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# METABOLISM OF THE ORAL CONTRACEPTIVE STEROIDS ETHYNYLESTRADIOL AND NORGESTIMATE BY NORMAL (HUMA 7) AND MALIGNANT (MCF-7 AND ZR-75-1) HUMAN BREAST CELLS IN CULTURE

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**Summary**—Human breast cancer cells are used extensively for the study of steroid hormone action. It is known that in both receptor positive and receptor negative cell lines there is considerable metabolism of the natural estrogens, estradiol ($E_2$) and estrone ($E_1$) with interconversion of the two steroids and formation of sulphate and glucuronide conjugates. The aim of the present work was to see if the commonly used oral contraceptive steroids (OCS) ethynylestradiol ($EE_2$) and norgestimate (Ngmate) were metabolized in human breast cancer cell lines (MCF-7 and ZR-75-1) and a normal breast cell line (Huma 7). MCF-7, ZR-75-1 and Huma 7 cells were maintained in Dulbeccos Modified Eagles Medium (DMEM) containing foetal calf serum (FCS) insulin and hydrocortisone. In addition, ZR-75-1 cells required epidermal growth factor (EGF) and $E_2$ while MCF-7 cells required only EGF. On reaching confluence cells were transferred to DMEM containing charcoal-stripped FCS, insulin and hydrocortisone. 48 h later this medium was renewed, radiolabelled steroid ([$^3$H]$E_1$; [$^3$H]$E_2$; [$^3$H]$EE_2$, [$^3$H]Ngmate; [$^3$H]$E_1$-$SO_4$; 1 nM; 0.2 $\mu$Ci) was added and incubation was for 24 or 48 h. Following incubation, the medium was removed and radioactive steroid extracted with ether. Metabolites were analysed by on-line radiometric HPLC.

All the cell lines were able to interconvert $E_1$ and $E_2$; the equilibrium favouring the formation of $E_2$ in MCF-7 and ZR-75-1 and $E_1$ in Huma 7 cells. $E_1$ and $E_2$ also underwent phase II metabolism to form their respective estrogen sulphates, this activity being most marked in the Huma 7 cell line. In addition to sulphotransferase activity, the study with $E_1$ sulphate demonstrated sulphatase activity in both normal and cancer cells. There appeared to be no difference in extent of hydrolysis, with both $E_1$ and $E_2$ formed. With $EE_2$ as substrate there was no evidence of phase I metabolism in any of the cell lines but there was conversion to the presumed 3-sulphate conjugate. The percentage formation of this metabolite was very much greater in Huma 7 cells ($64.1 \pm 9.6\%$ after 24 h) than in MCF-7 and ZR-75-1 cells ($7.4 \pm 5.3\%$ and $10.6 \pm 4.1\%$, respectively after 24 h). In all the cell lines deacetylation of the progestogen Ngmate to norgestrel oxime was complete within 24 h. In addition there was evidence of loss of the oxime moiety to give norgestrel.

These studies have shown that the OCS Ngmate and $EE_2$ are metabolized in both normal and malignant breast cell lines; in particular sulphotransferase and esterase activity is marked. In addition, there are both qualitative and quantitative differences in steroid metabolism between normal (Huma 7) and malignant (MCF-7, ZR-75-1) cells in culture.

## INTRODUCTION

Despite the fact that oral contraceptive steroids (OCS) have been available since the early 1960s and today are used by an estimated 80 million women worldwide, we know comparatively little about certain aspects of the pharmacokinetics and disposition of the steroids. Whilst pharmacokinetic parameters and metabolic profiles have been determined for individual steroids in clinical studies *in vivo* (reviewed

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*Trivial names*: Ethynylestradiol, 17α-ethynyl-1,3,5[10]-estratriene-3,17β-diol; estradiol, 1,3,5[10]-estratriene-3,17β-diol; estrone, 1,3,5[10]-estratriene-3-ol-17-one; estrone-3-sulphate, 1,3,5[10]-estratriene-3-ol-17-one sulphate; estradiol-3-sulphate, 1,3,5[10]-estratriene-3,17β-diol-3-sulphate; norgestimate, (+)-13-ethyl-17-acetoxy-18,19-dinor-17α-preg-4-en-20-yn-3-oxime; 3-keto norgestimate, (+)-13-ethyl-17-acetoxy-18,19-dinor-17α-preg-4-en-20-yn-3-one; 17-deacetyl norgestimate, (+)-13-ethyl-17-hydroxy-18-19,-dinor-17α-preg-4-en-20-yn-3-oxime; norgestrel, (+)-13-ethyl-17-hydroxy-18,19-dinor-17α-preg-4-en-20-yn-3-one.

in [1–3]) and in addition there have been *in vitro* metabolic studies involving the use of human liver [4, 5] and gastrointestinal mucosa [6, 7], little attention has been given to the potential for metabolism by the target organ. If we are to understand the mechanism(s) by which OCS produce both wanted and unwanted clinical effects, it is important to know if target organs such as breast and endometrium are capable of producing metabolites which could possibly act locally with autocrine/paracrine potential.

Human mammary cell lines have been used extensively to study steroid hormone action and metabolism [8–13]. For example, it is known that in both receptor positive and receptor negative cells there is considerable metabolism of the natural estrogens estradiol ($E_2$) and estrone ($E_1$) with interconversion of the two steroids by 17$\beta$-hydroxysteroid dehydrogenase and formation of conjugates [11]. The aim of the present work is to see if the OCS 17$\alpha$-ethynylestradiol ($EE_2$) and norgestimate are metabolized by human breast cancer cell lines (MCF-7 and ZR-75-1 [14, 15]) and a cell line (Huma 7) derived from normal human breast [16].

## EXPERIMENTAL

[$^3$H]$E_2$ (sp. act. 60.0 Ci·mmol$^{-1}$), [$^3$H]$E_1$ (sp. act. 54.6 Ci·mmol$^{-1}$), [$^3$H]$E_1$-3-sulphate (sp. act. 59.0 Ci·mmol$^{-1}$), [$^3$H]$EE_2$ (sp. act. 53.7 Ci·mmol$^{-1}$) were obtained from Du Pont U.K. Ltd, Stevenage. $E_2$, $E_1$, $E_1$-3-sulphate, $E_2$-3-sulphate and $EE_2$ were obtained from Sigma Chemical Co. (London). [$^3$H] norgestimate (sp. act. 44 Ci·mmol$^{-1}$), 17-deacetyl norgestimate (norgestrel oxime), 3-keto norgestimate (norgestrel 17-acetate) and levonorgestrel were all gifts from Ortho Pharmaceuticals Corp., NJ, U.S.A. Cell culture media and sera were obtained from Gibco Life Technologies Ltd, Paisley, Scotland. All other reagents were purchased from BDH Chemical Company Ltd, Poole and were of analytical grade.

### *Cells*

The MCF-7 and ZR-75-1 human breast cancer cell lines were obtained from Dr R. King (ICRF Breast Biology Group, Dept of Biochemistry, University of Surrey, Guildford) and the epithelial cell line (Huma 7) isolated from a simian virus-40 immortalized mammoplasty specimen of an otherwise healthy woman has been reported previously [16]. All cell lines were free of mycoplasma.

### *Cell culture and incubations*

MCF-7, ZR-75-1 and Huma 7 cells were maintained in 10 cm-dia Petri dishes with 10 ml Dulbecco's Modified Eagles Medium (DMEM) containing foetal calf serum (FCS; 5%), insulin (50 ng/ml) and hydrocortisone (50 ng/ml). In addition, for maximum growth, ZR-75-1 cells required epidermal growth factor (EGF; 5 ng/ml) and $E_2$ (2.7 ng/ml) while MCF-7 cells required only EGF (5 ng/ml). The hormones were not essential but enchanced growth. Also the addition of hormones to serum which can contain variable concentrations of endogenous hormones gives standardization of concentrations. On reaching confluence, cells were transferred to DMEM containing 5% FCS, depleted of endogenous steroids by dextran-coated charcoal (DCC; 0.05% dextran, 0.5% charcoal) treatment, insulin (50 ng/ml) and hydrocortisone (50 ng/ml). Forty-eight hours later this medium was renewed and radiolabelled steroid in ethanol added (0.2 $\mu$Ci; final concentration 1 nM; the final ethanol concentration was $<0.5\%$). Incubations were performed at 37°C in 10% $CO_2$ 90% air for 24 or 48 h. Controls were prepared simultaneously and consisted of growth medium containing similar steroid concentrations, incubation times and temperatures, but incubated without cells or with 5% trichloroacetic acid/ethanol-killed cells. Following incubation, the medium was removed and extracted with ether (2 × 2 vol). Pooled ether extracts were evaporated to dryness, reconstituted in a small volume of methanol and analysed by high performance liquid chromatography (HPLC). Ether-extracted culture medium (4 ml) containing polar, conjugated metabolites was applied to primed (5 ml methanol followed by 5 ml distilled, deionized water) Sep-Pak $C_{18}$ cartridges (Waters Assoc. Milford, MA, U.S.A.). Unwanted components were first eluted with distilled, deionized water (5 ml). Estrogen conjugates were then eluted with methanol (5 ml). The methanol eluate was subsequently evaporated to dryness and reconstituted in a small volume of methanol prior to analysis by HPLC. Total radioactivity was determined in the various fractions and in the cellular material by liquid scintillation spectrometry.

*HPLC analysis*

A Spectra-Physics SP 8700 solvent delivery system was used connected to a FLO ONE beta on-line radiometric detector (Radiomatic Instruments and Chemical Co., Tampa, FL, U.S.A.). Parent steroids and metabolites were quantified from peak areas. All chromatographic-separations were performed on a Techopak 10 $C_{18}$ column (3.9 mm i.d. × 30 cm; HPLC Technology). $E_1$, $E_2$, $EE_2$ and $E_1$-3-sulphate phase I metabolites were separated with a mobile phase of methanol–ammonium dihydrogen orthophosphate (0.5%; pH 3.0) (62:38). The flow rate was 0.7 ml·min$^{-1}$. Authentic standards were monitored by u.v. detection at 280 nM. Norgestimate phase I metabolite separation was achieved with a mobile phase of methanol–water (70:30). The flow rate was 1.5 ml·min$^{-1}$. Authentic standards were monitored by u.v. detection at 240 nM. Separation of estrogen phase II metabolites utilized a mobile phase gradient of methanol (initially 30% but increasing to 60% over 40 min) and ammonium acetate (10 mM; pH 6.9). The flow rate was 1.5 ml·min$^{-1}$. Authentic standards were monitored by u.v. detection at 280 nM.

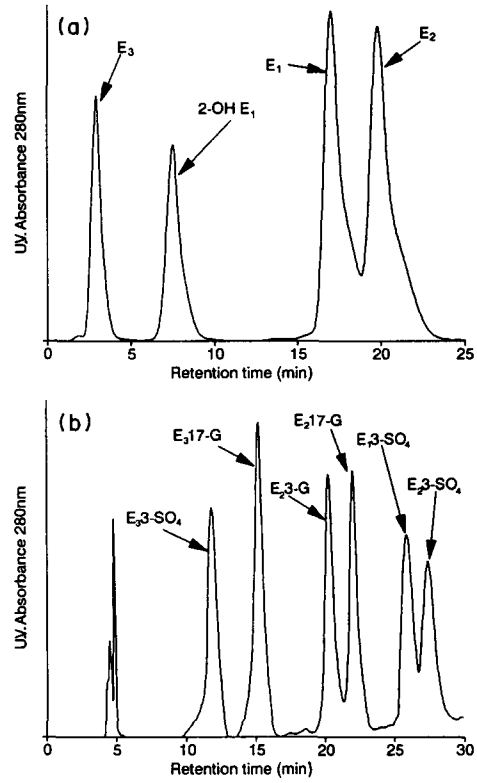


Fig. 1. U.V. chromatogram illustrating retention times of unconjugated estrogens (a) and conjugated estrogens (b).

*Data analysis*

Results are expressed as the percentage of total steroid added to the incubation which was recovered from the medium as parent steroid and metabolites or was bound to the cells (Tables 1–5). The data were analysed using a one-way analysis of variance. Significance levels were obtained using a modified *t*-test (Bodeferroni). Results are presented as mean ± standard deviation.

## RESULTS

*$E_2$ and $E_1$ metabolism by cell lines*

It should be noted that approximately equal numbers of cells ($3 \times 10^6$) were used in incubations irrespective of the cell line. The protein content of the cells was previously found to be: 0.25 mg/$10^6$ cells for Huma 7 and 0.5 mg/$10^6$ for MCF-7 and ZR-75-1.

Chromatograms (u.v.) showing the separation of a mixture of unconjugated and conjugated estrogens are shown in Fig. 1(a) and (b), respectively. Radiochromatograms illustrating the metabolism of the endogenous estrogens $E_2$ and $E_1$ by the MCF-7 human breast cancer cell line are presented in Figs 2 and 3, respectively. Phase I metabolism refers to the formation of metabolites that were extractable into diethyl ether, while phase II metabolism refers to the formation of non-ether-extractable, water-soluble metabolites. It can be seen that there is conversion of $E_2$ to $E_1$ [Fig. 2(a)] and the formation of predominantly $E_2$-3-sulphate with smaller amounts of $E_1$-3-sulphate [Fig. 2(b)]. An additional metabolite with a retention time of 30 min was present.

Tables 1 and 2 summarize the respective metabolism of $E_2$ and $E_1$ by the two human breast cancer cell lines MCF-7 and ZR-75-1 and the normal breast cell line, Huma 7. All the cell lines were able to interconvert $E_2$ and $E_1$. The data suggests that this interconversion favoured the formation of $E_2$ in the two cancer cell lines, whilst favouring $E_1$ formation in the normal breast cell line. There were quantitative and qualitative differences in the phase II metabolism of $E_2$ and $E_1$ between normal and cancer cell lines. The formation of estrogen sulphates was greater at 48 than 24 h in MCF-7 and ZR-75-1 cells while in Huma 7 cells sulphate formation was approximately the same at 24 and 48 h. A very much greater percentage of the

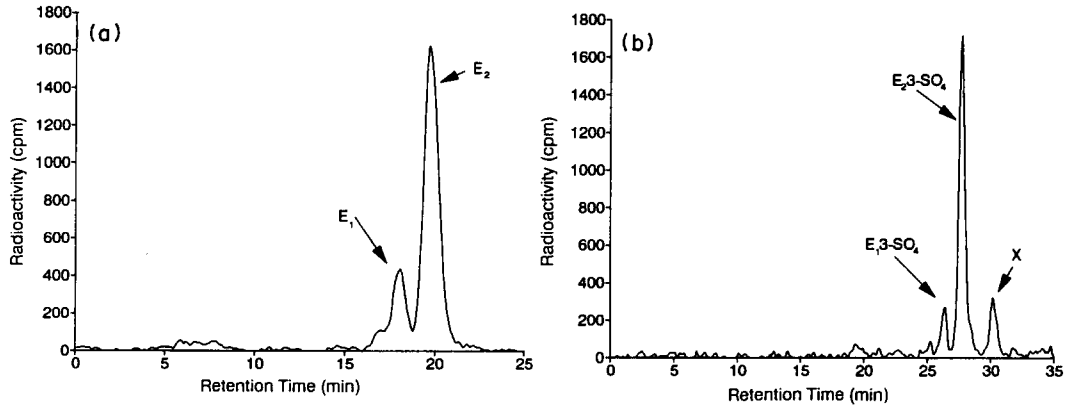


Fig. 2. Radiochromatogram showing phase I (a) and phase II (b) metabolism of $E_2$ by MCF-7 human breast cancer cell line.

total radioactive steroid added was converted to estrogen sulphates by Huma 7 cells (~60% after 24 h) than by MCF-7 and ZR-75-1 cells (~5–7% after 24 h). This difference was also reflected by the very much smaller percentage recovery of unconjugated estrogen in the Huma 7 cell line than in the cancer cell lines. In addition, unlike MCF-7 and ZR-75-1 cells, Huma 7 cells did not form the metabolite present at retention time 30 min.

*Metabolism of $EE_2$ by human breast cell lines*

Radiochromatograms illustrating the phase I and phase II metabolism of $EE_2$ by MCF-7 human breast cancer cells are illustrated in Fig. 4. It is apparent that no phase I metabolism of $EE_2$ occurs [Fig. 4(a)]. Using the system for phase II metabolite analysis a single sharp radioactive peak was observed at 29 min [Fig. 4(b)]. This peak vanished when the ether-extracted culture medium was incubated with sulphatase enzyme (Helix pomatia Type 2) prior to the isolation and analysis of the conjugate. This suggested that the single radioactive peak corresponded to a sulphate conjugate, most probably $EE_2$-3-sulphate.

The metabolism of $EE_2$ by human breast cancer lines (MCF-7 and ZR-75-1) and normal breast cell line (Huma 7) is summarized in Table 3. No phase I metabolism was detected in any of the cell lines. Over the time points studied $EE_2$-3-sulphate formation was dependent on time of exposure to radioactive precursor in the cancer cells but was the same at 24 and 48 h in the normal cells. As shown for $E_1$ and $E_2$, the percentage formation of $EE_2$ sulphate was very much greater (64.1 ± 9.6% after 24 h) in Huma 7 cells than in MCF-7 and ZR-75-1 cells (7.4 ± 5.3% and 10.6 ± 4.1%, respectively after 24 h). Again, this difference was reflected in the corresponding percentage recovery of unconjugated estrogen, Huma 7 (22.8 ± 11.2%), MCF-7 (53.9 ± 8.8%) and ZR-75-1 (52.3 ± 4.9%).

*Metabolism of norgestimate by human breast cell lines*

U.V. and radiochromatograms illustrating the phase I metabolism of the novel progestogen

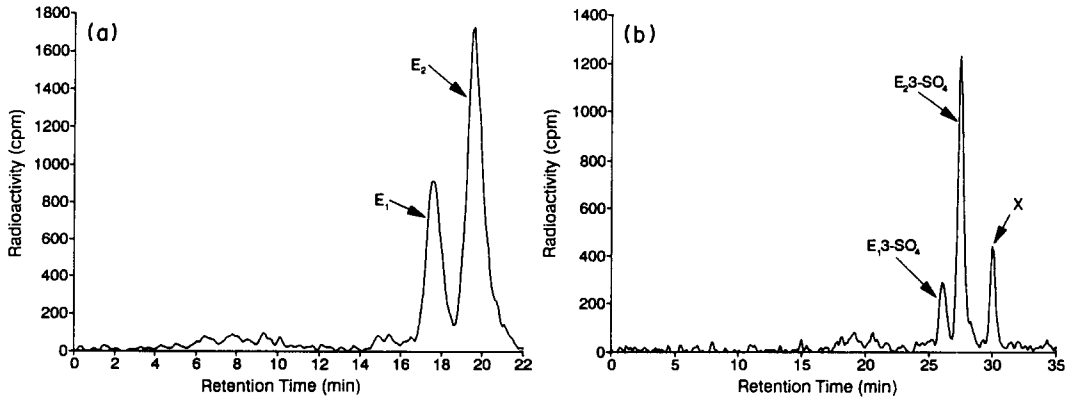


Fig. 3. Radiochromatogram showing phase I (a) and phase II (b) metabolism of $E_1$ by MCF-7 human breast cancer cell line.

Table 1. Metabolism of $E_2$ (0.2 $\mu$Ci; final concentration 1 nM) by MCF-7, ZR-75-1 and Huma 7 human breast cell lines

| Cell line | Incubation time (h) | Parent steroid $E_2$ | Phase I $E_1$ | $E_1$-3-$SO_4$ | Phase II $E_2$-3-$SO_4$ | x | Cell-bound steroid |
|---|---|---|---|---|---|---|---|
| MCF-7 | 24 | 48.0 ± 11.6 | 7.8 ± 2.1 | 0.2 ± 0.2 | 5.5 ± 0.9 | 1.5 ± 1.8 | 29.0 ± 12.4 |
| | 48 | 42.3 ± 7.0 | 7.9 ± 2.4 | 0.7 ± 1.0 | 12.8 ± 3.2 | 4.5 ± 3.6 | 23.9 ± 5.7 |
| ZR-75-1 | 24 | 41.0 ± 3.9 | 11.3 ± 1.2 | 0.2 ± 0.3 | 5.6 ± 3.3 | 1.6 ± 0.8 | 31.1 ± 8.5 |
| | 48 | 37.1 ± 7.2 | 14.0 ± 2.6[a] | 0.7 ± 0.6 | 12.7 ± 3.5 | 5.4 ± 0.3 | 23.1 ± 14.0 |
| Huma 7 | 24 | 10.4 ± 3.0[c,d] | 8.2 ± 2.3 | 16.1 ± 6.7[c,d] | 45.1 ± 3.5[e,f] | — | 15.1 ± 5.0 |
| | 48 | 8.9 ± 1.9[e,f] | 4.8 ± 1.0[d] | 18.6 ± 1.1[e,f] | 47.7 ± 1.2[e,f] | — | 15.6 ± 2.2 |

Results (mean ± SD; $n = 3$) are expressed as a percentage of total radioactive steroid added, x refers to the metabolite present at a retention time of 30 min.
[a]Significantly different from MCF-7, $P < 0.05$.
[b]Significantly different from ZR-75-1, $P < 0.05$.
[c]Significantly different from MCF-7, $P < 0.01$.
[d]Significantly different from ZR-75-1, $P < 0.01$.
[e]Significantly different from MCF-7, $P < 0.001$.
[f]Significantly different from ZR-75-1, $P < 0.001$. Statistical analysis was performed only at equivalent times.

norgestimate are illustrated in Fig. 5. Chromatographic separation of authentic standards of norgestimate, 3-keto norgestimate, 17-deacetyl norgestimate and norgestrel are shown in Fig. 5(a). Figure 5(b) is a typical radiochromatogram illustrating the phase I metabolism of norgestimate by MCF-7 human breast cancer cells. It is clear that norgestimate has been completely metabolized to 17-deacetyl norgestimate and norgestrel.

The metabolism of norgestimate by human breast cancer cell lines MCF-7 and ZR-75-1 and by a normal breast cell line (Huma 7), are summarized in Table 4. Metabolism of norgestimate by all cell lines was complete within 24 h. There was very little difference in the percentage of recovered metabolites after 24 and 48 h. Huma 7 cells formed a smaller percentage of 17-deacetyl norgestimate but a greater percentage of norgestrel than the MCF-7 and ZR-75-1 cells. A considerable percentage of the total added radioactivity (14.9 ± 3.9% after 24 h) was not extracted into diethyl ether from the culture medium of Huma 7 cells. This non-extractable steroid may represent conjugated metabolite(s), the identity of which is unclear at present.

### *Metabolism of $E_1$-3-sulphate by human breast cell lines*

The metabolism of $E_1$-3-sulphate by the human breast cancer cell lines MCF-7 and ZR-75-1 and normal breast cell line (Huma 7) is summarized in Table 5. It is clear that both normal and cancer cells were able to hydrolyse $E_1$-3-sulphate. The radioactive peaks detected co-chromatographed with authentic $E_1$ and $E_2$ standards. The formation of unconjugated estrogen was dependent on time of exposure to the conjugated estrogen in all cell lines over the time points studied. Normal and cancer cells did not differ in the extent to which they hydrolysed $E_1$-3-sulphate.

## DISCUSSION

The initial studies with $E_1$, $E_2$ and $E_1$-3-sulphate demonstrated that under the experimental conditions used the cell cultures exhibited metabolic activity. Consistent with the findings of Adams *et al.* [10], $E_2$ was metabolized to $E_1$, $E_2$-3-sulphate and $E_1$-3-sulphate in MCF-7 and ZR-75-1 cells. However, we were unable to demonstrate the presence of the glucuronide conjugates reported by Adams *et al.* [11] in MCF-7, ZR-75-1 and T47-D cells. Since our isolation procedure extracts effectively both sulphate and glucuronide conjugates, and the HPLC system readily separates most estrogen monosulphates, monoglucuronides and mixed conjugates, this metabolic difference between the two studies may reflect differences in the strains of MCF-7 and ZR-75-1 cells used or in the conditions of tissue cultures.

Table 2. Metabolism of $E_1$ (0.2 $\mu$Ci; final concentration 1 nM) by MCF-7, ZR-75-1 and Huma 7 human breast cell lines

| Cell line | Incubation time (h) | Parent steroid $E_1$ | Phase I $E_2$ | $E_1$-3-$SO_4$ | Phase II $E_2$-3-$SO_4$ | x | Cell-bound steroid |
|---|---|---|---|---|---|---|---|
| MCF-7 | 24 | 20.5 ± 3.0 | 39.3 ± 5.5 | 1.3 ± 0.2 | 3.5 ± 1.4 | 0.9 ± 0.5 | 25.7 ± 2.3[b] |
| | 48 | 11.4 ± 5.7 | 44.1 ± 2.4 | 2.0 ± 0.7 | 8.4 ± 2.0 | 2.8 ± 1.5 | 23.5 ± 7.3 |
| ZR-75-1 | 24 | 27.2 ± 5.6 | 23.6 ± 6.1[c] | 2.1 ± 1.8 | 2.8 ± 0.8 | 0.6 ± 0.6 | 34.0 ± 2.0 |
| | 48 | 18.5 ± 2.9 | 29.2 ± 3.7[c] | 2.8 ± 1.4 | 7.2 ± 0.3 | 4.0 ± 0.4 | 30.5 ± 8.6 |
| Huma 7 | 24 | 20.6 ± 5.0 | 9.6 ± 3.0[b,c] | 44.8 ± 3.6[e,f] | 11.7 ± 3.1[c,d] | — | 7.6 ± 4.8[e,f] |
| | 48 | 16.5 ± 2.8 | 4.4 ± 1.8[e,f] | 48.3 ± 2.5[e,f] | 14.8 ± 4.1[a,b] | — | 10.3 ± 5.4[b] |

Results (mean ± SD; $n = 3$) are expressed as a percentage of total radioactive steroid added, x refers to the metabolite present at a retention time of 30 min. For significance values [a-f] refer to legend of Table 1.

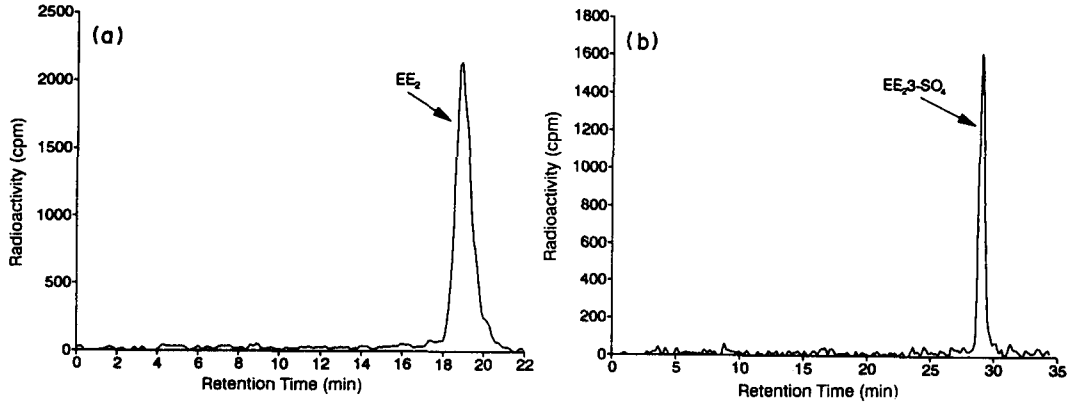


Fig. 4. Radiochromatogram showing phase I (a) and phase II (b) metabolism of $EE_2$ by MCF-7 human breast cancer cell line.

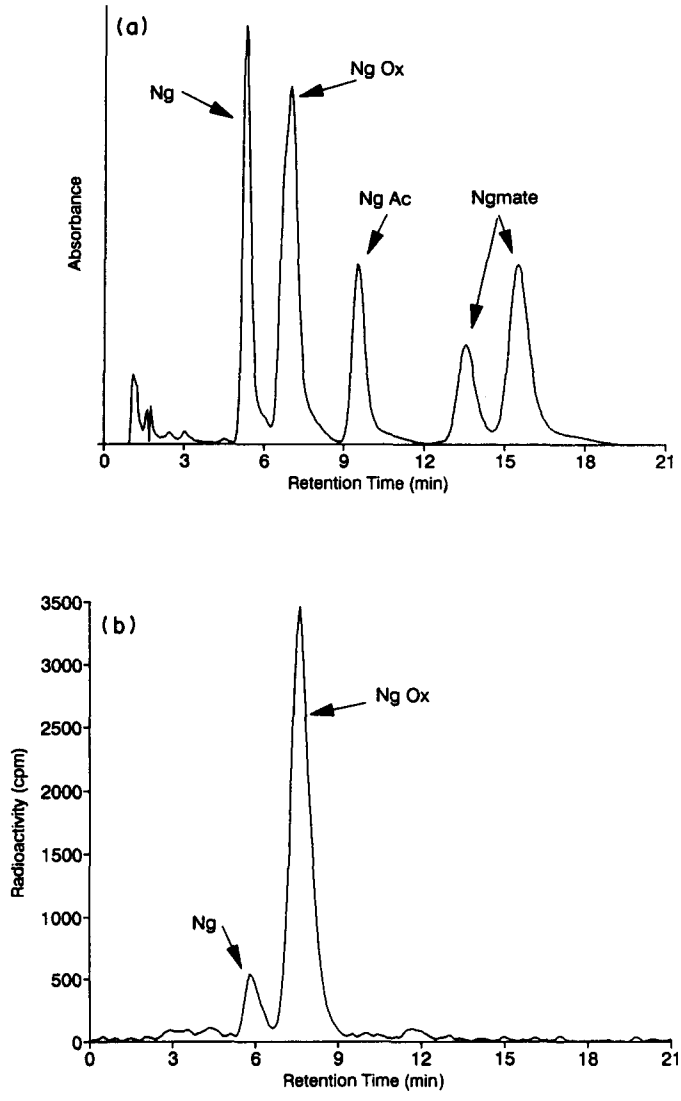


Fig. 5. (a) U.V. chromatogram illustrating retention times of authentic norgestimate and metabolites. Norgestimate is a racemate, hence two peaks, and (b) radiochromatogram showing metabolism of norgestimate by MCF-7 human breast cancer cell line.

Table 3. Metabolism of $EE_2$ (0.2 $\mu$Ci; final concentration 1 nM) by MCF-7, ZR-75-1 and Huma 7 human breast cell lines

| Cell line | Incubation time (h) | Parent steroid $EE_2$ | Phase I | Phase II $EE_2$-3-$SO_4$? | Cell-bound steroid |
|---|---|---|---|---|---|
| MCF-7 | 24 | 53.9 ± 8.8 | — | 7.4 ± 5.3 | 31.2 ± 10.7 |
| | 48 | 48.6 ± 4.5 | — | 14.5 ± 3.5 | 31.5 ± 7.0 |
| ZR-75-1 | 24 | 52.3 ± 4.9 | — | 10.6 ± 4.1 | 29.9 ± 11.3 |
| | 48 | 47.9 ± 4.9 | — | 24.3 ± 9.0 | 23.2 ± 14.4 |
| Huma 7 | 24 | 22.8 ± 11.2[c,d] | — | 64.1 ± 9.6[e,f] | 8.6 ± 3.7[a,b] |
| | 48 | 16.9 ± 11.1[c,d] | — | 66.8 ± 10.3[e,f] | 11.9 ± 5.0 |

Results (mean ± SD; $n = 3$) are expressed as a percentage of total radioactive steroid added. For significance values [a–f] refer to legend of Table 1.

Table 4. Metabolism of norgestimate (0.2 $\mu$Ci; final concentration 1 nM) by MCF-7, ZR-75-1 and Huma 7 human breast cell lines

| Cell line | Incubation time (h) | Parent steroid Ngmate | Phase I | | Phase II | Cell-bound steroid |
|---|---|---|---|---|---|---|
| | | | Ng | NgOx | | |
| MCF-7 | 24 | — | 9.0 ± 0.1 | 69.1 ± 4.4 | — | 19.1 ± 4.7 |
| | 48 | — | 11.6 ± 3.5 | 58.8 ± 2.3 | — | 25.8 ± 1.0 |
| ZR-75-1 | 24 | — | 8.0 ± 3.1 | 67.3 ± 9.3 | — | 22.4 ± 8.1 |
| | 48 | — | 10.6 ± 2.5 | 60.0 ± 3.3 | — | 27.0 ± 1.6 |
| Huma 7 | 24 | — | 22.1 ± 9.1[a,b] | 51.2 ± 4.7[a,b] | 14.9 ± 3.9 | 8.6 ± 0.9[b] |
| | 48 | — | 23.0 ± 3.1[c,d] | 42.0 ± 2.4[e,f] | 17.9 ± 3.2 | 14.2 ± 2.0[e,f] |

Results (mean ± SD; $n = 3$) are expressed as a percentage of total radioactive steroid added. For significance values [a–f] refer to legend of Table 1. Ng, norgestrel; NgOx, 17-deacetyl norgestimate.

In addition, we were unable to detect any hydroxylated estrogens and hence any $E_2$ hydroxylase activity in contrast to the studies of Niwa *et al.* [12, 13] who showed that $E_2$ was converted to the 2- and 16-hydroxy-metabolites in MCF-7 cells. However in order to demonstrate this they developed a radiometric assay based on the release of tritium from regiospecifically labelled estrogens [17] that is more sensitive than our HPLC system.

Although the cell lines Huma 7 and MCF-7/ZR-75-1 were isolated by immortalizing cultures of normal and malignant breast epithelial cells with simian virus-40 and spontaneously, respectively, nevertheless they retain many of the characteristics of the majority of the epithelial cells in the original cultures apart from their immortality [18, 19] and as such can be taken to be reasonable representatives of normal and malignant human breast epithelial cells [20]. In the present work, the normal breast cell line Huma 7 also interconverts $E_2$ and $E_1$ and forms $E_2$ and $E_1$-3-sulphates. However unlike MCF-7 and ZR-75-1 cells, the Huma 7 cell line did not form the additional metabolite at retention time 30 min. Since multiple forms of sulphotransferase exist [21] it may be that this is a sulphated estrogen and that different isozymes are involved in $C_3$ and $C_{17}$ conjugation, with the Huma 7 cells being deficient in certain isozyme(s).

It is apparent from Tables 1 and 2 that the ratio of $E_1$ to $E_2$ observed in the normal cell line Huma 7, was greater than that seen in the two cancer cell lines. Similarly, the ratio of $E_1$-3-sulphate to $E_2$-3-sulphate was greater in Huma 7 cells. Thus the interconversion of $E_1$ and $E_2$ favours the formation of $E_2$ in MCF-7 and ZR-75-1 cells whilst favouring $E_1$ formation in Huma 7 cells. This is consistent with results *in vivo* which have shown that endogenous concentrations of $E_2$ in human breast tissue from pre- and postmenopausal women are significantly higher in malignant tissues than in non-malignant tissues [22], and the ratio $E_2/E_1$ is significantly higher in breast cancer tissue than

Table 5. Metabolism of $E_1$-3-sulphate (0.2 $\mu$Ci; final concentration 1 nM) by MCF-7, ZR-75-1 and Huma 7 human breast cell lines

| Cell line | Incubation time (h) | Parent steroid $E_1$-3-$SO_4$ | Phase I | | Cell-bound steroid |
|---|---|---|---|---|---|
| | | | $E_1$ | $E_2$ | |
| MCF-7 | 24 | 50.2 ± 2.6 | 6.5 ± 0.9 | 6.9 ± 0.3 | 10.6 ± 1.0 |
| | 48 | 27.1 ± 1.7 | 11.5 ± 4.3 | 12.5 ± 1.5 | 20.4 ± 3.2 |
| ZR-75-1 | 24 | 55.1 ± 3.8 | 7.9 ± 0.4 | 5.4 ± 1.0 | 9.1 ± 0.4 |
| | 48 | 37.5 ± 2.1[a] | 15.2 ± 2.0 | 7.2 ± 0.9[c] | 16.7 ± 1.3 |
| Huma 7 | 24 | 43.4 ± 2.1[a,d] | 10.9 ± 3.0 | 3.5 ± 2.0 | 17.2 ± 4.1[a,d] |
| | 48 | 31.1 ± 2.9[b] | 21.0 ± 0.8[b,c] | 4.1 ± 1.7[b,c] | 18.2 ± 4.7 |

Results (mean ± SD; $n = 3$) are expressed as a percentage of total radioactive steroid added. For significance values [a–f] refer to legend of Table 1.

in normal tissue following an infusion of [$^3$H]$E_2$ [23]. 17$\beta$-Hydroxysteroid dehydrogenase (17$\beta$-HSDH) is the enzyme that catalyses the interconversion of $E_1$ and $E_2$. 17$\beta$-HSDH exists in multiple forms [24] in human breast tissue with the isozymes differing in their substrate affinities and co-factor requirements. Significant differences in co-factor concentrations between malignant and normal breast tissue have been found and this result is thought to account in part for the observed elevated $E_2$ (biologically more active than $E_1$) concentrations in malignant as opposed to normal tissue [25].

The metabolism of the OCS $EE_2$ has been fairly extensively studied both *in vivo* and *in vitro* [2, 4, 26–29] with the identification of hydroxylated metabolites and conjugates (methyl, glucoronic acid and sulphate derivatives) in urine and/or following incubations with human liver. In this, the first report of metabolism of $EE_2$ in a target organ, the breast, the presence of a single, water-soluble metabolite from both cancer (MCF-7 and ZR-75-1) and normal (Huma 7) cells has been demonstrated. Hydrolysis of the metabolite by the enzyme arylsulphatase indicated that it was a sulphate, most probably $EE_2$-3-sulphate. This metabolite awaits definitive characterization.

Norgestimate is a relatively new synthetic progestogen. The possibility has been raised, principally on the basis of studies carried out in rhesus monkeys [30], that metabolites of norgestimate rather than norgestimate itself may bind to the progestogen receptor. Sisenwine *et al.* [30] concluded that norgestimate was a pro-drug which is rapidly deacetylated to form 17-deacetyl norgestimate and that this metabolite can subsequently be hydrolyzed to norgestrel which may than undergo further metabolism. In one study in women [31] the urinary metabolites of norgestimate were monitored following administration of [$^{14}$C]norgestimate. Metabolites identified included norgestrel, tetrahydronorgestrel and various hydroxylated metabolites. Madden and Back [32] have recently demonstrated than norgestimate is rapidly deacetylated by both human gastrointestinal mucosa and liver and that the deacetylated metabolite can then be metabolized further. In the present study we have shown that norgestimate is completely metabolized by all three cell lines from the human breast within 24 h. The main metabolite identified was deacetylated norgestimate (norgestrel oxime) although norgestrel was also present. The presence of esterases in MCF-7 cells have been shown, previously [9] but the complete hydrolysis of norgestimate in this study is most striking. The ratio of norgestrel to norgestrel oxime in Huma 7 cells was greater than that observed in MCF-7 and ZR-75-1 and the Huma 7 cells were also able to form an appreciable percentage of an as yet unidentified water-soluble metabolite.

An examination of all the estrogen ($E_1$, $E_2$ and $EE_2$) data presented herein reveals that incubation of the steroids with Huma 7 cells yields a higher percentage of sulphate conjugates than after incubation with MCF-7 or ZR-75-1 cells. The formation of estrogen sulphates is dependent on the equilibrium between sulphate conjugation catalyzed by estrogen sulphotransferase and their hydrolysis back to the parent estrogen by sulphatase [33]. The observed increase in the formation of estrogen sulphates in Huma 7 cells could therefore be explained by either increased sulphotransferase activity, decreased sulphatase activity or a combination of both factors. Estrogen sulphatase activity has been shown to be considerably lower in normal breast tissue compared to malignant breast tissue [34, 35]. However, the data generated with $E_1$ sulphate (Table 5) suggests little difference in hydrolytic activity between normal (Huma 7) and cancer (MCF-7; ZR-75-1) cells. Estrogen sulphates are thought to be incapable of binding to the estrogen receptor and therefore to have no direct biological effect. The increased formation of inactive estrogen sulphate in normal breast cells (Huma 7) is suggestive of an increased clearance in normal versus malignant breast cells.

In conclusion, we have shown that the two OCS $EE_2$ and norgestimate are metabolized by normal and malignant cultured human breast cells. Further work to examine metabolism by normal and malignant human breast tissue is currently being undertaken. In addition, there are both qualitative and quantitative differences in steroid metabolism between normal and malignant breast cells in culture.

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