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Note

High-performance liquid chromatographic determination of imide inhibitors of aromatase in biological samples

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The most common form of cancer among women is carcinoma of the breast. Of the treatments available, aminoglutethimide (compound 5, Fig. 1) has proved to be an effective palliative therapy in postmenopausal women with the advanced disease whose tumor is oestrogen receptor positive [1]. The use of compound 5 is based on its ability to inhibit aromatase but it also inhibits desmolase and unless there is coadministration of glucocorticoid this leads to adrenal insufficiency [2,3]. We have previously reported the development of two new compounds (compounds 1 and 3, Fig. 1) analogous to compound 5 which are selective inhibitors of aromatase [4–6].

Although several chromatographic procedures have been described for the determination of compounds 5 and 6 in human plasma and/or urine [7–12], there are no established assay methods for the new compounds. To aid examination of the pharmacokinetics and disposition of compounds 1 and 3, a suitable high-performance liquid chromatographic (HPLC) method was developed.

EXPERIMENTAL

Apparatus and chromatographic conditions

Chromatographic analysis was performed on an automated system consisting of a Rheodyne 7125 injector fitted with a 100-μl injection loop, a 30 cm $\times$ 3.9 mm μBondapak C_{18} column (Waters; 5 μm particle size), an autosampler (Varian Assoc. Model 8000) with the capacity for 60 vials, an LDC Constametric pump (Model IIIG), an LDC Spectromonitor III with variable-wavelength detector set

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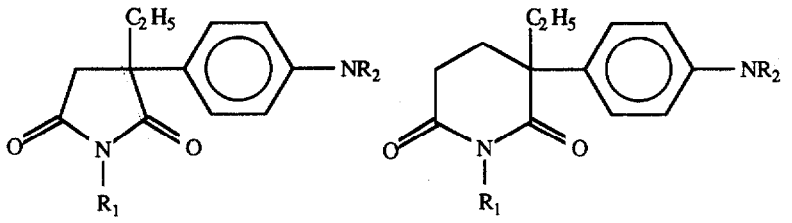

A:	R_1	R_2	B:	R_1	R_2
1	H	H,H	5	H	H,H
2	H	H,CH_3CO	6	H	H,CH_3CO
3	CH_3	H,H	7	H	H,CH_3CH_2CO
4	CH_3	H,CH_3CO			

Fig. 1. Structure of aromatase inhibitors 1, 3 and 5 and their N-acetyl derivatives (2, 4 and 6, respectively). Compound 7 was used as the internal standard for the HPLC assay. 1 = 3-(4′-Aminophenyl)-3-ethylpyrrolidine-2,5-dione; 2 = the acetylated metabolite of compound 1; 3 = 3-(4′-aminophenyl)-3-ethyl-1-methylpyrrolidine-2,5-dione; 4 = the acetylated metabolite of compound 3; 5 = 3-(4′-aminophenyl)-3-ethylpiperidine-2,5-dione (5 = aminoglutethimide); 6 = the acetyl metabolite of 5; 7 = 3-(4′-propanamidophenyl)piperidine-2,5-dione (internal standard).

at 245 nm, an LDC 301 computing integrator with Commodore SFD 1001 disc drive. The mobile phase (water–methanol–acetonitrile, 67:24:9, v/v/v) was filtered (Millipore type HA; 0.45-μm filter) before being delivered at a flow-rate of 1.25 ml/min. Cross-contamination of samples was eliminated by using alternate wash-vials containing mobile phase.

Materials and methods

Compounds 1 and 3 were synthesised according to the procedure described by Daly et al. [4]. Aminoglutethimide was a gift from Ciba-Geigy (Horsham, U.K.). Compounds 2, 4 and 6 were prepared from their corresponding parent compounds (1, 3 and 5, respectively) by refluxing the amine with acetic acid–acetic anhydride (1:1, v/v) for 2–6 h and recrystallising from ethanol. The internal standard (compound 7) was synthesised by mixing a solution of compound 5 in ethyl acetate (2.32 g per 30 ml) with propionic anhydride (1.4 ml) and recrystallising from ethanol.

All solvents used were of HPLC grade (Rathburn Chemicals, Walkburn, U.K.). For the biological work, laboratory bred ten-week-old female Wistar rats (200–250 g) were employed. Food but not water was withheld from the animals 16 h prior to experimentation. A single oral dose (25 mg/kg) of either compound 1 or compound 3 suspended in carboxymethyl cellulose (1%, w/v) and Tween 80 (0.1%, v/v) was administered. Blood was collected, 3 h after dosing, by cardiac puncture

under diethyl ether anaesthesia. It was placed immediately in heparinised tubes and centrifuged at 700 *g* and 4°C for 15 min. The separated plasma was stored frozen at −20°C until analysed. Tissues from animals receiving compound 1 were quickly dissected out and a 20% (w/v) homogenate in 0.1 *M* acetate buffer (pH 5.6) prepared and frozen until analysed. Urine was collected from four animals placed in pairs in two all-glass metabolism cages (Metabowl, Jencons) over a 72-h period following a dose (25 mg/kg) of compound 1. The concentrations of compounds 1 and 2 in the urine collected were then determined.

Biological fluids and tissue homogenates were also prepared from control rats that had not received any of the compounds. Aliquots of these samples were spiked with known amounts of compounds 1–6 dissolved in methanol (one compound per aliquot) and they were employed to determine the recovery of the compounds when the assay procedure was applied to the biological specimens.

Extraction

The frozen plasma and urine samples and the tissue homogenates were thawed at room temperature and 500-μl aliquots transferred to 12-ml solvent-resistant disposable test tubes each containing 100 μl of compound 7 in methanol as internal standard (stock solution of 10 μl/ml for plasma and homogenates, and 200 μg/ml for urine). To this, 500 μl of 0.1 *M* acetate buffer (pH 5.6) were added and mixed well before the addition of 5 ml of dichloromethane (DCM). The extraction was carried out using a Spiramix roller mixer (No. 10, Denley, Billinghurst, U.K.) for 1 h. After centrifugation (700 *g* for 5 min), the DCM layer was transferred to a clean test tube and evaporated under a stream of nitrogen. The dried residue was reconstituted by the addition of 500 μl of mobile phase and shaken well for 1 min on a vortex mixer. An aliquot (250 μl) of this solution was transferred to an autosampler vial (03-CV, Chromacol) from which 100 μl were injected automatically onto the column. The amount of compound (1–6) was determined by measuring the ratio of the AUC to that of the internal standard and reading off the corresponding value from the standard calibration curve.

RESULTS AND DISCUSSION

No endogenous component either of human plasma or of rat plasma, urine and tissue homogenate was found to interfere with this assay at the sensitivities employed. Fig. 2C and D present typical chromatograms for extracts of plasma and urine, respectively, from rats dosed with compounds 1 and 3 and also of plasma (Fig. 2E) from a breast cancer patient receiving treatment with compound 5. The presence of compounds 2 and 4 in the plasma and urine samples (Fig. 2C and D, respectively) indicates that N-acetylation of compound 1 and 3 is a route of metabolism of these compounds in the rat. In the urine extract from the rat receiving compound 3 the presence of compounds 1 and 2 indicates that demethylation of compound 3 occurs.

The retention times were 6.2, 7.7, 9.5, 12.4, 8.1, 10.1 and 15.2 min for compounds 1–7, respectively. Table I shows the inter-assay coefficients of variation ($n=4$) for the determination of compounds 1 and 2 in plasma (0.2–10 μg/ml for

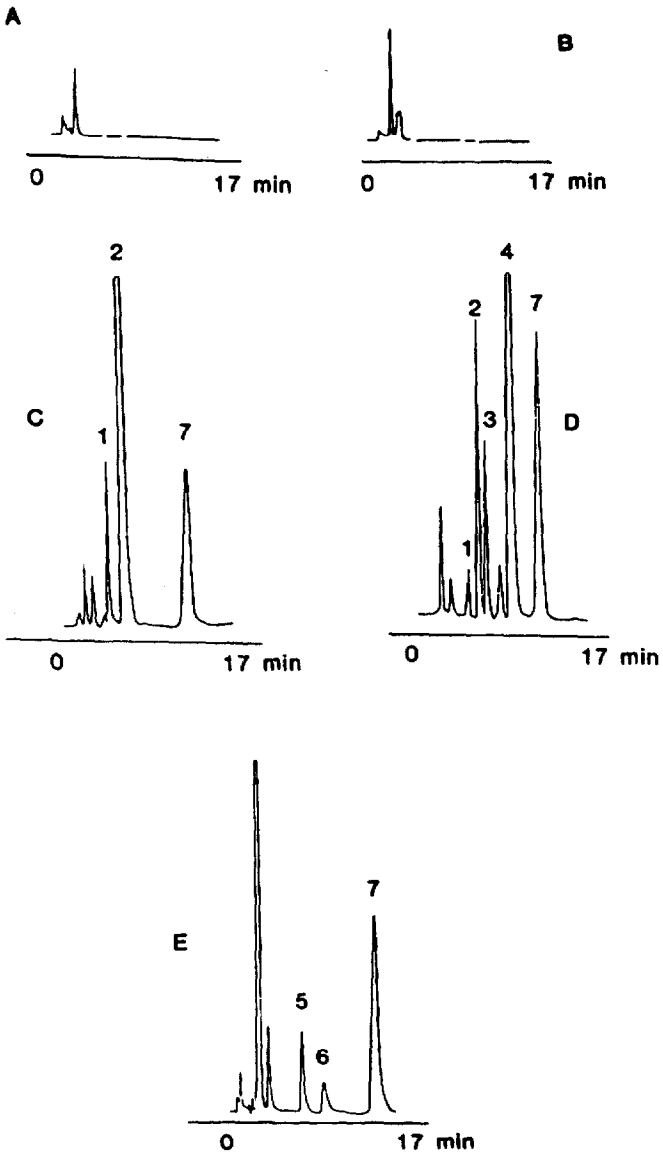

Fig. 2. Chromatograms of plasma and urine extracts which run from left to right over 0–17 min with monitoring at 245 nm. Numbers of peaks refer to compounds 1–7, respectively, which are identified in Fig. 1. (A) Extract of rat plasma (control); (B) extract of rat urine (control); (C) extract of plasma from rat treated with compound 1; (D) extract of urine from rat treated with compound 3; (E) extract of plasma from a patient receiving compound 5 (aminoglutethimide).

compound 1, 0.5–40 μg/ml for compound 2) and urine (1–80 μg/ml for compound 1 and 4–400 μg/ml for compound 2).

The calibration curves were linear for all six compounds in both plasma and urine over the concentration ranges studied.

In the recovery experiments with spiked samples the concentration range was

TABLE I

INTER-ASSAY COEFFICIENTS OF VARIATION FOR DETERMINATION OF COMPOUNDS 1 AND 2 IN RAT PLASMA AND URINE

Values are mean ± S.E.M. of four determinations. AUC ratio = AUC compound 1/AUC compound 2. C.V. = coefficient of variation.

Plasma			Urine		
Concentration (μg/ml)	Mean AUC ratio	C.V. (%)	Concentration (μg/ml)	Mean AUC ratio	C.V. (%)
Compound 1					
0.2	0.06	8.9	1.0	0.01	12.0
0.5	0.11	5.5	4.0	0.04	4.5
1.0	0.21	5.7	10.0	0.14	7.4
1.5	0.36	7.5	20.0	0.29	2.7
2.0	0.53	6.8	40.0	0.53	5.7
5.0	1.26	10.7	60.0	0.82	4.4
10.0	2.65	2.9	80.0	1.0	5.3
Compound 2					
0.5	0.22	5.2	4.0	0.09	9.5
1.0	0.40	1.8	10.0	0.24	8.8
2.0	0.89	7.9	40.0	0.91	3.3
5.0	2.31	10.5	100.0	2.46	6.5
10.0	3.93	13.0	200.0	4.65	5.3
20.0	7.90	1.8	300.0	6.70	5.3
40.0	16.37	3.2	400.0	8.52	7.4

0.2–20 μg/ml for compounds 1, 3 and 5 in tissue homogenates and plasma, and 1–80 μg/ml for urine. For compounds 2, 4 and 6 the range was 0.5–40 μg/ml for homogenates and plasma, and 4–400 μg/ml for urine. For each compound quadruplicate assays at each of seven concentrations in these ranges were employed. As for each compound there were no significant ($P > 0.05$) differences between the mean recoveries at each concentration, the results presented in Table II for percent recovery are the means ± standard error of the means (S.E.M.) of the pooled data for each compound in each type of biological specimen studied.

The mean recoveries for the six compounds in plasma ranged from 75 to 85%, the values for compounds 1 and 2 being 75.0 ± 4.9 and 75.4 ± 2.6, respectively. The recovery of these compounds from tissue homogenates was in the range 47–92%, the lowest being 47.0 ± 2.9% for the extraction of compound 5 and the highest being 92.8 ± 0.8% for the extraction of compound 4, both from the homogenate of kidney.

The minimum level of detection for all compounds was 40 ng per 100 μl of injection in plasma and homogenates and 800 ng per 100 μl for urine (because of high levels of endogenous compounds in the latter case). Table III shows the amounts of compounds 1 and 2 in various tissues related to the sites of action in the rat ($n = 4$) 3 h after a single oral dose of compound 1. The concurrent plasma

TABLE II

RECOVERIES OF COMPOUNDS 1-6 FROM VARIOUS BIOLOGICAL SAMPLES

Tissue homogenates and body fluids from rats were spiked with the compounds before extraction and assay. For compounds 1, 3 and 5 the concentration range was 0.2-20 μg/ml for homogenates and plasma, and for urine the range was1-80 μg/ml; with compounds 2, 4 and 6 the range was 0.5-40 μg/ml for homogenates and plasma, and 4-400 μg/ml for urine. For each compound quadruplicate assays at each of seven concentrations in the above ranges were employed to determine the recovery. Values are the means ± s.e.m. being calculated from the 28 determinations.

Sample	Recovery (%)					
	1	2	3	4	5	6
Adrenal	62.2 ± 1.6	81.0 ± 0.9	66.5 ± 2.6	82.6 ± 1.2	65.2 ± 2.7	89.3 ± 1.8
Brain	78.8 ± 1.1	75.9 ± 2.6	75.4 ± 1.3	83.7 ± 1.5	79.0 ± 1.4	75.0 ± 3.0
Fat	83.3 ± 1.5	80.4 ± 0.9	78.7 ± 2.1	90.8 ± 1.4	82.1 ± 3.8	84.8 ± 1.9
Kidney	53.3 ± 2.1	84.8 ± 1.3	55.7 ± 2.3	92.8 ± 0.8	47.0 ± 2.9	84.6 ± 2.4
Liver	60.6 ± 2.5	81.2 ± 1.4	54.1 ± 1.8	90.8 ± 1.8	52.2 ± 2.1	85.7 ± 1.3
Ovary	72.0 ± 3.4	79.1 ± 2.6	74.8 ± 1.8	88.1 ± 1.4	76.1 ± 2.4	82.1 ± 1.6
Plasma	75.0 ± 4.9	75.4 ± 2.6	76.3 ± 2.1	82.7 ± 2.1	77.4 ± 3.3	85.0 ± 1.9
Urine	78.0 ± 4.0	82.4 ± 1.9	81.4 ± 4.2	88.4 ± 1.2	84.4 ± 3.5	86.5 ± 2.3

levels and urinary elimination of compounds 1 and 2 are also shown. The results demonstrate that acetylation is a pathway for the metabolism of compound 1 and that the acetylated metabolite is widely distributed throughout the body tissues.

The assay has also been used for the determination of compounds 5 and 6 in the plasma of eleven breast cancer patients under treatment with compound 5. The peak plasma level of compound 5 (1.4 ± 0.3 μg/ml, mean ± S.E.M.) was observed 3 h after a single oral dose (250 mg) at the commencement of therapy. The 3-h plasma level after a single dose (250 mg) following a daily regime of 250

TABLE III

CONCENTRATION OF COMPOUNDS 1 AND 2 IN VARIOUS TISSUES OF RATS 3 h AFTER AN ORAL DOSE OF COMPOUND 1 (25 mg/kg)

For the assay, tissues were homogenised in acetate buffer (pH 5.6) and extracted with DCM as described in the text. Values are means from four rats.

Biological sample	Concentration (μg/g of tissue)	
	Compound 1	Compound 2
Adrenal	4.6	14.0
Brain	0.8	3.8
Fat	0.2	2.2
Kidney	0.8	12.8
Liver	3.9	12.2
Ovary	0.7	12.0
Plasma*	1.4	18.6
Urine**	247	7323

*μg/ml.

**Total amount (μg) excreted in urine over 72 h.

mg three times a day for three months had risen to 3.6 ± 1.2 μg/ml. This indicates a degree of accumulation of compound 5. The corresponding plasma levels for compound 6 were 0.9 ± 0.2 and 1.6 ± 0.6 μg/ml, respectively.

The assay procedure described above should be readily applicable to all tissues and biological fluids and this would enable a complete study of the pharmacokinetic profile of these compounds in the rat and other species. If either compound 1 or 3 is successful in reaching clinical trials, the method developed here would be invaluable for pharmacokinetic and dispositional studies in man.

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