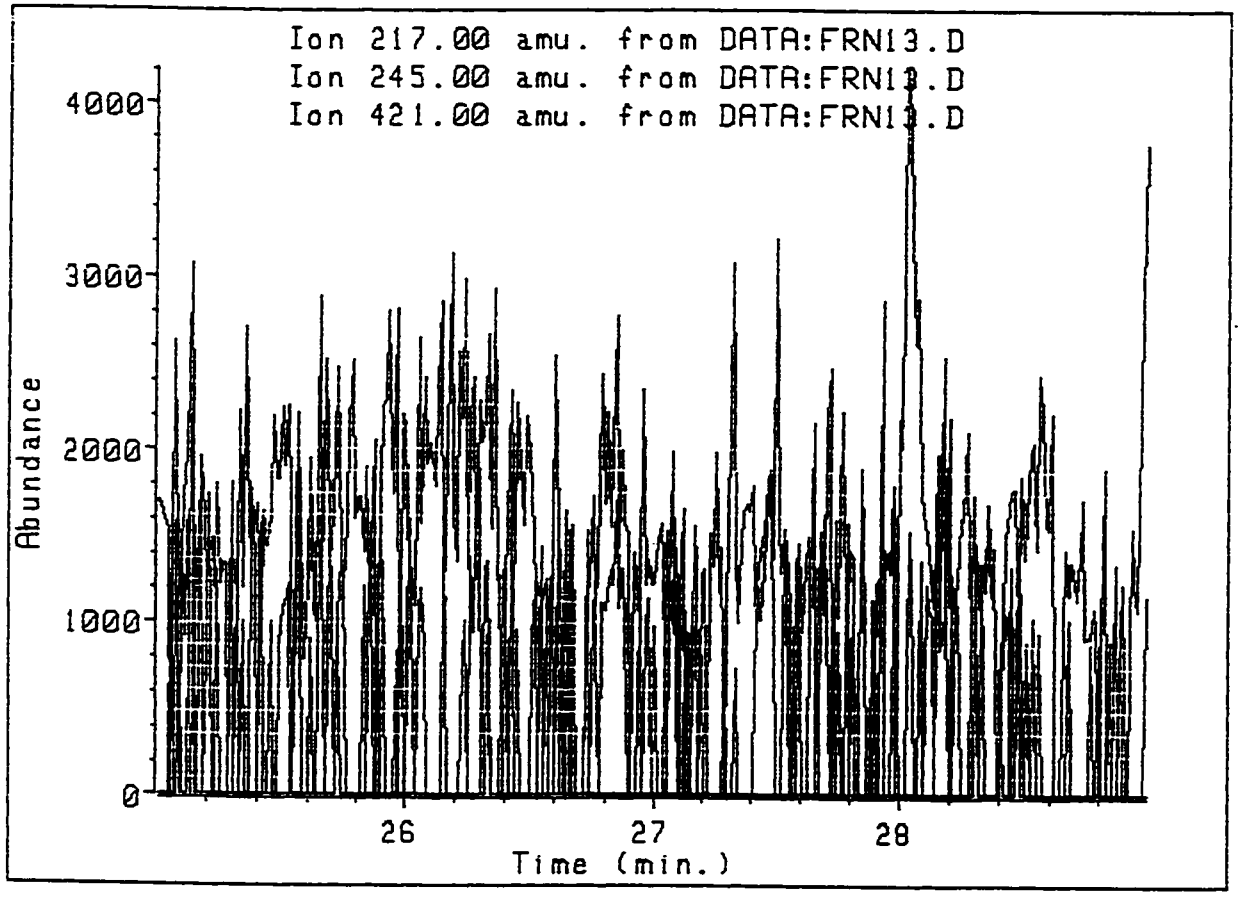


Figure 15. Extracted ion chromatograms m/z 217, 245 and 421 merged - pre-dose monkey urine full scan

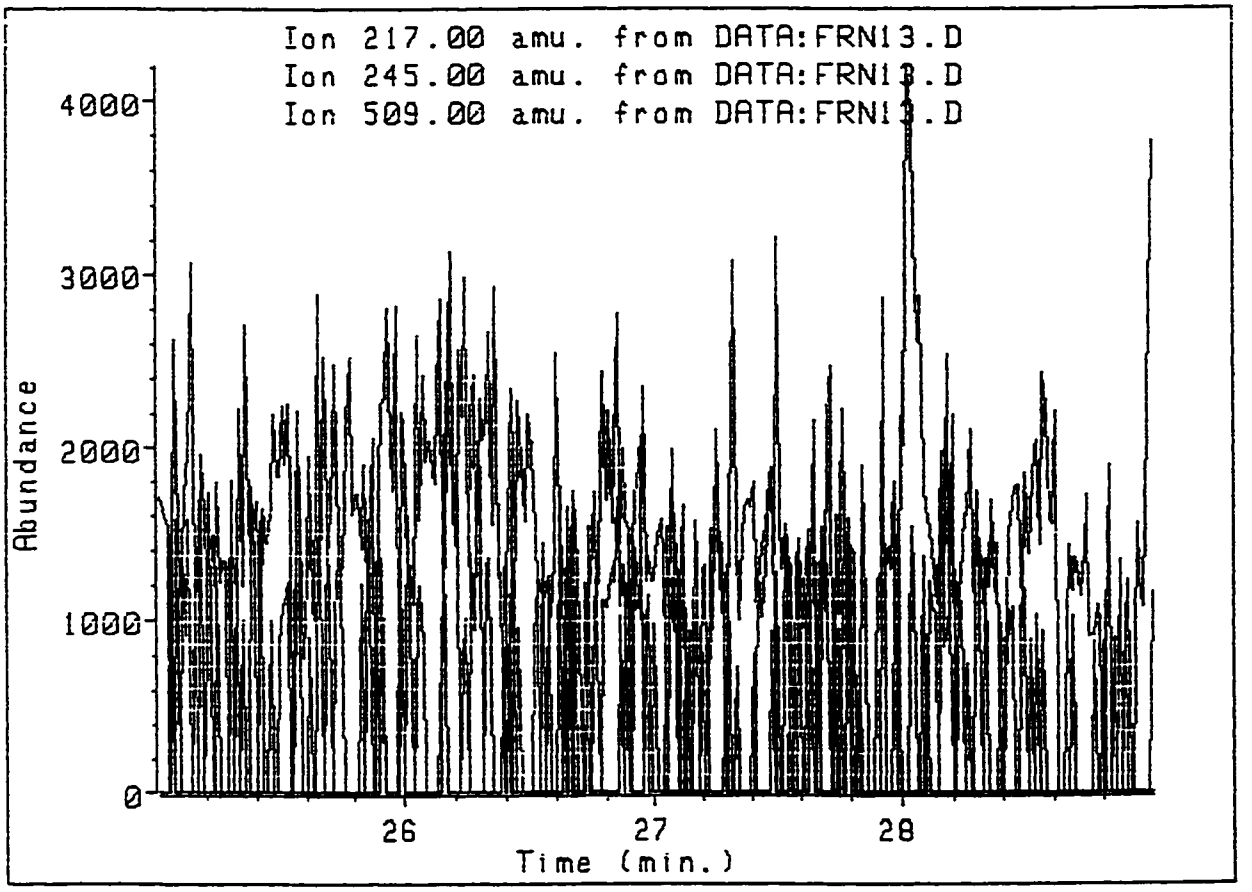


Figure 16. Extracted ion chromatograms m/z 217, 245 and 509 merged - pre-dose monkey urine full scan

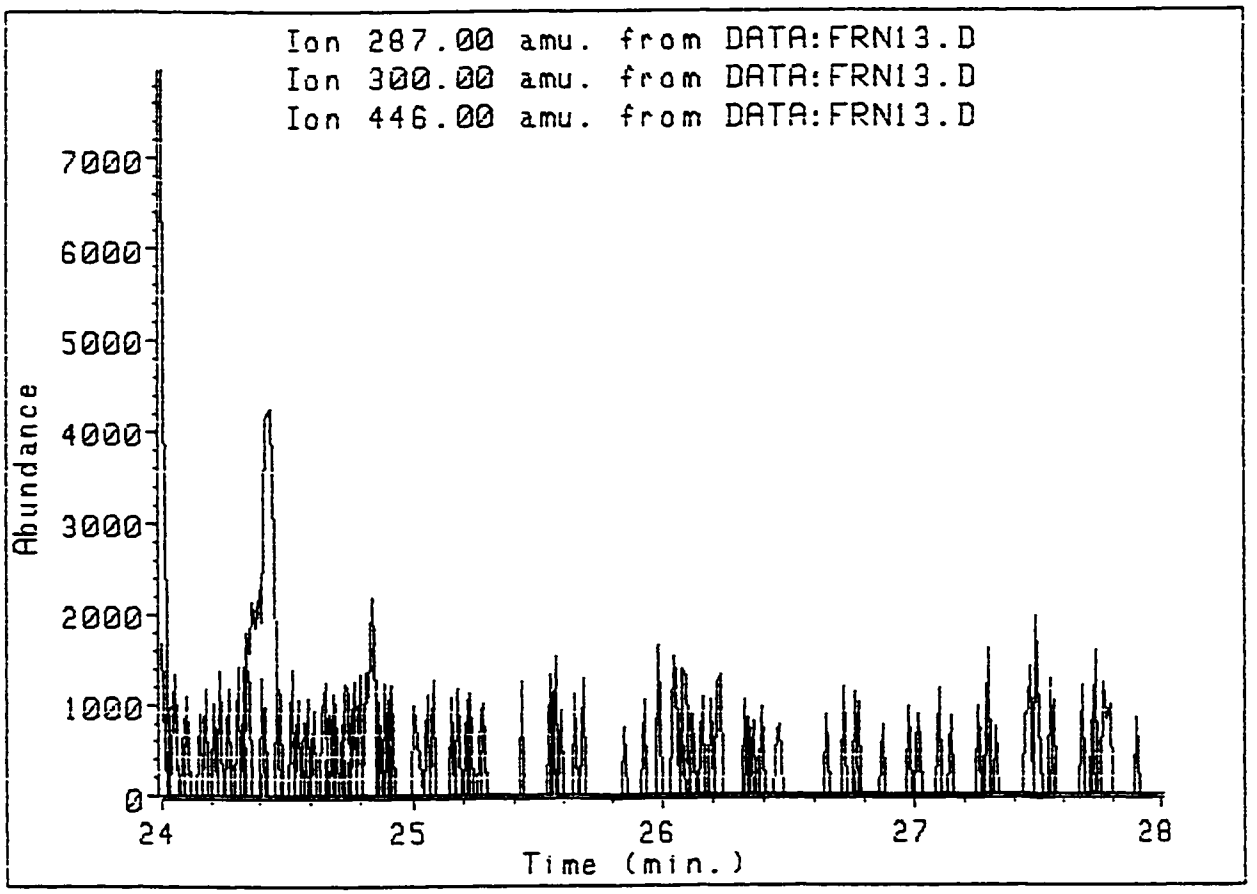


Figure 17. Extracted ion chromatograms m/z 287, 300 and 446 merged - pre-dose monkey urine full scan

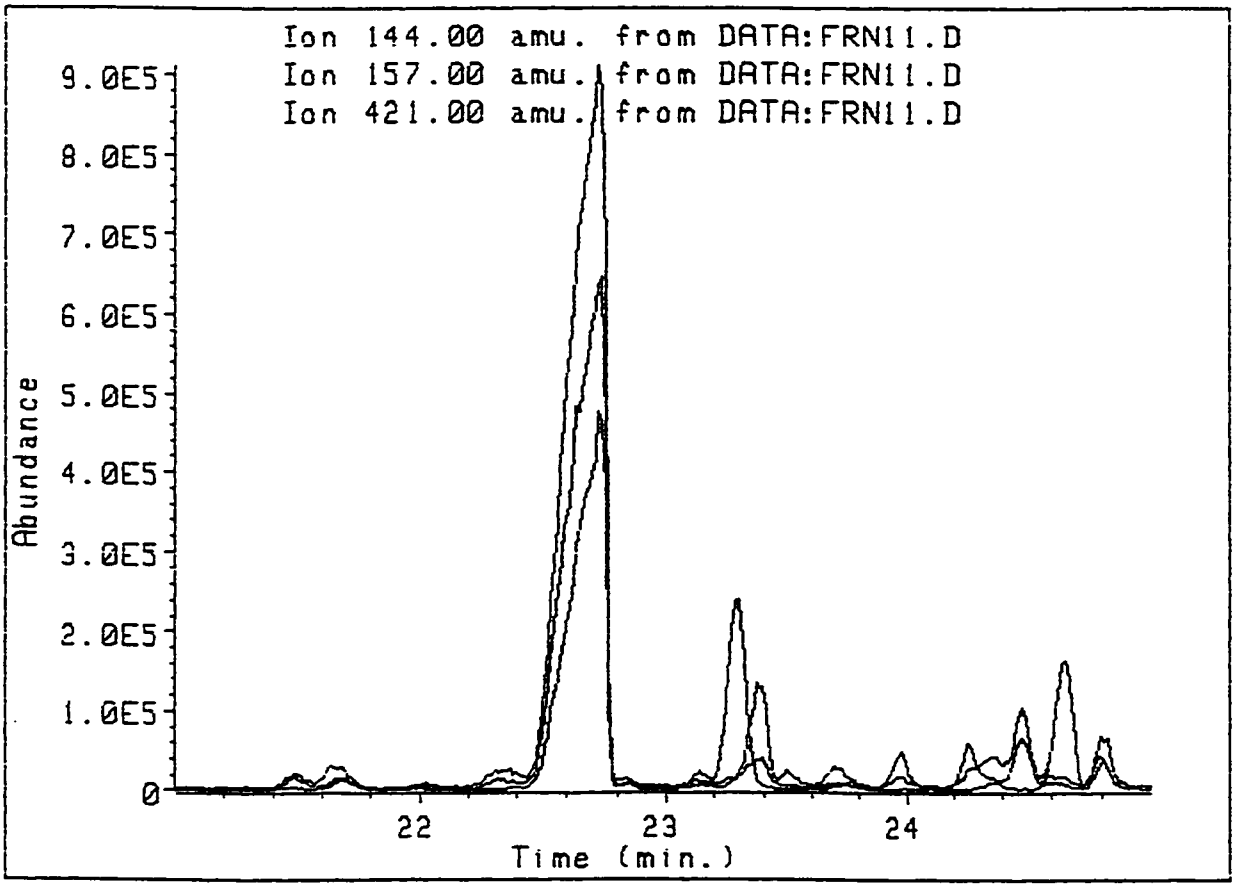


Figure 18. Extracted ion chromatograms m/z 144, 157 and 421 merged – (SP0003 M-F) ethylestrenol monkey urine full scan

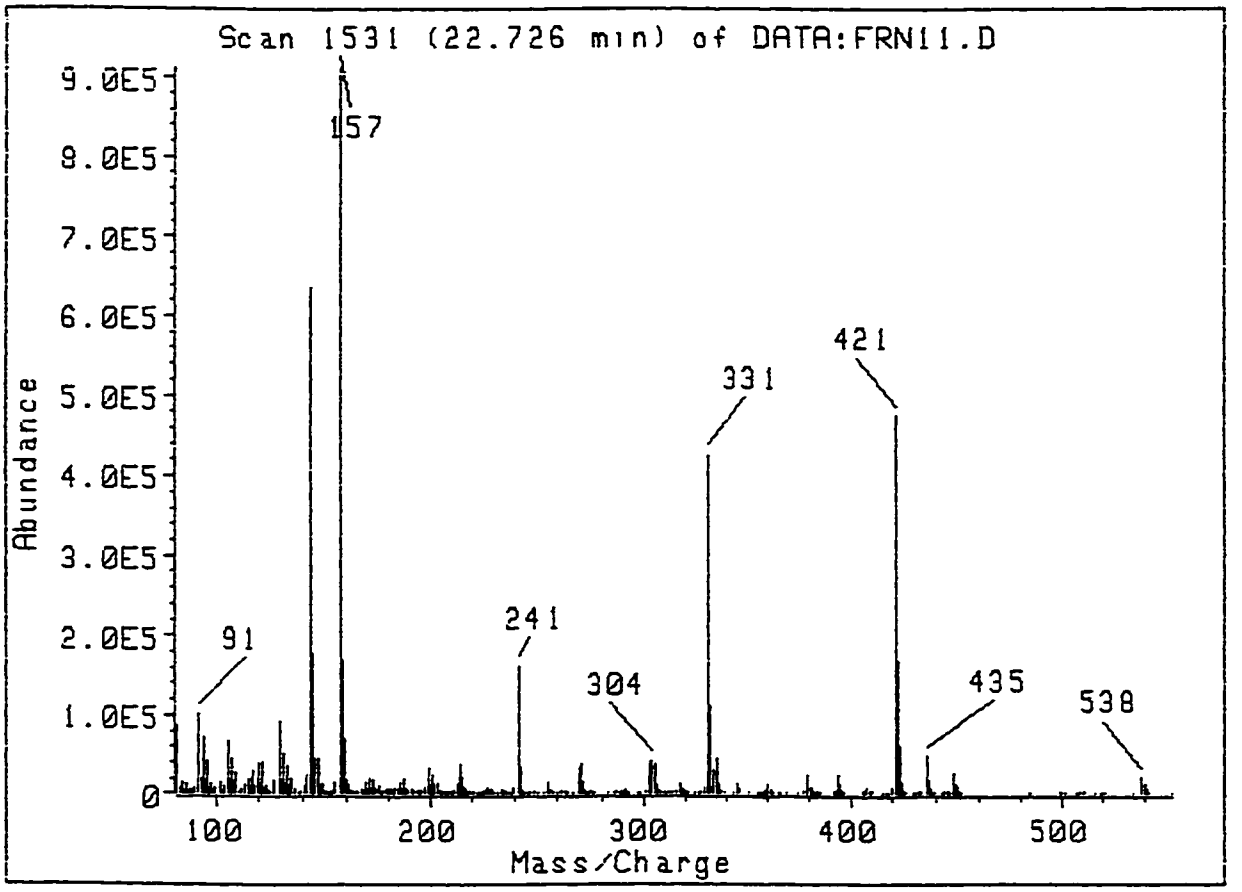


Figure 19. Di-hydroxy metabolite full scan mass spectrum – (SP0003 M-F) ethylestrenol monkey urine

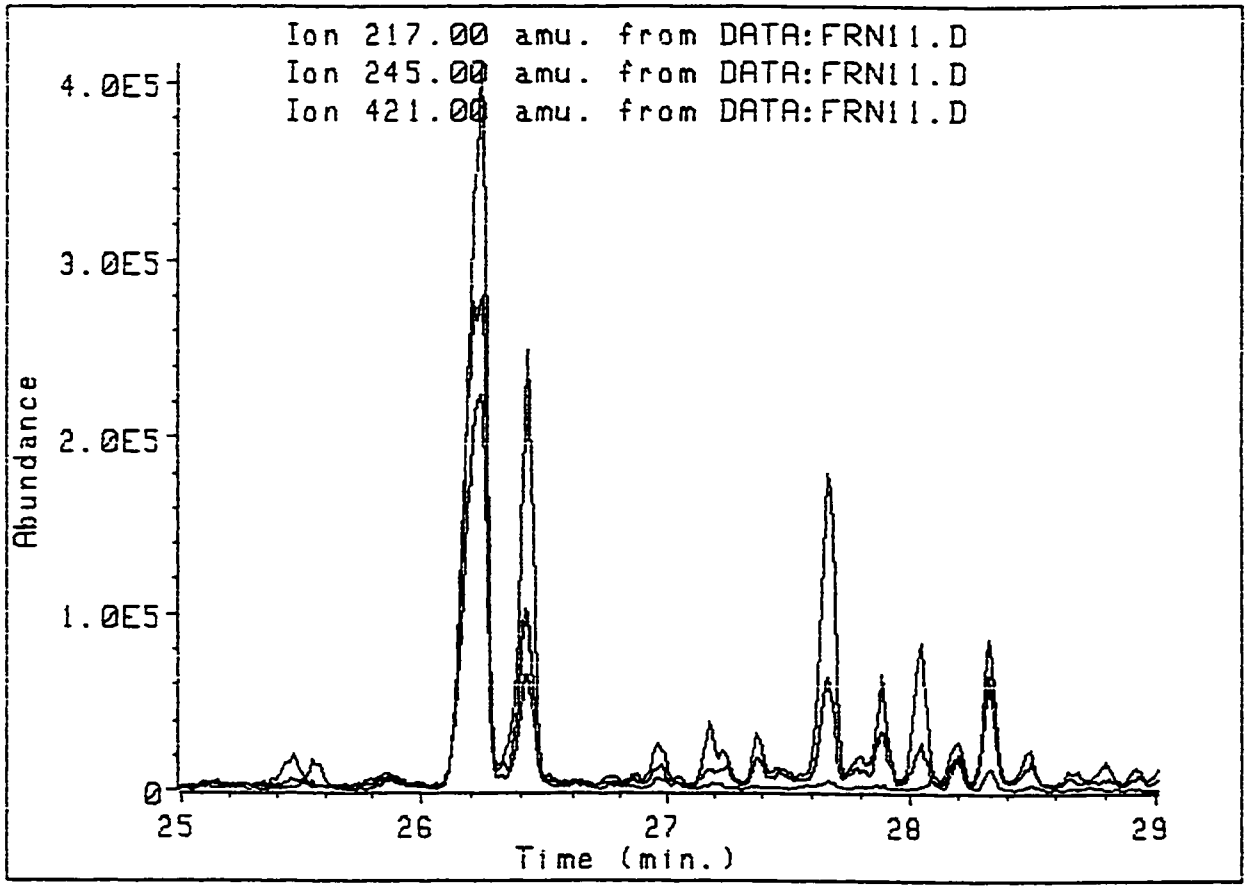


Figure 20. Extracted ion chromatograms m/z 217, 245 and 421 merged – (SP0003 M-F) ethylestrenol monkey urine full scan

prominent peaks have all three ions present and their mass spectra, the first one at retention time 26.234 and the second at 26.424, are shown in Figures 21 and 22.

The search for less abundant tri-hydroxy metabolites, with hydroxylation at the C-3, 17 and 16 positions, has been done by extracting ion profiles m/z 217, 245 and 509 from the TIC (Figure 23). In this monkey excretion urine we see one metabolite at retention time 27.882 and its mass spectrum is shown in Figure 24.

Norethandrolone peak was identified by extracting the ion chromatograms of m/z 287, 300 and 446 (Figure 25), at retention time 25.897 (mass spectrum in Figure 26).

To confirm that no ethylestrenol is excreted as parent the TIC was searched for ethylestrenol fragmentation ions m/z 241, 270 and 331, in the time window (13-17 minutes) where it is expected (Figure 27). The full scan mass spectrum of TMS derivative of ethylestrenol is shown in Figure 28.

5.A.3. Norethandrolone human metabolic urine

Ion chromatograms m/z 144, 157 and 421 in time window 21-25 minutes (Figure 29) show that there are two diols present at retention times 21.669 and 22.526. Their mass spectra are shown in Figure 30 and 31.

Figure 32 represents the search for C-3, 17, 21-tri-hydroxy metabolites, resulting in two metabolites at retention times 26.176 and 26.396. The mass spectra are shown in Figure 33 and 34.

One C-3, 17, 16-tri-hydroxy metabolite was detected at retention time 28.488 in extracted ion profiles in Figure 35, and its mass spectrum is shown in Figure 36.

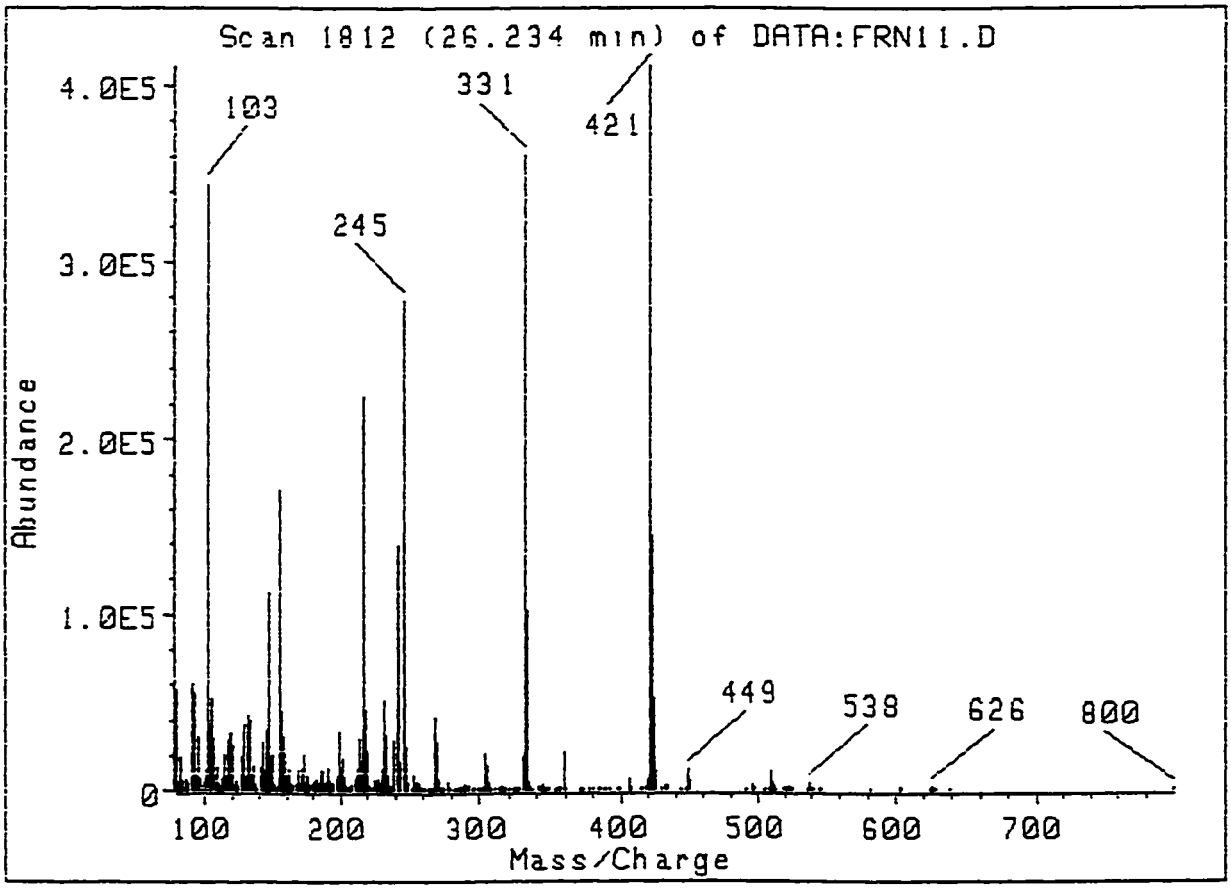


Figure 21. 3,17,21-Tri-hydroxy metabolite full scan mass spectrum – (SP0003 M-F) ethylestrenol monkey urine

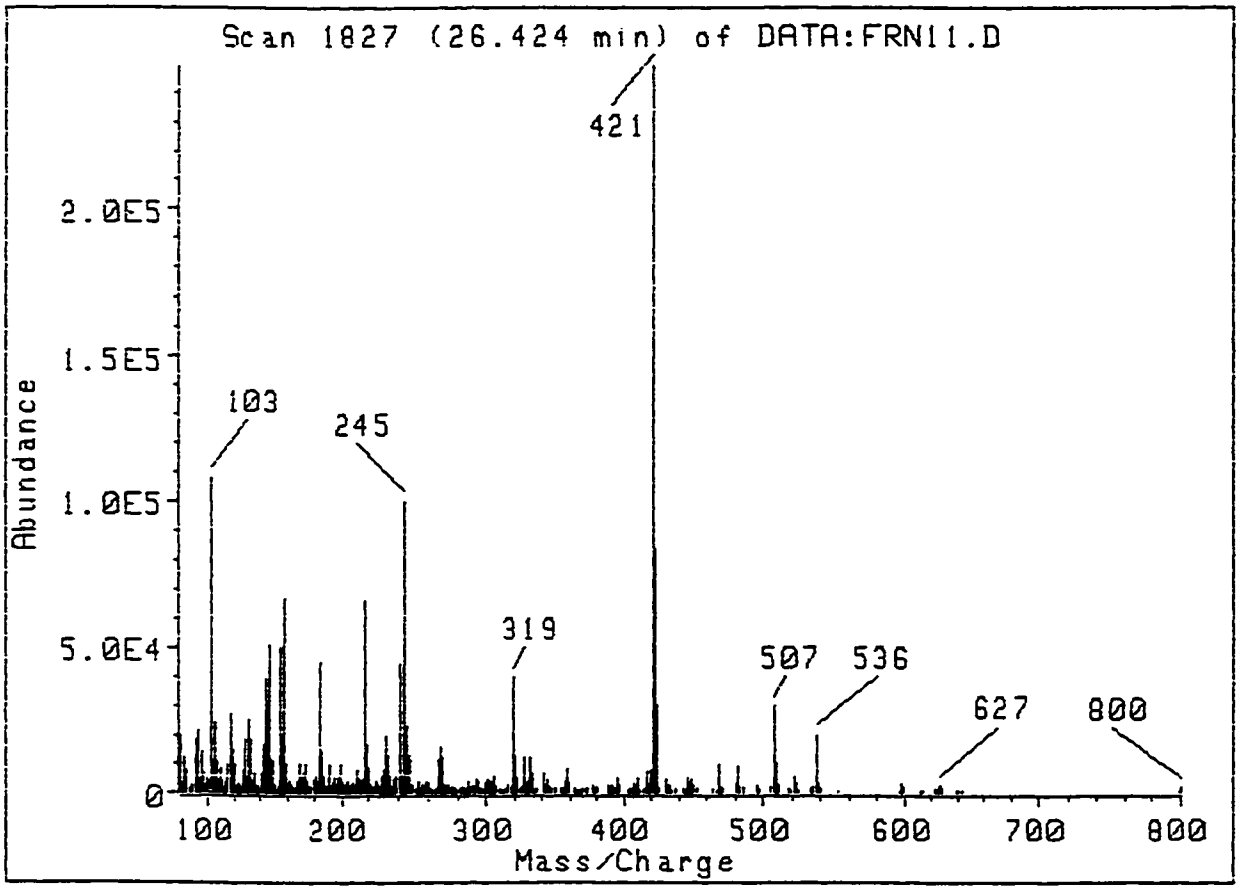


Figure 22. 3,17,21-Tri-hydroxy metabolite full scan mass spectrum – (SP0003 M-F) ethylestrenol monkey urine

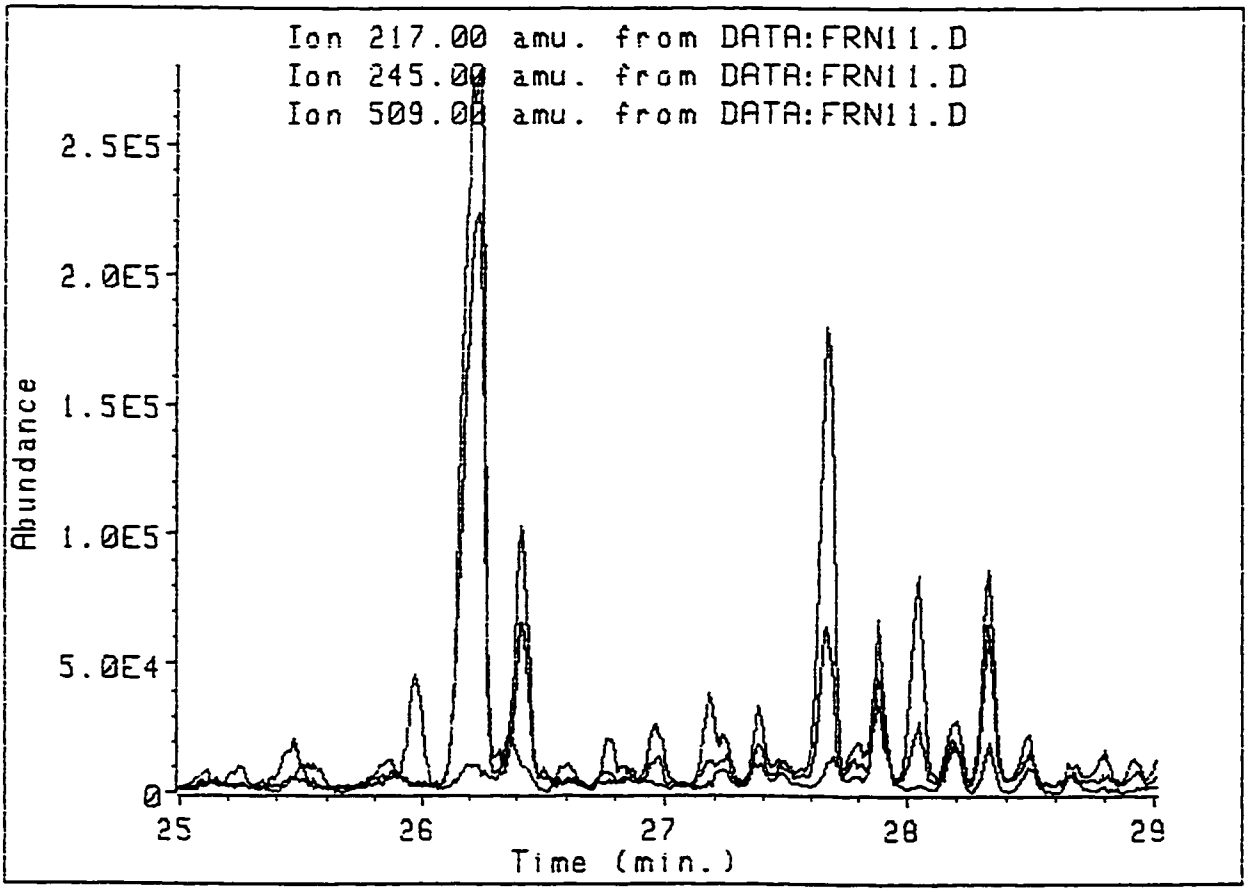


Figure 23. Extracted ion chromatograms m/z 217, 245 and 509 merged – (SP0003 M-F) ethylestrenol monkey urine full scan

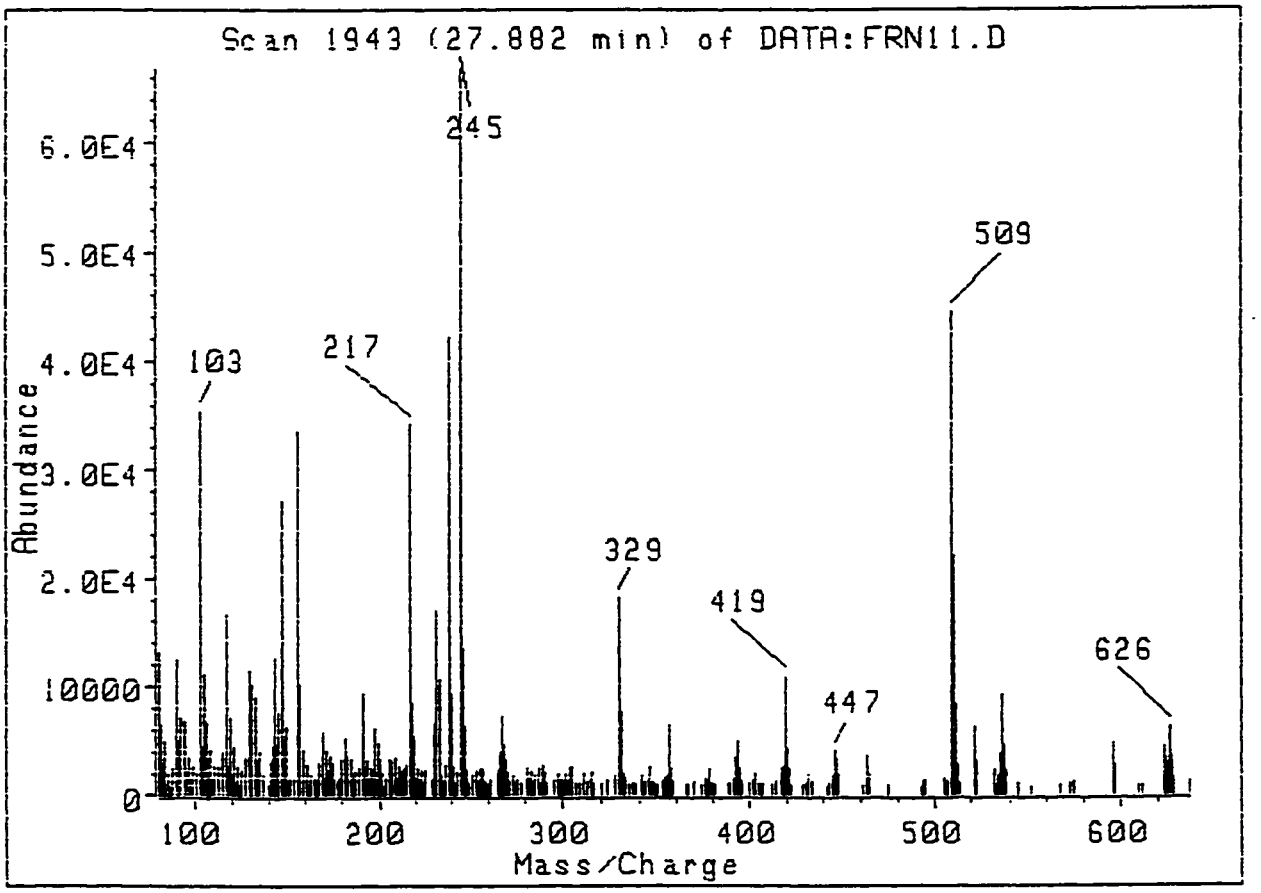


Figure 24. 3,16,17-Tri-hydroxy metabolite full scan mass spectrum – (SP0003 M-F) ethylestrenol monkey urine

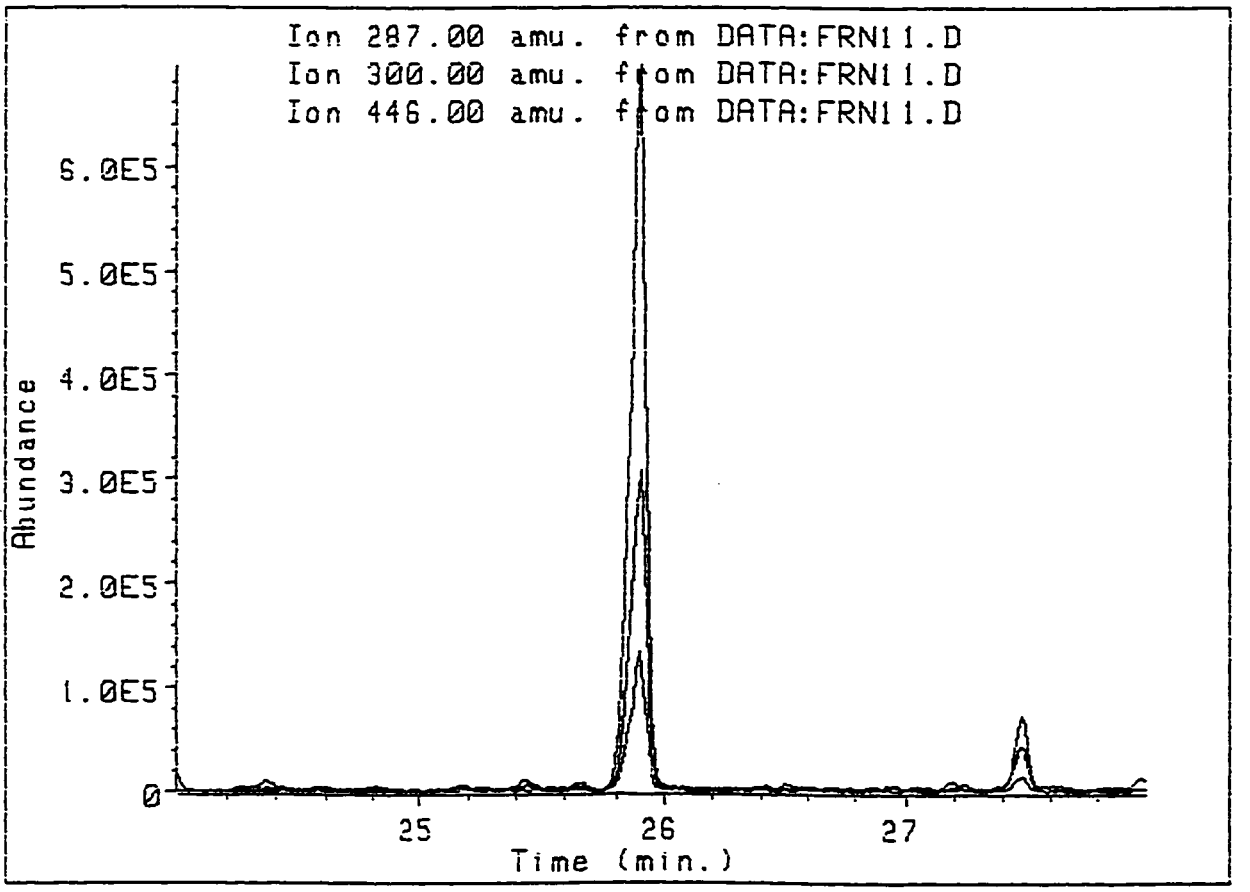


Figure 25. Extracted ion chromatograms m/z 287, 300 and 446 merged – (SP0003 M-F) ethylestrenol monkey urine full scan

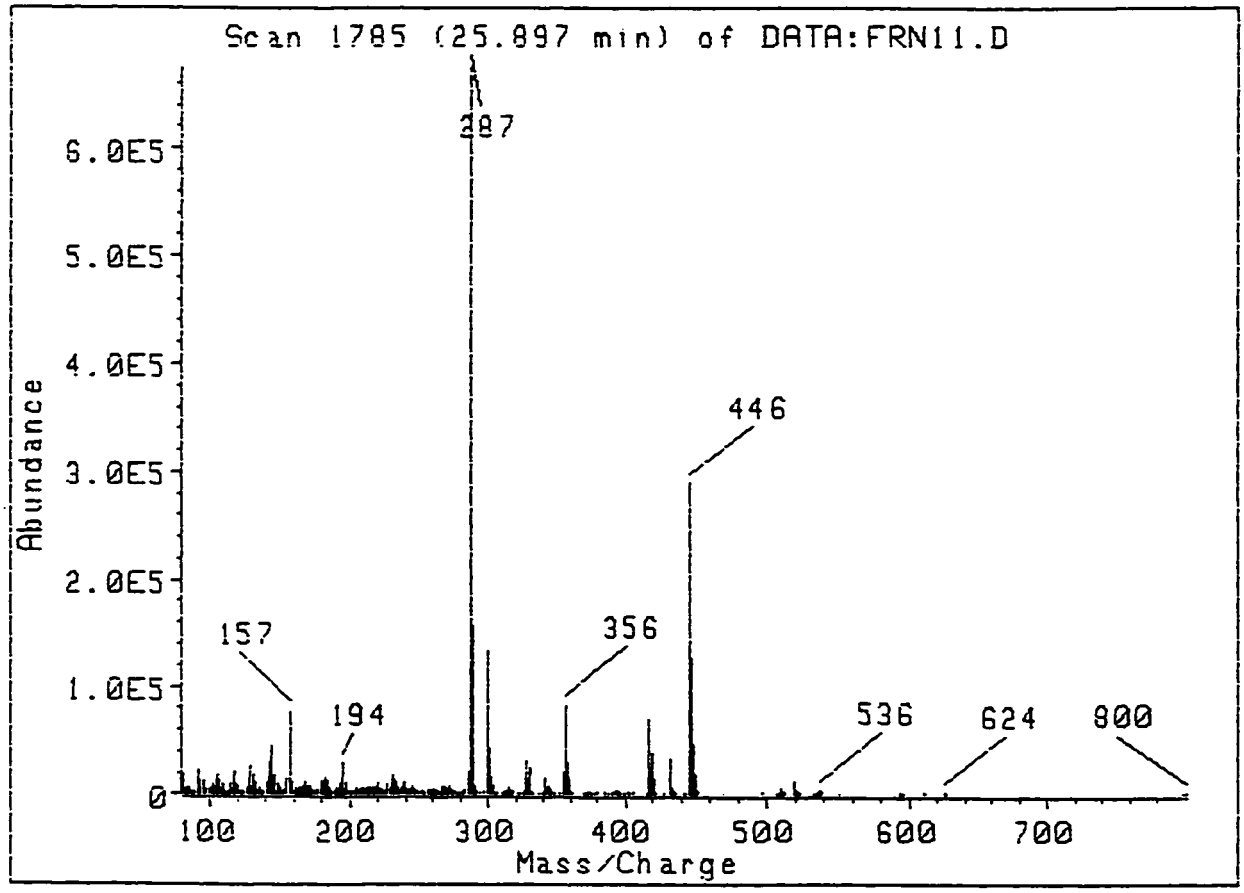


Figure 26. Norethandrolone full scan mass spectrum – (SP0003 M-F) ethylestrenol monkey urine

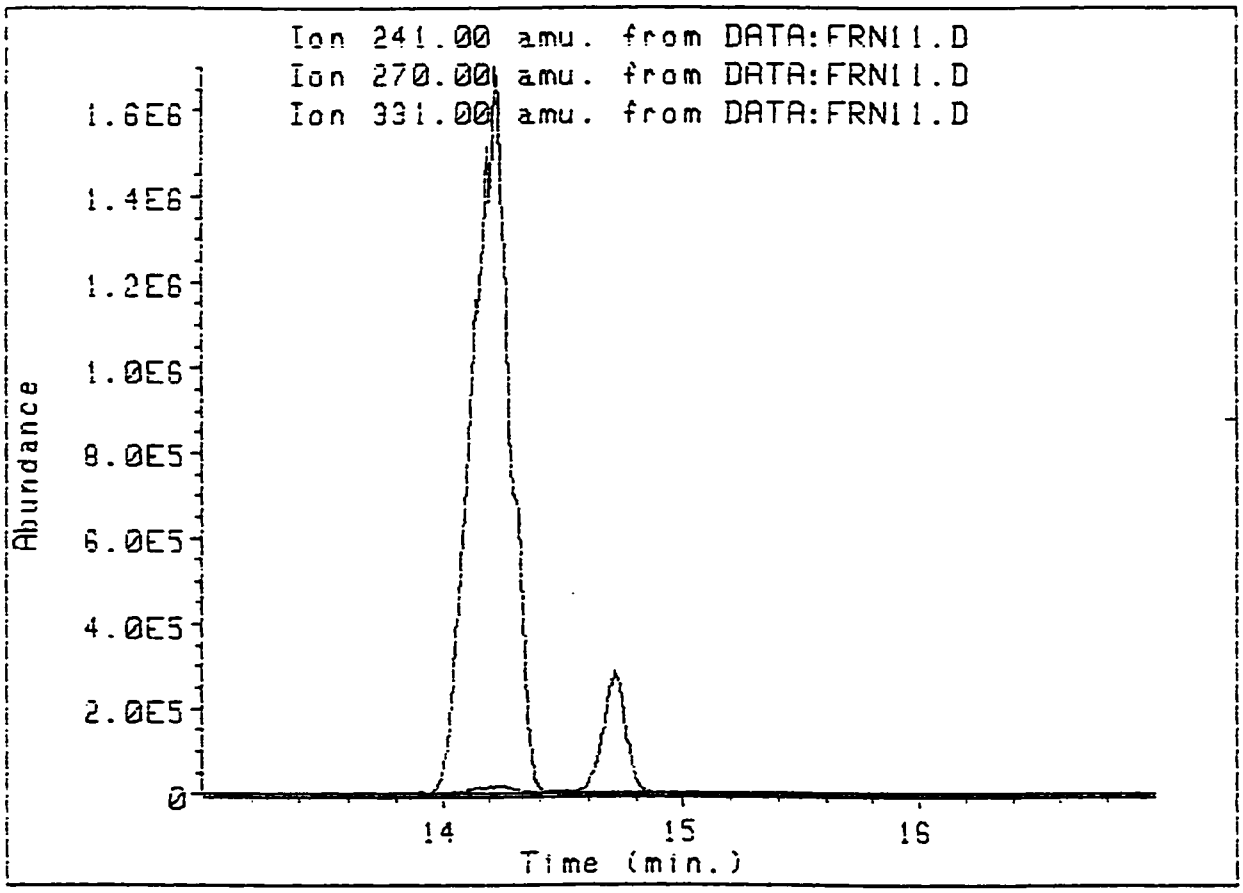


Figure 27. Extracted ion chromatograms m/z 241, 270 and 331 merged – (SP0003 M-F) ethylestrenol monkey urine full scan

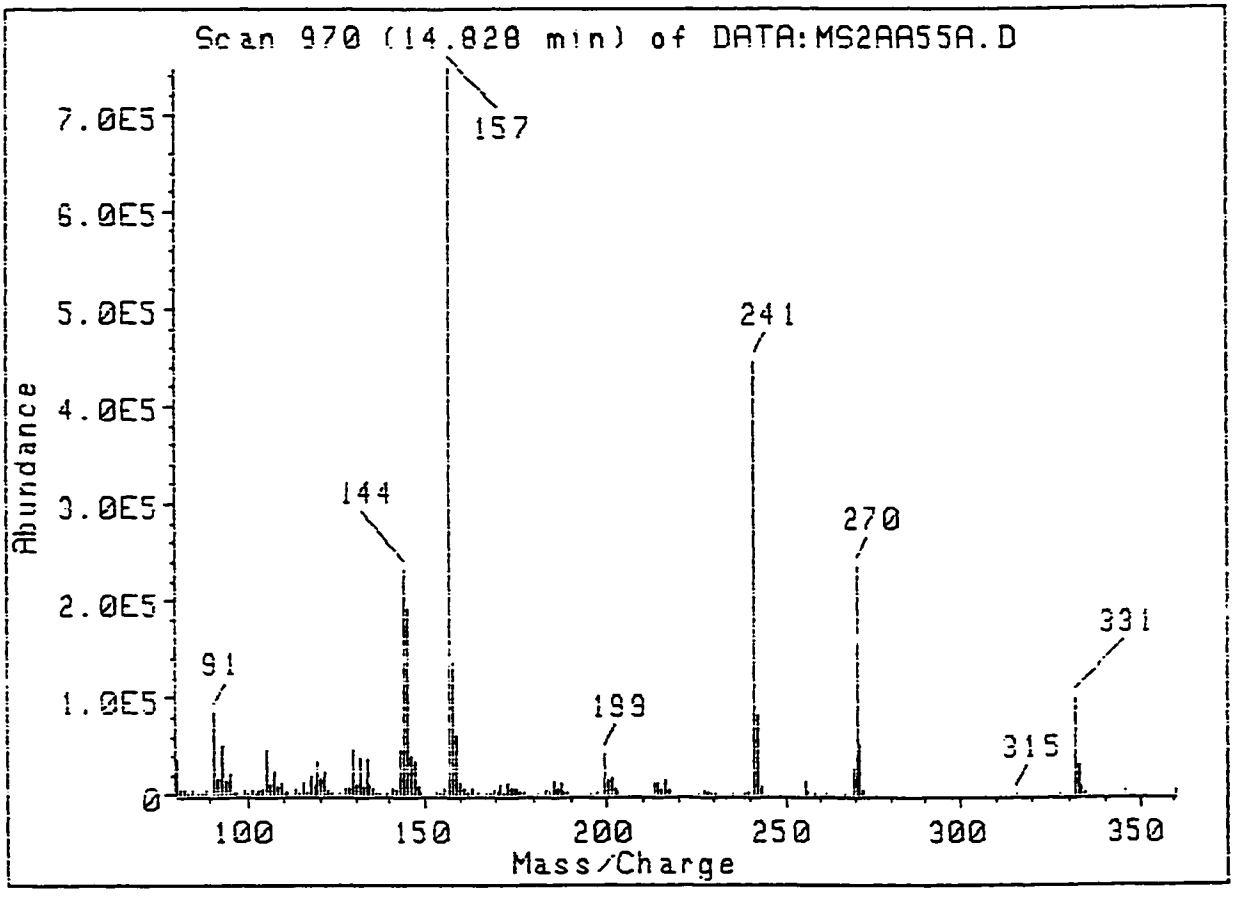


Figure 28. Full scan mass spectrum of ethylestrenol – TMS derivative

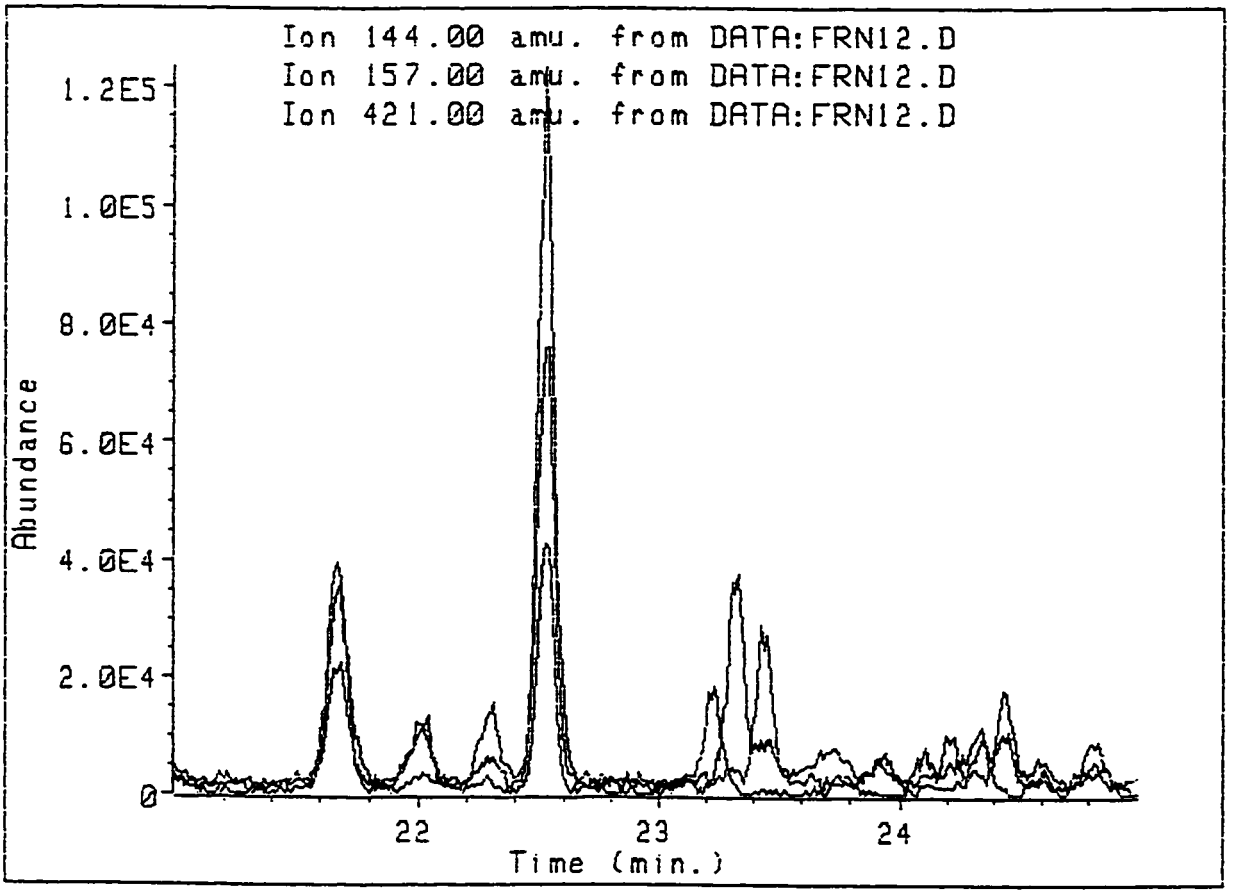


Figure 29. Extracted ion chromatograms m/z 144, 157 and 421 merged – norethandrolone human urine full scan

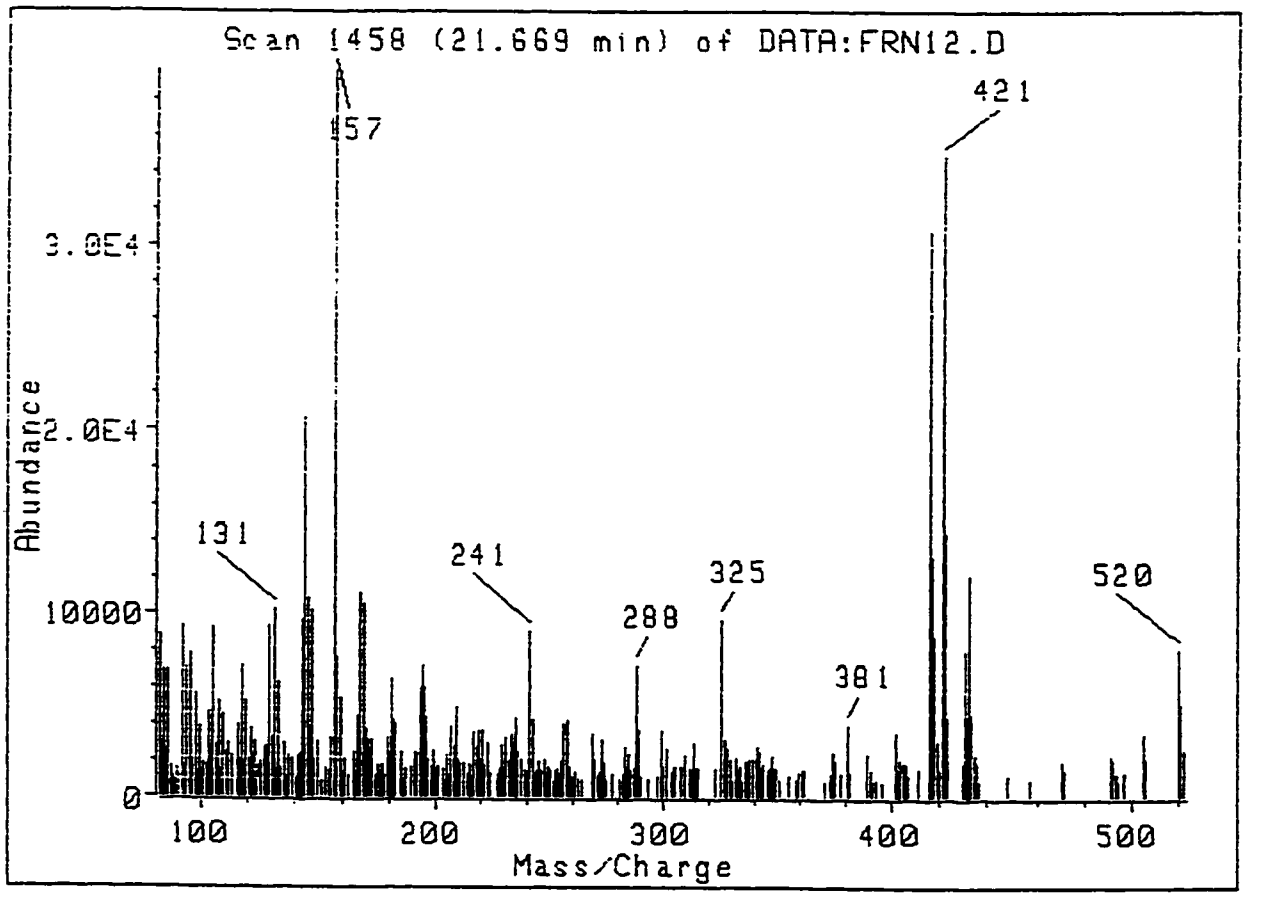


Figure 30. Di-hydroxy metabolite full scan mass spectrum – norethandrolone human urine

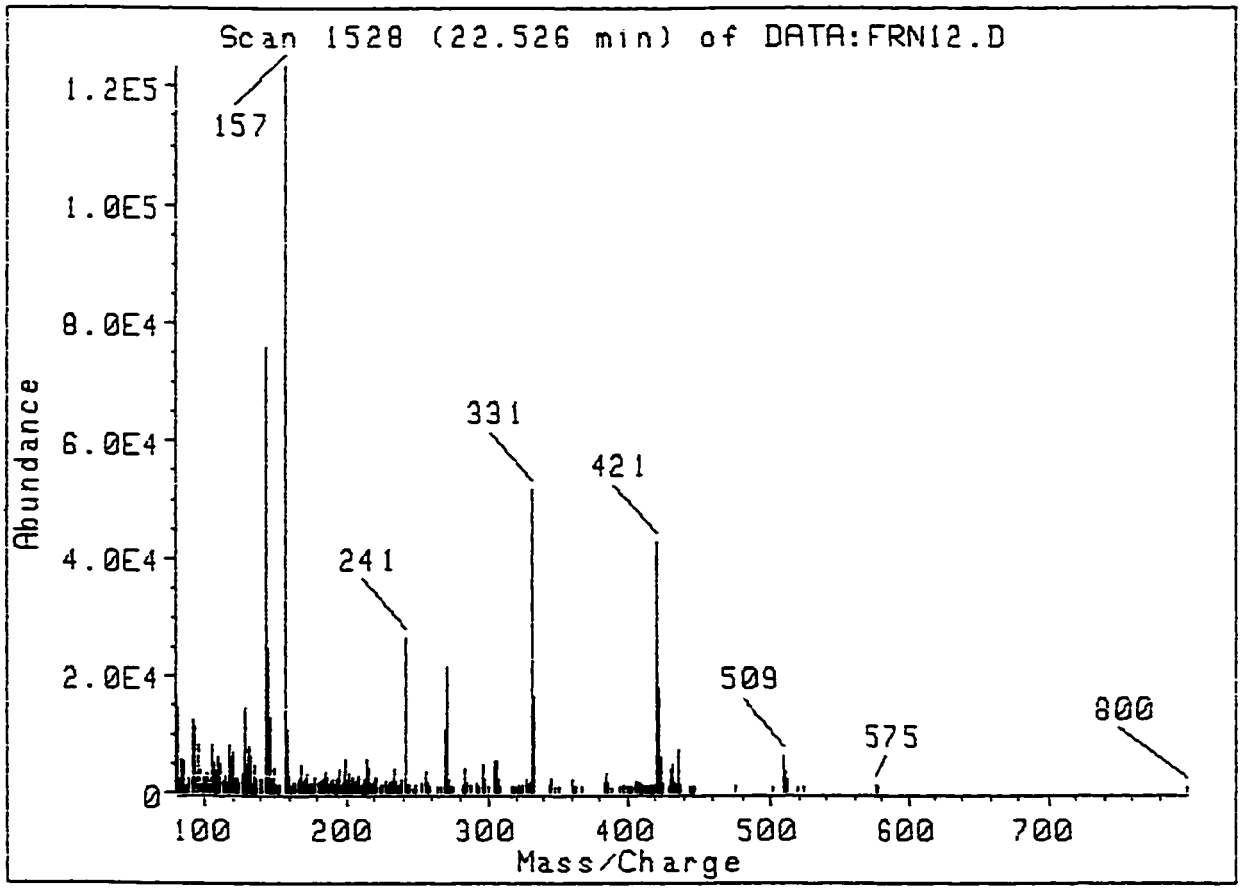


Figure 31. Di-hydroxy metabolite full scan mass spectrum – norethandrolone human urine

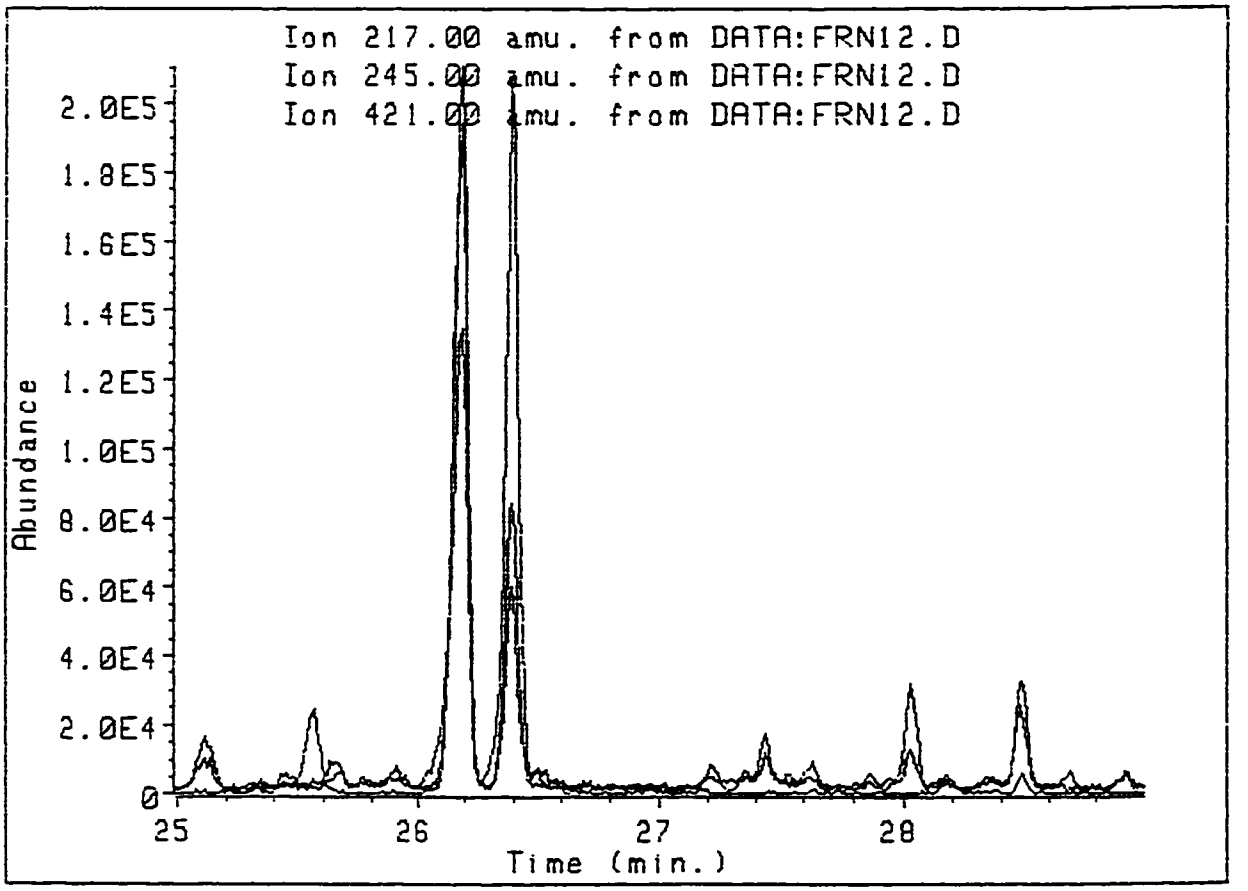


Figure 32. Extracted ion chromatograms m/z 217, 245 and 421 merged – norethandrolone human urine full scan

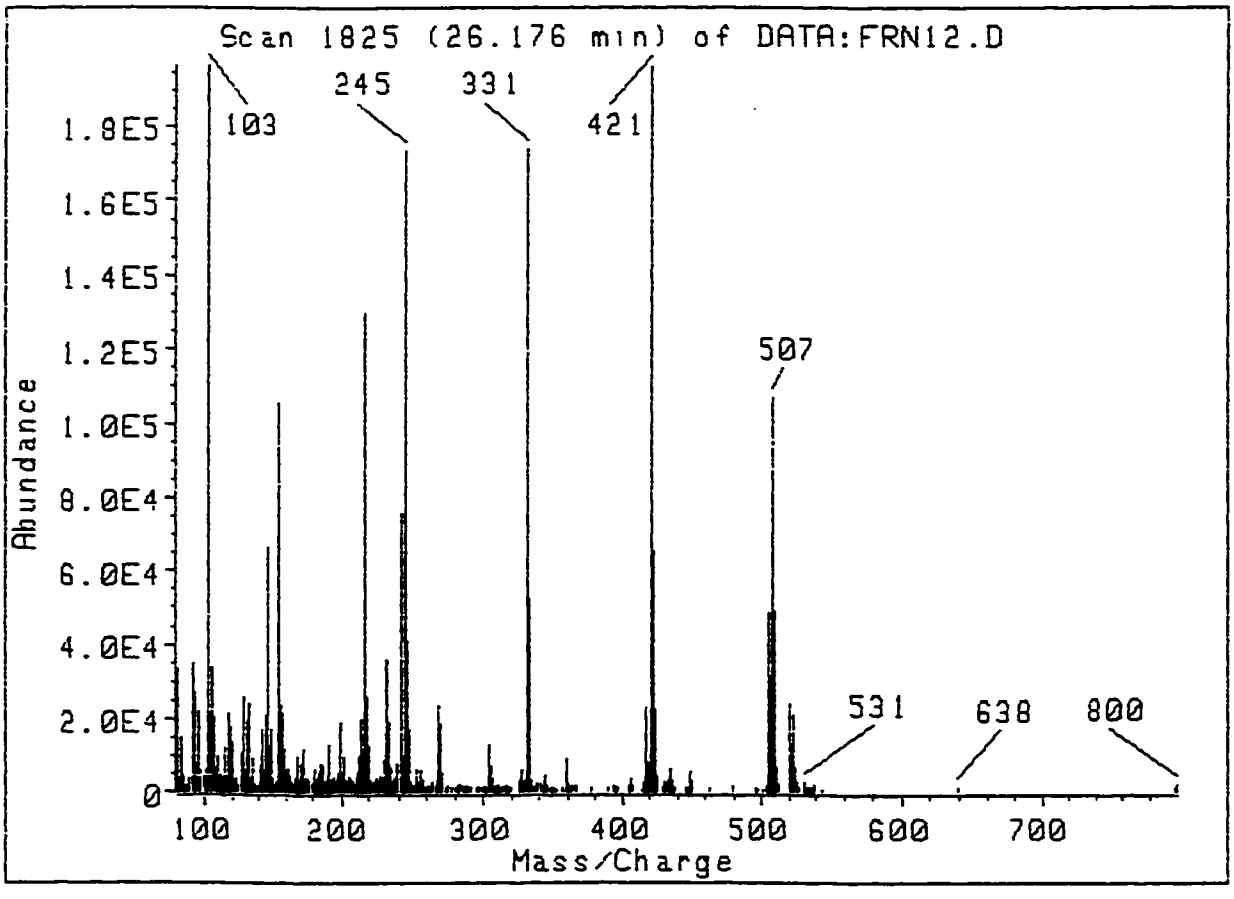


Figure 33. 3,17,21-Tri-hydroxy metabolite full scan mass spectrum – norethandrolone human urine

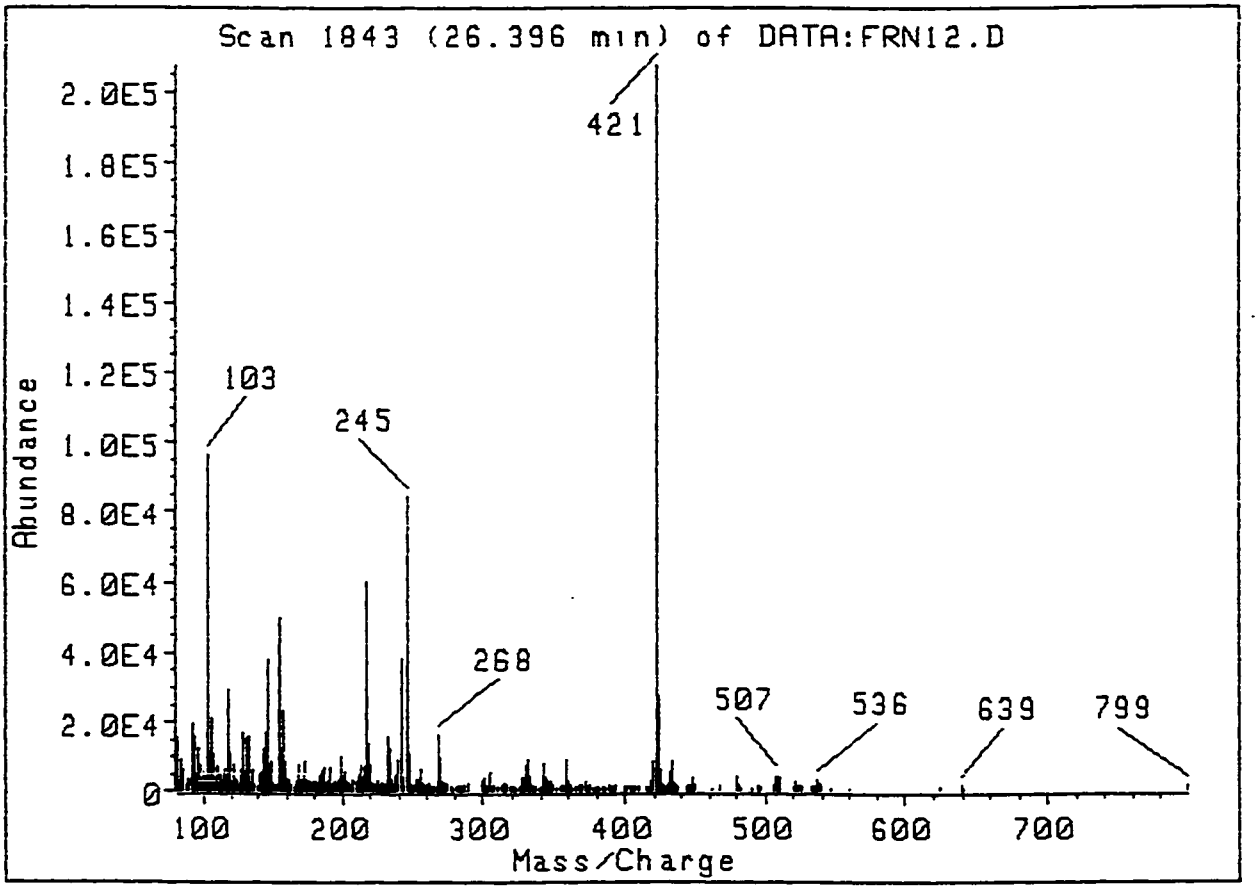


Figure 34. 3,17,21-Tri-hydroxy metabolite full scan mass spectrum – norethandrolone human urine

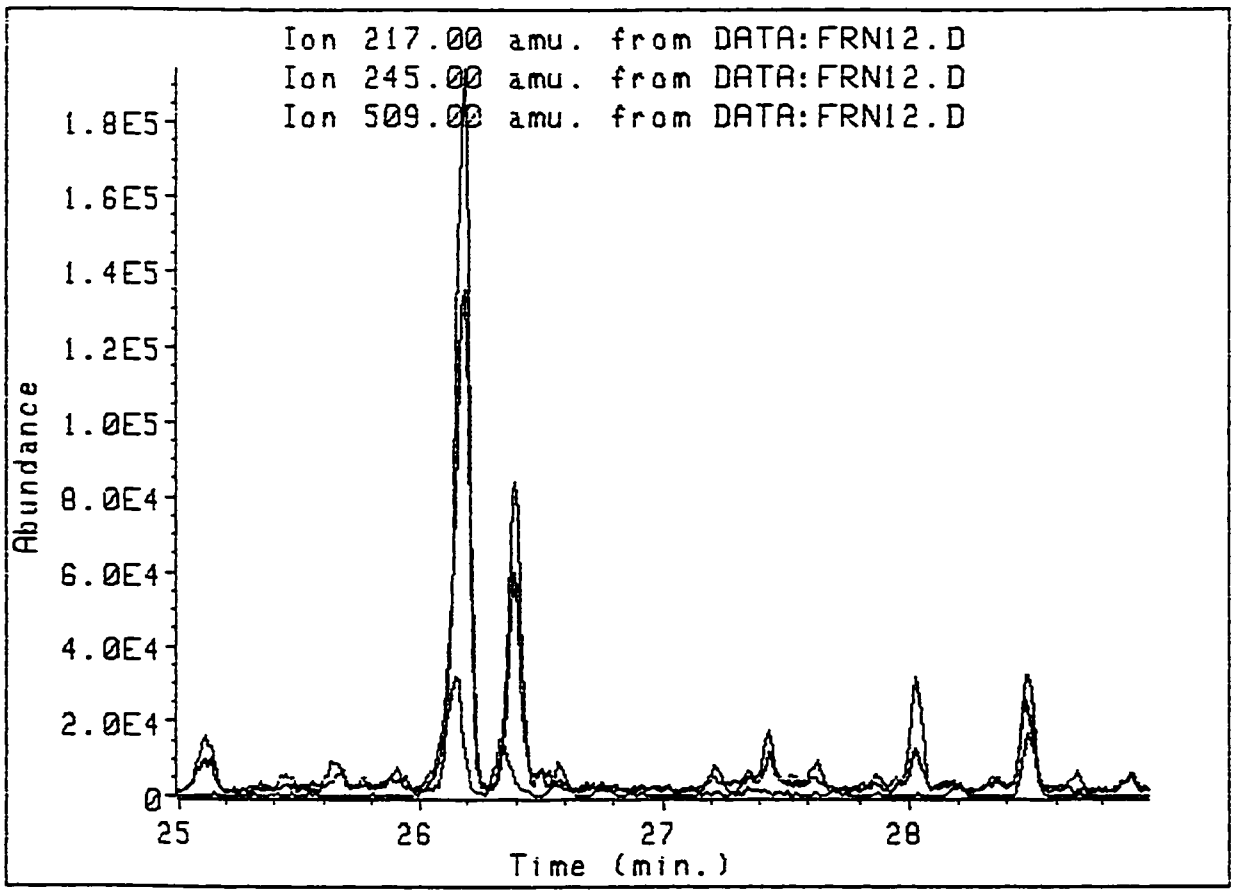


Figure 35. Extracted ion chromatograms m/z 217, 245 and 509 merged – norethandrolone human urine full scan

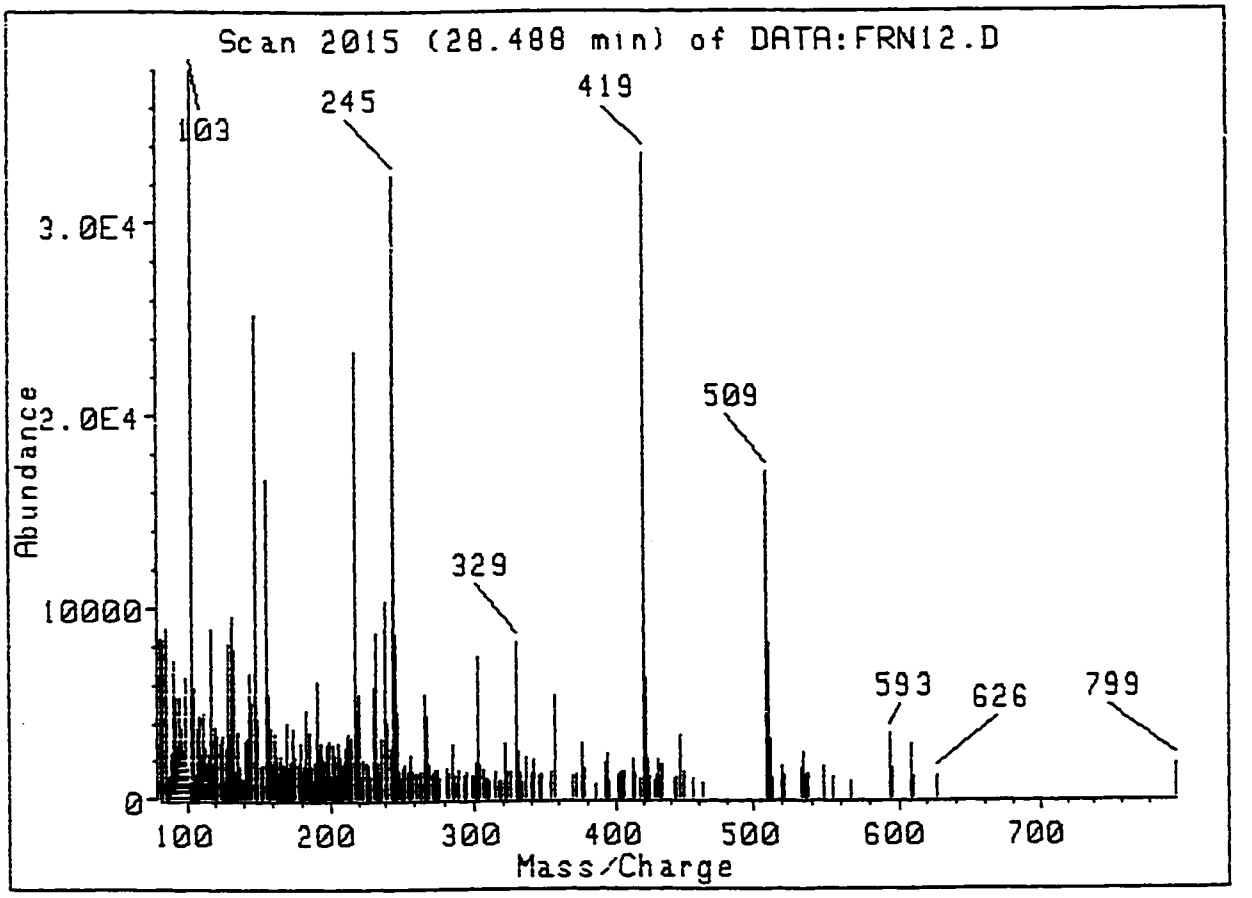


Figure 36. 3,16,17-Tri-hydroxy metabolite full scan mass spectrum – norethandrolone human urine

Fragment ion m/z 287, 300 and 446 chromatograms in Figure 37 indicate that peak at 25.878 with mass spectrum in Figure 38 is norethandrolone.

5.A.4. Norethandrolone monkey metabolic urine

Di-hydroxy metabolite search (Figure 39) identifies three metabolites at retention times 21.702, 22.643 and 22.796, with mass spectra shown in Figure 40, 41 and 42.

In this urine there are two C-3, 17, 21-tri-hydroxy metabolites (Figure 43) at retention times 26.236 and 26.458 and mass spectra shown in Figure 44 and 45.

Also, there is no C-3, 17, 16-tri-hydroxy metabolite (Figure 46).

Norethandrolone is found at retention time 25.923 (Figure 47) and its mass spectrum is shown in Figure 48.

5.A.5. Other ethylestrenol monkey metabolic urine

The metabolic pattern also changes depending on the dose of the anabolic steroid administered, length of administration, and time of collection of excretion urine. GC/MS analysis of ethylestrenol excretion urine from different time collection in the week and over the four-month period of administration of ethylestrenol has been done (different 3L pools of the M-F and S-S collection – see 4.2.).

Over the four months period six pools were collected as described in section 4.2. They were labeled:

1. SP0001 M-F

2. SP0002 S-S
3. SP0003 M-F
4. SP0004 S-S
5. SP0005 M-F
6. SP0006 M-F

In section 5.A.2. results of the GC/MS analysis of SP0003 M-F ethylestrenol excretion urine were presented. The major metabolites observed in all of the pools collected are the same as in SP0003 M-F. However, the presence and abundance of these metabolites varies from urine to urine. To present these differences in metabolic pattern, the extracted ion chromatograms (of representative ions) in specified windows for the search of different category of metabolites are shown in figures listed in Table 7. Furthermore, we see some additional minor metabolites in urine SP0005 M-F. The mass spectra of these minor metabolites are shown in Figures 69-74.

Table 7. Metabolite search figures

Excretion urine	3,17-diol search	3,17,21-triol search	3,17,16-triol search	Norethandrolone search
SP0001 M-F	Figure 49	Figure 50	Figure 51	Figure 52
SP0002 S-S	Figure 53	Figure 54	Figure 55	Figure 56
SP0004 S-S	Figure 57	Figure 58	Figure 59	Figure 60
SP0005 M-F	Figure 61	Figure 62	Figure 63	Figure 64
SP0006 M-F	Figure 65	Figure 66	Figure 67	Figure 68

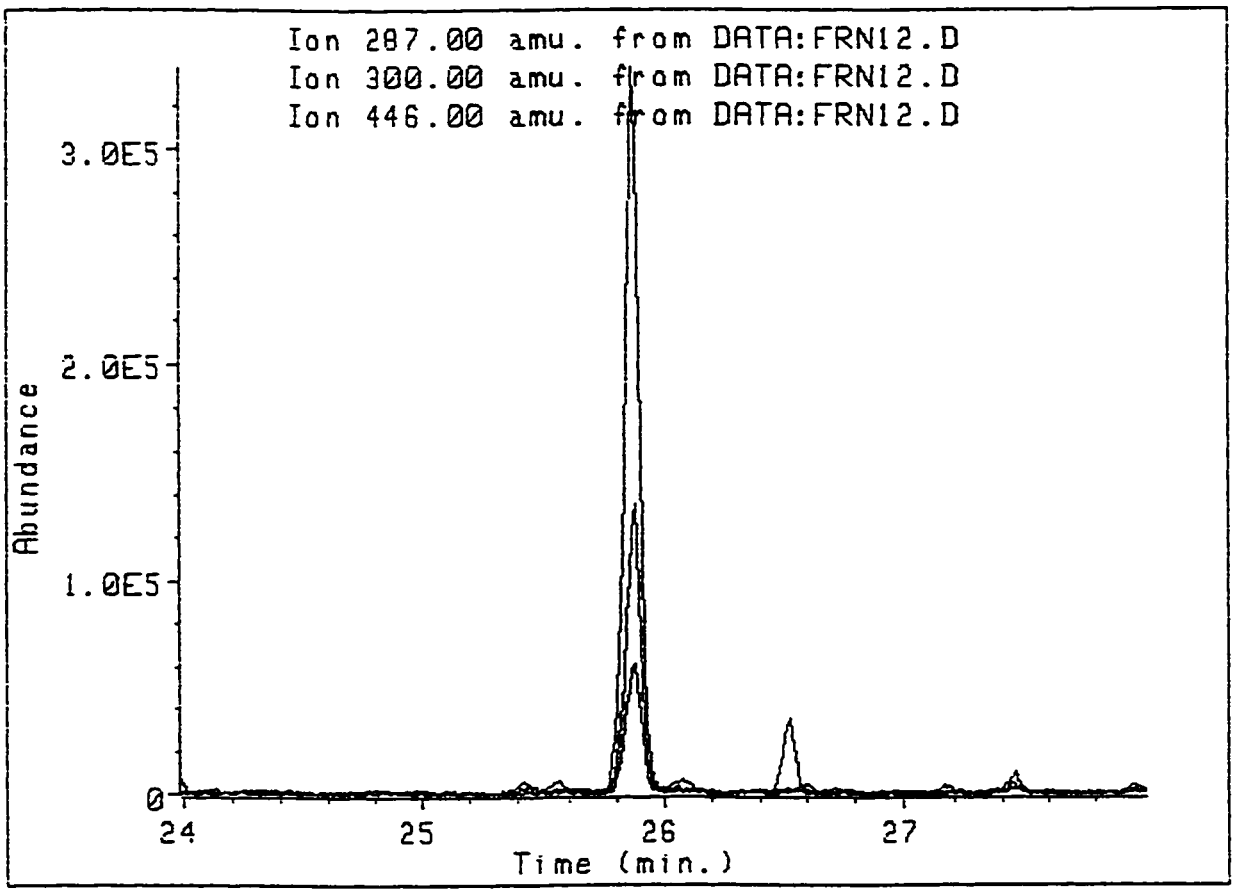


Figure 37. Extracted ion chromatograms m/z 287, 300 and 446 merged – norethandrolone human urine full scan

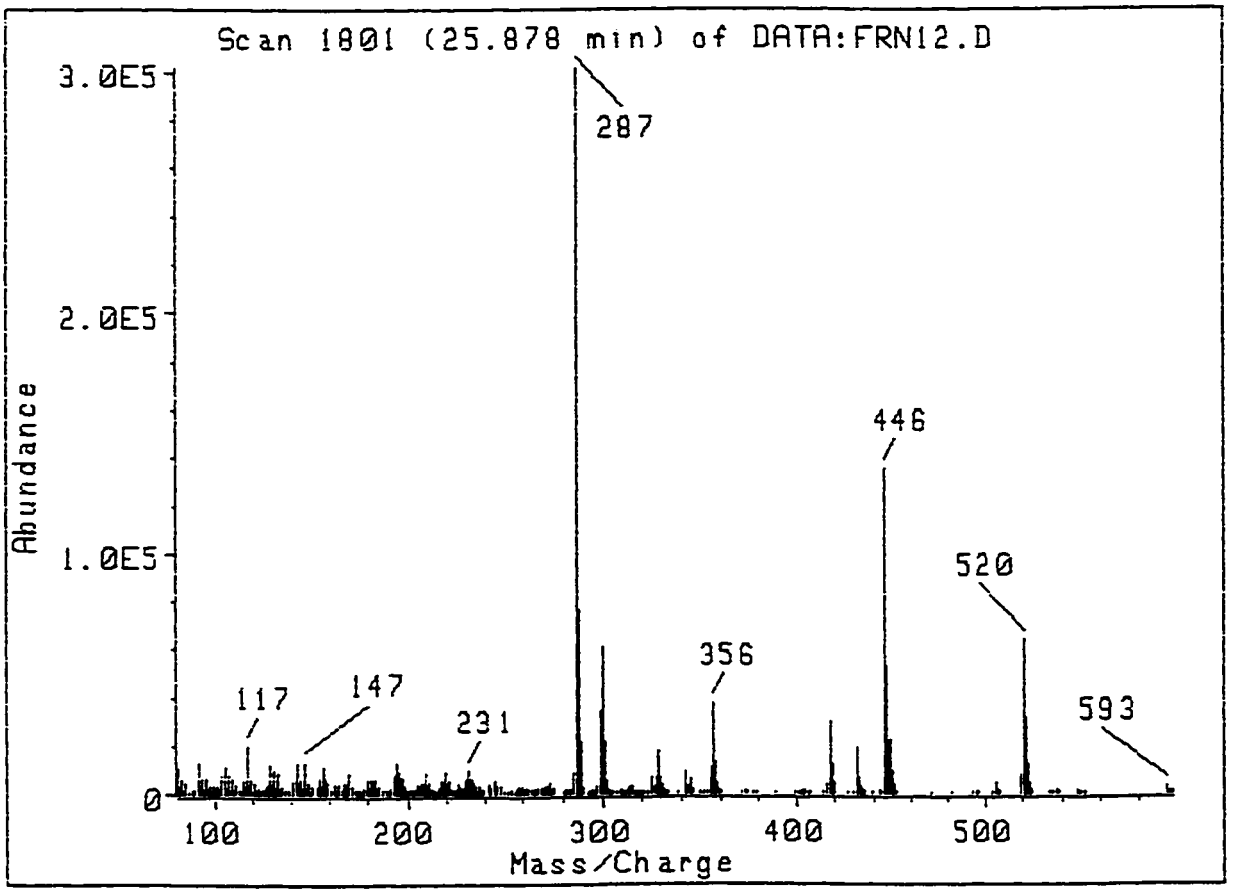


Figure 38. Norethandrolone full scan mass spectrum – norethandrolone human urine

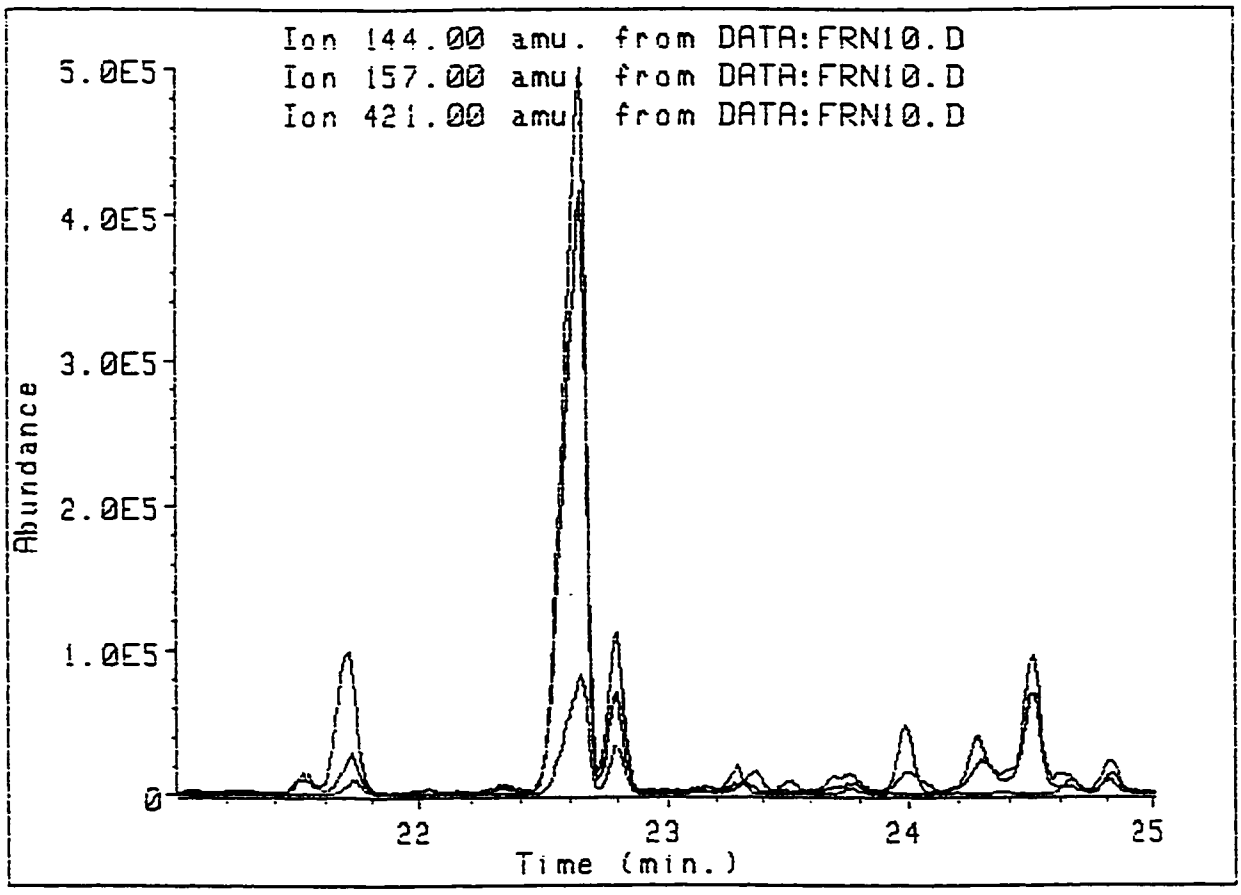


Figure 39. Extracted ion chromatograms m/z 144, 157 and 421 merged – norethandrolone monkey urine full scan

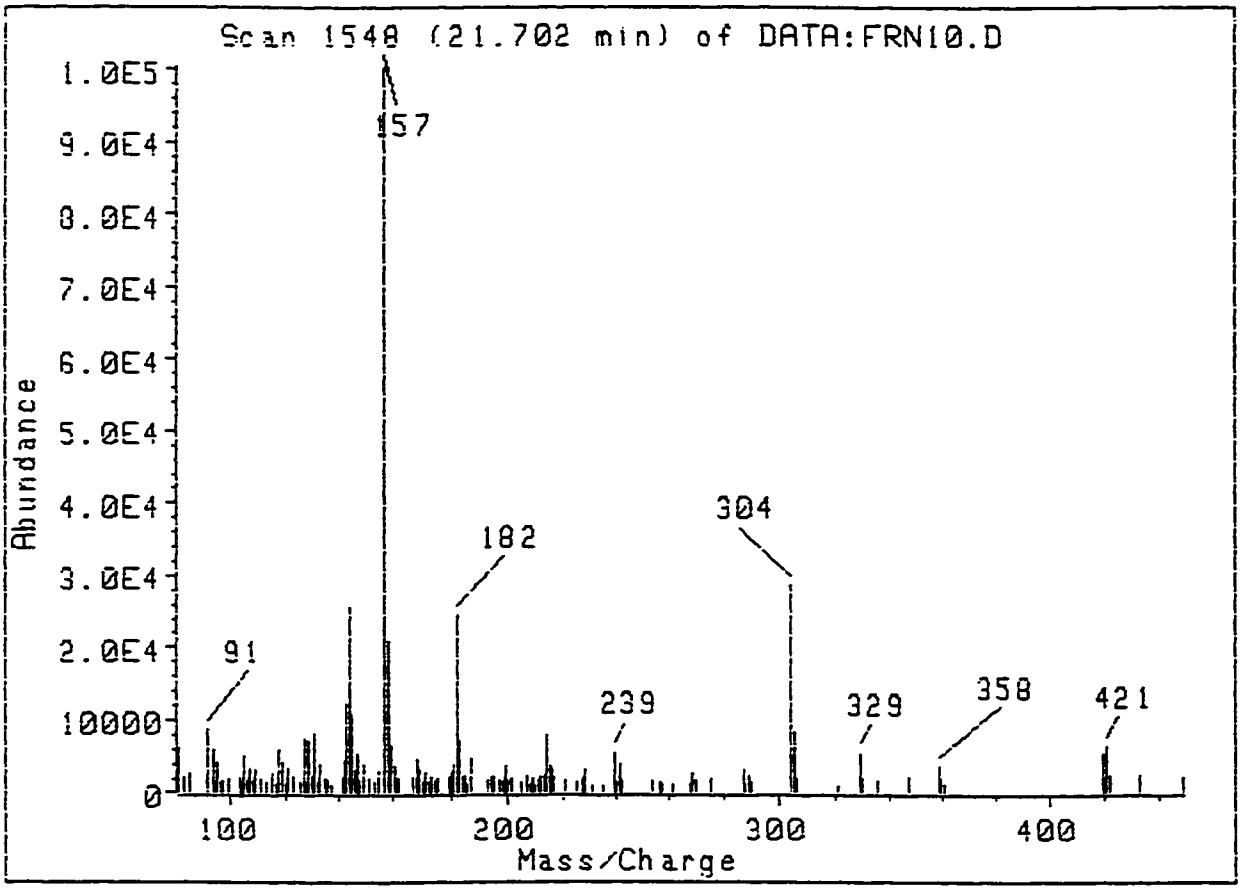


Figure 40. Di-hydroxy metabolite full scan mass spectrum – norethandrolone monkey urine

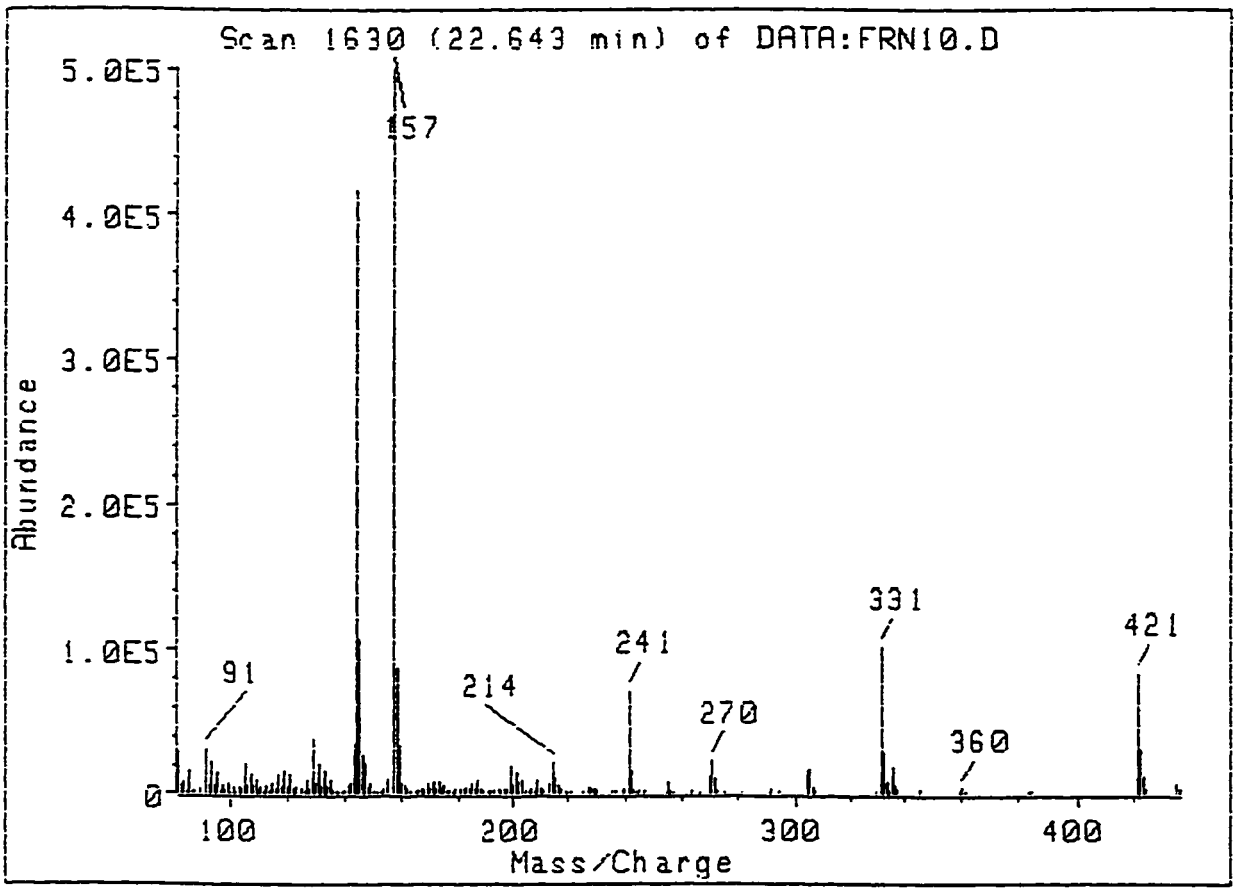


Figure 41. Di-hydroxy metabolite full scan mass spectrum – norethandrolone monkey urine

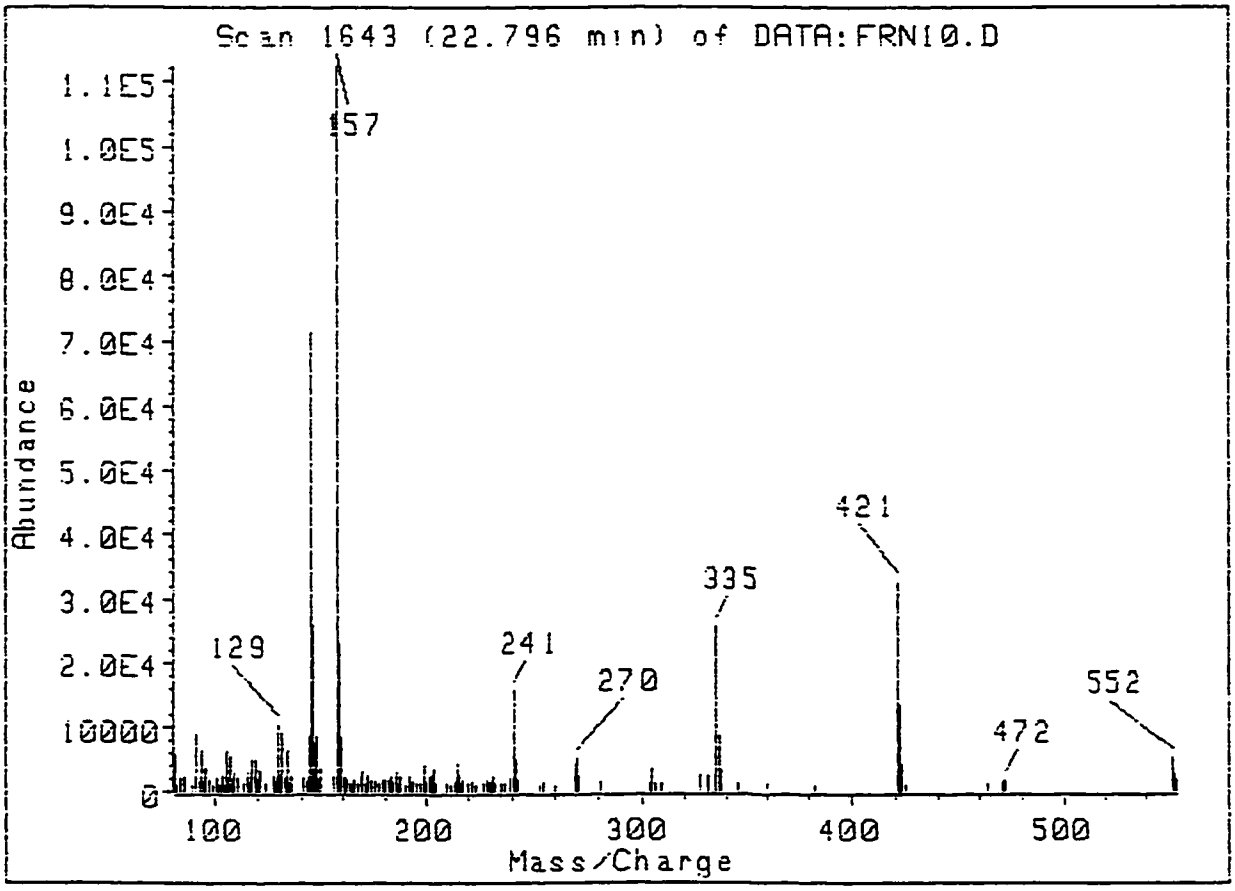


Figure 42. Di-hydroxy metabolite full scan mass spectrum – norethandrolone monkey urine

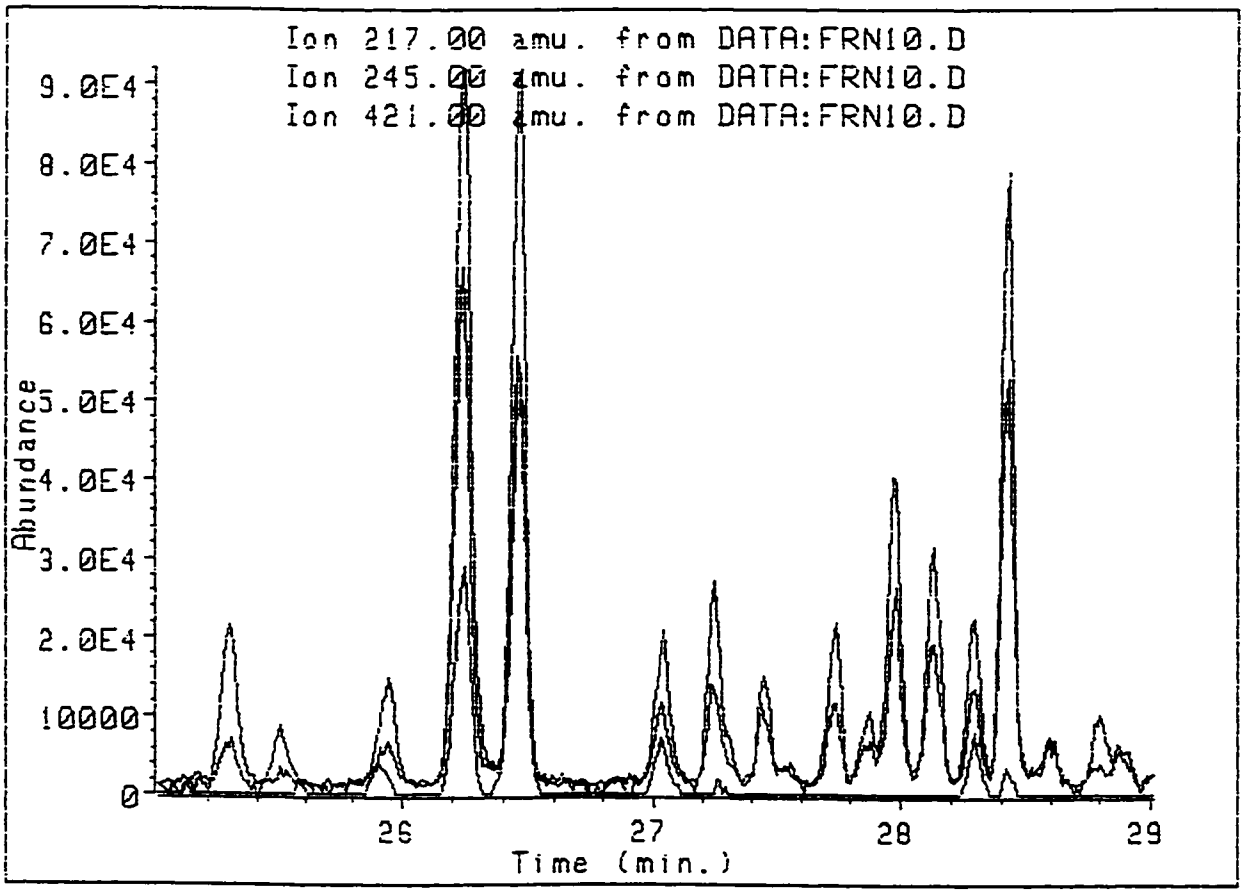


Figure 43. Extracted ion chromatograms m/z 217, 245 and 421 merged – norethandrolone monkey urine full scan

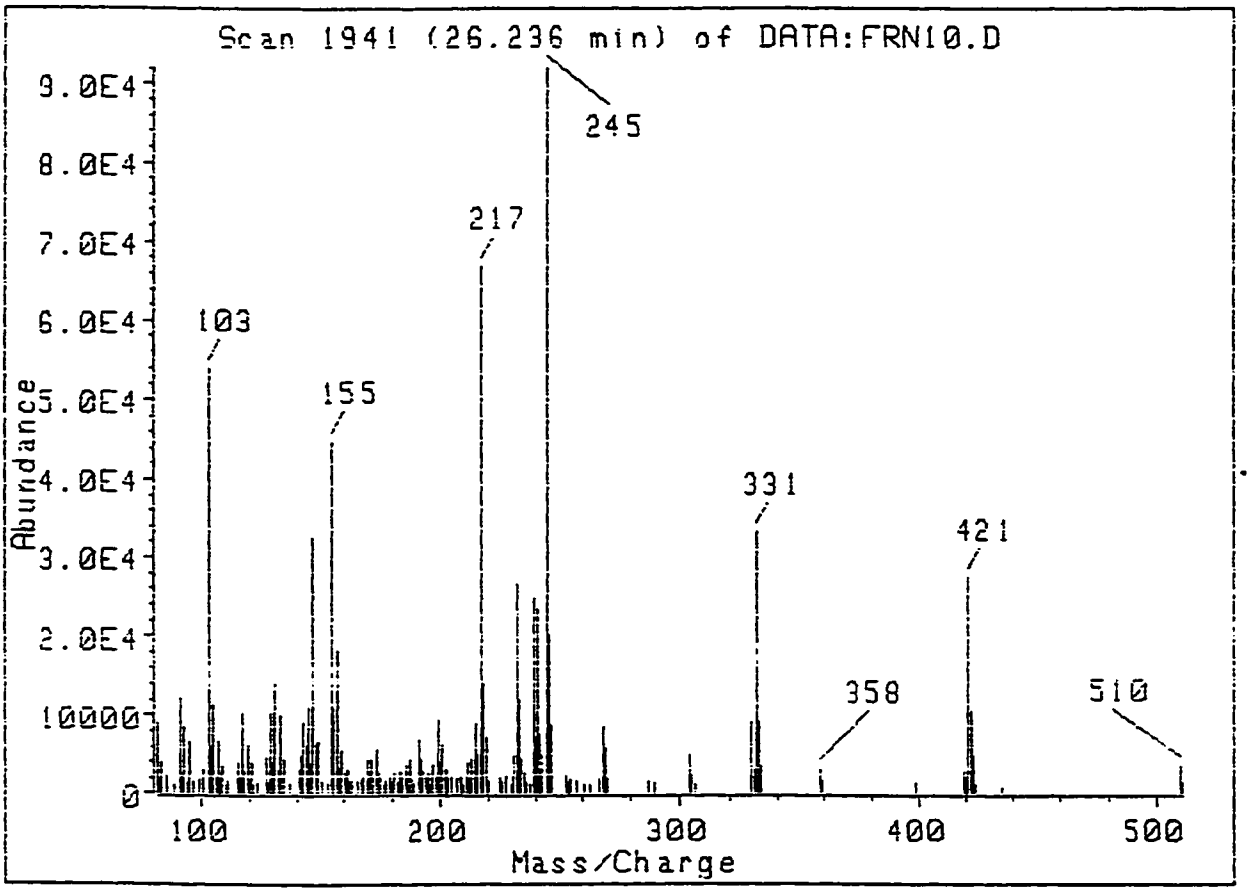


Figure 44. 3,17,21-Tri-hydroxy metabolite full scan mass spectrum – norethandrolone monkey urine

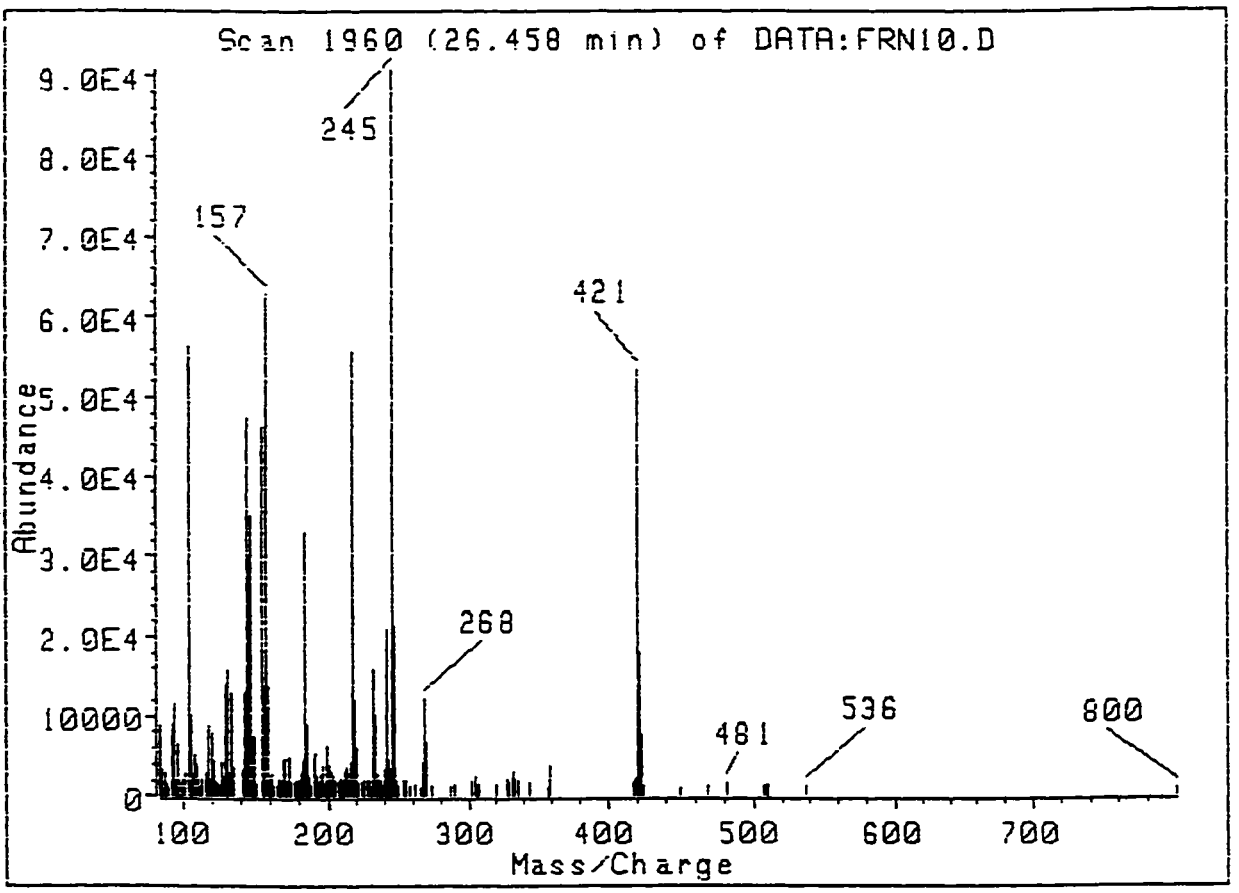


Figure 45. 3,17,21-Tri-hydroxy metabolite full scan mass spectrum – norethandrolone monkey urine

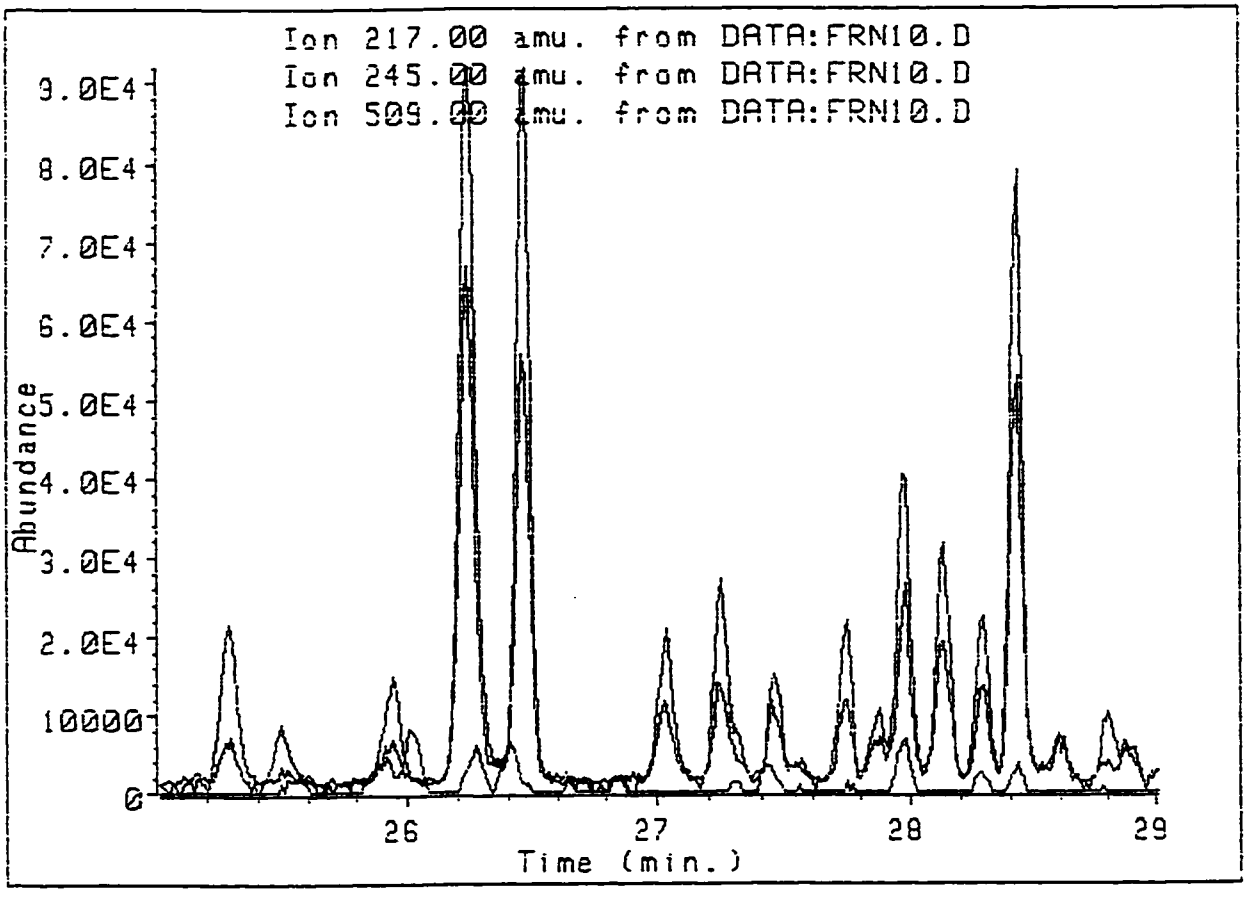


Figure 46. Extracted ion chromatograms m/z 217, 245 and 509 merged – norethandrolone monkey urine full scan

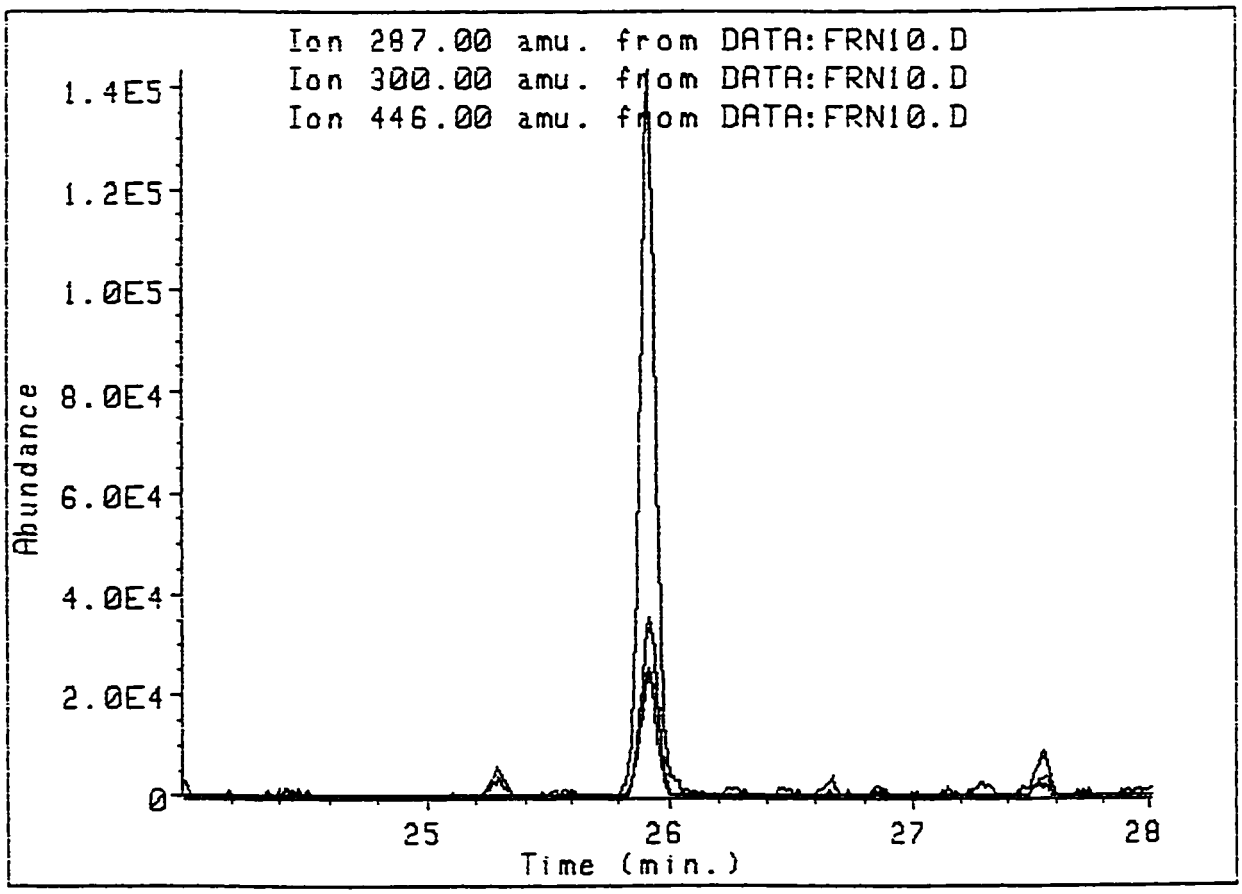


Figure 47. Extracted ion chromatograms m/z 287, 300 and 446 merged – norethandrolone monkey urine full scan

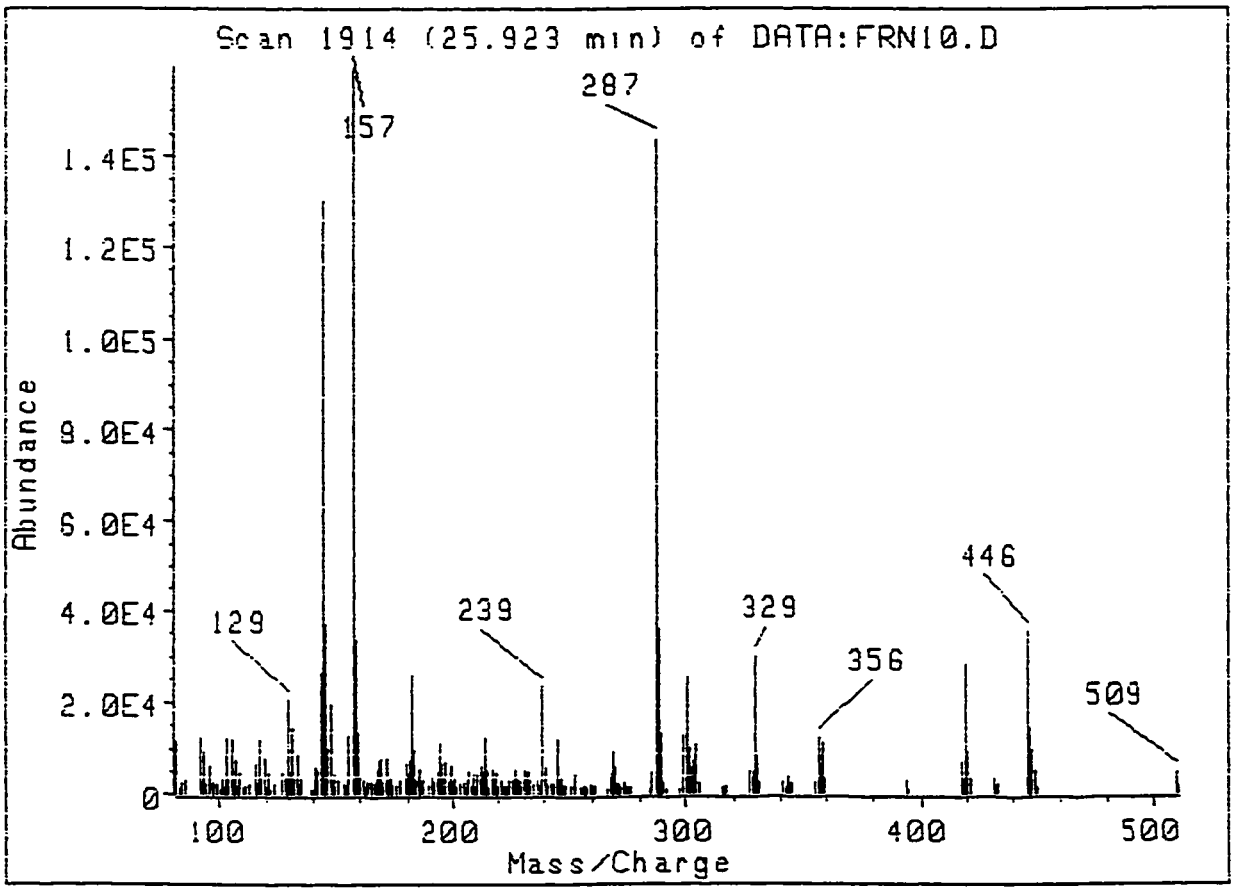


Figure 48. Norethandrolone full scan mass spectrum – norethandrolone monkey urine

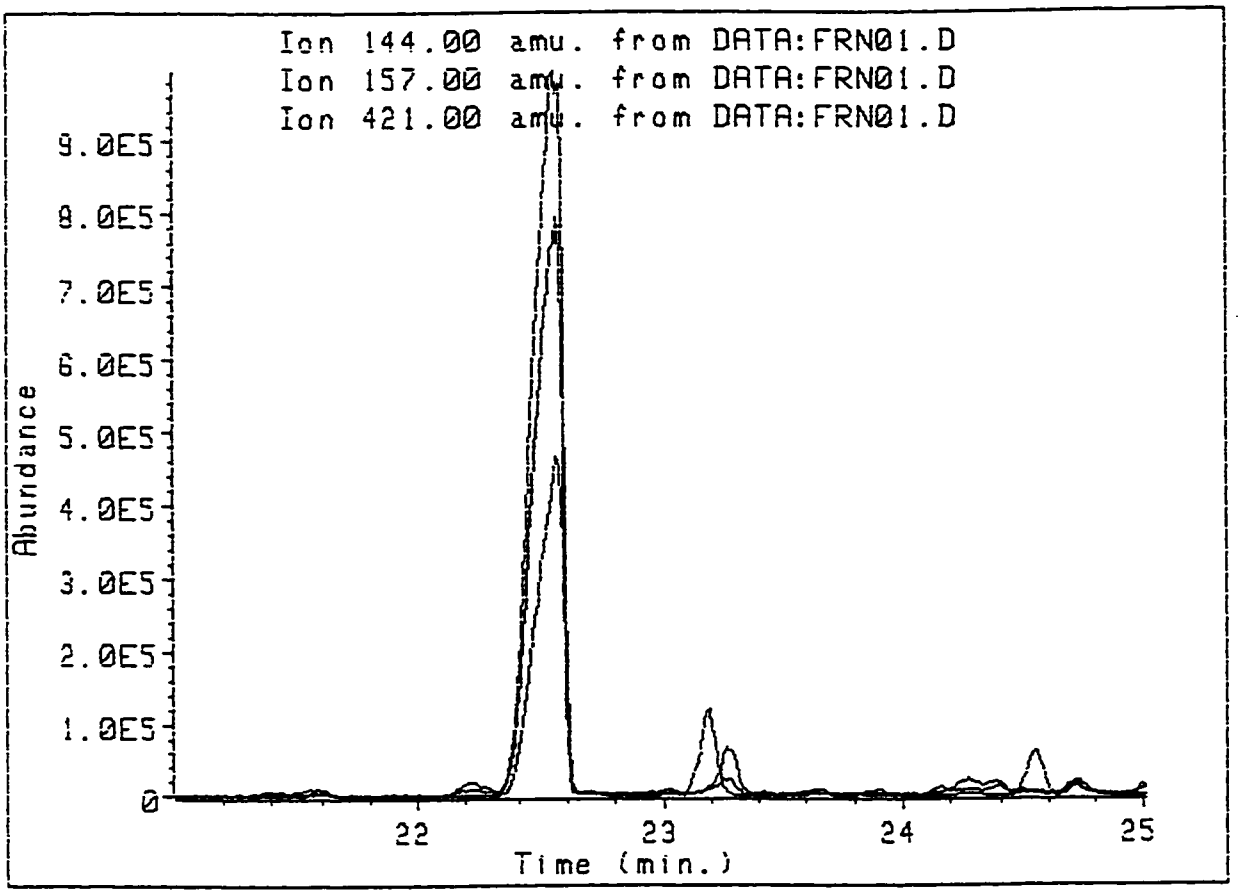


Figure 49. Extracted ion chromatograms m/z 144, 157 and 421 merged – (SP0001 M-F) ethylestrenol monkey urine full scan

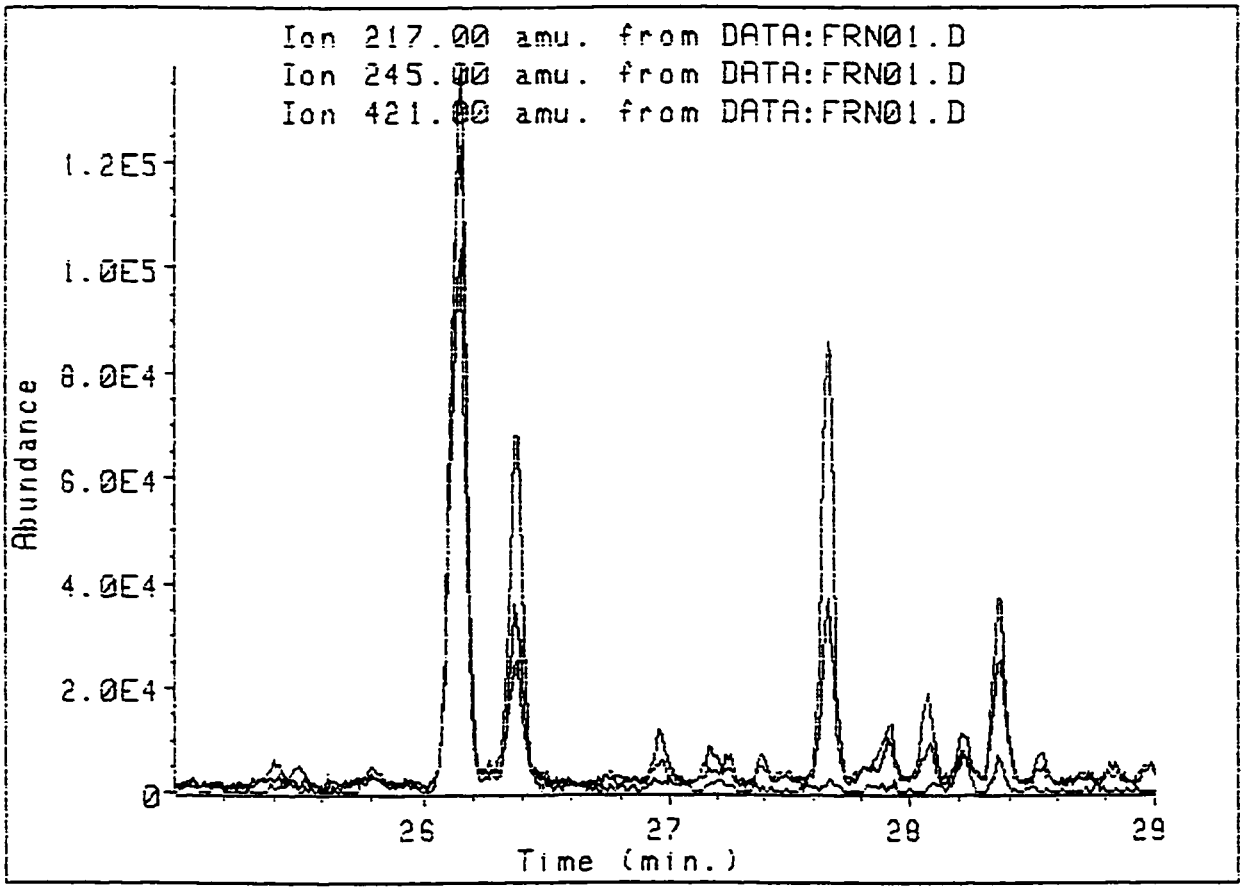


Figure 50. Extracted ion chromatograms m/z 217, 245 and 421 merged – (SP0001 M-F) ethylestrenol monkey urine full scan

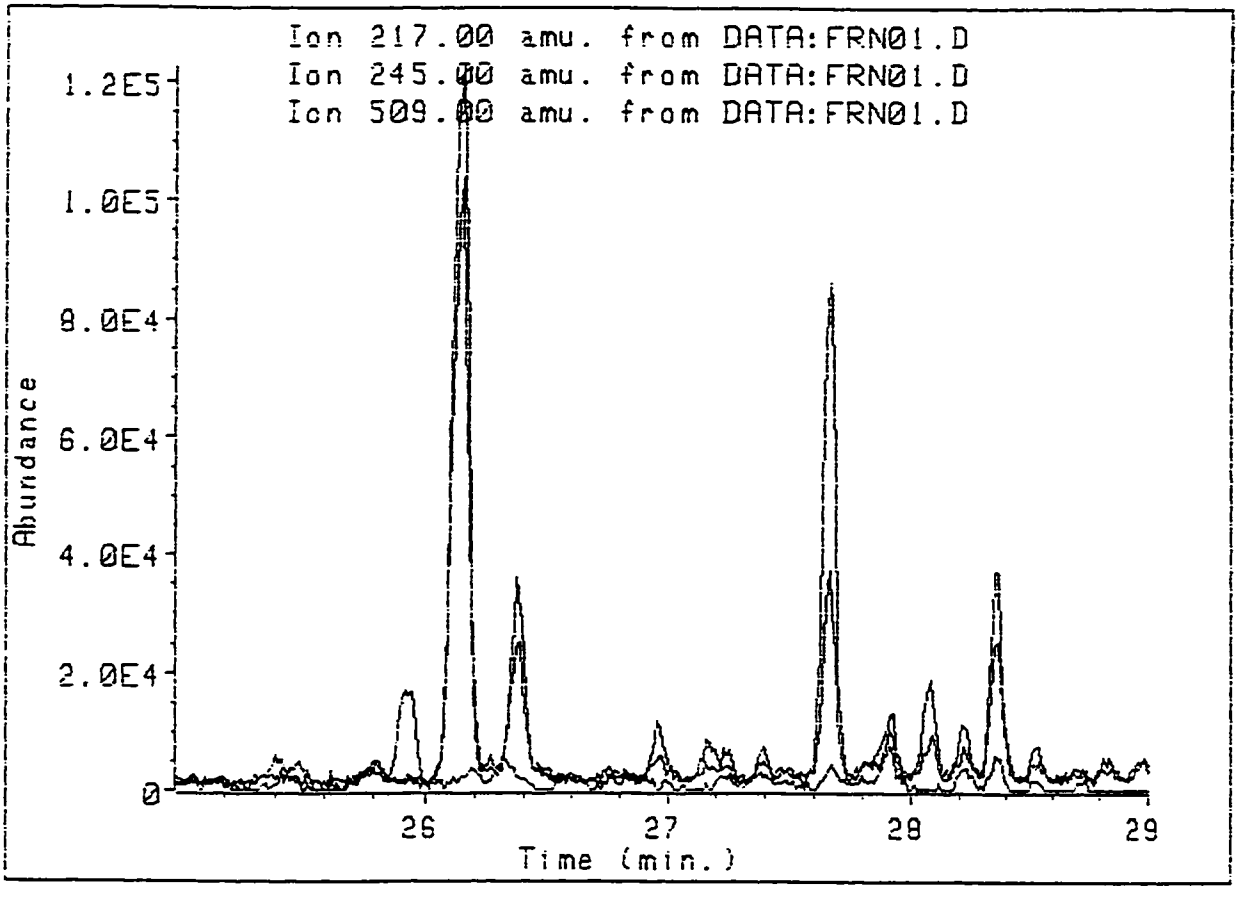


Figure 51. Extracted ion chromatograms m/z 217, 245 and 509 merged – (SP0001 M-F) ethylestrenol monkey urine full scan

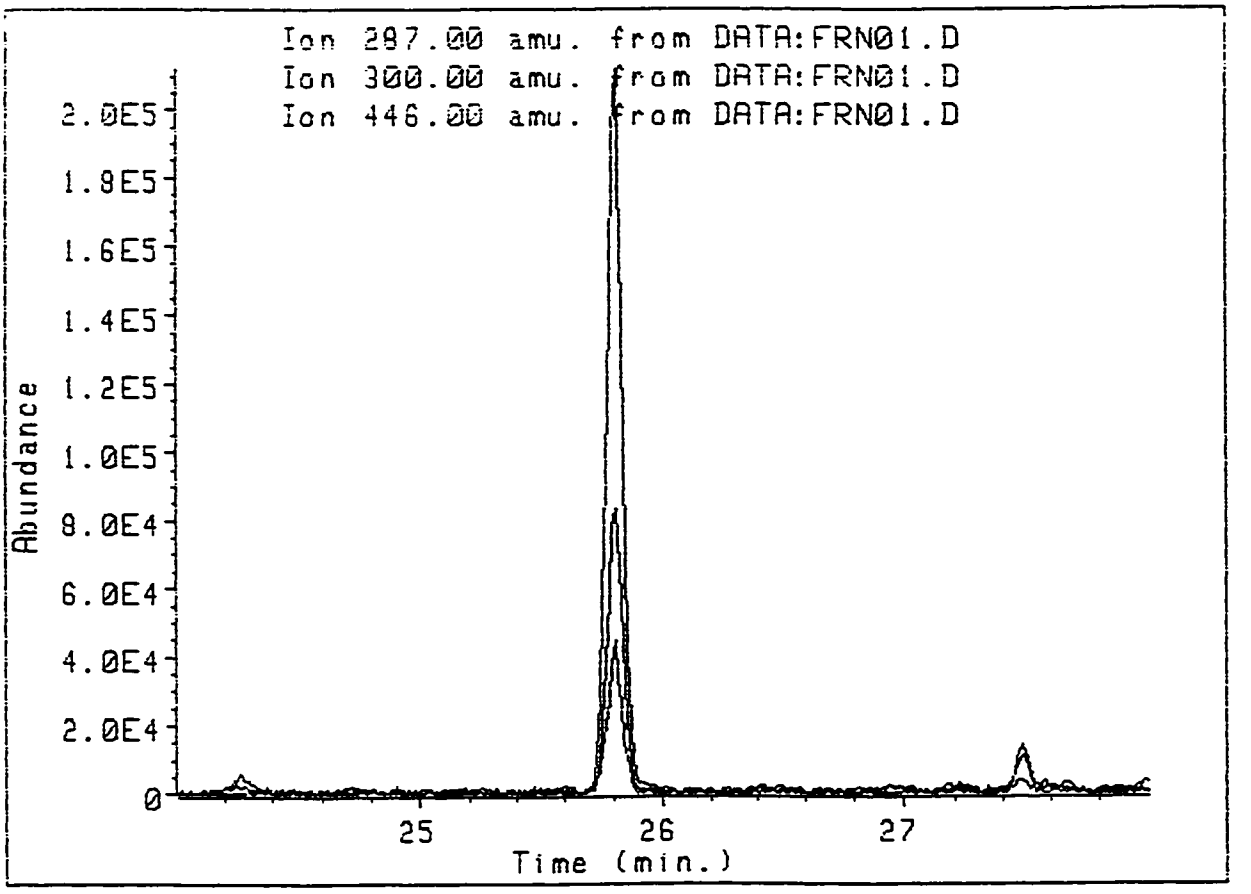


Figure 52. Extracted ion chromatograms m/z 287, 300 and 446 merged – (SP0001 M-F) ethylestrenol monkey urine full scan

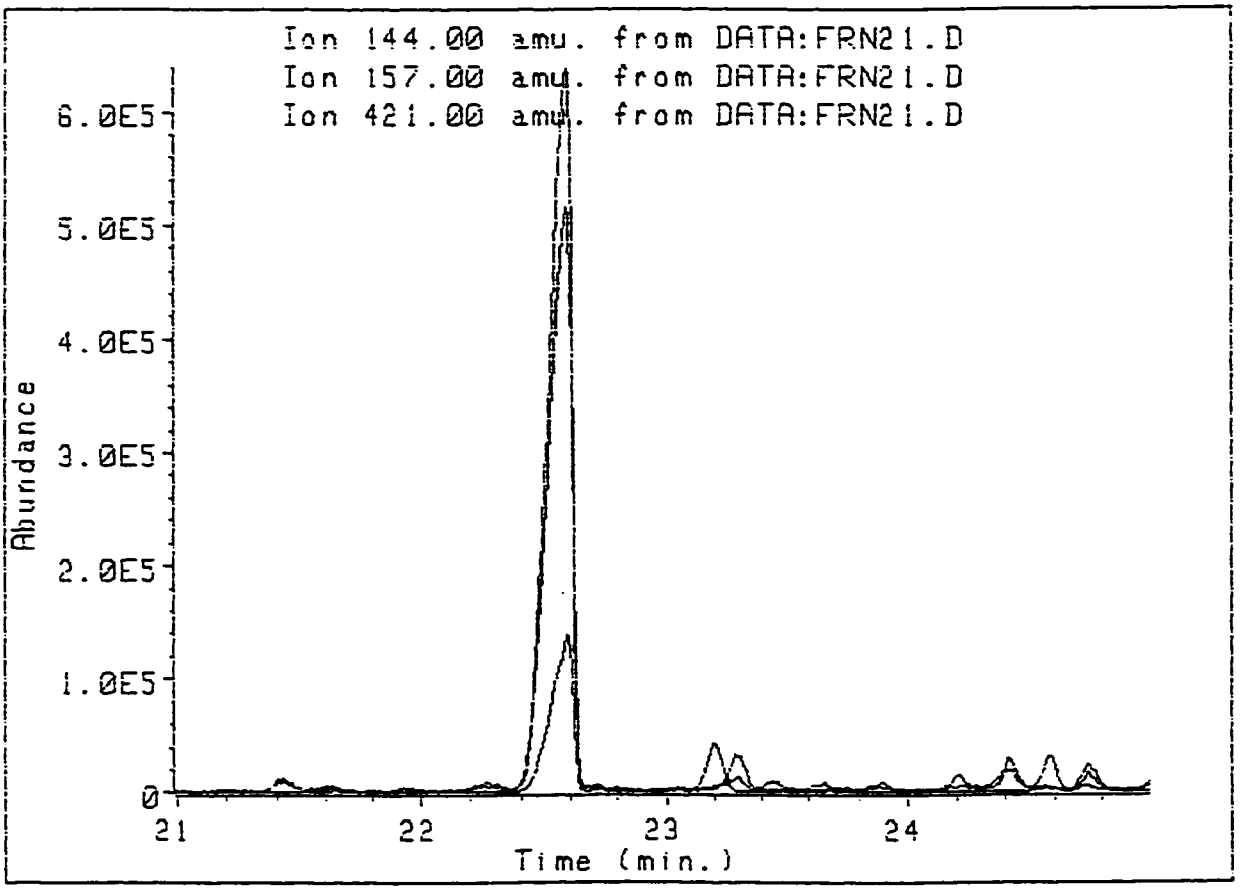


Figure 53. Extracted ion chromatograms m/z 144, 157 and 421 merged – (SP0002 S-S) ethylestrenol monkey urine full scan

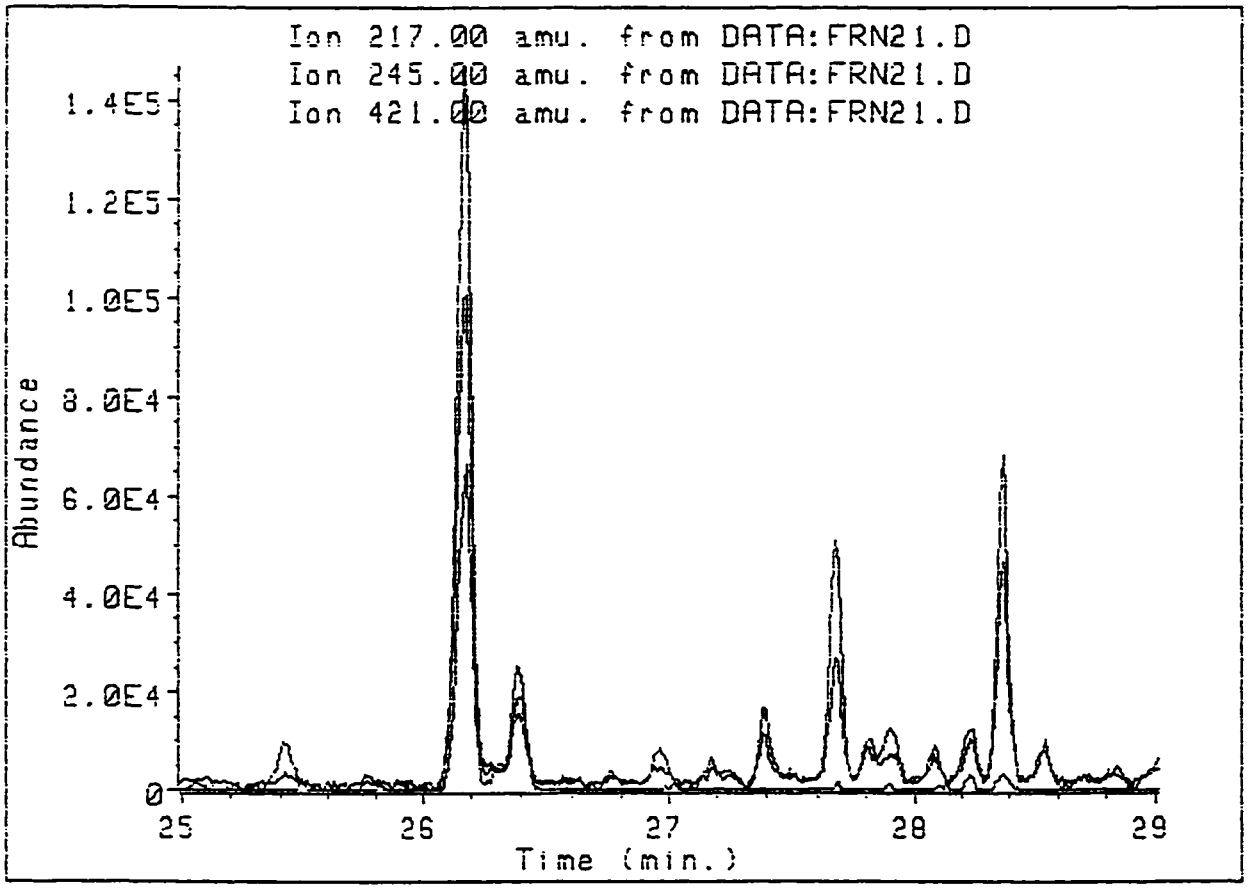


Figure 54. Extracted ion chromatograms m/z 217, 245 and 421 merged – (SP0002 S-S) ethylestrenol monkey urine full scan

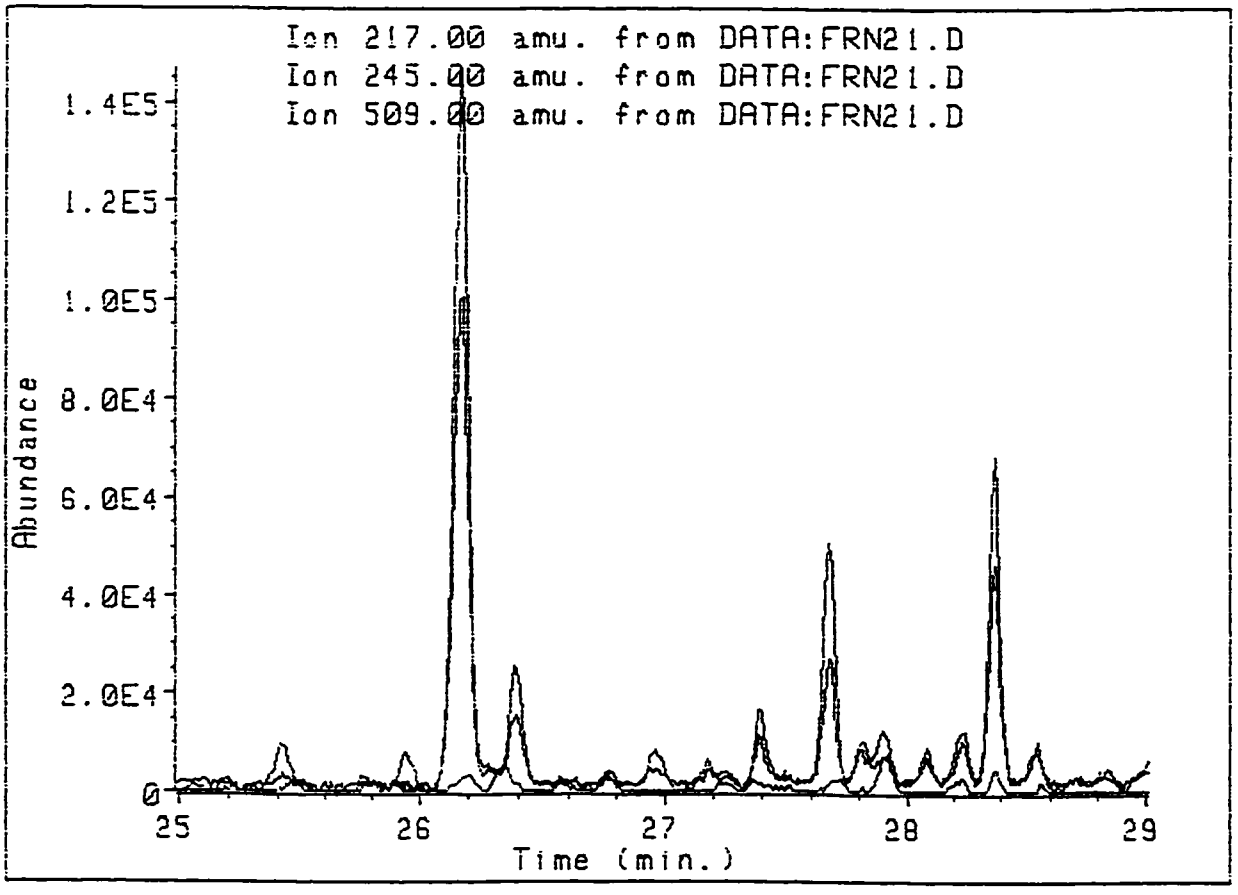


Figure 55. Extracted ion chromatograms m/z 217, 245 and 509 merged – (SP0002 S-S) ethylestrenol monkey urine full scan

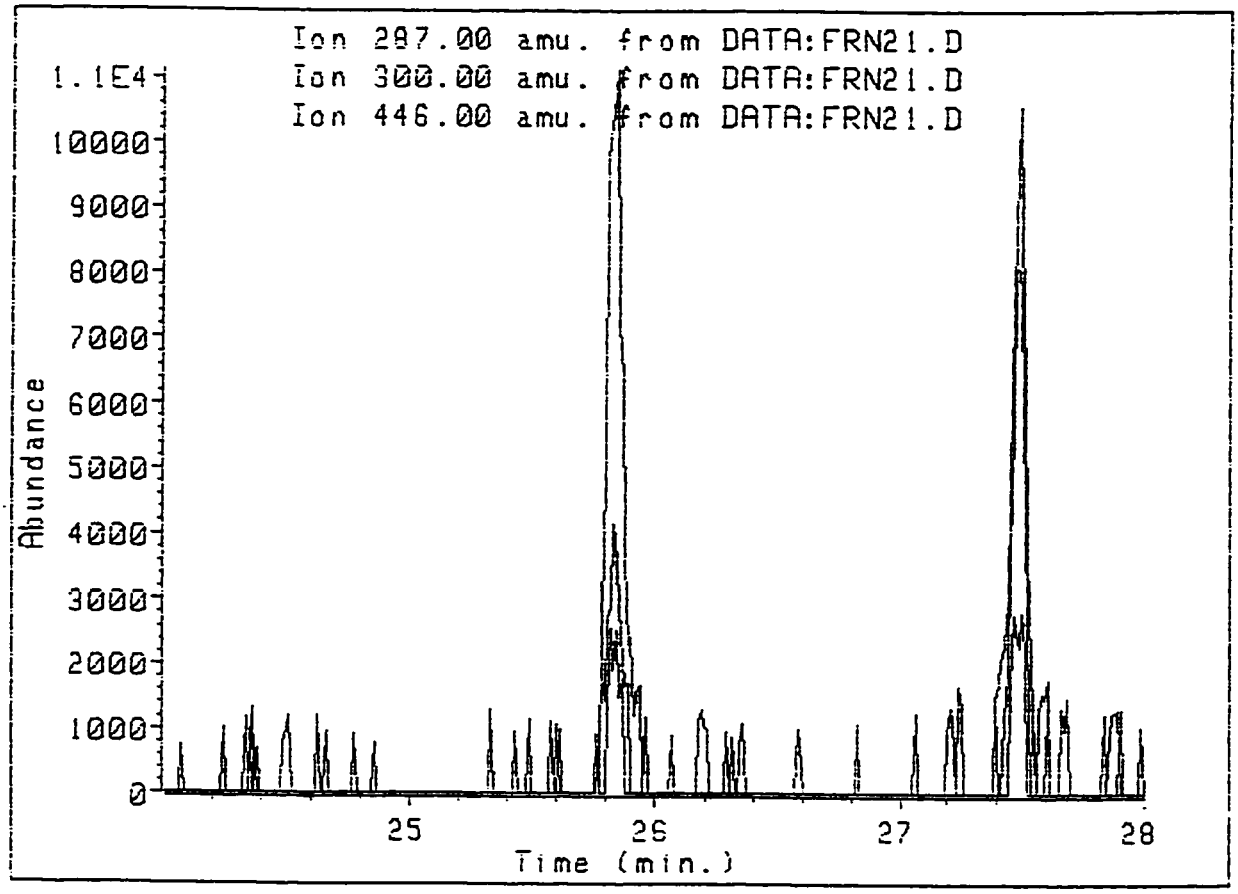


Figure 56. Extracted ion chromatograms m/z 287, 300 and 446 merged – (SP0002 S-S) ethylestrenol monkey urine full scan

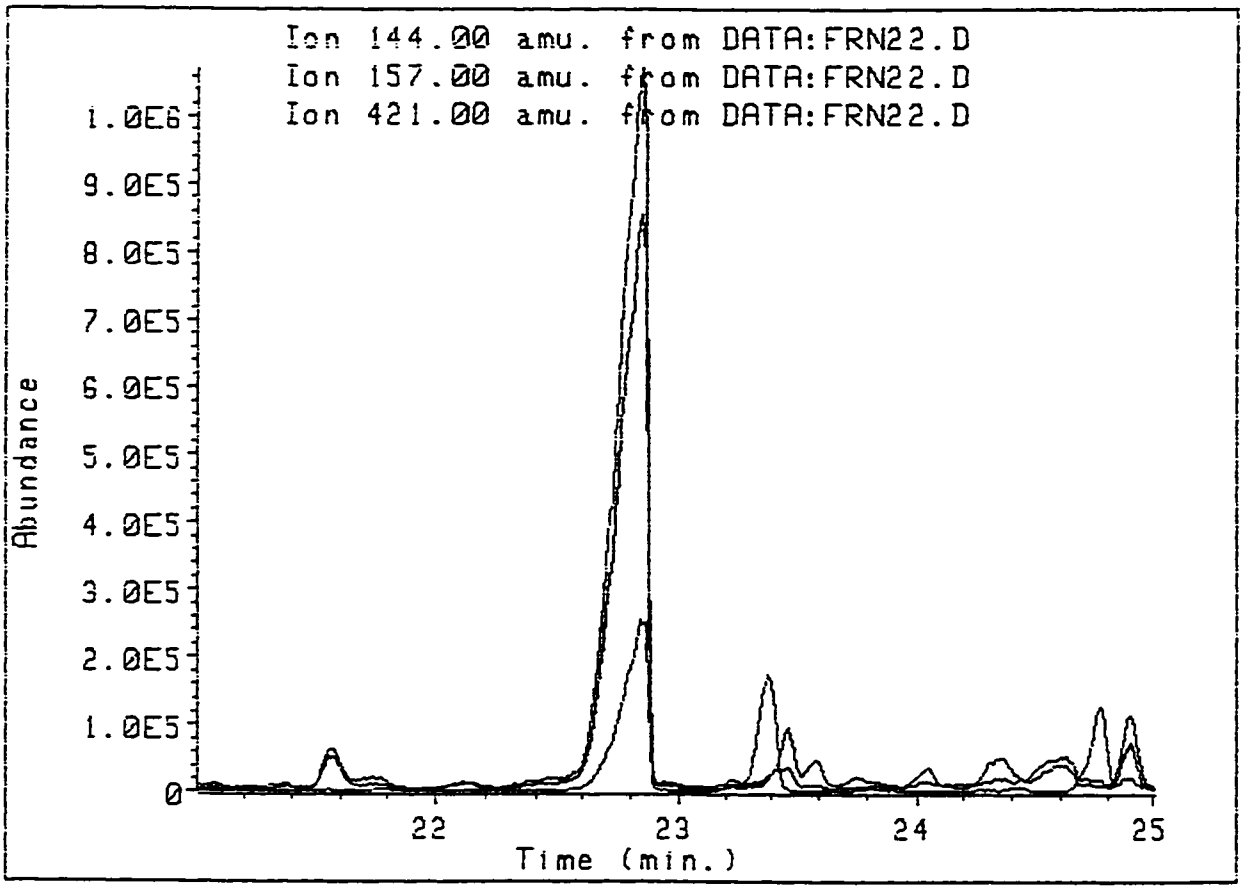


Figure 57. Extracted ion chromatograms m/z 144, 157 and 421 merged – (SP0004 S-S) ethylestrenol monkey urine full scan

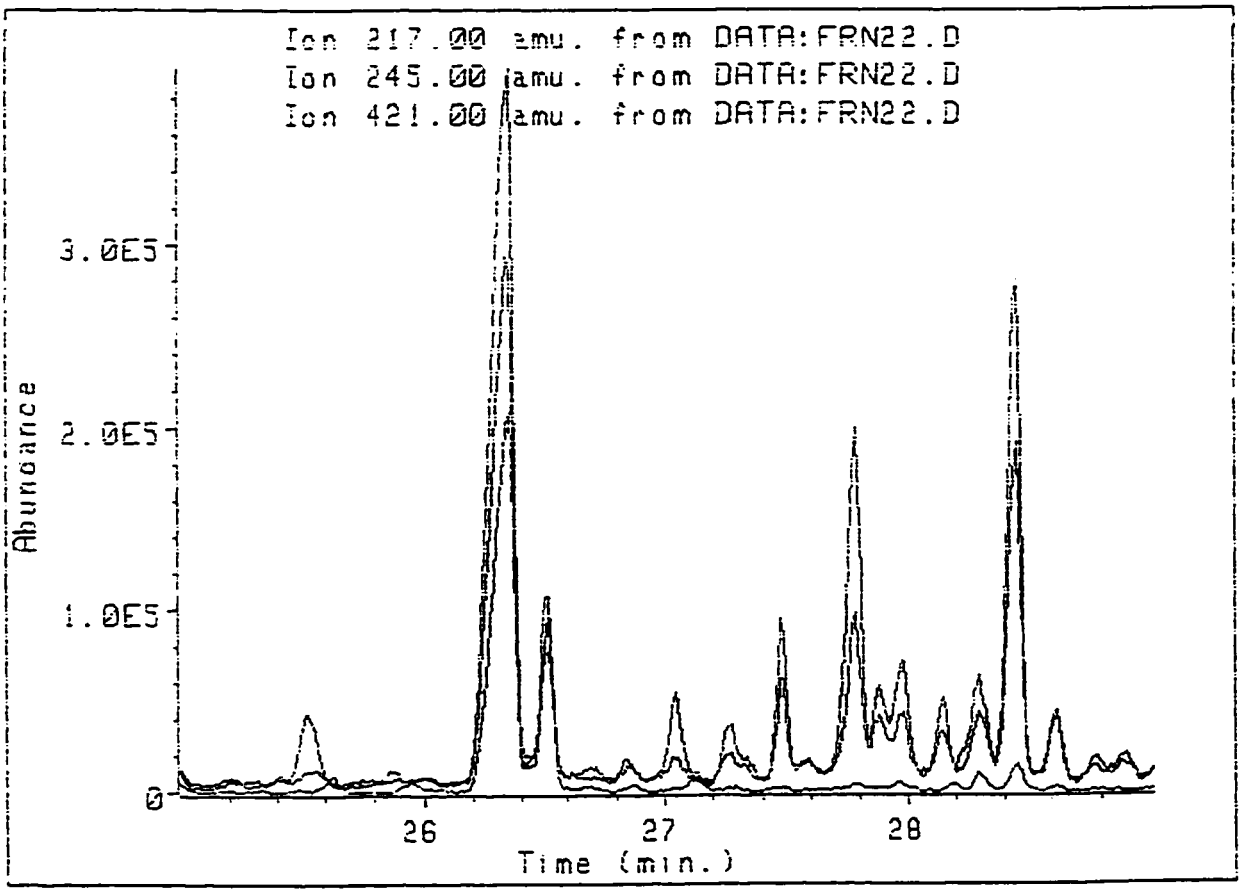


Figure 58. Extracted ion chromatograms m/z 217, 245 and 421 merged – (SP0004 S-S) ethylestrenol monkey urine full scan

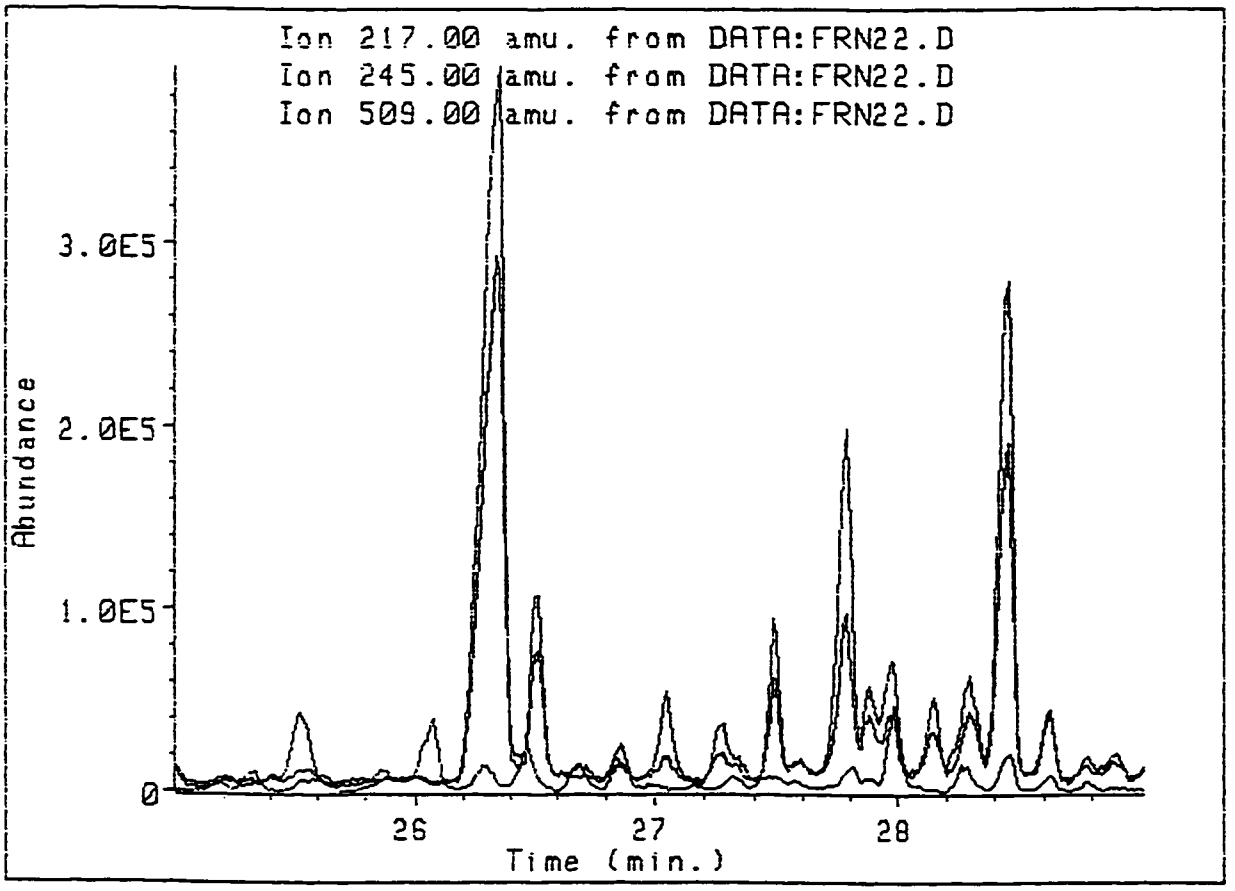


Figure 59. Extracted ion chromatograms m/z 217, 245 and 509 merged – (SP0004 S-S) ethylestrenol monkey urine full scan

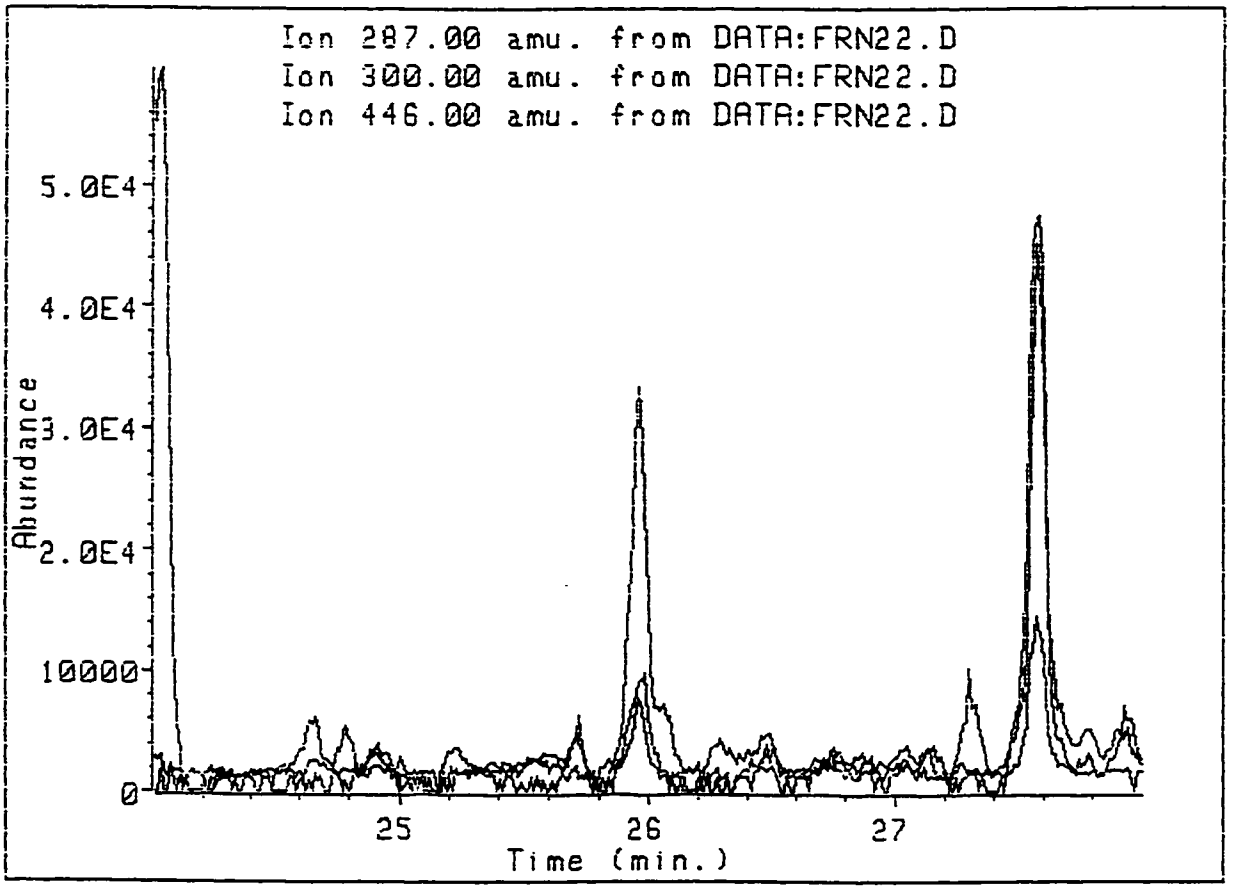


Figure 60. Extracted ion chromatograms m/z 287, 300 and 446 merged – (SP0004 S-S) ethylestrenol monkey urine full scan

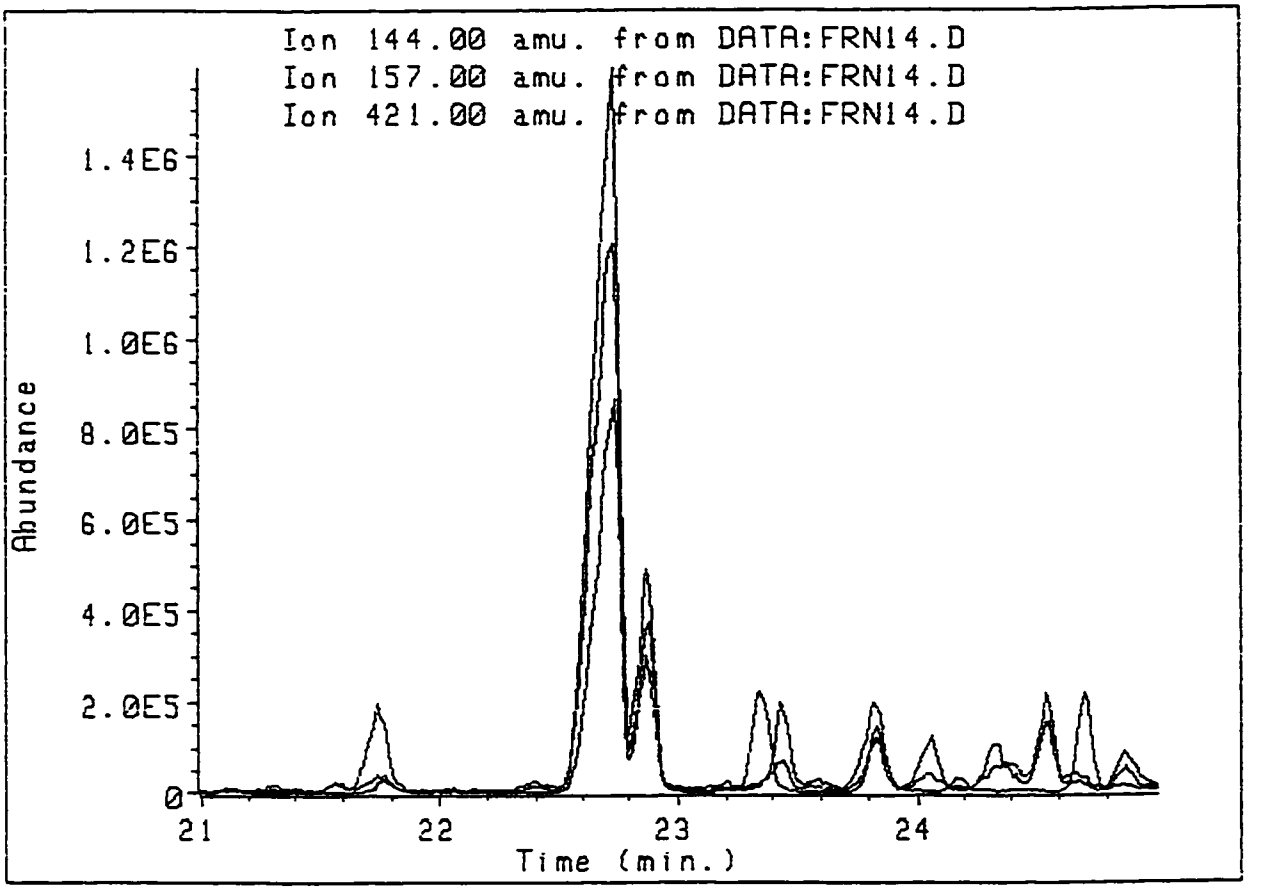


Figure 61. Extracted ion chromatograms m/z 144, 157 and 421 merged – (SP0005 M-F) ethylestrenol monkey urine full scan

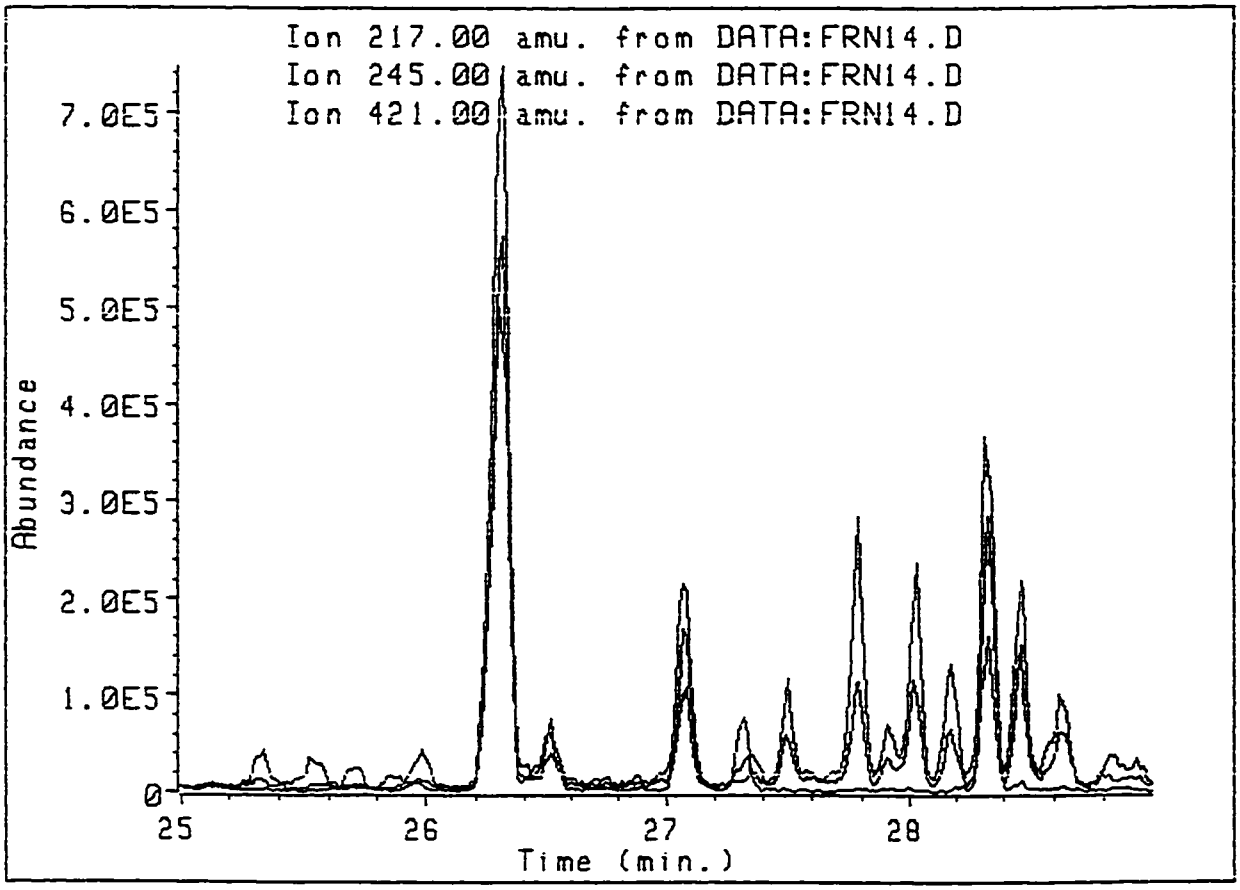


Figure 62. Extracted ion chromatograms m/z 217, 245 and 421 merged – (SP0005 M-F) ethylestrenol monkey urine full scan

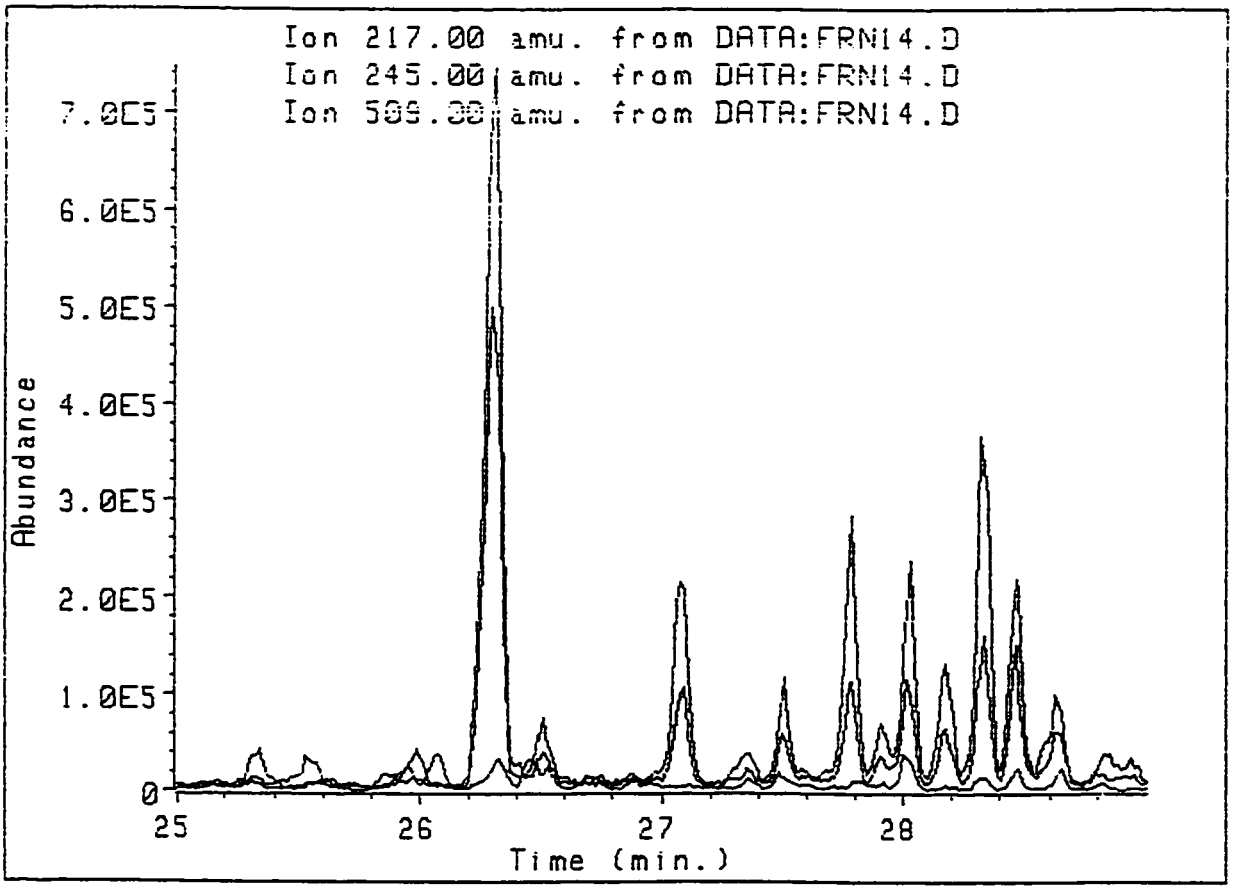


Figure 63. Extracted ion chromatograms m/z 217, 245 and 509 merged – (SP0005 M-F) ethylestrenol monkey urine full scan

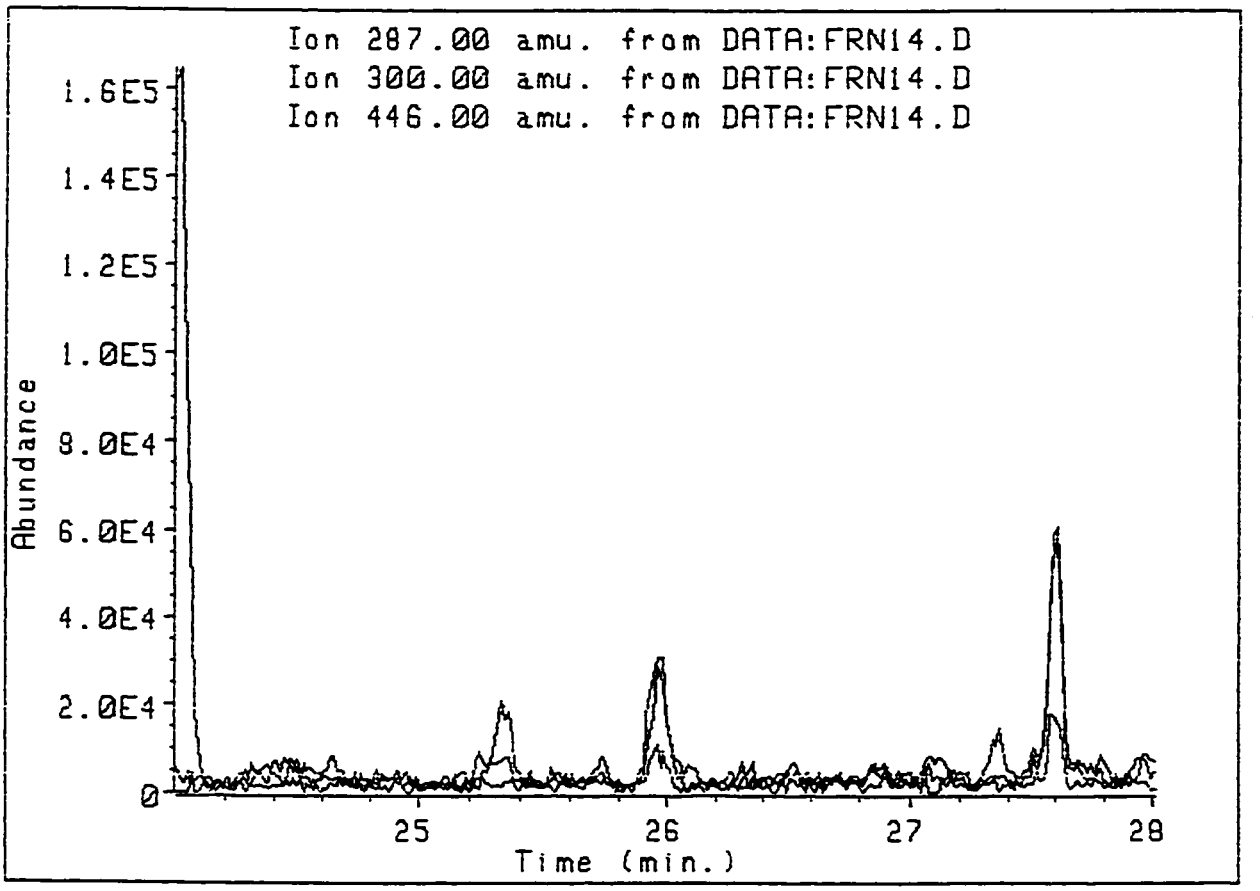


Figure 64. Extracted ion chromatograms m/z 287, 300 and 446 merged – (SP0005 M-F) ethylestrenol monkey urine full scan

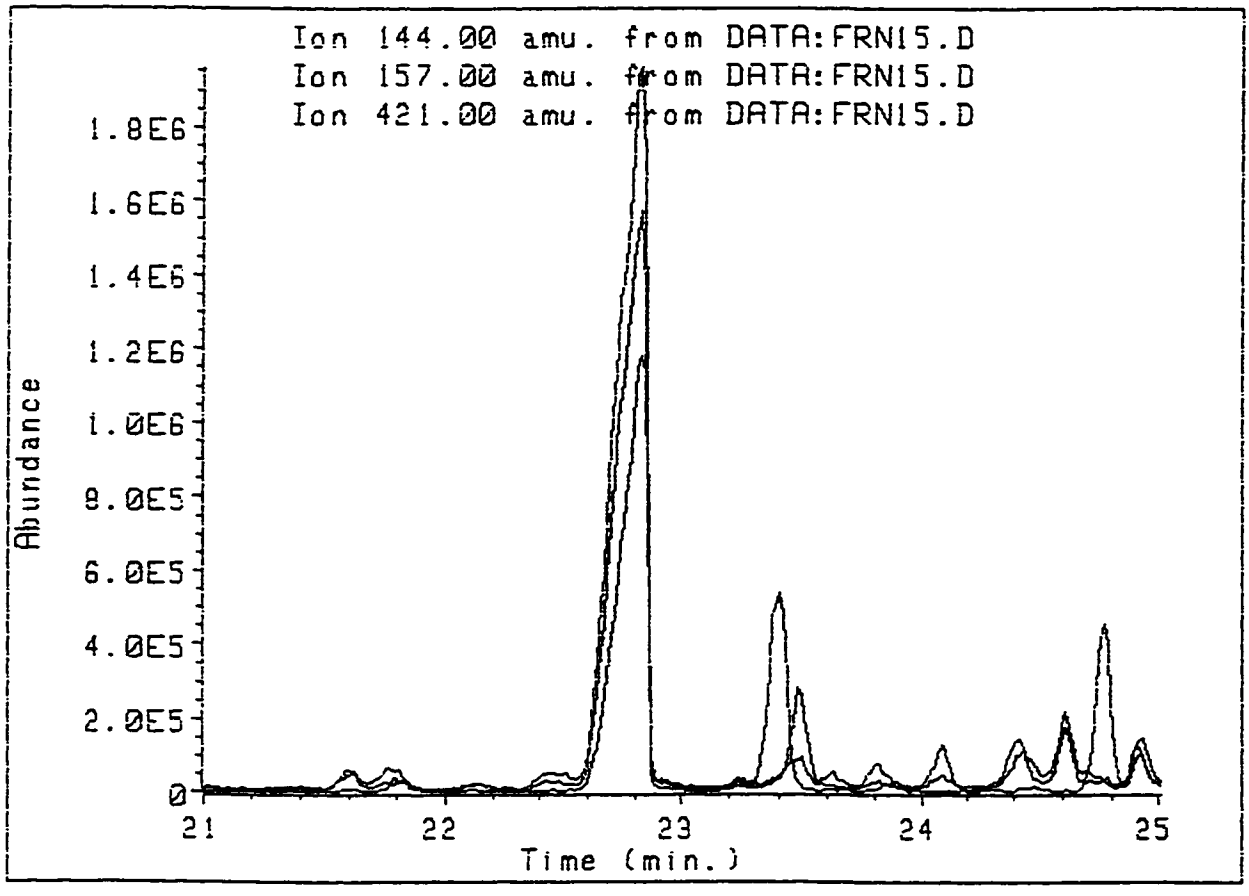


Figure 65. Extracted ion chromatograms m/z 144, 157 and 421 merged – (SP0006 M-F) ethylestrenol monkey urine full scan

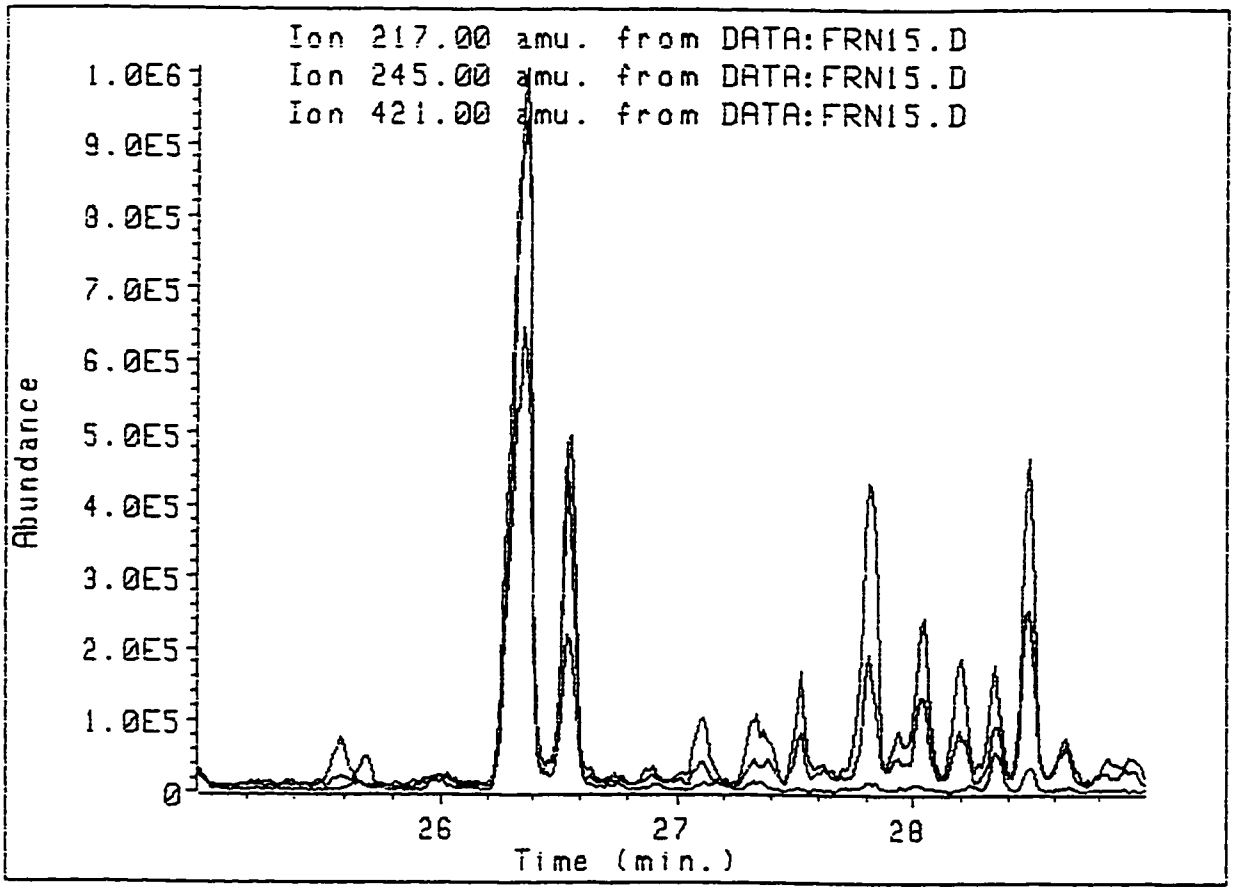


Figure 66. Extracted ion chromatograms m/z 217, 245 and 421 merged – (SP0006 M-F) ethylestrenol monkey urine full scan

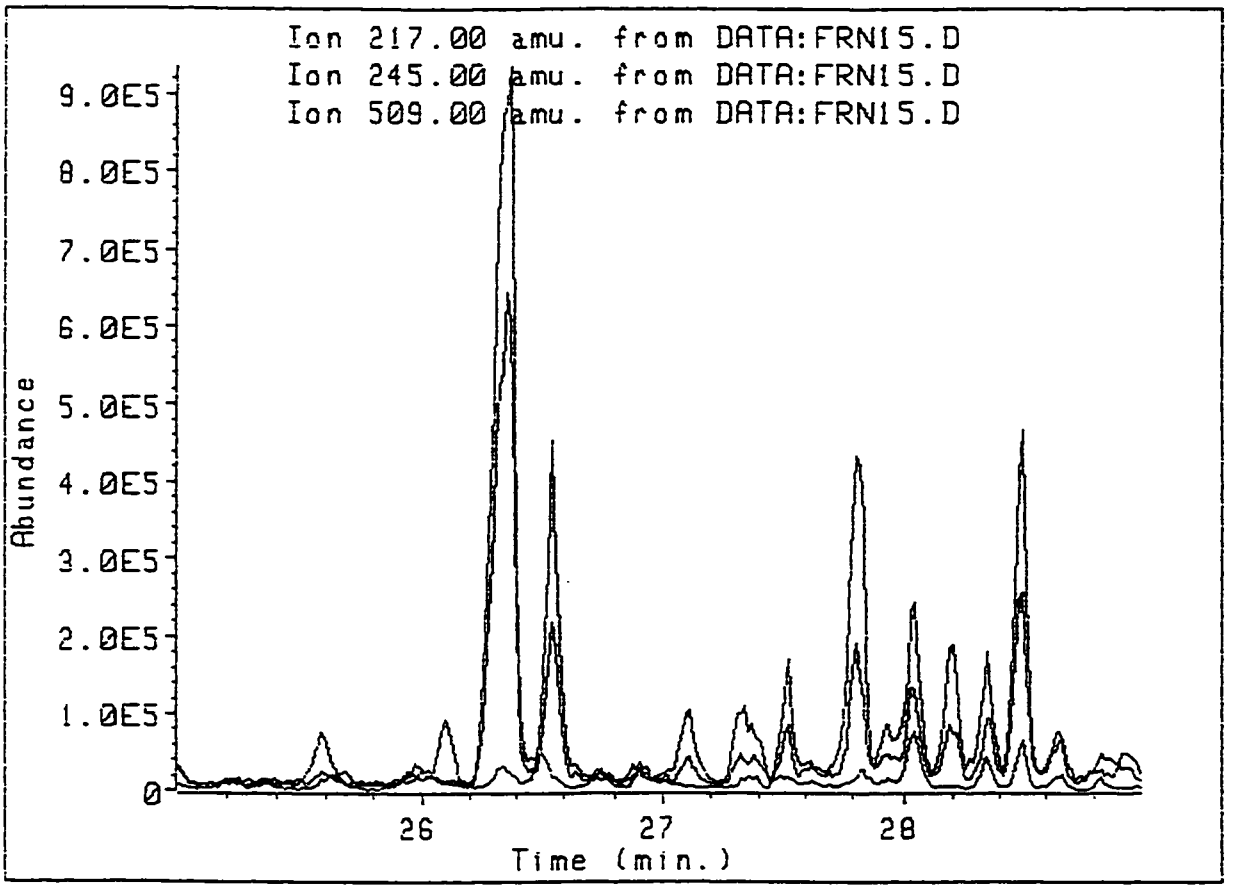


Figure 67. Extracted ion chromatograms m/z 217, 245 and 509 merged – (SP0006 M-F) ethylestrenol monkey urine full scan

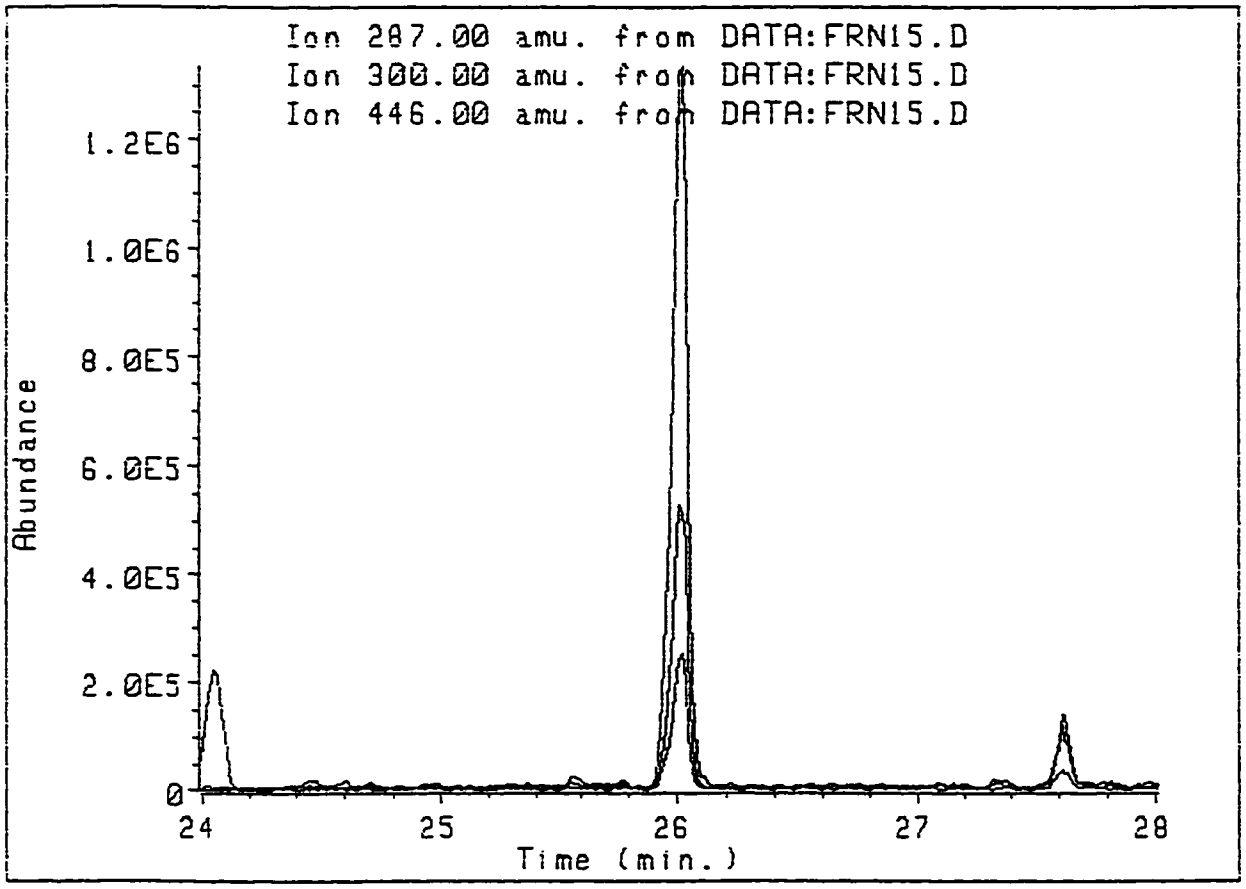


Figure 68. Extracted ion chromatograms m/z 287, 300 and 446 merged – (SP0006 M-F) ethylestrenol monkey urine full scan

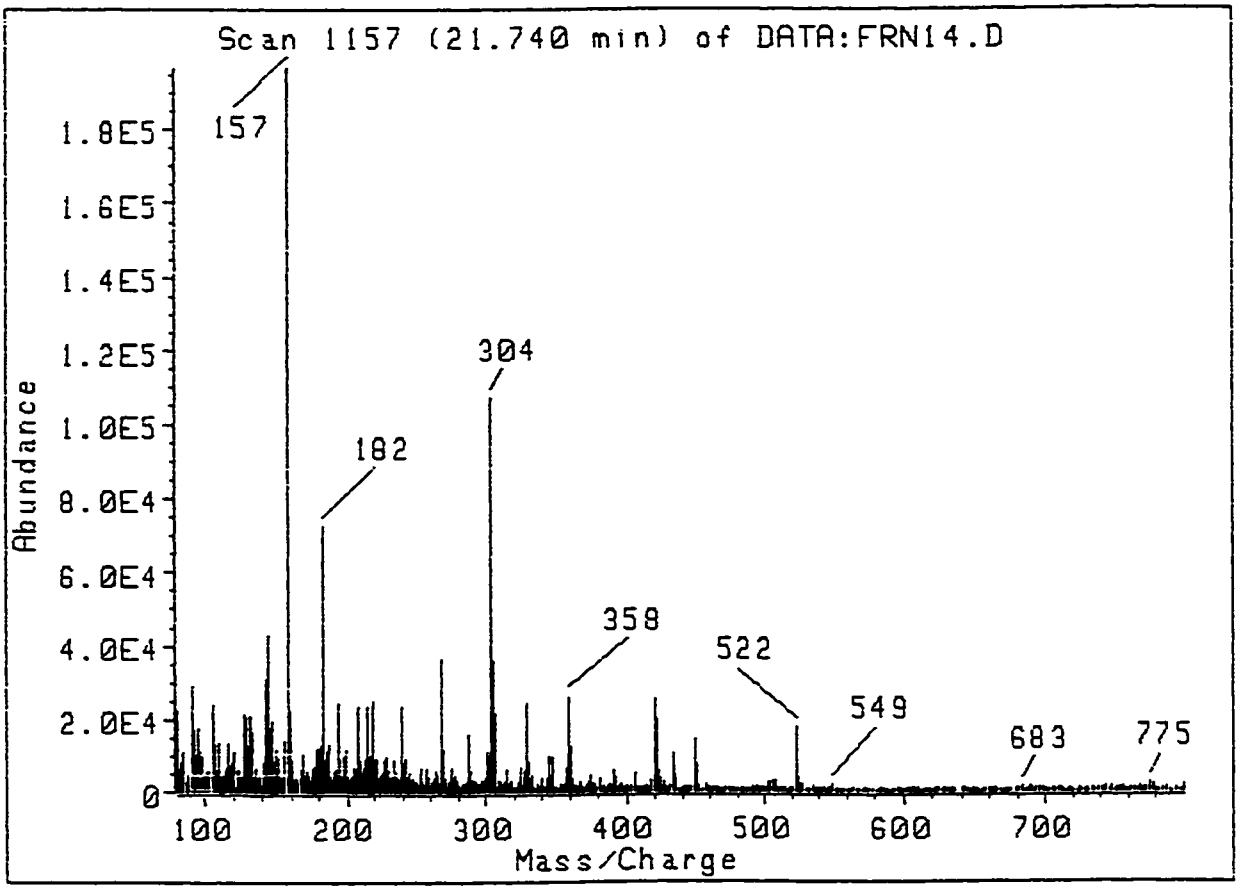


Figure 69. Di-hydroxy metabolite full scan mass spectrum – (SP0005 M-F) ethylestrenol monkey urine

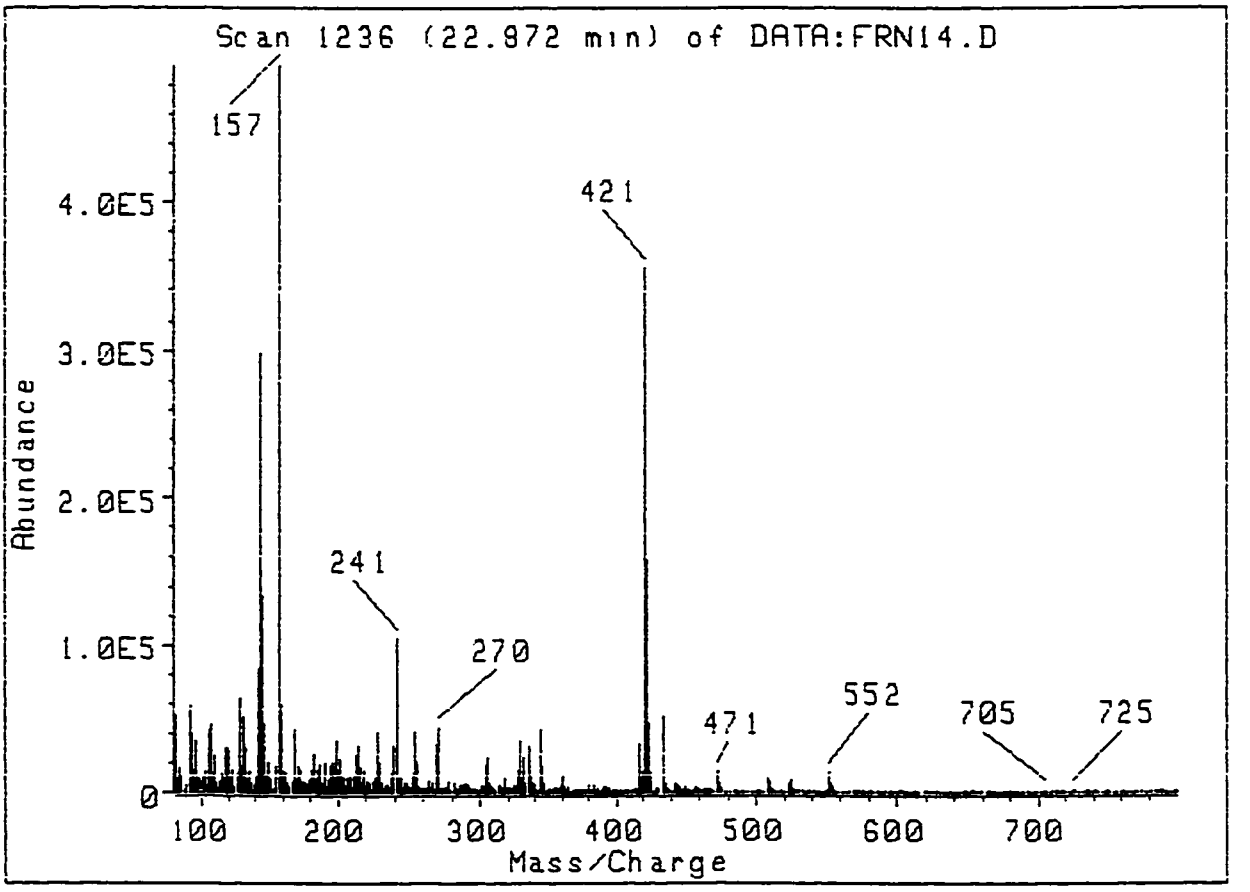


Figure 70. Di-hydroxy metabolite full scan mass spectrum – (SP0005 M-F) ethylestrenol monkey urine

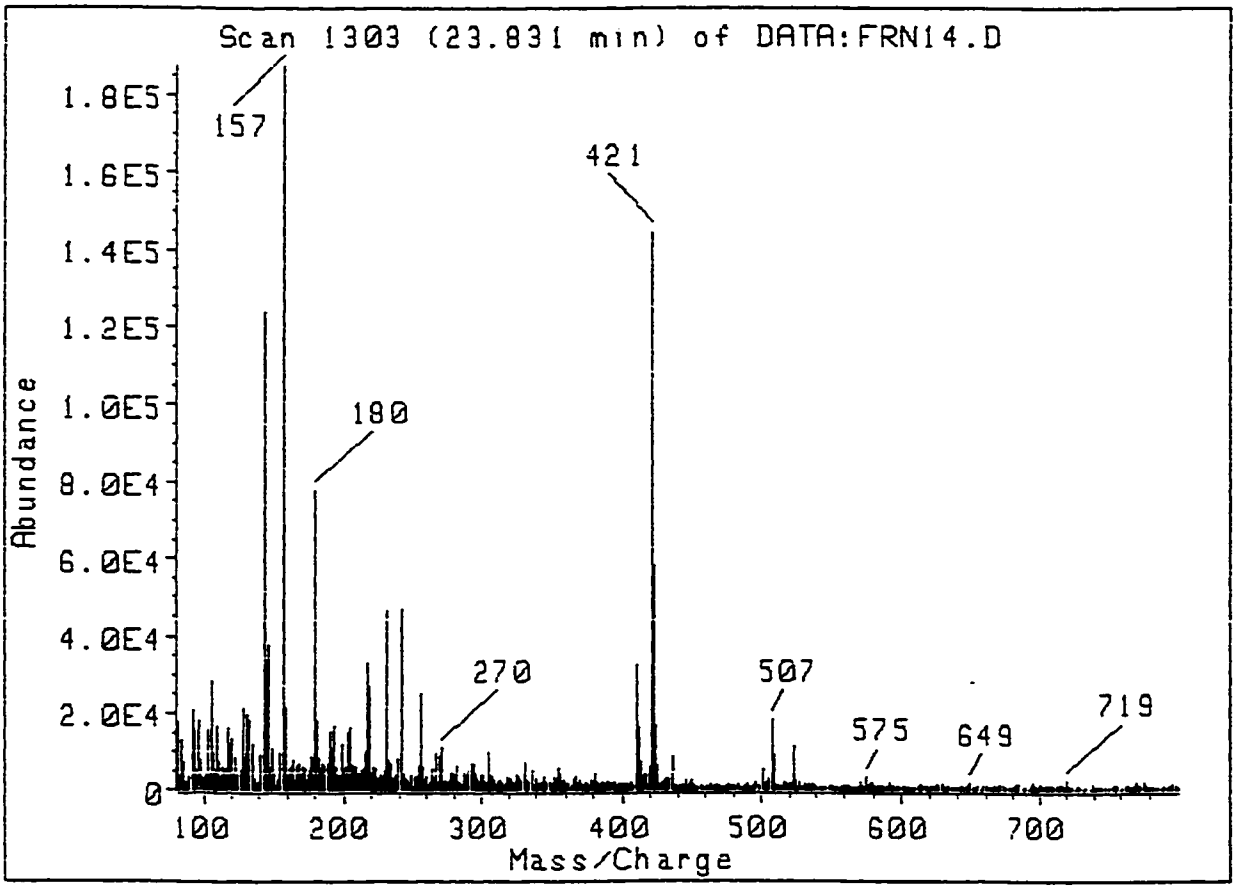


Figure 71. Di-hydroxy metabolite full scan mass spectrum – (SP0005 M-F) ethylestrenol monkey urine

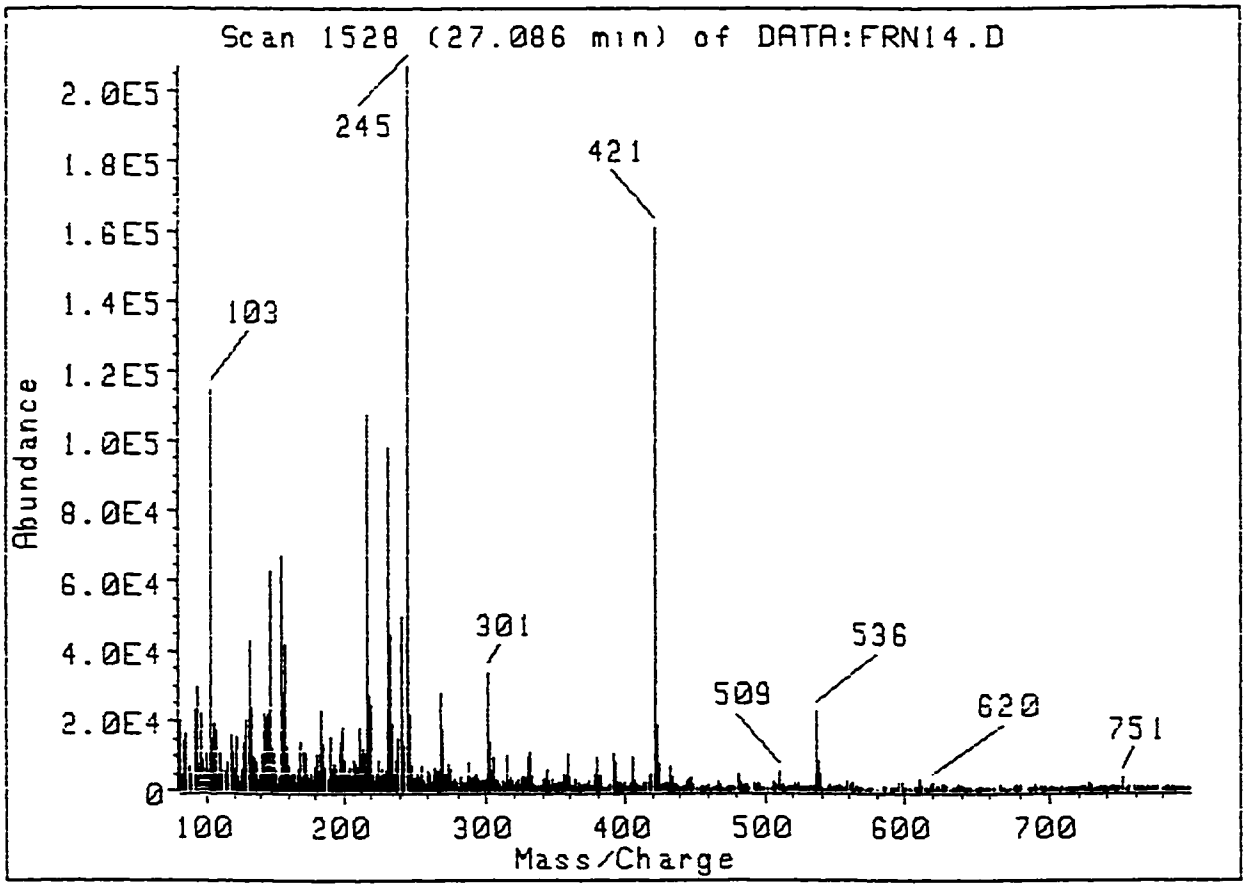


Figure 72. 3,17,21-Tri-hydroxy metabolite full scan mass spectrum – (SP0005 M-F) ethylestrenol monkey urine

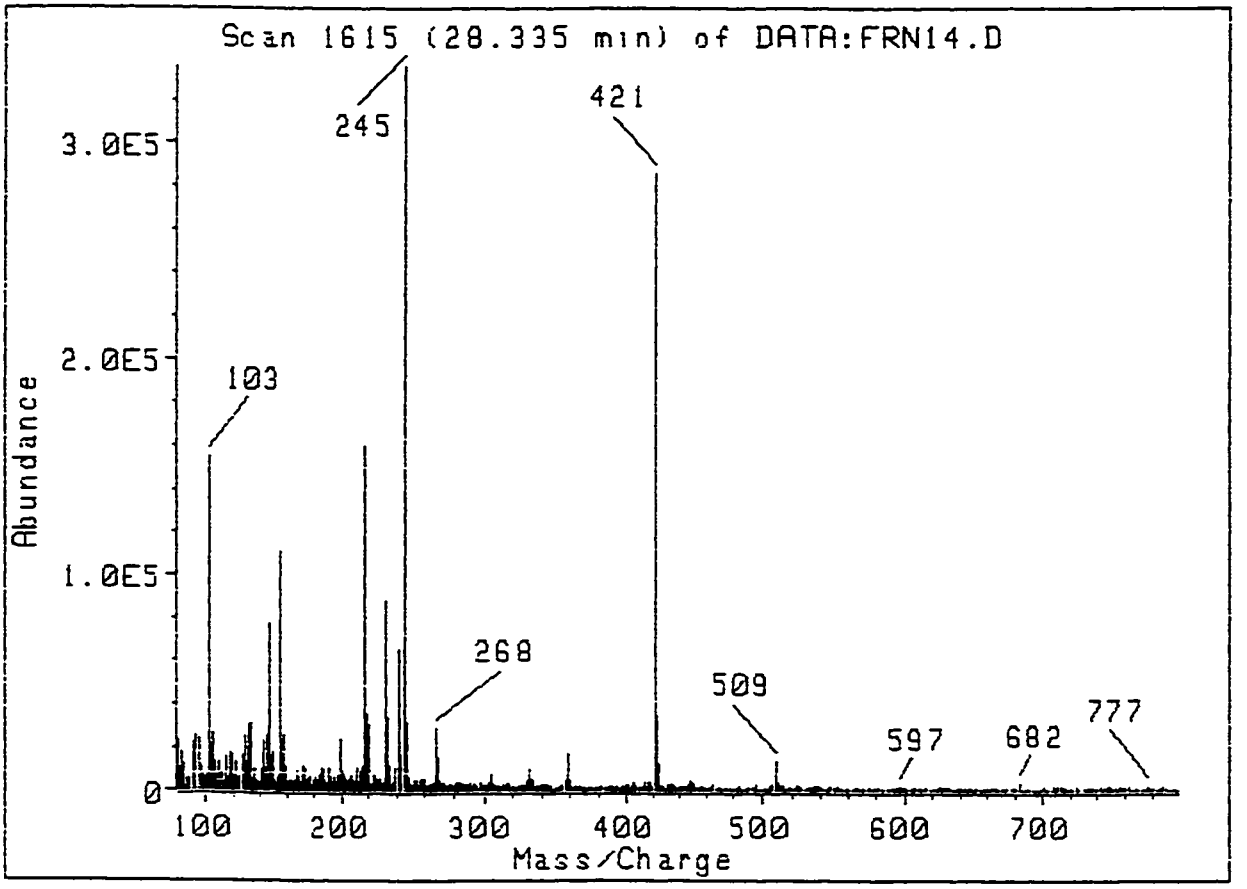


Figure 73. 3,17,21-Tri-hydroxy metabolite full scan mass spectrum – (SP0005 M-F) ethylestrenol monkey urine

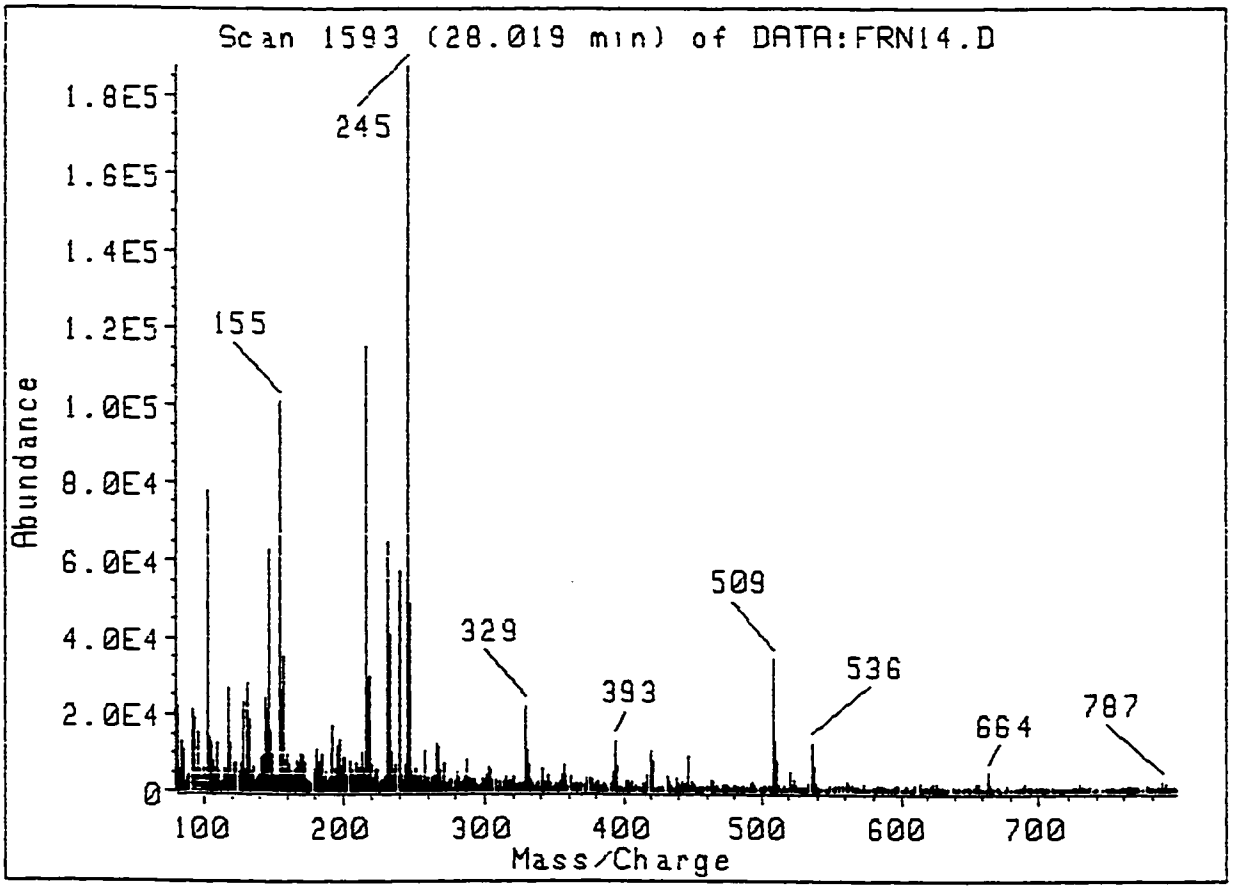


Figure 74. 3,16,17-Tri-hydroxy metabolite full scan mass spectrum – (SP0005 M-F) ethylestrenol monkey urine

5.B. ISOLATION OF ETHYLESTRENOL METABOLITE

5.B.1. Development of the isolation procedure

In the development of the solid phase extraction on the Amberlite XAD-2 column (section 4.4.A.1), we have performed a full scan GC/MS analysis of the wash fractions, to determine if ethylestrenol metabolites are eluted in these wash steps. The column was washed with 120 mL of: water, 20%, 30%, 40% and 50% solution of methanol in water. Ethylestrenol metabolites are recovered by elution with methanol. Different mixtures of methanol and water are applied to determine the highest methanol content that will wash unwanted substances and at the same time not elute the desired metabolites. After extracting the diagnostic ions for the different metabolites it was concluded that no metabolite was present in these fractions, and only the 50% MeOH:50% H_2O wash was used in subsequent experiments.

Also, in the development of the Sephadex LH-20 chromatography (section 4.4.A.4) full scan GC/MS analysis of the collected fractions was required to determine the contents of the fractions and the elution profile of the column chromatography. After searching for all different metabolites of ethylestrenol in the mass spectral data of each of the 45 fractions, it was concluded that the main component eluted was a 3,17-diol. The elution profile was determined by plotting the intensity of the base ion (m/z 157) of this diol in the different fractions 10 mL in volume each (Figure 75 – Graph 1). As the diagram shows, we can see that most of the diol is eluted between 150 mL and 350 mL.

Figure 75. Graph 1 - Sephadex LH-20 column elution profile

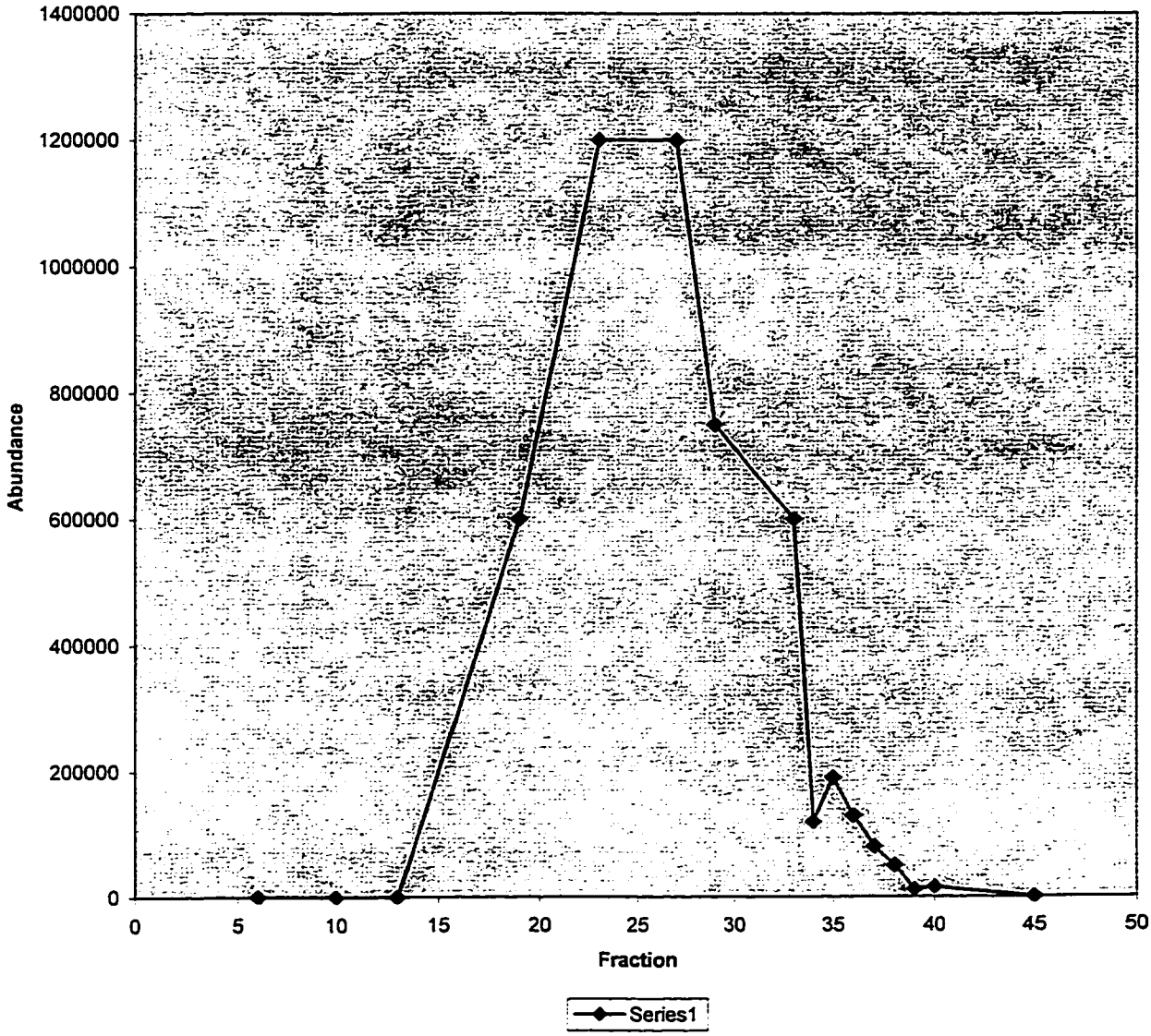


Figure 76 shows the extracted ion chromatograms of m/z 157 and 421 (base ion of 3,17-diol and 3,17,21-triol) in time window 21-29 minutes of fraction 13. Only base line noise was observed, and there was no metabolite in this fraction. The same ion profiles from the GC/MS analysis of fraction 19 and 27 are shown together with the mass spectrum of the diol present in these fractions (at retention times 22.570 and 22.611) in Figure 77 and 78. Also the TIC of the full scan analysis of fraction 27 is presented in Figure 79, showing that the main component in this fraction is the diol. No other ethylestrenol metabolites or endogenous steroids were present.

5.B.2. Isolation of ethylestrenol metabolite from 500 mL of urine

As previously described in section 4.4.B, after the solid phase extraction, steroid metabolites were subjected to hydrolysis and free steroids recovered by liquid-liquid extraction. This extract, fraction D, was reconstituted with 1 mL of MeOH. A 50 μL portion was dried, derivatized and analyzed by GC/MS.

The TIC of the full scan analysis of this portion of fraction D is shown in Figure 80, and it shows that this extract is a mixture of ethylestrenol metabolites and many other compounds. The extracted ion chromatograms of m/z 157 and 421 (base ion of 3,17-diol and 3,17,21-triol) and the mass spectrum of the peak of the 3,17-diol at retention time 23.055 are shown in Figure 81.

The isolation of the 3,17-diol was accomplished by Sephadex LH-20 column chromatography. Aliquots of the main fractions, I.2 and II.2 (section 4.4.B), of the two Sephadex LH-20 columns were analyzed by GC/MS. After searching the full scan of the

fraction I.2 it was concluded that only the 3,17-diol was present and there was no other steroid compound. The same conclusion was made about fraction II.2. The TIC of the full scan of fraction II.2 and that of the derivatizing mixture used to derivatize this fraction are shown in Figure 82.

The TIC of full scan mass spectral analysis of fraction II.2 shows only one major peak corresponding to that of the 3,17-diol. Some of the minor peaks originated from the derivatizing reagents, the TIC of which is also shown in Figure 82. The mass spectrum of the 3,17-diol at retention time 22.713 is shown in Figure 83.

5.B.3. Yield of the isolated metabolite

The isolation of the 3,17-diol from 500 mL of urine has been described in section 4.4.B. After solid phase extraction of the urine, hydrolysis of the conjugates of the metabolite and liquid-liquid extraction of the free metabolite, an extract was obtained that was concentrated and then reconstituted with 1 mL of MeOH. A 50 μL portion was used for GC/MS analysis. The remaining 950 μL was divided in two parts: I - 100 μL and II - 850 μL, which were further purified on two different Sephadex LH-20 columns. The 3,17-diol was collected in the second fraction in both instances (fractions I.2 and II.2). Fractions I.2 and II.2 were concentrated. Both were reconstituted with 1 mL of MeOH.

As mentioned in section 4.4.C the concentration of the 3,17-diol in excretion study urine SP0003 M-F was determined with the four-point Calibration Curve 1 (Table 8). The area ratios (area of the diol peak over the area of the internal standard peak) and the

concentrations of the calibrators are listed in the table. The Calibration Curve is shown in Figure 84. The concentration of 3,17-diol in urine SP0003 M-F was calculated to be 3450 ng/mL.

Table 8. Calibration Curve 1

Concentration	Area ratio
0 ng/mL	0
100 ng/mL	0.44
200 ng/mL	0.85
500 ng/mL	1.97

The quantitative report (section 4.4.C) for calibrator 200 ng/mL and the x20 dilution of the sample SP0003 M-F are shown on page 130-133.

Calibration Curve 2 was used for the quantitation of the 3,17-diol in fraction I.2. and II.2. In Table 9 the area ratios and concentrations are listed. Figure 85 shows Calibration Curve 2. Also the quantitative report for calibrator 500 ng and fraction II.2 is shown on page 135-138. It was determined that fraction I.2 contains 958.3 ng of the 3,17-diol in the 20 µL aliquot tested and the whole 1mL reconstituted fraction I.2 contains 47.9 µg.

Table 9. Calibration Curve 2

Concentration	Area ratio
0 ng	0
100 ng	0.11
200 ng	0.22
500 ng	0.59
1000 ng	1.22

For the quantitation of fraction II.2 first a x10 dilution was prepared by mixing 5µL of the fraction and 45 µL of methanol. Then 10 µL of this dilution was aliquoted for

quantitation. The 10 μL aliquot of the diluted fraction II.2 contained 650 ng, and the total fraction II.2 650 μg of the 3,17-diol.

The total amount of this diol present in 500 mL of urine was 1725 μg. Fractions I.2 and II.2 represent 10% and 85% of the initial volume of urine extracted. Therefore, the yield of the isolation procedure according to the amount found in fraction I.2 and II.2 was calculated to be 27.8% and 44.3%.

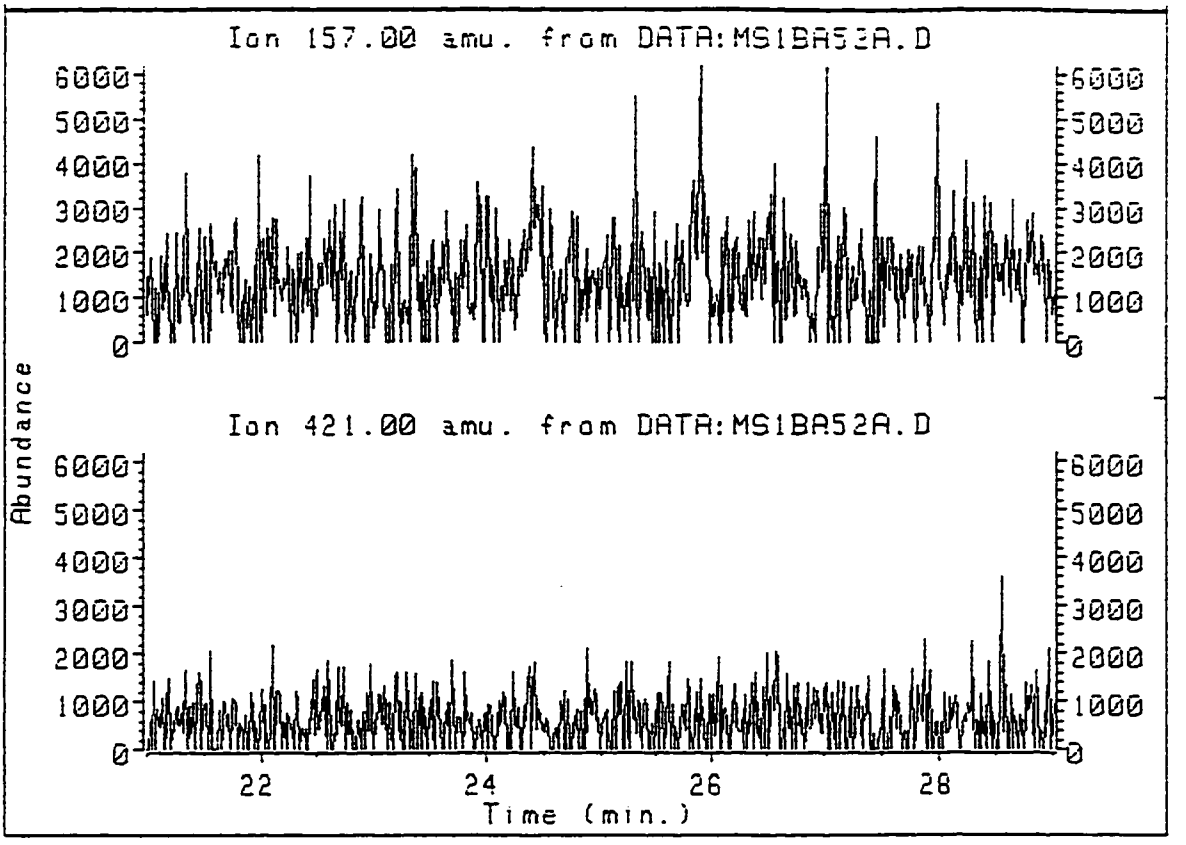


Figure 76. Extracted ion chromatograms m/z 157 and 421 in time window 21-29 minutes – Fraction 13 full scan

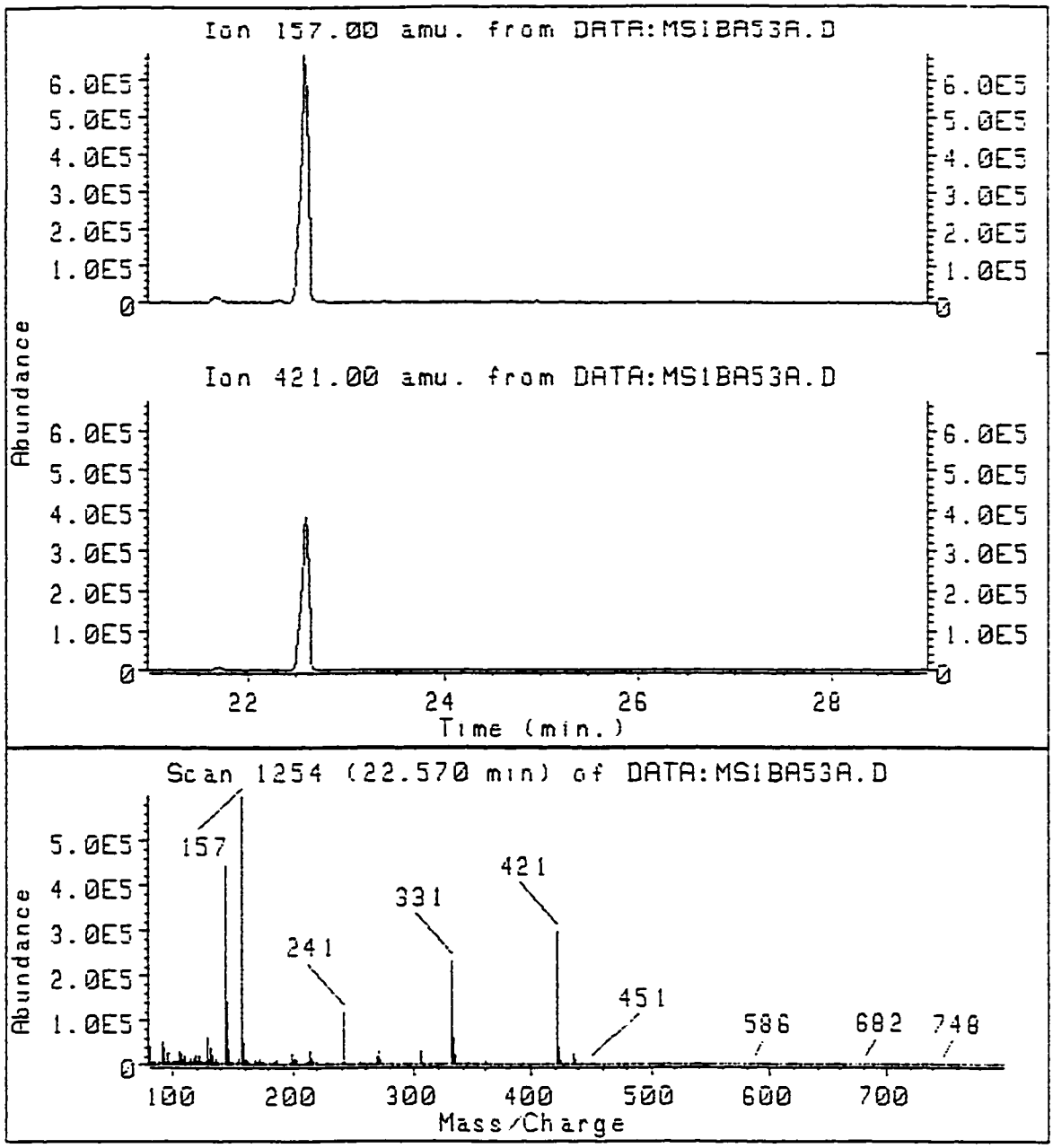


Figure 77. Extracted ion chromatograms m/z 157 and 421 in time window 21-29 minutes and di-hydroxy metabolite mass spectrum at RT 22.570 minutes – Fraction 19 full scan

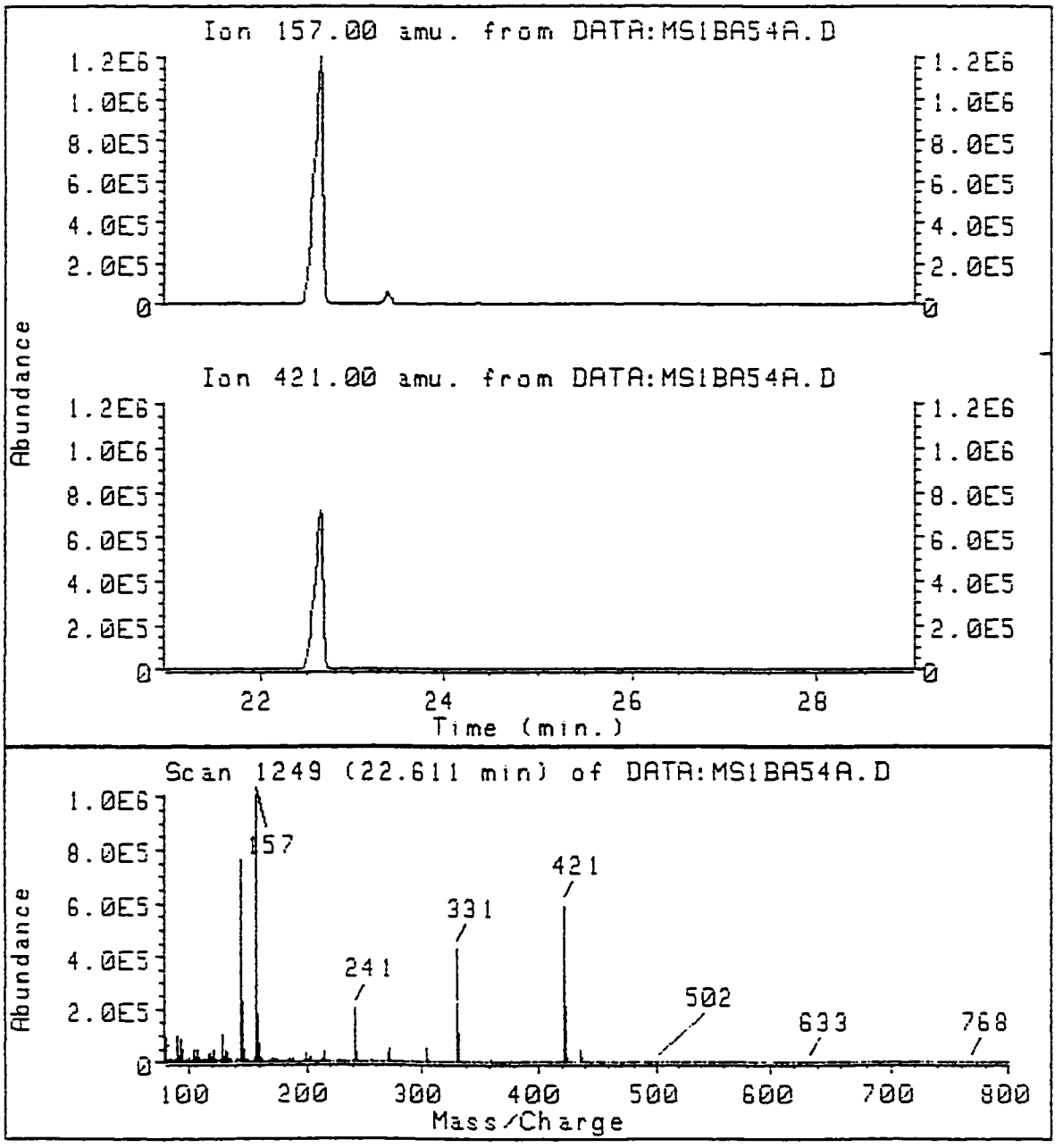


Figure 78. Extracted ion chromatograms m/z 157 and 421 in time window 21-29 minutes and di-hydroxy metabolite mass spectrum at RT 22.611 minutes – Fraction 27 full scan

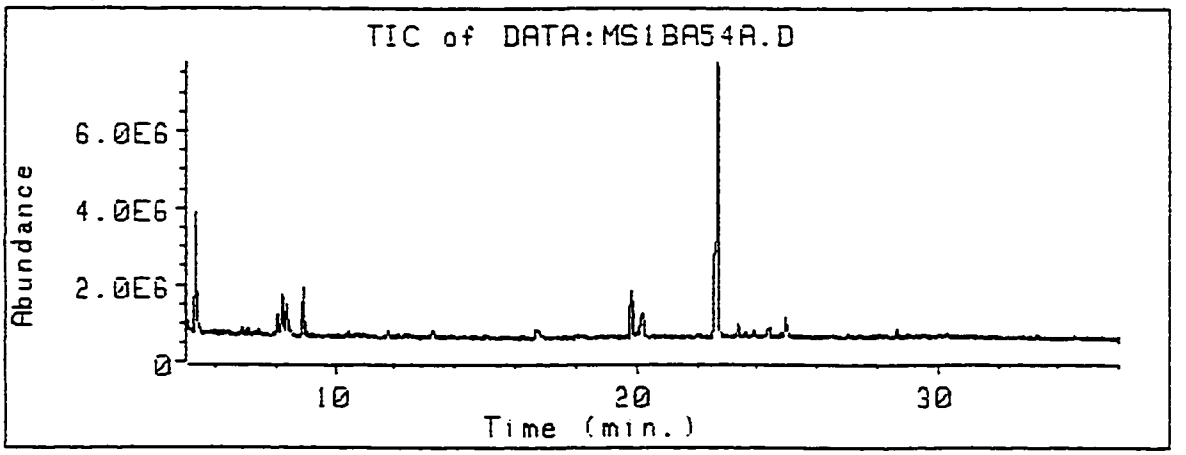


Figure 79. Total Ion Chromatogram – Fraction 27 full scan

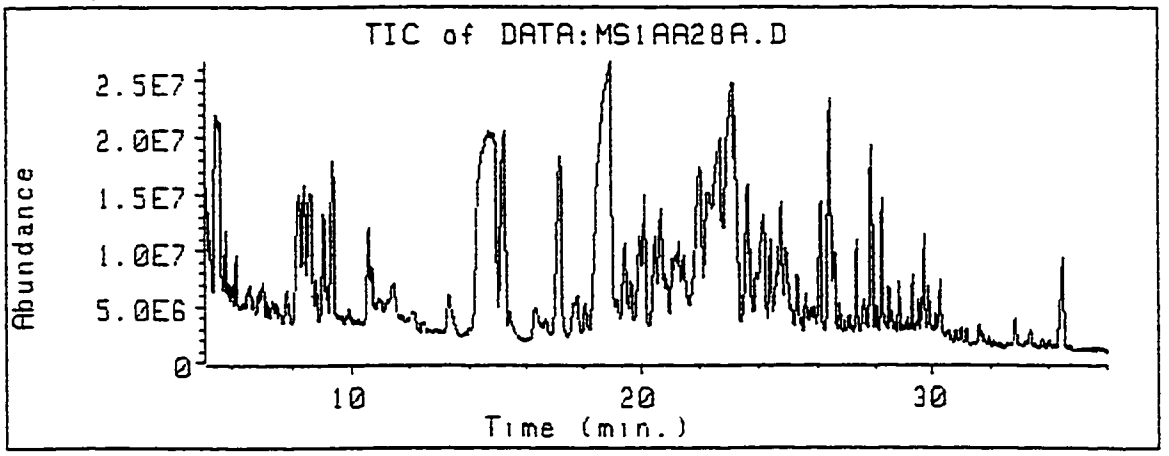
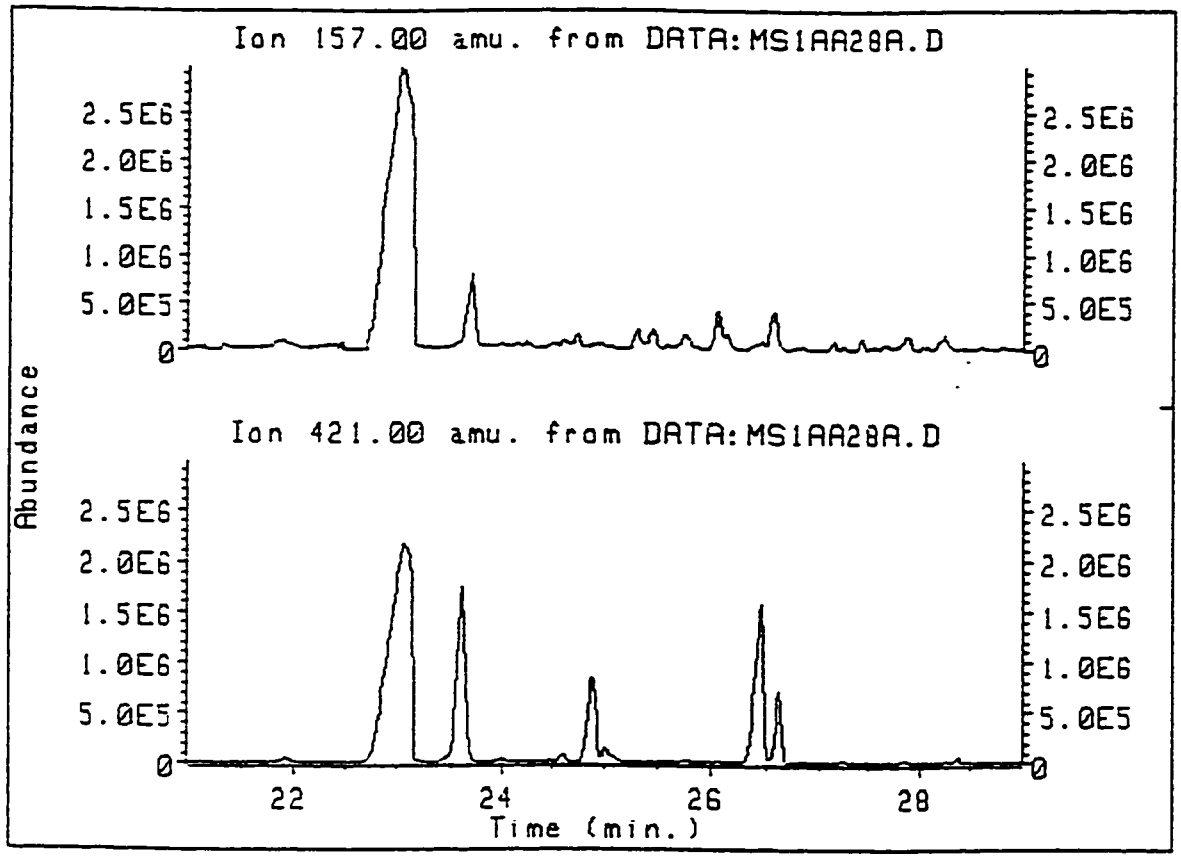


Figure 80. Total Ion Chromatogram – Fraction D full scan



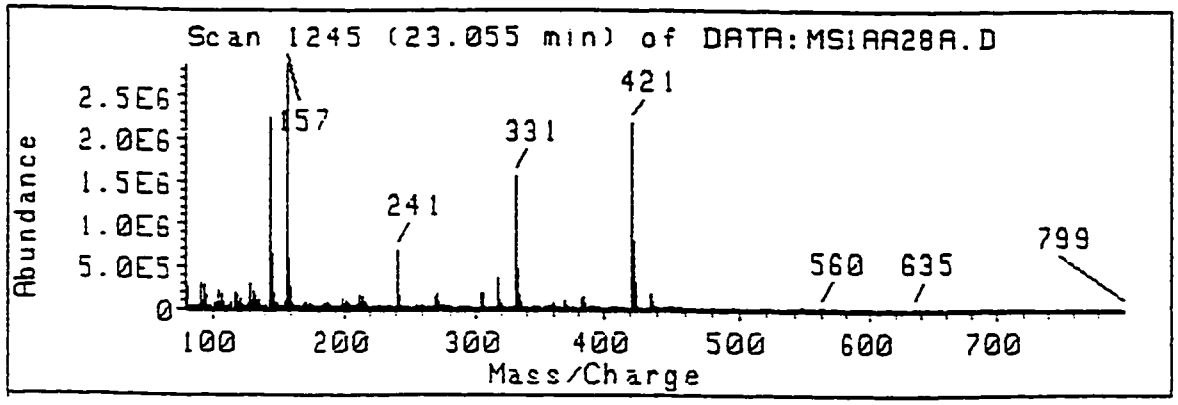


Figure 81. Extracted ion chromatograms m/z 157 and 421 in time window 21-29 minutes and di-hydroxy metabolite mass spectrum at RT 23.055 minutes – Fraction D full scan

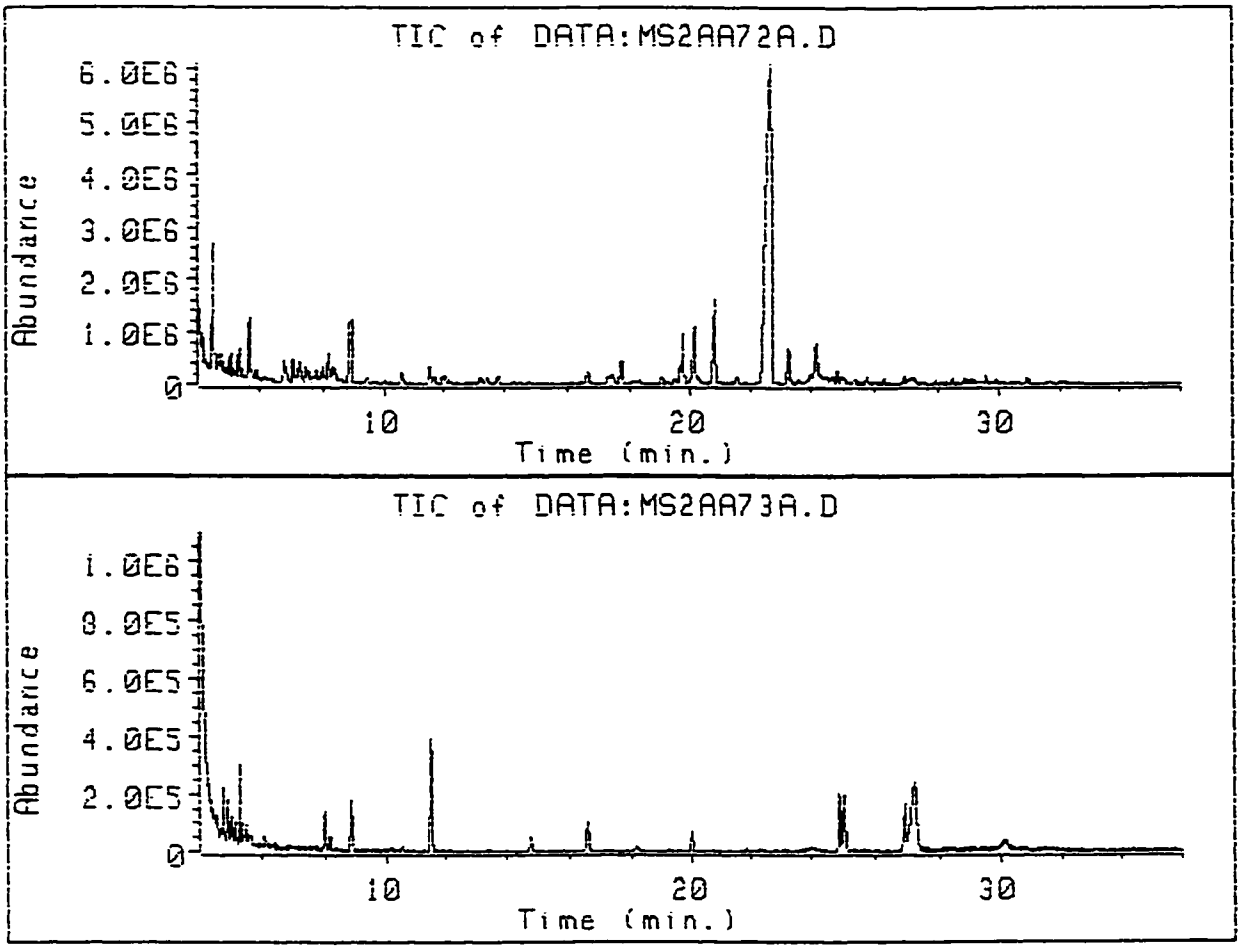


Figure 82. Total Ion Chromatograms – Fraction II.2 full scan (top) and derivatizing mixture full scan (bottom)

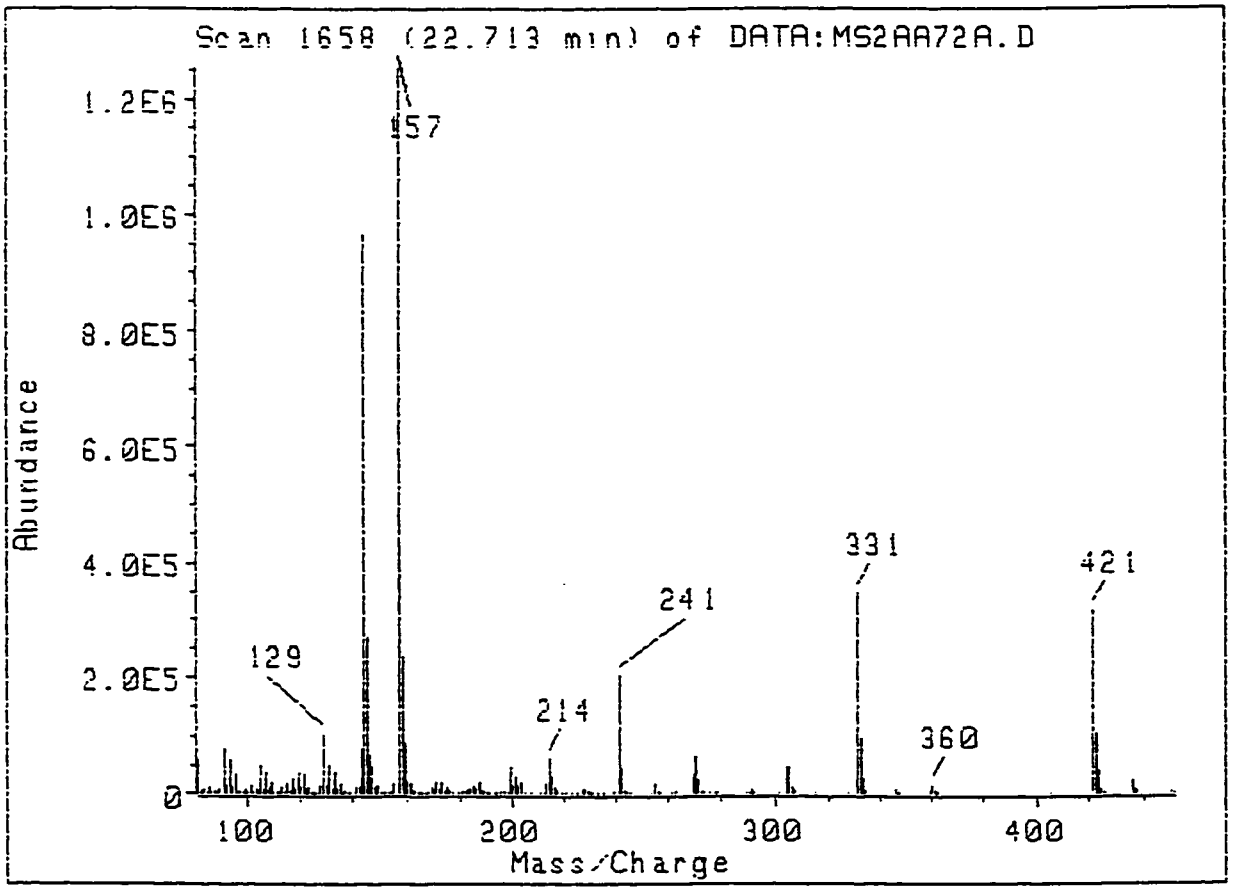


Figure 83. Di-hydroxy metabolite mass spectrum at RT 22.713 minutes – Fraction II.2 full scan

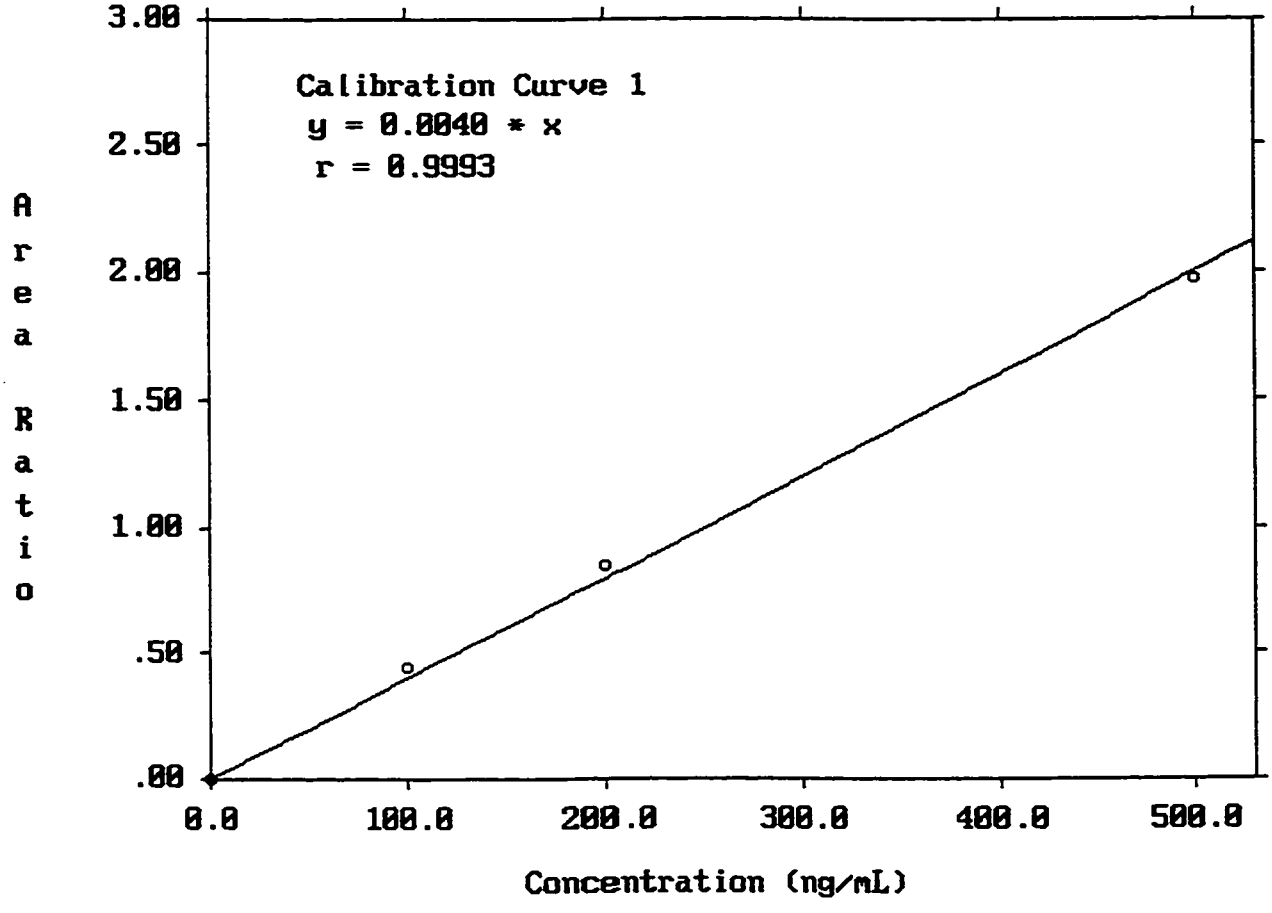


Figure 84. Calibration Curve 1 – Quantitation of the 3,17-diol in excretion study urine SP0003 M-F

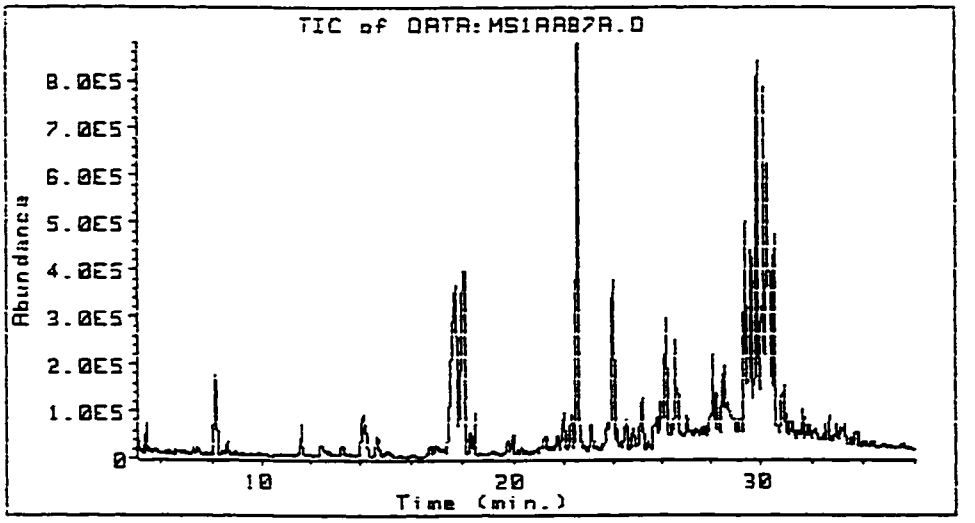
Data file: DATA:MS1AA87A.D
File type: GC / MS DATA FILE

Name Info: CAL 200 ng/mL
Misc Info:
Operator :

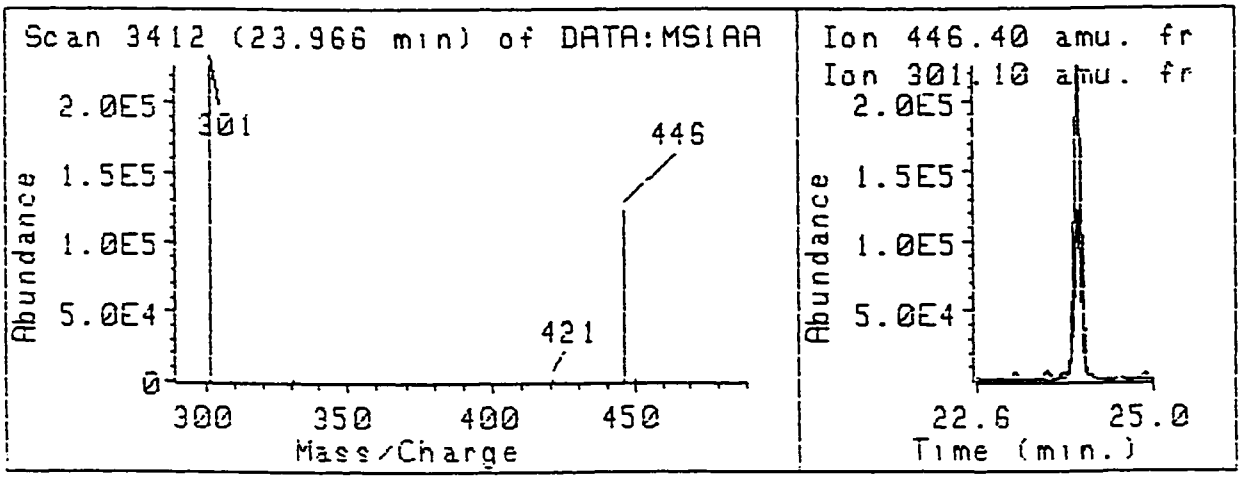
Date : 14 Dec 97 8:45 pm
Instrment: MS_5970
Inlet : GC

Sequence index : 1
Als bottle num : 87
Replicate num : 1

* ETHYLESTRENOL/NORETHANDROLONE METABOLITE CONFIRMATION *



INTERNAL STANDARD (Methyltestosterone) 23.8



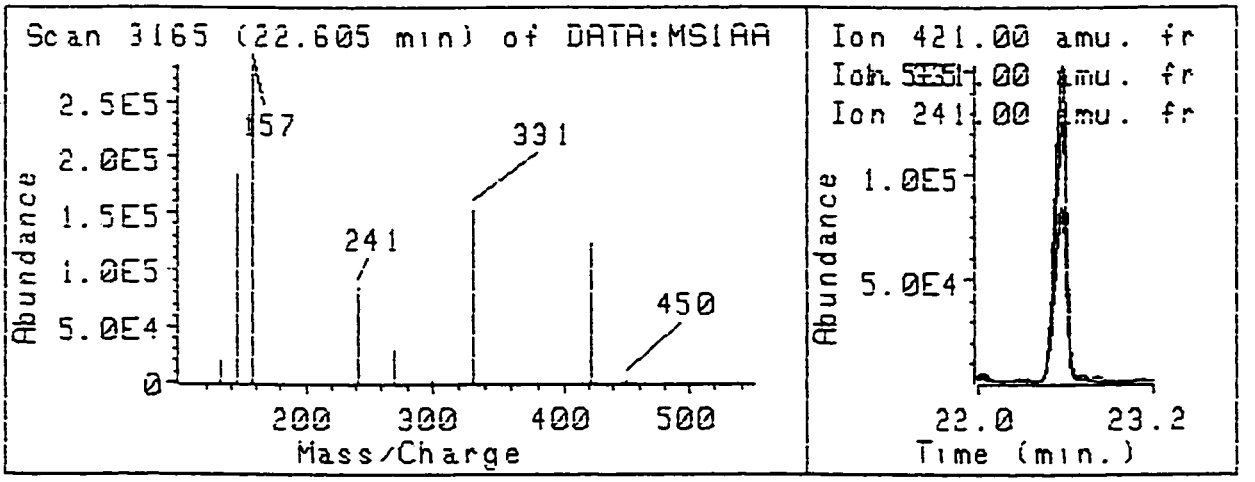
Data file: DATA:MS1AA87A.D
File type: GC / MS DATA FILE

Name Info: CAL 200 ng/mL
Misc Info:
Operator :

Date : 14 Dec 97 8:45 pm
Instrment: MS_5970
Inlet : GC

Sequence index : 1
Als bottle num : 87
Replicate num : 1

METABOLITE 1 (Norpregnanediol) 22.5



Norpregnanediol Retention Time: 22.51 Norpregnanediol Quant Ion: 421.00
Internal Standard Retention Time: 23.97 Int Std Quant Ion: 446.40
Relative Retention Time: 0.94

	Mass Ion	Ion Abundance	Ion Abund. Ratio	Area	Area Ratio
	421.00	123328.00	1.00	6853233.48	0.85
Metab 1	331.00	152768.00	1.24		
	241.00	84160.00	0.68		
	446.40	121672.00	1.00	8035870.89	
Int Std	361.10	226888.00	1.86		

ALPHACOPY
[OE]

T: null.
Z: null.
Y: Scan 3165 (22.605 min) of
X: Set of 3 GCMS

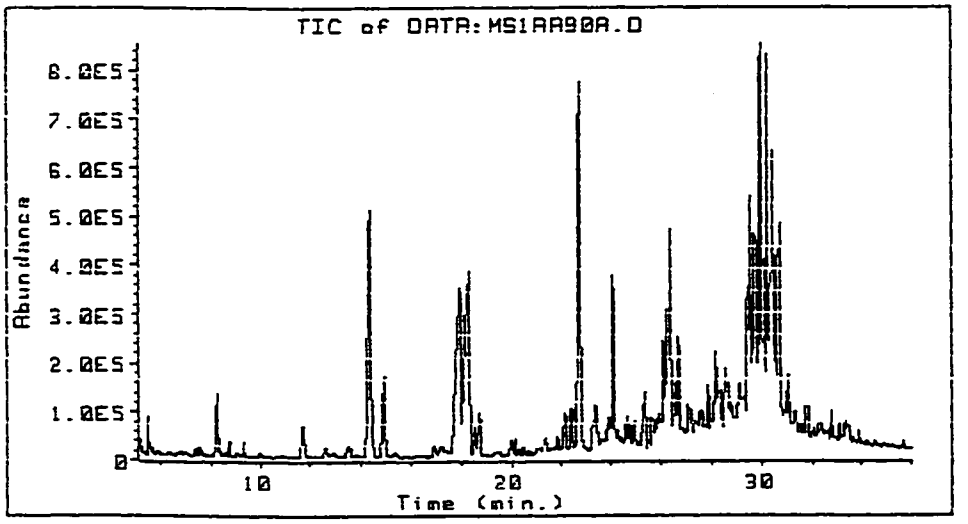
Data file: DATA:MS1AA90A.D
File type: GC / MS DATA FILE

Name Info: SP0003 (x 20 dilution)
Misc Info:
Operator :

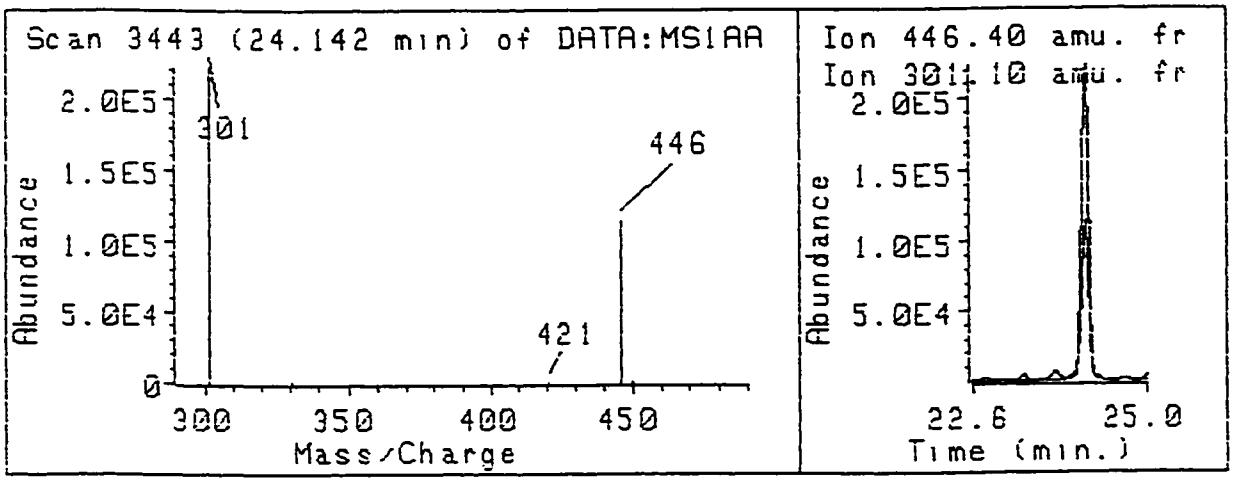
Date : 14 Dec 97 10:42 pm
Instrment: MS_5970
Inlet : GC

Sequence index : 1
Als bottle num : 90
Replicate num : 1

* ETHYLESTRENOL/NORETHANDROLONE METABOLITE CONFIRMATION *



INTERNAL STANDARD (Methyltestosterone) 23.8



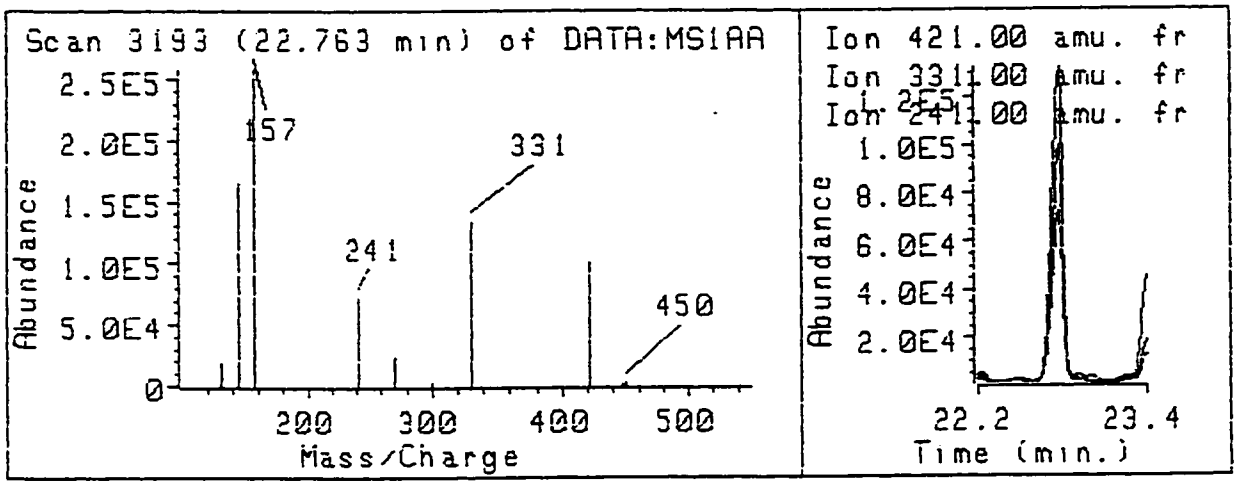
Data file: DATA:MS1AA90A.D
File type: GC / MS DATA FILE

Name Info: SP0003 (x 20 dilution)
Misc Info:
Operator :

Date : 14 Dec 97 10:42 pm
Instrment: MS_5970
Inlet : GC

Sequence index : 1
Als bottle num : 90
Replicate num : 1

METABOLITE 1 (Norpregnanediol) 22.5



Norpregnanediol Retention Time: 22.76 Norpregnanediol Quant Ion: 421.00
Internal Standard Retention Time: 24.14 Int Std Quant Ion: 446.40
Relative Retention Time: 0.94

	Mass Ion	Ion Abundance	Ion Abund. Ratio	Area	Area Ratio
	421.00	100104.00	1.00	5265552.26	0.69
Metab 1	331.00	133760.00	1.34		
	241.00	72304.00	0.72		
	446.40	114944.00	1.00	7631799.95	
Int Std	361.10	222016.00	1.93		

T: null.
Z: null.
Y: Scan 3193 (22.763 min) of
X: Set of 3 GCMS

ALPHACOPY
[DE]

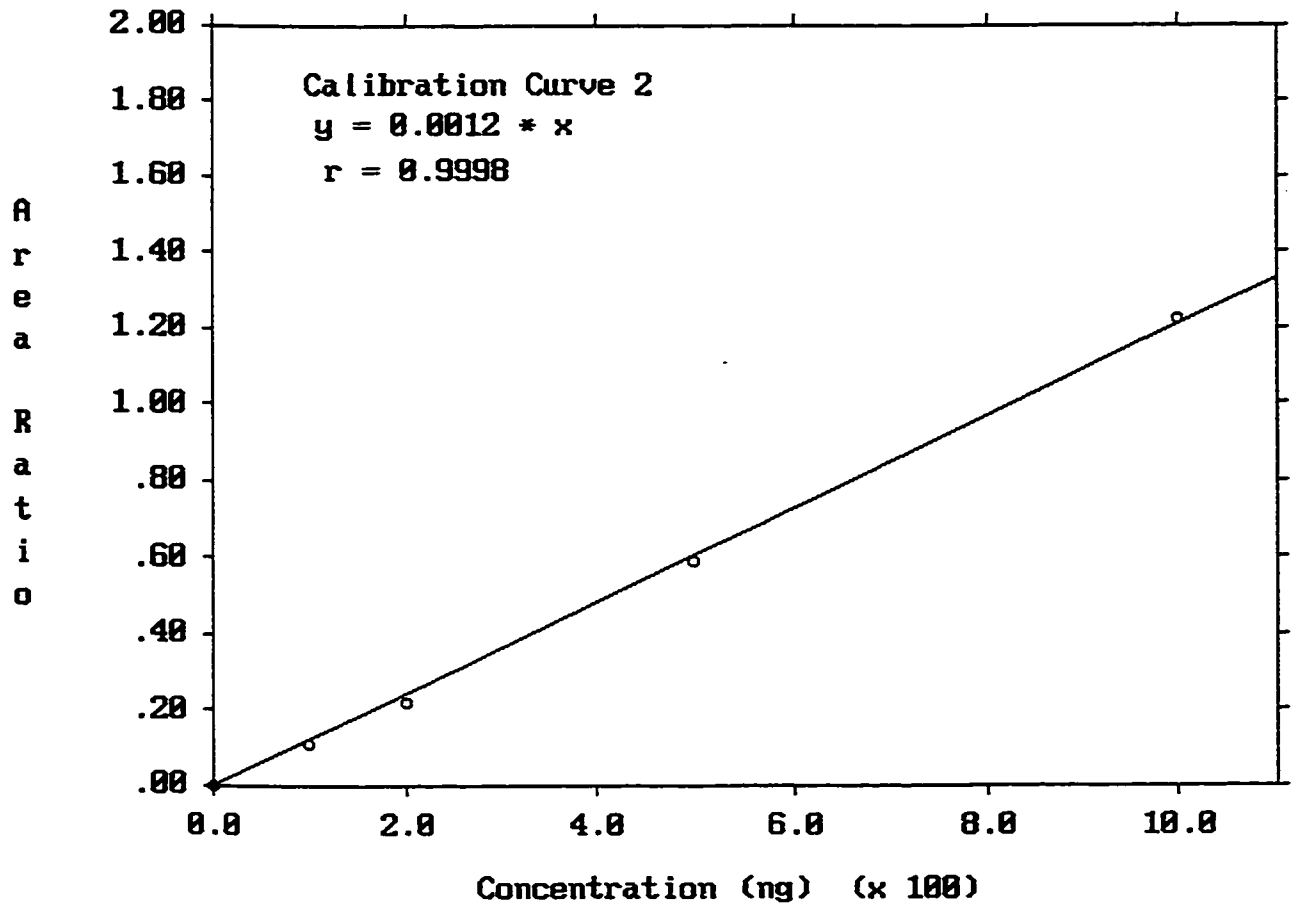


Figure 85. Calibration Curve 2 – Quantitation of the 3,17-diol in fractions I.2 and II.2

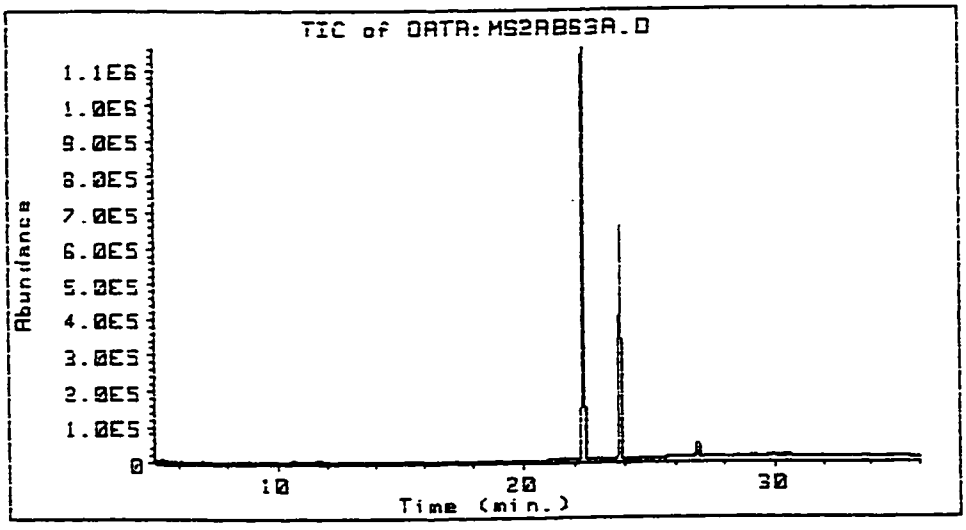
Data file: DATA:MS2AB63A.D
File type: GC / MS DATA FILE

Name Info: CAL 500 ng
Misc Info:
Operator :

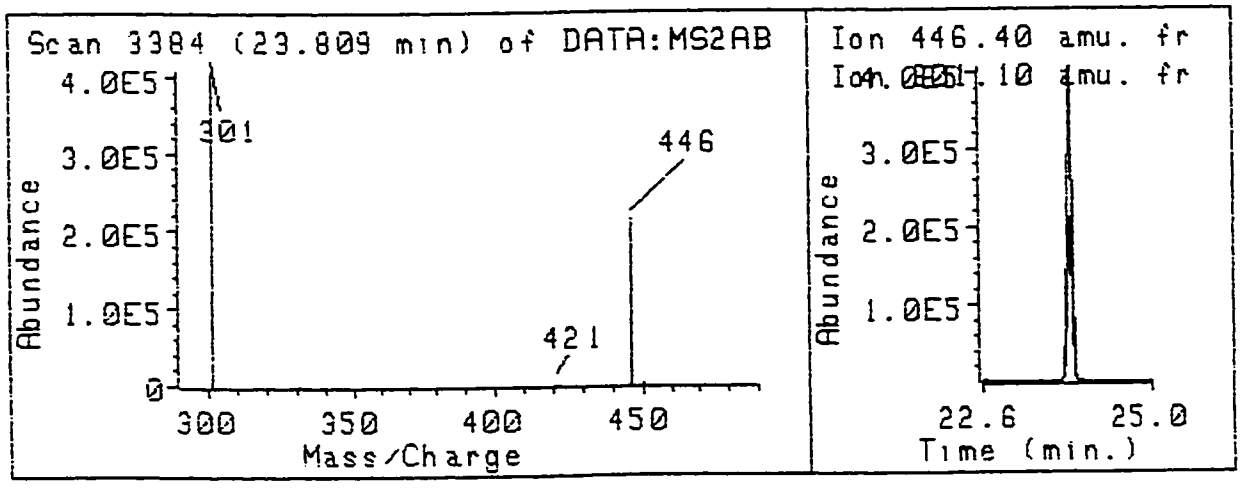
Date : 3 Jan 98 11:22 pm
Instrment: MS_5970
Inlet : GC

Sequence index : 2
Als bottle num : 63
Replicate num : 1

* ETHYLESTRENOL/NORETHANDROLONE METABOLITE CONFIRMATION *



INTERNAL STANDARD (Methyltestosterone) 23.8



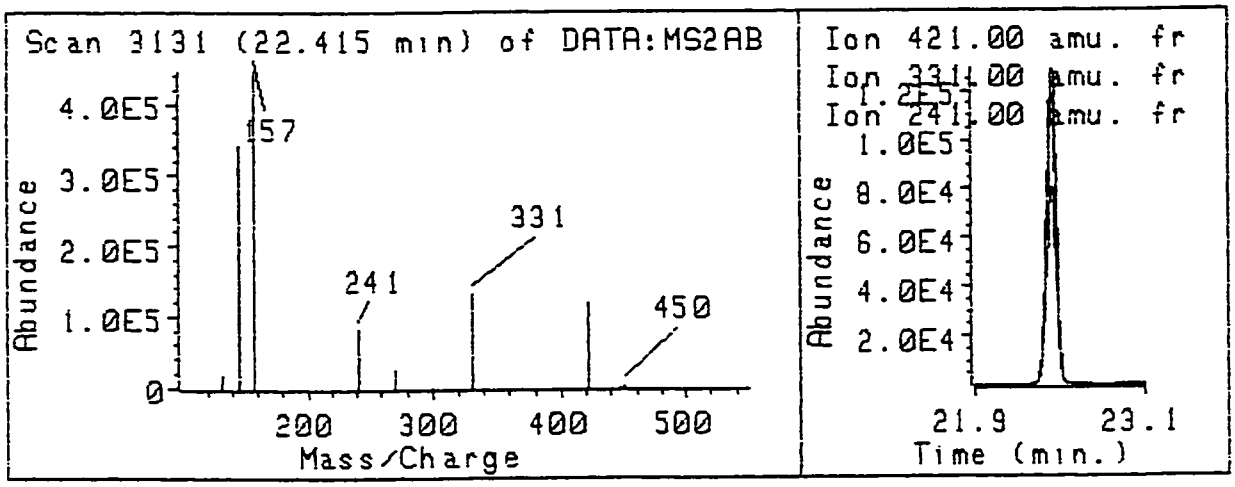
Data file: DATA:MS2AB63A.D
File type: GC / MS DATA FILE

Name Info: CAL 500 ng
Misc Info:
Operator :

Date : 3 Jan 98 11:22 pm
Instrment: MS_5970
Inlet : GC

Sequence index : 2
Als bottle num : 63
Replicate num : 1

METABOLITE 1 (Norpregnanediol) 22.5



Norpregnanediol Retention Time: 22.42 Norpregnanediol Quant Ion: 421.00
Internal Standard Retention Time: 23.81 Int Std Quant Ion: 446.40
Relative Retention Time: 0.94

	Mass Ion	Ion Abundance	Ion Abund. Ratio	Area	Area Ratio
	421.00	119064.00	1.00	5801436.83	0.59
Metab 1	331.00	138200.00	1.09		
	241.00	81248.00	0.68		
	446.40	212600.00	1.00	9848083.94	
Int Std	301.10	406384.00	1.92		

T: null.
Z: null.
Y: Scan 3131 (22.415 min) of
X: Set of 3 GCMS

ALPHACOPY
/

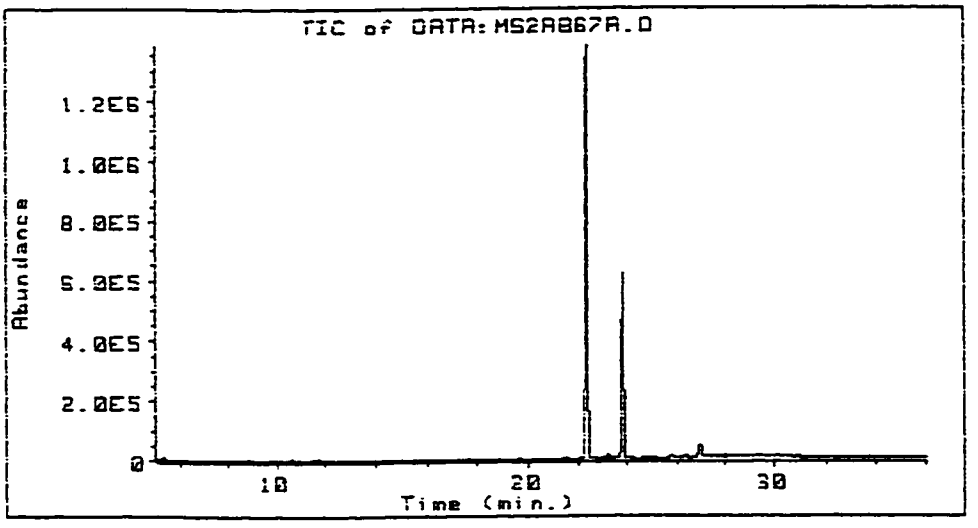
Data file: DATA:MS2AB67A.D
File type: GC / MS DATA FILE

Name Info: SAMPLE 2
Misc Info:
Operator :

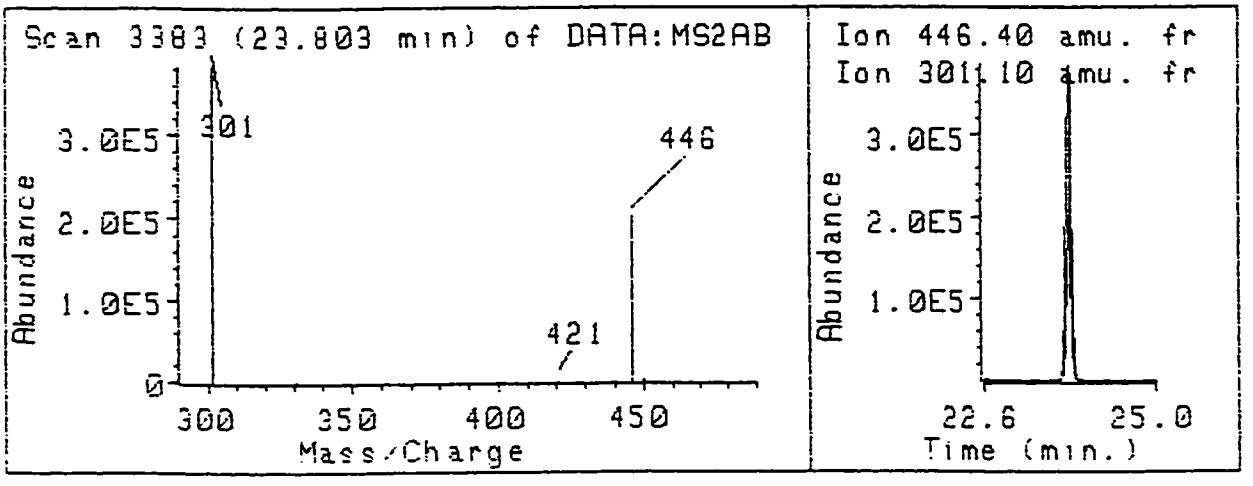
Date : 4 Jan 98 1:58 am
Instrment: MS_5970
Inlet : GC

Sequence index : 2
Als bottle num : 67
Replicate num : 1

* ETHYLESTRENOL/NORETHANDROLONE METABOLITE CONFIRMATION *



INTERNAL STANDARD (Methyltestosterone) 23.8



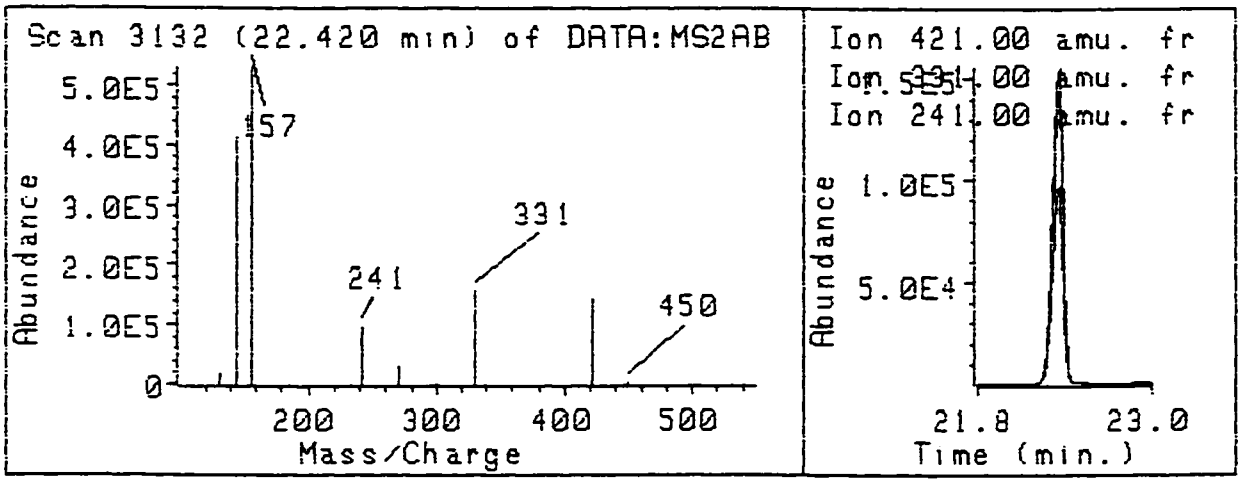
Data file: DATA:MS2AB67A.D
File type: GC / MS DATA FILE

Name Info: SAMPLE 2
Misc Info:
Operator :

Date : 4 Jan 98 1:58 am
Instrment: MS_5970
Inlet : GC

Sequence index : 2
Als bottle num : 67
Replicate num : 1

METABOLITE 1 (Norpregnanediol) 22.5



Norpregnanediol Retention Time: 22.42 Norpregnanediol Quant Ion: 421.00
Internal Standard Retention Time: 23.00 Int Std Quant Ion: 446.40
Relative Retention Time: 0.94

	Mass Ion	Ion Abundance	Ion Abund. Ratio	Area	Area Ratio
	421.00	144320.00	1.00	7392779.45	0.78
Metab 1	331.00	155648.00	1.08		
	241.00	96896.00	0.67		
	446.40	201344.00	1.00	9524340.98	
Int Std	301.10	385792.00	1.92		

ALPHACOPY
/

T: null.
Z: null.
Y: Scan 3132 (22.420 min) of
X: Set of 3 GCMS

6. DISCUSSION

6.A. METABOLISM OF ETHYLESTRENOL/NORETHANDROLONE IN THE CYNOMOLGUS MONKEY

To be able to determine relative retention times (RRT) of different metabolites, the retention time (RT) of the internal standard (IS), methyltestosterone (see section 4.3.), was determined in each GC/MS analysis of the urine extracts. The peak of methyltestosterone was located by extracting diagnostic ions m/z 446 (molecular ion) and m/z 301 (D-ring fragmentation –2H). Figure 86 represents an example of how the RT of the IS was determined for ethylestrenol monkey excretion urine SP0003 M-F (see section 4.2.), and also contains the mass spectrum of methyltestosterone. The IS retention times for each urine are listed in Table 10.

Table 10 represents a tabulation of RT and RRT of all di-hydroxy metabolite and norethandrolone peaks identified (see section 5.A. for identification criteria) in different excretion urines analyzed.

The metabolism of norethandrolone and ethylestrenol will not be discussed separately as it has been shown by Ward et al. [11], and confirmed in this thesis (see later discussion on norethandrolone), that they are identical.

Di-hydroxy metabolites as observed in the different excretion urine listed in Table 10, can be divided by their RT and RRT into four groups (Table 11). Therefore, four

different di-hydroxy metabolites have been identified. No major differences in mass spectra of the four metabolites have been identified, indicating that these metabolites are probably stereoisomers. The fragmentation ions are discussed in section 5.A. (Figure 8).

Table 10. Ethylestrenol/norethandrolone metabolites

SAMPLE	IS RT	3,17-Diol RT	RRT	Norethandrolone RT	RRT
SP0001 M-F	23.836	22.555	0.946	25.809	1.083
SP0002 S-S	23.887	22.582	0.945		
SP0003 M-F	23.995	22.726	0.947	25.897	1.079
SP0004 S-S	24.020	22.849	0.951		
SP0005 M-F	24.004	21.740	0.906		
		22.729	0.947		
		22.872	0.953		
		23.831	0.993		
SP0006 M-F	24.051	22.826	0.949	26.025	1.082
Norethandrolone monkey	23.947	21.702	0.906	25.923	1.082
		22.643	0.946		
		22.796	0.952		
Norethandrolone human	23.913	21.669	0.906		
		22.526	0.942		

Table 11. Grouping of di-hydroxy metabolites by RT and RRT

Group I		Group II		Group III		Group IV	
RRT	RT	RRT	RT	RRT	RT	RRT	RT
0.906	21.740	0.946	22.555	0.953	22.872	0.993	23.831
0.906	21.702	0.945	22.582	0.952	22.796		
0.906	21.669	0.947	22.726				
		0.951	22.849				
		0.947	22.729				
		0.949	22.826				
		0.946	22.643				
		0.942	22.526				

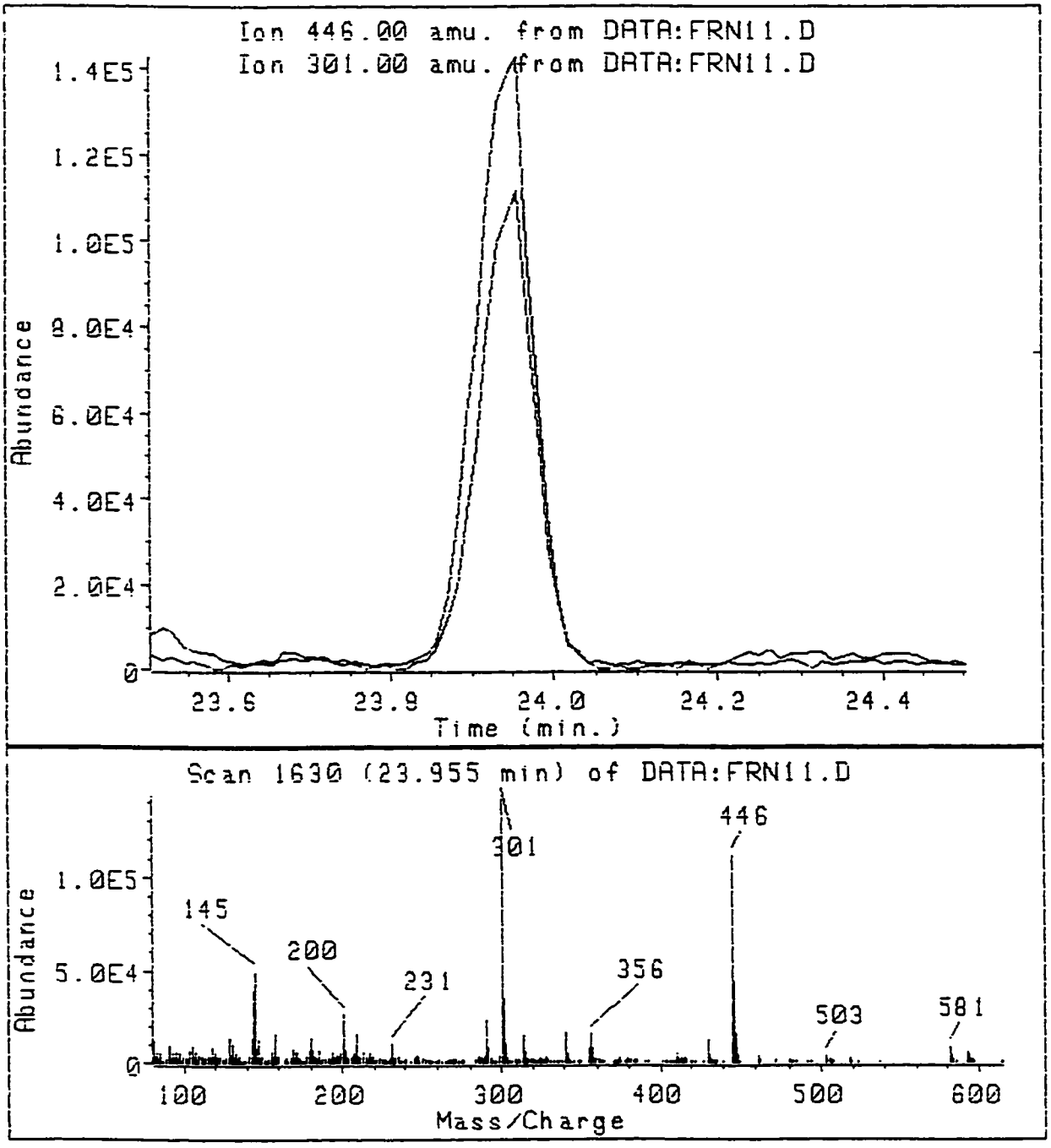


Figure 86. Internal standard diagnostic ions and mass spectrum – Full scan GC/MS analysis of SP0003 M-F

The major di-hydroxy metabolite, found in each urine (Group II), is 17α-ethyl-5β-estran-3α,17β-diol. The identity of this metabolite was verified by comparing with purchased authentic compound that has been synthesized by RADIAN. In addition to mass spectra, RT and RRT comparison, some of the standard was added to the diluted sample of ethylestrenol excretion urine SP0003 M-F and no additional peaks have been observed (Figure 87 shows extracted diagnostic ions, discussed in section 5.A., for the diol from the full scan analysis of SP0003 M-F urine diluted 10 times and Figure 88 from the analysis of the 1:1 mixture of this extract and extract of a calibrator containing 500 ng/mL of 17α-ethyl-5β-estran-3α,17β-diol). This metabolite was first identified in human norethandrolone excretion urine by Brooks et al. [10] and later by Schänzer and Donike [13]. In the norethandrolone human excretion, analyzed along with the monkey excretions (Table 10 and 11), 17α-ethyl-5β-estran-3α,17β-diol has also been identified.

To understand the configuration of the three minor metabolites identified, the metabolism of ethylestrenol/norethandrolone was related to the metabolism of testosterone and other 3-keto-4-ene anabolic steroids. The metabolism of 3-keto-4-ene steroids most likely follows that of testosterone, as the enzymes that convert testosterone to its distinct metabolites are also active towards these steroids [9]. In the A-ring metabolism, the initial and rate-limiting step is the reduction of the C-4,5 double bond, catalyzed by 5α- and 5β-reductase located mainly in the liver. It yields an asymmetric center at C-5, and two with 5α- and 5β-configuration are formed (Figure 89).

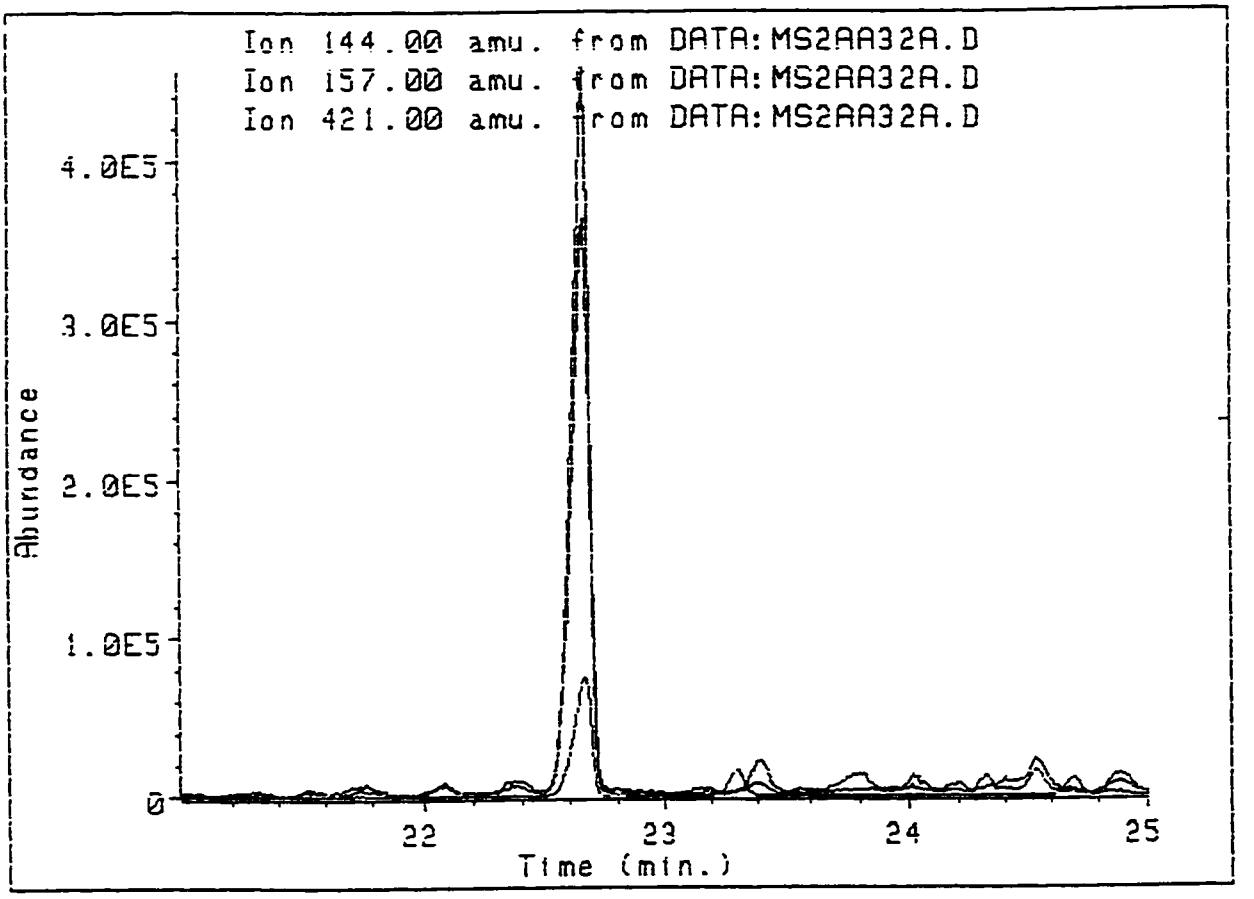


Figure 87. Full scan GC/MS analysis of SP0003 M-F (x10 dilution) – di-hydroxy metabolite diagnostic ions

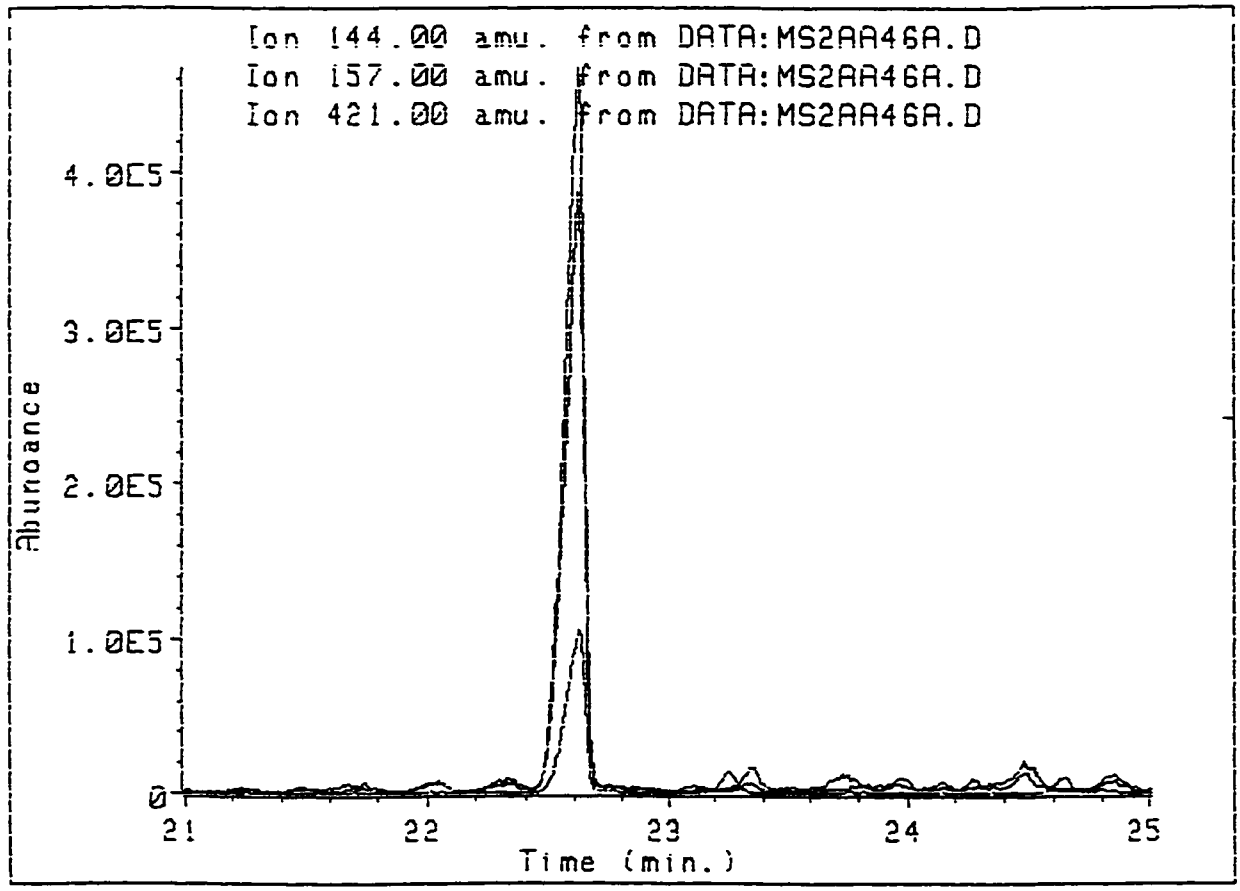


Figure 88. Full scan GC/MS analysis of SP0003 M-F (x10 dilution) + CAL 500 ng/mL – di-hydroxy metabolite diagnostic ions

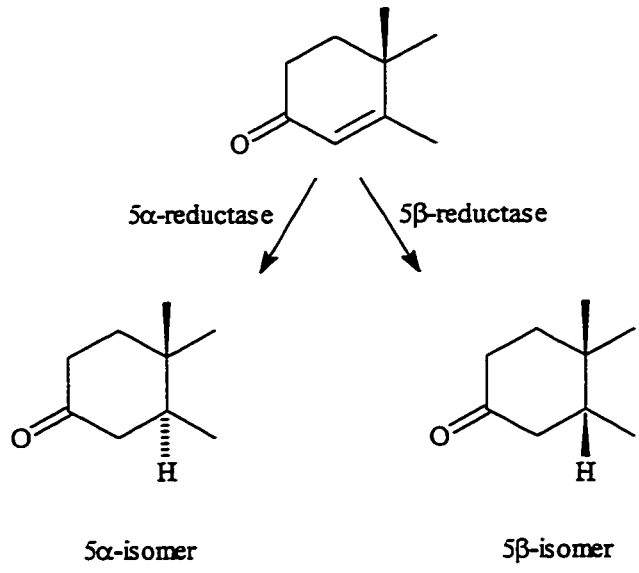


Figure 89. A-ring metabolism: 5α- and 5β-reduction of 3-keto-4-ene steroids

Metabolism of different 3-keto-4-ene anabolic steroids has been studied in human. In the metabolism of some of them the reduction to 5α- and/or 5β-isomer has been confirmed and for some only proposed. Published data has been compiled in Table 12 [9].

The extent of the 5α- and 5β-isomer produced is dependent on the structure of the steroid, as shown in Table 13 for several anabolic steroids in one male individual [9], however 5β-isomers are mainly produced.

Table 12. Reduction of the C-4,5 double bond in the metabolic pathway of 3-keto-4-ene anabolic steroids

Anabolic steroid	**5α and/or 5β- Reduced metabolites**
Bolasterone	5β-isomer*
Boldenone	5β-isomer*
Calusterone	5α and 5β-isomer*
4-Chloro-1,2-dehydro-17α-methyltestosterone	5β-isomer is proposed
Clostebol	Both isomers are proposed
Fluoxymesterone	5α and 5β-isomer
Formebolone	Not detected
Metandienone	5β-isomer*
Methyltestosterone	5α and 5β-isomer*
Mibolerone	5β-isomer is proposed
Nandrolone	5α and 5β-isomer*
Norclostebol	Both isomers are proposed
Norethandrolone	5α and 5β-isomer
Oxymesterone	Not detected
Testosterone	5α and 5β-isomer*
Trenbolone	Not detected

* See also Table 13.

Table 13. Stereospecific metabolism of 3-keto-4-ene steroids to 5α- and 5β-steroids

Substance	Applied amount (mg)	17β-Hydroxy D-ring structure	
		5α*	5β*
d_3-Testosterone	20	13	87
	2	9	91
Nandrolone	20	15	85
Methyltestosterone	10	17	83
	100	14	86
Bolasterone	20	0	100
Calusterone	40	22	78
Boldenone	22	0	100
	80	0	100
Metandienone	22	0	100
	40	0	100

*Results expressed as % of steroid in 5α and 5β form

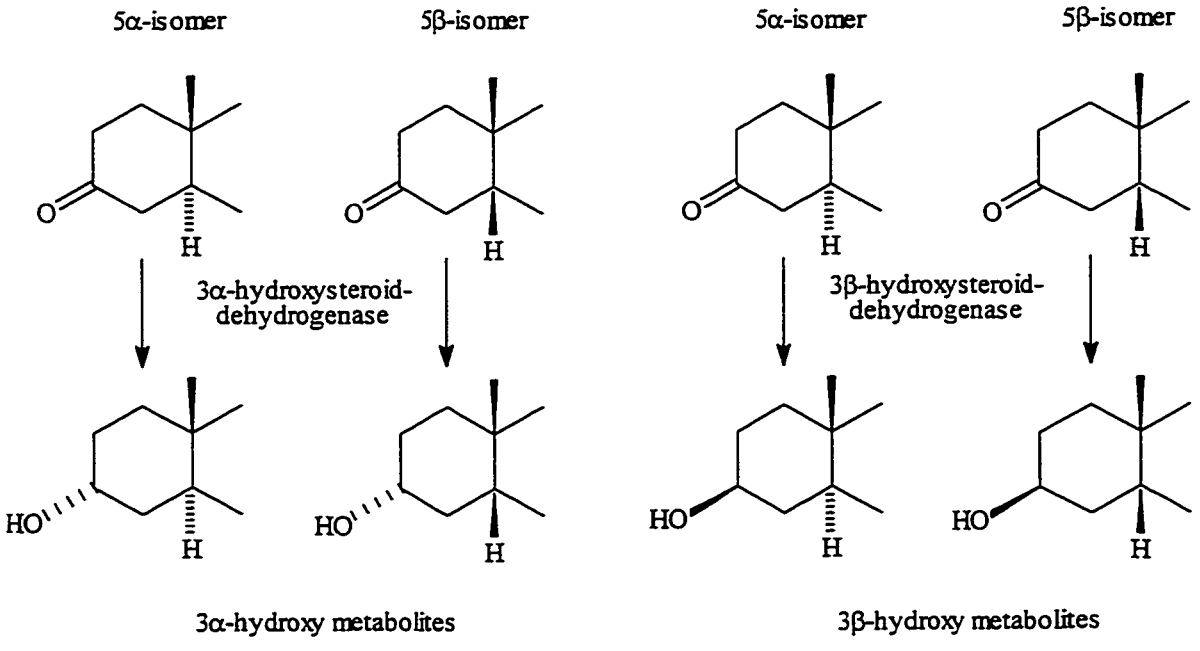


Figure 90. A-ring metabolism: reduction of 3-keto groups with 3α-hydroxysteroid dehydrogenase and 3β-hydroxysteroid dehydrogenase

After the nonreversible reduction of the C-4,5 double bond, the 3-keto group is rapidly reduced by either 3α-hydroxysteroid dehydrogenase or 3β-hydroxysteroid dehydrogenase (Figure 90), producing four stereoisomers. Several studies of testosterone metabolism [9], after oral administration or intramuscular injection, have shown that mainly 3α-hydroxy isomers are produced and only small amounts of the 3β-hydroxy-5α metabolite are generated. In the metabolism of nandrolone, drostanolone, mesterolone and clostebol, 3β-hydroxy-5α-androstan isomers are formed [9]. For clostebol it has been found that the 3β-sulfate was a long-term excreted metabolite. The formation of the 3β-hydroxy-5β isomer has not been reported, indicating that the reaction occurs to a very small extent.

Our finding that 17α-ethyl-5β-estran-3α,17β-diol is the major di-hydroxy metabolite in the ethylestrenol/norethandrolone metabolism both in human and monkey is in full agreement with data presented on the metabolism of testosterone and other 3-keto-4-ene anabolic steroids (Table 13). Therefore we can interpret the configuration of the three minor metabolites as the other possible stereoisomers.

The peak assignment for each stereoisomer was done by comparing the elution order of the four metabolites with the elution order of corresponding isomers determined in the analysis of other 3-keto-4-ene steroids, methyltestosterone and nandrolone, performed in our laboratory as part of the routine urine anabolic steroid testing. Table 14 lists RT and RRT of metyltestosterone and nandrolone metabolites. For both steroids, the elution order of the 3α-hydroxy-5α-isomer is first, 3α-hydroxy-5β-isomer second and for nandrolone the 3β-hydroxy-5α-isomer is eluted third. Figure 61 (see section 5.A.5.)

represents diagnostic ions for di-hydroxy metabolites for ethylestrenol monkey excretion urine SP0005 M-F that contains all four metabolites.

Table 14. Methyltestosterone and nandrolone metabolites

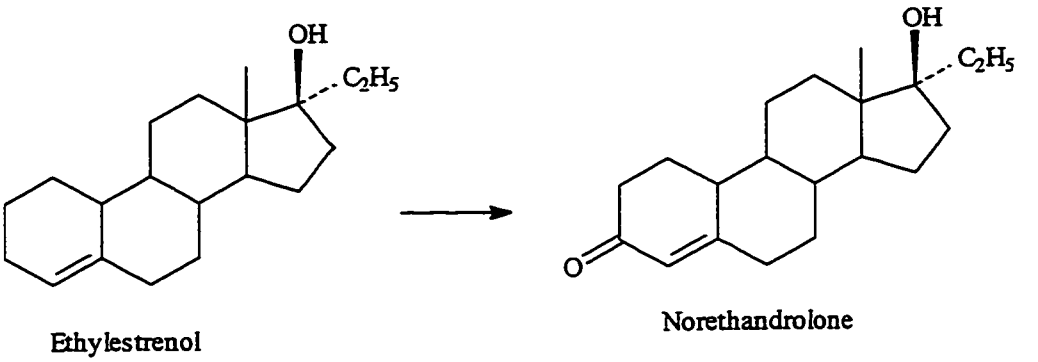
Anabolic steroid	3α-hydroxy-5α-isomer		3α-hydroxy-5β-isomer		3β-hydroxy-5α-isomer	
	RT	RRT	RT	RRT	RT	RRT
Methyltestosterone	20.3	0.85	20.5	0.86		
Nandrolone	14.6	0.61	16.5	0.69	17.1	0.72

Therefore, it was postulated that the first di-hydroxy ethylestrenol/norethandrolone metabolite by RT and RRT (Group I) is 17α-ethyl-5α-estran-3α,17β-diol. Brooks et al. [7] have identified this metabolite in a human norethandrolone urine. It is also present in the human excretion study we have analyzed (Table 10 and 11). The second metabolite was identified (described earlier) to be 17α-ethyl-5β-estran-3α,17β-diol. The ratio of the amount of 3α-hydroxy-5β- and 3α-hydroxy-5α-isomer produced (Figure 61) is comparable to ratios found for other 3-keto-4-ene anabolic steroids (Table 13), and to the ratio observed in the human norethandrolone excretion urine analyzed (Figure 29).

The third and fourth di-hydroxy metabolite by RT and RRT (Group III and IV) were postulated to be 17α-ethyl-5α-estran-3β,17β-diol and 17α-ethyl-5β-estran-3β,17β-diol respectively. These two minor metabolites seem to be long-term excreted metabolites as they are detected in excretion urine collected after prolonged administration of the drug

(SP0005 M-F - see section 4.2.) or after significantly increasing the dosage of the drug (norethandrolone monkey excretion urine – see section 4.2.). As described earlier, SP0005 M-F urine pool was collected in the third and fourth month of continuous administration of 50 mg of ethylestrenol two times a week. Norethandrolone monkey excretion urine was a urine collection over a week of administering 100 mg of norethandrolone every day.

The second major metabolite identified in both monkey ethylestrenol and norethandrolone excretion urines analyzed, is norethandrolone (Table 10). The identity of norethandrolone was verified by comparing with the authentic substance. The observation of norethandrolone in ethylestrenol excretion urine confirms that ethylestrenol is oxidized into norethandrolone prior to further metabolism (Figure 91). In addition, we have not been able to detect any ethylestrenol. Ward et al. [11] have studied the metabolism of norethandrolone and ethylestrenol in human and marmoset monkey. Both in man and marmoset monkey, the urinary metabolite patterns of ethylestrenol and norethandrolone were the same, confirming the conversion of ethylestrenol to norethandrolone as the first step in its metabolism. Norethandrolone has been identified as a metabolite for both man and monkey and no ethylestrenol was detected. Further, Liu et al. [12] have also identified norethandrolone as a metabolite in a human norethandrolone excretion.



Ethylestrenol

Norethandrolone

Figure 91. First step in the metabolism of ethylestrenol

Further ethylestrenol/norethandrolone metabolites identified are suspected to be tri-hydroxy metabolites by the fragmentation ions observed in their mass spectra as discussed in the Results section (5.A. – see Figure 9,10 and 11). The tabulation of RT and RRT of all tri-hydroxy metabolites observed in excretion urines analyzed is presented in Table 15.

Tri-hydroxy metabolites are produced by D-ring hydroxylation. Hydroxylation takes place at the ethyl side chain or at the C-16 position. The hydroxylated position was confirmed by D-ring fragment ions observed in the mass spectra of the tri-hydroxy metabolite TMS derivative [9-13].

Table 15. Ethylestrenol/norethandrolone tri-hydroxy metabolites

SAMPLE	IS RT	3,17,21-Triol RT	RRT	3,17,16-Triol RT	RRT
SP0001 M-F	23.836	26.152	1.097		
		26.371	1.106		
SP0002 S-S	23.887	26.169	1.096		
		26.380	1.104		
SP0003 M-F	23.995	26.234	1.093	27.882	1.162
		26.424	1.101		
SP0004 S-S	24.020	26.349	1.097		
		26.502	1.103		
SP0005 M-F	24.004	26.315	1.096	28.019	1.167
		26.514	1.104		
		27.086	1.128		
		28.335	1.180		
SP0006 M-F	24.051	26.372	1.096		
		26.545	1.104		
Norethandrolone monkey	23.947	26.236	1.096		
		26.458	1.105		
Norethandrolone human	23.913	26.176	1.095	28.488	1.191
		26.396	1.104		

Hydroxylation at the 17α-ethyl side chain has been proposed by several researchers [9-13]. Brooks et al., Ward et al. and Liu et al. did not suggest the configuration of this metabolite, 17α-ethyl-5ξ-estran-3ξ,17β,21-triol, detected in human norethandrolone [10-12] and human ethylestrenol excretion [11]. In 1992, Schänzer and Donike tentatively identified this metabolite as the 3α-hydroxy-5β-isomer in human norethandrolone excretion urine [13]. Four years later, in 1996 [9], Schänzer reported 17α-ethyl-5ξ-estran-3ξ,17β,21-triol has been confirmed as one of the main metabolites in the metabolism of ethylestrenol and norethandrolone. 3α-Hydroxy configuration is proposed based on the

conjugation of the metabolite [9] but weather it is a 5α- or a 5β-isomer remains unknown. We have detected two 3,17,21-tri-hydroxy metabolites in the human norethandrolone urine (Table 15).

The 3,17,21-tri-hydroxy metabolites listed in Table 15 have been further grouped by RT and RRT in Table 16.

Table 16. Ethylestrenol/norethandrolone 3,17,21- tri-hydroxy metabolites

Group I		Group II		Group III		Group IV	
RRT	RT	RRT	RT	RRT	RT	RRT	RT
1.097	26.152	1.106	26.371	1.128	27.086	1.180	28.335
1.096	26.169	1.104	26.380				
1.093	26.234	1.101	26.424				
1.097	26.349	1.103	26.502				
1.096	26.315	1.104	26.514				
1.096	26.372	1.104	26.545				
1.096	26.236	1.105	26.458				
1.095	26.176	1.104	26.396				

Similar to observing four isomers among the di-hydroxy metabolites, four isomers of 3,17,21- tri-hydroxy metabolites are observed. The configuration of these metabolites are undetermined, however it can be postulated they are: 3α-hydroxy-5α-, 3α-hydroxy-5β-, 3β-hydroxy-5α- and 3β-hydroxy-5β-isomers. It was observed that the mass spectra of the most abundant and first 3,17,21-tri-hydroxy metabolite (Group I) by RT and RRT has m/z 331 fragment ion (m/z 421 – TMSOH), which is not observed in the mass spectra of the other three isomers. The first and second isomer are observed in all monkey excretion urines as well as in the human norethandrolone urine (Table 15 and 16).

One 16-hydroxylated tri-hydroxy metabolite has been observed in two monkey excretion urines, as well as in the human norethandrolone urine (Table 15). The metabolites observed in the monkey urines have the same RRT. However, the RT and RRT of the metabolite observed in the human noretandrolone urine is different. The configuration of these metabolites is unknown, however it is likely that we have detected different isomers in monkey and in human. Liu et al. [12] have reported detecting two 16-hydroxylated metabolites in human norethandrolone urine.

In human, 16-hydroxylation is well described in the metabolism of estrogens [9]. One of the main pathways in the metabolism of estradiol and estrone is 16α-hydroxylation [25]. 16-hydroxylation has been reported for several anabolic steroids as a stereospecific hydroxylation, but the extent of formation of 16α- and 16β-isomers differs for different anabolic steroids [9]. In some cases only one isomer is excreted.

6.B. ISOLATION OF ETHYLESTRENOL METABOLITE

As it has been described in the Result section (5.B.1.), the isolation procedure was developed using a small ethylestrenol urine sample (50 mL). In the initial clean-up step, Amberlite XAD-2 solid phase extraction, different washes were applied to accomplish best separation of the desired steroid metabolites from other unwanted compounds present in the urine. Mixtures of water and methanol (20%, 30%, 40% and 50% MeOH : H_2O) were applied as washes, collected and analyzed.

As steroid metabolites are eluted with methanol in this column chromatography, it was important to determine the highest percentage of the methanol mixture that can be applied without eluting ethylestrenol metabolites in the wash. After the search for different metabolites, using their diagnostic ions (see section 5.A.) in the full scan analysis of the 50% MeOH : 50% H_2O wash, was completed it was concluded that no metabolite was present. Therefore, only this wash mixture was used in subsequent experiments.

The hydrolysis of steroid conjugates was successfully accomplished using the crude extract of Helix promatia. The free steroids were then extracted into the organic phase, diethyl ether at an alkaline pH.

In the purification and separation of the free metabolites on a Sephadex LH-20 column, difficulty was encountered in finding a elution solvent system that was easy to use and would give good separation (see section 4.4.A.4.). In the process of finding the adequate solvent system the column was repacked in different eluents until satisfactory flow was accomplished with chloroform 50% : heptane 50% : ethanol 1% : water to saturation. Then the sample was applied on this column and small (10 mL) fractions were collected. All fractions were analyzed for the presence of ethylestrenol metabolites. It was determined that only one metabolite, a di-hydroxy metabolite, was eluted with this solvent system. The elution profile was determined by plotting the intensity of the base ion of this metabolite in the different fractions collected (Figure 75 – section 5.B.1.). Attempts to elute other metabolites with other solvent systems were unsuccessful.

The developed isolation procedure was used to isolate the di-hydroxy metabolite from 500 mL of urine. The initial extract acquired after the solid phase extraction, hydrolysis of conjugates and liquid-liquid extraction was divided in two parts and the two were further purified on two Sephadex LH-20 columns separately (see section 4.4.B). However, the result of the GC/MS analysis of the main fractions (I.2 and II.2) containing the di-hydroxy metabolite, was the same: only one metabolite was present and no other steroid compound was observed. If we compare the TIC of the GC/MS analysis of a small portion of the initial extract (50 μL – see section 5.B.2.) in Figure 80 and TIC of the full scan of fraction II.2 and that of the derivatizing mixture used in the GC/MS analysis in Figure 82, we can observe that the di-hydroxy metabolite was isolated from many compounds present in the initial extract. Also, most of the additional peaks observed in TIC of the fraction II.2 (other than the peak at RT 22.713 which is the di-hydroxy metabolite) are present in the TIC of the derivatizing mixture.

The di-hydroxy metabolite isolated was identified to be 17α-ethyl-5β-estran-3α,17β-diol. Its identity was verified by comparing with purchased authentic compound that has been synthesized by RADIAN. This purchased standard was also used to quantify the amount of the metabolite present in the urine used for isolation, SP0003 M-F, and in the final fractions I.2 and II.2, and then the yield of the isolation procedure was calculated to be 27.8% and 44.3% (see section 5.B.3.).

7. CONCLUSION

The first objective of this thesis was to determine if the cynomolgus monkey has similar urinary metabolite patterns as human. This was acomplished by analyzing, by GC/MS, excretion study urine collected from the monkey after administering orally ethylestrenol and norethandrolone, and comparing with human ethylestrenol and norethandrolone excretion study urine.

In both human and the cynomolgus monkey the metabolism of ethylestrenol is identical to the metabolism of norethandrolone, as ethylestrenol is first oxidized to noretandrolone prior to further metabolism.

Major metabolites identified in human are also identified in the cynomolgus monkey: 17α-ethyl-5β-estran-3α,17β-diol, norethandrolone and two 17α-ethyl-5ξ-estran-3ξ,17β,21-triol metabolites. In addition, some minor metabolites observed in monkey have also been detected in human: 17α-ethyl-5α-estran-3α,17β-diol, and 17α-ethyl-5ξ-estran-3ξ,17β,16ξ-triol.

Further, the metabolic pathways of testosterone and other anabolic steroids in human can be used to explain the formation of the ethylestrenol/norethandrolone metabolites in the cynomolgus monkey.

In conclusion, the hypothesis of this thesis, that the cynomolgus monkey produces similar urinary metabolites of ethylestrenol/norethandrolone as human, has been proven. As a

result, the monkey excretion urine can be used in drug testing laboratories as positive control urine, to replace human excretion urine.

The second objective in this thesis was to develop a method for isolation of an ethylestrenol metabolite from the urine of the cynomolgus monkeys. A successful method was developed for isolation of the 17α-ethyl-5β-estran-3α,17β-diol.

8. FUTURE WORK

This thesis has provided a isolation method for one of the di-hydroxy metabolites identified in the monkey ethylestrenol excretion urine, 17α-ethyl-5β-estran-3α,17β-diol. Future studies should provide a method for isolation of the main tri-hydroxy metabolite, 17α-ethyl-5ξ-estran-3ξ,17β,21-triol, as it would be very interesting to determine the configuration of this metabolite. Determining the configuration of this metabolite might help to understand the configuration of the other tri-hydroxy metabolites detected.

More importantly, this thesis has answered the quest for an animal model that would metabolize anabolic steroid similar to man. Further research on the metabolism of other anabolic steroids in the cynomolgus monkey compared to human would be of great value, as it can show that all human excretion urine can be replaced by monkey.

This would answer all the ethical and legal problems involved in human production of anabolic steroid metabolic urine. Monkey excretion urine could be the source of "positive" urine required for the assay quality control in drug testing laboratories and for proficiency testing samples. And furthermore, monkey excretions could be used to determine the metabolic fate of anabolic steroids and structures of the metabolites.

9. REFERENCES

1. Taylor WN. Macho medicine, A history of the anabolic steroid epidemic. McFarland & Company, Inc., 1991
2. Johnson LC. History and development of anabolic steroids. In: Banks R, ed. Substance abuse in sport: The realities. United Sates Sports Academy, Kendall/Hunt Publishing Company, 1990: 13-23
3. Wadler GI, Hainline B. Drugs and the athlete. F. A. Davis Company, 1989: 55-69
4. Chan SC, Nolan SL. Urinary metabolites of anabolic steroids. Forensic Science Review 1993; 5: 53-66
5. Fuentes RJ, Rosenberg JM. Anabolic steroids and the athlete. In: Fuentes RJ, Rosenberg JM, Davis A, eds. Athletic drug reference '95. Clean Data, Inc., 1995: 111-39
6. Royal Canadian Mounted Police, Canadian Centre for Drug-Free Sport, Sport Medicine Council of B.C.. Drugs and sport: the score. Royal Canadian Mounted Police, 1993
7. Phillips WN. Anabolic reference guide. 6th ed. Mile High Publishing, 1991
8. Barry Sample RH, Baenziger JC. Gas chromatography/mass spectrometry screening for anabolic steroids. In: Deutsch DG, ed. Analytical aspects of drug testing. John Wiley & Sons, Inc., 1989: 247-72

9. Schänzer W. Metabolism of anabolic androgenic steroids. Clinical Chemistry 1996; 42(7): 1001-20

10. Brooks CJW, Thawley AR, Rocher P, Middleditch BS, Anthony GM, Stillwell WG. Characterization of steroidal drug metabolites by combined gas chromatography-mass spectrometry. Journal of chromatograpic science 1971; 9: 35-43

11. Ward RJ, Lawson AM, Shackleton CHL. Metabolism of anabolic steroid drugs in man and the marmoset monkey (*Callithrix Jacchus*) – I. Nilevar and Orabolin. Journal of Steroid Biochemistry 1977; 8: 1057- 63

12. Liu CS, Zhang J, Zhou TH. GC-MS Analysis of norethandrolone and its metabolites in man. Acta Pharmaceutica Sinica 1991; 26(10): 777-81

13. Schänzer W, Donike M. Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. Analytica Chimica Acta 1993; 275: 23-48

14. Williams CSF. Practical guide to laboratory animals. C. V. Mosby Company, 1976

15. Masse R, Ayotte C, Dugal R. Studies on anabolic steroids. I: Integrated methodological approach to the gas chromatograpic mass spectrometric analysis of anabolic steroid metabolites in urine. Journal of Chromatography 1989; 489:23-50

16. Chan SC, Totok-Both GA, Billay DM, Przybylski PS, Gradeen CY, Pap KM, Petruzelka J. Drug analysis at the 1988 Olympic Winter Games in Calgary. Clinical Chemistry 1991; 37: 1289-96

17. Gradeen CY, Chan SC, Przybylski PS. Urinary excretion of furazabol metabolite. Journal of Analytical Toxicology 1990; 14:120-2

18. Shackleton CHL. Profiling steroid hormones and urinary steroids. Journal of chromatography 1986; 379: 91-156

19. Eneroth P, Nyström E. A study on liquid-gel partition of steroids and steroid derivaties on lipophilic Sephadex gels. Biochim. Biophys. Acta 1967; 144: 149-61

20. Sippell WG, Putz G, Scheuerecker M. Convenient system for the simultaneous separation of 11-deoxycortisol and aldosterone by Sephadex LH-20 multiple column chromatography. Journal of Crtomatography Biomedical Applications 1978; 146:333-6

21. Setchell KDR, Shackleton CHL. The group separation of plasma and urinary steroids by column chromatography on Sephadex LH-20. Clinica Chimica Acta 1973; 47:381-8

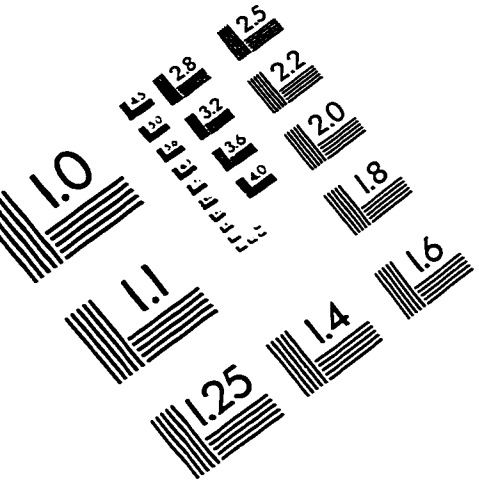
22. Bourne AR. A convenient system for the separation of some steroids on Sephadex LH-20. Journal of Chromatography 1974; 92: 465-6

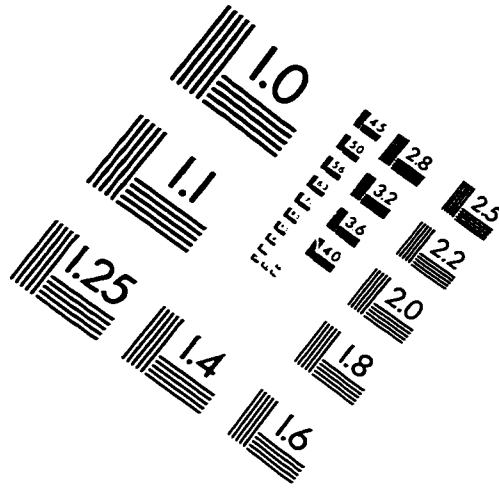
23. Van Baelen H, Heyns W, De Moor P. The separation of estrogens on Sephadex. Journal of Chromatography 1967; 30: 226-7

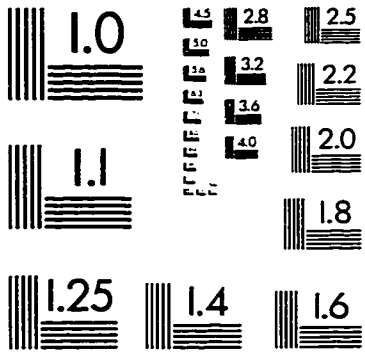
24. Golder WA, Sippell WG. Sephadex LH 20 Multiple-column chromatography for the simultaneous separation of progesterone, deoxycorticosterone and 17α-hydroxyprogesteron from small plasma samples. Journal of Chromatography 1976; 239-9

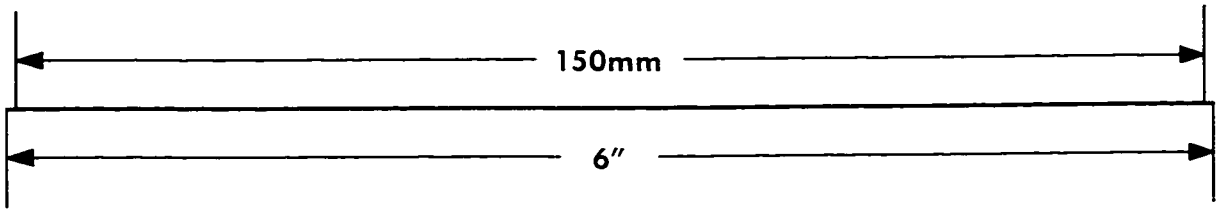
25. Lim SK, Won YJ, Lee JH, Kwon SH, Lee EJ, Kim KR, Lee HC, Huh KB, Chung BC. Altered hydroxylation of estrogen in patients with postmenopausal osteopenia. Journal of Clinical Endocrinology and Metabolism 1997; 82:1001-5

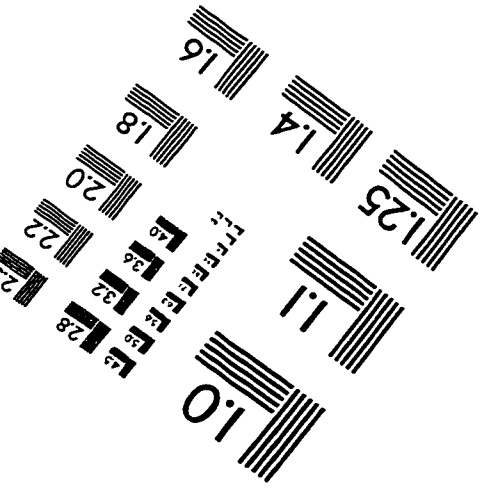
IMAGE EVALUATION
TEST TARGET (QA-3)

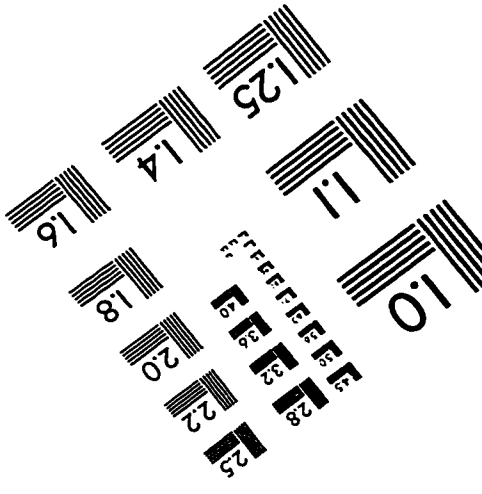












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved