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Identification of 4-hydroxyandrost-4-ene-3,17-dione metabolites in prostatic cancer patients by liquid chromatography–mass spectrometry☆

G. K. Poon*, M. Jarman and R. McCague

Cancer Research Campaign Laboratory, Drug Development Section, Institute of Cancer Research, Belmont, Sutton, Surrey (UK)

J. H. Davies

Department of Urology, St. George's Hospital, Tooting, London (UK)

C. E. M. Heeremans, R. A. M. van der Hoeven, W. M. A. Niessen and J. van der Greef

Division of Analytical Chemistry, Center for Bio-Pharmaceutical Sciences, P.O. Box 9502, 2300 RA Leiden (Netherlands)

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ABSTRACT

Liquid chromatography with thermospray mass spectrometry has proved to be an invaluable technique for the study of metabolic degradation of xenobiotics in complex biological fluids. This paper describes the detection of 4-hydroxyandrost-4-ene-3,17-dione and its metabolites in urinary extracts from prostatic cancer patients. Several metabolites were detected including 4β,5α-dihydroxyandrostan-3,17-dione, 3,17-dihydroxyandrostan-4-ones and 3α-hydroxy-5β-androstan-4,17-dione.

INTRODUCTION

4-Hydroxyandrostenedione (4OHA) (**1** in Table I) is an effective inhibitor of aromatase and reduces ovarian secretion of estrogen in rats [1]. 4OHA is under clinical trial for the treatment of patients with estrogen-dependent breast cancer [2] and, more recently, androgen-dependent prostatic cancer [3]. Metabolic studies of 4OHA have been carried out in animals dosed with radioactive material [4]. When [6,7-^{3}H]4OHA was administered intravenously to male rhesus monkeys, 6–7% of the total radioactivity in blood samples was recovered in the form of the free drug and its metabolite, 4-hydroxytestosterone (4OHT) (**2**). Marsh *et al.* [5] studied the metabolism of [6,7-^{3}H]4OHA in rats. The *in vitro* metabolism by ovarian microsomes gave mainly 4OHT (**2**), while *in vivo* metabolism by female rats (administered intravenously) gave 3β-hydroxy-5α-androstan-4,17-dione (**3**) as the major free metabolite (~18%), while 4OHT (**2**) and 4OHA (**1**) were detected in minor concentrations (1.4 and 0.5%, respectively). Foster *et al.* [6] reported the presence of several new metabolites after incubating [4-^{14}C]4OHA with rat hepatocytes, but no

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4OHT (**2**) was detected. The only study of metabolites of 4OHA in patients' urine is that reported by Goss *et al.* [7]. After oral administration of 4OHA in post-menopausal patients with metastatic breast cancer, they identified the 4OHA-glucuronide conjugate as the principal metabolite in the 24-h urine samples. However this metabolite accounted for only 20–45% of the administered dose, and no 4OHA-glucuronide was detected in any subsequent 24-h urine samples.

Previous methods of detection of 4OHA and its metabolites have included radioimmunoassay (RIA) [8], gas chromatography–mass spectrometry (GC–MS) [7,9] and thin-layer chromatography (TLC) used in conjunction with mass spectrometry [6]. However, the RIA lacks total specificity, showing some cross-reactivity with androstenedione and testosterone, GC–MS requires derivatization of samples prior to analysis, while TLC followed by MS requires a substantial quantity of material.

A variety of liquid chromatographic–mass spectrometric (LC–MS) techniques have been applied in the analysis of steroid-related compounds and their metabolites including steroid conjugates [10]; thermospray and ionspray prove to be most successful [11–14]. In this work a study is reported on the analysis of non-radioactive 4OHA and its metabolites in urine samples taken from prostatic cancer patients using LC–MS with a thermospray interface. Previously, the identification of 4OHA metabolites in urine samples of female patients has been reported [15]. The general analytical strategy is based on steroid metabolism studies with GC–MS and consists of the synthesis of possible metabolites, characterization with respect to chromatographic retention times and mass spectra, analysis of the biological sample after and before enzymatic hydrolysis of the conjugates, and by comparison of metabolites with synthetic standards (retention times and mass spectra) subsequent identification of the metabolites. This procedure was followed because the steroid metabolites show insufficient fragmentation in thermospray MS to achieve structural identification from the mass spectra and various isomers most definitively cannot be distinguished from the thermospray spectra. Unlike GC–MS no derivatization was necessary in LC–MS analysis.

EXPERIMENTAL

Chemicals

4OHA was provided by Ciba-Geigy Pharmaceuticals (Horsham, UK). Testosterone-β-glucuronide and 4-androsten-19-al-3,17-dione were purchased from Sigma (Poole, UK). Reference compounds were synthesized as described previously [5,6]. All solvents used were HPLC or analytical grade.

Sample collection and pretreatment

Urine was collected pre-treatment and 24 and 48 h after the treatment from patients who had been given a single dose of 4OHA (**1**) (500 mg intramuscularly). Different procedures were followed to obtain (i) urine extracts wherein 4OHA and metabolites are present in their unconjugated form and (ii) extracts where they are also present as their glucuronide conjugates.

(i) The mixture of free and conjugated steroids was converted entirely into free steroids by hydrolysing an aliquot of each urine sample (5 ml) at 37°C (for 22 h) using β-glucuronidase (500 μl) (Boehringer Manheim). At the end of the hydrolysis ethyl acetate was used (3 × 10 ml) to extract the compounds of interest. The organic extracts were concentrated to dryness and the residue was redissolved in 100 μl of methanol. From this solution 20 μl were submitted to LC–MS analysis.

(ii) Free steroids already present in the urine were recovered by extracting an aliquot of each untreated urine sample (20–25 ml) with ethyl acetate (3 × 30 ml). The organic phase was concentrated to dryness. Conjugated metabolites present in the aqueous phase were treated by first acidifying this phase to pH 1.5 using 5 *M* hydrochloric acid followed by ethyl acetate (3 × 30 ml) extraction. The organic phase was concentrated *in vacuo*. Previous studies [6], using ^{14}C-labelled 4OHA, demonstrated this method removes 80% of the original radioactivity losing the remaining 20% of the most polar metabolites in the aque-

ous phase which contained 3β,4β-dihydroxyandrostan-17-one and 2,4,17β-trihydroxyandrostan-3-one. Neither of these two metabolites were present in the enzyme-hydrolysed fractions of these urine samples. Each organic extract was redissolved in 1 ml of methanol and from this solution 20 μl were submitted to LC–MS analysis.

Synthesis of model compounds

A number of possible 4OHA metabolites are shown in Table I. These have been synthesized as previously described [6] except for compounds **5** and **6**.

*Synthesis of 3β,17β-dihydroxy-5α-androstan-4-one (**5**) and 3α,17β-dihydroxy-5β-androstan-4-one (**6**)*

4OHT (**2**) (50 mg) in ethanol (20 ml) was stirred with ammonium formate (250 mg) and 5% palladium/carbon (20 mg) for 30 min at room temperature. At the end of the reaction, the mixture was filtered and the filtrate was concentrated *in vacuo*. The residue was purified by HPLC using an APEX II 5ODS column (Jones Chromatography, Hengoed, UK), 250 mm × 10 mm I.D., with a mobile phase composed of a 65:35 (v/v) mixture of methanol–water (0.01% TFA) isocratically at a flow-rate of 3.5 ml/min. The first isomer (**5**) was eluted at 10.15 min (15 mg) and the second isomer (**6**) was eluted at 14.5 min (20 mg).

The stereochemistry of the two products **5** and **6** were deduced by ^{1}H NMR spectroscopy (Bruker AC-250 spectrometer, Switzerland Spectrospin, Faellander, Switzerland) on solutions in methanol. The most noticeable difference in their spectra were the positions of the singlets for the angular methyl groups C-18 and C-19; those of **5** were virtually coincident at δ 0.81 and δ 0.82, whereas those for **6** were widely separated at δ 0.81 (C-18) and δ 1.24 (C-19). The difference in the positions of the C-19 resonance for the two compounds can be assigned to a different mode of fusion of the A and B rings, being *trans*-fused in **5** with H-5α and *cis*-fused in **6** with H-5β. This is as reported [16] for corresponding microbial metabolites of 4-OHA which have a ketone instead of hydroxy at C-17. Confirmation of the 5β-stereochemistry in **6** is made from a nuclear Overhauser enhancement study. The proton at C-5 which resonates at δ 2.42 is enhanced in intensity by 5.3% after pre-irradiation of the C-19 methyl group showing their close proximity. Both compounds gave a signal for H-3 with a spread of 18 Hz, this relatively high value indicating an axial proton. This is the same value as in the reported 17-keto metabolites in which the axial configurations of the protons were rigorously established [16]. For both compounds homocoupling relationships were determined by COSY-45 2D NMR experiments. These revealed, for both compounds, that H-3 was coupled not only to the two C-2 protons as expected but also to a third proton at δ 2.00 in **5** and δ 2.08 in **6**. The cross peaks for this coupling were slanted approximately parallel to the diagonal as expected for a [1,4]-coupling relationship, and the presence of the coupling at all indicates that the C–H bonds are coplanar, *i.e.* H-1β in **6** and H-1α in **5**. In the case of compound **6**, this same proton (H-1β) was enhanced by 1.9% upon pre-irradiation of the proximate C-19 methyl group. These assignments add further confirmation to the proposed structures and support the previously reported assignments for the 17-ketones [16].

LC – MS analysis

The HPLC system was manufactured by LKB (Model 2150) with a gradient controller (Model 2152). The column used was a 150 mm × 4.6 mm I.D. APEX II 5ODS (Jones Chromatography) column. The mobile phases were either 60:40 (v/v) mixtures of methanol–water (containing 0.01% formic acid) isocratically (system 1) for the hydrolysed urine, or 50:50 (v/v) mixtures of methanol–water (containing 0.01% formic acid) isocratically (system 2) for the acidic extracts, both at a flow-rate of 1.2 ml/min. A Finnigan MAT (San Jose, CA, USA) TSQ 700 triple quadrupole mass spectrometer equipped with a Finnigan MAT thermospray interface was used. The system was operated in discharge-on mode (1000 V) and in positive-ion mode. The source block temperature was kept at 200°C. The vaporiser

temperature and the repeller potential were optimized daily to give the maximum response for the parent compound; typical values were 100°C and 50V, respectively. The scan speed was 2.5 s/scan.

RESULTS AND DISCUSSION

Synthesized 4OHA-related compounds

All synthetic 4OHA-related compounds (Table I) have been analyzed under the conditions of high-performance liquid chromatography (HPLC) system 1. The retention times are displayed in Table I. The compounds with insufficient sensitivity for detection at 254 nm because of the lack of the 4,5 double bond were analyzed at 225 nm.

4OHA and related compounds have been submitted to thermospray MS (bypassing the column). The major ions observed are summarized in Table I. The general appearance of these mass spectra is exemplified by that of the parent compound 4OHA (Fig. 1). The protonated molecule ($[4OHA + H]^+$) at m/z 303 is the base peak of the spectrum. The main fragments, which may be useful diagnostic ions in the identification of 4OHA-related metabolites, are due to the loss of a hydrogen molecule (not always observed) and of one or two water molecules. In the spectrum of the parent compound 4OHA these fragments are found at m/z 301, 285 and 267, respectively.

Hydrolysed urine extracts

The hydrolysed blank, 24-h and 48-h urine extracts were analyzed in LC–MS using system 1. To locate the metabolites selected ion chromatograms have been extracted at the m/z values of the protonated molecules of the possible metabolites presented in Table I (see Fig. 2). The spectra of the major peaks are shown in Fig. 3; these are discussed in more detail below. All peaks indicated in the chromatograms and in the mass spectra were carefully checked against the pre-treatment control urine samples. The peaks indicated in the chromatograms in Fig. 2 were not present in the pre-treatment control urine extract. More detailed remarks on some peaks observed in the mass spectra that were also observed in the pre-treatment control samples are made below.

Peak A (see Fig. 2 and the mass spectrum in Fig. 3a) was identified as a hydroxylated, reduced 4OHA, namely 4β,5α-dihydroxyandrostan-3,17-dione (**4**) and is found only in the 48-h urine sample. The protonated molecule $[M + H]^+$ is observed at m/z 321. The peaks at m/z 309 and 278 were also detected in the pre-treatment control urine extract.

Peaks B and C (Fig. 2 and the mass spectra in Fig. 3b and c) were identified as the isomers of 3,17-dihydroxyandrostan-4-one (**5** and **6**) and detected in both 24- and 48-h samples. The protonated molecule $[M + H]^+$ is found at m/z 307; fragment ions are due to the loss of one, two or three water molecules which corresponds to m/z 289, 271 and 253, respectively. The mass spectrum of metabolite C contains extraneous peaks at m/z 287 and 329 which are also present at the same retenton time in the pre-treatment control urine extract. The other synthetic compound with M = 306 a.m.u. (see Table I), 3β,4β-dihydroxyandrostan-17-one (**7**), elutes at another retention time (6.1 min). It does not correspond to any of the detected metabolites.

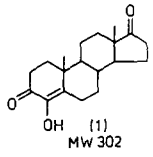
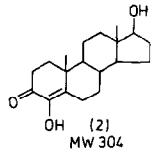
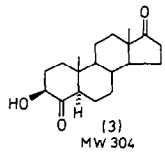
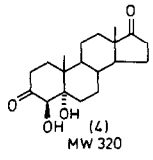
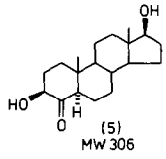
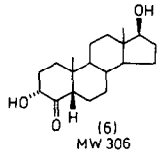
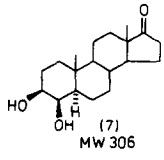
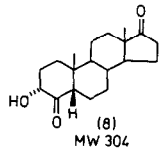
Peak D (see Fig. 2 and the mass spectrum in Fig. 3d) was identified as 3α-hydroxy-5β-androstan-4,17-dione (**8**) which can be formed from 4OHA by a reduction of the 4,5 double bond followed by isomerisation to give structure (**8**). The protonated molecule was observed at m/z 305 as well as the typical fragment ions at m/z 303, 287 and 269. The more polar isomer of compound **8**, 3β-hydroxy-5α-androstan-4,17-dione (**3**), elutes earlier (at 4.5 min), but was not detected in the urine extract.

Peak E (Fig. 2 and the mass spectrum in Fig. 3e) was identified as the parent compound 4OHA.

Finally, for peak F (Fig. 2 and the mass spectrum in Fig. 3f), the parent ion could be observed at m/z 301. Possible structures include 4-hydroxyandrost-1,4-diene-3,17-dione (**9**), 4-hydroxyandrost-4,6-diene-3,17-dione (**10**) and 4-androsten-19-al-3,17-dione (**11**). However, the retention times of these compounds do not match with the retention time of peak F. HPLC analysis

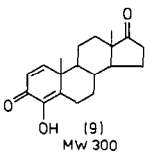
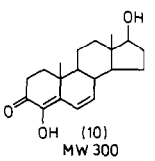
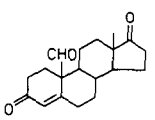
TABLE I

NAMES, STRUCTURES, RETENTION TIMES IN SYSTEM 1, AND MASS SPECTRAL DATA OF THE SYNTHESIZED POSSIBLE METABOLITES OF 4OHA

No.	Name	Structure	Retention time (min)	$[M + H]^+$	Characteristic fragments
1	4-Hydroxyandrost-4-ene-3,17-dione	 (1) MW 302	8.1	303	301, 285, 267
2	4-Hydroxytestosterone	 (2) MW 304	9.0	305	303, 287, 269
3	3β-Hydroxy-5α-androstane-4,17-dione	 (3) MW 304	4.5	305	303, 287
4	4β,5α-Dihydroxyandrostane-3,17-dione	 (4) MW 320	3.8	321	319, 303, 285
5	3β,17β-Dihydroxy-5α-androstan-4-one	 (5) MW 306	4.4	307	289, 271, 253
6	3α,17β-Dihydroxy-5β-androstan-4-one	 (6) MW 306	7.4	307	289, 271, 253
7	3β,4β-Dihydroxy-5α-androstan-17-one	 (7) MW 306	6.1	307	289, 271
8	3α-Hydroxy-5β-androstane-4,17-dione	 (8) MW 304	6.7	305	303, 287, 269

(Continued on p. 240)

TABLE I *(continued)*

No.	Name	Structure	Retention time (min)	$[M + H]^+$	Characteristic fragments
9	4-Hydroxyandrost-1,4-diene-3,17-dione	 (9) MW 300	6.5	301	283
10	4-Hydroxyandrost-4,6-diene-3,17-dione	 (10) MW 300	7.8	301	283
11	4-Androsten-19-al-3,17-dione	 (11) MW 300	4.1	301	Not determined

showed a strong UV absorption at 254 nm, indicating the possible presence of an extra double bond. The precise structure of this metabolite is still uncertain.

Non-hydrolysed urine extracts

Pre-treatment and 48-h post-treatment urine samples were first extracted with ethyl acetate under neutral conditions in order to isolate the phase I (unconjugated) metabolites, and subsequently after acidification to isolate the phase II (conjugated) metabolites. Both extracts were analyzed by LC–MS using system 2. From the retention times and mass spectra, three unconjugated metabolites and the parent compound could be identified in the neutral ethyl acetate extract; they were the metabolites C (**6** in Table I), D (**8**) and F (Fig. 2c).

Prior to the analysis of the acidic ethyl acetate extract, the LC–MS system was tested with a conjugated compound, *i.e.* testosterone glucuronide, as no synthetic 4OHA-glucuronide stan-

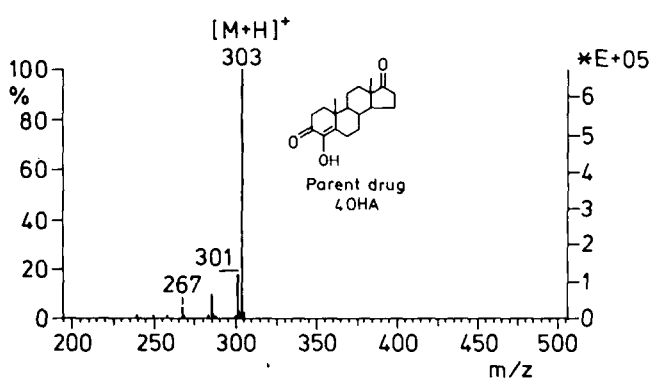


Fig. 1. Thermospray mass spectrum of 4OHA.

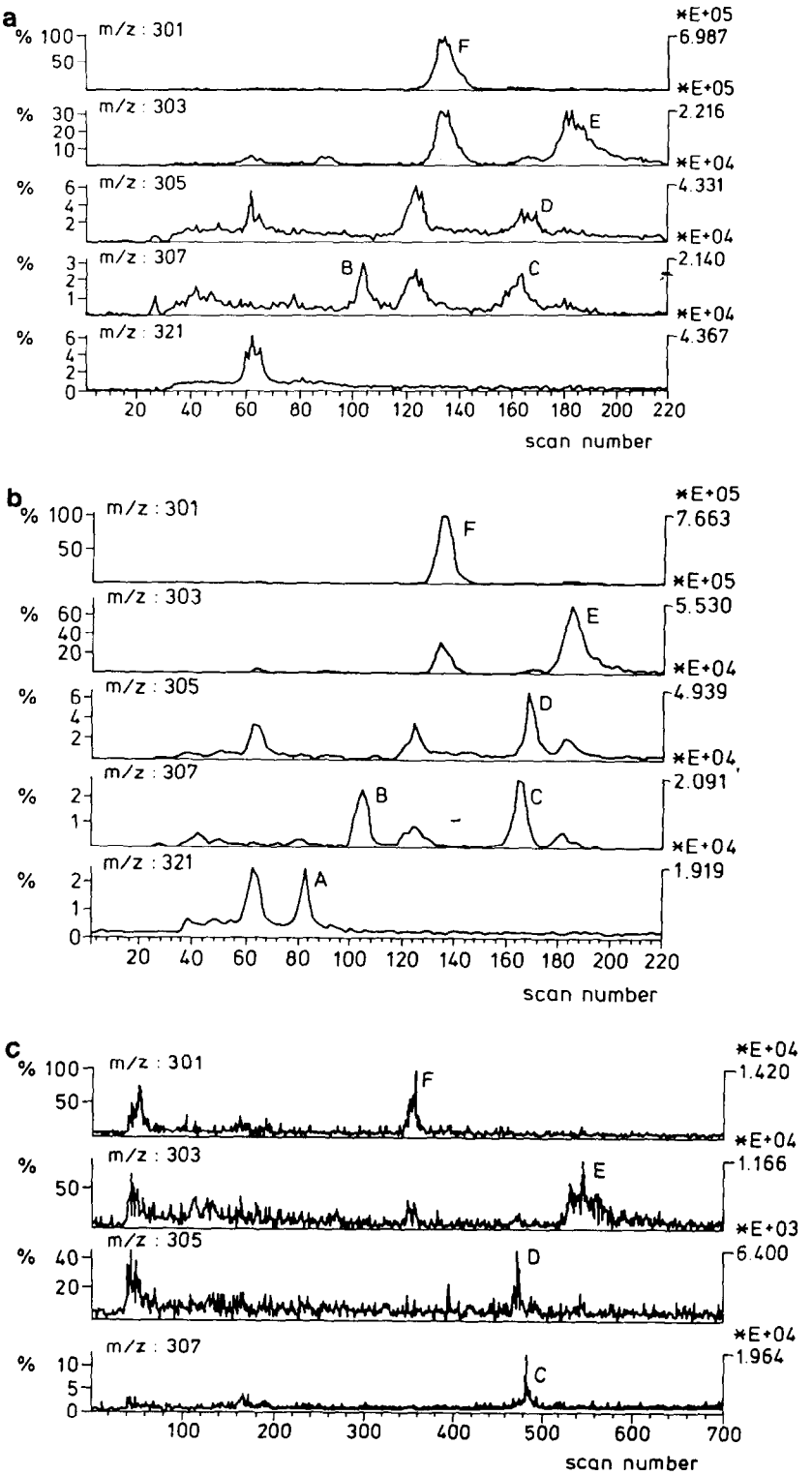


Fig. 2. LC–MS mass chromatograms of the hydrolysed urinary extracts: (a) 24-h and (b) 48-h post-treatment of 4OHA using system (c) LC–MS mass chromatograms of the 48-h post-treatment neutral urine extracts using system 2.

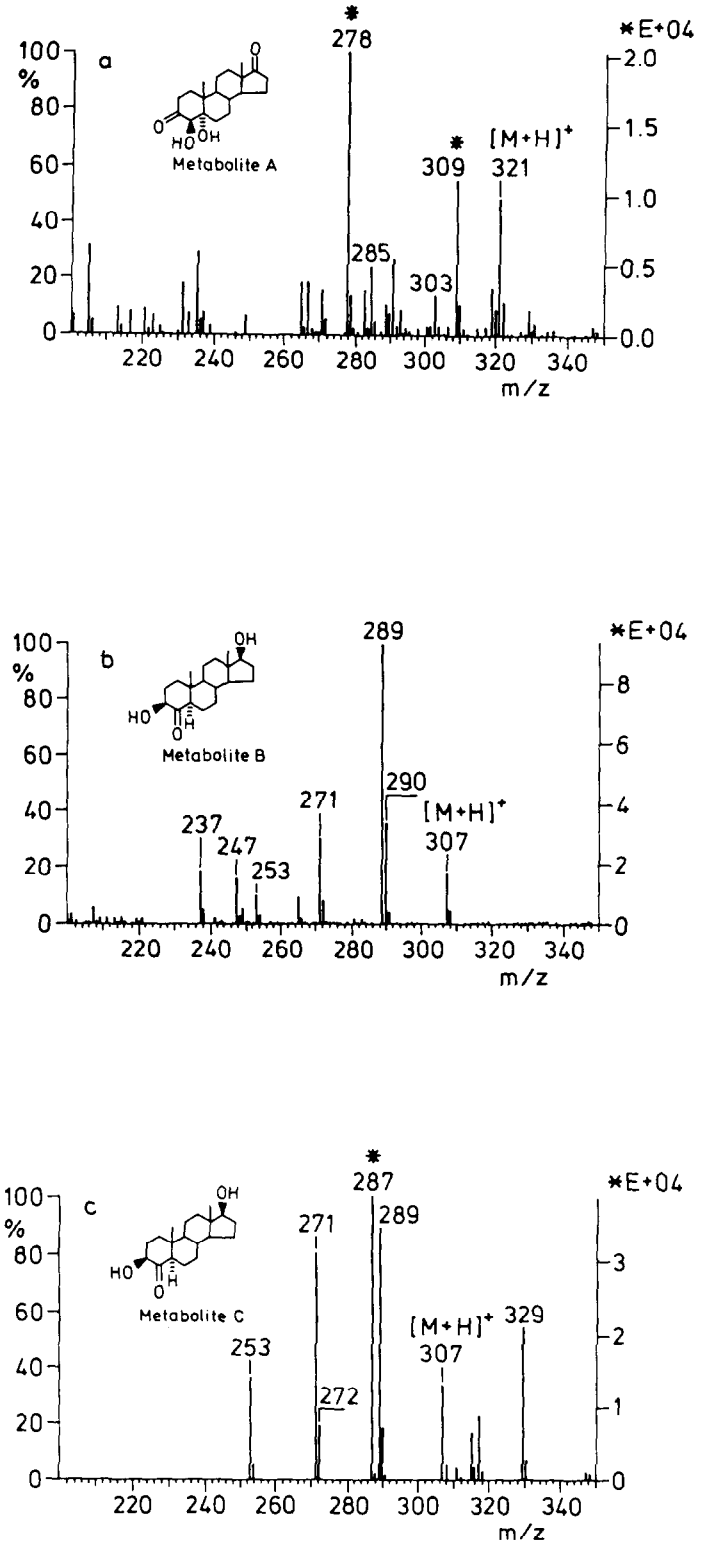

a
Metabolite A
278
309
[M+H]+
321
285
303
*E+04
2.0
1.5
1.0
0.5
0.0
100
%
80
60
40
20
0
220 240 260 280 300 320 340
m/z
b
Metabolite B
289
290
271
237
247
253
[M+H]+
307
*E+04
8
6
4
2
0
c
Metabolite C
287
271
289
253
272
[M+H]+
307
329
*E+04
3
2
1

Fig. 3.

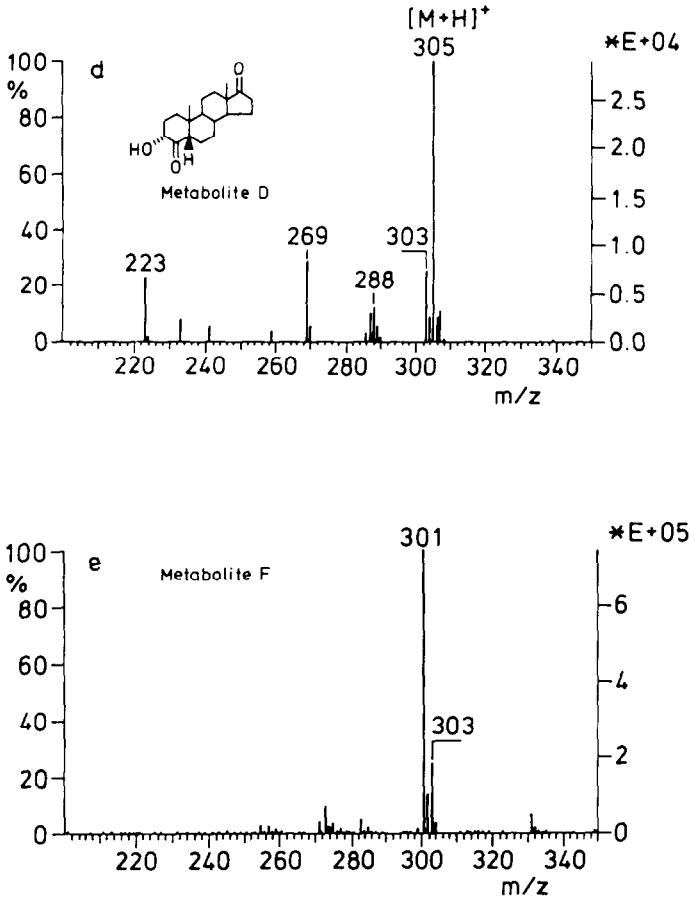


Fig. 3. Mass spectra of the peaks A–F found in the urine extracts, and their proposed structures. The asterisk represents endogenous materials.

dard was available. The intact protonated molecule of testosterone glucuronide could only be observed at low vaporiser temperatures (<80°C), while at a vaporiser temperature of 100°C only the aglycone is observed as well as a peak at *m/z* 177, which is characteristic for glucuronides, probably arising from thermally induced solvolysis of the glycoside bond, as is frequently observed in thermospray spectra of these types of compounds. However, considerably better sensitivity (15×) was achieved at higher vaporiser temperatures.

The detection of intact glucuronides of 4OHA in the acidic ethyl acetate extracts of post-treatment urine samples was attempted in vain (vaporiser temperature 80°C). At a vaporiser temperature of 100°C, two conjugated metabolites could be detected with a poor signal-to-noise ratio with the aglycone peaks at *m/z* 303 and 305, respectively; the glucuronide-characteristic peak at *m/z* 177 was also observed. The unconjugated metabolite F was also extracted under acidic conditions, as it was detected at the same retention time as in the neutral extract (*cf.* Fig. 2c). Most metabolites of 4OHA are expected to be eliminated in the urine as their conjugates. For the present study we solely concentrated on trying to identify the glucuronide conjugates. Despite our inability to detect these glucuronides by thermospray LC-MS, other LC–MS methods, such as continuous-flow fast atom bombardment and electrospray, might be more appropriate. More

recently, sulphate conjugates of 4OHA have been isolated in some experiments using an improved technique, but all their steroid moieties were equivalent to those of the glucuronide conjugates found in this study.

Comparison with previous studies

Conjugated 4OHA metabolites A (**4**) and D (**6**) were previously detected in rat hepatocytes after 4OHA incubation [6], whereas the previously reported 4-hydroxytestosterone (**2**) [4,5] and 3β-hydroxy-5α-androstan-4,17-dione (**3**) were not observed in this study.

In the previous study on 4OHA metabolism in female patients a series of phase II metabolites were observed [15]. Besides the parent compound (**1**), metabolites **4, 5, 6** and **8** were detected in both studies, whereas metabolites **2, 7** and **10** were identified in the urine samples of female patients but not in the prostatic cancer patients.

CONCLUSION

Thermospray LC–MS offers a minimum sample preparation, improved selectivity in the detection of overlapping peaks and the detection of poorly UV-absorbing compounds. It is a powerful tool in steroid phase I metabolism studies. However, since most urinary metabolites are present as phase II metabolites, other LC–MS methods must be evaluated in further studies. The major metabolic pathways of 4OHA include reduction of the 4,5 double bond for all observed metabolites except one (peak F) together with either a further reduction of the C-17-keto function (peaks B and C), a hydroxylation at C-5 (peak A) or an isomerisation (peak D).

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