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IDENTIFICATION AND MEASUREMENT BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF NORETHINDRONE AND METABOLITES IN HUMAN URINE AND BLOOD

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SUMMARY

Urinary metabolites of norethindrone (17β-hydroxy-17α-ethynyl-4-estren-3-one) in the free, sulfate, and glucuronide fractions were identified by gas chromatography–mass spectrometry following treatment of a volunteer with a 25 mg dose of norethindrone. Compounds identified in the three fractions were norethindrone, the four ring A reduced isomers, a reduced and hydroxylated metabolite, and ethynyl estradiol (17α-ethynylestra-1,3,5(10)-triene-3, 17β-diol).

Plasma free, sulfate and glucuronide conjugated metabolites of norethindrone were identified and quantified by mass fragmentography. Blood metabolites identified in the three fractions 3 h following oral administration of 25 mg of norethindrone were unchanged drug, and ring A reduced metabolites 17α-ethynyl-5α-estrane-3α,17β-diol, 17α-ethynyl-5β-estrane-3α,17β-diol, 17α-ethynyl-5β-estrane-3β, 17β-diol, and 17β-hydroxy-17α-ethynyl-5β-estrane-3-one. These plasma metabolites were also quantified using mass fragmentography in blood obtained from a volunteer undergoing daily treatment with 2 mg norethindrone, 0.1 mg mestranol (17α-ethynylestra-1,3,5(10)-triene-3,17β-diol-3-methyl ether) (Ortho-Novum, 2 mg).

INTRODUCTION

Use of the current regimens of oral contraceptives can lead to a number of undesirable side effects, the most serious being hypertension and thromboembolic phenomenon. At present, no valid evidence has been provided to show what features of the contraceptive steroids cause them to differ from the naturally occurring compounds in their ability to generate the undesirable effects. Recent evidence obtained in our laboratory indicates a slow disappearance of blood borne metabolites of ^{3}[H]norethindrone (17β-hydroxy-17α-ethynyl-4-estrene-3-one) and ^{3}[H]mestranol (17α-ethynylestra-1,3,5(10)-triene-3, 17β-diol-3-methyl ether) [1–3] in spite of rapid clearance of the parent steroids. Since the unknown metabolites appear to stay in the circulation for a long period of time and reach continually higher levels with prolonged administration, it is of interest to investigate their role in the generation of the untoward effects. Norethindone (NE, Fig.1) is extensively used as the progestational component in a number of oral contraceptive preparations, but only a few studies have been directed toward characterization of the blood borne metabolites. Of these, Gerhards *et al.* [4] identified several plasma metabolites after a single dose of tracer amounts of NE, and Cook *et al.* [5] described plasma metabolites of NE arising from administered ethynodiol diacetate (17α-ethynyl-4-estrene-3β, 17β-diol-diacetate). Much remains to be learned regarding the pharmacokinetics of metabolite elimination, and the buildup of specific metabolites after prolonged administration of NE.

In the work described here, we have separated urinary metabolites from a patient treated with NE into free, sulfate and glucuronide fractions, and identified the aglycones by gas chromatography–mass spectrometry (GC–MS). In order to identify and quantitate these metabolites in blood, plasma samples of one volunteer treated with 25 mg of NE and another volunteer treated with 2 mg NE daily were analyzed by mass fragmentography. We show here that it is possible to use mass fragmentography to quantitate metabolites of NE in the free, sulfate and glucuronide fractions of blood during chronic treatment with normal doses of the contraceptive pill.

EXPERIMENTAL

Chemicals

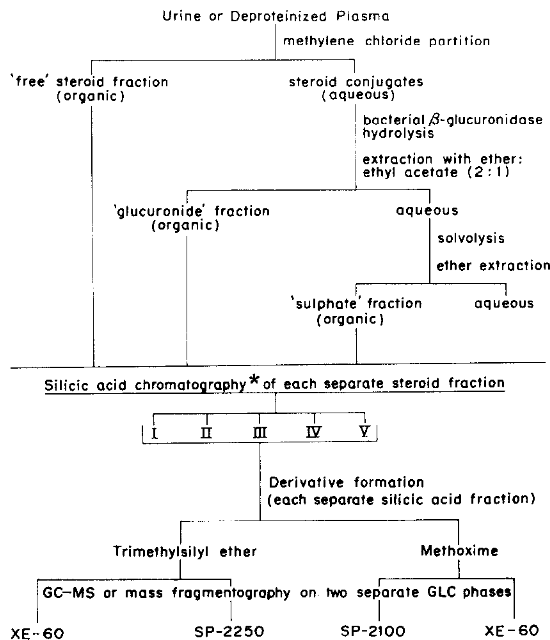
Reagent grade chemicals and solvents were used without further treatment. Steroid reference compounds were provided through the courtesy of the G.D. Searle Co.

Biological extracts

Urinary Fractions. A normal female volunteer was administered 25 mg of NE per day for 4 days, the urine collected over boric acid and refrigerated. For the identification of metabolites, a composite was prepared containing 2.5% of each of the 4 days urine. Considerable amount of preliminary work was done to work out a method of hydrolysis and extraction in the initial studies of Mills *et al.* [1]. The total ^{3}H in urine was determined after hot acid hydrolysis, followed by ether extraction. The separation of the free, 'sulfate' and 'glucuronide' fractions was carried out by a methylene chloride extraction, followed by

solvolysis, followed by enzymatic hydrolysis using liver β-glucuronidase. The recovery of ^{3}[H] using the above procedures was significantly lower than that obtained by direct Insta-Gel (Packard) counting with quench correction. Subsequent work indicated that exposure to acid significantly reduced the recovery of ^{3}H in material extractable by organic solvents. The sequence of extraction of the 'free fraction' followed by β-glucuronidase hydrolysis, followed by solvolysis gave the best overall recoveries. Furthermore, the use of bacterial β-glucuronidase rather than liver β-glucuronidase further improved the recovery in the 'glucuronide fraction'. The following procedure gave the best overall recoveries and was used in this and subsequent studies. The urine was extracted twice with 2 vol. of methylene chloride to obtain the free steroid fraction. The aqueous phase was then brought to pH 6.5 with 1 M phosphate buffer (1 ml for every 14 ml urine), 0.3 g of bacterial β-glucuronidase were added (total 33,300 units; 360 units per ml) and the mixture incubated at 37° for 24 h. A second addition of β-glucuronidase (0.3 g) was then made and the mixture incubated for another 24 h. After a third addition of 0.3 g of β-glucuronidase, the mixture was incubated for an additional 48 h prior to extraction. The urine was then extracted 3 times with 2 vol. of ether: ethyl acetate mixture (2:1 vol:vol) to yield the glucuronide fraction. The aqueous phase remaining was acidified to pH 1 with 4 N H_2SO_4 and after saturating with sodium chloride was extracted 3 times with 2 vol. of ethyl acetate. The combined ethyl acetate extract was incubated at 37° for 16 h, neutralized with ammonium hydroxide and evaporated to dryness. Ten ml distilled water were added to the dry residue, and the solution was extracted 3 times with 3 vol. of ether. This was called the sulfate fraction. Diagram 1 is a general outline of the extraction procedure described above, and the purification and analytical procedures described below.

A urinary glucuronide fraction representing 7.5% of the original urine obtained following NE administration and a sulfate fraction representing 5% were each subjected to a toluene–1 N NaOH partition procedure [6] and a neutral and phenolic fraction obtained. The phenolic glucuronide fraction was further purified by silicic acid chromatography (see below) and examined for aromatized metabolites by GC-MS. The phenolic sulfate fraction was purified by silicic acid chromatography, and further purified by chromatography on Amberlite XAD-2. A column of Amberlite XAD-2 was prepared by packing the Amberlite, washed in distilled water to remove fines, in a 10 ml disposable pipette to a bed vol. of 7 ml. The sample was dissolved in 40 μl of acetone: 1 ml of water and applied to the top of the column, then rinsed in with an additional 1 ml of water. The column was eluted with 25 ml of water and the eluate discarded. The estrogen fraction was then removed by elution with two 5 ml aliquots of methanol. The methanol fractions were combined and evaporated



* I, 10% ethyl acetate in benzene; II, 26% ethyl acetate in benzene; III, 35% ethyl acetate in benzene; IV, ethyl acetate; V, methanol.

Diagram 1. Outline of method for isolation and identification of norethindrone and metabolites from blood and urine.

with N_2. This fraction was examined for ethynyl estradiol (17α-ethynylestra-1,3,5(10)-triene-3, 17β-diol) as the bis-trimethylsilyl (TMS) derivative using mass fragmentography (see below).

Plasma fractions. A normal female volunteer was administered 25 mg of NE orally, blood was drawn 3 h after the oral ingestion, and the blood plasma separated. A second female volunteer was treated daily with 2 mg norethindrone and 0.1 mg mestranol (Ortho-Novum, 2 mg). A blood sample was drawn 3 h after ingestion of pill 10, on day 14 of the cycle (day zero was the first day of menstruation). To 10 ml aliquots of plasma (8 ml actual plasma + 2 ml of anticoagulant solution used during collection) were added 25 ml of methanol to precipitate the proteins. The mixture was centrifuged and the aqueous methanol solution decanted. The precipitated protein fraction was washed twice with 5 ml methanol and the washings added to the original aqueous methanol solution. The combined solution was evaporated to dryness under vacuum at 45° using a rotary evaporator. The residue was taken up in 5 ml water and subjected to extraction and hydrolysis using the method described above for the urine.

Silicic acid chromatography. Free, sulfate and glucuronide fractions representing either 2.5% of the original four day total urine sample or 8 ml of plasma were each further purified by silicic acid chromatography [7] and the following fractions collected: FI—10% ethyl acetate in benezene; FII—27% ethyl acetate in benezene; FIII—35% ethyl acetate in benzene; FIV—ethyl acetate; FV—methanol. Each fraction was dried, and steroids partitioned between 90%

methanol–hexane. The methanol layer was then evaporated with a stream of N_2.

Gas chromatography–mass spectrometry

Urinary fractions. Trimethylsilyl ethers (TMS) of silicic acid fractions I–V were formed by reaction with hexamethyldisilazane and trimethylchlorosilane in pyridine (Tri Sil, Pierce Chemical Co.) for 30 min at 70°. The compounds were chromatographed on a Finnigan 1015D gas chromatograph–mass spectrometer interfaced with a Systems Industries System 150 data processing and control system. Spectra were obtained at an ionization energy of 70 eV, scanning the quadrupole mass spectrometer in the mass ranges 50–150; 151–400; 401–550 at 3; 6; 17 ms/a. m.u. respectively. The columns used were 1.5 m by 2 mm I.D. glass, packed with either (a) 1% SP-2250 on 100/120 mesh Supelcoport, which was programmed from 190–240° at 2°/min, or (b) 1% XE-60 on 80/100 mesh Gas Chrom Q, programmed from 170–240° at 1°/min. Gas flow was 30 ml/min. Methylene unit values [8] were determined with n-alkanes as references. Quantitative approximations were obtained by measuring peak heights relative to the C_{28} hydrocarbon.

Plasma fractions. For chromatography of the tetrahydro metabolites and 3β-NE, aliquots of silicic acid fractions I, II and III were derivatized as TMS ethers. 5α-Androstane-17α-vinyl-3β,17β-diol bis-TMS was used as a reference standard for measurement of relative retention times and relative peak heights (for quantitation). Additional aliquots were derivatized as O-methyloximes (MO) by reacting with methoxyamine hydrochloride in pyridine for 30 min at 60° for gas chromatography of NE and 5β-NE. The steroid–MO derivatives were partitioned into ethyl acetate and dried with a stream of N_2. They were then dissolved in 20 μl of dry ethyl acetate and 1–2 μl samples injected onto the chromatograph. 17α-Ethynylestr-5(10)-en-17β-ol-3-one MO was used as reference compound for measurement of relative peak heights and relative retention times during chromatography of the MO derivatives on XE-60 stationary phase.

Mass fragmentography of the plasma fractions was performed at an ionization energy of 70 eV, scanning the selected ions for 500 ms each (400 ms during mass fragmentography of MO-derivatives on XE-60). The columns used were 1.5 m × 2 mm I.D. glass, packed with (a) 1% XE-60 on 80/100 mesh Gas Chrom Q, operated isothermally at 185° for TMS derivatives and 215° for MO derivatives; (b) 1% SP-2250 on 100/120 mesh Supelcoport, operated at 230°; (c) 1% SP-2100 on 100/120 mesh Supelcoport, operated at 220°.

Positive identification of a compound by mass fragmentography required the presence of the selected ion peak at a relative retention time identical to the standard, determined on two separate stationary phases. Quantitation was accomplished by measurement of peak height relative to the reference compound, and interpolation from a standard curve prepared the same day. Samples of standard were alternated with samples of unknown during the mass fragmentographic determinations to account for slight changes in operating parameters of the GC–MS throughout the day [9].

RESULTS AND DISCUSSION

Urinary metabolites

GC–MS identification of neutral steroids. The compounds in the urinary free, sulfate, and glucuronide fractions were identified by comparison of the mass spectra of the TMS derivatives with those of the authentic reference compounds. Characteristic ions for the TMS derivatives of the tetrahydro metabolites of NE at $M^+ = 446$, M-15 (431), M-29 (417), M-90 (356), M-15-90 (341), M-29-90 (327), M-90-90 (266), m/e 125, 140, 153, 196 and 209 were as described by Stillwell *et al.* [10]. The similarity of the spectra obtained on our quadrupole mass spectrometer of the four stereoisomers did not allow unequivocal identification on this basis alone. Therefore methylene unit values (MU) were obtained on two different stationary phases (Table 1) in order to make the final assignments of configuration (Fig. 1 contains the structures of compounds identified). A total ion current chromatogram of silicic acid fraction II of the urinary glucuronide fraction chromatographed on stationary phase XE-60 is shown in Fig. 2. The location of the reduced metabolites is shown by a computer reconstructed chromatogram of ion m/e 431. This can be compared with the same urinary glucuronide fraction chromatographed on SP-2250, (Fig. 3). Although the tetrahydro metabolites were more cleanly separated on XE-60, several other 17α-ethynyl compounds were identified on the SP-2250 column (Fig. 3). From Table 1, it can be seen that all four of the possible tetrahydro metabolites of NE could be identified in the sulfate and glucuronide fractions of urine, while the 5β, 3α-NE (17α-ethynyl-5β-estrane-3α,17β-diol) and 5α, 3α-NE (17α-ethynyl-5α-estrane-3α,17β-diol) metabolites were present in the free fraction. 5β, 3α-NE was the most adundant metabolite in all fractions, 5α, 3α-NE and 5β, 3β-NE (17α-ethynyl-5β-estrane-3β, 17β-diol) were present in lesser quantities, and 5α, 3β-NE (17α-ethynyl-5α-estrane-3β,17β-diol) was observed in trace amounts. Stillwell *et al.* [10] previously identified 5β, 3α-NE as the major metabolite of NE in a total urinary hydrolysate, and 5α, 3α-NE as a minor metabolite, using GC–MS. With radioactive tracer techniques, 5β, 3α-NE was also identified as the main metabolite of NE in the glucuronide fraction of urine, 5α, 3α-NE a minor metabolite and 5α, 3β-NE and 5β, 3β-NE as trace metabolites [4]. These authors, who used sulfatase cleavage rather than solvolysis, found 5β, 3β-NE to be the main metabolite in the sulfate fraction [4].

Chromatography on SP-2250 (Fig. 3) also revealed a hydroxylated-tetrahydro metabolite of NE in the

Table 1. Urinary metabolites of norethindrone

Fraction	Methylene Units (Standards) SP-2250	XE-60	Total (2.5%) μg
Free			
5α, 3α-NE	26.03(26.09)	26.20(26.29)	Trace
5β, 3β-NE	—		—
5β, 3α-NE	26.57(26.60)	27.25(27.34)	0.24
5β NE	—	—	—
NE	—	—	—
Sulfate			
5α, 3β-NE	25.53	25.49	Trace
5α, 3α-NE	26.05(26.09)	26.20(26.29)	0.20
5β, 3β-NE	{26.57(26.60)	26.79(26.93)	0.37
5β, 3α-NE		27.27(27.34)	4.93
5β NE	—	—	—
NE	29.41(29.41)	—	0.76
Ethynyl Estradiol*	—	—	—
Glucuronide			
5α, 3β-NE	25.53	—	Trace
5α, 3α-NE	26.02(26.09)	26.26(26.29)	0.56
5β, 3β-NE	{25.54(26.60)	26.85(26.93)	0.24
5β, 3α-NE		27.23(27.34)	4.09
5β NE	—	—	—
NE	29.42(29.41)	—	1.52
OH-5ξ, 3ξ-NE	29.01	—	—
Ethynyl Estradiol	29.12(29.24)		—

* Determined by mass fragmentography on SP-2100. Retention time relative to the C_{28} standard was 0.92 (ref. 0.91).

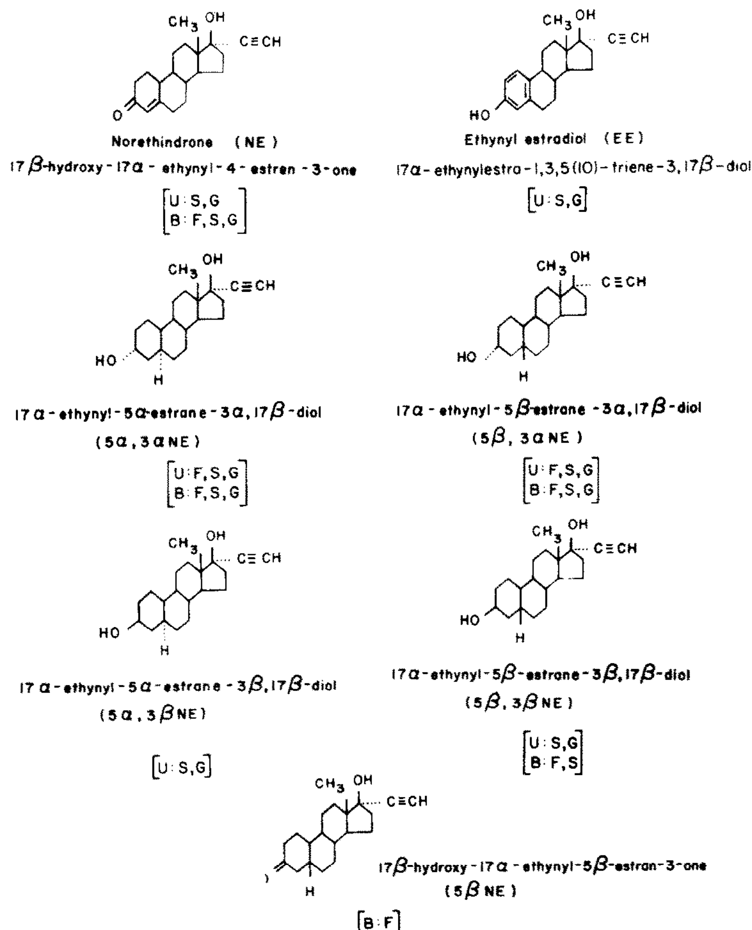


Fig. 1. Structures of norethindrone and metabolites identified in blood and urine by gas chromatography–mass spectrometry or mass fragmentography. The body fluid and fraction where each compound was identified are designated: U, urine; B, blood; F, free; S, sulfate; G, glucuronide.

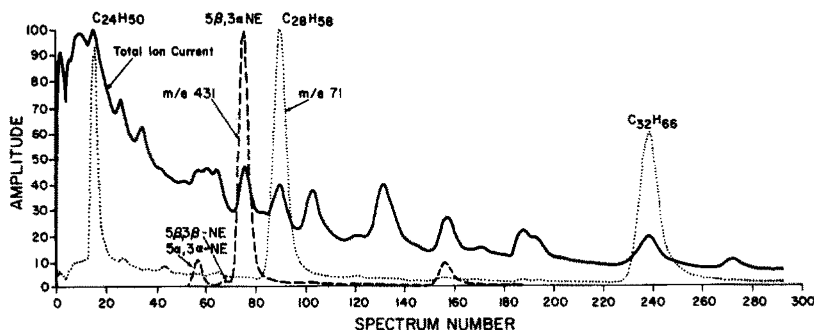


Fig. 2. Chromatogram on XE-60 of Silicic Acid Fraction II of the urinary glucuronides following NE administration. The tetrahydro metabolites of NE are shown by the reconstructed specific ion chromatogram of m/e 431 (---) and the n-alkane reference compounds by m/e 71 (····).

glucuronide fraction. This compound ($M^+ = 534$ as the tris–TMS) could be identified as a metabolite of NE by the group of ions at 125, 140, 153, 196, which are indicative of the 17α-ethynyl group of the synthetic steroids [10]. The position of the extra hydroxyl group, and the stereoconfiguration have not been deduced from the spectrum obtained.

Unchanged NE was found in significant quantities in urine as both the sulfate and the glucuronide conjugate (Table 1). Thus, conjugation is possible at the hindered 17β-hydroxyl of the 17α-ethynyl progestogens. Williams *et al.* [11] previously found that the orally administered estrogen, mestranol was recovered in urine solely in the conjugated fraction.

Silicic acid fractions I–V were examined by GC–MS for the presence of NE metabolites. The bulk of the 17α-ethynyl compounds were found in silicic acid fraction, II, but small amounts of metabolites were also seen to overlap into fractions I and III.

Identification of an estrogenic metabolite. GC–MS examination of the phenolic fraction of the urinary glucuronides and sulfates provided evidence that aromatization of norethindrone to ethynyl estradiol occurred. The total ion current chromatogram of the phenolic glucuronide fraction (silicic acid FII) is shown in Fig. 4 with the computer reconstructed chromatogram of ions $M^+ = 440$ and m/e 425 (M-15). The peak at MU 29.12 (ref. standard 29.24) indicates that ethynyl estradiol bis–TMS is located on the leading edge of a larger peak. The background subtracted mass spectrum of the compound at MU 29.12 is compared with reference ethynyl estradiol–bis–TMS in Fig. 5. Evidence that ethynyl estradiol was also present in the sulfate fraction was obtained by mass fragmentography. Four characteristic fragment ions of the ethynyl estradiol–bis–TMS spectrum (Fig. 5) m/e 196, 285, 425 and 440 and two ions of the C_{28} alkane spectrum, m/e 71 and 85 were selectively focused upon. The mass fragmentogram of a sample of the phenolic urinary sulfate fraction (silicic acid FII) in Fig. 6 shows a peak containing the characteristic ions of ethynyl estradiol–bis–TMS at the same relative retention time as the reference steroid shown below it. Others have provided evidence using radiolabeled precursors that NE and related compounds are aromatized *in vivo* to ethynyl estradiol [12–15]. Breuer [16] however was not able to demonstrate aromatization of 17α-ethynyltestosterone or 17α-ethynyl-19-nortestosterone by an *in vitro* placental aromatase system or by placental perfusions, and postulated that the earlier findings were a result of dehydration of 1β-hydroxylated products during the analytical workup. We have been able to obtain further evidence of the *in vivo* conversion of norethindrone to an estrogenic compound by biological experiments [17]. Using a castrate female rat model, it was demonstrated that progesterone, megestrol acetate (17α-acetoxy-6-methyl-4, 6-pregnadiene 3, 20-dione)

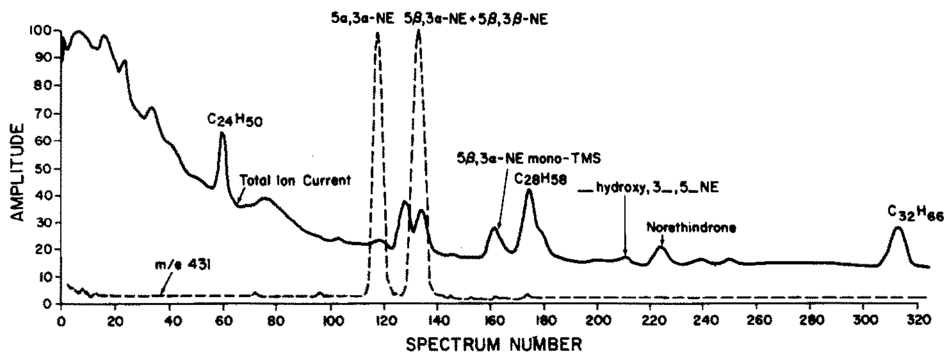


Fig. 3. Total ion current chromatogram on SP-2250 of silicic acid fraction II of the urinary glucuronides following NE administration. Tetrahydro metabolites of NE are shown by the reconstructed chromatogram of ion m/e 431 (---).

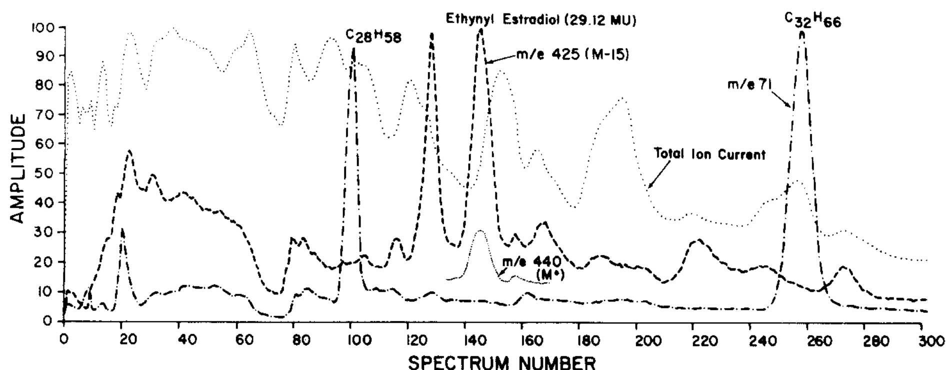


Fig. 4. Chromatogram on SP-2250 of the phenolic glucuronide fraction (silicic acid fraction II) of urine following NE administration. The reconstructed specific ion chromatograms of m/e 440 and 425 indicate the presence of ethynyl estradiol–bisTMS at M.U. 29.12.

and low doses of NE did not significantly modify serum FSH, LH and uterine weight in the absence of estrogens. However, larger doses of NE caused a dramatic fall in serum gonadotrophin levels without estrogen treatment. These were correlated with a dose related increase in uterine weight, an expression of estrogenic activity. This implies that NE was aromatized, since other progestogens were not estrogenic at high doses.

Plasma Metabolites

Identification and quantification of plasma metabolites after a 25 mg dose of NE. Following identification of norethindrone and metabolites in the urinary fractions, mass fragmentography was used to identify and quantitate plasma NE and metabolites after an oral dose of 25 mg of NE. Although 5β-NE and 3β-NE were not identified in the urinary fractions these compounds are possible intermediates in the pathway to the tetrahydro-reduced metabolites, so mass fragmentographic analysis for these compounds also was carried out on the plasma fractions. The best separation of the 5α, 3α-NE, 5β, 3β-NE, 5β, 3α-NE and 3β-NE (17α-ethynyl-4-estrene-3β, 17β-diol) metabolites was achieved on XE-60, so this stationary phase was used for identification and quantitation. SP-2250 was used to verify the presence of compounds identified on XE-60.

The 3-keto compounds, NE and the 5β reduced metabolite 5β-NE, (17β-hydroxy-17α-ethynyl-5β-estrane-3-one) migrated too slowly as the TMS derivatives on XE-60 and SP-2250 for a practical assay, but did chromatograph well as the MO derivatives on XE-60 and SP-2100. The MO derivative of 17α-ethynylestr-5(10)-en-17β-ol-3-one served as an ideal reference compound for these steroids on XE-60, with a retention time midway between the two.

Figs. 7–10 are mass fragmentograms of TMS and MO derivatives of plasma free, sulfate and glucuronide fractions compared with corresponding mass

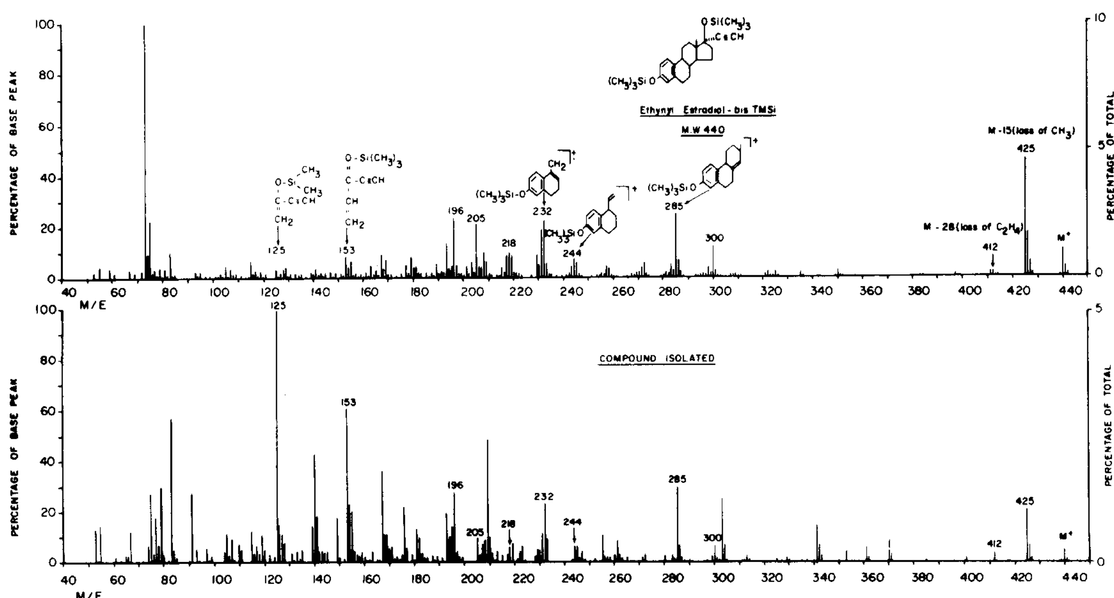


Fig. 5. Mass spectra (70 eV) of authentic ethynyl estradiol–bisTMS (upper) and peak containing ethynyl estradiol in urinary glucuronide fraction following administration of NE (lower).

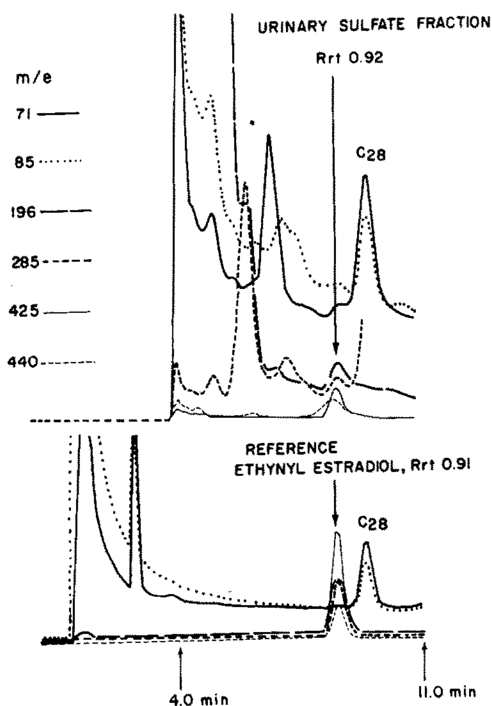


Fig. 6. Mass fragmentography of the phenolic urinary sulfate fraction following administration of NE. Samples were chromatographed on SP-2100 at 225°.

fragmentograms of the reference steroids. The presence of two or more characteristic ions at the proper relative retention time served to confirm the identity of isolated metabolites. Such was the case of ions 301 (M-26) and 270 (M-57) of NE, Figs. 8 and 10, and ions m/e 417, 419 and 431 of the ring A reduced metabolites, Fig. 9. NE was further characterized by the separation of the syn and anti isomers of the MO derivative on XE-60, Fig. 8. Metabolites identified by mass fragmentography in the plasma fractions following a single 25 mg dose of NE are shown in Table 2, with their relative retention times on the two separate phases, and those of the authentic reference compounds determined the same day. The compounds eluted from the silicic acid column in fraction II. Only traces of these NE metabolites could be seen in FI and III.

5β-NE identified in the free plasma fraction was not detected in the urinary fractions. Presumably it is completely reduced (mainly to 5β, 3α-NE) before excretion. The 3β-ol metabolite and ethynyl estradiol were not identified in any of the plasma fractions using the present methodology.

After oral administration of NE, Gerhards *et al.* [4] identified 5β, 3α-NE, 5β, 3β-NE and 5α, 3α-NE in the glucuronide fraction of plasma and 5β, 3β-NE following sulfatase cleavage. Ethynodiol diacetate is rapidly metabolized to NE in the human [18] and Cook *et al.* [5] found NE as the major metabolite in the plasma free fraction, in addition to 5α, 3α-NE and 5β, 3α-NE. The hydrolyzed plasma fraction contained NE, 5α, 3α-NE, 5α, 3β-NE and 5β, 3α-NE.

Quantitation of the plasma metabolites was accomplished by interpolation of relative peak heights from a standard curve prepared on the same day. The blood levels of metabolites in the free, sulfate and glucuronide fractions following an oral dose of 25 mg NE are shown in Table 2. As would be expected 3 h after ingestion, unchanged NE comprised the greatest portion of the free fraction. The most abundant urinary metabolite, 5β, 3α-NE was also the most abundant compound in the plasma sulfate fraction. NE glucuronide and sulfate were present in plasma in considerable quantities, confirming our observation on the urinary metabolites that conjugation at the hindered 17β position can indeed occur with the 17α-ethynyl progestogens.

Quantification of plasma metabolites during daily treatment with 2 *mg NE.* Others have used mass fragmentography to measure blood levels of large doses of synthetic progestogens [9] but measurement of blood levels of the parent drug and metabolites was not attempted following administration of normal contraceptive dose levels. In the present study, following oral administration of a large dose of NE, it is evident (in Figs. 7–10) that the steroid metabolites are present in these purified plasma fractions in quantities that give extremely high signal to noise ratios, and in fact, the samples often had to be diluted to get the points in the range of the standard curves. Thus it appeared feasible to measure metabolites in plasma from patients treated with the usual contraceptive dosage of NE. Fig. 11 is a mass fragmentogram of MO derivatives of the free steroid fraction

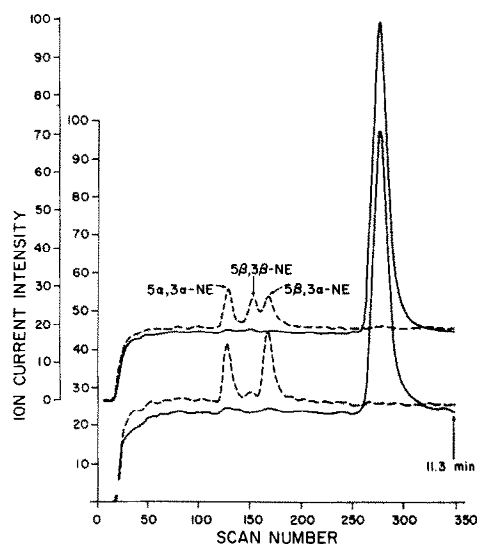


Fig. 7. Mass fragmentogram of TMS derivatives of 0.5 ng each of the 5α, 3α-NE, 5β, 3β-NE and 5β, 3α-NE reference compounds (above) and 2.5% of the free steroid fraction obtained from an 8 ml plasma sample following administration of 25 mg NE (below). Norethindrone metabolites are indicated by m/e 431 (---), while m/e 419 (——) indicates the internal standard 17α-vinyl-5α-androstane-3β, 17β-diol-bisTMS. The compounds were chromatographed on 1% XE-60 (1.5 m × 2 mm) at 185°.

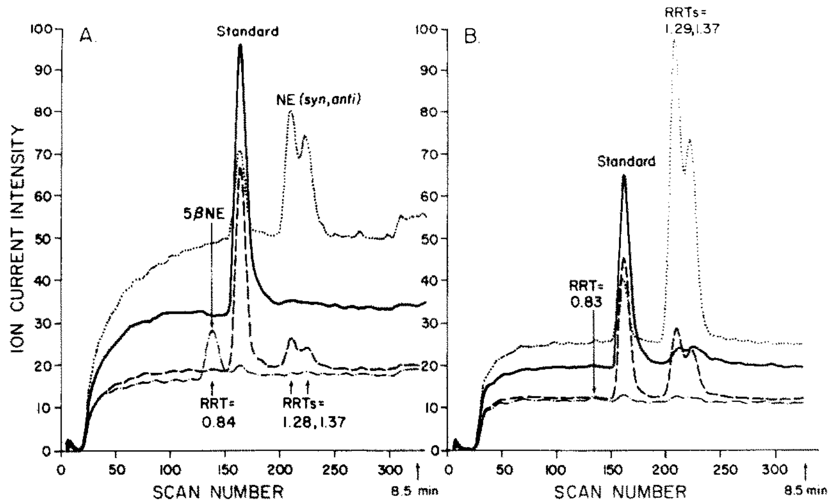


Fig. 8. A: Mass fragmentogram of the methoxime derivatives of 5 ng each of the reference compounds NE, represented by ions m/e 270 (— —) and 301 (···); 5β-NE, indicated by m/e 272 (-·-·); and the internal standard 17α-ethynylestr-5(10)-en-3-on-17β-ol, m/e 269 (——), 270 and 301. The compounds were chromatographed on 1% XE-60 (1.5 m × 2 mm) at 215°. NE-methoxime is separated into its syn and anti isomers by these conditions. B: Mass fragmentogram of the methoxime derivatives of 2.5% of the free fraction from 8 ml of plasma following administration of 25 mg of NE.

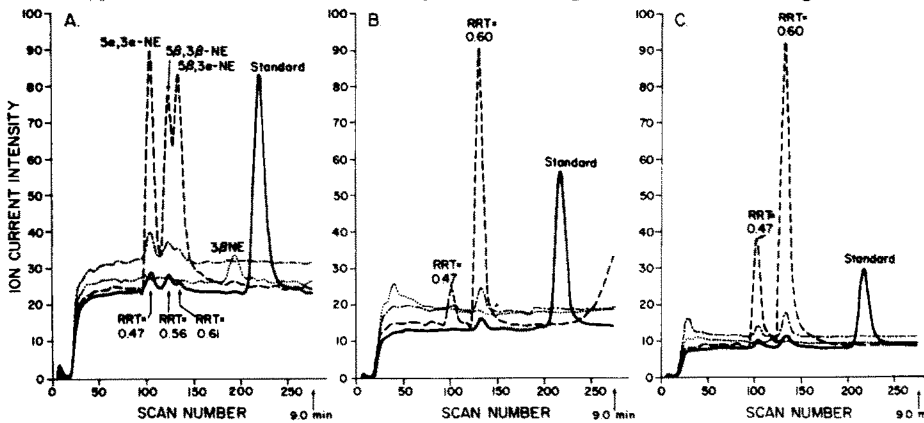


Fig. 9. A: Mass fragmentogram of the TMS derivatives of 2.5 ng each of the reference compounds 5α, 3α-NE, 5β, 3β-NE, 5β 3α-NE, indicated by ions m/e 417 (—·—·), 419 (——) and 431 (----), and 3β-NE, indicated by m/e 429 (····). The internal standard 17α-vinyl-5α-androstane-3β, 17β-diol (5 ng) is indicated by m/e 419. The compounds were chromatographed on 1% XE-60 (1.5 × 2 mm) at 185°. B: Mass fragmentogram of the TMS derivatives of 2.5% of the glucuronide fraction from an 8 ml plasma sample following administration of 25 mg of NE. Conditions were the same as described in the legend to Fig. 9A. C: Mass fragmentogram of the TMS derivatives of 1.25% of the sulfate fraction from an 8 ml plasma sample following administration of 25 mg of NE. Conditions were the same as described in the legend to Fig. 9A.

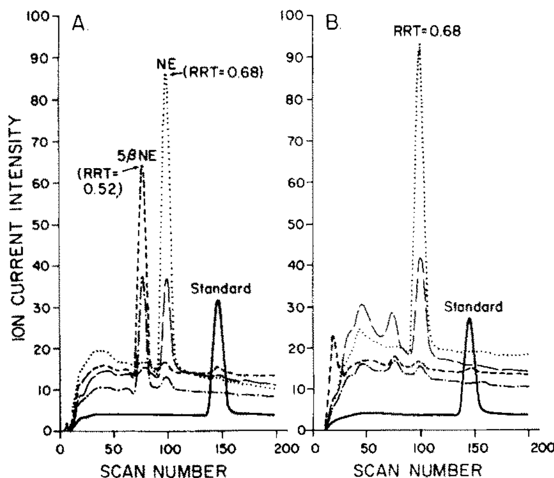


Fig. 10. A: Mass fragmentogram of the methoxime derivatives of 10 ng each of the reference compounds NE, indicated by ions m/e 270 (— —), 272 (-·-·) and 301 (····); 5β-NE, indicated by ions m/e 270, 272 and 303 (----); and 1.25 ng of the internal standard 17α-vinyl-5α-androstane-3β, 17β-diol-bisTMS, ion m/e 419 (——). The compounds were chromatographed on 1% SP-2100 (1.5 m × 2 mm) at 220°. B: Mass fragmentogram of the methoxime derivatives of 2.5% of the glucuronide fraction from 8 ml of plasma following administration of 25 mg of NE. Conditions were the same as described in the legend to Fig. 10A.

Table 2. Identification and quantification by mass fragmentography of norethindrone and metabolites in plasma following administration of 25 mg NE. Retention time of authentic reference compounds are given in parentheses.

Plasma Fraction	Relative Retention Time (Standards) XE-60	SP-2250	SP-2100	Plasma level (ng/ml)
Free				
5α, 3α-NE	0.45(0.45)	0.54(0.54)		4
5β, 3β-NE	0.53(0.54)	0.64(0.64)		4
5β, 3α-NE	0.60(0.60)			8
5β-NE	0.83(.084)		0.50(0.51)	9
NE	1.29(1.29)		0.65(0.65)	104
Sulfate				
5α, 3α-NE	0.47(0.47)	0.57(0.57)		46
5β, 3β-NE	0.57(0.56)*	0.65(0.65)		0.2
5β, 3α-NE	0.60(0.61)			110
5β-NE	—		—	—
NE	1.29(1.28)		0.67(0.67)	23
Glucuronide				
5α, 3α-NE	0.47(0.47)	0.57(0.57)		5
5β, 3β-NE	0.54(0.56)	0.64(0.65)		—
5β, 3α-NE	0.60(0.61)			27
5β-NE	—		—	—
NE	1.29(1.28)		0.68(0.68)	69

* Identified in silicic acid fraction I.

from a patient treated daily with Ortho-Novum (2 mg NE, 0.1 mg mestranol), and Fig. 12 a mass fragmentogram of TMS derivatives of the sulfate fraction from the same patient. It is apparent that the parent compound and resulting metabolites can readily be measured by this method. Adlercreutz *et al.* [9] pointed out that difficulties in accurate quantitation of synthetic progestogens can occur during mass fragmentographic measurement due to instrument instability. Replicate determinations on our quadrupole mass spectrometer of tetrahydro NE metabolites in a plasma sample (sulfate fraction) indicated the fol-

Table 3. Plasma levels of NE and metabolites in a volunteer treated daily with 2 mg NE, 0.1 mg mestranol. The sample was obtained 3 h after treatment with the tenth pill, on day 14 of the cycle

	ng/ml Free	Sulfate
5α, 3α-NE	0.3	151
5β, 3β-NE	0.3	8.4
5β, 3α-NE	0.6	44
5β-NE	20	0
NE	42	7.9

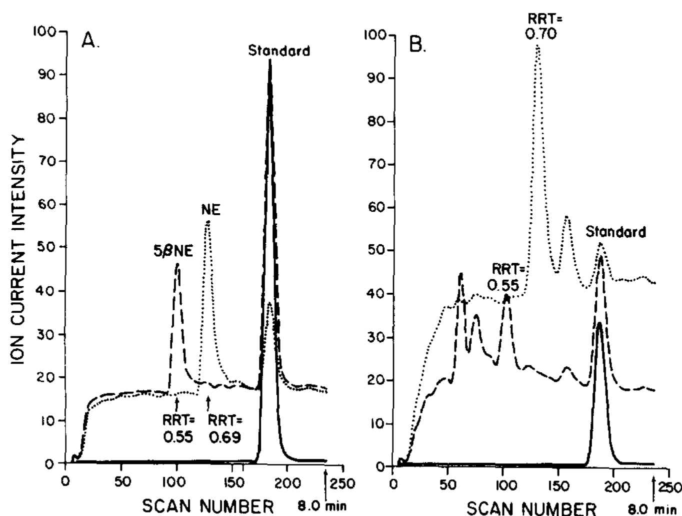


Fig. 11. A: Mass fragmentogram on 1% SP-2100 of the MO derivatives of 5 ng each of reference NE, indicated by m/e 301 (····) and 5β-NE, indicated by m/e 303 (---). The internal standard 17α-vinyl-5α-androstane-3β, 17β-diol-bisTMS is shown by m/e 419 (——). B: Mass fragmentogram of the reduced metabolites from the serum sulfate fraction following administration of a normal 2 mg dose of NE.

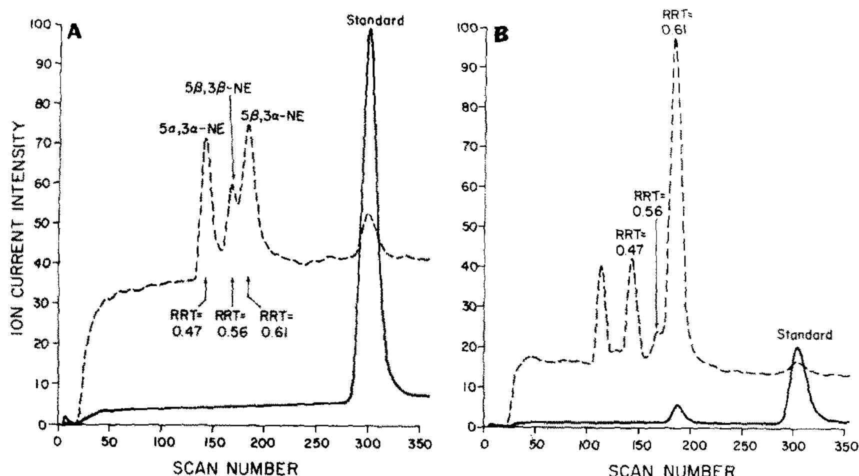


Fig. 12. A: Mass fragmentogram on 1% XE-60 of the TMS derivatives of 1 ng each of the tetrahydro NE reference compounds, indicated by m/e 431 (---). The internal standard 17α-vinyl-5α-androstane-3β, 17β-diol-bisTMS is shown by m/e 419 (——). B. Mass fragmentogram of the reduced metabolites from the serum sulfate fraction following administration of a normal 2 mg dose of NE.

lowing mean values and standard deviations ($n = 4$): 5β, 3α-NE, 21.4 ± 1.5 ng/ml; 5α, 3α-NE, 5.9 ± 0.2 ng/ml; 5β, 3β-NE, 3.8 ± 0.6 mg/ml. The recovery of 50 ng of NE and 5α, 3α-NE through the silicic acid chromatography, derivatization and mass fragmentography procedures was quantitative. Blood levels of metabolites found in the free and sulfate fractions of the above patient are shown in Table 3. The glucuronide fraction of this patient treated with therapeutic amounts of NE contained only traces of NE metabolites. The results indicate that the study of blood concentrations of NE and metabolites during long term administration of therapeutic levels can be accomplished using mass fragmentography. Such studies are now being undertaken in this laboratory.

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