Journal of Chromatography, 570 (1991) 293–299
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6014

Isocratic high-performance liquid chromatographic measurement of optimal 5α-steroid reductase activity in Hep-G2 cells

A. P. STUBBS, G. M. MURPHY and M. L. WILKINSON*

Gastroenterology Unit, U.M.D.S. of Guy's and St. Thomas's Hospitals, Guy's Campus, London SE1 9RT (UK)

(First received February 13th, 1991; revised manuscript received May 21st, 1991)

ABSTRACT

Measurement of 5α-reductase activity usually involves quantitation of the radiolabelled products of [^{3}H]testosterone. Recently, however, it has been claimed that the activity of 5α-reductase is masked by the activities of 17β-hydroxysteroid dehydrogenase and 3-ketosteroid reductase. Therefore in determining 5α-reductase activity in Hep-G2 cells, we have monitored the concentration of androstenedione to ensure that the conditions for measurement of optimum enzyme activity are maintained. Using a polar (cyano) bonded-phase column and hexane–isopropanol (9:1, v/v) as eluent, the ratio of relative retention times (methyl lithocholate used as the reference standard) of the closest peaks, dihydrotestosterone and estradiol, was 1.2, whilst the highest inter-assay coefficient of variation was 2.7%. Therefore this technique appears suitable for the evaluation of 5α-reductase in cell and tissue samples.

INTRODUCTION

The presence of the enzyme 5α-reductase (5αR; EC 1.3.1.22), which catalyses the conversion of 4-androsten-17β-ol-3-one (testosterone; T) to the more potent androgen 5α-androstan-17β-ol-3-one (dihydrotestosterone; DHT) and thence other 5α-reduced metabolites (5α-androstan-3β,17α(β)-diol; DIOLS), is characteristic of androgen-responsive tissues [1,2].

There is an increasing amount of evidence, albeit indirect, that 5αR activity plays an important role in the activation of androgen receptors [2] and may be implicated in the actiology of hepatocellular carcinoma (HCC) [3]. Hep-G2 cells, which were originally isolated from a male Caucasian with HCC [4], contain androgen receptors [5], but it is unknown whether they contain 5αR.

The optimum pH for 5αR has been extensively characterised [6] and found to lie between 5.5 and 7.4, however, at pH 5.5 and higher, conversion of T to 4-androstene-3,17-dione (androstenedione; AD), 1,3,5(10)-estratrien-3-ol-17-one (estrone; E1) and 1,3,5(10)-estratrien-3,17β-diol (estradiol; E2) also occurs [6] (Fig. 1). This conversion of T to products other than DHT means that the steady-state assumptions necessary for the accurate measurement of enzyme activity

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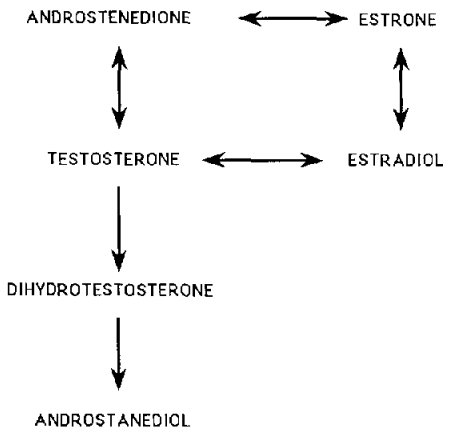

Fig. 1. Pathway of testosterone metabolism in the hepatocellular carcinoma cell line (Hep-G2).

may not apply. Therefore, it is essential when determining 5αR activity to monitor the conversion of T to AD, E1 and E2. The conversion of T to the DIOLS is taken as being a measure of 5αR since the precursor to these steroid products is DHT.

To measure 5αR in Hep-G2 cells we have used isocratic normal-phase high-performance liquid chromatography (HPLC) which, compared with isocratic reversed-phase HPLC, has the advantage of isolating both androgens and oestrogens from the same sample.

EXPERIMENTAL

Materials

All chemicals were of the highest purity. [1,2-α-^{3}H]Testosterone (specific activity 1.96 TBq/mmol) was obtained from Amersham International (Aylesbury, UK) and T, DHT, E2, E1, AD, 5α-androstane 3α(β),17β-diols, NADPH (disodium salt) and ethanol (HPLC grade) were all obtained from Sigma (Poole, UK). N,O-Bis(trimethylsilyl)trifluoroacetamide (BTSFA) was obtained from Pierce (Chester, UK), Tris–HCl, sucrose, $MgCl_2 \cdot 6H_2O$ and KCl from BDH (Poole, UK), diethyl ether (HPLC grade) and isopropanol (HPLC grade) from Aldrich (Poole, UK) and hexane (HPLC grade) from Rathburn (Walkerburn, UK).

Cell culture

Hep-G2 cells were obtained from the American Type Culture Collection (Rockville, MD USA) and routinely cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum at 95% humidity and 5% CO_2 in a Leec MkII proportional temperature incubator.

Enzyme assay

Cells at confluence were harvested at 4°C, then spun at 110 *g* (IEC Centra 4 × centrifuge) for 5 min. The resultant pellet was homogenised at 4°C in Tris–sucrose (5 m*M* Tris–HCl, 250 m*M* sucrose, 5 m*M* $MgCl_2 \cdot 6H_2O$ and 25 m*M* KCl), pH 7.4. The homogenate was stored as 125-μl aliquots at −70°C until being defrosted at 4°C before use at 37°C. Each incubation tube contained 2 m*M* NADPH, 12.6 n*M* [1,2-^{3}H]testosterone and either 0.1, 0.5, 1, 5 or 10 μ*M* unlabelled T or 126 n*M* [1,2-^{3}H]testosterone and 20 μ*M* unlabelled T, the reaction being started by the addition of 100 μl of homogenate and performed at 37°C. The reaction was stopped after 15 min by the addition of 3 ml of ice cold diethyl ether, vortexed and the organic phase removed. The incubate was subject to organic extraction with 3 ml of diethyl ether twice more, the combined organic phases were pooled and evaporated to dryness and then reconstituted in 100 μl of ethanol and stored at −20°C.

Chromatography

Products stored in ethanol at −20°C were evaporated to dryness at 37°C under nitrogen and reconstituted in 60 μl of hexane–isopropanol (9:1, v/v), the mobile phase for subsequent HPLC, containing the internal standard, 2.56 m*M* methyl lithocholate (MLCA), 6.8 m*M* DHT, 0.096 m*M* E2, 42.7 μ*M* T, and 0.85 μ*M* AD. These products were separated on a Hi-Chrom (Reading, UK) cyano bonded-phase 250 mm × 5 mm I.D. column (5 μm), with a mobile phase of hexane–isopropanol (9:1, v/v) and flow-rate of 1.0 ml/min. Ketosteroids (T, AD and DHT) and steroids with unreduced A rings (E1 and E2) were detected using an ACS 210 UV detector measuring absorbance at 215 nm. The effluent was collected in 0.166-ml aliquots, in a Gilson fraction collector linked directly to the UV detector. A 4-ml volume of Quickszint 2000 was added to each aliquot before counting in an LKB 81000 liquid scintillation counter for 5 min.

Gas chromatography–mass spectrometry (GC–MS)

Positive identification of steroids, after isolation by normal-phase HPLC, was achieved by derivatisation with BTSFA followed by GC–MS on a Hewlett-Packard P5670 bench-top quadrapole GC–MS system with a HP1 column with a temperature gradient of 0 150°C (10°C/min).

RESULTS

Resolution of standards is shown in Fig. 2a and retentions (relative to MLCA) of the closest peaks (DHT and E2) gave a ratio of 1.2 indicating that all known peaks were efficiently separated. That precise isolation of selected androgens and estrogens can be achieved by this method is substantiated by the data for intra- and inter-assay variability (Table I) from which the maximum coefficient of variation is 2.7%. The addition of sample whilst adding a "UV-absorptive" front had

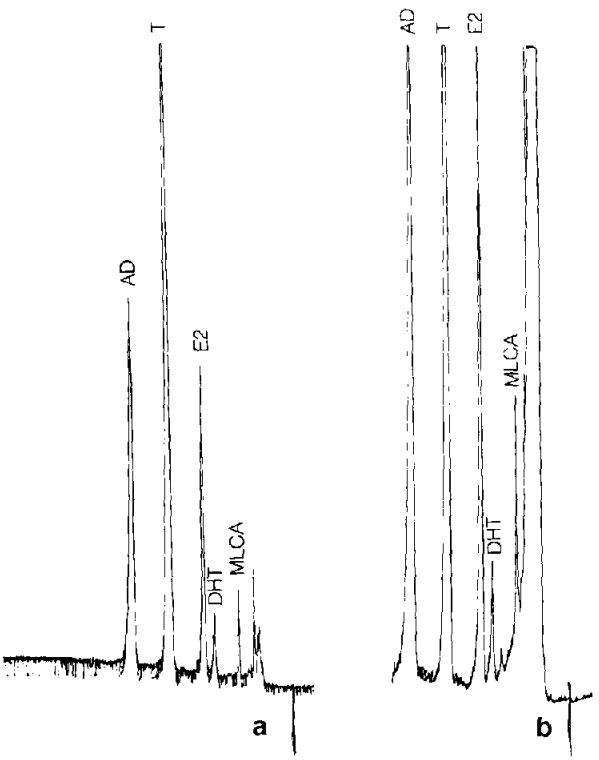

Fig. 2. (a) Chromatogram of steroid standards 2.56 m*M* methyl lithocholate (MLCA), 6.8 m*M* dihydrotestosterone (DHT), 0.096 m*M* 17*β* estradiol (E2), 42.7 *μM* testosterone (T) and 0.85 *μM* androstenedione (AD). (b) Chromatogram of steroid standards MLCA, DHT, T and AD extracted from Hep-G2 whole cell homogenate.

no effect on R_F values (Fig. 2b). Peak identification of DHT and DIOLS was confirmed by GC–MS (Fig. 3); the E2 and AD preparations each gave single peaks corresponding to labelled products.

TABLE I

RELATIVE RETENTIONS OF STANDARDS TO MLCA AND INTRA- AND INTER-ASSAY VARIABILITY (COEFFICIENT OF VARIATION)

Steroid	Relative retention (mean ± S.D.)	Coefficient of variation (%)	
		Intra-assay	Inter-assay
DHT	1.48 ± 0.02	0.22	1.04
E2	1.69 ± 0.03	0.33	0.6
T	2.33 ± 0.04	0.44	1.33
AD	3.02 ± 0.07	0.77	2.77

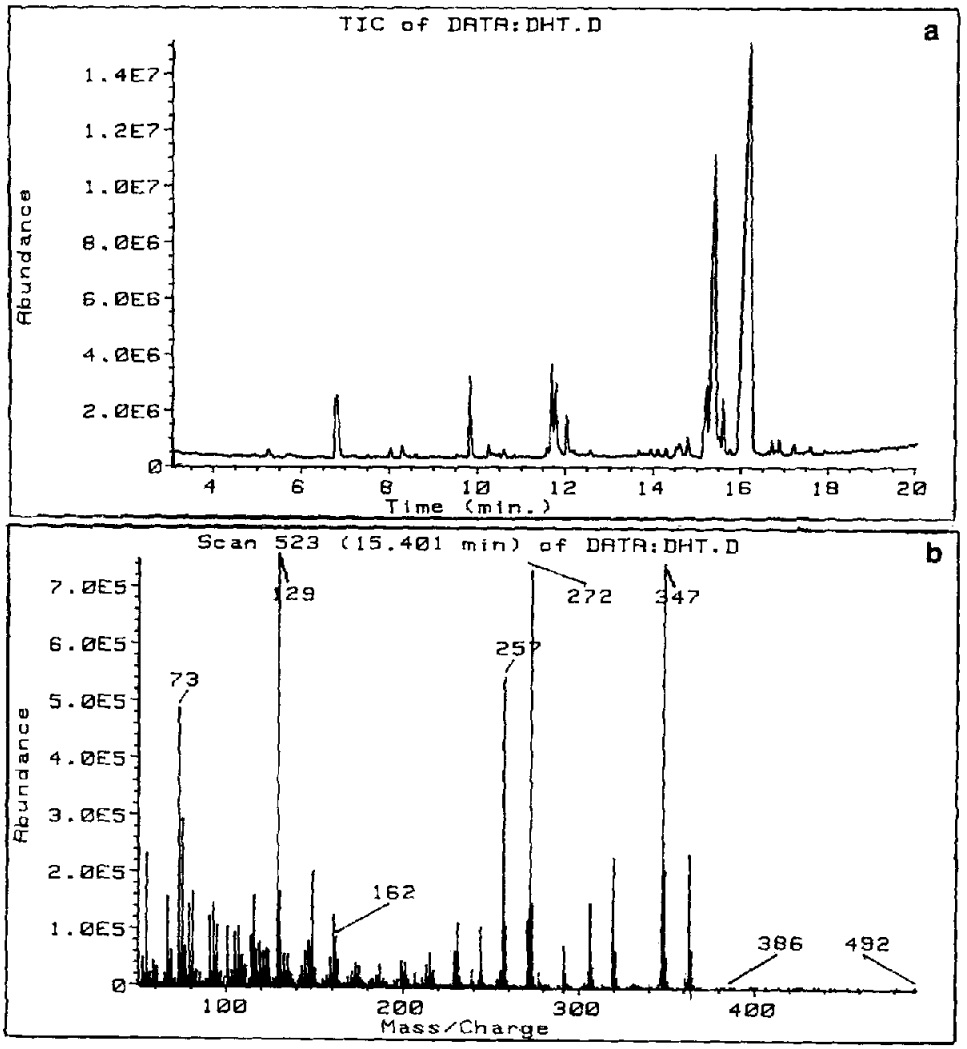

Fig. 3. (a) GC of DHT. (b) Mass spectrum of DHT. The prominent fragmentation ions observed have mass/charge ratios attributable to loss from molecular ion (362) of C_{18} methyl group (Me; 347), O-trimethylsilyl (O-TMS; 272) at C_{17} and simultaneous loss of Me and O-TMS (257) from C_{18} and C_{17}, respectively.

Time course studies showed that at pH 7.4 (at which pH the activity of acid proteases and the spontaneous hydrolysis of NADPH is negligible) the accumulation of DHT and AD was linear over 20 min, and 15 min was chosen as a convenient incubation period (Fig. 4). The Michaelis constant (K_M) and the maximum rate (V_{max}) for 5αR were 3.63 μM T (at 2 mM NADPH) and 6.4 pmol/min/mg protein (Lineweaver–Burk analysis).

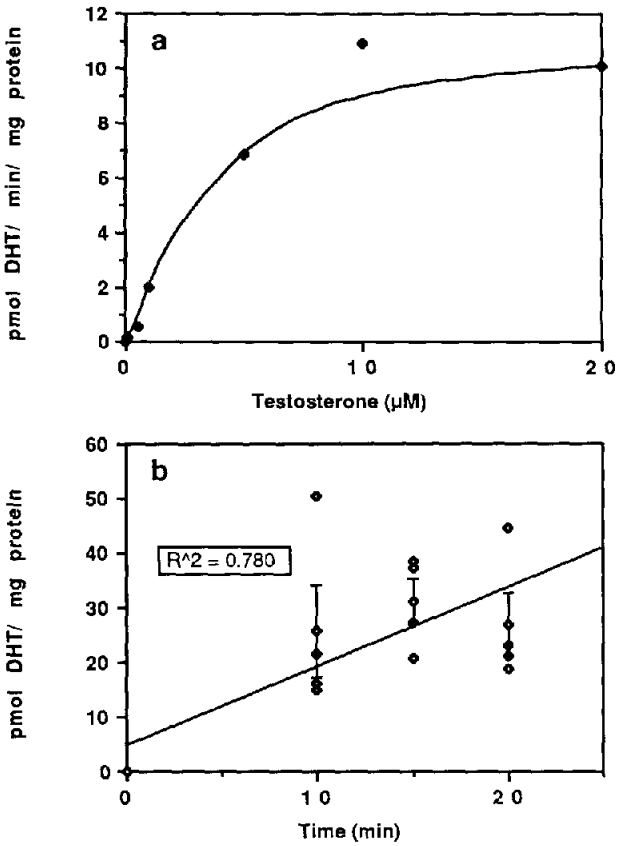

Fig. 4. Effect of testosterone concentration (a) and time (b) on the accumulation of radiolabelled DHT after incubation of Hep-G2 homogenate with testosterone at 37°C.

The minimum detection of DHT necessary for this assay was 0.06 pmol DHT per 15 min at 37°C which corresponds to a sensitivity of 0.24 U of 5αR (1 U 5αR = formation of 1 pmol DHT in 1 h at 37°C). This sensitivity was comparable to that used in studies of prostatic 5αR (LeGoff *et al.* [7]: 0.3 U; Houston *et al.* [8]: 0.2 U).

CONCLUSIONS

Measurement of 5αR activity not only requires the quantitative separation of the reaction products DHT and DIOLS, which represent 5αR activity, but it also necessitates the identification and separation of E1, E2 and AD (Fig. 1). Such studies have been performed on known androgen-dependent tissue such as the epididymis [9] and the prostate [6,7].

The kinetic mechanisms of 5αR in both rodent [10] and normal human liver tissue [8] have been characterised. However, this appears to be the first determination of 5αR activity in human primary liver cancer cells.

ACKNOWLEDGEMENTS

The authors gratefuly acknowledge the Cancer Research Campaign, The Special Trustees of Guy's Hospital and the New Moorgate Trust for financial support, and Dr. H. L. J. Makin for the assistance with the GC–MS analysis.

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