Journal of Chromatography, 219 (1981) 152–155
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,204

Note

High-performance liquid chromatography of progesterone and its metabolites

DAVID G. WALTERS*, PAUL M. D. FOSTER and RICHARD C. COTTRELL
The British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey SM5 4DS (Great Britain)

(First received May 22nd, 1981; revised manuscript received July 6th, 1981)

The estimation of progesterone and its three principal metabolites in testis, ovary, adrenal gland and placenta is required for the determination of the activity of the enzymes involved in the biosynthesis of testosterone from this precursor (Fig. 1). Steroid 17α-monooxygenase (E.C. 1.14.99.9) converts progesterone to 17α-hydroxyprogesterone the substrate for 17α-hydroxyprogesterone aldolase (E.C. 4.1.2.30). The product of this stage, androst-4-ene-3,17-dione, is reduced by testosterone 17β-dehydrogenase (NADP, E.C. 1.1.1.64) to testosterone.

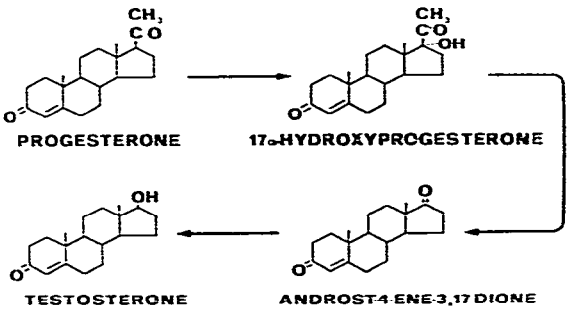

Fig. 1. Microsomal metabolism of progesterone to testosterone.

We were concerned to develop a suitable procedure to measure rapidly and reproducibly the stages in this metabolic process in rat testis as a replacement for the time consuming and somewhat variable thin-layer chromatographic (TLC) methods[1,2] previously in use in our laboratory.

Although high-performance liquid chromatography (HPLC) has been widely applied to the analysis of steroid hormones in general[3] and to the separation[4,5] and quantification[6,7] of this type of hormone, a robust and rapid reversed-phase method of adequate resolution was not available. This paper describes a satisfactory technique for determining these four steroids in radiolabelled form in incubations of interstitial and germinal cells of the testis or in sub-cellular fractions.

0021-9673/81/0000–0000/$02.50

EXPERIMENTAL

Materials

[4-^{14}C]Progesterone, [4-^{14}C]androst-4-ene-3,17-dione (The Radiochemical Centre, Amersham, Great Britain), [4-^{14}C]-17α-hydroxprogesterone (NEN Chemicals, Dreieich, G.F.R.), testosterone, progesterone, 17α-hydroxyprogesterone and androstenedione (Sigma London, Poole, Great Britain) were used as supplied. Identity and radiochemical purity was confirmed as >98% by TLC using silica gel plates with benzene–acetone (4:1, v/v) as solvent. HPLC grade methanol and tetrahydrofuran were obtained from Rathburn Chemicals, Walkerburn, Great Britain. Prepared solvent mixtures were sparged with helium and maintained under a helium atmosphere during use.

Equipment

An Applied Chromatography Systems (Luton, Great Britain) LC750 high-performance liquid chromatograph fitted with 250 × 4.6 mm I.D. columns packed with Partisil PXS 10 ODS, Partisil PXS 10 ODS-2, or Partisil PAC was used for all separations. A pre-column of CO:PELL:ODS (Whatman Labsales, Maidstone, Great Britain) was fitted for biological samples. Detection was either by UV absorbance at 240 nm using a Cecil Instruments CE2012 variable-wavelength UV monitor (Cecil Instruments, Cambridge, Great Britain) or by scintillation counting[8] of collected effluent fractions[9] in Tritosol[10] scintillant.

RESULTS AND DISCUSSION

Initial attempts to achieve an adequate separation within a practicable analysis time using water–methanol mixtures and gradients and ODS, ODS-2 or PAC stationary phases proved unsuccessful. With tetrahydrofuran–water mixtures and ODS-2 as stationary phase more promising results were obtained. Complete resolution of the 4 steroids was possible in tetrahydrofuran–water (25:75) at 25°C but the analysis time of 50 min was unacceptably long. It was not found to be possible to shorten the analysis time and retain adequate resolution under isocratic conditions. The introduction of a linear gradient following an isocratic phase produced the desired result in that satisfactory resolution was achieved with a total analysis turnaround time of 20 min (Fig. 2).

Biological application

Sample preparation methods of known efficiency were already available from TLC studies of biological matrices[1,2]. In order to avoid problems arising from incompatibility of the extraction solvent (chloroform) with the eluent, the samples were dried under a stream of nitrogen and redissolved in a methanol solution containing carrier steroids. Recoveries were checked with radiolabelled materials. Fig. 3 shows the separation of radiolabelled metabolites following incubation of [4-^{14}C]progesterone with testis microsomes. A comparison of the TLC and HPLC systems for replicate incubations of testes microsomes showed correlation coefficients of 0.98 for 17α-hydroxyprogesterone (n = 10), 0.95 for androstenedione (n = 12 and 0.99 for testosterone (n = 11) in enzyme assays using the appropriate radiolabeled

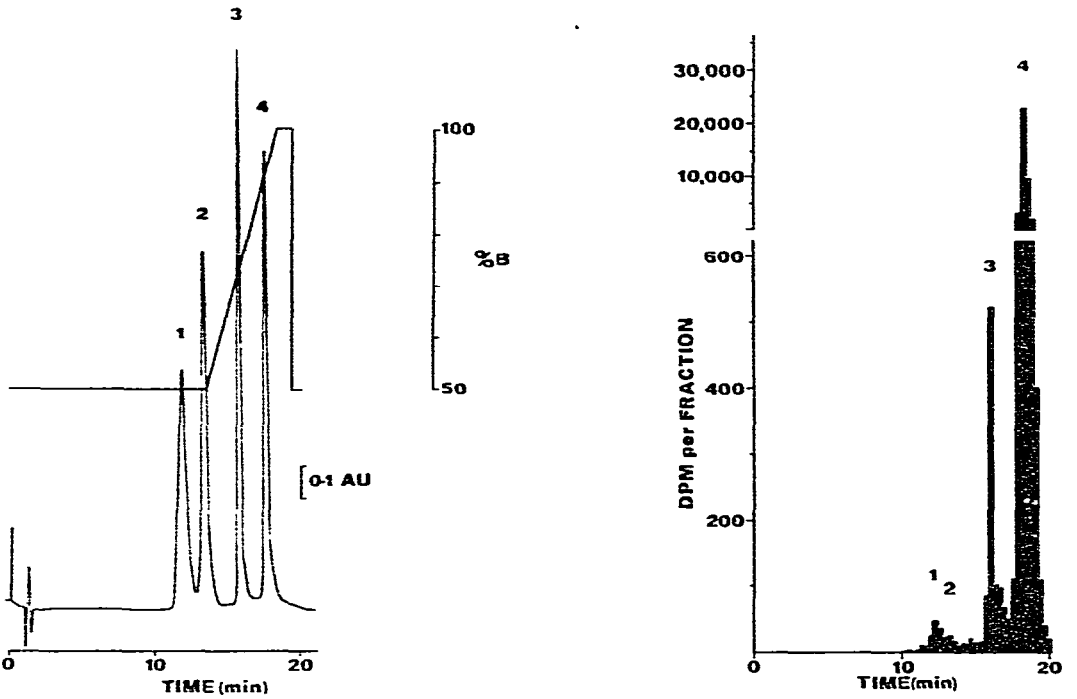

Fig. 2. Chromatogram obtained with Partisil PXS 10 ODS-2 eluted with a linear gradient of tetrahydrofuran-water; flow-rate 2 ml/min. Injection (6 μl) contained 24 μg of each steroid: androstenedione (peak 1), testosterone (peak 2), 17α-hydroxyprogesterone (peak 3), progesterone (peak 4). Reservoir A contained 1% tetrahydrofuran. Reservoir B contained 50% tetrahydrofuran. Elution conditions were: 50% B for 9 min followed by an increase of 10% B per min for 5 min then held at 100% B for 1 min. 3 min to reset starting condition (50% B).

Fig. 3. Separation of metabolites of progesterone from an incubation of [4-^{14}C]progesterone with rat testis microsomes in the presence of an NADPH generating system. Peaks identified and chromatographic conditions correspond to Fig. 2. Injection 80 μl, contained carrier steroids at 0.2 mg/ml of each.

precursors. The reproducibility of the determination of progresterone was assessed by replicate incubations with rat testis microsomes which yielded an overall coefficient of variance of 7.5% on subsequent HPLC analysis.

ACKNOWLEDGEMENT

This work forms part of a research project sponsored by the U.K. Ministry of Agriculture, Fisheries and Food to whom our thanks are due.

REFERENCES

1 A. O. Brinkmann, F. G. Leemborg, E. M. Roodnat, F. H. DeJong and H. J. van der Molen, *Biol. Reproduction*, 23 (1980) 801.
2 M. Shikita and B. Tamaoki, *Endocrinology*, 76 (1965) 563.
3 F. A. Fitzpatrick, *Adv. Chromatogr.*, 16 (1978) 37.
4 R. C. Cochran and L. L. Ewing, *J. Chromatogr.*, 173 (1979) 175.

5 M. Schöneshöfer and H. J. Dulce, *J. Chromatogr.*, 164 (1979) 17.
6 R. C. Cochran, K. J. Darney, Jr. and L. L. Ewing, *J. Chromatogr.*, 173 (1979) 349.
7 M. J. O'Hare, E. C. Nice, R. Magee-Brown and H. Bullman, *J. Chromatogr.*, 125 (1976) 357.
8 J. C. Phillips, D. Mendis, C. T. Eason and S. D. Gangolli, *Food Cosmet. Toxicol.*, 18 (1980) 7.
9 D. G. Walters, B. G. Lake and R. C. Cottrell, *J. Chromatogr.*, 196 (1980) 501.
10 U. Fricke, *Anal. Biochem.*, 63 (1975) 555.