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Determination of 4-hydroxyandrost-4-ene-3,17-dione metabolism in breast cancer patients using high-performance liquid chromatography–mass spectrometry

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ABSTRACT

A sensitive procedure for studying the metabolism of the steroidal aromatase inhibitor 4-hydroxyandrost-4-ene-3,17-dione (4OHA) was developed based on enzyme hydrolysis, liquid–liquid extraction and reversed-phase liquid chromatography coupled with a mass spectrometer (LC–MS) using a thermospray interface. Seven metabolites were identified from the hydrolysed urine samples together with the parent drug. The major routes of metabolism were via dehydrogenation, reduction of the ketone functional groups, reduction at the C-4–C-5 double bond and hydroxylation at the C-5 position. Confirmation of the identity of 4OHA and its metabolites isolated from female patients' urine samples was accomplished by comparison of the retention times of their corresponding synthetic standards on LC–MS. We have demonstrated that this technique is particularly suitable for studying the metabolism of steroidal drugs.

INTRODUCTION

One of the approaches of chemotherapy in the treatment of breast cancer in postmenopausal patients is to suppress their estrogen production in the body. The major pathway of estrogen production in postmenopausal women is the conversion of androstenedione and testosterone into estrone and estradiol, respectively, by the action of the enzyme aromatase. Drugs which are capable of blocking this biosynthetic step (generally known as aromatase inhibitors) have been found to be effective in the treatment of breast cancer.

Aminoglutethimide (Fig. 1) is an existing non-steroidal drug, which is >95% effective in inhibiting the aromatisation of androstenedione to estrone [1]. However this drug also inhibits the cholesterol side-chain cleavage enzyme system [2]

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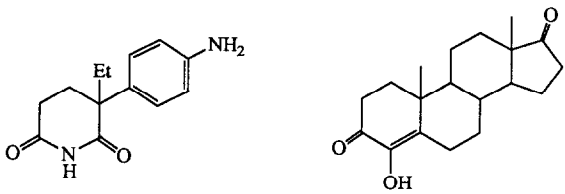


Aminoglutethimide 4-Hydroxyandrostenedione

Fig. 1. Structures of aminoglutethimide and 4-hydroxyandrostenedione (4OHA).

and moreover it can cause unpleasant side-effects in patients receiving the treatment [3]. Hence it has been necessary to search for a more selective aromatase inhibitor. Several such candidates are now available and are undergoing Phase I/II clinical trials [4–7]. One of the candidates, 4-hydroxyandrost-4-ene-17-dione (4OHA, Fig. 1) [8] has been shown in studies to be a more potent aromatase inhibitor than aminoglutethimide both *in vitro* and *in vivo* [9,10]. When radioactive 4OHA was administered to male rhesus monkeys [11], only 6–7% of the total radioactivity in blood samples was present in the form of the free drug and its metabolite, 4-hydroxytestosterone (E, 4OHT). *In vivo* metabolism by female rats gave 3α-hydroxy-5β-androstan-4,17-dione (C) as the major free metabolite (~ 18%), with 4OHT and 4OHA in minor concentrations (1.4 and 0.5%, respectively) [12], whereas an *in vitro* study demonstrated that ~ 20% of the 4OHA had been converted into 4OHT [12]. Foster *et al.* [13] detected several new metabolites after incubating 4-[^{14}C]OHA with rat hepatocytes. Hydroxylated, reduced and conjugated metabolites were found, but no 4OHT was present.

Even though 4OHA has been on clinical trial and for several years in treating patients with estrogen-dependent breast cancer [14], no detailed studies of its metabolism in patients have been carried out. Goss *et al.* [15] have identified the 4OHA glucuronide conjugate as the principal metabolite in the patients' urine samples after they have been given 4OHA, by oral administration, but this metabolite accounted for only 20–45% of the administered dose. In the past, 4OHA analysis comprised of chromatographic isolation of the steroid from plasma, followed by radioimmunoassay (RIA) [16–18], electron-impact mass spectometry (EI-MS) [13] or measurement by direct gas chromatography–mass spectrometry (GC–MS) [15,19]. These methods have several drawbacks. GC–MS required sample derivatization, RIA lacks total specificity and in the case of 4OHA, some cross-reactivity with 4OHT and androstenedione occurs [16].

High-performance liquid chromatography (HPLC) with on-line coupling to MS using a thermospray interface (LC–TSP–MS) is an invaluable tool for the identification of drugs and their metabolites in biological fluid [20–22]. This technique offers the potential for direct determination of polar, non-volatile and thermally unstable molecules, it avoids isolating pure samples for the analysis and complements mixture analyses that are characterised by incomplete HPLC separations. The combination of chromatographic separation and tandem mass

spectrometry (MS–MS) not only provides molecular weight information for the unknown compounds, but also allows unequivocal identification of products in urine or plasma extracts of patients. Recently we have successfully employed this technique to study the metabolism of 4OHA in prostatic cancer patients [23]. Now we have utilized this method to study the metabolism of 4OHA in breast cancer patients.

EXPERIMENTAL

Chemicals

4OHA was provided by Ciba-Geigy Pharmaceuticals (Horsham, U.K.). Reference compounds and metabolites were synthesized as previously described [12,13,23]. β-Glucuronidase was obtained from Boehringer Mannheim (East Sussex, U.K.). All solvents and reagents were analytical or HPLC grade.

Isolation of metabolites

Three patients were given a single oral dose of 4OHA (500 mg). Their urine samples were collected 4 h after 4OHA treatment and were kept frozen at −20°C until analysis. Each sample (0.5 ml) was extracted by ethyl acetate (2 x 1 ml). The organic phase was concentrated under a stream of nitrogen. The aqueous phase was hydrolysed by β-glucuronidase (50 μl) at 37°C for 22 h. At the end of the hydrolysis, ethyl acetate (3 x 1 ml) was used to extract the sample. The organic phases were combined and concentrated under nitrogen. The residues from organic fractions were redissolved in 40 μl of methanol. Aliquots (1 and 20 μl) were analysed by HPLC or HPLC–MS.

HPLC analysis

The HPLC system used was manufactured by Applied Chromatography System (HPLC Technology, Cheshire, U.K.) consisting of a single pump (Model 350/04), a gradient controller (Model 351/04) and a variable-wavelength detector (Model 702/12). Separation was achieved using a 150 mm x 4.6 mm I.D. Spherisorb 3ODS2 column (Jones Chromatography, Mid Glamorgan, U.K.). The mobile phase was composed of methanol–water (containing 0.01% trifluoroacetic acid, v/v) at a flow-rate of 1.0 ml/min isocratic. UV detection was at 254 nm.

LC–MS analysis

The HPLC system used was a Waters 600MS solvent delivery module, with a Waters 484 variable-wavelength UV detector (Millipore Assoc., Milford, MA, U.S.A.). The column used was the same as described above. The mass spectrometer employed was a triple-stage quadrupole (TSQ 70 Series) manufactured by Finnigan MAT (San Jose, CA, U.S.A.), fitted with a Finnigan TSP II interface. The ion source was operated in the "discharge on" positive-ion mode and the discharge voltage was set to 1200 V. The ion source block temperature was set at

250°C and the vaporiser temperature at 105°C. Mass spectra were scanned from m/z 170 to 350 by the first quadrupole (Q_1) at a scanning speed of 2 s/scan. MS–MS was effected by collision activations in the second quadrupole (Q_2) using argon as collision gas at a pressure of 0.6–0.8 mTorr and a collision energy of 20 eV. Daughter ion spectra were acquired in the third quadrupole (Q_3). The system was set up such that it could simultaneously monitor 1 s in full scan mode and 2 s at collision-induced dissociation (CID) mode during the chromatographic separation.

Aromatase assay

The metabolites of 4OHA were assayed for inhibitory acitivity against the human placental microsomal aromatase enzyme using the procedure described previously [24], with [1,2-^{3}H]testosterone as the substrate. Compounds were screened at final concentrations of 0.5, 1, 2, 5 and 10 μM. The IC_{50} value is the concentration of inhibitor required to reduce the activity of the enzyme to 50% of the control value.

RESULTS AND DISCUSSION

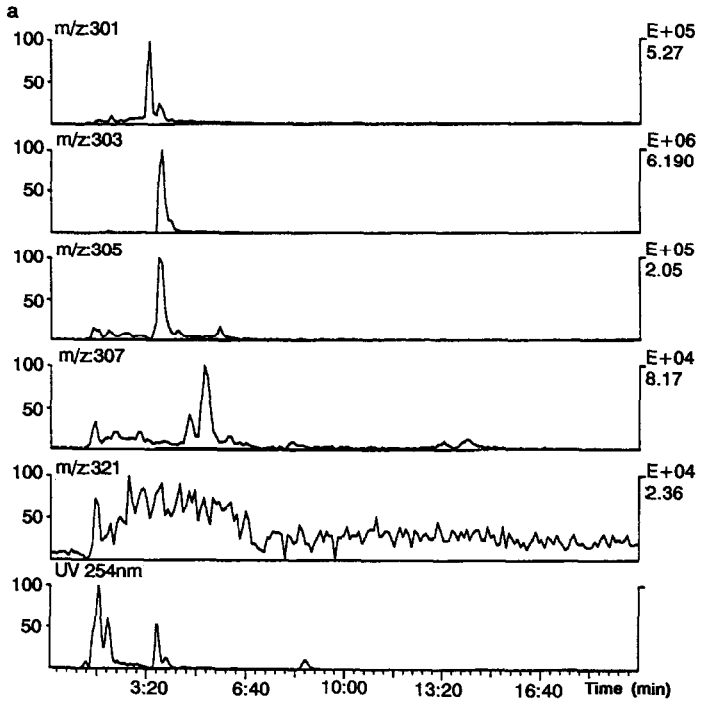
Based on previous studies [11–13,23], steroidal metabolism of 4OHA is mostly to 3α-hydroxy-5β-androstan-4,17-dione (C), 4OHT (E) and their corresponding hydroxylated products. In order to check for their presence, the $[M+H]^+$ ions of each expected metabolite (m/z 300, 303, 305, 307 and 321) were used in the extracted ion monitoring (SIM) mode.

Phase I metabolism

The SIM and HPLC traces of an unhydrolysed sample of patient's urine are shown in Fig. 2a. None of the parent drug nor its metabolites was detectable in the free form, indicating the lack of Phase I metabolites. This is not surprising as the drug was administered orally, and therefore conjugation becomes a major metabolic pathway in the elimination process forming mostly Phase II metabolites. However around 3:20 and 3:40 min, m/z 300, 303 and 307 were detected, their early elution indicating that they must be conjugated metabolites, which decomposed under the thermospray condition to give the steroid moiety (Fig. 2b–d). The exact nature of these components has yet to be determined by means of continuous-flow fast atom bombardment mass spectrometry, a technique more appropriate for analysing conjugated metabolites [25].

Phase II metabolism

The reconstructed ion chromatogram (RIC), HPLC trace and SIM trace of the urine extract after it had been treated with β-glucuronidase is shown in Fig. 3a and b. Several metabolites were identified as summarized in Fig. 4. The parent drug, $[M+H]^+$ of m/z 303 (peak B, see Fig. 6a), was present in high concentra-



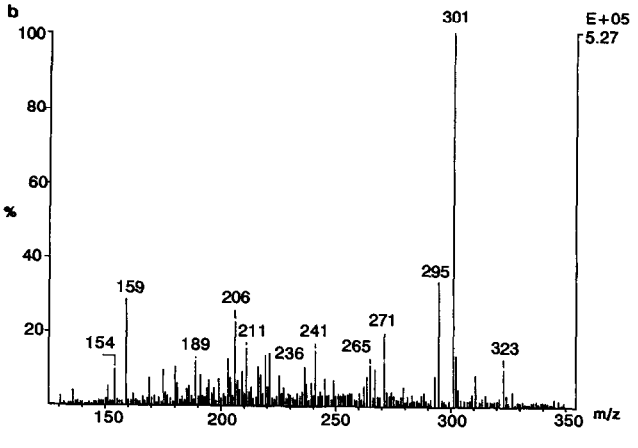


Fig. 2.

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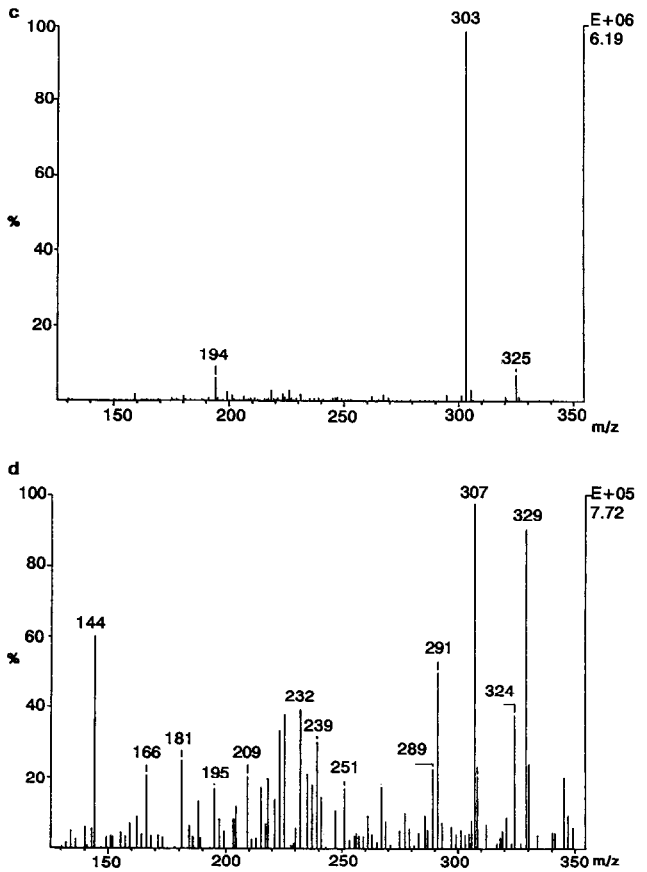

Fig. 2. (a) Reconstructed ion chromatogram of 4OHA metabolites and HPLC trace of patients' urine sample on LC–TSP–MS after ethyl acetate extraction. (b–d) LC–TSP–MS spectra indicating that polar and conjugated 4OHA metabolites were present in this fraction of urine extract.

tion. Eluting very close to compound (B) is a metabolite with *m/z* 301 (peak A, see Fig. 6b). MS–MS analysis of this component gave a daughter spectrum as shown in Fig. 5a. The CID daughter ion mass spectra were obtained for the authentic 4-hydroxyandrost-1,4-diene-3,17-dione (4OHA-1,4-diene) and 4-hydroxyandrost-4,6-diene-3,17-dione (4OHA-4,6-diene, A) under the described experimental conditions. For 4OHA-1,4-diene (Fig. 5b), the CID daughter ion mass spectrum consisted of fragments at *m/z* 283, 151 and 137. We assume the fragment ion at *m/z* 283 corresponds to loss of H_2O from MH^+. Assuming protonation at the 3-carbonyl or 4-hydroxyl position, the ions at *m/z* 151 and 137 can be explained by the cleavage of ring B at C-7–C-8, C-9–C-10 and C-6–C-7, C-9–C10 positions, respectively. The CID daughter ion mass spectrum for compound A (Fig. 5c) has one dominating fragment ion at *m/z* 149, resulting from the cleavage of the ring B between C-7–C-8 and C-9–C-10. The close resemblance of the CID daughter ion

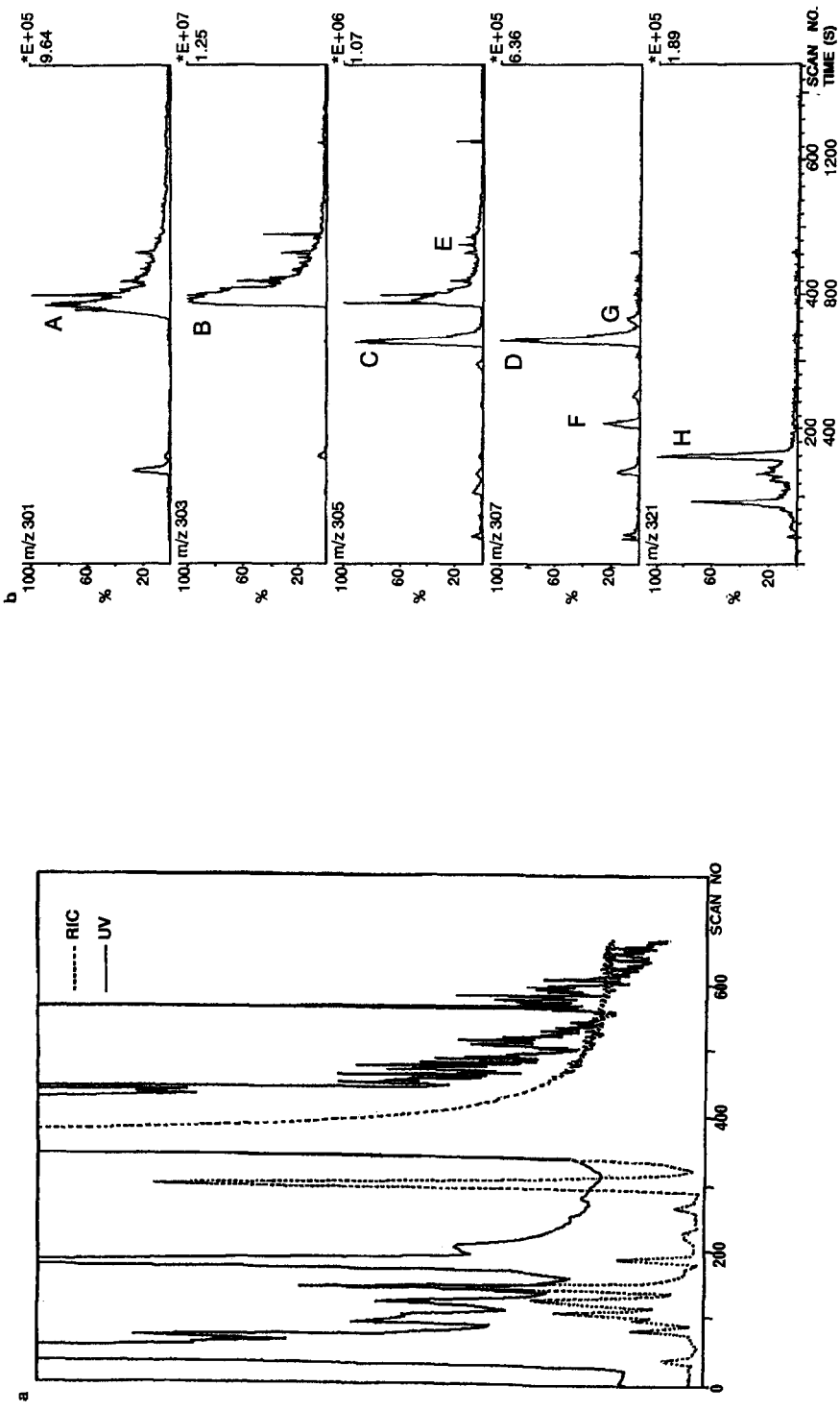

Fig. 3. (a) RIC trace and UV chromatogram of 4OHA-treated patient's urine extract after enzyme hydrolysis. (b) Extracted ion chromatograms of 4OHA metabolites (A–F). Unlabelled peaks represent endogenous materials.

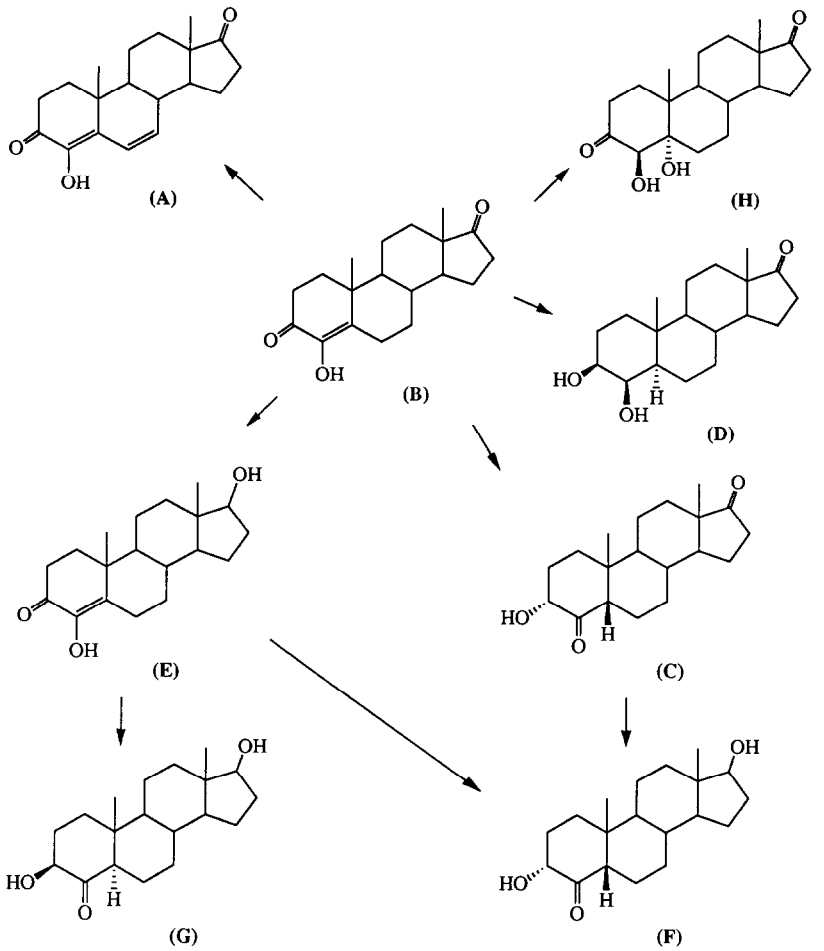

Fig. 4. Proposed scheme for metabolism of 4OHA metabolites showing the $[M+H]^+$ ions.

spectrum and identical elution profile on the HPLC provide strong evidence that metabolite A was 4OHA-4,6-diene. Such a metabolite has been reported before, Nagata *et al.* [26] found 17β-hydroxy-4,6-androstadien-3-one to be a metabolite of testosterone. They suggested that cytochrome P-450 isozymes in the liver microsomes are responsible for the dehydrogenation of testosterone to form a double bond between the C-6 and C-7 position. A similar component of m/z 301 has been isolated but not yet identified in the male urine sample after 4OHA treatment intramuscularly [23]. However, when comparing the two metabolites, compound A has a different retention time and MS–MS spectrum to the unknown metabolite present in the male patients' urine sample. Consequently it can be concluded that these two metabolites are of similar nature but not identical due to different routes of administration of the drug or differences between the sex of the patients.

Since the majority of the metabolites and their corresponding authentic synthetic materials lack the double bond across the C-4–C-5 position, they have poor absorption at wavelength 254 nm, we have to rely on the mass spectrometer for their detection.

Metabolite C and metabolite D (with $[M+H]^+$ of m/z 305 and 307, respectively) co-eluted at 11.2 min (Fig. 6c). Metabolite C was identified to be 3α-hydroxy-5β-androstan-4,17-dione as demonstrated in previous studies [12,13,23]. Metabolite D was determined to be 3β,4β-dihydroxy-5α-androstan-17-one. This metabolite has been reported in the rat hepatocytes studies but not in the male patients' urine. The retention time of metabolite E (Fig. 6d) was in good agreement with that of 4OHT. Even though this metabolite has been reported on several occasions [11,12], this is the first time that a mass spectrum was shown for structural confirmation. The other two metabolites (F and G) with $[M+H]^+$ of m/z 307 were the two isomers of 3,17-dihydroxyandrostan-4-one (Fig. 6e and f). These metabolites had been described in detail previously [23]. Finally the metabolite H at 5.3 min with an $[M+H]^+$ of m/z 321 had been identified as 4β,5α-dihydroxyandrostane-3,17-dione, as found in the male patients' urine samples.

Aromatase assay

All the metabolites identified in the patients' urine were tested for aromatase inhibition. Metabolites which do not possess any inhibitory activity against the aromatase enzyme are C, D, F, G and H. Compounds which exhibit inhibitory activity are listed in Table I. However, compounds A and E are less active as aromatase inhibitors than 4OHA, consistent with previous reports on these compounds [12,27].

CONCLUSION

4OHA is metabolised extensively in the body and similar metabolites were identified from the three samples analysed. Since the drug was administered orally, no Phase I metabolites were observed in the 4 h urine samples. Instead the metabolites were extensively conjugated and could be detected in the samples after they had been hydrolysed enzymatically (see Fig. 4). There are some variations in the metabolic profiles between female patients and male patients though further studies are required to determine if these are related to sex difference or to route of administration. Compounds A, D and E were absent in the male patients' samples [23]. Future studies will be carried out to monitor the rate of the formation of these metabolites over a period of 48 h in order to have a better understanding of their biotransformations within the body.

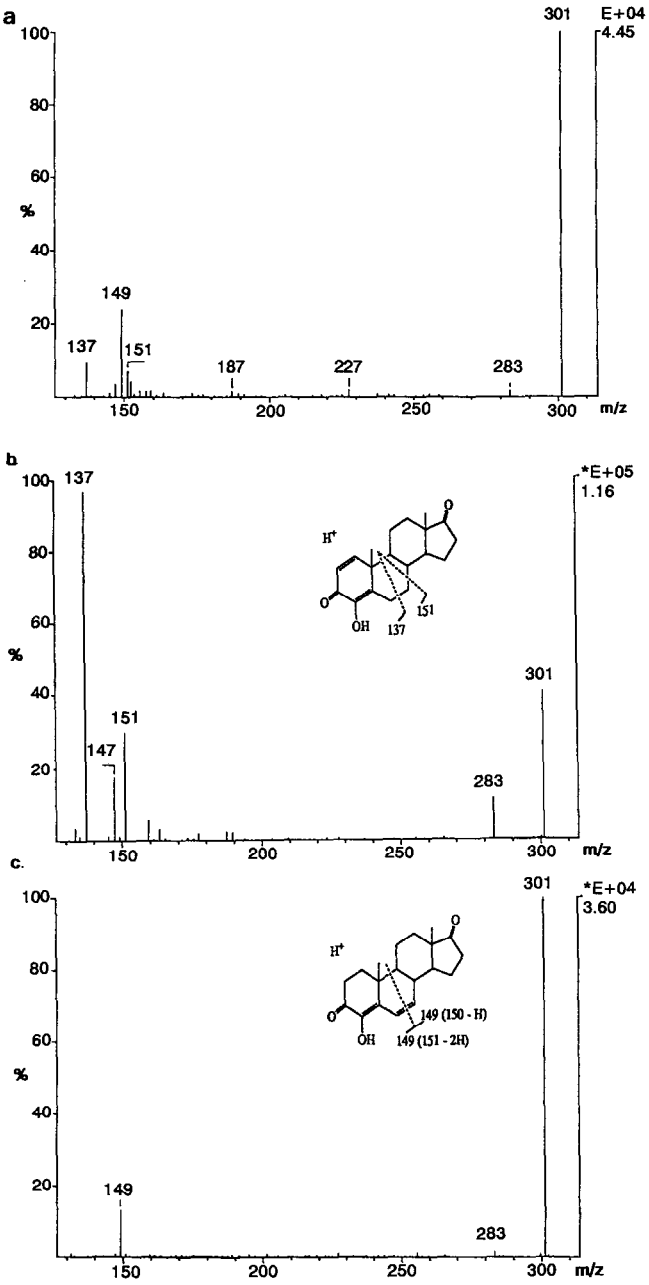

Fig. 5. CID daughter ion spectra of (a) metabolite A, (b) authentic 4OHA-1,4-diene and (c) authentic 4OHA-4,6-diene.

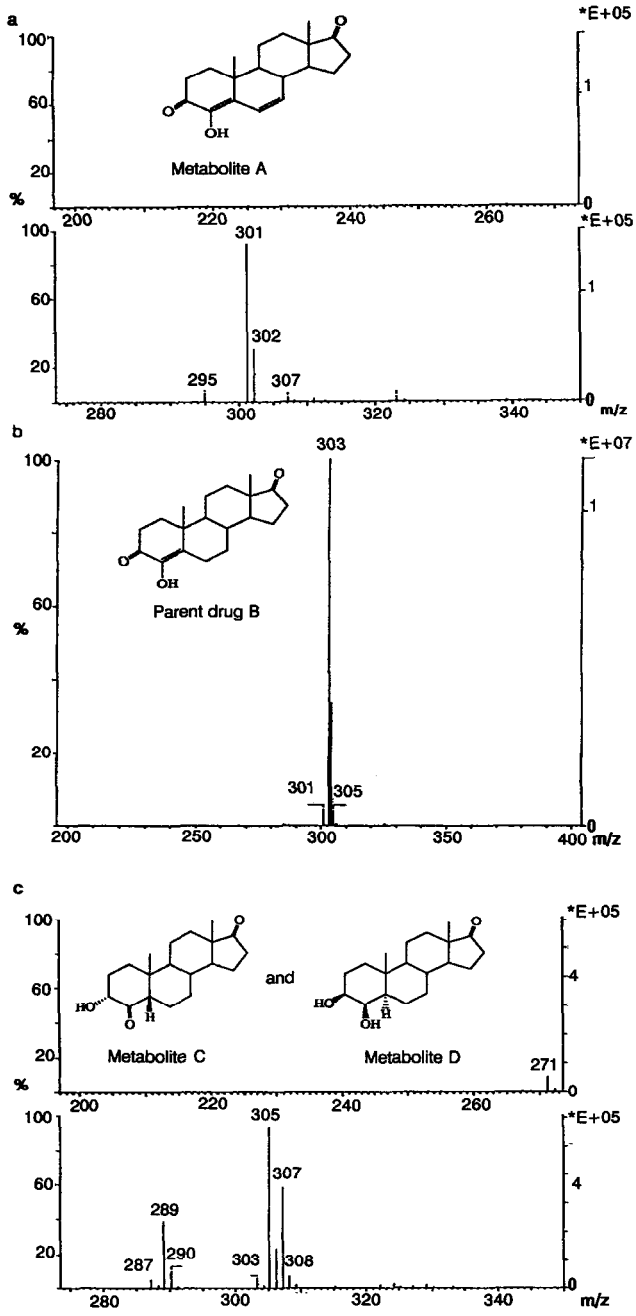

Fig. 6.

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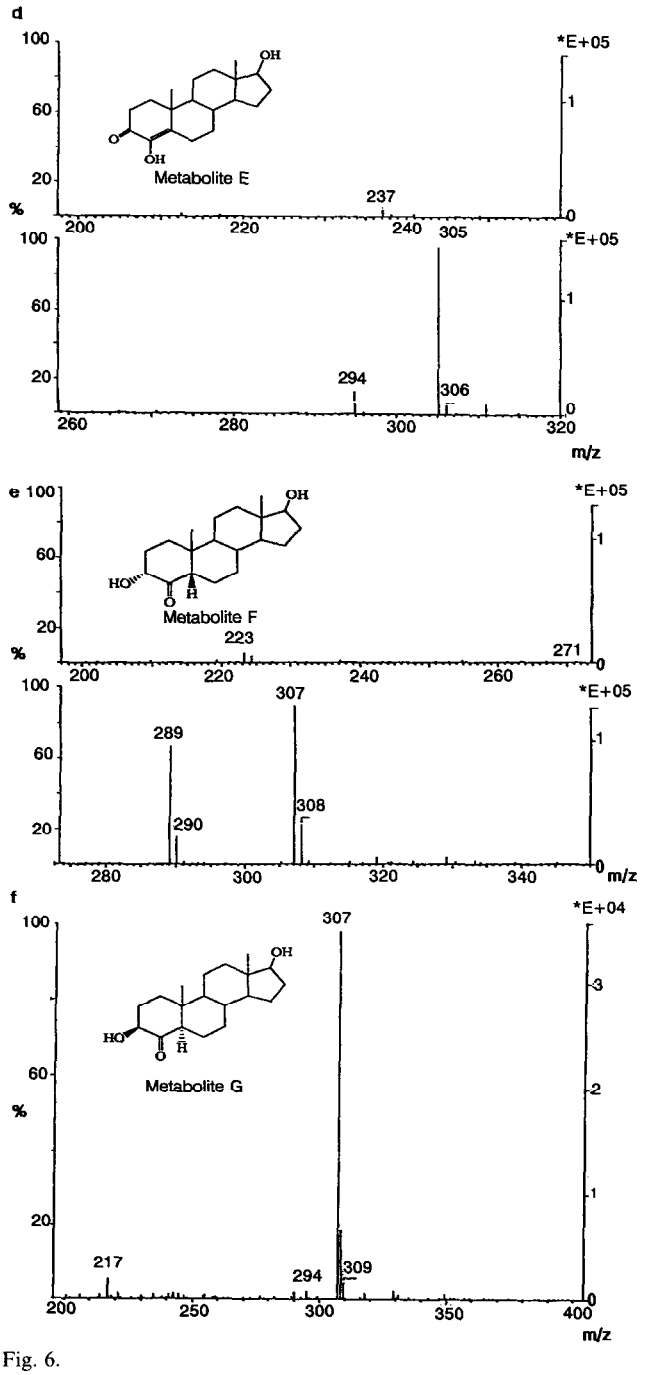
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Metabolite E
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Fig. 6.

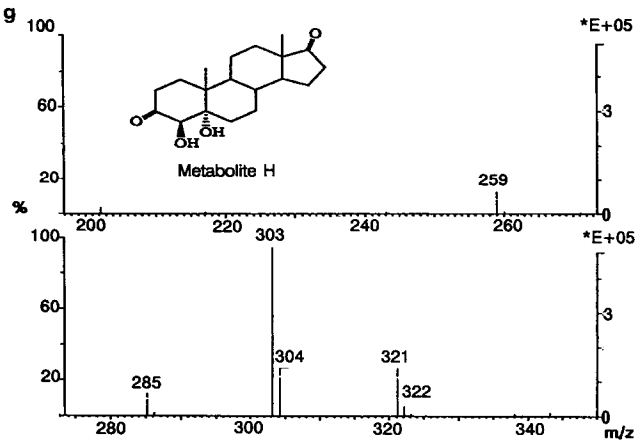

Fig. 6. LC–TSP–MS spectra of 4OHA metabolites showing the $[M+H]^+$ ions.

TABLE I

INHIBITION OF AROMATASE ACTIVITY BY ANALOGUES OF 4OHA

Compound	IC_{50} value (μM)
4OHA (B)	0.2
4OHT (E)	1.9
4OHA-4,6-diene (A)	1.0

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