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Simple approach to measure metabolic pathways of steroids in living cells[a]

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ABSTRACT

A simple, rapid approach to the study of conversion rates and metabolic patterns of the steroids testosterone and estradiol is presented. It includes an optimized isocratic high-performance liquid chromatographic procedure in the reversed-phase mode and radioactive on-line detection. The purpose was to estimate the activity of key enzymes of steroid pathways, such as 17β-hydroxysteroid dehydrogenase and 5α-reductase, in *in vivo* conditions. Using this system, we obtained good efficiency and linearity of radio detection, under continuous flow conditions. Sensitivity limits were of the order of 50 and 70 cpm for [^{3}H]estradiol and [^{14}C]estrone, respectively, even though the efficiency was quite dissimilar (17.3% *versus* 56.2%). The applicability of this approach to studies of steroid metabolic pathways in growing cancer cells in culture is illustrated with examples of the conversion rates of both testosterone and estradiol. The high reproducibility (coefficients of variation of 2.7 and 5.1% for ^{3}H and ^{14}C, respectively) and good extraction efficiency (ranging from 86 to 94%) indicate the feasibility and reliability of this approach.

INTRODUCTION

In recent years much attention has been focused on peripheral steroid metabolism in target tissues [1]. These hormones represent the main growth factors for

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the so-called "endocrine-related" tumours, accounting for more than 30% of all human neoplasias, which orginate from epithelial target cells. It has been previously observed that steroids accumulate in normal and, to a greater extent, in neoplastic target tissues [2,3].

The main enzymes involved in steroidogenesis have been extensively studied; they have been in turn solubilized and purified to homogeneity. Most of their molecular structures and actions have been clarified, and some have also been recently cloned and their amino acid sequences deduced [4,5].

However, despite the large amount of accumulated data, we are not able to elucidate functions nor to attribute a meaningful biological role to many enzymic activities involved in metabolic pathways of steroids. For instance, it is still uncertain whether 17β-hydroxysteroid dehydrogenase (17β-HSD), which also governs the interconversion of estrone (E_1) and estradiol (E_2) and then can strongly affect the proliferative activity of epithelial cells, represents a single reversible enzymic activity or is composed, as already suggested for other enzyme systems, of two distinct, independently modulated, components [6,7].

Another example in point is the 5α-reductase enzyme which is responsible for the conversion of testosterone (T) into its more biologically active form, dihydrotestosterone (DHT). It has been hypothesized, but only partially confirmed, that two different 5α-reductase isoenzymes exist, one in the epithelial and the other in the stromal compartment of target tissues, such as the prostate [8,9].

In addition, separate studies on several non-steroidal enzyme systems, which have become available in recent years, have suggested that it is unlikely that the *in vivo* enzyme activity can be predicted by using data obtained from studies of crude extracts [10].

We have adopted a simple and rapid procedure, which uses high-performance liquid chromatography in the reversed-phase mode (RP-HPLC) and "on-line" radioactive (RA) detection, as already applied to different substrate–enzyme systems [11–13] and extensively reviewed [14], in order to: (a) evaluate the overall activity of 17β-HSD and 5α-reductase enzymes in living cancer cells — during log-phase growth — more than their potential activity in cell-free systems, as previously studied with the classical enzymology approach [15,16]; (b) focus attention on the epithelial cells, which are subject to much cancer development; (c) quickly compare hormone responsive with unresponsive cancer cells to ascertain their different abilities in metabolizing steroids.

This approach is unique in that it allows the use of close to physiological concentrations of precursor(s), the evaluation of several, more than single, enzyme activities, and the extension of observation times compared with those commonly used in classical enzymology studies.

EXPERIMENTAL

Materials

All the unlabelled standards, estrogens and androgens (Table I), were from Sigma (St. Louis, MO, USA).

The radioactive steroids used as precursors, [6,7-^{3}H]E_2 (specific activity 60 Ci/mmol), [4-^{14}C]E_1 (specific activity 55 mCi/mmol) and [1,2,6,7-^{3}H]T (specific activity 82 Ci/mmol), were obtained from Amersham (Little Chalfont, UK).

The Ultrasphere ODS column (250 mm × 4.6 mm I.D., particle size 5 μm) was from Beckman (Fullerton, CA, USA).

Ascorbic acid, sodium ascorbate, diethyl ether, acetone, HPLC-grade acetonitrile and HPLC-grade water were from Farmitalia Carlo Erba (Milan, Italy).

TABLE I

SYSTEMATIC NAMES, TRIVIAL NAMES, ABBREVIATIONS AND RELATIVE RETENTION TIMES OF ESTROGENS AND ANDROGENS

Systematic name	Trivial name	Abbreviation	Relative retention time
Estrogens			
1. 1,3,5(10)-Estratriene-3,16α,17β-triol	Estriol	E_3	0.23
2. 1,3,5(10)-Estratriene-3,16α-diol-17-one	16α-Hydroxyestrone	16αOH-E_1	0.34
3. 1,3,5(10)-Estratriene-2,3,17β-triol	2-Hydroxyestradiol	2OH-E_2	0.45
4. 1,3,5(10)-Estratriene-3,4,17β-triol	4-Hydroxyestradiol	4OH-E_2	0.49
5. 1,3,5(10)-Estratriene-2,3-diol-17-one	2-Hydroxyestrone	2OH-E_1	0.57
6. 1,3,5(10)-Estratriene-3,4-diol-17-one	4-Hydroxyestrone	4OH-E_1	0.66
7. 1,3,5(10)-Estratriene-3,17β-diol	17β-Estradiol	E_2	0.88
8. 1,3,5(10)-Estratriene-3,4,17β-triol 4-methyl ether	4-Methoxyestradiol	4MeO-E_2	0.96
9. 1,3,5(10),7-Estratetraen-3-ol-17-one	Equilin	Eq	1.00
10. 1,3,5(10)-Estratriene-2,3,17β-triol 2-methyl ether	2-Methoxyestradiol	2MeO-E_2	1.07
11. 1,3,5(10)-Estratrien-3-ol-17-one	Estrone	E_1	1.18
12. 1,3,5(10)-Estratriene-3,4-diol-17-one 4-methyl ether	4-Methoxyestrone	4MeO-E_1	1.29
13. 1,3,5(10)-Estratriene-2,3-diol-17-one 2-methyl ether	2-Methoxyestrone	2MeO-E_1	1.40
Androgens			
1. 5-Androstene-3β,17β-diol	Androstenediol	Delta5A-diolo	0.53
2. 4-Androsten-17β-ol-3-one	Testosterone	T	0.63
3. 5-Androsten-3β-ol-17-one	Dehydroepiandrosterone	DHA	0.82
4. 4-Androstene-3,17-dione	Androstenedione	Delta4A-dione	0.87
5. 5α-Androstan-3β-ol-17-one	Epi-androsterone	Epi-A	1.00
6. 5α-Androstan-17β-ol-3-one	Dihydrotestosterone	DHT	1.10
7. 5β-Androstan-3α-ol-17-one	Etiocholanolone	Et	1.12
8. 5α-Androstan-3α-ol-17-one	Androsterone	A	1.50
9. 5α-Androstane-3,17-dione	5α-Androstanedione	5α-A-dione	1.57

Ready-Gel and Ready-Flow scintillation cocktails were purchased from Beckman.

Dulbecco modified eagle medium (DMEM), fetal calf serum (FCS), L-glutamine, streptomycin–penicillin, trypsin (0.25%, w/v)–EDTA (0.02%, w/v), Biggers-BJG and mycoplasma stain kit were obtained from Flow Labs. (Rickmansworth, UK).

Well established human cancer cell lines, derived from endometrium (HEC-1A and Ishikawa) and prostate (PC3 and DU-145), were purchased from the American Tissue Culture Collection (ATCC) (Rockville, MD, USA) or as otherwise stated (see Acknowledgements).

Apparatus

Glass–glass homogenizers were from Kontes Scientific (Vineland, NJ, USA). A refrigerated benchtop centrifuge, Model 100-2 KTi, with a swing-out rotor (r_{max} 11.5 cm) and tube holders was acquired from Runne Zentrifugen (Heidelberg, Germany). An LS-1801 beta-counter was obtained from Beckman Instruments (Irvine, CA, USA).

The chromatographic apparatus used was a Beckman Model 324 HPLC system equipped with a 112A pump, a sample injection valve (Model 210) and a UV detector (Model 160), set at 280 nm (for estrogens) or 214 nm (for androgens), an on-line (right after UV detector) Flo-One/beta (Model IC) three-channel radioactive detector (Radiomatic Instruments, High Wycombe, UK), with an interactive internal computer, a built-in liquid scintillator pump (set at 6 ml/min) and a liquid 2.5-ml flow-cell. Two counting channels are dedicated for radioactivity measurements (^3H and ^{14}C), a third counting channel is provided to accept and process an analogue signal from the UV, or any other detector. The system can handle data from all detectors simultaneously.

An ultrasonic solvent degasser and a FIAtron HPLC temperature-control system were from Biolabo Instruments (Milan, Italy).

High-performance liquid chromatography

Steroids were separated by RP-HPLC, using an Ultrasphere ODS column thermostatted at 20 ± 1.0°C, under isocratic conditions with a flow-rate of 1.0 ml/min. The following mobile phases were used: (a) 40% acetonitrile in 0.01 *M* citric acid, to separate the estrogen metabolites, with a total analysis time of 25 min; and (b) 45% acetonitrile in water, for androgen compounds, with a total analysis time of 30 min. All eluents were degassed before use in an Ultrasonic solvent degasser. In order to establish the optimal resolution in the minimum time, the mobile phase was optimized using a computerized simulation approach, as extensively described elsewhere [17–19]. The relative retention times obtained for estrogens and androgens are reported in Table I.

Integration of data was achieved using a FLO-ONE/beta F1B IC program (Radiomatic, Tampa, FL, USA). Routine integration was computed in net cpm,

which represent raw counts, corrected for both sample residence time (calculated as cell size to flow-rate ratio) and background subtraction. The latter was maintained at constant cut-off levels of 40 and 70 net cpm for ^{3}H and ^{14}C, respectively. In our hands, chemiluminescence phenomena were not observed when this chromatographic system was used.

Cell lines

Well established human cancer cell lines, derived from endometrium (HEC-1A and Ishikawa [20,21]) and prostate (PC3 and DU-145 [22,23]), were previously characterized for their hormone sensitivity [24–26] and responsiveness [22,23,27,28].

All cell lines were maintained in DMEM, supplemented with 10% FCS, 200 n*M* L-glutamine and antibiotics. Cells growing in the log phase were trypsinized in trypsin–EDTA solution (1:4, v/v) for 3–5 min, counted in a haemocytometer and subsequently seeded onto Petri dishes (diameter 60 mm) at a density of *ca.* $1 \cdot 10^6$ cells in fresh medium. After 24 h, the medium was replaced with 5 ml of phenol red and FCS-free Biggers medium in order to avoid any interference by cells due to both the presence of effective amounts of steroid hormones in FCS and the weak estrogenic activity exerted by phenol red at the concentrations commonly used in culture media [29,30]. To prevent oxidation of the hydroxylated compounds, sodium ascorbate was added to the medium (10 μg/ml final concentration), when appropriate.

Cell lines were routinely tested for both bacterial and mycoplasma contamination.

Metabolic studies in living cells

The experiments were carried out using either constant final concentrations of radioactive precursor, *i.e.* E_2 ($8.3 \cdot 10^{-8}$ *M*) and T ($4.8 \cdot 10^{-9}$ *M*) for *ca.* $1 \cdot 10^6$ cells, or constant precursor molarity/cell number ratio [24–26]. Each labelled steroid was checked and periodically purified by HPLC before metabolic experiments.

The formed metabolites were expressed as crude cpm or percentage conversion rate (%CR) or as pmol/cell number in relation to incubation time (30 min, 1.5, 3, 6, 12, 24, 72, 96 h).

Two controls per each cell line were set up to test both cell growth and viability.

Extraction procedures

At the end of the incubation period the medium was collected separately and cells were harvested in trypsin–EDTA. If separate extractions were to be performed, the cell sample was then resuspended in an equal volume of fresh medium. Prior to manipulation of samples, all the glassware to be used for the extraction was precoated with 4 μg of the same radio-inert steroid used in the

incubation in order to prevent adhesion to the walls. Cell plus medium or cell samples were disrupted in glass–glass homogenizers. For estrogen extraction, only in catechol–estrogens studies, 10 mg/ml ascorbic acid was added to lower the pH below 4.0 and to prevent oxidation of these metabolites. The temperature was kept constant at 4°C. A 1-ml aliquot of cell homogenate was transferred to a scintillation vial for reading the total radioactivity per ml processed (TR). The homogenate was then spun at 1000 *g* for 5 min. The supernatant was collected in a separate tube and used for extraction. The pellet was resuspended in 1 ml of distilled water and transferred to a scintillation vial (P) for reading radioactivity still bound to cell structures.

Aliquots of supernatant (three 1-ml volumes) were sampled into glass scintillation vials (A). For estrogen extraction, 10 ml of diethyl ether–acetone (9:1, v/v) were added. In order to facilitate androgen extraction using 10 ml of diethyl ether, the pH of the aqueous phase was raised to *ca.* 10.0 by addition of 20 μl of 2 *M* sodium hydroxide.

Samples were thoroughly mixed for a few seconds and allowed to stand for 5 min in a water-bath at 4°C. The aqueous phase was then removed from the glass vial and put in another one for counting the non-extracted radioactivity (B). The ether phase was evaporated to dryness under a stream of nitrogen; the residue was then removed from the vial with three 500-μl volumes of acetone and transferred to a test-tube, dried again with nitrogen, and resuspended in 30 μl of acetonitrile. A 20-μl aliquot was used for HPLC analysis, and 5 μl were used to determine the total extracted counts (C).

The percentage extraction efficiency (%EE) was estimated as follows:

$$\%EE = [cpm(C) \times 6] \times 100 / \{cpm(A) + cpm(B) + [cpm(C) \times 6] + cpm(P)\}.$$

The read-out of vial (TR) was used as an interna! quality control.

RESULTS AND DISCUSSION

We tested the performance of the RA detector "on line", before applying it to the study of metabolic pathways of steroids. Fig. 1a and b illustrate the linearity of response of the radio detector when tested with $[^3H]E_2$, in the range 0.7–184 pg (regression equation: $y = 1.824 + 0.968x$; correlation coefficient = 0.994, $p < 10^{-6}$ Pearson's test) and with $[^{14}C]E_1$, in the range 0.3–48.8 pg (regression equation: $y = 2.439 + 1.071x$; correlation coefficient = 0.999, $p < 10^{-6}$ Pearson's test), under the protocol chromatographic conditions.

The efficiency of radio detection under continuous-flow conditions is shown in Table II, where net cpm, experimentally detected by a single assay, are compared with expected dpm. Efficiency values under stopped-flow conditions, between 42% and 46% for ^3H, are not far from those observed for the LS-1801 beta-counter used (61%).

The combination of good linearity and efficiency in radio detection enables the

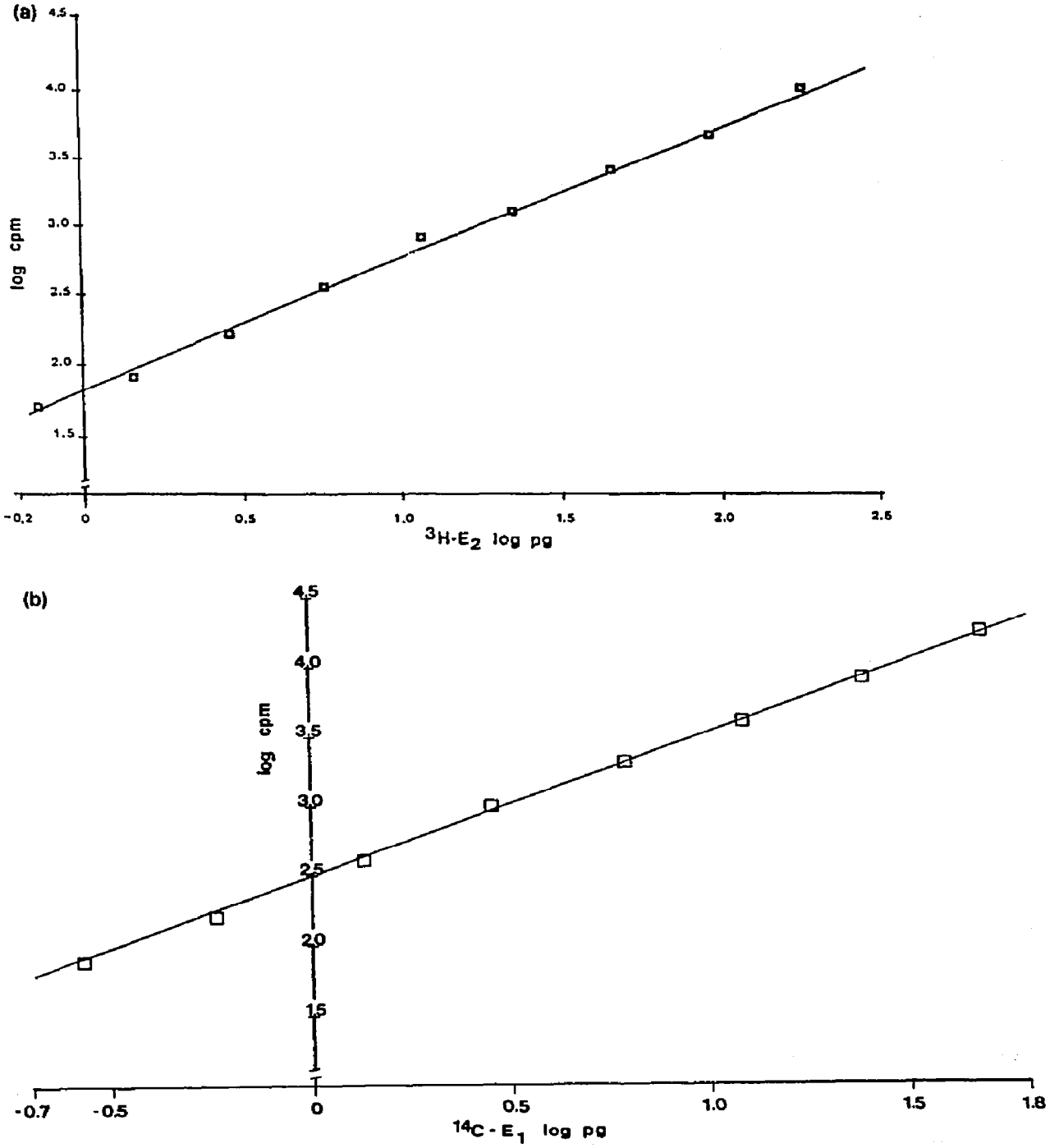

Fig. 1. Linearity of response of the on-line radiometric detector for (a) [^{3}H]E_2 and (b) [^{14}C]E_1.

detection of less than 2.5 fmol for pure [^{3}H]E_2 and less than 1.2 pmol for pure [^{14}C]E_1 standards, as reported in Table III; these values are compatible with the physiological concentrations of both estrogens (mainly E_2 and E_1) [31] and estrogen receptors [24,32]. The greater values obtained for the ^{14}C-labelled compound

TABLE II

EFFICIENCY OF TRITIATED ESTRADIOL DETECTION IN CONTINUOUS FLOW AT STANDARD CONDITIONS

dpm (theoretical)	cpm (observed)	Efficiency (%)
75 187	13 463	18
37 593	6125	16
18 798	3097	16
9318	1524	16
4699	987	21
2349	433	18
1174	192	16
587	81	14
294	52	18

must be attributed to its lower specific activity, despite the higher efficiency of radio detection.

This very low level of sensitivity has to be considered as an essential prerequisite for *in vivo* studies of the metabolic pathways of steroids, in order to expose cells to physiological concentrations of precursors.

The three-channel detector permits the simultaneous UV evaluation of cold internal standards. Consequently, it can confirm the identification of any single metabolite (Fig. 2).

Overall, as shown in Tables IV and V and illustrated in Fig. 3, the reproducibility appears to be satisfactory. It concerns in first place the radio detection of separately or simultaneously injected ^{3}H and ^{14}C isotopes (see Table IV); in second place the retention time (t_R) of either [^{3}H]E_2 (10.5 ± 0.1, C.V. = 0.6%)

TABLE III

DETECTION LIMITS OF β-RADIODETECTION ON-LINE TO RP-HPLC

Compound	Detection limit		Efficiency (%)
	mol	cpm	
[6,7-^{3}H]E_2	2.4[a]	51.3 ± 2.1[c]	17.3 ± 1.2[c]
[4-^{14}C]E_1	1.1[b]	70.2 ± 4.1[c]	56.2 ± 5.2[c]

[a] Femtomoles.
[b] Picomoles.
[c] Values represent means ± S.D. of six different analyses, processing Biggers-BJG medium spiked with pure radiolabelled compounds.

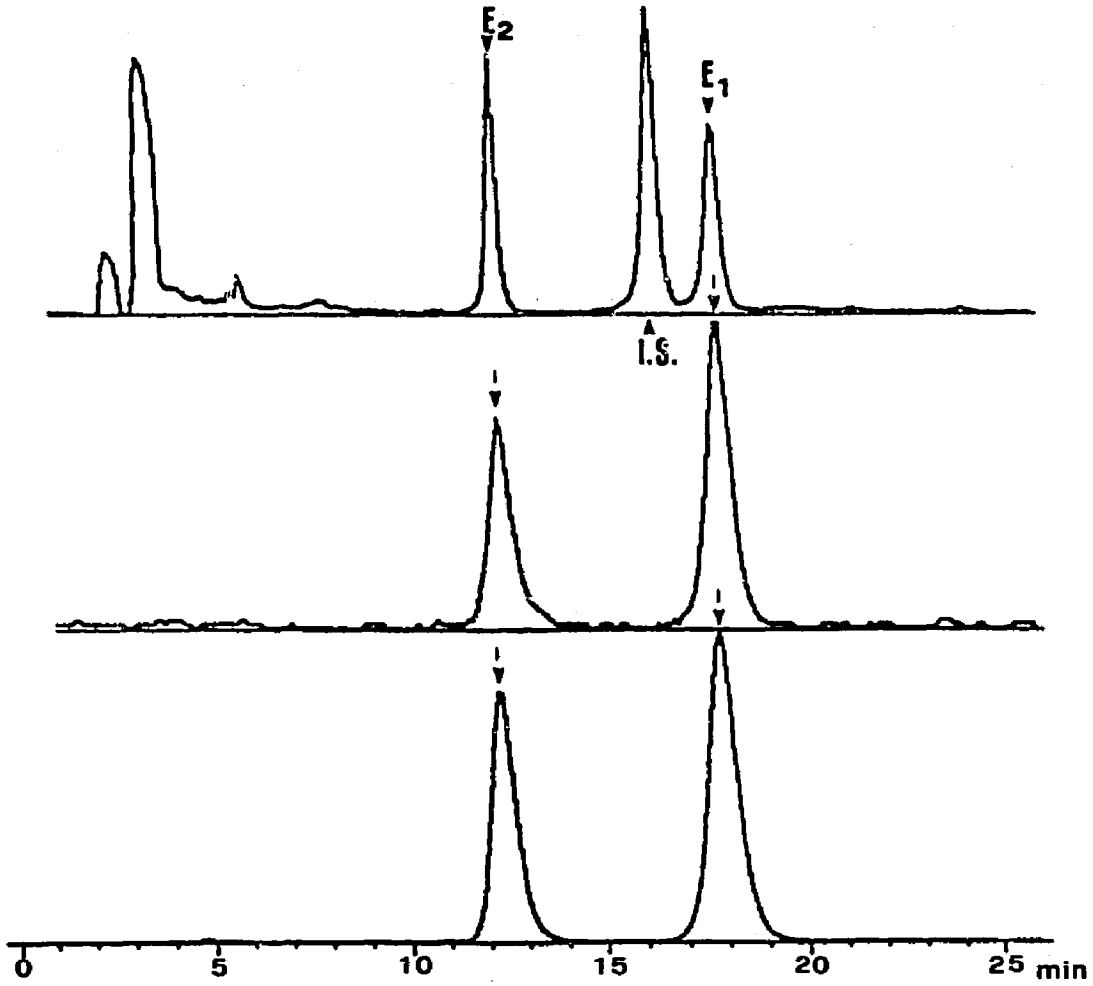

Fig. 2. Three-channel analysis of estrogen metabolic pathways from HEC-1A human endometrial cancer cell line after 24 h incubation. UV (top) and on-line radiometric detection of [^{14}C]E_1 (centre) and [^{3}H]E_2 (bottom) precursors. I.S. = equilin (internal standard).

TABLE IV

REPRODUCIBILITY DATA OF β-RADIODETECTION ON-LINE TO RP-HPLC (n = 7)

Compound	cpm								C.V. (%)
	1	2	3	4	5	6	7	Mean ± S.D.	
[6,7-^{3}H]E_2	30 598	31 030	30 759	32 343	31 097	32 040	29 896	31 109 ± 842	2.7
[6,7-^{3}H]E_2[a]	4727	4779	4636	4501	4530	4723	4827	4675 ± 124	2.6
[4-^{14}C]E_1[a]	4659	4949	4948	4861	5056	4985	4916	4911 ± 126	2.7
[4-^{14}C]E_1	406	420	411	432	454	463	418	429 ± 22	5.1

[a] Both isotopes were injected simultaneously.

TABLE V

RETENTION TIMES VARIATION OF SEVERAL ANDROGEN STANDARDS

Values represent retention times obtained from eight different UV profiles. T = testosterone; DHA = dehydroepiandrosterone; delta4A-dione = androstenedione; Epi-A = epiandrosterone; DHT = dihydrotestosterone; 5α-A-dione = 5α-androstanedione.

Experiment	T	DHA	Delta4A-dione	Epi-A	DHT	5α-A-dione
1	9.67	12.67	13.33	15.37	16.93	24.17
2	9.70	12.72	13.37	15.40	16.96	24.21
3	9.90	12.90	13.60	15.70	17.30	24.60
4	9.70	12.72	13.40	15.50	17.10	24.40
5	9.70	12.79	13.50	15.60	16.90	24.40
6	9.65	12.66	13.45	15.55	16.85	24.35
7	9.90	12.88	13.70	15.70	17.30	24.50
8	9.70	12.68	13.50	15.50	17.10	24.30
Mean	9.74	12.75	13.48	15.54	17.06	24.37
S.D.	0.10	0.09	0.12	0.12	0.18	0.14
C.V. (%)	1.03	0.71	0.92	0.79	1.01	0.60

and [^{14}C]E_1 (24.3 ± 0.1, C.V. = 0.4%) (Table IV) as well as that of pure androgens (see Table V).

In addition, the chromatographic procedure has been optimized for both estrogens [17,18] and androgens in order to achieve an optimal resolution for a proper identification and quantification of single metabolites, as shown by the UV profile of Fig. 3.

The spill-over of ^{14}C in ^{3}H was calculated by counting the [^{14}C]E_1 pure stan-

TABLE VI

TIME-COURSE OF E_1 PRODUCTION RATES FROM [^{3}H]E_2 IN HUMAN ENDOMETRIAL CANCER CELL LINES

Seven Petri dishes (five different observation times plus two controls) per cell line (just one experiment for any time-step) were set up simultaneously under the same experimental conditions. Control dishes were used for cell counting and viability at the beginning (time 0) and the end (24 h) of incubation.

Cell type	cpm				
	1.5 h	3.0 h	6.0 h	12 h	24 h
HEC-1A					
E_1	22 092	46 705	104 785	129 377	133 505
Total	(189 882)	(188 205)	(222 038)	(199 305)	(186 651)
Ishikawa					
E_1	3391	6793	21 182	39 027	61 893
Total	(178 205)	(186 681)	(230 073)	(325 249)	(283 914)

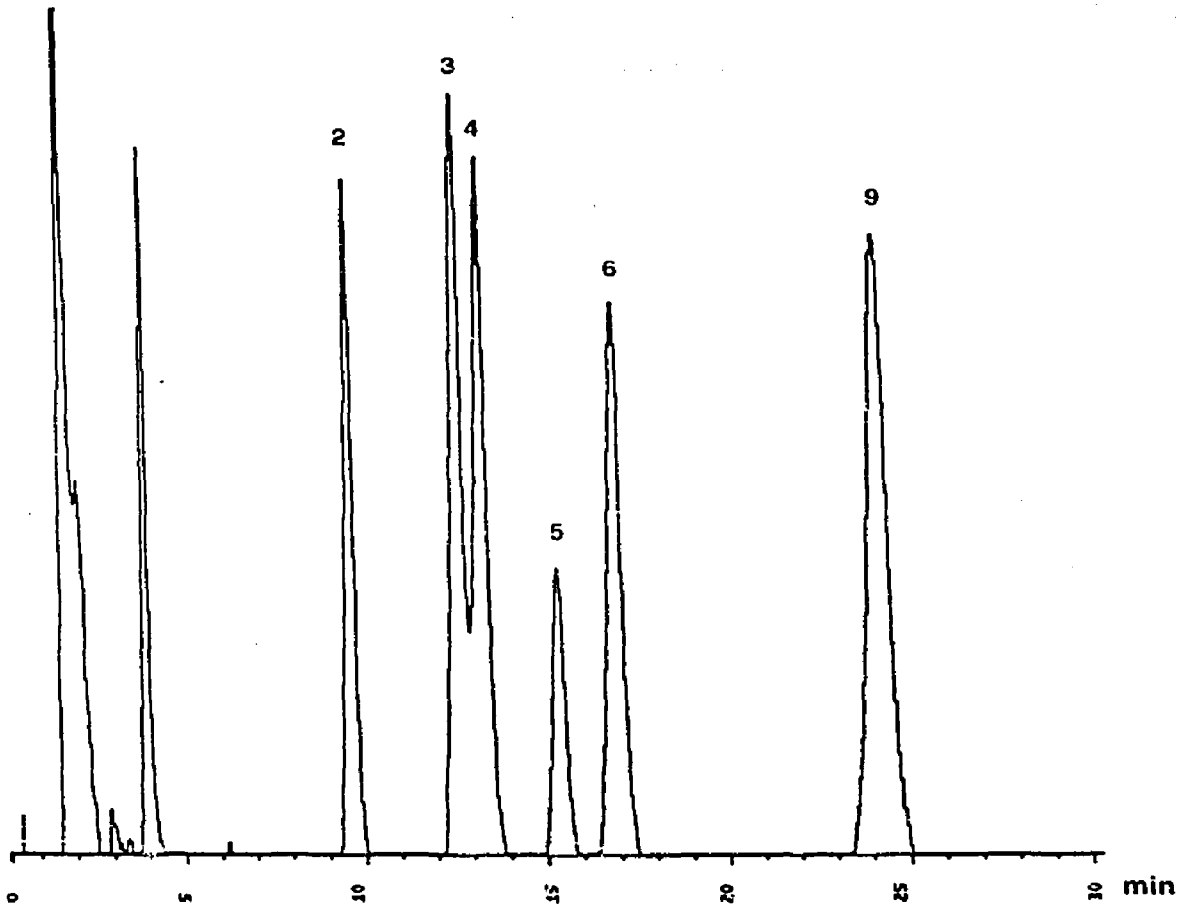

Fig. 3. UV profile of androgen standards in RP-HPLC. For peak identification numbers see Table I. DHA (peak 3) is used as internal standard.

TABLE VII

5α-REDUCED RADIOMETABOLITES OF [^{3}H]TESTOSTERONE BY HUMAN PROSTATE CANCER CELLS

Values represent mean ± S.D. of three different experiments at 24 h incubation with $4.6 \cdot 10^{-9}$ *M* [^{3}H]T. Values in parentheses are coefficients of variation (%). N.D. = not detectable.

Cell type	DHT		5α-A-dione	
	t_R (min)	Net cpm	t_R (min)	Net cpm
DU-145	17.23 ± 0.12 (0.7)	2225.0 ± 210.2 (9.4)	24.50 ± 0.10 (0.4)	398.0 ± 37.7 (9.5)
PC-3	–	N.D.	24.43 ± 0.06 (0.2)	6184.3 ± 157.8 (2.6)

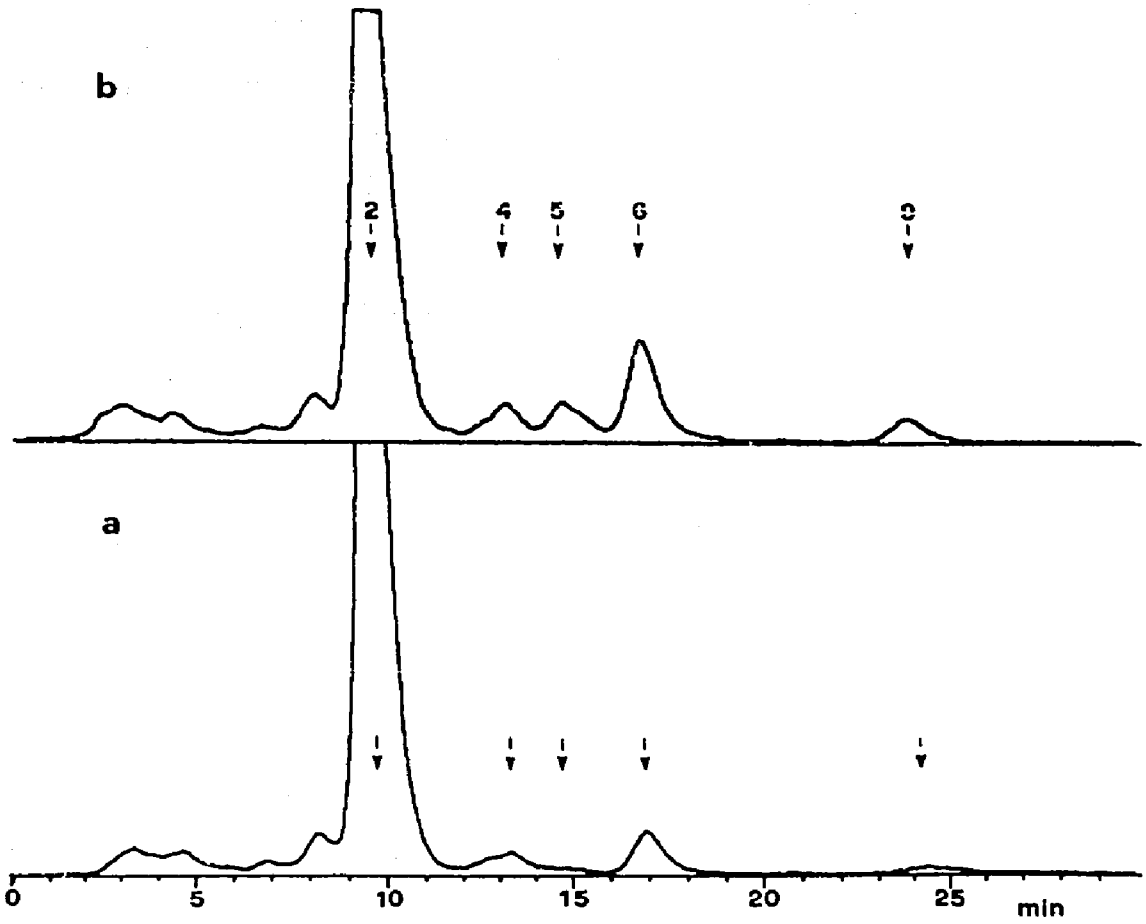

Fig. 4. Androgen metabolic profiles at (a) 24 and (b) 72 h by DU-145 human prostate cancer cell line, incubated with $9.2 \cdot 10^{-9}$ *M* [^{3}H]T. For peak identification numbers, see Table I. Net cpm and t_R values were: (a) 2 = 66 333 (9.70); 4 = 2998 (13.40); 5 = N.D.; 6 = 4019 (17.10); 9 = 1016 (24.40); (b) 2 = 57 437 (9.70); 4 = 3684 (13.40); 5 = 3838 (15.37); 6 = 8894 (17.00); 9 = 2542 (24.30).

dard in double-label counting mode and using the following formula: cpm in channel 1 (^{3}H)/cpm in channel 2 (^{14}C) × 100. It was fairly constant, given the isocratic conditions used, and was not higher than 12.5%, using a window setting of 1–40 for ^{3}H and of 51–100 for ^{14}C.

Extraction efficiency (EE) values, related to data shown in Tables VI and VII and illustrated in Figs. 2 and 4, were very close to those previously reported [24–26], ranging between 86 and 94% (mean 89%). Both Tables VI and VII report two series of experiments that studied the rate of conversion of E_2 and T by endometrial and prostate epithelial cancer cells.

As shown in Table VI, the time-course experiments on long-term epithelial cells from human endometrial adenocarcinomata, namely Ishikawa and HEC-1A cell lines, reveal different conversion rates of [^{3}H]E_2. In fact, non-hormone-responsive HEC-1A cells display very fast precursor degradation (close to 50%), the [^{3}H]E_2 almost matching at 6 h formed [^{3}H]E_1; conversely, in hormone-re-

sponsive Ishikawa, production of [^{3}H]E_1 does not attain these levels, being less than 10 and 22% at 6 and 24 h, respectively.

Table VII shows the conversion rates of [^{3}H]T by PC-3 and DU-145, human prostate epithelial cancer cells. It is evident that in DU-145, an androgen-receptor-positive cell , both products of 5α-reductase enzyme activity, namely the DHT and 5α-Adione, are present. Fig. 4 clearly shows that the amounts of both products increase between 24 and 72 h in DU-145 cells. This is true for several metabolites, including Epi-A.

In contrast PC-3, an androgen-receptor-negative cell line, shows only one product of 5α-reductase activity, namely 5α-Adione. Meanwhile, the amount of DHT formed, if any, is below the limit of detection; no evidence for other 5α-reduced products, such as androstanediols, has been obtained. Of course, any DHT formed could be quickly converted into 5α-Adione by 17β-HSD. This, however, reinforces the aforementioned experimental indications.

Concerning the formation of DHT, there is strong indication that the prostate epithelial cancer cell exhibits two distinct 5α-reductase activities [26]. Two enzyme forms were previously attributed to stromal and epithelial prostate cells, respectively [8,9,33]. Other studies defined the 5α-reductase behaviour as "non-michaelian", ascribing it to two different stabilized states of the same enzyme [34].

On the other hand, 17β-HSD activity may well privilege more oxidative pathways of E_2, as for HEC-1A with respect to Ishikawa cells, at least in the experimental conditions studied, which were close to physiological [24,25]. An oxidative metabolism of T prevails also in PC-3 cells with respect to DU-145 prostate cancer cells. Both of the above aspects can be hardly recognized using the crude extract approach.

The present methodology, in absolute terms, might be not original, nor the first to be used in the analysis of steroid enzymes. Nevertheless, it appears to be very promising for a new approach, at the same time alternative and complementary to classical enzymology, to the study of steroid metabolic pathways in *in vivo* conditions.

The most striking feature of this procedure is represented by the very low limits of detection attained. In terms of a more comprehensive evaluation, the simultaneous analysis of sequential enzyme activities is of interest for a better understanding of a given metabolic pathway. Among the advantages of such an approach, using radiolabelled precursors, are the potential use of double-labelling techniques and a more accurate identification and quantification of losses.

It is to be noted that our results on enzyme activity in *in vivo* conditions show some deviation from the prediction given by V_{max} studies and models obtained through classical enzymology, in that the observed 17β-HSD activity is much lower than expected in Ishikawa cells [35,36]. However, this discrepancy has been already observed in many other non-steroidal enzyme systems [10,15,16].

The data presented in this paper are an original contribution concerning enzymes involved in steroid metabolism, even though it has been previously shown

that a maximum enzyme activity established *in vitro* may well correspond to a minimum activity observed *in vivo* [38,39].

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