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Note

A specific reversed-phase liquid chromatographic method for analysis of steroids in Y-1 adrenal cell cultures

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The Y-1 cell line derived from mouse adrenal tumor was established by Buonassisi et al. in 1962 [1]. These cells retain steroidogenic activity and responsiveness to adrenocorticotropic hormone (ACTH) and other secretagogues, and have been used as an important model for studying hormonal effects on adrenal steroidogenesis and other functions [2–8]. Our laboratory has been involved in studies with these cells for over 20 years and has elucidated ACTH effects on glycolytic activity, cholesterol biosynthesis and metabolism, mitochondrial enzyme functions, and other alterations in specific subcellular organelles [9–13].

In contrast to normal mouse adrenocortical cells, these cells lack microsomal 21-hydroxylase activity [14] and express increased 20α-steroid dehydrogenase activity. Therefore, the major end-product steroid of the cells is 11β,20α-dihydroxy-4-pregnen-3-one. Depending on the level of mitochondrial 11β-hydroxylase activity in the cell line used and the experimental conditions, varying levels of 20α-hydroxy-4-pregnen-3-one may also be accumulated. These two steroids are both fluorescent at similar wavelengths of excitation (475 nm) and emission (525 nm) in ethanolic–sulfuric acid solution. Since authentic 11β,20α-dihydroxy-4-pregnen-3-one is not commercially available, a fluorometric assay using 20α-hydroxy-4-pregnen-3-one as standard has been developed that permits quantitation of total fluorescent steroids output by the cells [9]. Nevertheless,

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this method cannot determine the individual levels of the two steroids contributing to the fluorescence being measured.

Another method frequently used for determination of Y-1 steroidogenesis is radioimmunoassay using specific antibody against 20α-hydroxy-4-pregnen-3-one [4,7]. This method is accurate, yet the specificity limits the assay to the steroid intermediate rather than to the end-product steroid. In cell lines with active mitochondrial 11β-hydroxylation, 20α-hydroxy-4-pregnen-3-one synthesized by the cells is rapidly metabolized to form 11β,20α-dihydroxy-4-pregnen-3-one. Consequently, the radioimmunoassay method could result in an underestimate of the steroidogenic capacity of these cell lines.

Faust et al. [3] employed gas chromatography (GC) to determine steroids in Y-1 cultures. 11β,20α-Dihydroxy-4-pregnen-3-one could be separated from other intermediates and quantified. However, 20α-hydroxy-4-pregnen-3-one could not be separated from progesterone. The content of 20α-hydroxy-4-pregnen-3-one was presented, therefore, as the sum of this steroid and progesterone.

We have developed a method for analysis of steroids in Y-1 cell cultures using reversed-phase high-performance liquid chromatography (HPLC) which permits simultaneous separation and determination not only of the end-product steroid but also of other 3-keto steroid intermediates.

EXPERIMENTAL

Materials

Authentic 11β,20α-dihydroxy-4-pregnen-3-one and 11β,20β-dihydroxy-4-pregnen-3-one were obtained from Steroid Collections (London, U.K.). The former steroid was also prepared from stimulated Y-1 cultures in this laboratory using the authentic compound as a marker. The purity of our prepared steroid has been ascertained by comparing the retention time of the authentic sample in reversed-phase HPLC and by determining the molecular mass of 332 in 70-eV electron-impact mass spectral analysis using a Hewlett-Parkard 5985B gas chromatograph–mass spectrometer. The following chemicals were purchased from commercial sources: the components of the culture medium, Gibco (Grand Island, NY, U.S.A.); $ACTH_{1\text{-}24}$ (Cortrosyn), Organon (West Orange, NJ, U.S.A.); HPLC-grade solvents, J.T. Baker (Sanford, ME, U.S.A.); steroids, Sigma (St. Louis, MO, U.S.A.).

Cell culture and steroidogenesis

Methods of cell culture and tumor transplantation to LAF-1 mice have been detailed previously [9–13]. Cells were plated in 60 × 15 mm plastic culture dishes and grown to confluence prior to each experiment. On the day of the experiment, the serum-containing medium was replaced by 4 ml serum-free, phosphate-Ringer-N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid medium [11], pH 7.5. Cells were incubated with or without 100 mU/ml ACTH for 4 and 24 h to ensure maximum steroidogenesis. After incubation, the medium was collected and kept in a −20°C freezer until analysis.

Steroid analysis by reversed phase HPLC

Ethanol (5 μl) containing 2.5 μg 17α-hydroxyprogesterone, the internal standard, was added to 1 ml culture medium immediately prior to extraction. The culture medium was extracted at room temperature with a five-fold volume of dichloromethane by vigorously vortexing for 1 min. After centrifugation at 2000 *g* for 10 min, the acqueous phase was removed and the organic phase was washed with 0.5 ml triply distilled water. After centrifugation, the upper water phase was removed, and 4 ml of the organic phase were dried under a stream of nitrogen. The dried residues were dissolved in 100 μl methanol, and 50 μl were injected into a reversed-phase HPLC system as described below.

The HPLC system (Waters Assoc., Milford, MA, U.S.A.) used consisted of dual pumps (Model 510), a computer (Model 680), a UV detector (Model 450), and a 10-μm μBondapak C_{18} reversed-phase column (30 cm$\times$3.9 mm I.D.) with a guard column containing Bondapak C_{18}/Corasil (particle size 37–50 μm). An integrator–recorder (Shimadzu, Model CR3A, Tokyo, Japan) was used for data analysis. The mobile phase employed was a solvent mixture composed of acetonitrile–methanol–water (35:11:54, v/v/v). This composition was a modification of the solvent mixture acetonitrile–methanol–0.1% phosphoric acid (35:11:54, v/v/v), recommended by Supelco (Bellefonte, PA, U.S.A.). Replacement of 0.1% phosphoric acid by water did not significantly change the efficiency of separation, but reduced the time for preparation of the solvent mixture. HPLC-grade water was filtered once before use. Solvent mixtures were sonicated in an ultrasound bath for 15 min at room temperature to remove trapped air-bubbles from the mixtures before experiments. Isocratic flow-rate was set at 1.0 ml/min. The separation of steroids was monitored at 254 nm. Authentic steroids were added either internally or externally to samples to verify the identify of the steroid peaks in chromatograms. Quantitation of steroids was obtained by extrapolation of the data on a linear curve which shows the relationship between the area ratios of authentic steroids to the internal standard and the amount of steroids added before extraction.

RESULTS

The major steroidogenic pathways of Y-1 cells [10] is outlined as follows: cholesterol→pregnenolone→progesterone→20α-hydroxy-4-pregnen-3-one→ 11β, 20α-dihydroxy-4-pregnen-3-one; and, alternatively, progesterone→ 11β-hydroxyprogesterone→11β,20α-dihydroxy-4-pregnen-3-one.

The reversed-phase HPLC system described in this study is able to separate 11β,20α-dihydroxy-4-pregnen-3-one from its stereoisomer 11β,20β-dihydroxy-4-pregnen-3-one (Table I). It confirms that the peak produced by the cells is exclusively of 11β,20α-dihydroxy-4-pregnen-3-one. The coefficients of variation of the retention time of 11β,20α-dihydroxy-4-pregnen-3-one among intra- and inter-assays are 0.33 and 2.38%, respectively. By including 17α-hydroxyprogesterone, which is not a steroid intermediate of Y-1 cells, as internal standard, the system is capable of separating (Fig. 1) and quantifying 11β,20α-dihydroxy-4-

TABLE I

SEPARATION OF STEREOISOMERS OF 11β,20α-DIHYDROXY-4-PREGNEN-3-ONE AND 20α-HYDROXY-4-PREGNEN-3-ONE BY REVERSED-PHASE HPLC

The chromatographic method and HPLC system are detailed under Experimental.

Steroids	Retention time (min)
11β,20α-Dihydroxy-4-pregnen-3-one	11.99
11β,20β-Dihydroxy-4-pregnen-3-one	24.15
20α-Hydroxy-4-pregnen-3-one	37.83
20β-Hydroxy-4-pregnen-3-one	56.87

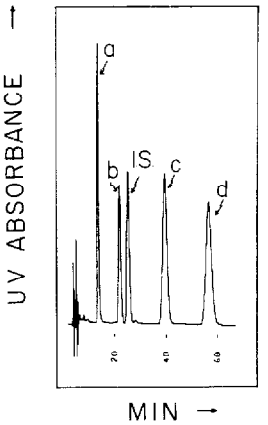

Fig. 1. Separation of authentic steroids. The peaks a–d represent the authentic steroids 11β,20α-dihydroxy-4-pregnen-3-one, 11β-hydroxyprogesterone, 20α-hydroxy-4-pregnen-3-one, and progesterone, respectively. The peak I.S. represents the internal standard 17α-hydroxyprogesterone. Detection wavelength, 254 nm; a.u.f.s. range, 0.02.

pregnen-3-one (see Experimental) and all 3-keto steroid intermediates in one chromatogram.

The steroid chromatograms and content of a set of unstimulated and ACTH-stimulated cultures are presented in Figs. 2 and 3, respectively. The level of 11β,20α-dihydroxy-4-pregnen-3-one in the 4-h stimulated culture (B) is markedly elevated compared to that in the unstimulated (A). Further incubation to 24 h increases the level of this steroid (D versus B, C). The levels of 20α-hydroxy-4-pregnen-3-one are either similar to (in A) or lower than (in B, C, and D) those of 11β-hydroxyprogesterone indicating the mitochondrial 11β-hydroxylation of the cells is very active.

In a separate experiment (data not shown), cultures were stimulated for 4 h in the presence or absence of 150 μM aminoglutethimide (AG), a specific inhibitor of the cholesterol side-chain cleavage reaction. The stimulated level of 11β,20α-dihydroxy-4-pregnen-3-one was inhibited more than 90% and no trace of intermediates could be detected in the presence of AG. This added further validation that the peaks observed in the chromatograms of Fig. 2 are steroids.

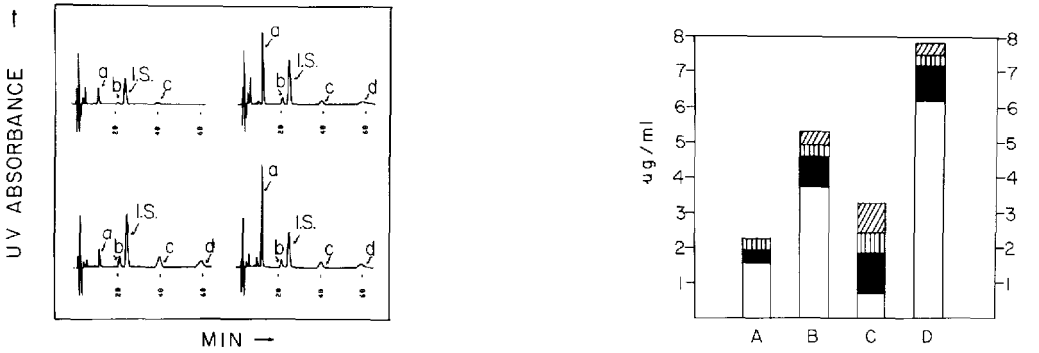

Fig. 2. Steroid chromatograms of Y-1 adrenal cell cultures. Chromatograms show the analysis of the extracts of 1 ml medium from a set of cultures after the following treatment: (A) 4 h incubation without stimulation (upper left); (B) 4 h stimulation with ACTH (upper right); (C) 24 h incubation without stimulation (lower left); and (D) 24 h stimulation with ACTH (lower right). The dose of ACTH for stimulation was 100 mU/ml. The symbols a–d and I.S. assigned for steroids are the same as those described in Fig. 1; a.u.f.s. range, 0.1.

Fig. 3. Steroid content of Y-1 adrenal cell cultures. The experimental conditions of cell cultures A–D correspond to the descriptions in the legend of Fig. 2. (□) 11β,20α-Dihydroxy-4-pregnen-3-one; (■) 11β-hydroxyprogesterone; (▥) 20α-hydroxy-4-pregnen-3-one; (▨) progesterone; which correspond to steroids a–d in Fig. 2.

DISCUSSION

The reversed-phase HPLC method described herein provides a highly specific method for the determination of the levels of 11β,20α-dihydroxy-4-pregnen-3-one in Y-1 cell cultures (as low as 0.26 μg per 1 ml medium) and also permits the separation and quantification of all the 3-keto steroid intermediates. The present method is more applicable for characterizing the steroidogenic capacity of subcultured cell lines and mutants than the aforementioned radioimmunoassay [4,7] which assays only 20α-hydroxy-4-pregnen-3-one. This method of separation is preferable to GC [3] which cannot separate 20α-hydroxy-4-pregnen-3-one from progesterone. Furthermore, the present method is particularly useful in studying steroidogenesis of culture cells subjected to various treatments with inhibitors or other chemicals which might interfere with the determination by acid fluorescence [11]. For instance (data not shown): (a) metyrapone, a specific inhibitor of mitochondrial 11β-hydroxylation which inhibits acid fluorescent 11β,20α-dihydroxy-4-pregnen-3-one synthesis of the cells, also induces an accumulation of the acid fluorescent intermediate 20α-hydroxy-4-pregnen-3-one; (b) 25-hydroxycholesterol, frequently used as an alternative substrate for steroidogenesis, also causes a considerable background of fluorescence.

The principal disadvantage of the method is the relatively longer time required for an assay. If interest is limited to the content of 11β,20α-dihydroxy-4-pregnen-3-one, one analysis takes 30 min to elucidate the levels of the steroid and the internal standard. If the levels of all 3-keto steroid intermediates are of interest,

one analysis takes 65 min. Presently, we have not tried to increase the flow-rate to reduce the times for an analysis.

Finally, during the course of the present work, Duch et al. [15] employed reversed-phase HPLC to determine steroids for studying regulation of tetrahydrobiopterin biosynthesis in Y-1 cell cultures, but did not report the chromatographic characterization of the steroids. In conclusion, we have provided a reversed-phase HPLC method for both qualitative and quantitative analysis of steroids which is specially designed for studying steroidogenesis in Y-1 cell cultures.

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