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The Utility of the Deuterated Spin Label in the Spin Immunoassay of Cortisol

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A deuterated spin-labeled derivative of cortisol (F3-SLD) was prepared by coupling cortisol-3-(*O*-carboxymethyl)oxime with 4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-d_{17} and used to improve the sensitivity of spin immunoassay (SIA) of cortisol. The linewidth of the high-field peak of F3-SLD was 1.4 G at the modulation amplitude (H_m) of 1.6 G, and that of the undeuterated spin-label was 1.9 G at $H_m = 2.0$ G. When 200 μl of cortisol standards was used for the assay, the minimum detectable concentration of cortisol was reduced from 10 ng/200 μl to 2.5 ng/200 μl by the use of F3-SLD. Although the increase in the sensitivity of SIA is not yet sufficient, the use of deuterated spin-labels is considered to be useful for enhancing SIA sensitivity.

Keywords——spin immunoassay; cortisol; ESR; spin-label; nitroxide; hydrocortisone; deuterated spin-label; spin-labeled cortisol

In the previous papers we reported on the spin immunoassay (SIA) of urinary testosterone[1a] and cortisol.[1b] Since SIA does not require that the free and bound label be physically separated, this assay has the advantages of speed, simplicity, and easiness of complete automation.[2] The main weakness of SIA is its lack of sensitivity. Although enhancement of SIA sensitivity can be achieved by improvement of the electron spin resonance (ESR) spectrometer, it may also be possible to enhance SIA sensitivity by the use of deuterated spin-labels. Venkataramu *et al.*[3] have synthesized perdeuterio-*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide; much narrower ESR lines were observed, resulting from the reduced hyperfine coupling between the unpaired electron of the nitroxide and the deuterium nuclei, and thus a five-fold increase in signal amplitude was obtained.

In this study we have synthesized a new deuterated spin-label, used it for the SIA of urinary cortisol, and compared the sensitivity of the SIA to that of SIA employing the undeuterated spin-label.

Experimental

Reagent——Cortisol-3-(*O*-carboxymethyl)oxime (F3-CMO) and cortisol-3-(*O*-2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-carbamoylmethyl)oxime (F3-SL) were prepared as described previously.[1b]

Cortisol-3-(*O*-2,2,6,6-tetramethylpiperidine-1-oxyl-4-carbamoylmethyl)oxime (F3-SL6): F3-CMO (0.7 g) and 4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine (0.27 g, Aldrich Co.) were dissolved in absolute ethanol (32 ml). *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.43 g) was added to the solution at room temperature, and the mixture was allowed to stand for 60 h, then evaporated to dryness under reduced pressure. The residue was dissolved in ethyl acetate, washed successively with 5% HCl, water, saturated $NaHCO_3$, and water, and dried over $MgSO_4$. The ethyl acetate solution was concentrated under reduced pressure, and developed on a thin-layer of Silicagel H (Merck Co., Type 60, 1 mm) with ethyl acetate. The yellow band was scraped off and extracted with ethyl acetate. Evaporation of the ethyl acetate extract gave an orange powder. mp *ca.* 150 °C (dec.). *Anal.* Calcd for $C_{32}H_{50}N_3O_7$: C, 65.28; H, 8.56; N, 7.14. Found: C, 64.56; H, 8.72; N, 7.11. IR ν_{max}^{KBr} cm^{-1}: 3470 (OH), 1720 (C=O), 1670 (CONH), 1115 (C–O), 1060 (C–O).

4-Amino-1-oxyl-2,2,6,6-tetramethylpiperidine-d_{17} was prepared by the method of Venkataramu *et al.*[3]

Cortisol-3-(*O*-2,2,6,6-tetramethylpiperidine-1-oxyl-d_{16}-4-carbamoylmethyl)oxime (F3-SLD) was prepared from F3-CMO and 4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-d_{17} by the same method as applied to the preparation of F3-SL6. Orange powder. mp *ca.*147 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3470 (OH), 2245 (CD_3), 2130 (CD_2), 1720 (C=O), 1670 (CONH), 1115 (C–O), 1060 (C–O). Thin-layer chromatography of F3-SLD (Silicagel H, ethyl acetate) gave a single spot identical with that of the undeuterated counterpart (F3-SL6).

Antibody——A cortisol-3-oxime/bovine serum albumin conjugate was prepared by the method of Erlanger *et al.*[4] Young male New Zealand white rabbits were immunized by the method of Furuyama *et al.*[5] Blood was drawn from the rabbits after 3 months. The antiserum was precipitated at 0 °C by addition of an equal volume of saturated ammonium sulfate. The pellet obtained after centrifugation was redissolved in water by the addition of a minimal amount of NaOH. After dialysis against water, the total volume of the antibody solution was adjusted to twice the volume of the original serum by the addition of water. Sodium azide (0.1%) was added to the antibody stock solution, which was then stored in a refrigerator. The antibody stock solution bound 58% of 109 pg of [^{3}H]cortisol at a final dilution of 1 : 2500 in 0.06 M phosphate buffer (pH 7.4) containing 0.01 M EDTA-2Na and 0.1% gelatin. The cross reactivity of this antibody with other closely related steroids was as follows: 11-deoxycortisol 55%; corticosterone 7.8%; cortisone 16%; 17-hydroxyprogesterone 2.8%; 11-deoxycorticosterone 6.3%; progesterone 2.4%.

The assay buffer was 0.06 M sodium phosphate buffer (pH 7.4) containing 0.01 M EDTA-2Na and 0.002% Triton X-100. Water was purified by the use of a Millipore MILLI-R/Q system. All other chemicals used were of reagent grade.

Apparatus——ESR spectra were recorded on a JEOL FE-1X spectrometer, equipped with 100 kHz field modulation, at room temperature (24±1 °C). Samples of test solution (40 μl) were aspirated into thin-walled capillaries of 1 mm inside diameter. The ESR settings were: microwave power 50 mW; receiver gain 10 × 1000; time constant 10 s. For analytical purposes, the peak-to-peak amplitude of the high-field peak was measured.

Assay Procedure——One and a half ml of urine sample was put into a 10 ml glass centrifuge tube and saturated with NaCl. Three ml of ether was added to the sample, and the tube was vigorously shaken for 10 min. The tube was dipped in a cooling bath (−40 °C) and the water was allowed to freeze. The ether layer was transferred to a 5 ml siliconized conical flask and evaporated to dryness in a stream of nitrogen. Then 600 μl of the assay buffer was added to the flask, and the contents were stirred with a Vortex mixer. A 200 μl aliquot of the solution was transferred to another flask, and 50 μl of the antibody-spin label mixture, which had been prepared by mixing equal volumes of the diluted antibody (1 : 35), and F3-SLD (2.45×10^{-6} M) or F3-SL (2.23×10^{-6} M), was added to the flask. The mixture was stirred and allowed to stand for 10 min, then aspirated into a capillary. The end of the capillary was sealed with a plugged silicon tube. The capillary was introduced into the ESR cavity and the amplitude of high-field peak was determined.

The F3-SLD solution (2.45×10^{-6} M) was stable on storage over a period of six months in a refrigerator.

Results and Discussion

ESR Signal Intensity of F3-SLD

The high-field derivative amplitude of the deuterated spin-label, F3-SLD, was measured at various modulation amplitudes (H_m) and compared with that of undeuterated spin-label (F3-SL). The results are shown in Fig. 1. The amplitude of F3-SL increased with increase in H_m up to 2.0 G, whereas that of F3-SLD leveled off at an H_m value of 1.6 G. This is because the linewidth (ΔH_{pp}) of F3-SLD is narrower than that of F3-SL. It has been shown that the derivative amplitude is maximum when $H_m \approx 2\Delta H_{pp}$ for Lorentzian lines and when $H_m \approx \Delta H_{pp}$ for Gaussian lines, and that at these settings the lines are considerably broadened and distorted.[6] Since, sensitivity is of prime concern in SIA, H_m should be adjusted for maximum derivative amplitude. In the previous paper,[1b] H_m was set to 2.0 G to measure F3-SL. However, for the measurement of F3-SLD a setting of H_m over 1.6 G does not result in an increase in sensitivity. Thus use of the deuterated spin-label brought about only a 1.28-fold increase in the derivative amplitude, in contrast to the results of Venkataramu *et al.*[3] The linewidth of the high-field peak of F3-SLD was 1.4 G at H_m=1.6 G, and that of F3-SL was 1.9 G at H_m=2.0 G.

The derivative amplitudes of F3-SLD and F3-SL were also measured at various scan rates. The results are shown in Fig. 2. The derivative amplitude of F3-SLD slightly increased even beyond a scan rate of 128 min/100 G. However, the use of a scan rate of 256 min/100 G is too time-consuming for routine analysis, so the scan rate was set at 128 min/100 G.

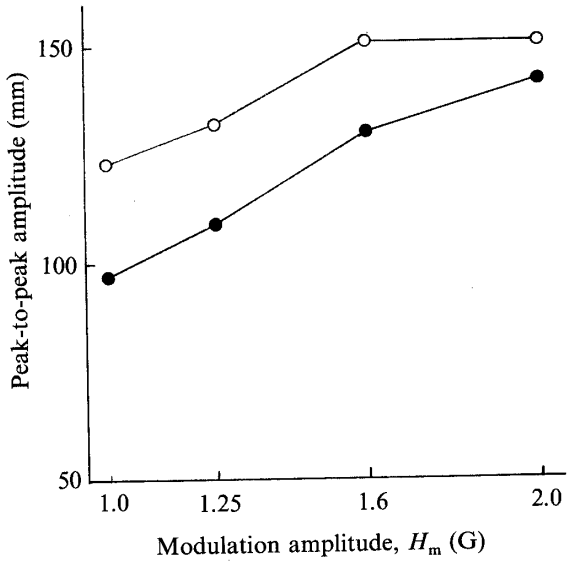

Fig. 1. Dependence of the Peak-to-Peak Amplitude on the Modulation Amplitude, H_m

○, F3-SLD (2.45×10^{-7} M); ●, F3-SL (2.97×10^{-7} M); scan rate, 128 min/100 G.

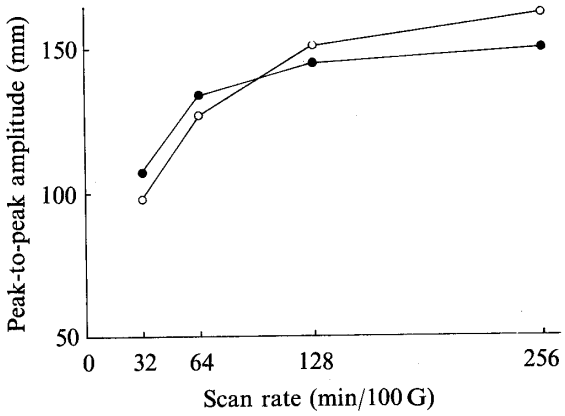

Fig. 2. Dependence of the Peak-to-Peak Amplitude on the Scan Rate

○, F3-SLD (2.45×10^{-7} M, $H_m = 1.6$ G); ●, F3-SL (2.97×10^{-7} M, $H_m = 2.0$ G).

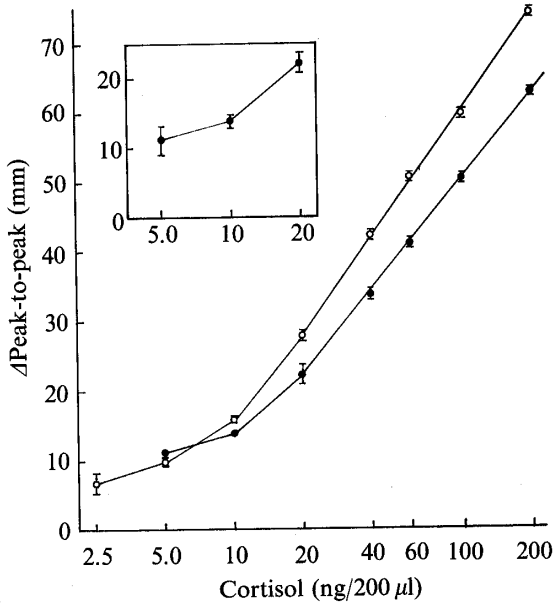

Fig. 3. Standard Curves for the Assay of Cortisol

Cortisol standards (200 μl) prepared in the assay buffer were mixed with 50 μl of antibody-spin label complex. ΔPeak-to-peak represents high-field peak height minus blank resonance. The points represent the means of 5 replicates. The vertical bars indicate 2 S.D. on either side of the mean.

○, F3-SLD (2.45×10^{-7} M, $H_m = 1.6$ G); ●, F3-SL (2.23×10^{-7} M, $H_m = 2.0$ G).

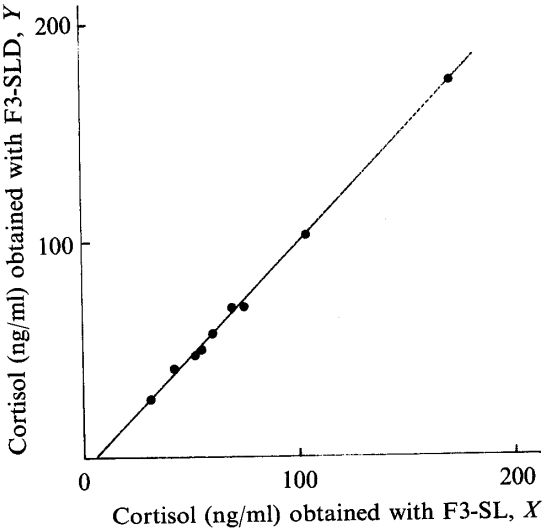

Fig. 4. Comparison of Urinary Cortisol Values Obtained by Spin Immunoassay with the Use of F3-SLD (Y) and F3-SL (X)

$Y = 1.045X - 5.80$; $r = 0.999$.

Standard Curves of Spin Immunoassay

A standard curve of SIA for cortisol with the use of F3-SLD is shown in Fig. 3. ΔPeak to peak (ΔP–P) represents high-field peak height minus blank resonance. Included in Fig. 3 for comparison is a standard curve of SIA with the use of F3-SL. Since the dilution of the antibody solution for the latter was set at the same value as that for the former, which is

1.33 times that used in the previous paper,[1b] the final concentration of F3-SL was reduced to 2.23×10^{-7} M in order to obtain a better standard curve.

With the use of F3-SLD, when the concentration of cortisol (C) is 10 ng/200 μl or above, a plot of ΔP–P *vs.* log C gives a straight line ($r=0.9996$). On the other hand, with the use of F3-SL a plot of ΔP–P *vs.* log C is linear ($r=0.9998$) when C is 20 ng/200 μl or above, and the slope of the plot is gentle between $C=5$ and 10 ng/200 μl. Moreover the standard deviation (S.D.) at $C=5$ ng/200 μl is fairly large. Therefore, the concentration of 5 ng/200 μl cannot be distinguished from that of 10 ng/200 μl with 99% confidence in the assay. The minimum detectable concentration of cortisol was arbitrarily defined as the minimum concentration of cortisol which could be distinguished from double that concentration with 99% confidence. The values obtained were 2.5 ng/200 μl for SIA with the use of F3-SLD and 10 ng/200 μl for SIA with the use of F3-SL. A four-fold increase in sensitivity was obtained by the use of the deuterated spin-label. Since the increase in the derivative amplitude caused by the deuteration was only a factor of 1.28, some of the increase in sensitivity may be ascribed to a difference in the antibody-spin label binding strength.

Cortisol levels were determined in nine normal urine samples simultaneously by SIA with the use of F3-SLD and F3-SL. The results are shown in Fig. 4. The coefficient of correlation was 0.999, and the slope was 1.045. With the use of F3-SLD, the recovery of various amounts (60 to 240 ng) of cortisol added to 1.5 ml of urine was on average $83 \pm 2.9\%$. These results indicate that there is essentially no difference between the results obtained by the two methods. Since, in the previous paper,[1b] a good correlation ($r=0.988$) was found between the results obtained by radioimmunoassay and SIA with the use of F3-SL, SIA with the use of F3-SLD is also considered to be useful for measuring free cortisol in human urine. Although the increase in the sensitivity of SIA is not yet sufficient, the use of deuterated spin-labels is considered to be useful for enhancing SIA sensitivity.

References and Notes

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