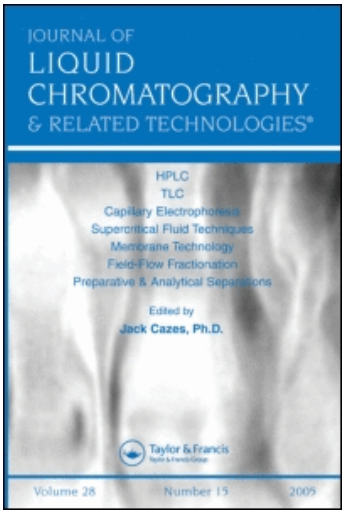

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An Isocratic Reversed-Phase High Performance Liquid Chromatographic Analysis of 5α-Reduced Androgen Metabolites Formed by Rat Ventral Prostate Cells in Culture

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AN ISOCRATIC REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF 5α-REDUCED ANDROGEN METABOLITES FORMED BY RAT VENTRAL PROSTATE CELLS IN CULTURE

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ABSTRACT

Androgen actions in the rat prostate gland are complicated because of extensive metabolism of the androgens. Traditional methods for separating and quantitating the androgens and their metabolites involved thin layer and paper chromatographic steps which are laborious and time-consuming. An isocratic reversed-phase high performance liquid chromatographic system has been developed which separates 11 androgens. This system has been applied to the analysis of the androgen metabolites formed by rat ventral prostate cells in culture and these compounds quantitatively account for all significant metabolism. Using a flow-through radioactivity detector experiments are performed with ^{3}H-substrates. Previously reported systems did not achieve separation of all the compounds.

INTRODUCTION

Androgen actions in male reproductive tract organs, such as the prostate, are complicated by

extensive androgen metabolism (for review see Ref 3). Metabolism of androgens, such as testosterone (T) or Δ^4-androstenedione (A), by prostatic tissue produces exclusively 5α-reduced metabolites with further conversions being concerned with alterations of the 5α-reduced products. A number of these metabolites have significant androgenic activity. Recent studies (4-6) on human prostate tissue have suggested that abnormal regulation of the enzymes involved in androgen metabolism may play a role in the pathogenesis of benign prostatic hyperplasia. In an effort to understand the biochemistry and biology of this steroid metabolizing system, an efficient method for separating the various androgens and their metabolites was needed.

Traditional methods for separating and purifying 5α-reduced androgens are based mainly on thin layer silica gel and paper chromatography (7,8). These methods have the disadvantage of being time-consuming and laborious. Recent research has focused on high performance liquid chromatographic (HPLC) methods for separating various C_{21}-, C_{19}-, C_{18}-steroids (9-18). However, very few of these systems are capable of resolving the numerous stereoisomers in the androgen or C_{19}-steroid group (ie, T, A and their respective 5α-reduced metabolites). The efficiency of the few systems available are restricted by a number of factors, including the use of two columns (12), two solvent systems (12,13), extremely long elution times (14), or the resolution of only a limited number of the androgens (13,14-18).

This report describes a single-step, isocratic reversed phase HPLC system for simultaneously separating eleven androgens found in varying amounts in primary cultures of rat ventral prostate epithelial cells following incubation with various androgens.

MATERIALS

Radioinert steroids were obtained from Steraloids (Wilton, NH) and Sigma Chemical Co. (St. Louis, MO). Radioactive steroids were purchased from New England Nuclear (Lachine, PQ) or prepared as previously described (8) with the exception of the [^{3}H] and [^{14}C] hydroxylated derivatives of 5α-androstane-3β,17β-diol (3β-Adiol). The latter steroids were prepared using either [^{3}H] or [^{14}C] 3β-Adiol as substrate and rat ventral prostate epithelial cells as the hydroxylase source. The steroids were purified by HPLC using methanol:H_2O (45:55) at 1 ml/min. All other radioactive steroids were purified prior to use by thin layer silica gel and paper chromatography (7,8). For use as recovery standards, solutions containing 5000 DPM [4-^{14}C] steroid and 25 μg of radioinert steroid per 100 μl were prepared in methanol.

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade solvents were obtained from Fisher Scientific Co. (Toronto, Ont). All other solvents, reagents and supplies used for cell culture were as previously described (19).

METHODS

Primary cultures of ventral prostate epithelial cells from immature (21-22 d) rats were established as previously described (19) with the following modifications. Briefly, following the isolation of epithelial-enriched cell fractions from Percoll isopycnic gradients, the cells were seeded onto 10 cm^2 plastic dishes and cultured in F12/DME medium supplemented with 10% fetal bovine serum, 10 mM HEPES,

10 mM $NaHCO_3$, insulin (5 μg/ml), transferrin (5 μg/ml), 50 nM testosterone, 50 nM dexamethasone, 500 nM retinoic acid, penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (1 μg/ml). Cells reached confluency in 6-7 d. Cultures were maintained in a humidified incubator at 37°C in air:carbon dioxide (98%:2%). The medium was changed every 2 d. Deoxyribonucleic acid (DNA) levels in the culture dishes were measured (20).

Androgen metabolism was studied in cultures of epithelial cells after they reached confluency (6-7 d). The culture medium was removed and replaced with serum-free and steroid-free medium for 24 h. The medium was then replaced with fresh F12/DME medium containing [^{3}H]-radiolabelled androgens (50 nM; 0.25 μCi). Following a further 6 h incubation, medium and cells were extracted with methylene chloride. [^{14}C]-Steroids were added prior to extraction to monitor losses and identify the androgens on the HPLC chromatogram. The extracts were filtered through anhydrous sodium sulfate and evaporated to dryness under nitrogen. The dried residue was stored at room temperature prior to analysis by reverse-phase HPLC.

High Performance Liquid Chromatography

To analyse the androgen metabolites formed during culture, the following system was developed. A Beckman HPLC System (Beckman Scientific Inc., Toronto, Ont) was utilized. A Beckman pump (Model 100A) was connected to a reverse-phase Beckman C-18, 5 μm spherical packing, ultrasphere-ODS column (4.6 mm I.D. x 250 mm) using a degassed isocratic mobile phase of acetonitrile:methanol:water (1:3:3). The flow rate was

1.0 ml/min with an operating pressure of 2.7 x 10^3 psi. The effluent from the column was monitored using a high sensitivity, variable wavelength, spectrophotometer (Altex Instruments, Model 155) (wavelength = 254 nm) and a Flo-one/Beta radioactive flow detector (Model IC) equipped with a 0.25 ml flow cell (Radiomatic Instruments and Chemical Co. Inc., Tampa, FL). Using the latter detector, the effluent from the HPLC column was directly coupled to a splitter and mixed with 3 volumes of liquid scintillant. The scintillation fluid was Flo-Scint II, also obtained from Radiomatic Instruments and Chemical Co., Inc. The Flo-one/Beta radioactive flow detector was utilized to quantitate ^3H and ^{14}C in the single-label or double-label modes. All operating conditions and procedures were microprocessor controlled. Radioactive measurements were tabulated using a Qume Microcomputer (model QVT-102A) (Qume Corporation, San Jose, CA) and data reduction software package (Radiomatic Instruments and Chemical Co., Inc.). Data was stored on floppy diskettes and printed using a dot matrix serial impact printer (Model 8501A;C. Itoh & Co. Ltd., Los Angeles, CA).

The dried residues from the incubations were redissolved in 200 µl HPLC grade methanol and taken up in 1 ml syringes. 20 µl aliquots were injected into the HPLC instrument through Spartan ^{TM}-3 disposable syringe filters (Schleicher & Schuell Inc., Keene, NH) with a pore size of 0.45 µm. Also, twenty µl aliquots were removed to measure total radioactivity prior to HPLC. The latter aliquots were mixed with 10 ml Scintiverse as the scintillation fluid (Fisher Scientific, Toronto, Ont) and counted using a Beckman Scintillation Counter (Model LS 5801).

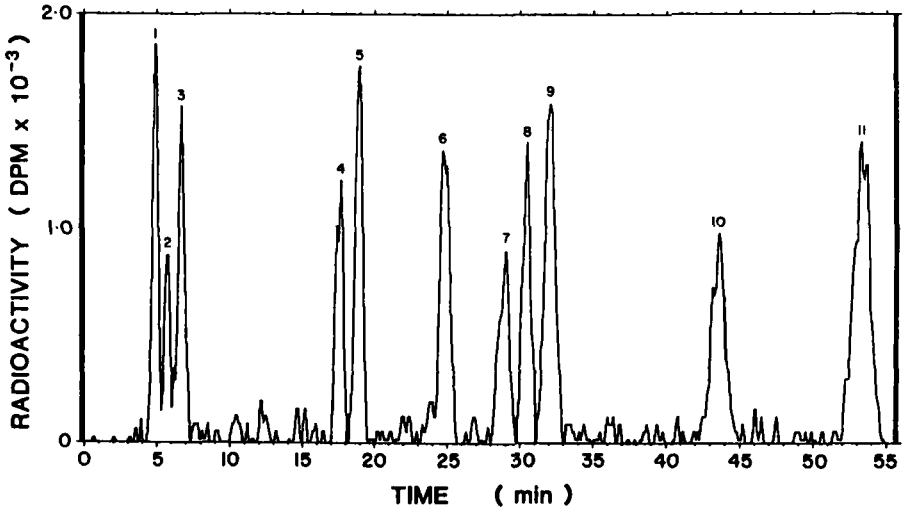
RESULTS AND DISCUSSION

Table 1 lists the eleven androgens separated simultaneously in a single chromatographic run. A sample radiochromatogram is shown in Figure 1. The HPLC system allowed for complete separation of the eleven [^{3}H]-radiolabelled androgen standards. The polar metabolites are simply referred to as Polar I, II and III because of the lack of standards for confirmation of identification. Based on the studies of others (eg. 6) they are probably 6- and 7-hydroxylated metabolites of 3β-Adiol. The analysis time was approximately 55 min at an optimal flow rate of 1 ml/min. An increased flow rate resulted in decreased resolution of the androgens and reduced

TABLE 1

Androgens Separated by Reverse-Phase HPLC System

Steroid No.	Systematic Name	Trivial Name	Abbreviation
1	Polar metabolite I		Polar I
2	Polar metabolite II		Polar II
3	Polar metabolite III		Polar III
4	4-Androstene-3,17-dione	Androstendione	A
5	4-Androsten-17β-ol-3-one	Testosterone	T
6	5α-Androstane-3β-17β-diol	3β-Androstandeiol	3β-Adiol
7	5α-Androstan-3β-ol-17-one	Isoandrosterone	IsoAn
8	5α-Androstane-3,17-dione	5α-Androstanedione	5α-A
9	5α-androstan-17β-ol-diol	5α-Dihydrotestosterone	5α-DHT
10	5α-Androstane-3α,17β-diol	3α-Androstanediol	3α-Adiol
11	5α-Androstan-3α-ol-17-one	Androsterone	An



Radiochromatogram profile of separated androgens and metabolites. Peaks are numbered 1 - 11 based on list in Table 1.

counting efficiency due to the shortened residence time in the 0.25 ml flow-cell (data not shown). Increasing the flow-cell size (up to 2.5 ml) will increase the counting efficiency but decrease the radiochromatographic resolution. The counting efficiencies of the detector for ^{3}H and ^{14}C were 30% and 70% respectively, which is comparable to liquid scintillation counters. Background activity for the detector is normally 65 cpm for ^{3}H and 25 cpm for ^{14}C in the double label counting mode.

Using a ternary solvent system of acetonitrile:methanol:water (1:3:3), the androgens in the chromatogram (Fig 1) eluted sequentially in 5 distinct groups: (I) $C_{19}O_3$ (hydroxylated 5α-reduced-$C_{19}O_2$) (II) 3-oxo-4-ene-$C_{19}O_2$ (III) 3β-hydroxy-5α-

reduced-$C_{19}O_2$ (IV) 3-oxo-5α-reduced-$C_{19}O_2$ and (V) 3α-hydroxy-5α-reduced-$C_{19}O_2$. The following observations on the reactivity of the various functional groups were noted. As expected, the $C_{19}O_3$ steroids (I) which contain three hydroxyl groups were eluted first due to their high polarity. The 3-oxo-4-ene-$C_{19}O_2$ steroids (II) eluted before the 3-oxo-5α-reduced-$C_{19}O_2$ steroids (IV). This suggested that the bonding electrons increase steroid polarity and hence decrease the retention values in a reverse-phase system, as previously observed (21). The 3-hydroxyl epimers (III & V) are not separated by thin layer silica gel chromatography but this can be accomplished with paper chromatography. Using HPLC, the 3-hydroxyl epimers are easily separated with the 3β-hydroxyl epimers eluting before the 3α-hydroxyl epimers. This indicated that the 3β-hydroxylated position conferred a greater degree of polarity to the molecule. Within groups III and V, the steroids with the 17β-hydroxyl group eluted before the steroids with the 17-oxo group. This is as expected since the additional hydroxyl group at the 17-position should increase the polarity of the steroid. In contrast, within groups II and IV, the steroids with the 17-oxo group eluted before the steroids with the 17β-hydroxyl group. This is interpreted to indicate that the conformation of these steroids with the 3-oxo and 17-oxo groups results in decreased retention times even though these steroids are considered to be less polar than the steroids containing 3-oxo and 17β-hydroxy groups using this solvent system.

Table 2 summarizes the capacity factors (K) which are a measure of retention for the separated androgens on the column. The values range from 0.67 to 17.57. While the general, allowable range (22) for efficient and rapid analysis is $1 \leq K \leq 10$, we have adopted a

TABLE 2

Capacity Factors For The Separated Androgens

Steroid No.	Trivial Name	Capacity Factor (K)[a]
1	Polar I	0.67 ± 0.02
2	Polar II	0.93 ± 0.03
3	Polar III	1.32 ± 0.03
4	A	5.11 ± 0.07
5	T	5.54 ± 0.22
6	3β-Adiol	7.60 ± 0.16
7	IsoAn	9.02 ± 0.13
8	5α-A	9.62 ± 0.15
9	5α-DHT	10.26 ± 0.17
10	3α-Adiol	14.32 ± 0.38
11	An	17.57 ± 0.33

a: The capacity factor (K) is determined by subtracting the void column from the elution volume of the particular steroid and dividing this difference by the void volume.Results are expressed as the mean ± SD(n=15).

system with a larger range to encompass all eleven androgens formed by the rat prostate. Usually values of K>10 result in more variable retention data making peak identification more difficult. However, in this system we are also using [^{14}C]-steroid standards to monitor the peaks, thereby alleviating the need to rely solely on the retention data for peak identification.

Table 3 summarizes the separation factors (a) for adjacent androgens. In our experience, the general range for efficient separation is a =1.05-1.5. A value of 1.1 represents good separation which usually produces baseline resolution. The results indicate that all the adjacent androgens are efficiently separated although three pairs of steroids (ie, T/A, 5α-A/IsoAn and 5α-DHT/5α-A) may not be completely

TABLE 3
Separation Factors for Adjacent Androgens

Steroid (a)[a]	Trivial	Separation Factor
2/1	Polar I/Polar II	1.39
3/2	Polar II/Polar III	1.42
4/3	A/Polar III	3.89
5/4	T/A	1.09
6/5	3β-Adiol/T	1.37
7/6	IsoAn/3β-Adiol	1.19
8/7	5α-A/IsoAn	1.07
9/8	5α-DHT/5α-A	1.07
10/9	3α-Adiol/5α-DHT	1.40
11/10	An/3α-Adiol	1.23

a: The separation factor (a) is defined as the ratio of the capacity factors of two steroids in a given chromatographic system ($a=K_2/K_1$; K_2 represents the steroid with the longer retention volume).

resolved to baseline. Resolution of the individual peaks, however, was adequate based on the radiochromatogram (Figure 1). All peaks could be identified and separately measured.

As an example of the use of this system, the HPLC analysis of androgen metabolites following the in vitro incubation of rat ventral prostate epithelial cells with [^{3}H] radiolabelled androgens (T, 5α-DHT, 3α-Adiol and 3β-Adiol) is tabulated in Table 4. The efficiency of the methylene chloride extraction procedure was 80-95% depending on the steroid composition of the extract. Following correction for losses, the androgen metabolites formed in the cell cultures were accounted for by using our isocratic HPLC system. The distribution of androgen metabolites obtained using

TABLE 4

Analysis of Androgen Metabolism by Rat Ventral Prostate Epithelial Cell Cultures Using Reversed-Phase HPLC

Substrates	T	5α-DHT	3α-Adiol	3β-Adiol
% SUBSTRATE RECOVERED	28.5 5.4	51.7 ± 6.1	1.6 ± 0.2	11.1 ± 4.7
% METABOLITES				
Polar I	1.7 ± 0.5	1.5 ± 0.8	1.4 ± 0.1	44.8 ± 2.1
Polar II	1.0 ± 0.5	0.7 ± 0.3	0.7 ± 0.3	14.2 ± 2.1
Polar III	0.4 ± 0.2	0.5 ± 0.4	0.5 ± 0.3	9.5 ± 3.9
A	11.8 ± 2.5	nd	nd	nd
3β-Adiol	1.6 ± 1.1	3.1 ± 0.7	nd	—
IsoAn	2.3 ± 0.2	1.6 ± 1.1	0.4 ± 0.3	3.0 ± 2.5
5α-A	19.5 ± 5.1	38.8 ± 5.0	45.5 ± 4.0	6.4 ± 1.7
5α-DHT	29.0 ± 4.6	—	49.2 ± 3.4	8.0 ± 0.1
3α-Adiol	2.0 ± 1.6	0.4 ± 0.4	—	nd
An	1.6 ± 0.5	1.8 ± 1.1	0.3 ± 0.3	nd
Aqueous Metabolites	1.7 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	3.1 ± 0.4

nd: not detected. The average DNA content of the culture dishes was 17.1 ± 2.8 μg/culture dish. Results are expressed as the mean ± SD (n=3).

HPLC were similar to those obtained in other studies (23) using more conventional thin layer chromatography.

The advantages of this chromatographic system for separating androgens are simplicity (single column and solvent system), high resolving power (separates a large number of androgens in a single step) and reproducibility. A minor disadvantage is the moderately long elution time required to resolve the androgens. However, this is more than compensated for by the advantages. The HPLC system is at least 10 times faster than conventional chromatographic

techniques. Furthermore, the radioactive detector instrument also features a built-in electronic stream splitter that enables the user to direct and collect a fraction of the HPLC effluent for further analysis (ie, RIA, mass spectrometry, crystallization).

In conclusion, this chromatographic system is a powerful tool for studying androgen metabolism in prostate cell cultures with the potential for other steroid analyses.

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