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COMBINED PAPER AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE STUDY OF PREGNENOLONE AND PROGESTERONE METABOLITES

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SUMMARY

A method for the separation and quantitation of steroid metabolites, obtained from enzymatic conversions of tritiated pregnenolone or progesterone, is described. As the first step, paper chromatography is used and the different zones obtained, as monitored by radiochromatogram scanning, are then eluted separately from the paper. The final resolution is achieved by reversed-phase high-performance liquid chromatography in 35% acetonitrile with UV detection at 215 nm. The method has been successfully applied to the study of metabolite patterns obtained from steroid conversion produced by testicular biopsy material under conditions of in vitro incubations.

INTRODUCTION

Methods for the investigation of androgen biosynthesis in the testis have been developed by Steinberger and Ficher [1] and by Tcholakian and Eik-Nes [2], using in vitro incubations of testicular biopsy specimens with a radiolabelled Δ^4-steroid as precursor. After extraction the metabolites were separated by paper and thin-layer chromatography (TLC) and quantified by means of liquid scintillation counting. However, the transfer of the radioactive material from TLC plates requires exact localization of the spots, collection of the layer containing the activity and elution, a very tedious and time consuming procedure. To accomplish adequate separation between different steroids, derivatization is required in certain cases. When [^{3}H]pregnenolone is used as precursor the increased number of possible metabolites requires further TLC steps in order to obtain complete separation. In our opinion, reversed-phase high-performance liquid chromatography (HPLC) as a separation tool should possess definite

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advantages compared to TLC due to its superior resolution, speed and reproducibility. O'Hare et al. [3] in 1976 published a method which combines different gradient elution HPLC systems to separate testicular steroids. A recent report by Cochran and Ewing [4] describes a study on the use of HPLC after Celite column chromatography for the separation of 14 testicular steroids.

In the present paper a systematic investigation of the use of paper chromatography, followed by reversed-phase HPLC for the separation and quantitation of metabolites formed from [^{3}H] pregnenolone and [^{3}H] progesterone via the Δ^5 and Δ^4 pathways, is described in detail. An improved method is proposed for separation and quantitation of the metabolites formed after enzymatic conversions by means of testicular tissue in vitro.

MATERIAL AND METHODS

Biopsy techniques and biopsy material sources

Testicular material was obtained from infertile men by means of regular testicular biopsy. The biopsy specimen was kept cool and taken for enzyme assay within 30 min.

Incubation technique

Teased testicular biopsy specimens (wet weight ca. 50 mg) were incubated in 3 ml of a chemically well defined medium for 3 h in an atmosphere of carbon dioxide—oxygen (5:95) at 37°C under constant shaking and in the presence of 10 μCi of [^{3}H] pregnenolone (3.0 nmol). The medium used consisted of 3.0 ml of Krebs—Ringer bicarbonate buffer (KRBB), pH 7.4, including glucose (11 m*M*), pyruvic acid (3 m*M*), nicotinamide (30 m*M*), NADP (0.4 m*M*), glucose-6-phosphate (3.5 m*M*), NADH (0.4 m*M*) and magnesium chloride (1.5 m*M*) together with ca. 2.0 units/ml of each of the enzymes glucose-6-phosphate dehydrogenase and lactate dehydrogenase necessary for recycling. The reactions were stopped by addition of 0.1 ml 1 *M* hydrochloric acid. To protect the metab-

TABLE I

TRIVIAL NAMES AND ABBREVIATIONS USED

Trivial name	Abbreviation	Chemical name
Androstenediol	A_5	Androst-5-ene-3β,17β-diol
Androstenedione	A_4	Androst-4-ene-3,17-dione
Dehydroepiandrosterone	DHEA	3β-Hydroxyandrost-5-en-17-one
20α-Dihydroprogesterone	20αDH-P_4	20α-Hydroxypregn-4-en-3-one
17β-Estradiol	E_2	Estra-1,3,5(10)-triene-3,17β-diol
Estrone	E_1	3-Hydroxyestra-1,3,5(10)-trien-17-one
17α-Hydroxy-20α-dihydroprogesterone	17α,20α(OH)$_2$-P_4	17α,20α-Dihydroxypregn-4-en-3-one
17α-Hydroxypregnenolone	17αOH-P_5	3β,17α-Dihydroxypregn-5-en-20-one
20α-Dihydropregnenolone	20αDH-P_5	3β, 20α-Dihydroxypregn-5-ene
17α-Hydroxyprogesterone	17αOH-P_4	17α-Hydroxypregn-4-en-3-one
Pregnenolone	P_5	3β-Hydroxypregn-5-en-20-one
Progesterone	P_4	Pregn-4-ene-3,20-dione
Testosterone	T	17β-Hydroxyandrost-4-en-3-one

olites formed, 10 μg of each of the following unlabeled steroids were added: progesterone (P_4), 20α-dihydroprogesterone (20αDH-P_4), 17α-hydroxyprogesterone (17αOH-P_4), androstenedione (A_4), testosterone (T), estrone (E_1), 17β-estradiol (E_2), pregnenolone (P_5), 17α-hydroxypregnenolone (17αOH-P_5), dehydroepiandrosterone (DHEA), androstenediol (A_5) and 20α-dihydropregnenolone (20αDH-P_5) (cf. Table I).

Extraction procedure and paper chromatography

The incubate was repeatedly extracted (8 times) with 25-ml portions of diethyl ether—chloroform (4:1, v/v). The extract, after evaporation under a stream of dry nitrogen, was then chromatographed on a 50-cm paper strip (Whatman no. 1, Clifton, NJ, U.S.A.) in hexane saturated with formamide to the front. The paper strip was impregnated with formamide—methanol (1:2, v/v) beforehand. After evaporation the paper strip was rechromatographed in hexane—benzene—formamide (10:10:1, v/v) to the front as described by Zaffaroni and Burton [5]. The chromatogram was dried overnight at 45°C and then scanned for radioactivity. The zones with radioactive material in the paper strip were then cut out, eluted completely and the eluates evaporated to dryness. Each sample of dried material was then redissolved in 2.00 ml of methanol and an exact volume of each taken to liquid scintillation counting for quantitation of radioactivity. The final separation of the conversion products was achieved using reversed-phase HPLC.

Thin-layer chromatography

The TLC separations were performed on glass plates (20 × 20 cm) coated with a 0.25-mm layer of silica gel G F_{254} (Merck, Darmstadt, G.F.R.) using four different solvent systems as developing media: A, chloroform—acetone (4:1, v/v); B, chloroform—acetone (5:1, v/v); C, chloroform—acetone—ethyl acetate (3:1:1, v/v); D, toluene—ethyl acetate (4:1, v/v). To accomplish separation of T from 17αOH-P_4 derivatization of T to T-acetate was necessary [6].

Reversed-phase HPLC

Instrumentation. The chromatograph was constructed from a Constametric III constant-flow solvent pump, a Rheodyne Model 7125 injection valve provided with a 20-μl loop, a Lichroma 150 × 4.6 mm stainless-steel column, slurry-packed in our laboratory with 5-μm Nucleosil C18, a Schoeffel Model 770 variable-wavelength UV detector and a Linear Model 264 potentiometric recorder.

Chromatographic conditions. All separations were performed under isocratic conditions with acetonitrile—water mixtures as the mobile phase. The flow-rate was 2.0 ml/min.

Measurement of radioactivity

The paper chromatograms were scanned with a Packard Model 7200 radiochromatogram scanner and the TLC plates with a Berthold thin-layer scanner Model LB 2723.

Liquid scintillation counting was performed with a Packard Model 2450 Tri-Carb spectrometer. The scintillation medium was composed of Permablend III

(Packard, Downers Grove, IL, U.S.A.) dissolved in toluene (5.5 g/l) and the counting efficiency for tritium on single isotope analysis was 50%.

Chemicals

Radioactive steroids. Δ^5-[7(n)-^{3}H] Pregnenolone and [1,2(n)-^{3}H]-progesterone were obtained from the Radiochemical Centre (RCC) (Amersham, Great Britain) or from New England Nuclear (NEN) (Boston, MA, U.S.A.). 17α-Hydroxy[7(n)-^{3}H]-pregnenolone and dehydro-[7(n)-^{3}H]-epiandrosterone were from RCC and androst-5-ene-3β,17β-diol[1,2(n)-^{3}H], estrone-[2,4,6,7(n)-^{3}H], estradiol-[2,4,6,7(n)-^{3}H] and estriol-[2,4,6,7(n)-^{3}H] were from NEN.

Unlabeled steroids. These compounds were obtained from different commercial suppliers.

Solvents. All solvents were of pro analysi quality. The acetonitrile of HPLC grade S was obtained from Rathburn Chemicals (Walkerburn, Great Britain).

RESULTS AND DISCUSSION

Separation efficiency

The complete separation scheme is outlined in Fig. 1. As shown, all paper zones could be taken directly to HPLC after elution. However, under the conditions used, optimal column performance was always a prerequisite for complete resolution of A_4 from DHEA (paper zone VI).

Chromatographic retention data for the various steroids obtained in the final steps are summarized in Table II. The TLC systems used supplemented with derivatization of T permit separation of the Δ^4-metabolites. However, the Δ^4- and Δ^5-metabolites in paper zone VI can not be adequately separated with the TLC systems used as seen in Table II. The HPLC system permits adequate separation of T and 17αOH-P_4 without previous acetylation.

The column efficiency as monitored by simultaneous peak tracing by UV and by liquid scintillation counting is shown in Fig. 2. At the detector wavelength 215 nm the Δ^4- as well as the Δ^5-metabolites could be visualized, thus permitting an exact localization of the peaks as required for eluate collection.

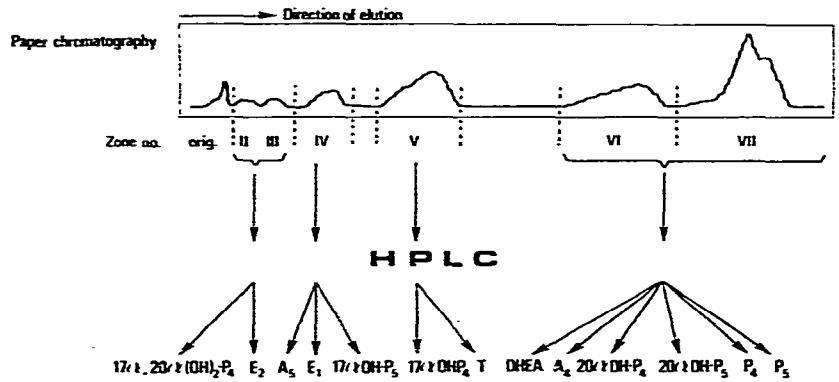

Fig. 1. The complete separation scheme, permitting the determination of 13 identified testicular steroid metabolites.

TABLE II

CHROMATOGRAPHIC RETENTION DATA

Solvent systems: HPLC, 35% acetonitrile; TLC, as indicated, full details in Materials and Methods.

Zone No.	Steroid	HPLC	TLC	
		Capacity factor, k'	R_F	Solvent system
II—III	17α,20α(OH)$_2$-P$_4$	13.5	0.20	B
	E$_2$	16.1	0.38	
IV	A$_5$	19.3	0.37	C
	17αOH-P$_5$	22.8	0.46	
	E$_1$	18.1	0.69	
V	T	19.5	0.35 (acetate)	D
	17αOH-P$_4$	22.3	0.14	
VI	A$_4$	22.3	0.60	A
	DHEA	24.8	0.46	
	20αDH-P$_4$	57.8	0.44	
	20αDH-P$_5$	62.8	0.45	
VII	P$_4$	83.3	0.64	A
	P$_5$	96.0	0.48	

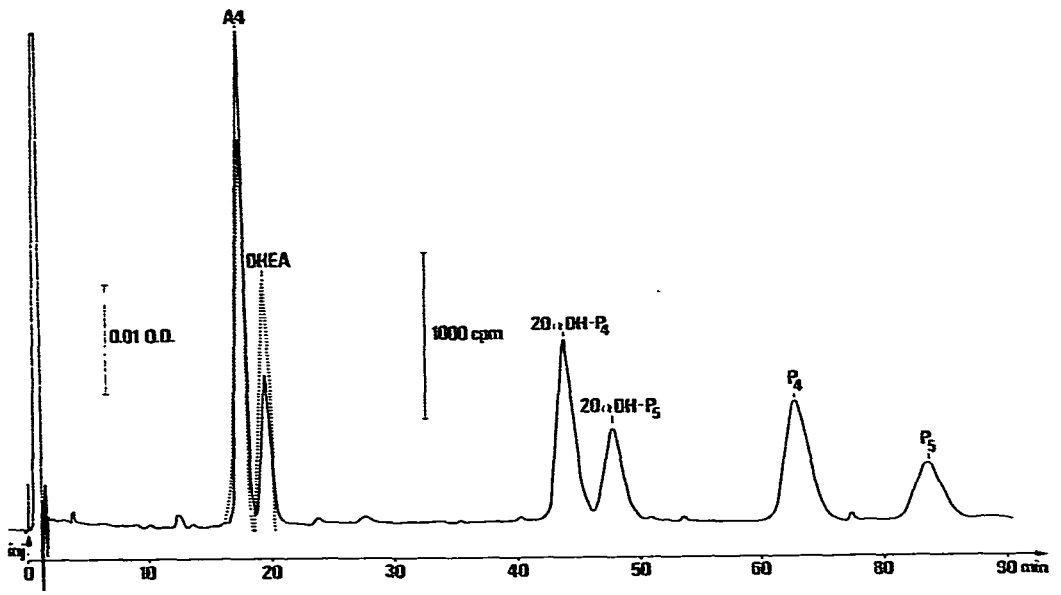

Fig. 2. Illustration of column efficiency and chromatographic resolution. The solid and dashed lines refer to monitoring of UV absorption at 215 nm and to liquid scintillation counting, respectively. For the latter case fractions were collected at intervals of 10 sec.

Extremely purified solvents are absolutely necessary at this wavelength to avoid UV absorbance by impurities.

Reproducibility and methods comparison

The reproducibilities obtained on incubation, extraction, paper chromatography and TLC separation have been reported previously [6].

Several experiments were performed to compare the results of steroid quantitation via TLC and HPLC, respectively, of the zones after the initial paper chromatography. Thus, the paper zones from a given biopsy incubation experiment were eluted and the eluate from each zone divided into two identical parts, one for TLC and the other for HPLC analysis.

Table III shows the results of such an experiment, which demonstrate clearly the satisfactory agreement between the two methods. However, the slightly larger amounts of unidentified radioactive material obtained by HPLC should be consistent with the higher separation efficiency of this method.

Repeated separations of a steroid mixture obtained from paper zones VI and VII gave the results shown in Table IV. ^{14}C-Labelled A_4 was used in order to demonstrate its complete chromatographic resolution from DHEA (Table IV and Fig. 2).

TABLE III

COMPARISON OF RESULTS USING TLC AND HPLC AS FINAL QUANTITATION STEPS

Data obtained from a testicular biopsy incubation with P_5 as the precursor.

Zone No.	Steroid	Radioactivity (%) obtained according to	
		TLC	HPLC
II + III	17α,20α(OH)$_2$-P_4	5.4	4.7
	ui*	4.1	4.5
IV	—	—	—
V	T	4.2**	2.1
	17αOH-P_4	16.2	19.4
	ui	0.5	2.9
VI	A_4	1.3	0.9
	DHEA	—	0.2
	20αDH-P_4	6.0	5.0
	20αDH-P_5	—	0.2
	ui	1.0	—
VII	P_4	38.8	34.8
	P_5	1.4	0.4
	ui	6.0	9.5
Recovery of activity (%)		84.9***	84.6***

*ui denotes unidentified compounds.

**Derivatized to acetate.

***The remaining activity is completely located at the origin (zone I) and at the solvent front in the paper chromatogram.

TABLE IV

ILLUSTRATION OF THE REPRODUCIBILITY ON THREE SUBSEQUENT HPLC SEPARATIONS OF STEROIDS FROM PAPER ZONES VI AND VII (FIVE ^{3}H-LABELLED Δ^{5}- AND ONE ^{14}C-LABELLED Δ^{4}-SPECIES)

Results are presented as percent recovered radioactivity of each steroid related to the total amount of radioactivity injected on the column.

Run No.	1	2	3	Mean	Range
[^{3}H]DHEA	18.7	18.1	17.6	18.1	17.6—18.7
[^{3}H]20αDH-P_4	13.7	13.3	12.8	13.3	12.8—13.7
[^{3}H]20αDH-P_5	18.9	17.9	17.2	18.0	17.2—18.9
[^{3}H]P_4	18.3	20.2	21.8	20.1	18.3—21.8
[^{3}H]P_5	24.5	26.6	25.3	25.5	24.5—26.6
[^{3}H]ui*	5.9	3.9	5.3	5.0	3.9—5.9
	100	100	100	100	
[^{14}C]A_4	99.7	100.5	102.9	101.0	99.7—102.9

*ui denotes unidentified compounds.

CONCLUSIONS

Compared to TLC, HPLC methods are generally more adequate for quantitative work. Retention parameters can be obtained with high precision, and measurements on the eluate can be performed without destroying the chromatographic system as is the case on elution from TLC plates. The continuous operation of HPLC systems represents a definite advantage, especially if clinical demands of sample processing capacity are taken into consideration.

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