Determination of progesterone and 17-hydroxyprogesterone by high performance liquid chromatography after pre-column derivatization with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionohydrazide

Masatoki Katayama,*[a] Rieko Nakane,[a] Yoshifumi Matsuda,[a] Satoru Kaneko,[b] Izumi Hara[c] and Hirohisa Sato[c]

[a] *Department of Functional Bioanalysis, Meiji College of Pharmacy, 1-35-23, Nozawa, Setagaya-ku, Tokyo 154, Japan*
[b] *Department of Obstetrics and Gynecology, Ichikawa Genaral Hospital, Tokyo Dental College, Ichikawa, Chiba, Japan*
[c] *Department of Obstetrics and Gynecology, Tachikawa Kyosai Hospital, Tachikawa, Tokyo, Japan*

Received 3rd June 1998, Accepted 10th August 1998

Progesterone, 17-hydroxyprogesterone and four other 3-keto steroids were determined by high performance liquid chromatography with fluorescence detection. Each steroid was derivatized with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionohydrazide (BODIPY FL hydrazide) and separated on a Wakosil 5C4 column with acetonitrile–water (7 + 3) as mobile phase. The limits of detection of progesterone, 17-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, testosterone and 17-methyltestosterone were 550–3700 fmol per 10 μl injection (signal-to-noise ratio = 5) serum. The calibration curves were linear up to 1000 ng/ml serum. The proposed method was most sensitive among the available high performance liquid chromatographic methods after fluorescence and chemiluminescence pre-labeling with dansylhydrazine.

Introduction

Progesterone, 17-hydroxyprogesterone and other 3-keto steroids (*e.g.*, dehydroepiandrosterone) have been monitored for the diagnosis of metabolic disorders. As simultaneous determinations were needed for each metabolite, chromatographic methods were the main approach used. Gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS)[1–5] and high performance liquid chromatography (HPLC)[6–11] have been reported for the simultaneous determination of progesterone, 17-hydroxyprogesterone and other 3-keto steroids. HPLC is widely used because simple extraction and derivatization methods can be employed. Using HPLC, each steroid was separated on a normal- or reversed-phase column after pre-labeling with 1-dimethylaminonaphthalene-5-sulfonylhydrazine (dansylhydrazine).[6–11] These steroids were detected by fluorescence[6–7,10,11] or chemiluminescence detection.[8,9]

We have developed an HPLC method for monitoring estrogens[12–14] in biological fluids from pregnant women. Progesterone and 17-hydroxyprogesterone change in concentration during pregnancy and it was expected that monitoring progesterone and 17-hydroxyprogesterone could help in diagnosis during pregnancy and in hormone diseases. The aim of this work was to develop an HPLC method for monitoring progesterone and 17-hydroxyprogesterone in the serum from pregnant women. The concentration of these compounds in serum from pregnant women is in the range 10–200 ng ml^{-1}. but the detection limits of reported HPLC methods with dansylhydrazine were 60–4000 ng ml^{-1} in serum or plasma.[6–11] Hence it was necessary to improve the sensitivity after pre-labeling for monitoring progesterone and 17-hydroxyprogesterone in serum from pregnant women. We therefore studied new pre-labeling reagents and HPLC conditions.

Experimental

Apparatus

The HPLC conditions were as follows: HPLC pump, Shimadzu, Kyoto, Japan LC-6A; analytical column, Wakosil 5C4 (300 × 4.0 mm id) (Wako , Osaka, Japan); mobile phase, acetonitrile–water (7 + 3); flow rate, 0.8 ml min^{-1}; detector, Shimadzu RF-535 fluorescence detector (λ_{ex} 495 nm, λ_{em} 516 nm); integrator, Shimadzu CR-5A; and injection volume, 10 μl.

Reagents

The HPLC mobile phase and solvents were of HPLC grade (Wako). Dansylhydrazine was purchased from Tokyo Kasei (Tokyo, Japan) and 7-dimethyaminocoumarin-3-carbohydrazide (DCCH), 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionohydrazide (BODIPY FL hydrazide), fluorescein-5-thiocarbazide (FTCB), *N*-(fluorenylmethoxycarbonyl)hydrazine (FMOC hydrazine), 4-hydrazino-7-nitro-2,1,3-benzoxadiazole (NBD-hydrazine), 1-pyrenebutanohydrazide (PBAH) and Texas Red hydrazide from Molecular Probes (Eugene, OR, USA). Progesterone (P), 17-hydroxyprogesterone (17-OHP), dehydroepiandrosterone (DHEA), testosterone (T), 17-α-methyltestosterone (17-MeT; internal standard) and androstenedione (A-dione) were supplied by Sigma (St. Louis, MS, USA). BODIPY FL hydrazide solution (1 μg ml^{-1}) was prepared by dissolving 0.5 mg of the reagent in 5.0 ml of ethanol. Trifluoroacetic acid solution (0.08% v/v) was prepared by dissolving 83 μl of trifluoroacetic acid in 100 ml of ethanol. BODIPY FL hydrazide solution could be used for 3 h.

Extraction and derivatization procedure

A 10 μl volume of steroid solution (containing P, 17-OHP, DHEA, T, 17-MeT as internal standard and A-dione) was added to 1 ml of serum (final concentration 100 ng ml^{-1}) from a non-pregnant woman. The spiked sample was diluted to 3 ml with water and 10 ml of dichloromethane were added. This serum was mixed in a Vortex mixer for 1 min and allowed to stand for 10 min. An 8 ml volume of the dichloromethane layer was collected in a test-tube and evaporated to dryness in a Zymark 111B test-tube evaporator. A 0.2 ml volume of BODIPY FL hydrazide solution and 0.2 ml of 0.08% trifluoroacetic acid solution were added and the test-tube was allowed to stand for 15 h at room temperature. A 10 μl volume of the solution was injected into the HPLC apparatus. The derivatization reaction is shown in Fig. 1.

Results and discussion

Extraction conditions

The extraction conditions used for progesterone and other steroids were those described by Kawasaki *et al.*[6,7] with some modifications. The recoveries of progesterone and other steroids (100 ng ml^{-1} serum) were 90.6–93.8%.

Derivatization conditions

Trifluoroacetic acid concentration. The derivatization reaction of dansylhydrazine and steroids forms hydrazones. Hydrochloric acid and trifluoroacetic acid have been used as catalysts in methanol or ethanol solution.[6–9] Recently, the use of triflouromethanesulfonic acid was also reported,[10,11] but it is very hazardous and not easy to use. Moreover, it was necessary to decompose the excess trifluoromethanesulfonic acid with 2 M aqueous ammonia. We planned to use an autosampler, but methanol is a strong denaturant of serum proteins, so facile precipitation and evaporation could occur. Therefore, we selected trifluoroacetic acid as a catalyst owing to its ease of handling and ethanol was selected as an alternative solvent to methanol for BODIPY FL hydrazide derivatization. The highest and constant detector response was obtained when trifluoroacetic acid concentrations in the range 0.01–0.12% v/v were used and 0.08% v/v was therefore adopted.

Effect of BODIPY FL hydrazide concentration. The molar ratios of dansylhydrazine and progesterone have been discussed.[11] High molar ratios were effective in improving sensitivity. We plotted the molar ratios of BODIPY FL hydrazide to progesterone (molar ratios 20–2000, BODIPY FL hydrazide concentration 10–1000 μg ml^{-1}; Fig. 2). The highest and most constant detector response was obtained with molar ratios in the range 120–1000 with 60–500 μg ml^{-1} BODIPY FL hydrazide solution. Therefore, a molar ratio of 200 and a 100 μg ml^{-1} concentration were selected.

Effect of reaction time and temperature. The reaction time was varied from 0.5 to 24 h and the reaction temperature was varied in the range 20–50 °C. A constant detector response was obtained at each temperature (Fig. 3), *e.g.*, over 1.5 h at 50 °C and over 11 h at 25 °C. Room temperature was selected in all previous work using dansylhydrazine[6–11] to avoid the decomposition of steroids and metabolites in biological samples. We therefore selected 15 h at room temperature (about 22 °C)

Comparison of derivatizing reagents. We derivatized progesterone (1000 ng ml^{-1}) with several fluorescent derivatization reagents reported for HPLC. Dansylhydarazine, BODIPY FL hydrazide, DCCH, FMOC hydrazide, FTCB, NBD-hydrazine, PBAH and Texas Red hydrazide were tested with trifluoroacetic acid as catalyst. Each derivative was separated on a Wakosil 5C4 column with acetonitrile–water as eluent and detected at the excitation maximum and emission maximum wavelengths of each derivative in acetonitrile–water (7 + 3). Table 1 shows the effect of the derivatization reagent and Fig. 4 shows the excitation and emission spectra of the progesterone derivative with each reagent. FTCB, Texas Red hydrazide and FMOC hydrazide did not give fluorescent

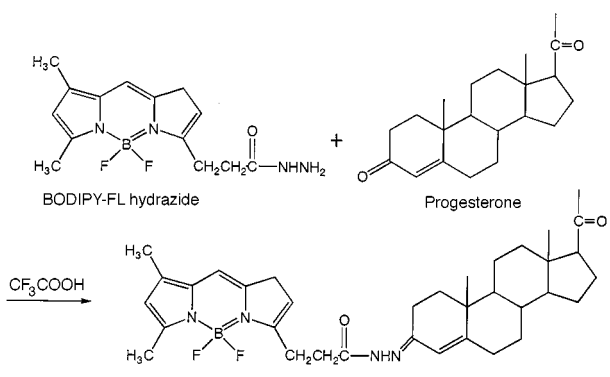

Fig. 1 Derivatization reaction of BODIPY FL hydrazide with progesterone.

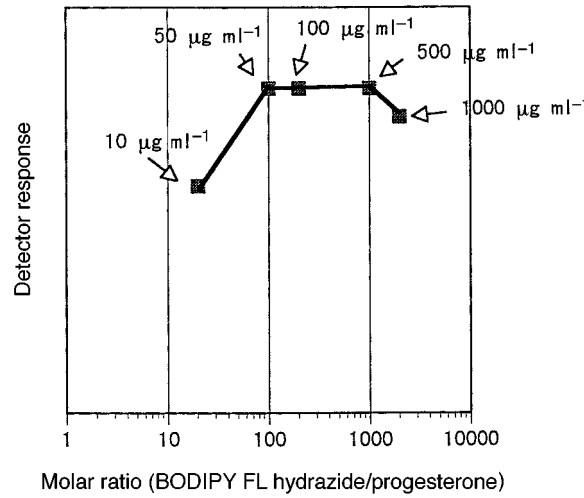

Fig. 2 Effect of BODIPY FL hydrazide concentration: 100 ng ml^{-1} progesterone was reacted at room temperature for 15 h. BODIPY FL hydrazide concentrations are indicated using arrows.

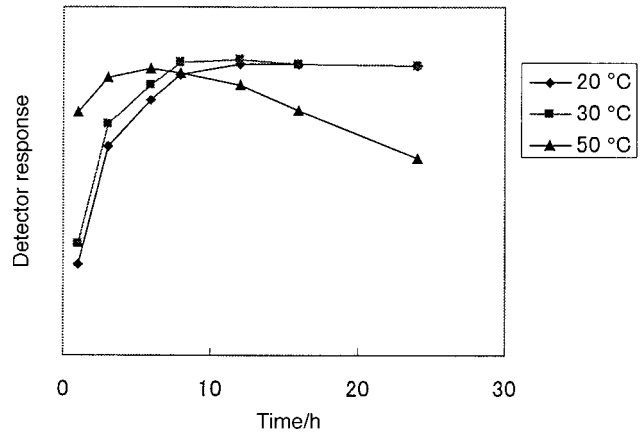

Fig. 3 Effect of reaction time and temperature: 100 ng ml^{-1} progesterone was reacted.

products under the proposed conditions. BODIPY FL hydrazide gave the highest detector response and was therefore selected as the derivatization reagent for progesterone, 17-hydroxyprogesterone and other 3-keto steroids.

Chromatographic conditions and chromatogram

HPLC using normal-phase[6,7] and reversed-phase analytical columns[8–11] has been reported. We selected the reversed-phase mode for convenience of handling biological samples. A C_{18} column has usually been reported for HPLC systems with dansylhydrazine derivatization of progesterone, 17-hydroxyprogesterone and other 3-keto steroids with methanol–water and acetonitrile–water as mobile phase. We tested commercial reversed-phase columns, Zorbax ODS, Zorbax TMS, Zorbax C_8, Nucleosil C_{18}, LiChrosorb C_{18}, TSK ODS-120T, Wakosil 5C4, Wakosil 5C8 and Wakosil 5C18. The sharpest peaks were obtained when a Wakosil 5C4 column was used and this was adopted in subsequent work. The reported problem was peak splitting of dansylhydarzine derivatives. In the proposed BODIPY FL hydrazide method, peak splitting was also observed when high concentrations of progesterone and other 3-keto steroids (over 1000 ng ml^{-1}) were used. A wider calibration range was obtained with acetonitrile–water than methanol–water as the mobile phase and single peaks of progesterone and other 3-keto steroids were observed.

Table 1 Comparison of derivatization reagents. Progesterone (1000 ng ml^{-1} serum) was used. The derivatization conditions were as follows: reagent concentration, 0.005%, m/v; trifluoroacetic acid solution, 0.1 %, v/v; reaction temperature, room temperature; reaction time, 15 h. Average values were obtained from six runs. The calculated peak area of BODIPY FL hydrazide derivatives was taken as 100. Each progesterone derivative was detected at the wavelength that showed maximum fluorescence intensity in acetonitrile–water (7 + 3).

	Detection wavelength/nm		
Reagent	λ_{ex}	λ_{em}	Peak Area
Dansylhydrazine	360	550	25.2
BODIPY FL hydrazide	495	516	100.0
DCCH	336	531	1.2
FMOC hydrazine	298	322	ND[a]
FTCB	472	510	ND
NBD-hydrazine	465	515	5.1
PBAH	341	376	51.0
Texas Red hydrazide	531	612	ND

[a] No response on detector.

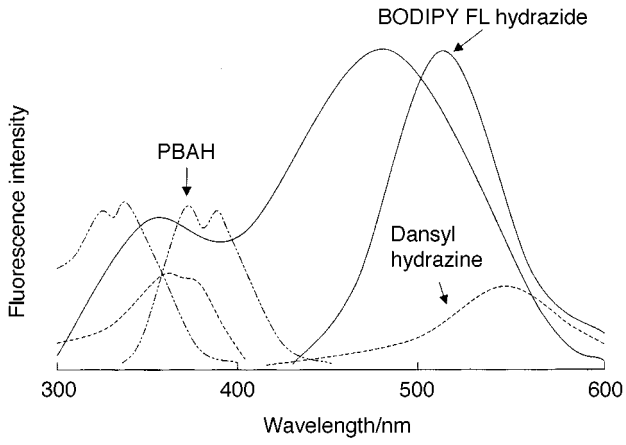

Fig. 4 Excitation and emission spectra of the derivatization product of progesterone. Progesterone (1000 ng ml^{-1}) was derivatized with each fluorescent reagent. Each derivative was separated by HPLC and its spectrum measured.

The detection limits were studied by addition of steroids to serum from a non-pregnant woman: 550 fmol (10 μl injection, signal to noise ratio = 5) for 17-hydroxyprogesterone, 550 fmol for progesterone, 1.2 pmol for dehydroepiandrosterone, 1.2 pmol for testosterone and 3.7 pmol for androstenedione. The calibration ranges were up to 1000 ng ml^{-1} (for example, $y = 0.3992x - 0.0914$, $r^2 = 0.9993$ for progesterone). Fig. 5 shows the chromatogram of six steroids (100 ng ml^{-1}, 17-MeT as internal standard) added to serum from a non-pregnant woman. The within-day relative standard deviations (RSDs) of steroids (100 ng ml^{-1}) were 5.9–6.4% and the day-to-day RSDs were 6.7–7.6% (average of six runs). The proposed BODIPY FL hydrazide method could determine 550 fmol of progesterone on-column (10 μl injection, signal-to-noise ratio = 5) in serum. In comparison, for example, the limit of detection in a recently reported HPLC method for progesterone[11] with dansylhydrazine was 12 pmol per 500 μl injection. The proposed method was the most sensitive among reported HPLC methods with fluorescence[6,7,10–11] and chemiluminescense detection[8,9] with dansylhydrazine. These reported HPLC methods with dansylhydrazine were not sensitive enough for monitoring progesterones (10–200 ng ml^{-1}) in serum from pregnant women. In contrast, the proposed HPLC method is sufficiently sensitive for monitoring progesterone and 17-hydroxyprogesterone in serum from pregnant women.

Conclusions

A new HPLC method for the determination of progesterone, 17-hydroxyprogesterone and other 3-keto steroids has been reported. Each steroid was derivatized with BODIPY FL hydrazide at room temperature. The proposed HPLC method was the most sensitive among reported HPLC methods with fluorescence and chemiluminescence derivatization with dansylhydrazine.[6–11] The linear calibration range was up to 1000 ng ml^{-1}. The detection limits of progesterone and 17-hydroxyprogesterone were improved over 50-fold compared with reported HPLC methods with dansylhydrazine, so it can be used for monitoring progesterone and 17-hydroxyprogesterone in serum from pregnant women without preconcentration of the sample. The proposed method is now being applied to

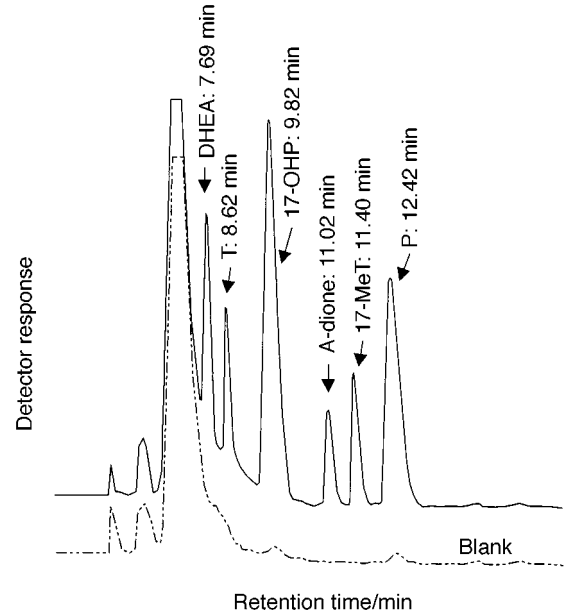

Fig. 5 Chromatogram of steroids derivatized with BODIPY FL hydrazide. Each steroid was added to serum from a non-pregnant woman (100 ng ml^{-1}).

monitoring progesterone and 17-hydroxyprogesterone in serum from *in vitro* fertilization embryo transfer (IVF-ET) patients.

References

1 W. G. Stillwell, A. Hung and M. G. Horning, *Anal. Lett.*, 1973, **6,** 407.
2 M. Ishibashi, H. Takayama, Y. Nakagawa and N. Harima, *Chem. Pharm. Bull.*, 1988, **36,** 845.
3 H. Ichimiya, B. Egestad, H. Nazar, E. S. Baginski, P. T. Clayton and J. Sjovall, *J. Lipid. Res.*, 1991, **32**, 829.
4 K. D. R. Setchell, F. J. Suchy, M. B. Welsh, L., Zimmer-Nechemias, J. Heubi and W. F. Balistrerei, *J. Clin. Invest.*, 1988, **82,** 2148.
5 L-J. Meng and J. Sjovall, *J. Chromatogr. B,* 1997, **688,** 11.
6 T. Kawasaki, M. Maeda and T. Tsuji, *Yakugaku Zasshi*, 1980, **100,** 925.
7 T. Kawasaki, M. Maeda and T. Tsuji, *J. Chromatogr.*, 1982, **232,** 1.
8 K. Imai, S. Higashidate, A. Nishitani, Y. Tukamoto, M. Ishihashi, J. Soda and T.Osuga, *Anal. Chim. Acta*, 1989, **227,** 21.
9 K. Imai, S. Higashidate, Y. Tsukamoto, S. Uzu and S. Kanda, *Anal. Chim. Acta*, 1989, **225,** 421.
10 M. Hyytiainen, P. Appelblad, E. Ponten, M. Stigbrand, K. Irgum and H. Jaegfeldt, *J. Chromatogr. A*, 1996, **740,** 279.
11 P. Appelblad, E. Ponten, H. Jaegfeldt, T. Bachstrom and K. Irgum, *Anal. Chem.*, 1997, **69,** 4905.
12 M. Katayama and H. Taniguchi, *J. Chromatogr*., 1993, **616,** 317.
13 T. Mukaida, M. Yemini, Y. Matsuda, S. Akihama, M. Katayama, H. Taniguchi, Y. Kuroda, S. Kaneko and S. Nozawa, *Jpn. J. Fertil. Steril.*, 1995, **40,** 26.
14 K-L. Lee, M. Katayama, H. Taniguchi, Y. Matsuda, S. Akihama, I. Hara and H. Sato, *Jpn. J. Fertil. Steril.*, 1996, **41,** 311.

Paper 8/04201B