*Journal of Steroid Biochemistry*, 1975, Vol. 6, pp. 1187–1191. Pergamon Press. Printed in Great Britain

# IDENTIFICATION OF 2-HYDROXYOESTRADIOL AND THE PATTERN OF CATECHOL OESTROGENS IN HUMAN PREGNANCY URINE*

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(*Received* 14 *October* 1974)

SUMMARY

2-Hydroxyoestradiol was identified in human pregnancy urine and quantitatively determined. The isolation procedure involved four chromatographic purification steps of 2-hydroxyoestradiol on ascorbic acid impregnated stationary phases and the formation of its phenazine derivative. Following two further chromatography, mass spectrometry and by a microchemical reaction. The catechol oestrogens so far spectrometry. The identity of the product finally obtained was established by U.V.-spectrometry, gas chromatography–mass spectrometry and by a microchemical reaction. The catechol oestrogens so far isolated from human urine were quantitatively determined in several pregnancy urines; the amounts excreted were found to vary within the following ranges: 110–2100 μg of 2-hydroxyoestrone/24 h, 20–180 μg of 2-hydroxyoestradiol/24 h and 35–240 μg of 2-hydroxyoestriol/24 h.

## INTRODUCTION

During recent years it has not only been shown that 2-hydroxyoestrogens play an important role in the metabolism of oestrogens in man [1, 2], but also that the enzymatic inactivation of catecholamines is strongly inhibited by 2-hydroxyoestrogens [3–6]. This interaction between catechol oestrogens and catecholamines might be relevant to the occurrence of hypertension of unknown origin in pregnancy. To study the clinical significance of this phenomenon it was necessary to investigate the excretion of catechol oestrogens in the urine of pregnant women.

So far only 2-hydroxyoestrone† [7, 8] and 2-hydroxyoestriol [9] have been identified and quantitatively determined in human pregnancy urine, whereas no data concerning the occurrence of 2-hydroxyoestradiol in human urine are available. However in 1959, strong evidence for the urinary excretion of 2-methoxyoestradiol was obtained by Frandsen[10], indicating that 2-hydroxyoestradiol might also be present in the urine of pregnant women, since it is well established that catechol oestrogens are precursors in the biogenesis of 2-methoxyoestrogens [11]. Furthermore, Femino *et al.* have claimed that radioactive oestrone and oestradiol are transformed *in vivo* to 2-hydroxy- and 2-methoxyoestradiol to some extent in man, but definite experimental proof has not yet been given [12]. Knuppen and Ball[13] have shown unequivocally by *in vitro* experiments that 2-hydroxy- and 2-methoxyoestradiol are important products of oestrogen metabolism.

Investigation of the presence of 2-hydroxyoestradiol in human pregnancy urine was considered necessary, and also of the excretion pattern of that steroid and the related oestrogens, 2-hydroxyoestrone and 2-hydroxyoestriol.

## EXPERIMENTAL

### *Steroids*

2-$OHE_3$ was obtained from Steraloids Inc., N.Y., and 5β-cholanic acid-3α,7α,12α-triol methyl ester from Koch-Light Laboratories Ltd., England. 2-$OHE_2$, [4-$^{14}$C]-2-$OHE_2$ (50 mCi/mmol) and [4-$^{14}$C]-2-$OHE_3$ (50 mCi/mmol) were synthesised by oxidation of the monophenolic oestrogens with potassium nitrosodisulfonate [14]. Stock solutions of the radioactive catechol oestrogens were prepared by dissolving the steroids in methanol–standard ascorbic acid solution–glacial acetic acid (80:20:1, by vol.). Preparation of $PhE_1$ and $PhE_2$ was carried out according to Gelbke and Knuppen [15].

### *Chemicals and solutions*

Organic solvents were redistilled before use with the exception of chloroform (DAB 7) used for the conversion of 2-$OHE_2$ to $PhE_2$. All chemicals were purchased from E. Merck, Darmstadt, Germany, and were of analytical grade.

The standard ascorbic acid solution contained ascorbic acid (15 g) and glacial acetic acid (4 ml) in methanol (400 ml). The ascorbic acid solution of pH

* Part of this investigation was presented at the Joint Meeting of the Biochemical Societies of Belgium, the Federal Republic of Germany and the Netherlands, Düsseldorf 2–5 October 1974.

† The following abbreviations and trivial names have been used: 2-$OHE_1$ = 2-hydroxyoestrone = 2,3-dihydroxy-1,3,5(10)-oestratrien-17-one; 2-$OHE_2$ = 2-hydroxyoestradiol = 1,3,5(10)-oestratriene-2,3,17β-triol; 2-$OHE_3$ = 2-hydroxyoestriol = 1,3,5(10)-oestratriene-2,3,16α,17β-tetrol; $PhE_1$ = phenazine of 2-$OHE_1$ = 1,3,5(10)-oestratrien-17-one-[2,3-b]-quinoxaline; $PhE_2$ = phenazine of 2-$OHE_2$ = 1,3,5(10)-oestratrien-17β-ol-[2,3-b]-quinoxaline.

10·5 was prepared by dissolving ascorbic acid (30 g) and $NaHCO_3$ (45 g) in water (600 ml); shortly before use 15 ml of 15 M NaOH were added and the solution was then saturated with NaCl.

*Chromatographic methods*

Paper chromatography (2043 b Mgl paper, Schleicher and Schüll) and silica gel column chromatography (Kieselgel 60; particle size 0·063–0·2 mm; E. Merck) on ascorbic acid impregnated stationary phases were carried out as described by Gelbke and Knuppen[16]. The phenazine derivatives were chromatographed on papers impregnated with formamide–dimethylformamide–methanol (2:3:5, by vol.). Alumina t.l.c. was performed on MN-Polygram Alox-N/U.V.$_{254}$-precoated plates (Machery and Nagel, Germany), and alumina for column chromatography (Aluminium Oxide, basic, activity grade I) was obtained from Woelm, Germany, and deactivated with water (3 ml/100 g).

Gas–liquid chromatography was carried out with a Pye Unicam gas chromatograph, Series 104, equipped with a flame ionization detector. For silylation the material was treated with 30 $\mu$l of the silylating reagent (N,O-bis(trimethylsiyl)-trifluoroacetamide containing 5% of trimethylchlorosilane) for 1 h at 120°C. The gas chromatographic conditions were for:

(a) 2-$OHE_3$-TMS-ether: silicone OV-101 and silicone OV-17 as described previously [9];

(b) $PhE_2$-TMS-ether: coiled glass tubes (180 × 0·3 cm.), stationary phase 3% silicone OV-17 on chromosorb W HP 100/120, carrier gas flow 25 ml $N_2$/min, column temperature 290°C, injection port temp. 330°C, detector temp. 340°C, retention time 23 min; or: coiled glass tubes (150 × 0·3 cm.), stationary phase 3% silicone OV-101 on chromosorb W HP 100/120, carrier gas flow 25 ml $N_2$/min, column temp. 265°C, injection port temp. 285°C, detector temp. 290°C, retention time 20 min.

(c) $PhE_1$: the same conditions as described for (b); retention time when using silicone OV-17: 32 min; retention time when using OV-101: 19 min.

*Apparatus*

Radioactivity was counted either by liquid scintillation spectrometry (Intertechnique liquid scintillation spectrometer, Model SL 36) or directly on the paper chromatograms (Radiochromatogram Scanner, Berthold, Model LB 280) or t.l.c. plates (Dünnschicht–Scanner, Berthold, Model LB 2723). Ultraviolet spectra were recorded with a Shimadzu double-beam spectrometer, U.V.-200. Mass spectra were obtained using a gas chromatograph–mass spectrometer, LKB 9000.

*Determination of 2-hydroxyoestradiol in urine*

In order to avoid the autoxidation of 2-$OHE_2$ the pregnancy urines were acidified with 6 M HCl (60 ml) during the collection period [9]. The 24 h-specimens were either directly analysed or stored at −15°C. Portions of 100 ml of each urine were used for the determination of 2-$OHE_1$ [8] and of the total oestrogens, while 2-$OHE_2$ and 2-$OHE_3$ were determined in the remaining urine as follows:

To 100 ml of urine 1 g of KI, 4 ml of a freshly prepared aqueous sodium bisulfite solution (1% w/v) and 18 ml of concentrated HCl (37%) were added. Nitrogen was bubbled through the urine for 10 min and the mixture was refluxed for 1 h. After cooling to room temp. 100 000 d.p.m. (0·3 $\mu$g) each of [4-$^{14}$C]-2-$OHE_3$ and [4-$^{14}$C]-2-$OHE_2$ were added. The hydrolysed urine was saturated with NaCl and extracted three times with half the vol. of ethyl acetate. The combined ethyl acetate extracts were washed with the ascorbic acid solution of pH 10·5 (2 × 0·25 vol.) and then with a 10% aqueous acetic acid solution (2 × 0·05 vol.). Standard ascorbic acid solution (1 ml) was added and the organic extract was evaporated *in vacuo*.

The residue was dissolved in glacial acetic acid (5 ml) and after addition of 20 ml of chloroform–cyclohexane (1:1, v/v) the mixture was subjected to column chromatography (2·0 cm dia.) on ascorbic acid impregnated silica gel. Elution was carried out with:

(1) 600 ml of chloroform–cyclohexane–acetic acid (2:2:1, by vol.),

(2) 600 ml of chloroform–cyclohexane–acetic acid (1·9:1·9:1, by vol.),

(3) 800 ml of chloroform–acetic acid (2:1, v/v) and

(4) 1200 ml of chloroform–acetic acid (7:5, v/v).

Each of these solvent systems was saturated with ascorbic acid.

Fractions were collected and the radioactivity was determined by liquid scintillation counting. The first 700 ml eluted from the column were discarded; the radioactive 2-$OHE_2$ was found within the following 400 ml of solvent system(2). The fractions eluted by solvent system(3) and the first 350 ml of solvent system(4) were also discarded; the radioactive 2-$OHE_3$ was present within the following 550 ml of the eluant. The fractions containing 2-$OHE_2$ and 2-$OHE_3$ were evaporated *in vacuo*.

Further purification and determination of the crude 2-$OHE_3$ was carried out as described previously [9], and 2-$OHE_2$ was determined as follows: The material obtained by the above described column chromatography was dissolved in 10% aqueous acetic acid (2·5 ml) which was extracted 3 times with ethyl acetate (3 ml). The combined organic extracts were washed once with water (3 ml) and evaporated *in vacuo* after addition of 1 ml of the standard ascorbic acid solution.

Further purification of the crude 2-$OHE_2$ was carried out by chromatography on formamide–ascorbic acid impregnated papers (16 cm. wide) in the system chlorobenzene–ethyl acetate (3:1, v/v). After development for 12 h the radioactive 2-$OHE_2$ was located (migration rate 1·9 cm./h) and the appropriate areas were thoroughly extracted with methanol containing 10% standard ascorbic acid solution. After evaporation the residue was subjected to a partition between 10% aqueous acetic acid and ethyl acetate as

described above: standard ascorbic acid solution (0·4 ml) was added and the ethyl acetate was evaporated. The residue was then purified by paper chromatography (8 cm. wide papers) in the system water–acetic acid–1,2-dichloroethane–methylcyclohexane (60:140:125:75, by vol.) for 7 h (migration rate: 4 cm./h). The 2-$OHE_2$ was eluted from the paper and partitioned between 10% aqueous acetic acid and ethyl acetate. Standard ascorbic acid solution (0·2 ml) was added and the ethyl acetate extract was evaporated *in vacuo*.

The residue was subjected to a second chromatography on a silica gel column (1·2 cm. dia.) prepared as follows: a slurry of unimpregnated silica gel (2 g) in n-hexane–ethyl acetate (2·5:1, v/v) was poured into the column first, followed by a slurry of ascorbic acid-impregnated silica gel (5 g) in the same solvent system. (In this way the ascorbic acid eluted from the upper part of the column during chromatography was retained by the unimpregnated silica gel resulting in fractions of 2-$OHE_2$ without any ascorbic acid, which decreases the yield of the conversion of 2-$OHE_2$ to $PhE_2$ [8].) The material obtained from urine was then dissolved in methanol (100 µl) and transferred to the column after addition of 5 ml of n-hexane–ethyl acetate (2·5:1, v/v). Elution of the column was carried out first with 80 ml of n-hexane–ethyl acetate–methanol (70:29:1, by vol.) and then with n-hexane–ethyl acetate–methanol (66:33:2, by vol.). Fractions of 5 ml were collected. As determined by liquid scintillation counting, fractions 15–31 contained 2-$OHE_2$; these were combined and evaporated *in vacuo*.

The 2-$OHE_2$ was then immediately converted to the phenazine derivative to prevent autoxidation. This reaction was carried out exactly as described in detail for 2-$OHE_1$ [8].

Further purification of the $PhE_2$ was achieved by paper chromatography in the system formamide–dimethylformamide (2:3, v/v)-methylcyclohexane–chlorobenzene (4:1, v/v) for 5 h ($R_F$ = 0·45). The radioactive phenazine was located and after elution from the paper (twice with methanol and twice with methanol–chloroform (1:1, v/v)) the material was partitioned between water and chloroform. The chloroform was evaporated *in vacuo* and the residue obtained finally purified by alumina column chromatography.

Basic alumina (1 g) was poured into a column (0·6 cm. dia.) containing benzene. $PhE_2$ was dissolved in benzene (3 × 1 ml) and transferred to the column. Chromatography was carried out with 20 ml of benzene and 25 ml of benzene containing 0·4% of methanol. Fractions of 5 ml were collected; fractions 6–8 contained the radioactive phenazine.

For quantitative evaluation the obtained $PhE_2$ was dissolved in 96% ethanol (3 ml). An aliquot was subjected to liquid scintillation counting to determine the overall recovery. The U.V.-absorptions at 242 nm, 257 nm ($\lambda_{max}$) and 272 nm were recorded and the $E_{corr}$-value was calculated using the Allen equation [17] $E_{corr} = 2\,E_{257}-(E_{242} + E_{272})$. As $E_{corr}$ = 0·548 corresponds to a concentration of 1 µg of $PhE_2$/1 ml of ethanol [15], the final amount of $PhE_2$ could be determined.

*Microchemical oxidation of $PhE_2$ to $PhE_1$*

After spectrometric determination the ethanolic solution of $PhE_2$ was evaporated *in vacuo* and the dry residue was dissolved in acetone (2 ml). The solution was cooled with ice and 30 µl of Jones reagent [18] (26·72 g of $CrO_3$ in 23 ml of concentrated $H_2SO_4$ diluted with water to 100 ml) were added. After 5 min the excess of $CrO_3$ was reduced with methanol (approx. 1 ml). Water (5 ml) was added, acetone and methanol were removed under $N_2$ at 60°C and the remaining aq. phase was extracted 3 times with chloroform. The combined organic extracts were washed twice with 1 M NaOH, twice with 1 M HCl, and twice with water, and the chloroform was evaporated *in vacuo*. The $PhE_1$ thus obtained was purified by column chromatography (1 g of basic alumina) allowing the separation of $PhE_1$ and $PhE_2$ as reported previously [8]. Quantitative evaluation of $PhE_1$ by liquid scintillation counting and spectrometry was carried out as described above for $PhE_2$.

## RESULTS

*Isolation and identification of 2-hydroxyoestradiol from human urine*

2-$OHE_2$ was isolated from several 24 h-urines of different pregnant women following the procedure presented in the flow diagram (Fig. 1). After purification 2-$OHE_2$ was oxidized to the corresponding o-quinone and subsequently converted to the phenazine derivative with o-phenylenediamine as described previously [15] (Fig. 2). The identity of the $PhE_2$ was established as follows:

1. The U.V.-spectrum of the phenazine isolated was identical with the spectrum of authentic $PhE_2$ ($\lambda_{max}^{ethanol}$: 257 nm).
2. When the phenazine derivative was silylated and subjected to gas chromatography on silicone OV-101 and OV-17, a single symmetrical peak was found with the same retention times as silylated authentic $PhE_2$.
3. Final identification of the gas chromatographic peak was achieved by mass spectrometry.
4. Oxidation of the $PhE_2$ by $CrO_3$ in acetone yielded the corresponding 17-oxo compound ($PhE_1$; phenazine derivative of 2-$OHE_1$). After column chromatography on alumina the identity of this reaction product was established by U.V.-spectrometry and gas chromatography on silicone OV-101 and OV-17.

*Quantitative determination of 2-hydroxyoestradiol*

To obtain reliable quantitative results for the determination of 2-$OHE_2$ the very laborious multistep work-up procedure shown in Fig. 1 was found to be absolutely necessary. Omission of different purification steps led to a decrease of the specificity as indi-

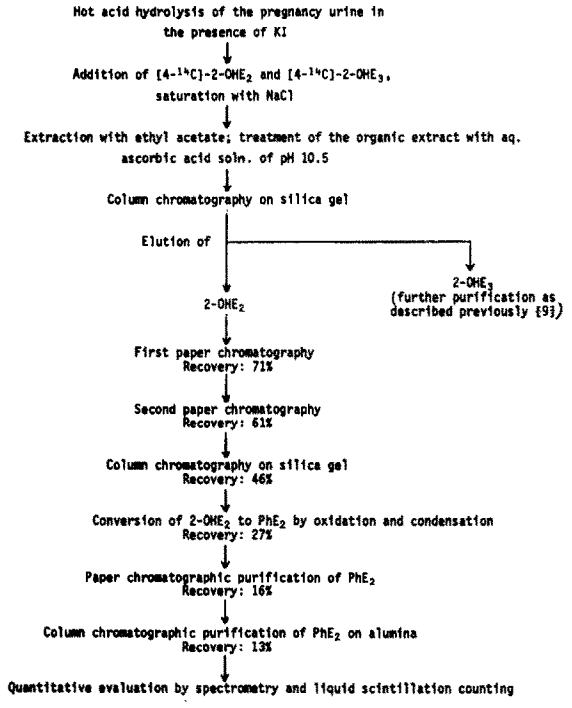


Fig. 1. Flow diagram for the isolation of 2-$OHE_2$ and the quantitative estimation of 2-$OHE_2$ and 2-$OHE_3$ in pregnancy urine. Recoveries are related to the originally added [4-$^{14}$C]-2-$OHE_2$ and represent the mean of four determinations.

cated by markedly increased values found for the urinary excretion of 2-$OHE_2$.

The addition of [4-$^{14}$C]-2-$OHE_2$ after hydrolysis as internal standard enabled an exact calculation of the recovery, since the radiochemical purity of the $PhE_2$ was found to be better than 95%. This was checked by paper chromatography in two different systems (formamide–dimethylformamide (2:3, v/v)-methylcyclohexane, and formamide–dimethylformamide (2:3, v/v)-chlorobenzene–methylcyclohexane (1:4, v/v)) and by alumina t.l.c. (benzene–ethanol (3:1)).

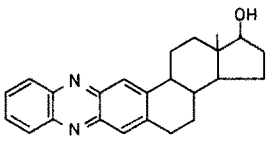


Fig. 2. Phenazine derivative of 2-$OHE_2$.

Specificity was examined by a microchemical reaction: the 2-$OHE_2$ in urine was determined by U.V.-spectrometry of the $PhE_2$ derivative. The $PhE_2$ was subsequently oxidized to $PhE_1$ which was subjected to a further alumina column chromatography. The amount of 2-$OHE_2$ in urine was then independently calculated by U.V.-spectrometry of $PhE_1$ and liquid scintillation counting. The yield of this reaction including the column chromatography of $PhE_1$ was approximately 50% and the radiochemical purity of the $PhE_1$ finally obtained was better than 95% as shown by alumina t.l.c. in the system benzene–ethyl acetate (2:1, v/v). When 2-$OHE_2$ in urine was determined *via* the phenazine derivatives $PhE_2$ and $PhE_1$ results were found to differ by less than 10%.

*Pattern of the excretion of catechol oestrogens in human pregnancy urine*

In order to demonstrate the relation of the different 2-hydroxyoestrogens isolated so far from human pregnancy urine the excretion of 2-$OHE_1$, 2-$OHE_2$ and 2-$OHE_3$ was determined in the urines of several pregnant women. The results obtained are listed in Table 1.

Table 1. Excretion of 2-$OHE_1$, 2-$OHE_2$, 2-$OHE_3$ and total oestrogens in human pregnancy urine.

| Patient | Week of pregnancy | Excretion of 2-$OHE_1$[µg/24h] | 2-$OHE_2$[µg/24h] | 2-$OHE_3$[µg/24h] | total oestrogens[mg/24h] |
|---|---|---|---|---|---|
| K | 24 | 2100 | 180 | 140 | 13 |
| | 36 | 1900 | 170 | 160 | 26 |
| | 38 | 1500 | 60 | 240 | 32 |
| | 39 | 2100 | 110 | 230 | 38 |
| | 39 | 1600 | 65 | 165 | 35 |
| G | 26 | 1600 | | 120 | 10 |
| | 29 | 950 | 120 | 240 | 8.5 |
| | 34 | 650 | 80 | 115 | 9 |
| E | 22 | 600 | 25 | 55 | 9 |
| | 25 | 450 | 95 | 40 | 8 |
| | 29 | 650 | 30 | 55 | 11 |
| M | 35 | 170 | 30 | 50 | 15 |
| | 38 | 350 | 25 | 85 | 25 |
| Sch | 26 | 680 | 20 | 40 | 8 |
| | 29 | 710 | 20 | 35 | 14 |
| P | 30 | 110 | 30 | 60 | |
| B | 36 | 270 | 25 | 60 | 12 |
| L | 38 | 600 | 40 | 55 | 33 |
| Sp | 38 | 280 | 25 | 100 | 16 |
| Si | 40 | 690 | 40 | 130 | 43 |
| P | 41 | 880 | | 110 | 25 |
| F | 42 | 310 | | 70 | 17 |

## DISCUSSION

The results reported here present unequivocal evidence that 2-$OHE_2$ is excreted in the urine of pregnant women. The final identification and quantitative determination of 2-$OHE_2$ was carried out after the formation of the corresponding phenazine derivative. The identity of this phenazine compound was established by gas chromatography, mass spectrometry, U.V.-spectrometry and by oxidation to the corresponding 17-oxo compound. The microchemical oxidation was also used to investigate the reliability of the quantitative data. Thereby it was found that the determination of 2-$OHE_2$ with sufficient specificity was only guaranteed by the very laborious purification procedure described here, whereas reliable results for the urinary excretion of 2-$OHE_1$ and 2-$OHE_3$ can be obtained by assay procedures more practicable [8, 9].

As the determinations of 2-hydroxyoestrogens involved different chromatographic purification steps and microchemical reactions, it seemed necessary to use radioactive internal standards for the correction of procedural losses. Since so far no information is available concerning the conjugates of 2-$OHE_2$ and 2-$OHE_3$, the unconjugated catechol oestrogens were used as internal standards, which can be easily prepared with high specific radioactivities [14]. With regard to the fact that definite amounts of the unconjugated steroid are present only at the end of the hydrolytic procedure, the internal standard was added after hydrolysis of the urine. Obviously, any losses incurring during hydrolysis cannot be assessed, but it should be mentioned that only negligible amounts of unconjugated 2-hydroxestrogens are decomposed in the course of acid hydrolysis [8, 9].

The results obtained so far on the concentration of catechol oestrogens in the urine of pregnant women show that the excretions of 2-$OHE_2$ and 2-$OHE_3$ are definitely lower than those of 2-$OHE_1$, in contrast to the predominant excretion of oestriol within the group of the classical oestrogens. The amount of 2-$OHE_1$ was 110-2100 $\mu$g/24 h, whereas the excretion of 2-$OHE_3$ was 35–240 $\mu$g/24 h and that of 2-$OHE_2$ was even lower, 20–180 $\mu$g/24 h (Table 1).

*Acknowledgements*—This work was supported by the Deutsche Forschungsgemeinschaft. The authors thank Dr. B. Spiegelhalder, Bonn, for recording and interpreting the mass spectra.

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