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MEASUREMENT OF AROMATISATION BY A URINE TECHNIQUE SUITABLE FOR THE EVALUATION OF AROMATASE INHIBITORS *IN VIVO*

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By modification of a recently developed method for separation of radio-labelled urinary oestrogens we were able to separate oestrogen metabolites and measure their isotope ratios in urine following injections of [^{3}H]Δ^4-androstenedione and [^{14}C]oestrone. This method provides a useful tool for studying *in vivo* aromatisation of Δ^4-androstenedione into oestrone in breast cancer patients before and during treatment with aromatase inhibitors.

KEY WORDS: Aromatase, aromatase inhibition, Hplc, breast cancer, oestrogens.

INTRODUCTION

Endocrine therapy for breast cancer is generally aimed at eliminating oestrogenic stimulation of the tumour cell. This can be achieved by one of two treatment modalities: blocking of oestrogen synthesis, or administration of anti-oestrogens.

In postmenopausal women, peripheral aromatisation of androgens, mainly androstenedione ($\Delta^4 A$) into oestrone (E_1), is the major pathway of oestrogen synthesis[1]. Aromatase inhibition is an important endocrine treatment modality for advanced breast cancer, and several new aromatase inhibitors are in preclinical or early clinical stages of development[2–8]. Measurement of serum oestrogen levels provides only indirect information about the production rate of oestrogens and the inhibition of this production. Due to low levels of circulating oestrogens in postmenopausal women, it is difficult to compare the efficacy of different drugs by measuring their effect on serum oestrogen levels alone due to problems related to method sensitivity[9]. In addition, serum oestrogen levels could be modified by alterations in oestrogen metabolism as well as changes in oestrogen production rate[10]. It is therefore essential to be able to measure and compare aromatase inhibition by isotopic tracer studies *in vivo* for each new aromatase inhibitor considered for clinical use.

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To evaluate the impact of these different drugs on *in vivo* aromatisation it is important to have a sensitive method, since only 1–3% of circulating Δ^4 *A* is aromatised into E_1^9. The methods applied so far have all had several disadvantages. Serum measurement methods have a poor sensitivity because of the limited amount of blood which can be sampled. Previous urine methods for measuring aromatisation have used multiple solvent partition and chromatographic purification steps to isolate single metabolites. Conflicting results have been published on whether an identical isotope ratio is found in different urine metabolites[9]. Thus, a straightforward urine method which measures the isotope ratio is different excretory metabolites would be the method of choice.

One of the authors has previously reported a method to separate different urine oestrogen metabolites by HPLC[11]. In this paper we report how this method may be modified to separate dual-labelled (^{3}H and ^{14}C) urine oestrogen metabolites in the presence of high concentrations of labelled androgens.

MATERIALS AND METHODS

The study protocol was approved by the hospital ethical committee and informed consent was obtained from all patients.

Women who were to receive treatment with different aromatase inhibitors for advanced breast cancer were injected with [6,7-^{3}H] Δ^4A (Ciba-Geigy 500 μCi, 41 Ci/mmol) and [4-^{14}C]E_1 (NEN 5 μCi, 50 mCi/mmol) dissolved in 50 ml saline containing 8% ethanol. Three aliquots (50 μl) were retained for counting later. Urine was collected for 4 days and pooled, aliquots were frozen and stored at -20°C until analysis.

The procedure for purification and separation of the urinary oestrogens is shown diagramatically in Figure 1.

Urine samples (800 ml) were thawed, centrifuged, and the supernatant was removed and mixed with sodium acetate buffer (80 ml, 1.5 M, pH3). The sample was then absorbed on Sep-Pak C18 cartridges (16 in parallel per sample), washed with sodium acetate (10 ml, 0.15 M, pH3) buffer and eluted with methanol (3 ml for each cartridge). The methanol eluents were pooled and the methanol evaporated. Water (10 ml) was added to the residue and the glucuronides were isolated on a DEAE Sephadex column (Pharmacia, 3 cm diameter × 6 cm length) and eluted by a salt concentration gradient as previously described[11]. The glucuronide fractions were pooled, acetate buffer (1.5 M, pH3) was added (10% by volume in proportion to the sample) and the sample was concentrated on 4 Sep-Pak C18 cartridges in parallel followed by elution with methanol as described above. The methanol fractions were pooled, evaporated and the sample was redissovled in sodium acetate buffer 0.1 M pH4 with 1 ml (144,000 units) of β-glucuronidase (Sigma, G-8885). Hydrolysis was performed at 37°C for 48 h, whereafter the sample was extracted 3 times with equal volumes of ether. The ether extracts were pooled, the solvent was evaporated, and the sample redissolved in 1 ml of methanol.

The samples were purified prior to HPLC in a two or three step process as follows:

System 1

DEAE Sephadex was washed successively with water and then methanol (25%, 50% and 75% v/v) for 1 h each and stored at 4°C until use[12]. Columns (1 cm × 6 cm) were

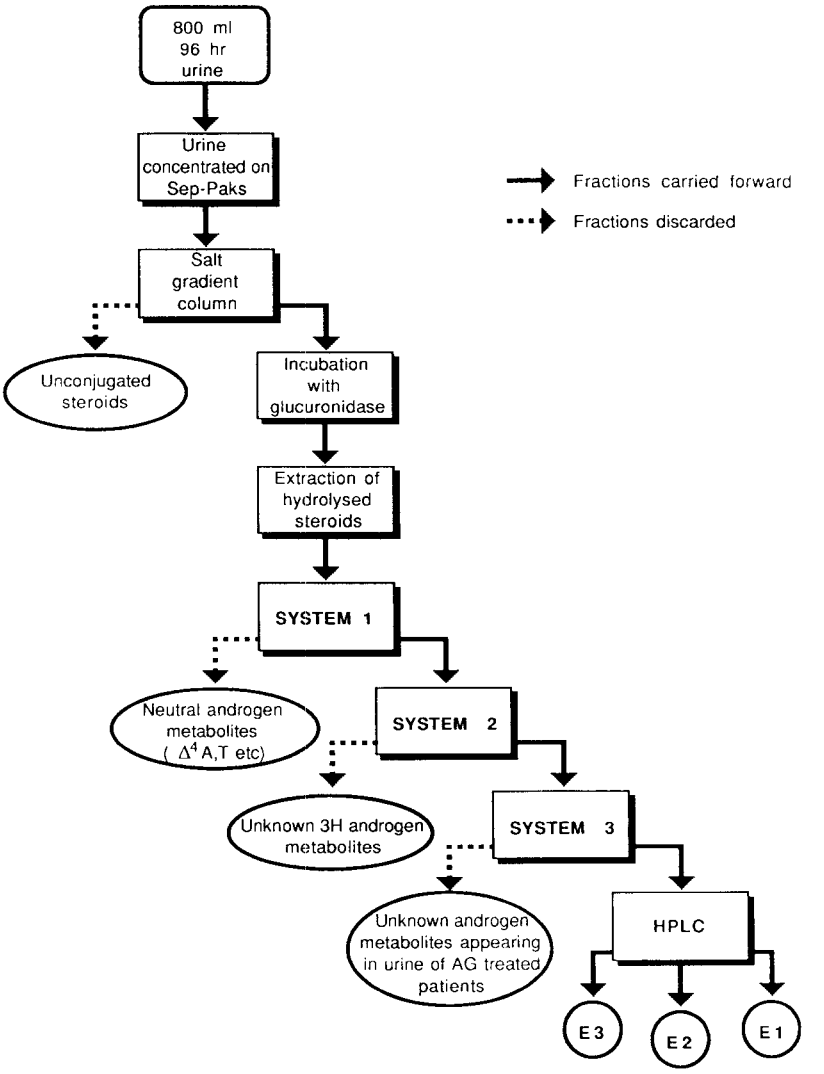

FIGURE 1 Flow diagram of the method for the purification and separation of radio-labelled oestrone, oestradiol and oestriol.

washed with 30 ml of ethanol/0.1 M NaOH (75:25) followed by 20 ml of methanol. The sample was loaded onto the column in 1 ml + 1 ml of methanol. The column was then eluted with another 14 ml of methanol followed by 22 ml methanol/acetate 0.5 M, pH3 (99.5:0.5). Fractions (2 ml) were collected and from these 50 μl aliquots were counted on a beta counter (Packard Tri-Carb 1900CA) to give a radiochromatographic profile of the eluates (Figure 2a.). In this system, neutral steriods (Δ^4A and testosterone and most of their metabolites) were eluted in the first 8 ml of the methanol fraction and the oestrogens in the methanol/acetate fractions. After elution

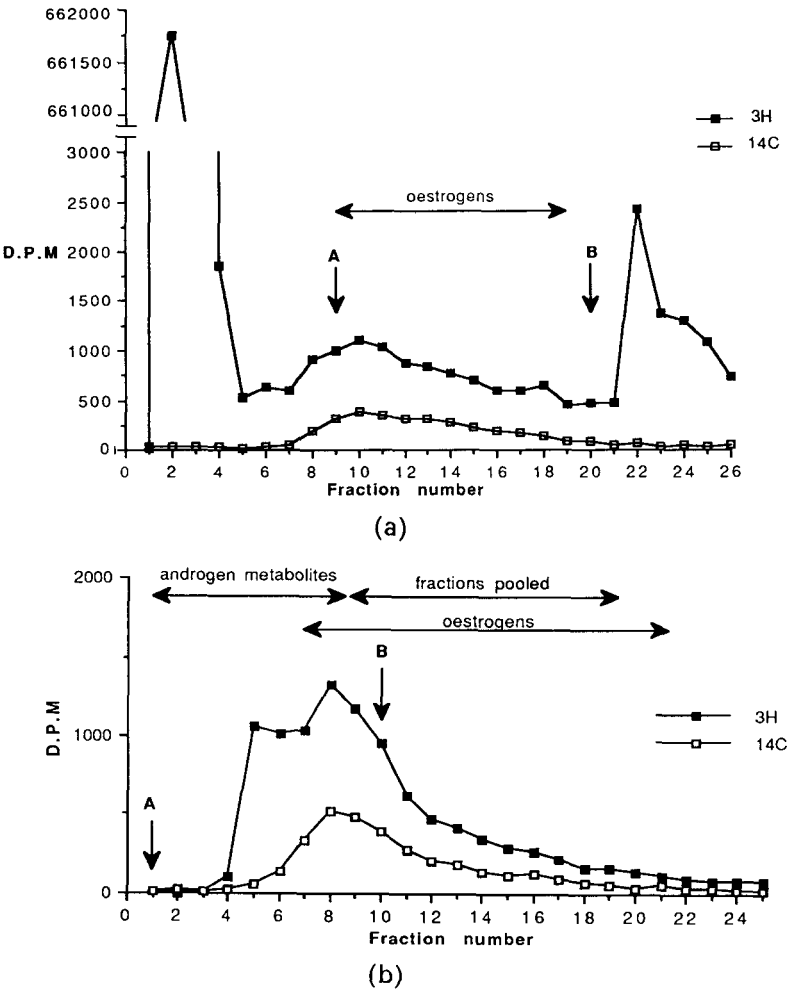

FIGURE 2 (a) Radiochromatogram of system 1. After elution of [^{3}H] androgens by methanol the oestrogens are flushed from the column with methanol/acetate buffer 0.5 M, pH3 (99.5:0.5) (A). Further [^{3}H]-metabolites with no corresponding [^{14}C] activity were eluted by acetate buffer 0.3 M, pH3 (B).
(b) Radiochromatogram of system 2. Washing the column with methanol/acetate buffer (0.5 M, pH12 (25:75)) (A) elutes [^{3}H]-metabolites. The oestrogens (shown by a constant [^{3}H]/[^{14}C] ratio are eluted with (B) methanol/acetate buffer (0.05 M, pH9.5 (25:75))

of the oestrogens, unidentified [^{3}H]-labelled metabolites with no corresponding [^{14}C]-radioactivity were eluted from the column by acetate buffer (30 ml, 0.3 M, pH3). A minor amount of non-identified [^{3}H]-labelled metabolites were eluted together with the oestrogen fraction in this system. Most chromogenic compounds were retained on the column. The fractions containing the oestrogens were pooled and the solvent was evaporated on a rotavapor. The sample was redissolved in acetate buffer (0.15 M, pH3), concentrated on a Sep-Pal C18 column and eluted with methanol as described.

System 2

Columns (1 cm × 5.5 cm) were made up from DEAE Sephadex washed with methanol/acetate buffer (0.05 M pH12 (25:75)). The samples was redissolved and applied to the column in 1 ml methanol/acetate buffer (0.05 M, pH12 (25:75)). The column was eluted by another 9 ml of this solution, followed by 16 ml methanol/acetate buffer (0.05 M, pH9.5 (25:75)). Fractions (1 ml) were collected and 50 μl aliquots taken and counted to obtain a radiochromatographic profile of the eluate (Figure 2b). In this system, the unidentified [^{3}H]-labelled metabolites were eluted in front of the estrogen fraction. Again, this chromatogaphic system provided a further separation of the oestrogen fraction from chromogenic compounds. Some oestrogen (eluted with [^{3}H]-labelled metabolites) was discarded to ensure increased purity in the retained portion.

System 3

This system is similar to the one developed by Fotsis and Adlercreutz for separating vicinal oestrogens from other oestrogen metabolites[13]. Briefly, QAE Sephadex (Pharmacia, Sweden) is swollen in water for 24 h, whereafter it is washed with methanol in increasing concentrations (20%, 50% and 100% v/v) for 1 h each. The Sephadex gel is converted to its borate form by washing for 1 h with 0.1 M sodium hydroxide in methanol (70% v/v), methanol (70% v/v), 0.5 M boric acid in methanol (70% v/v) and finally stored in methanol (70% v/v) at 4°C. Columns were prepared in a pasteur pipette, plugged with glass wool, to a height of 2.5 cm. The column was flushed with methanol (10 ml), the sample reconstituted and applied in 0.5 ml of methanol and the oestrogens eluted from the column with 2 ml methanol. This system was used to get rid of unidentified tritium-labelled androgen metabolites which appeared in the urine of patients on aminoglutehimide therapy.

Different oestrogens and oestrogen metabolites were separated by reverse phase HPLC on a Hypersil ODS 5 μm (Chrompack) 4.6 × 250 mm column using acetonitrile/phosphate buffer (0.05 M, pH3, 35:65). To confirm the purity of the oestrogen peaks a purified sample was also run in a normal phase HPLC system (5 μm Apex 2 diol column, Jones Chromatography, 4.6 × 250 mm) with chloroform: isoocaten: n-hexane (50: 11: 39) as the mobile phase. The [^{3}H]/[^{14}C] ratios in the oestrogen peaks from the two systems were then compared[11].

CALCULATIONS

^{3}H and ^{14}C activity in each fraction from the HPLC were counted on a Packard Tri-Carb 1900CA beta counter and expressed as D.P.M. Percentage aromatisation was calculated for each of the three oestrogens E_1, E_2 and E_3 as below.

$$\% \text{ Aromatisation} = \frac{[^3\text{H}/^{14}\text{C}] \text{ Oestrogen peak ration}}{[^3\text{H}/^{14}\text{C}] \text{ Injection ratio}} \times 100$$

$$\% \text{ Inhibition} = 100 - \left[\frac{\% \text{ Aromatisation (On treatment)}}{\% \text{ Aromatisation (Pre treatment)}}\right] \times 100$$

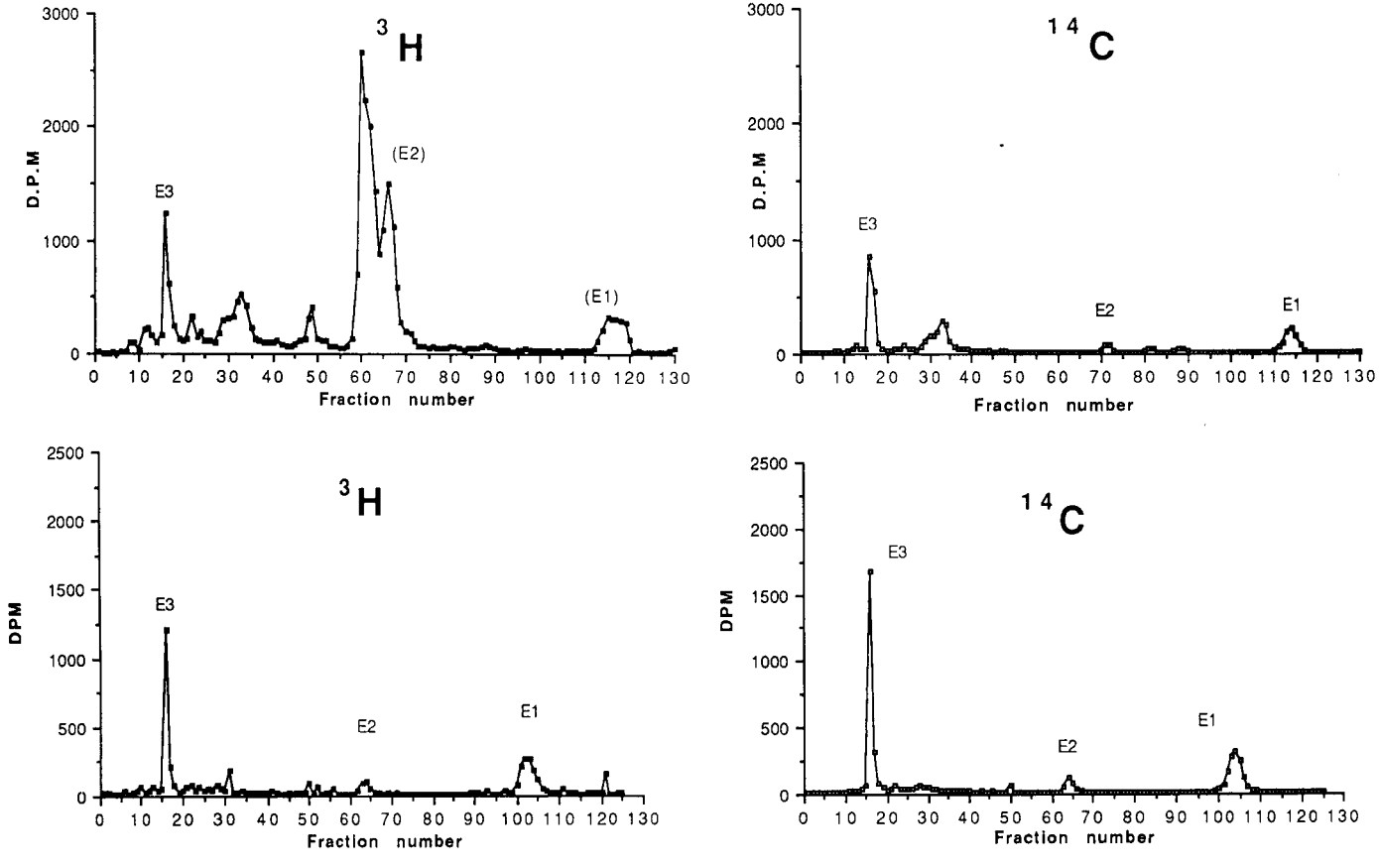

FIGURE 3 (a)Radiochromatogram of urine from an untreated patient following purification of the extract by use of system 1 with elution of the oestrogens with acetate buffer 0.3 M, pH3 and no further purification. There are [^{3}H]- contaminations in the (E_1) and (E_2) (and to a lesser extent E_3) fractions.

(b) Radiochromatogram of urine from an untreated patient following purfication of the extract by use of system 1 with elution of the oestrogens with methanol/acetate buffer 0.5 M, pH3 (99.5:0.5) followed by purification in system 2.

RESULTS

Using non-radioactive standards it was found that Δ^4A and testosterone (T) had retention times which partly overlapped E_1 and oestradiol (E_2) in the HPLC system. Thus, it was necessary to eliminate androgens by anion exchange chromatography before HPLC.

The task of purifying urine samples before HPLC was different for patients investigated during treatment with the aromatase inhibitor CGS 16949A compared to the situation from patients treated with aminoglutethimide. Thus, the results from urine samples obtained in patients on aminoglutethimide therapy are presented separately.

Measurement of urinary oestrogen metabolites in urine from patients not receiving aminoglutethimide therapy

A simple separation of neutral steroids and oestrogens was found to be insufficient. If during system 1 (an anion exchange column) androgens were eluted by methanol and the oestrogen fraction by 0.3 M, pH3 acetate buffer instead of the methanol/acetate mixture, unidentified [^{3}H]-labelled metabolites with retention times close to and contaminating the fractions of E_2 and oestriol (E_3) were found (Figure 3a). In contrast, if the oestrogen fraction was eluted in stage 1 by methanol/acetate buffer 0.5 M pH3 (99.5;0.5) and further purified through system 2, the HPLC chromatograms gave no indication of contamination of any of the three "classic" oestrogens (Figure 3b). The chromatograms presented here, are representative of all the samples tested.

While there was corresponding [^{3}H]/[^{14}C] radioactivity in fractions between E_3 and E_2 (16-OHE$_1$,, 16-Epi-E_3 etc) no attempts were made to purify these metabolites with other HPLC-systems[2]. The amount of radioactivity corresponding to catechol oestrogens (eluted between E_3 and E_2) was very low, because no attempts were made to preserve them by adding ascorbic acid during the analytical steps[11].

Purity of the oestrogen peaks were confirmed as follows: (a) No change in the isotope ratios were found following repeated chromatography on the DEAE Sephadex columns. (b) When isolated by different HPLC systems a constant [^{3}H]/[^{14}C] ratio in the oestrogen fractions E_1 and E_2 was found (Figure 4). (c) The isotope ratio did not change if the sample was further purified through system 3.

The last of these was investigated in a patient treated with the aromatase inhibitor CGS 16949A (1 mg b.d.). Urine samples (obtained immediately prior to treatment and after one month on treatment) were analysed both with and without purification by system 3. Aromatase inhibition was measured to be 86.8% and 83.5% for E_3 and E_1 respectively, and 85.4% and 83.5% after purification in system 3.

The reproducibility of the method for measuring aromatase inhibition was estimated in urine from one patient. The analysis was repeated and for each oestrogen the results varied by less than 5%: aromatase inhibition was measured to be 74.6% and 70.7% for E_3 and E_1 respectively, and 74.2% and 72.2% for the repeat analysis. In this patient, as in some others, the peak of $^3HE_2$ in the on treatment sample was too low to be quantified. In addition the variation in the [^{3}H]/[^{14}C] ratio was less than 5% between the 3 different oestrogens when isolated from the same urine sample.

The magnitude of aromatisation in the first six patients investigated was between 1.5% and 3.3% before treatment with an aromatase inhibitor.

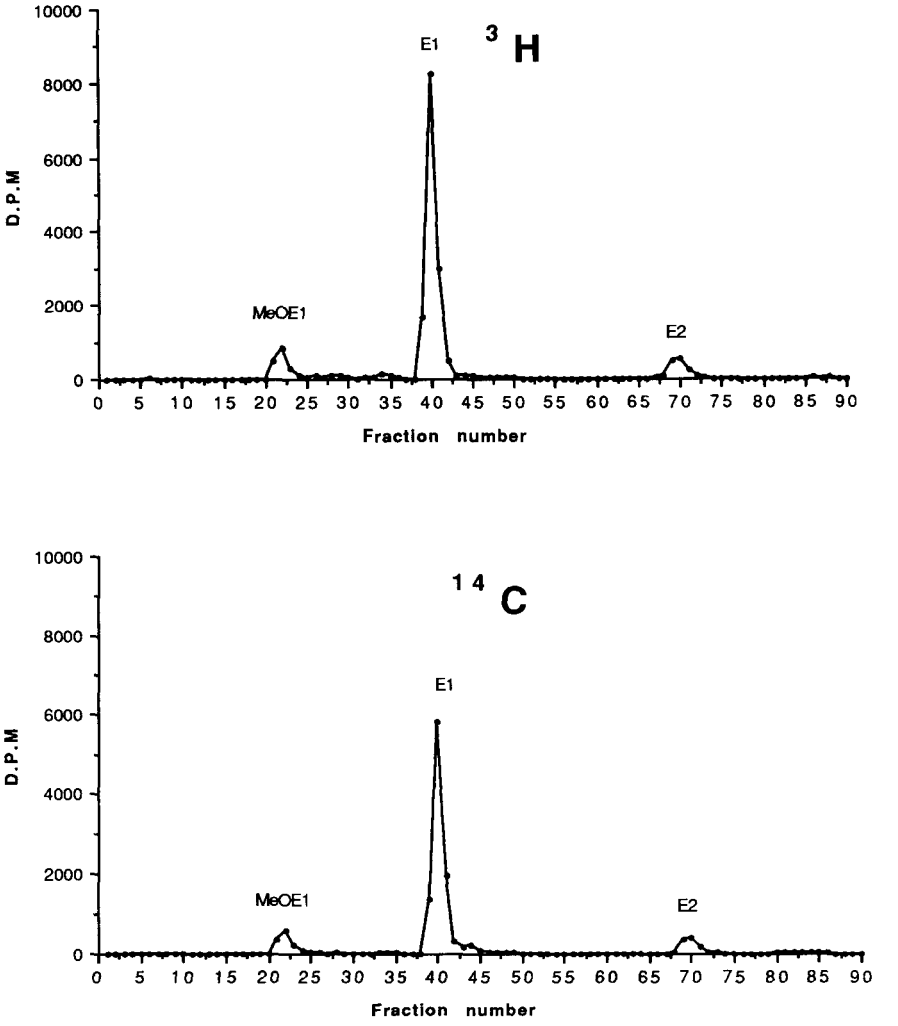

FIGURE 4 Radiochromatograms of urine following purification by normal phase HPLC. E_3 elutes very late in this system.

Measurement of urinary oestrogen metabolites in urine from patients receiving aminoglutethimide therapy

When urine samples were obtained from patients on treatment with aminoglutethimide, radiochromatograms indicated the presence of impurities following purfication in system 1 and 2, as there were peaks of ^{3}H which were not associated with ^{14}C activity (Figure 5a). Thus, aminoglutethimide seems to induce production of certain androgen metabolites which co-chromatograph with the oestrogen fraction. When the sample was purified by system 3 as well as systems 1 and 2, all ^{3}H peaks were associated with corresponding ^{14}C activity in the area of E_3, E_2, and E_1 (Figure 5b).

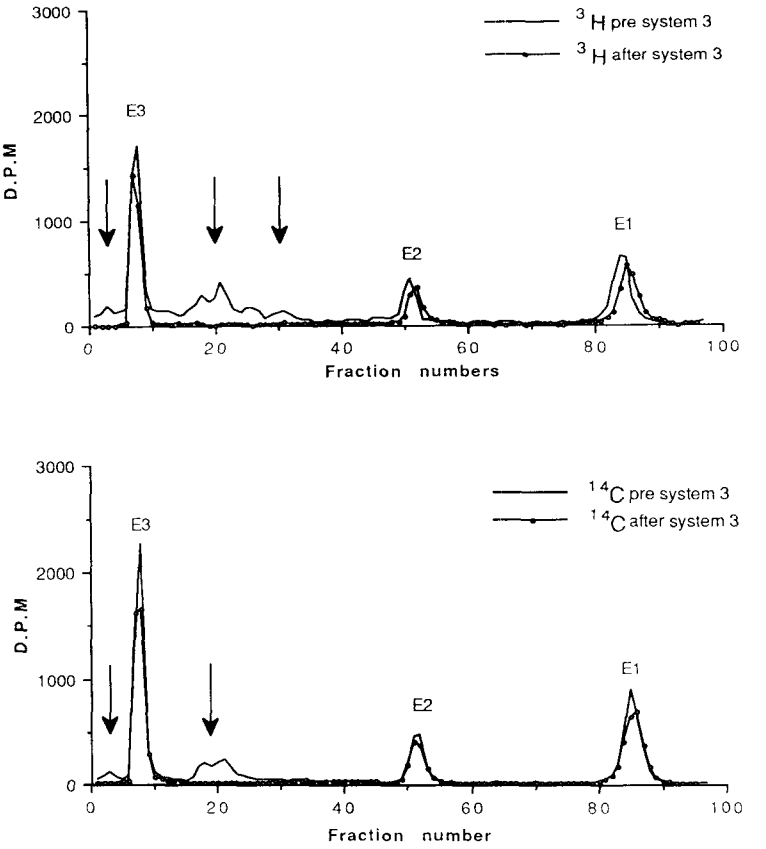

FIGURE 5a and 5b Radiochromatograms of urine from a patient treated with aminoglutethimide. One half of the extract was purified by system 2 but not system 3 (pre system 3). The other half was subjected to additional purification by systems 3 (after system 3). ^{3}H activity not assocaited with ^{14}C is indicated by ↓.

DISCUSSION

Over the next few years several new aromatase inhibitors will be introduced in the clinic for treatment of advanced breast cancer. In phase I/II investigations, biochemical evaluation of drug efficacy should be conducted in concert with toxicity measurement and early response rate evaluation to select suitable drugs for further trials. While it is essential to measure the suppression of serum oestrogen levels, it is important to confirm the underlying mechanism whereby this occurs. Recent findings[14] that the "prototype" aromatase inhibitor aminoglutethimide also accelerates oestrogen metabolism emphasise the need for proper radioactive tracer studies to assess oestrogen disposition during therapy with different drugs.

The finding that certain androgen metabolites were eluted with the oestrogens during anion chromatography has not been described previously; currently, work is being undertaken to identify these compounds. So far we have little information on

their nature other than that from their behaviour in the chromatography systems. While it might be somewhat surprising that these metabolites have not been identified in previous studies of androgen metabolism, the amount of [^{3}H] radioactivity corresponding to such metabolites is at the same level as the amount of [^{3}H] corresponding to oestrogen metabolites. Thus, these metabolites together may account for only a small percentage of Δ^4A metabolism. The interesting finding that certain androgen metabolites seem to be induced during aminoglutethimide treatment may be due to this drug's ability to act as a potent hepatic microsomal inducer[10]. Aminoglutethimide therapy has been reported to change urinary androgen excretion in man[15]. The fact that these metabolites could be separated by a column system designed for the separation of vicinal oestrogens suggests that these metabolites could be polyhydroxylated substances.

This paper reports a suitable method for studying aromatisation *in vivo*. It allows separation of E_1, E_2 and E_3 with good reproducibility. The HPLC system used in this investigation also separates catechol oestrogens, while certain metabolites such as 16α-OHE_1 and 16-Epi-E_3 could be separated in other systems[11]. Radioactivity corresponding to the catechol oestrogen fractions was very low. It is likely these metabolities were destroyed during the analytical steps because no antioxidants were used[11]. It was impossible to include antioxidants such as ascorbic acid as they would intefere in the anion exchange chromatography steps.

Conflicting results have been reported as to whether an identical isotope ratio is found in different urinary oestogen metabolites after injection of tracer Δ^4A and E_1[9]. This hypothesis can be evaluated by measuring the isotope ratio in the E_1, E_2 and E_3 fractions. Oestrogen metabolism occurs by two main pathways, hydroxylation of the steroid nucleus in the 2- or 16α-position[16], producing 2-OHE_1 and E_3 as its main metabolites respectively. The two pathways are competitive. Thus, if there is any significant difference in isotope ratio related to oestrogen metabolism, this should be reflected in a different isotope ratio between E_3 and the other two oestrogens. In our study, the ^{3}H/^{14}C ratios in the three oestrogens isolated from the same urine varied by less than 5%.

To our knowledge, reproducibility has not been reported for any of the methods previously used for aromatisation studies *in vivo*. Aromatase inhibitors have been reported to cause more than 90% inhibition of conversion of Δ^4A into E_1[17]. It is therefore essential to have a method of high sensitivity and reproducibility to study aromatisation under such conditions. The method described here meets these requirements with the amount of radioactivity collected in each estrogen fraction before treatment equal to 2000–4000 DPM of each isotope. Theoretically, aromatase inhibition could be measured reproducibly up to 99%.

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