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Note

Rapid analysis of C_{19}-steroid metabolism by high-performance liquid chromatography and in-line monitoring of radioactivity

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Analysis of androgen metabolism has traditionally been based on paper chromatography [1] or thin-layer chromatography (TLC) [2, 3] for the separation and isolation of metabolites. Such assays are generally time-consuming and laborious. Studies of androgen metabolism have therefore often been restricted to a few enzymatic reactions and a limited number of biological specimens at a time. In order to understand the biological role of the numerous steroid-metabolizing enzymes, a large number of assays have to be done simultaneously on the same biological sample [4, 5].

We have devised a fast and reproducible method for the automatic analysis of radioactive metabolites of C_{19}-steroids from incubation studies, based on high-performance liquid chromatography (HPLC) and in-line radiomonitoring (RM). In the present report, this method is compared with a traditional method based on TLC.

MATERIALS AND METHODS

Radioactively labelled steroids were obtained from The Radiochemical Centre (Amersham, U.K.). Unlabelled steroids were supplied by Steraloids (U.S.A.). Labelled steroids were purified as described by Rosness et al. [6]. Scintillation fluid (M299) was obtained from Packard (U.S.A.). From Sigma were obtained: β-NAD, Grade III; NADP, Sigma grade; glucose-6-phosphate; glucose-6-phosphate dehydrogenase, Type XV; and 2,7-dichlorofluorescein. Solvents for HPLC were from Rathburn Chemicals (Walkerburn, U.K.). Ultrapure water was obtained by filtering distilled water through a Gelman Water-I filtration unit (Gelman, Ann Arbor, MI, U.S.A.). All other chemicals and

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solvents were obtained from E. Merck (Darmstadt, F.R.G.) and were of analytical grade.

Incubations

The preparation of homogenates, conditions of incubation and methods for isolation and characterization of steroids have been described in detail previously [3, 6], and only a brief review will be presented here. The tissues were homogenized in Tris—HCl buffer. The enzymatic studies were done on the 800 *g* supernatant fraction. The ^{3}H-labelled steroids used as substrates were dissolved in Tris—HCl buffer together with the cofactors used, and incubations were started by adding tissue homogenate. Incubations were carried out for 5—30 min at 32°C (testis) or at 37°C (prostate and coagulating gland) and were terminated by the addition of 2 ml of ice-cold ethyl acetate. The ethyl acetate contained several reference steroids in a concentration of 15 μg/ml [6]. The incubates were further extracted with ethyl acetate containing no added steroids.

Chromatography

TLC was performed on 20 × 20 cm preformed silica gel plates, obtained from Merck or from Schleicher & Schüll, Dassel, F.R.G. (Table I). The TLC systems have been described previously [3, 6].

The TLC chromatograms were divided into zones corresponding to the reference steroids, and the radioactivity in each zone was measured by liquid scintillation counting (Rackbeta, 1215, LKB, Sweden).

HPLC was performed on a chromatograph consisting of a Constametric III reciprocal pump [Laboratory Data Control (LDC), U.S.A.], a Spectromonitor-III variable-wavelength ultraviolet detector (LDC), and a Refractomonitor-III refractive index (RI) monitor (LDC). The column system consisted of a 50 × 4.6 mm I.D. guard column, dry packed with 40-μm pellicular packing (Pelliguard LC-18; Supelco, U.S.A.) and a 250 × 4.6 mm I.D. reversed-phase analytical column (Supelcosil LC-18; 5-μm spherical packing; Supelco). Development of a separation system for androgens based on reversed-phase HPLC has been published previously [7].

For in-line detection of radioactive metabolites, the eluent from the HPLC column was directly coupled to an SM200 splitter mixer [Nuclear Enterprises

TABLE I

SUMMARY OF CHROMATOGRAPHIC SYSTEMS USED

System No.	Type	Stationary phase	Eluent	Purpose
1*	TLC	Silica Gel 60 (Merck)	Toluene—methanol (9:1)	Separation of 4-ene- and 5α-reduced androgens
2*	TLC	Silica Gel F-1500 (Schleicher & Schüll)	Dichloromethane—ethyl acetate (9:1)	Separation of 5α-reduced androgens
3	HPLC	Supelcosil® LC-18 (Supelco)	Methanol—acetonitrile—water (14:43:43)	Separation of 4-ene- and 5α-reduced androgens
4**	HPLC	Supelcosil® LC-18 (Supelco)	Methanol—acetonitrile—water (33:26:41)	Separation of 5α-reduced androgens

*Two developments [3, 6].
**Ref. 7.

(NE), U.K.], and there continuously mixed with three times its volume of liquid scintillant (scintillator 299, Packard). This mixture was led to an Isoflo (NE) radiomonitor equipped with a flow-cell of 1 ml volume. The content of radioactivity in the flow-cell was normally counted in intervals of 10 sec. The data collected by the radiomonitor were fed to an ABC-80 microcomputer (Scandia-Metric, Norway). This computer was programmed to collect the chromatographic data. The computer performed all peak integrations and the data were presented on a printer (8300, Scandia-Metric) and on a strip-chart recorder.

The eluents used in HPLC were prepared in batches of 10 l. Each batch of eluent was tested and elution volumes of various authentic radiolabelled C_{19}-steroids were recorded.

RESULTS AND DISCUSSION

Separation and quantification of radioactively labelled C_{19}-steroids by a TLC assay and our new HPLC—RM assay gave similar results (Tables II and III). Total time for analysing one sample, including chromatography and quantitation of recovered radioactivity in each isolated fraction, is about 80 min using the TLC assay. In our new HPLC—RM assay, the total analysis time is 10—35 min, depending on the flow-rate in the analytical column. An increase in flow-rate will result in decreased chromatographic resolution and reduced dynamic counting efficiency due to shortened sample residence time in the RM unit.

Each sample analysed by the TLC assay requires about 40 min of manual labour, compared to our new HPLC—RM assay which requires only 2—3 min attendance and manual labour per sample. The HPLC analysis may also be run with an automatic sample injection and computer-based automatic peak integration. This further reduces the manual work required per sample and enables 24 h per day operation giving a high sample through-put.

The static counting efficiency of the radiomonitor is comparable to that of an ordinary liquid scintillation counter. The baseline activity of the detector is normally about 10—15 cpm. Peaks containing about 350 dpm or more of a tritiated compound may be detected and quantitated with good accuracy in our standard assay.

TABLE II

A COMPARISON OF SEPARATION AND QUANTIFICATION OF RADIOLABELLED REFERENCE STEROIDS BY TLC AND HPLC ASSAYS

A mixture of 4-[^{14}C]androstene-3,17-dione (2.2 • 10^6 dpm), 17β-hydroxy-4-[^{14}C]androsten-3-one (5 • 10^5 dpm) and 17β-hydroxy-5α-[^{14}C]androstan-3-one (5 • 10^5 dpm) together with unlabelled reference steroids was divided into eight portions; four of these were analysed by TLC (system 1) and the other four by HPLC (system 3). Values are presented as percentage recovered radioactivity in each fraction (mean ± S.D., n = 4).

Isolated steroids	HPLC	TLC
4-Androstene-3,17-dione	68.4 ± 0.5	68.6 ± 0.1
17β-Hydroxy-4-androsten-3-one	15.5 ± 0.3	15.3 ± 0.1
17β-Hydroxy-5α-androstan-3-one	15.4 ± 0.2	15.3 ± 0.2

TABLE III

QUANTIFICATION OF METABOLITES FROM VARIOUS TISSUES BY HPLC AND TLC ASSAYS

(a) 5α-[^{3}H]Androstane-3α,17β-diol (1 μg) was incubated for 15 min at 37°C with a homogenate from the rat ventral prostate (17.0 mg protein). NAD was added as cofactor. (b) 5α-[^{3}H]Androstane-3β,17β-diol (1 μg) was incubated for 5 min at 32°C with a rat testicular homogenate (21.4 mg protein) in the presence of an NADPH-generating system. (c) 17β-[^{3}H]Hydroxy-4-androsten-3-one (1 μg) was incubated for 60 min at 37°C with a homogenate from the rat coagulating gland (10.5 mg protein) in the presence of an NADPH-generating system. The metabolites were extracted as described in Material and methods. The extracts were each divided into eight (c) or ten (a, b) aliquots. Half of the corresponding aliquots were analysed by TLC (a, b: system 2; c: system 1) or by HPLC (system 4). Values are presented as percentage recovered radioactivity in each fraction [mean ± S.D., n = 5 (a, b) n = 4 (c)].

Metabolite	Ventral prostate (a)		Testis (b)		Coagulating gland (c)	
	HPLC	TLC	HPLC	TLC	HPLC	TLC
5α-Androstane-3α,17β-diol	19.5 ± 0.5	18.7 ± 0.7	3.3 ± 0.2	3.8 ± 0.7	1.6 ± 0.1	1.9 ± 0.1
5α-Androstane-3β,17β-diol	—	—	78.0 ± 0.9	81.9 ± 0.3	—	—
3α-Hydroxy-5α-androstan-17-one	—	—	0.46 ± 0.37	0.16 ± 0.09	—	—
3β-Hydroxy-5α-androstan-17-one	—	—	0.60 ± 0.10	1.25 ± 0.16	—	—
17β-Hydroxy-5α-androstan-3-one	80.5 ± 0.5	81.3 ± 1.0	11.7 ± 0.8	11.8 ± 0.1	11.6 ± 0.2	11.0 ± 0.2
5α-Androstane-3,17-dione	—	—	0.72 ± 0.10	0.58 ± 0.20	—	—
17β-Hydroxy-4-androsten-3-one					86.8 ± 0.2	87.0 ± 0.2

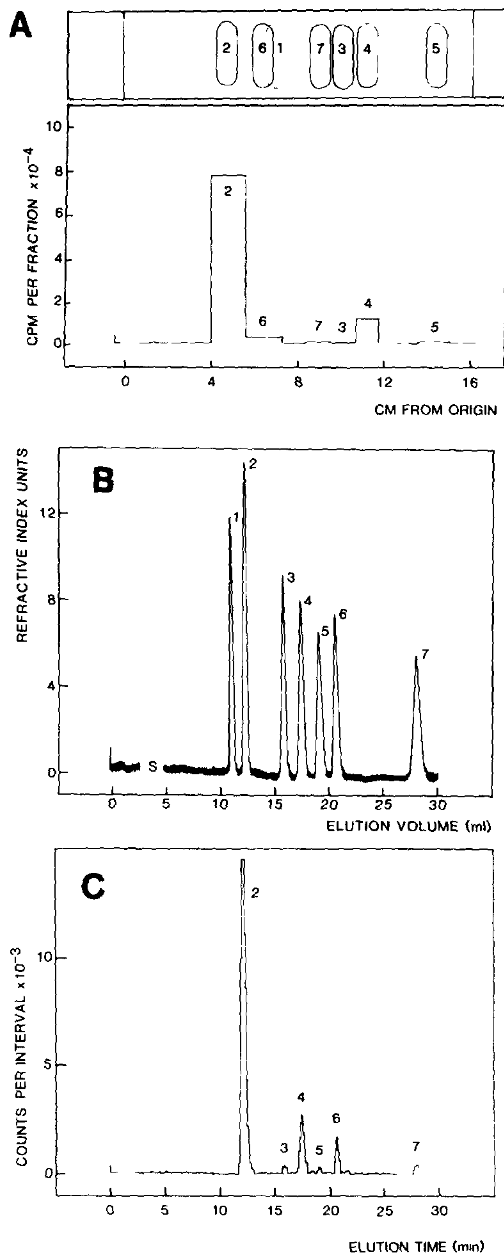

Fig. 1. Comparison of TLC and HPLC—RM separation and quantitation of radiolabelled C_{19}-steroids. Tritiated 5α-androstane-3β,17β-diol was incubated with a testicular homogenate as described in Materials and methods. Radiolabelled metabolites were extracted and separated by TLC or HPLC (systems 2 and 4, respectively, Table I). (A) TLC chromatogram and distribution of radioactivity in isolated fractions determined by liquid scintillation counting (see Materials and methods). (B) HPLC chromatogram of a mixture of authentic C_{19}-steroids. (C) Radiochromatogram of radiolabelled C_{19}-steroids separated by HPLC and quantitated by an Isoflo radiomonitor (see Materials and methods). Peaks: 1 = testosterone; 2 = 5α-androstane-3β,17β-diol; 3 = 3β-hydroxy-5α-androstan-17-one; 4 = 17β-hydroxy-5α-androstan-3-one; 5 = 5α-androstane-3,17-dione; 6 = 5α-androstane-3α,17β-diol; 7 = 3α-hydroxy-5α-androstan-17-one.

An HPLC—RM unit will generate a substantially increased amount of information compared to traditional TLC assays (Fig. 1). In extracts from incubations of radioactive C_{19}-steroids with rat and human prostatic tissues we have observed several minor metabolites yet to be structurally identified.

C_{19}-Steroids display functional group specific solvent selectivity which can be used to increase chromatographic selectivity [8]. We have used such selective solvent effects to gain structural information and thus tentative identification of unknown metabolites by running the chromatographic analysis with different mobile-phase compositions [8].

In conclusion, addition of a radiomonitor to a conventional HPLC system gives a fast and reproducible method for the separation and quantitation of radiolabelled gonadal steroids as well as a variety of other labelled compounds.

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