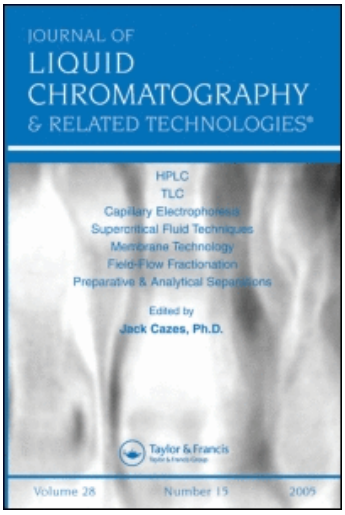

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High-Performance Liquid Chromatographic Determination of Progesterone

Bulent Güven[a]; Figen Ünlü Erkoç[a]; Semin Özsar[a]; Zişan Emre[a]; Ergül Ugrar[a]
[a] Turkish Atomic Energy Authority Lalahan Nuclear Research Institute of Animal Health Lalahan, Ankara, Turkey

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PROGESTERONE

Bulent Güven, Figen Ünlü Erkoç, Semin Özsar, Zişan Emre, and Ergül Ugrar
Turkish Atomic Energy Authority
Lalahan Nuclear Research Institute of Animal Health
Lalahan, Ankara, 06852, Turkey

ABSTRACT

A practical, rapid, reliable and isocratic reversed-phase HPLC method is described for the determination of progesterone; a method important for determining in cows (1) early pregnancy, (2) reproductive disorders, and (3) timing of artificial insemination. The method is reproducible with a detection limit of 0.5 ng/peak (400 pg/µl) at 254 nm (0.005 AUFS); down by nearly 10-fold from other methods and using simple 254 nm detection. Radioimmunoassay of all eluting fractions demonstrated specificity.

INTRODUCTION

Progesterone, 4-pregnene-3,20-dione, is a progestational hormone mainly secreted by the corpus luteum which helps gestation in early pregnancy in all mammals. Progesterone appears after ovulation and causes extensive development of the endometrium, preparing the uterus for the reception of the embryo and for its nutrition.

Progesterone levels in cows provide an early estimate of pregnancy, offer the possibility of examining ovarian activity during post partum and enable characterization of reproductive disorders. This need led to the development of a simple, rapid, nondestructive, isocratic high-performance liquid chromatographic method (LC) for the determination of progesterone.

Natural and synthetic steroids with a wide variety of functional groups traditionally have been separated and determined by thin-layer chromatography and gas chromatography (1). Recently, with the development of normal and reversed-phase HPLC methods, numerous publications on LC determination of progesterone and other steroids have appeared (2-8). The conjugated double bond found in most biologically active steroids shows maximum UV absorption around 240 nm: Detection sensitivities of 1-10 ng are found typically for the corticosteroids, which have a high UV molar extinction coefficient. The hormones of the reproductive cycle which have a lower UV molar extinction coefficient are usually detected in the 10-40 ng range (9-11). Sensitivity of 1-2 ng/peak has been reported for the 4-ene-3-one steroids at 246 nm (4). Dolphin and Pergande (7) detected 2,500 ng estriol/ml pregnancy urine at 280 nm. The LC method reported here reaches the 0.5 ng/peak level.

Conventional radioimmunoassay (RIA) and enzyme immunoassay (EIA) have limits of detection in the pg range for serum and milk progesterone (12,13). The excellent specificity of LC has been used to measure the specificity of RIA and EIA methods (8,14). Each peak may be collected individually to be analyzed by RIA, leading to a qualitative and quantitative analysis of the immunoassayable material. In addition, Jawad et al. (5) repurified, by reversed-phase LC the radiolabeled progesterone from contaminating steroids and degradation products.

This study reports a practical, rapid, nondestructive and reliable reversed-phase LC method using an acetonitrile-water mobile phase with simple 254 nm UV detection for the determination of progesterone to 0.5 ng/peak (400 pg/µl).

EXPERIMENTAL

Apparatus

A Waters Associates (Milford, MA) 6000A pump with a U6K injector was used with a reversed-phase column and a Radial Compression Separation System (RCSS) cartridge in a Model RCM 100 compression module (Waters Assoc.). Maximum pressure limit of the column was 2,000 psi; flow was 1.5-2 ml/min with the system at ambient temperature. A Model 441 fixed wavelength UV absorbance detector (Waters Assoc.) was used with either 254 or 280 nm filters. A BBC Goerz-Metrawatt SE-120 Recorder (Vienna, Austria) was used to monitor detector response. {^{3}H}-Radiolabeled materials were detected with an ICN coru/mat 2700 Liquid Scintillation Spectrometer (ICN Tracerlab Div., Mechelen, Belgium).

Samples and Reagents

Progesterone (4-pregnene-3,20-dione) (E. Merck, Darmstadt, W. Germany) stock solution was prepared in methanol (1 mg/ml), appropriate dilutions in methanol, were used for HPLC analyses. Radiolabeled {1,2,6,7-^{3}H}-Progesterone (S.A. 80 Ci/mmol; 254 X 10^{-3} μCi/ng, Amersham International, Amersham, Bucks, UK) was diluted in methanol for all experiments.

HPLC grade solvents (Fisher Scientific, Fair Lawn, NJ) were filtered through 0.5 μm filters and vacuum degassed before use. Double glass-distilled water was passed through Norganic Water Purification System (Waters Assoc., Milford, MA).

The samples were dissolved and injected in methanol (50 μl for analytical; 200 μl for HPLC-RIA runs). For the RIA runs, 0.5 ml fractions were collected and analyzed by RIA according to the procedure of Hoffmann *et al.* (12), with the LC modification of Enzenhöfer (15). The antibody was raised in rabbits against 11α-OH-progesterone-HS-BSA in our laboratory and used at a dilution of 1:12,000. The antiserum has cross reactions: 0.028% testosterone, 0.004% cortisol, and lower than 0.001% against others (estradiol-17β, estrone and estrone sulfate).

Column effluents were assayed for ^{3}H-radioactivity using Xyloflour aqueous cocktail (J.T. Baker Chemical Co., Phillipsburg, NJ).

Mobile Phase

Isocratic eluents were 50% and 90% (v/v) acetonitrile in water (3). Fractions of 0.5 ml were collected at a flow rate of 1.5 ml/min for HPLC-RIA; 2 ml/min for the analytical runs.

RESULTS AND DISCUSSION

Retention times of progesterone were 6.8 min ($k' = 5.4$) in 50% acetonitrile and 3.0 min ($k' = 2.4$) in 90% acetonitrile. Reproducibility of retention times was consistent over a period of three weeks, a total of 20 individual runs. The position of progesterone was confirmed by injecting 200 ng of non-radioactive progesterone spiked with approximately 1,500 cpm tritiated-progesterone; collecting each UV-absorbing peak and counting for {^{3}H}-radioactivity. Figure 1 shows such a typical chromatogram. The 90% acetonitrile eluent was prefered over the 50% acetonitrile because of shorter retention time.

Radiochemical purity and specificity of the tritium-labeled progesterone, routinely used for RIA in our laboratory, was checked by injecting known amounts of {^{3}H}-progesterone on the column, and collecting and assaying each 0.5 ml fraction for progesterone. Figure 2 shows this elution pattern both with UV detection at 254 nm (solid) and RIA (dashed). Average analytical LC recovery of injected radioactivity was above 95%. All of the radioactivity was under the UV absorbing peak (shown with an arrow) with 3 min retention time; corresponding to progesterone. Maximum binding in fraction 15 led to the conclusion that the antibody against progesterone used in our RIA system was specific for progesterone.

The limit of detection for progesterone was 0.5 ng/peak (UV at 254 nm, 0.005 AUFS, signal/noise ratio = 3). The calibration curve for non-radioactive progesterone is shown in Figure 3. Detector response is linear from 2 ng to 14 ng but with calibration results can be used to 20 ng. The values in the calibration curve

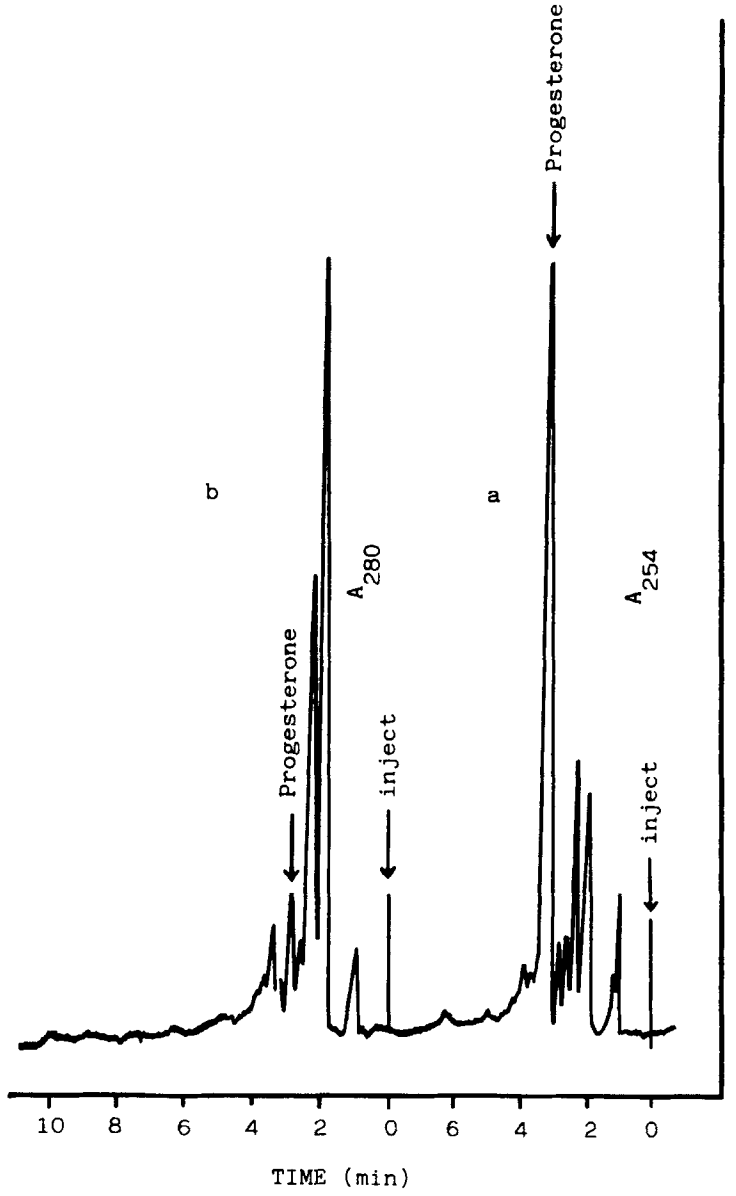

FIGURE 1. ^{3}HPLC of 200 ng non-radioactive progesterone spiked with 1,500 cpm ^{3}H-progesterone. Mobile phase is 90% acetonitrile in water (v/v); at a flow of 2 ml/min; recorder speed 1 cm/min. (a) UV detector at 254 nm; (b) UV detector at 280 nm, both chromatograms with attenuation 0.02 AUFS. Arrow indicates position of progesterone.

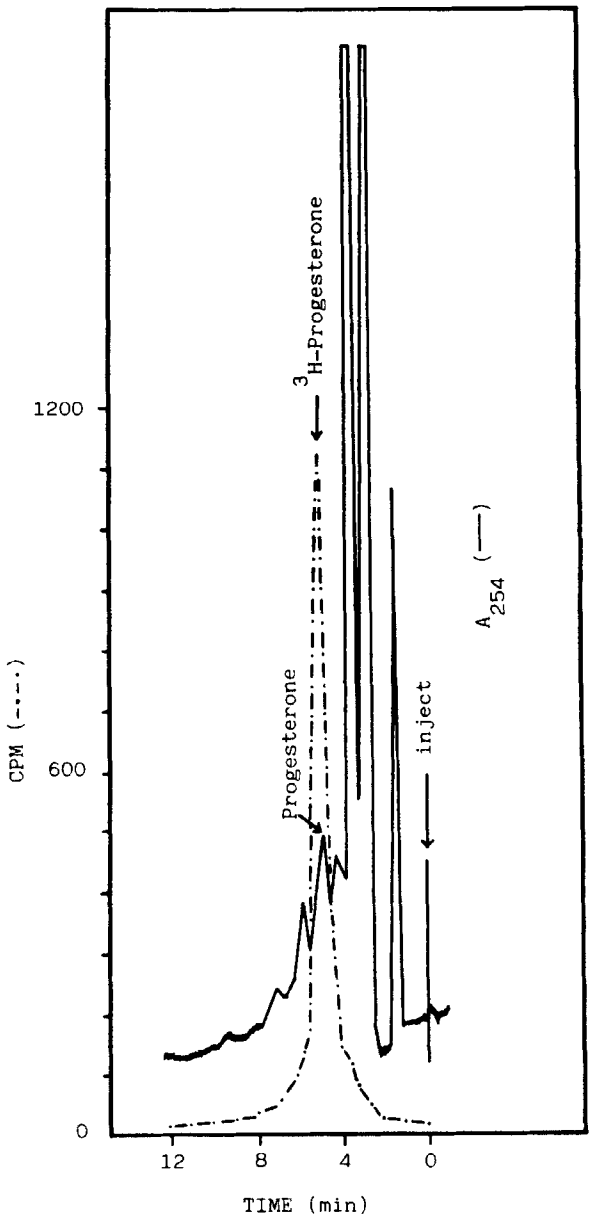

FIGURE 2. HPLC-RIA of ^{3}H-progesterone (32,000 cpm; 200 µl, Specific Activity 80 Curies/mmol). Mobile phase same as Figure 1. Detection by UV absorbance at 254 nm; 0.01 AUFS (———). Each fraction, corresponding to 0.5 ml, was subjected to RIA (.-.-.-) as described in MATERIALS AND METHODS. Flow 1.5 ml/min; recorder chart speed 0.5 cm/min; at ambient temperature.

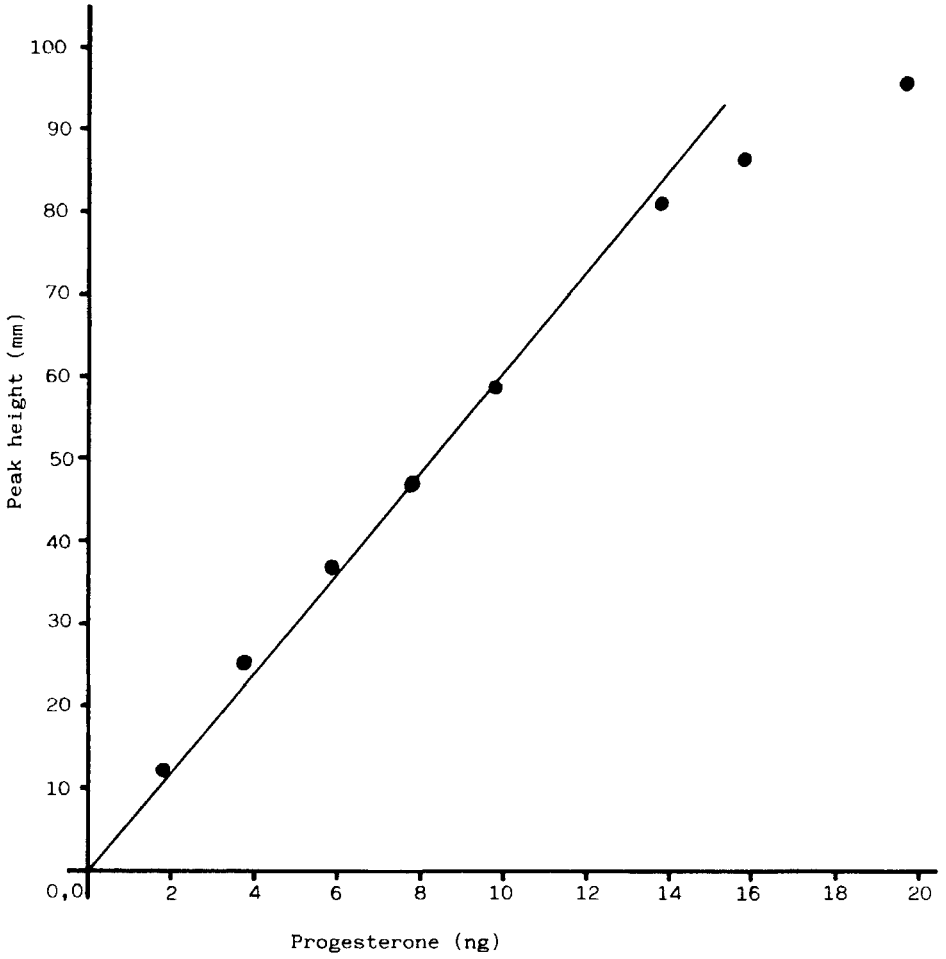

FIGURE 3. Typical calibration curve for non-radioactive progesterone (injected in methanol). LC conditions same as FIgure 1, UV detection at 254 nm, attenuation 0.005 AUFS.

are averages of six runs. As expected, the detection limit at 280 nm (20 ng) is much poorer than at 254 nm. Therefore, detection at 254 nm is better.

In conclusion, the reversed-phase LC method described here offers a practical, rapid, nondestructive, reliable determination of progesterone in the ng range. Although, the detection limit does not reach the pg range of RIA and EIA (12,13) this LC method may be used with RIA to (1) check radiochemical purity, (2) identify metabolite(s), (3) quantify degradation products, and (4) measure specificity of RIA methods. The nondestructive property of reversed-phase LC permits individual peaks or fractions to be collected and used for other purposes.

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