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Abstract

Using a novel isotopic dilution gas chromatography-mass spectrometry method, the plasma kinetics of 4-hydroxyandrostenedione (4-OHA) after a single i.m. injection of 250 or 500 mg of the drug have been studied in two groups of postmenopausal women affected by advanced breast cancer. Although the mean values of C_{Max} (the maximum concentration), $t_{1/2}$ and AUC (the area under the curve) for the 500 mg dose group were higher, the differences were not statistically significant, possibly because of the high intersubject variability.

In addition, the 4-OHA levels in the breast adipose tissue of the quadrant where the tumor was located were measured in another group of women affected by breast cancer who received a 250 mg dose of drug 2, 7 and 15 days before surgical intervention. The tissue concentrations of 4-OHA were more than five times higher than the corresponding plasma concentrations; in addition they correlated significantly with the circulating levels of the drug and showed an exponential disappearance pattern.

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Introduction

4-Hydroxyandrostenedione (4-OHA) is a suicide inhibitor of aromatase and is more potent than aminoglutethimide *in vitro*. 4-OHA has been demonstrated to be useful for the treatment of postmenopausal breast cancer (Miller 1990). The administration schedule (one injection every 2 weeks) was based on the observation that suppression of plasma estrogens persists for 14 days. The dose usually employed is 250 or 500 mg every 2 weeks. However, the incidence of side-effects with pain and inflammation at the injection site was less with the dose of 250 mg every 14 days, and this schedule has been adopted for parenteral use (Dowsett *et al.* 1989). A randomised trial in 40 postmenopausal women with breast cancer compared the effect of the two doses (250 or 500 mg every 2

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weeks) on plasma estradiol levels showing the same degree of suppression (Dowsett *et al.* 1989). However the clinical non-randomised studies performed with the two schedules gave contradictory results. In one clinical study (Dowsett *et al.* 1989) there was no difference in the response rate between the two dose levels, while in another study the objective responses were 28% in the 250 mg group and 46% in the 500 mg group (Bajetta *et al.* 1994).

Recently, Bulun & Simpson (1994), using competitive polymerase chain reaction following reverse transcription, have demonstrated that the highest expression of P450 aromatase in the stromal cell compartment of adipose tissue is in the quadrant where the tumor is located. Since we have recently developed an isotopic dilution gas chromatography-mass spectrometry (GC-MS) method for the determination of 4-OHA in human plasma (Guarna *et al.* 1989, Danza *et al.* 1993) we have set up a similar method for the measurement of this drug in the breast adipose tissue of postmenopausal women affected by breast cancer.

The present study was therefore organised to re-investigate the plasma kinetics of 250 and 500 mg doses of 4-OHA and, in addition, in another group of patients treated with an i.m. injection of 250 mg 4-OHA at different intervals before surgery, to measure the concentration of 4-OHA in adipose breast tissue taken from the quadrant where the tumor was located.

Materials and Methods

Patients

For the plasma pharmacokinetic study, eight postmenopausal women with advanced breast cancer received 250 or 500 mg 4-OHA. The mean(±S.D.) age and body weight of the first group were 57(±6.4) years and 63.5(±7.3) kg respectively, those of the second group were 64.7(±11.5) years and 63(±8.7) kg respectively. Blood samples were collected before and 4, 24, 48, 72, 96 192, 240 and 336 h after the injection of the drug. Immediately after collection, the samples were centrifuged and plasma was stored at −20 °C until analysis.

For the tissue pharmacokinetic study, eight patients with breast cancer (see Table 2 for clinical details), undergoing breast surgery, were treated with an intramuscular injection of 250 mg 4-OHA at 2, 7 or 15 days before the operation. Tissue samples were obtained at operation and immediately frozen at −80 °C. Blood samples were taken before the injection of the drug and just before the operation; samples were centrifuged and plasma stored at −20 °C until analysis.

This protocol has been approved by the ethical committee of USL 10D, Florence, Italy, and written informed consent was obtained from the patients.

Materials

4-OHA was a kind gift of Dr A Brodie (University of Maryland, Baltimore, MD, USA). 7,7′-[^{2}H]$_2$-4-Hydroxyandrostenedione (D_2-4-OHA) was synthesised in our laboratories as previously described (Guarna *et al.* 1989). The solvents used for the purification procedure were analytical grade and were purchased from J T Baker (Phillisburg, NJ, USA). Extrelut 3 columns were purchased from Merck (Darmstadt, Germany) and the chromatographic stationary phase Bio-beads SX3 was obtained from Bio-Rad (Richmond, CA, USA). The HPLC instrument was a Perkin-Elmer LC series 3B. All GC-MS analyses were performed using a Hewlett-Packard (Palo Alto, CA, USA) GC-MS system with a 5890 series II gas chromatograph equipped with a 5971A mass spectrometry detector and a 7673A automatic injector. The GC capillary column used was an HP1 (12 m × 0.2 mm × 0.33 μm) (Hewlett-Packard).

Internal standard and stock and working solutions

D_2-4-OHA, synthesised as previously described (Guarna *et al.* 1989), was used as internal standard (IS). Working solutions of 4-OHA and D_2-4-OHA in ethanol were prepared by dilution of stock solutions (1 mg/ml in ethanol).

Analysis of plasma and adipose tissue samples

Plasma samples were purified and analysed as previously described (Danza *et al.* 1993). For the analysis of adipose tissue samples a new method was established. The extraction of 4-OHA was performed according to the following procedure: a tissue sample of 0.5-1.0 g was minced with scissors and 20 ng IS were added (20 μl of 1 ng/μl solution). Minced tissues were extracted with 3 × 5 ml methanol: dichloromethane (90:10, v/v); the extracts were concentrated to 1 ml under nitrogen, then 3 ml

cyclohexane:dichloromethane (85:15, v/v) were added and the solutions were dried on short columns of anhydrous Na_2SO_4. The vials and the Na_2SO_4 columns were then washed with 2 × 3 ml cyclohexane:dichloromethane (85:15). The solutions were evaporated to dryness under nitrogen; the residues were redissolved with 0.5 ml cyclohexane:dichloromethane (85:15) and the resultant solutions were injected into a semipreparative low-pressure gel permeation column (Bio-Beads SX3), gelified with cyclohexane:dichloromethane (85:15), connected to the HPLC instruments. The column was eluted at a flow rate of 1 ml/min and the fractions containing 4-OHA and D_2-4-OHA (23-33 min) were collected, evaporated to dryness under nitrogen and derivatised for 1 h at

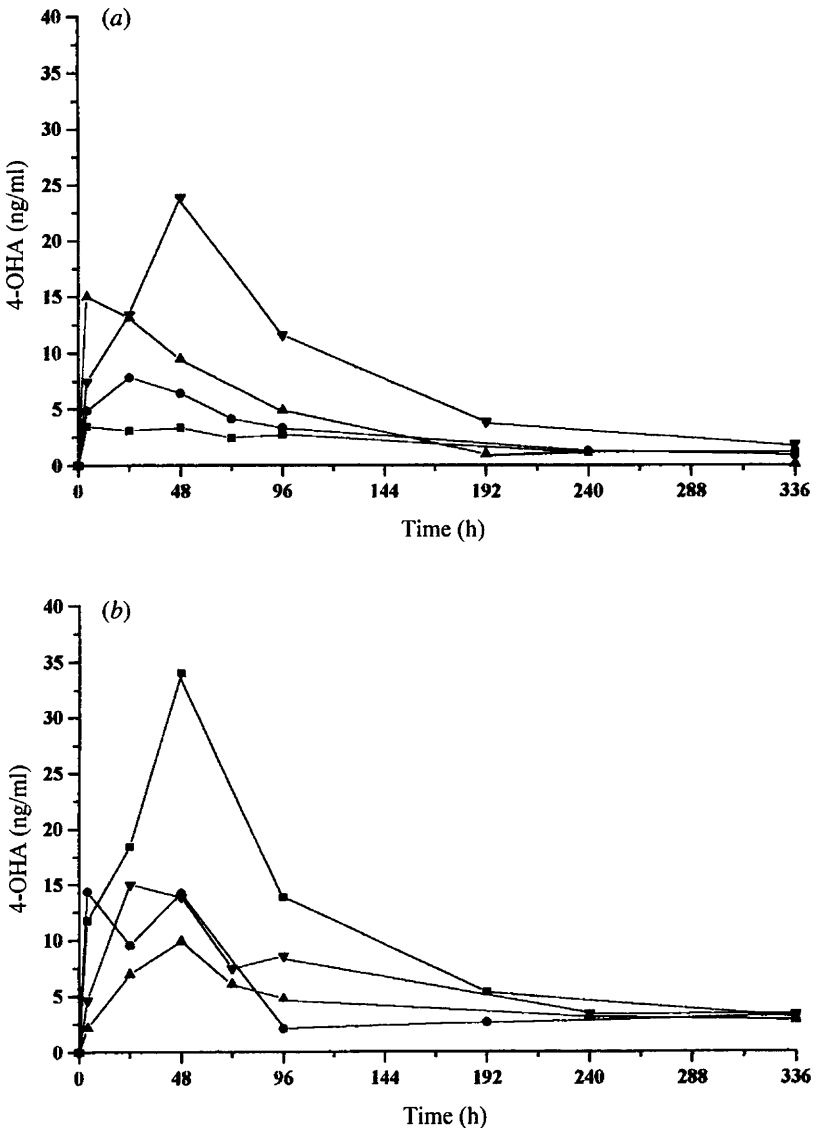

Figure 1 Plasma pharmacokinetic profiles of 4-OHA after a single i.m. injection of (*a*) 250 mg or (*b*) 500 mg of the drug.

Table 1 Mean values of plasma kinetic parameters for the two doses of 4-OHA (250 and 500 mg)

Parameter	**4-OHA** (250 mg)	**4-OHA** (500 mg)
C_{Max} (ng/ml)	10.78	17.9
t_{Max} (h)	48	48
$t_{½}$ (h)	99.0	173.3
AUC_{0-336} (ng h/ml)	1279	2075.8
C_{336} (ng/ml)	1.011	3.05*

*P=0.006 compared with result for 250 mg 4-OHA.

room temperature with 25 µl bis-trimethylsilyl-trifluoroacetamide to obtain the 4-O-trimethylsilyl (4-O-TMS) derivative. The sample solutions were evaporated to dryness under nitrogen, dissolved in 30 µl *n*-heptane and 3 µl were autoinjected into the GC-MS instrument.

The mean analytical recovery of 4-OHA from adipose tissue, calculated as previously described (Guarna *et al.* 1995), was 63%.

The selectivity of the method was evaluated by analysing blank samples and 4-OHA-free tissue samples. The calibration curve was made with seven points in the range 0-70 ng with 20 ng IS. Peak area ratios of 4-OHA and IS were plotted versus 4-OHA concentration and a good linearity was obtained (r=0.997)

The precision and accuracy of the method were determined by replicate interday analyses (n=3) of tissue samples (0.5 g) at a 4-OHA concentration of 20 ng/g. Precision, evaluated by the coefficient of variation, was 13%, and accuracy, calculated as mean measured concentration versus nominal concentration, was 92%.

The sensitivity limit of the instrument was evaluated by injection of decreasing amounts of 4-OHA and calculating the signal-noise ratio (S/N); 10 pg of injected 4-OHA gave S/N=50. The sensitivity limit of the assay was evaluated analysing tissue samples (1 g) at decreasing concentrations of 4-OHA and calculating S/N; samples at 500 pg/g gave S/N=30.4 when 3 µl of the 30 µl final volume were injected.

Analyses were performed using an HP1 (12 m × 0.2 mm × 0.33 µm) GC capillary column with a 100% methylsilicone phase. The temperature program was: 70 °C for 1 min, then an increase of 40 °C/min to 220 °C, 220 °C for 0.5 min, then an increase of 3 °C/min to 300 °C. The transfer line temperature was 280 °C. The carrier gas was helium with an inlet pressure of 35 kPa. In these conditions, the retention times of 4-OHA and D_2-4-OHA were 14.14 min and 14.12 min respectively. Injections were performed in the unsplit mode with a purge-off time of 1 min and an injector temperature of 280 °C. Selected ion monitoring analyses were made acquiring the ions at 359.2 and 361.2 m/z corresponding to the M-15 fragment of the 4-O-TMS derivatives of 4-OHA and D_2-4-OHA respectively.

Plasma and tissue pharmacokinetic profiles

Plasma pharmacokinetic parameters for the two doses of 4-OHA (250 and 500 mg) were calculated as follows: C_{Max} was the maximum concentration of

Table 2 Comparison between adipose breast tissue and plasma 4-OHA concentrations at different intervals after a single 250 mg injection of the drug. The age, body weight, height and TNM staging system according to the UICC classification are also reported for individual patients

Patient	**Age** (years)	**Body weight** (kg)	**Height** (cm)	**TNM**	**Days after injection**	**Breast adipose 4-OHA** (ng/g)	**Plasma 4-OHA** (ng/ml)
1	72	68	163	$T_1N_0M_0$	2	134.6	21.4
2	64	54	165	$T_1N_1M_0$	2	104.7	21.4
3	63	59	158	$T_2N_0M_0$	2	57.4	10.7
4	62	84	160	$T_{4d}N_1M_x$	2	77.4	23.9
5	56	65	155	$T_1N_1M_0$	2	97.8	9.46
6	64	65	155	$T_2N_2M_1$	7	20.2	4.7
7	49	55	158	$T_1N_0M_0$	15	2.82	0.08
8	71	85	160	$T_2N_1M_x$	15	1.26	0.00

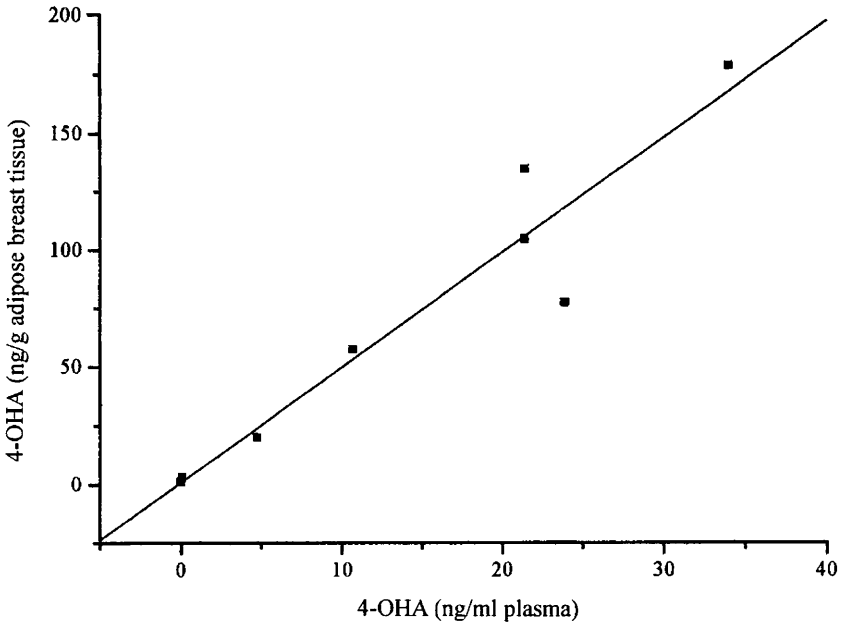

Figure 2 Correlation between adipose breast tissue and plasma concentrations of 4-OHA after a single 250 mg injection (y=0.75+4.91x, r=0.96).

the pharmacokinetic profile and t_{Max} was the time at which the maximum concentration was reached. The $t_{1/2}$ was calculated as $t_{1/2}$=0.693/*Kel* where *Kel* is the elimination constant. The $AUC_{0\text{-}336}$ (area under the curve) was calculated with the trapezoidal rule. C_{336} was the 4-OHA concentration at 336 h after the drug injection. All the parameters for the two doses were compared with Student's *t*-test.

For the tissue pharmacokinetic profile only the elimination phase was valuable and the AUC was not determined.

Results

The plasma kinetics of 4-OHA measured after the injection of the two doses of 4-OHA are shown in Fig. 1*a* and *b* and the mean values of the relevant parameters are reported in Table 1. In spite of the large intersubject variability, the highest concentration was obtained at 48 h with both doses. For that reason, 2 days before surgical intervention was chosen as the first point for the tissue study. Although the C_{Max}, $t_{1/2}$ and AUC obtained with the 500 mg dose were higher than those obtained with the 250 mg dose, the results were not significantly different (P>0.05) when compared by Student's *t*-test. Only the levels measured at 336 h (14 days) were significantly higher (3.05 versus 1.011 ng/ml of plasma, P=0.006) for the 500 mg dose. The tissue concentrations of 4-OHA were five times higher than the corresponding plasma concentrations (Table 2); they correlated significantly with circulating levels of the drug (r=0.96) (Fig. 2) and showed an exponential disappearance pattern (Fig. 3). The C_{Max} in breast adipose tissue and the apparent $t_{½}$ were respectively 94.4 ng/g and 57.8 h.

Discussion

The plasma kinetics of 4-OHA after a single injection of 250 or 500 mg of the drug have also been studied by Dowsett *et al.* (1989) using an RIA. Their results are in agreement with those of the present study showing a maximal concentration of about 10 ng/ml for the 250 mg dose and about 18 ng/ml for the 500 mg dose on the second day after injection. The concentration of 4-OHA in breast adipose tissue from the quadrant where the tumor is located has not

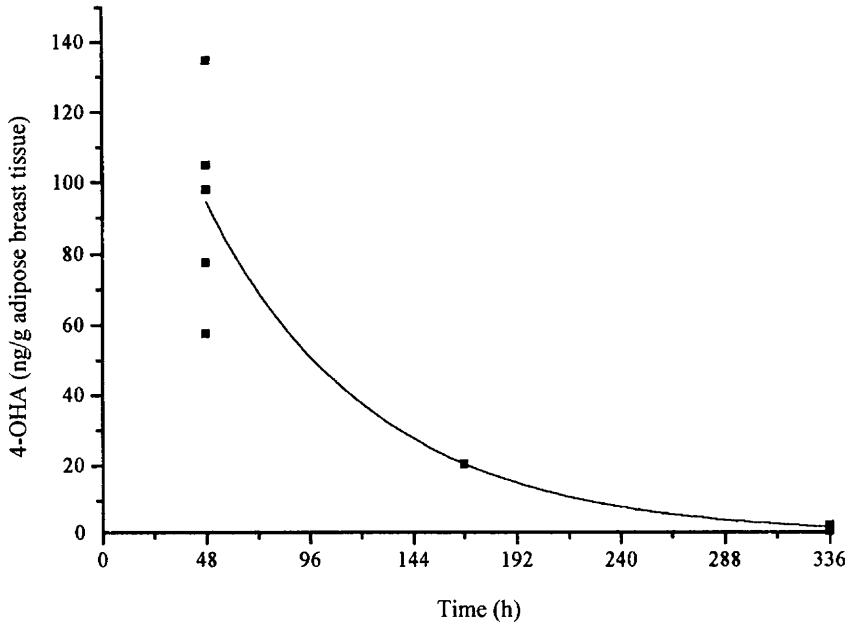

Figure 3 Exponential reduction of the concentration of 4-OHA in adipose breast tissue after a single 250 mg injection of the drug.

been measured before. However, Reed *et al.* (1991) measured 4-OHA levels in normal and tumor breast tissue. They found that the concentration of drug in breast tumor tissue was similar to that in plasma while that in normal breast tissue was lower. They found also a significant correlation between plasma and tissue concentrations of the drug. In the present study the circulating levels of 4-OHA still correlated with the tissue levels but the concentration in adipose tissue was five times higher than that in plasma. In breast adipose tissue the concentration of 4-OHA needed to reduce the aromatase activity to 50% has been calculated to be 30 ng/g (Perel *et al.* 1981), which is threefold lower than that which we measured on the second day. On the basis of the apparent tissue half-life we can calculate that 4-OHA levels which are able to reduce the aromatase activity to 50% persist in breast adipose tissue for 6 days.

In conclusion, the ability to measure concentrations of 4-OHA in both plasma and breast adipose tissue allows us to obtain new information regarding the disposition of this potent and useful drug.

Acknowledgements

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References

Bajetta E, Zilembo N, Buzzoni R, Noberasco C, Di Leo A, Bartoli C, Merson M, Sacchini V, Moglia D, Celio L & Nelli P 1994 Endocrinological and clinical evaluation of two doses of formestane in advanced breast cancer. *British Journal of Cancer* **70** 145-150.

Bulun SE & Simpson ER 1994 Breast cancer and expression of aromatase in breast adipose tissue. *Trends in Endocrinology and Metabolism* **5** 113-120.

Danza G, Muratori M, Guarna A, Occhiato EG, Sadri R & Serio M 1993 Pharmacokinetics of 4-hydroxyandrostenedione in man after intramuscular injection of different formulations and the effect of this drug on plasma aromatizable androgens and 17β-estradiol concentrations. *Journal of Steroid Biochemistry and Molecular Biology* **46** 373-379.

Dowsett M, Cunningham DC, Stein RC, Evans S, Dehennin L, Hedley A & Coombes RC 1989 Dose-related endocrine effects and pharmacokinetics of oral and intramuscular 4-hydroxyandrostenedione in postmenopausal breast cancer patients. *Cancer Research* **49** 1306-1312.

Guarna A, Moneti G, Prucher D, Salerno R & Serio M 1989 Quantitative determination of 4-hydroxy-4-androstene-3,17-dione (4-OHA), a potent aromatase inhibitor, in human plasma, using isotope dilution mass spectrometry. *Journal of Steroid Biochemistry* **32** 699-702.

Guarna A, Danza G, Bartolucci G, Marrucci A, Dini S & Serio M 1995 Synthesis of 5,6-[$^{2}H_{3}$]finasteride and quantitative determination of finasteride in human plasma at picogram level by an isotope-dilution mass spectrometric method. *Journal of Chromatography B* **674** 197-204.

Miller WR 1990 Endocrine treatment for breast cancer: biological rationale and current progress. *Journal of Steroid Biochemistry and Molecular Biology* **37** 467-480.

Perel E, Davis SP, Covey DF & Killinger DW 1981 Effects of 4-hydroxy-4-androstene-3,17-dione and 17-propargylestr-4-ene-3,17-dione on the metabolism of androstenedione in human breast carcinoma and breast adipose tissues. *Steroids* **38** 397-402.

Reed MJ, Aherne GW, Ghilchik MW, Patel S & Chakraborty J 1991 Concentrations of oestrone and 4-hydroxyandrostenedione in malignant and normal breast tissues. *International Journal of Cancer* **49** 562-565.