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ANALYSIS OF ISOMERIC ETHYNYLESTRADIOL GLUCURONIDES IN URINE

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SUMMARY

A method for separation and analysis of conjugates of ethynylestradiol in urine is described. Steroid conjugates are separated on a lipophilic strong anion exchanger (triethylaminohydroxypropyl Sephadex LH-20), and phenolic steroids released by enzyme hydrolysis or solvolysis are isolated by chromatography on the same ion exchanger. Steroids carrying an ethynyl group are isolated by chromatography on SP-Sephadex (Ag^+). Ethynylestradiol is analyzed by gas chromatography–mass spectrometry of the trimethylsilyl ether, using [9,11,11,12,12-$^2H_5$]ethynylestradiol as internal standard.

INTRODUCTION

Steroids carrying an ethynyl group are widely employed for contraception. The metabolic fate of these steroids has been studied with the aid of radioactively labelled compounds, and several metabolites have been identified[1–9]. Gas chromatography–mass spectrometry (GC–MS) has also been used in a few studies of unlabelled steroids[10,11]. None of the methods was designed for routine use, and administration of radioactively labelled steroids has to be limited for ethical reasons. Thus, there is a need for a generally applicable analytical method which can be used in metabolic studies of larger groups of women. Such studies are important in order to define metabolic differences between individuals possibly related to side effects of the steroids.

We recently developed selective isolation procedures for GC–MS analysis of ethynyl steroids in urine and plasma[12]. These methods did not include group separation of A- and D-ring glucuronides of phenolic steroids. Such a step has now been included, and the quantitative aspects of the method have been evaluated by analysis of the urinary excretion of metabolites of an orally administered mixture of $^3H_2$- and $^2H_3$-labelled and unlabelled ethynylestradiol [19-nor-17α-pregna-1,3,5(10)-trien-20-yn-3-ol].

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EXPERIMENTAL

Urine samples

A mixture of 25 μg each of unlabelled ethynylestradiol (Leo, Hälsingborg, Sweden) and ethynyl[9,11,11-$^2H_3$]estradiol[13] and a tracer amount (16.8 μCi, $19.4 \cdot 10^6$ cpm) of ethynyl[6,7-$^3H_2$]estradiol (40.9 Ci/mmol; NEN Chemicals, Dreieich, G.F.R.) were dissolved in a few millilitres of aqueous ethanol and given orally to a 50 year old healthy male. Urine was collected for 24 h, each portion being immediately frozen in a polyethylene bottle kept on dry-ice. The 24-h sample was thawed, mixed thoroughly and frozen in 200-ml portions. Aliquots were taken for counting in a Packard Tri-Carb liquid scintillation spectrometer using Instagel as scintillating liquid.

Steroid analysis

All solvents were of reagent grade and redistilled. Chromatography columns and washing procedures for the chromatographic media have been described[12,14–16]. A flow scheme of the method is shown in Fig. 1. The first part is a 40-fold scale-up of a method for analysis of steroid profiles in urine[17].

Extraction. Steroids were extracted from urine (200 ml) on a column of Amberlite XAD-2 (50 × 1 cm). Following a wash with water, the steroids were eluted with methanol. Water was added to this eluate to give a 70% methanolic solution which was then passed through a column (20 × 1 cm) of SP-Sephadex C-25 (H^+) (Pharmacia, Uppsala, Sweden).

Group separation. The effluent from the SP-Sephadex column was passed through a column (18 × 1 cm) of triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20) (OH^-) packed in 70% methanol[17]. Following a rinse with 70% methanol to recover all neutral material, the column was eluted with 250–300 ml each of a series of solutions of electrolytes in 70% methanol to recover groups of steroids. Unconjugated phenolic steroids were obtained by saturation of the solvent with CO_2, glucuronides of neutral steroids and A-ring glucuronides of phenolic steroids with 0.8 *M* acetic acid, glucuronides having a free phenolic hydroxyl group (D-ring glucuronides) with 0.4 *M* formic acid, steroid monosulphates with 0.3 *M* potassium acetate buffer, pH 6.5, and disulphates with 0.5 *M* potassium acetate–hydroxide, pH 10.

To study overlap between A- and D-ring glucuronide fractions, aliquots (1/20) were taken to dryness and rechromatographed on small columns of TEAP-LH-20 (6 × 0.4 cm)[17].

Hydrolysis. To aid in the subsequent quantitation by GC–MS, a suitable amount of ethynyl[9,11,11,12,12-$^2H_5$]estradiol[13] was added to the main part (3/4) of the glucuronide fractions. Following evaporation of the solvents, hydrolysis was performed with a purified[17] solution of 2 ml *Helix pomatia* digestive juice (Pharmindustrie, Clichy, France) in 30 ml 0.2 *M* acetate buffer, pH 4.5, at 62°C for 1 h. The hydrolyzed steroids were extracted with Amberlite XAD-2 (14 × 0.8 cm) and a phenolic fraction was isolated by chromatography on TEAP-LH-20 (16 × 0.4 cm).

In another experiment the D-ring glucuronide fraction was hydrolyzed with a purified preparation of β-glucuronidase free of sulphatase activity (B-10, from bovine liver; Sigma, St. Louis, MO, U.S.A.). Incubation was performed with 10,000 units of enzyme in 5 ml acetate buffer, pH 4.5, at 37°C for 24 h. The mixture was then passed through a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.)[18], steroids

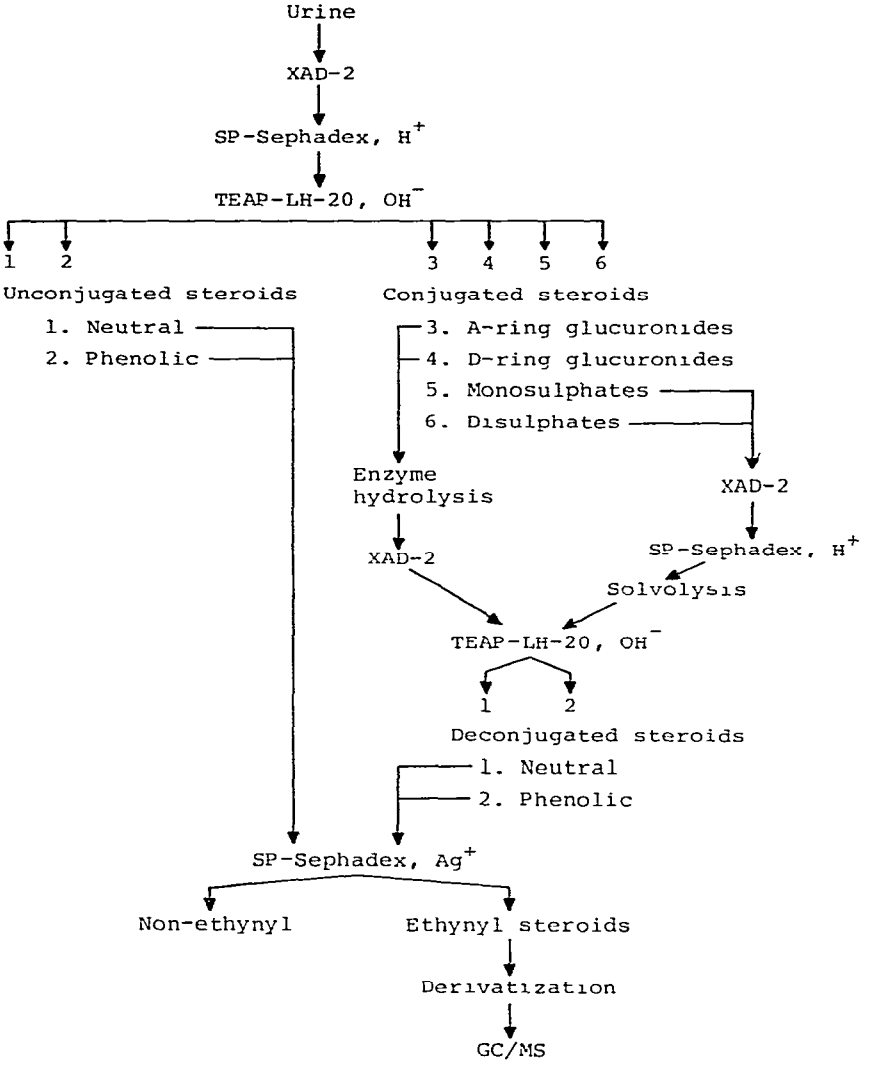

Fig. 1. Flow scheme of the analytical method.

were eluted with methanol and a phenolic fraction was isolated by chromatography on TEAP-LH-20.

Solvolysis. Ethynyl[9,11,11,12,12-$^2H_5$]estradiol was added to the sulphate fractions, which were concentrated to remove methanol and desalted by extraction with Amberlite XAD-2 (8 × 1 cm). The column was washed with 0.2 *M* sodium sulphate and water to ensure quantitative yield of steroid sulphates in the subsequent elution with methanol[19]. The latter eluate was passed through SP-Sephadex (10 × 0.4 cm). Following evaporation to about 0.1 ml, solvolysis was performed in 10 ml of 4 m*M* sulphuric acid in freshly distilled tetrahydrofuran at 50°C for 1 h. One millilitre of water was added and the tetrahydrofuran was evaporated. Methanol was added to

give a 70% solution which was passed through TEAP-LH-20 (OH^-) (16 × 0.4 cm). Phenolic steroids were recovered as above.

Ethynyl steroids were isolated as previously described[12] using a bed of SP-Sephadex (Ag^+) (2 × 0.8 cm) on top of another bed (H^+) (3 × 0.8 cm). Following elution of non-ethynyl steroids with 70% methanol, ethynyl steroids were eluted with 2.5% (v/v) of freshly distilled 1-hexyne in 70% methanol.

GC–MS analyses. The phenolic ethynyl steroids were analyzed as trimethylsilyl (TMS) ethers using a modified LKB 9000 instrument equipped with a 25-m open-tubular glass capillary column coated with SE-30 (see ref. 20). The temperature of the column, adjustable single-stage jet separator and ion source were 250°C, 250°C and 310°C, respectively. The electron energy was 22.5 eV, the ionizing current 60 μA and the initial accelerating voltage 3.5 kV. Partial mass spectra, *m/z* 446–422, were obtained by repetitive accelerating voltage scanning (2 sec per scan) using a data sampling rate of 10 kKz. Intensity readings were bunched to give eleven values per mass spectrometric peak which were recorded on magnetic tape[21]. Chromatograms representing *m/z* 440, 443 and 445 (M^+ for unlabelled, tri- and pentadeuterated species, respectively) and *m/z* 425, 428 and 430 ([$M - 15^+$] for the respective species) were constructed by the computer. Amounts were calculated from the peak areas, both by comparison with the peak area of the internal (pentadeuterated) standard and the peak area given by a known amount of external (unlabelled) standard.

RESULTS

Group fractionation of metabolites of ethynylestradiol

About 16% of the administered radioactivity was excreted in urine in 24 h. The relative distribution of the different groups of conjugates is shown in Table I. Most of the radioactivity was found in the two glucuronide fractions. Following enzyme hydrolysis, about 80% of this radioactive fraction had the mobility of unconjugated phenolic steroids (Table I), the remainder probably representing unhydrolyzed conjugates. Similar results were obtained following solvolysis of the monosulphate fraction (Table I).

TABLE I

RELATIVE DISTRIBUTION OF RADIOACTIVE METABOLITES OF ETHYNYLESTRADIOL IN DIFFERENT FRACTIONS FROM URINE BEFORE AND AFTER HYDROLYSIS (SOLVOLYSIS)

Original fraction from TEAP-LH-20	*Radioactivity as % of total*	*Distribution following hydrolysis*			
		Neutral	*Phenolic*		*Acidic**
			Ethynyl	*Non-ethynyl*	
Neutral	<1	–	–	–	–
Phenolic	1	–	1	–	–
Glucuronide, A-ring	31	2	11	10	8
Glucuronide, D-ring	46	–	34	7	5
Monosulphate	21	–	11	6	4
Disulphate	1	–	–	–	–

* Probably due in part to incomplete hydrolysis (solvolysis).

The phenolic steroids released by hydrolysis and solvolysis were separated on SP-Sephadex (Ag^+) into metabolites with and without an ethynyl group. As seen in Table I, about half of the radioactivity from the A-ring glucuronide fraction represented non-ethynyl compounds. The same was true of one third of the activity in the sulphate fraction and one sixth of that in the D-ring glucuronide fraction. Re-chromatography of the non-ethynyl material confirmed that it was not sorbed by SP-Sephadex (Ag^+), thus excluding a chromatographic artefact. The possibility that non-ethynyl steroids were formed as artefacts at some stage of the procedure was tested by carrying labelled ethynylestradiol through the different steps of the procedure. Chromatography on SP-Sephadex (Ag^+) showed that the ethynyl group was not lost.

Analysis of the glucuronide fractions

To establish that the separation of radioactivity into A-ring and D-ring glucuronide fractions was not due to a chromatographic artefact, aliquots of these fractions were subjected to rechromatography on TEAP-LH-20. The separation into two fractions, having mobilities of A-ring and D-ring glucuronides of phenolic steroids, respectively[17], was confirmed (Fig. 2). Some overlap occurred, probably because of slight overloading in the first chromatography on TEAP-LH-20.

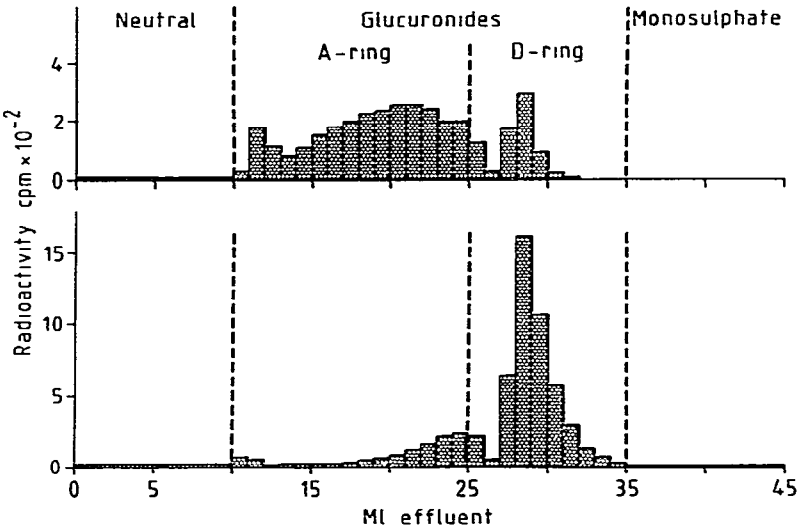

Fig. 2. Rechromatography of the A-ring (top) and D-ring (bottom) glucuronide fractions from urine on TEAP-LH-20 (OH^-).

The small amounts of material available did not permit direct GC–MS analysis of derivatized glucuronides. To confirm the glucuronide structure, the fractions were treated with a purified preparation of β-glucuronidase from bovine liver. This resulted in conversion of 92% of the radioactivity of the D-ring glucuronide fraction into material having a similar mobility on TEAP-LH-20 to unconjugated phenolic steroids.

The phenolic ethynyl fractions from the hydrolyzed A- and D-ring glucuronide

fractions were analyzed by GC–MS after formation of TMS ethers. Peaks having the retention time of the TMS ether of ethynylestradiol were obtained in the chromatograms of *m/z* 440 and 425 ($^2H_0$-molecules), 443 and 428 ($^2H_3$-molecules) and 445 and 430 ($^2H_5$-molecules). Partial separations occurred as expected, depending on the number of ^2H atoms (Fig. 3).

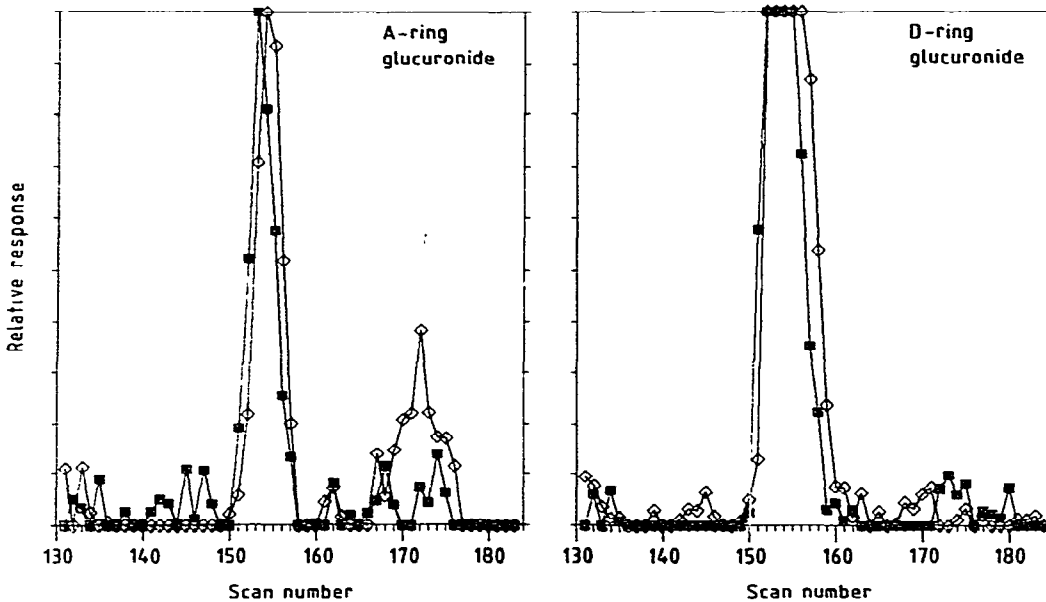

Fig. 3. Chromatograms of fragment ions *m/z* 425 (◇—◇) and 428 (■—■) obtained in the GC–MS analysis of the TMS ether of ethynylestradiol from the A-ring (left) and D-ring (right) glucuronide fractions from urine of a subject given a 1:1 mixture of unlabelled and trideuterated ethynylestradiol. A glass capillary column was used and the retention time of the compound was about 10 min.

The quantitative results are summarized in Table II. It is seen that the amount of ethynylestradiol determined by GC–MS corresponds to about 85% of the total content of ethynylestradiol and its metabolites calculated from the radioactivity. The ratio between unlabelled and trideuterated ethynylestradiol was the same as for the administered material.

GC–MS of the phenolic ethynyl fraction from the solvolyzed monosulphate fraction failed to reveal the presence of ethynylestradiol. This means that less than 5–10% of the radioactivity in this fraction was due to ethynylestradiol. The nature of the labelled metabolites was not further studied.

The non-ethynyl steroids from the different conjugate fractions were not analyzed by GC–MS. The amounts of metabolites were too small for a search for mass spectra showing the ion clusters expected of metabolites of the administered steroid mixture. For example, the A-ring glucuronide fraction contained about 0.4 ng of non-ethynyl metabolites per ml of urine (calculated from the radioactivity). Furthermore, chromatography on Sephadex LH-20 showed that the radioactivity was distributed between several different metabolites.

TABLE II

AMOUNTS OF GLUCURONIDATED ETHYNYL STEROIDS AND ETHYNYLESTRADIOL EXCRETED IN 24 h FOLLOWING ORAL ADMINISTRATION OF 50 μg OF A MIXTURE OF UNLABELLED, TRIDEUTERATED AND TRITIATED ETHYNYLESTRADIOL

Conjugate fraction	*Total ethynyl steroids** (μg)	*Ethynyl-estradiol*** (μg)
A-ring glucuronide	0.88	0.77
D-ring glucuronide	2.72	2.31

* Calculated from total radioactivity.
** Sum of $^2H_0$- and $^2H_3$-species, determined by GC–MS.

DISCUSSION

Previous studies have indicated that the main part of ethynylestradiol and its metabolites in urine is conjugated with glucuronic acid[5,7–9]. However, the pattern of conjugated metabolites is quite variable and at least two types of ethynylestradiol glucuronides have been detected[7]. One of these had the same mobility as synthetic 3-glucuronide, and the other was suggested to be the 17β-glucuronide. This assumption was supported by the previous finding of a conjugate of the 3-methyl ether of ethynylestradiol that could be hydrolyzed by β-glucuronidase[22]. Our study provides further support by showing that two conjugates of ethynylestradiol have the same mobilities as A-ring and D-ring glucuronides of phenolic steroids, respectively, on a lipophilic ion exchanger. The difference in mobility between the two types of conjugates depends on the presence of a free phenolic hydroxyl group in the D-ring glucuronide[17,23].

It is obvious that other types of conjugates may be eluted in the A-ring and D-ring glucuronide fractions. The pattern of elution of radioactivity shown in Fig. 2 indicates that this might be the case. However, treatment with β-glucuronidase converted most of the radioactive metabolites into compounds with the mobility of free phenolic steroids. Since the amount of steroid in the two fractions could be determined both from the radioactivity and by GC–MS analysis, it could be shown that ethynylestradiol constituted about 85% of the total amount of metabolites in the two fractions. The quantitative GC–MS analysis could be validated by monitoring ions from both the unlabelled and trideuterated molecules of ethynylestradiol, which had been administered as a 1:1 mixture. The use of this twin-ion technique[24] greatly increases the specificity of the analyses.

The principle of the sample purification procedure is simple and based on ion exchange before and after hydrolysis or solvolysis of the conjugates. When phenolic steroids are studied this results in highly purified fractions which can be obtained in a reasonably short time. The most time-consuming steps are the extractions with Amberlite XAD-2 which can now be carried out much faster using Sep-Pak C_{18} cartridges[18]. Studies can be focused on fractions of particular interest which reduces the time of analysis further.

The method permits analysis of ethynylestradiol in the A-ring and D-ring

glucuronide and monosulphate fractions using about 20 ml urine. While ethynylestradiol was not found in the monosulphate fraction in the present study, it can be a major compound in this fraction in other subjects[12]. In the subject studied, most of the ethynylestradiol was excreted in a form compatible with a 17β-glucuronide structure. Conjugation at this site is unexpected since the hydroxyl group is tertiary and hindered. It is interesting to note that a chemical glucuronidation of ethynylestradiol yielded the 17β-glucuronide as the main product[25]. It appears that the structure of the side chain at C-17 may have a profound influence on both the formation and enzymatic hydrolysis of 17β-glucuronides.

The method will permit studies of individual variation in the excretion of conjugates of ethynylestradiol in urine as well as kinetic studies in the steady state using deuterated ethynylestradiol. However, the sensitivity of the repetitive scanning GC–MS is still insufficient for analyses of complete profiles of ethynylestradiol metabolites. Many 2-, 6α-, and 16β-hydroxylated ethynyl and non-ethynyl metabolites have been shown to be excreted in urine (see ref. 8) and the complexity of the mixture is clearly seen when high-performance liquid chromatography is used to separate radioactive metabolites[26]. Each metabolite constitutes only a small percentage of the total and only half of the ethynylestradiol in a contraceptive pill (30 or 50 μg) is eliminated via the kidneys. The amounts of individual metabolites will therefore be in a range requiring selected ion monitoring of only a few m/z values.

Part of ingested ethynylestradiol loses the ethynyl group or is converted into D-homosteroids prior to excretion (see ref. 8). This part was large in the present study. Control experiments ruled out the possibility that the non-ethynyl radioactivity was formed as an artefact. The method may therefore be used for analysis of this type of metabolites. Due to the small amounts excreted it will be necessary to use selected ion monitoring of a limited number of preselected m/z values.

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