Journal of Chromatography, 308 (1984) 11—18
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2072

QUANTITATIVE METABOLIC PROFILING OF TESTICULAR STEROID SECRETIONS WITH BONDED-PHASE CAPILLARY GAS CHROMATOGRAPHY

CURTIS CHUBB* and CATHERINE NOLAN

Department of Cell Biology, Southwestern Medical School, The University of Texas Health Science Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75235 (U.S.A.)

(First received October 7th, 1983; revised manuscript received January 16th, 1984)

SUMMARY

A procedure is described for quantitative metabolic profiling of nine steroids secreted by mouse testes. The procedure incorporates Celite column chromatography and a gas chromatograph equipped with a bonded-phase capillary column. Validation tests demonstrated that the procedure is precise, efficient, sensitive, and capable of providing information about the spectrum of steroids secreted by testes perfused in vitro. The procedure was used to determine the effect of the hemimelic extra toes gene mutation on the steroidogenic potential of inbred mouse testes perfused in vitro.

INTRODUCTION

The evolution of capillary columns has dramatically increased the resolution power of gas chromatography (GC) [1]. The high resolution of capillary columns coupled with the universal detection ability of flame ionization detectors provide a valuable method for the metabolic profiling of steroids. Metabolic profiles are "multicomponent GC analyses that define or describe metabolic patterns for a group of metabolically or analytically related metabolites" [2, 3].

Previously, investigators have analyzed urinary steroids [4—6] and, to a lesser extent, plasma steroids [7, 8] with capillary GC. The current report describes an efficient and practical procedure utilizing capillary GC for the metabolic profiling of steroids secreted by mouse testes perfused in vitro. Although a spectrum of steroids occurs naturally [9], nine steroids were selected for metabolic profiling because they account for more than 85% of the total steroid secretion of mouse [10], rat [11], and rabbit [11] testes

0378-4347/84/$03.00

perfused in vitro. The selected steroids were profiled quantitatively by a combination of Celite column chromatography and capillary GC.

MATERIALS AND METHODS

Animals

B10.D2/nSn (F43) male mice with (Hx/+) or without (+/+) the hemimelic extra toes (Hx) gene mutation were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) at six weeks of age and housed in sibling groups for two months before use. Lab-Blox (Wayne Feed Division, Chicago, IL, U.S.A.) and water were available at all times. The animal room was maintained at 23 ± 1°C with 14 h of light per 24 h.

Chemicals and steroids

Isooctane (Spectra-Analyzed, Fisher, Pittsburgh, PA, U.S.A.), benzene and chloroform (Omnisolv, MCB, Cincinatti, OH, U.S.A.), and hexane (Pesticide Grade, J.T. Baker, Phillipsburg, NJ, U.S.A.) were redistilled and stored in glass-stoppered bottles. Acetic anhydride (Pfaltz and Bauer, Stamford, CT, U.S.A.) and pyridine (Certified ACS, Fisher) were also redistilled and the pyridine stored in a desiccator. Ethylene glycol (Fisher) was used without modification. Celite Analytical Filter Aid (Fisher) was heated at 550°C for at least 18 h before use and allowed to cool to room temperature in a desiccator.

Reference samples of the steroids selected for analysis were obtained from Steraloids (Wilton, NH, U.S.A.) and recrystallized three times. Ethanolic solutions (2—4 mg/ml) of the following steroids were stored at —20°C: pregnenolone (PREG, 3β-hydroxy-5-pregnen-20-one); dehydroepiandrosterone (DHA, 3β-hydroxy-5-androsten-17-one); androstenediol (DIOL, 5-androstene-3β,17β-diol); progesterone (PROG, 4-pregnene-3,20-dione); androstenedione (DIONE, 4-androstene-3,17-dione); testosterone (T, 17β-hydroxy-4-androsten-3-one); dihydrotestosterone (DHT, 17β-hydroxy-5α-androstan-3-one); 3α-androstanediol (3α-DIOL, 5α-androstane-3α,17β-diol); 3β-androstanediol (3β-DIOL, 5α-androstane-3β,17β-diol); 5α-cholestane.

Two radio-labelled steroids were purchased from New England Nuclear (Boston, MA, U.S.A.): [1,2,6,7,16,17-^{3}H]testosterone (135 Ci/mmol) and [1,2-^{3}H]androstenediol (45 Ci/mmol). The ^{3}H-labelled steroids were purified by Sephadex LH-20 chromatography prior to use.

In vitro testis perfusion

Testes were perfused in vitro for 4 h at 32°C via the capsular artery with an artificial medium containing 0.2% glucose, 3% bovine serum albumin and 25% bovine erythrocytes [12]. Luteinizing hormone (LH; NIAMDD-oLH-24) was infused at 100 ng/ml in order to maximally stimulate steroidogenesis. After an initial 1-h equilibration period, three consecutive 60-min samples of venous effluent were collected. Supernatants of the venous effluent and saline washes of the erythrocytes were combined and stored at —20°C prior to steroid analysis by GC.

Steroid extraction

Two 60-min collections of supernatant plus wash from each testis were

combined and [^{3}H]testosterone (approximately 1000 dpm) and [^{3}H]-androstenediol (approximately 1000 dpm) were added for recovery determinations. For validation tests, aliquots of each reference steroid solution were dissolved in 3 ml of perfusion medium supernatant. Steroids were extracted from the analytical and validation samples by adding 6 ml benzene—hexane (2:1) and vortexing for 90 sec. After centrifugation (500 *g* for 10 min), aqueous phases were frozen in an acetone—dry ice bath. Organic phases were decanted into 13 × 100 mm disposable test tubes and evaporated under nitrogen in a water bath (47°C). Residues were then redissolved in 0.5 ml of isooctane (saturated with ethylene glycol) and applied to the bed of a Celite column.

Glassware was silanized and subsequently rinsed with methanol and chloroform before use. Re-usable glassware was chromic acid-washed.

Celite column chromatography

Celite column chromatography was performed as described previously [10, 13] with the exception that only two consecutive fractions of eluent were collected: fraction A = 3.5 ml of isooctane + 3.5 ml of 5% benzene in isooctane + 5 ml of 20% benzene in isooctane; fraction B = 6 ml of 20% benzene in isooctane + 10 ml of 35% benzene in isooctane.

The collected fractions were transferred with chloroform to 1-ml reaction vials, dried under nitrogen (60°C), and acetylated (0.2 ml pyridine—acetic anhydride (5:1) at 60°C for 1 h) [14]. Afterwards, the contents of the reaction vials were evaporated under nitrogen at 60°C and redissolved in 100 μl of chloroform. Aliquots (10 μl) of analytical samples were removed for recovery determination. Acetylated samples were stored at −20°C until GC analysis.

Gas chromatography

Acetylated samples were transferred to injection tubes (6 × 50 mm disposable test tubes) containing 100 ng of the internal standard, 5α-cholestane. The injection mixture was evaporated under nitrogen in a 60°C water bath and redissolved in 4 μl of chloroform. After vortexing for 5 sec, 2 μl of the contents were injected into a Spectra-Physics SP7100 gas chromatograph (Spectra-Physics, San Jose, CA, U.S.A.) equipped with a capillary inlet and dual flame ionization detectors. Steroids were separated on a WCOT bonded-phase vitreous silica capillary column (12 m × 0.2 mm, 12 QC2/BP1-0.25, Scientific Glass Engineering, Austin, TX, U.S.A.) coated with non-polar dimethyl silicone. The conditions selected for steroid analyses are presented in Table I.

TABLE I

GC CONDITIONS FOR SEPARATION OF NINE TESTICULAR STEROIDS

Injector temperature: 250°C	Carrier gas: helium
Detector temperature: 300°C	Capillary inlet: splitless mode
Oven temperature: 160°C initial, hold for 1 min;	Splitless injection time: 0.6 min
240°C final, hold for 5 min;	Linear gas velocity: 25 cm/sec
4°C/min ramp	Capillary column head pressure: 100 kPa
Electrometer: $1 \cdot 10^{12}$ A/mV	Septum purge: 1.3 ml/min
Attenuation: 32	Hydrogen flow-rate: 30 ml/min
	Air flow-rate: 400 ml/min

Peaks were integrated by a built-in programmable data system/printer plotter. Component peaks were identified by their relative retention times and quantified by comparison to the internal standard peak area.

RESULTS AND DISCUSSION

Steroid extraction and preliminary purification

Organic solvent extraction was selected because of the resulting quantitative recoveries as reported in an earlier study [10]. The extracted steroids were chromatographed on a Celite column to provide a preliminary purification step and to divide the steroids into two fractions: fraction A steroids are DIONE, DHA, DHT, T, PROG, PREG; fraction B steroids are 3α-DIOL, DIOL, 3β-DIOL.

Steroid derivatization

Acetylation stabilized the steroids, resulted in single peaks, and was simple to perform. Additionally, the residue remaining after evaporation of the reaction mixture did not significantly contribute to the background of the GC analyses. In contrast, the formation of methoxime—trimethylsilyl ether derivatives [15] of 3-oxo-4-ene steroids (PROG, DIONE, T) produced isomers [3] and required additional clean-up columns as reported by Axelson and Sjövall [8].

Two steroids, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, were not acetylated under the stated conditions. The sterically hindered hydroxyl at the 17-position has been reported to be difficult to derivatize [14].

Precision of retention times and peak identification

Because of the high resolution of capillary GC, retention times can be used to identify peaks if the retention times can be proven to be stable [16]. The

TABLE II

PRECISION OF RETENTION TIMES

Retention times (min) are of steroids analyzed by the described GC method on three different days. Steroid concentrations ranged from 10 to 200 ng.

Steroid	n	$\bar{x}$	Coefficient of variation $(\frac{\text{S.D.}}{\bar{x}} \times 100)$
Androstenedione	16	12.54	0.3
Dehydroepiandrosterone	16	13.50	0.3
Dihydrotestosterone	16	14.02	0.3
Testosterone	16	15.25	0.3
Progesterone	16	15.84	0.3
Pregnenolone	16	16.71	0.2
3α-Androstanediol	16	15.58	0.3
Androstenediol	16	16.16	0.3
3β-Androstanediol	16	16.30	0.3
5α-Cholestane	32	17.26	0.2

highly reproducible and stable retention times of steroids analyzed on our gas chromatograph (Table II) support our decision to use retention times for peak identification.

Sensitivity and range of detection

The sensitivity and range of detection were evaluated by recording the detector response to different amounts of injected steroids (Fig. 1). The sensitivity was 2 ng for each of the derivatized steroids. Detector response was linear from 0 to 200 ng as indicated by the high correlation coefficient (r = 0.98). However, the increased error for masses above 100 ng suggests that column overloading occurred.

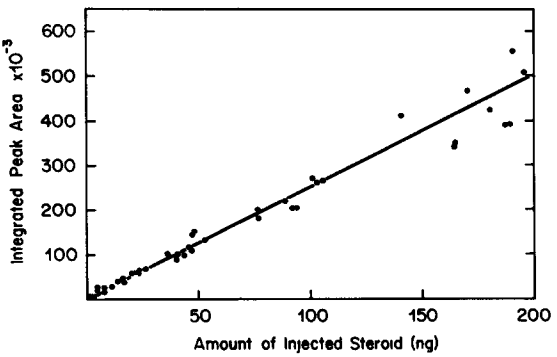

Fig. 1. Linear-regression analysis of the relationship between steroid mass injected and detector response. In total 44 data points were analyzed. The data points represent nine steroids and five mass ranges (5, 25, 50, 100, and 200 ng). The correlation coefficient (r = 0.982) was highly significant ($p < 0.001$) as determined by Student's t test; $y = 2089 + 1260x$.

Recovery and precision

The recovery and precision of the procedure were assessed by extracting known amounts of all nine steroids from 2 ml of water or perfusion medium supernatant. Examples of the chromatograms are presented in Figs. 2 and 3. The results (Table III) demonstrate that the recovery and precision of the method compare favorably with previously reported capillary GC analyses of plasma steroids [7, 8, 17]. The presence of a close-eluting background peak may explain the high recovery for androstenedione. The background peak has been subsequently diminished by a more defined distillation of isooctane.

Metabolic profile of nine steroids secreted by mouse testes perfused in vitro

The hemimelic extra toes (Hx) gene mutation causes infertility in male mice [18, 19]. Since successful spermatogenesis requires a milieu of steroids, we hypothesized that the Hx gene mutation may alter steroidogenesis. The hypothesis was tested by comparing the metabolic profile of steroids secreted by sibling inbred mice that were coisogenic for the Hx gene mutation. Testicular venous effluent was analyzed for steroids by the described GC procedure. The results (Fig. 4) suggest that the Hx gene mutation does not deleteriously affect steroidogenesis. Additionally, the steroid secretion profile presently

TABLE III

RESULTS OF ASSAYS TO DETERMINE RECOVERY AND PRECISION

Identical amounts of a solution containing the nine selected steroids were added to 2 ml water (n = 3), 2 ml perfusion medium supernatant (n = 3), and two reaction vials. The steroids in the reaction vials were used to determine recoveries of the steroids extracted from the aqueous solutions and analyzed by the described method.

Steroid	n	Mass ($\bar{x}$, ng)	Recovery percentage (%, $\bar{x}$ ± S.E.M)	Precision coefficient of variation ($\frac{S.D.}{\bar{x}} \times 100$)
Androstenedione	5	61 7	102 ± 7	14 7
Dehydroepiandrosterone	6	100 5	82 ± 3	8.4
Dihydrotestosterone	6	68 1	83 ± 3	7 8
Testosterone	6	83.4	81 ± 2	7 7
Progesterone	6	49 4	84 ± 3	9 9
Pregnenolone	6	55.5	86 ± 3	8 4
3α-Androstanediol	5	86 3	93 ± 4	8 2
Androstenediol	6	74 2	93 ± 4	12 9
3β-Androstanediol	6	47 9	86 ± 3	7 7

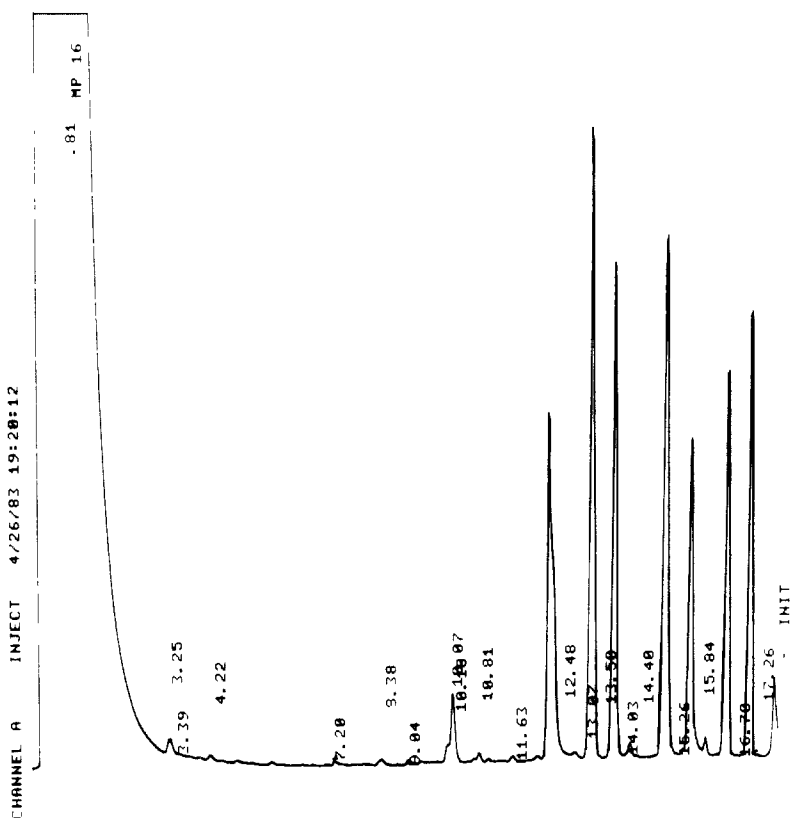

Fig. 2. A chromatogram of the six fraction A steroids extracted from 2 ml of perfusion medium supernatant containing the nine selected steroids and processed by the described procedure. Steroids were identified by retention times as specified on the chromatogram· androstenedione, 12.48 min; dehydroepiandrosterone, 13.50 min; dihydrotestosterone, 14.03 min; testosterone, 15.26 min; progesterone, 15.84 min; pregnenolone, 16.70 min; 5α-cholestane, 17.26 min The 5α-cholestane peak represents 50 ng

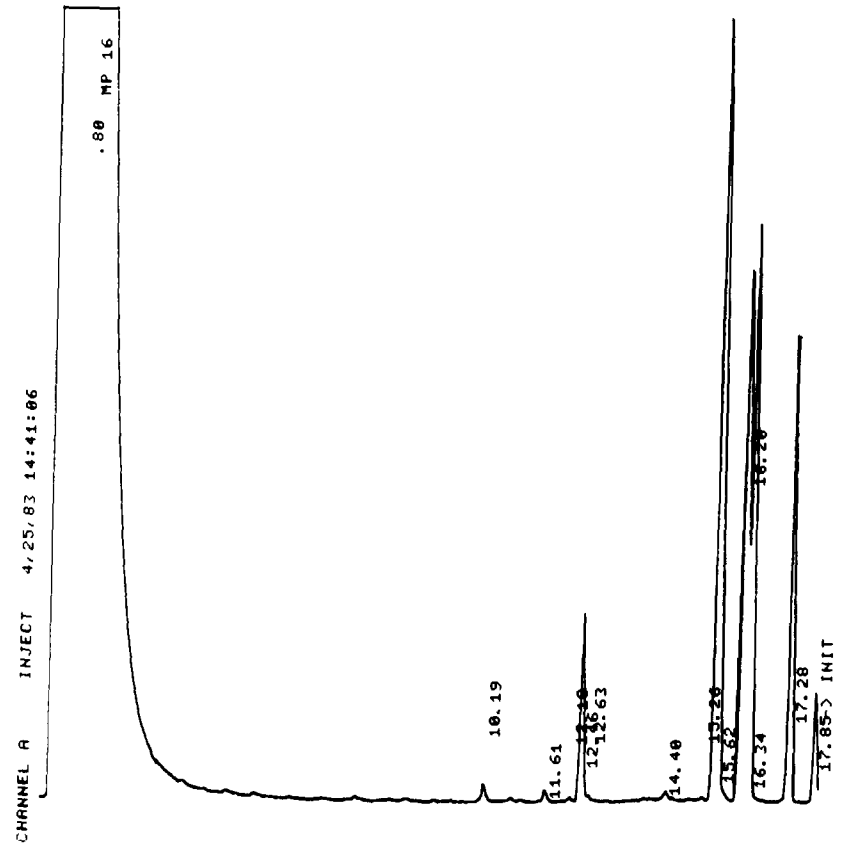

Fig. 3. A chromatogram of the three fraction B steroids extracted from 2 ml of perfusion medium supernatant containing the nine selected steroids and processed by the described procedure. The steroids were identified by retention times as specified on the chromatogram: 3α-androstanediol, 15.62 min; androstenediol, 16.20 min; 3β-androstanediol, 16.34 min; 5α-cholestane, 17.28 min. The 5α-cholestane peak represents 50 ng.

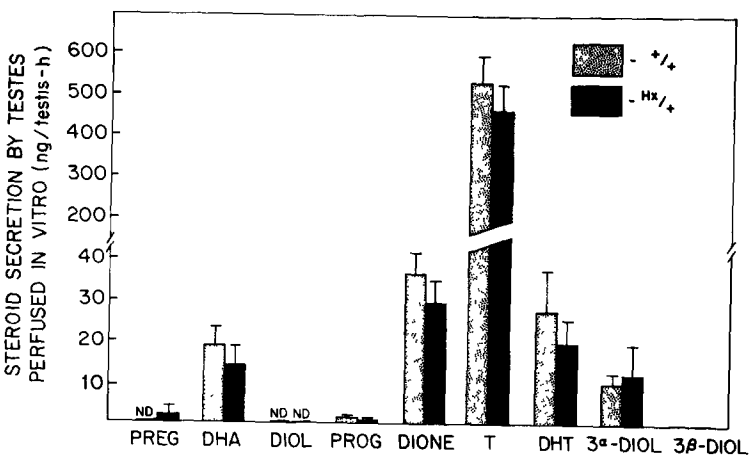

Fig. 4. Metabolic profile of steroids secreted by LH-stimulated testes of hemimelic extra toes mutant mice (Hx/+) and control siblings (+/+). Testes were perfused in vitro for 4 h and the venous effluent was analyzed for steroids by the described capillary GC procedure. The steroid abbreviations are defined in Materials and methods. Each value represents the mean ± standard error. $n = 6$; ND = non-detectable

reported is comparable to an earlier report of steroid secretion by CBF_1 mouse testes perfused in vitro [10]. The previous study was accomplished with column chromatography and radioimmunoassay procedures. Differences in absolute secretion rates may be due to the different strains of mice used in the two studies.

CONCLUSION

Together, the data support the applicability of the described procedure employing capillary GC for the metabolic profiling of steroids secreted by testes. All of the "most desirable achievements of a GC analysis" as listed by Schomburg [20] are attained: adequate resolution, reasonable analysis time, sensitive detection, highly precise and accurately obtained data.

ACKNOWLEDGEMENTS

Luteinizing hormone, NIAMDD-oLH-24, was obtained from the National Hormone and Pituitary Program, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD, U.S.A.

The research was supported, in part, by Public Health Service Research Grant HD-15594 from the National Institute of Child Health and Human Development.

REFERENCES

1 A.L. German, C.D. Pfaffenberger, J.-P. Thenot, M.G. Horning and E.C Horning, Anal Chem., 45 (1973) 930.
2 E.C. Horning and M.G. Horning, Clin. Chem., 17 (1971) 802.
3 C.D. Pfaffenberger, in W.G. Jennings (Editor), Applications of Glass Capillary Gas Chromatography, Marcel Dekker, New York, 1981, p. 203.
4 M. Axelson, B.-L. Sahlberg and J. Sjövall, J Chromatogr., 224 (1981) 355.
5 C.D. Pfaffenberger, L.R. Malinak and E.C Horning, J. Chromatogr., 158 (1978) 313
6 P. Pfeifer and G. Spiteller, J. Chromatogr., 223 (1981) 21.
7 M. Axelson and B.-L Sahlberg, J. Steroid Biochem., 18 (1983) 313
8 M. Axelson and J Sjövall, J. Steroid Biochem., 8 (1977) 683.
9 R.I. Dorfman and F. Ungar, Metabolism of Steroid Hormones, Academic Press, New York, 1965.
10 C. Chubb and C. Desjardins, Amer. J. Physiol., 244 (1983) E575
11 C Chubb and L.L. Ewing, Amer. J. Physiol., 237 (1979) E231.
12 C Chubb and C. Desjardins, Comp. Biochem. Physiol., 74A (1983) 231
13 R.J Moore, J.M. Gazak, J.F. Quebbeman and J.D. Wilson, J. Clin. Invest., 64 (1979) 1003.
14 O.V. Dominguez, J.R. Seely and J. Gorski, Anal. Chem., 35 (1963) 1243.
15 C.D. Pfaffenberger and E.C. Horning, Anal. Biochem., 80 (1977) 329.
16 F. Rowland, Amer. Lab , (March 1982) 110.
17 R.G. Frith and G. Phillipou, Ann. Clin. Biochem., 18 (1981) 84.
18 M.M. Dickie, Mouse News Lett., 38 (1968) 24.
19 M.C. Green, in M.C. Green (Editor), Genetic Variants and Strains of the Laboratory Mouse, Gustav Fischer Verlag, Stuttgart, 1981, p. 8
20 G. Schomburg, J. Chromatogr. Sci., 21 (1983) 97.