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Determination of Phenobarbital in Human Serum by Spin Immunoassay

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A deuterated spin-labeled derivative of phenobarbital was prepared by coupling 5-ethyl-5-phenylbarbituryl-1-butyric acid (PB-BUA) with 4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-d_{17} and used to develop a method for measuring phenobarbital levels in human serum. Antiserum was raised in rabbits immunized with the PB-BUA/bovine serum albumin conjugate. With 2 μl of serum, the sensitivity limit of the assay was 10 ng of phenobarbital. Phenobarbital levels in Q-PAK therapeutic drug-monitoring control sera were determined by spin immunoassay (SIA). The values obtained by SIA were in good agreement with those presented by the manufacturer. The method was found to be simple and suitable for routine use, though one of the expected advantages of SIA, *i.e.*, rapidity of analysis, was not realized.

Keywords——spin immunoassay; electron spin resonance; phenobarbital; spin-labeled phenobarbital; drug assay; deuterated spin-label; nitroxide; drug monitoring

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 was achieved to some extent by the use of a deuterated spin-label,[1c] it is not yet sufficient to determine clinically important steroids such as aldosterone, whose concentration in blood is very low.

Recently, the determination of therapeutic drug concentrations in human serum has become very important, and various methods have been devised for this purpose. The ideal clinical laboratory technique for measuring drugs in biological fluids should be simple, specific, readily automated, and applicable to a wide range of compounds. SIA has been hitherto applied only to the determination of morphine[3a,b] and phenytoin[3b,c] in human serum. Phenobarbital (PB) is one of the most commonly used antiepileptic drugs. Its dose must be adjusted by monitoring the drug level in th serum. Therefore, a convenient analytical method with high sensitivity and specificity is required for routine measurement of PB in serum. In this paper we have synthesized a new deuterated spin-lable, and used it for the SIA of PB in human serum.

Experimental

Reagent——5-Ethyl-5-phenylbarbituryl-1-butyric acid (PB-BUA) was prepared essentially according to the method of Deleide *et al.*[4] Ethyl 5-ethyl-5-phenylbarbituryl-1-butyrate, prepared from phenobarbital sodium salt and ethyl γ-chlorobutyrate, was purified by preparative thin-layer chromatography (Merck, Silica gel 60 PF_{254}, 1 mm, hexane–ethyl acetate 3 : 1). The product was suspended in 20% HCl and refluxed for 8 h. The mixture was cooled and decanted, and the residual oil was dissolved in ethyl acetate. The organic phase was extracted three times with one-half volume of saturated $NaHCO_3$ solution. The aqueous solution was adjusted to pH 2.0 with concentrated HCl and extracted with ethyl acetate. Evaporation of the ethyl acetate gave a heavy sirup, which crystallized gradually on standing in a desiccator. The crystals were recrystallized from hexane–ethyl acetate. mp 115 °C. *Anal.* Calcd for

$C_{16}H_{18}N_2O_5$: C, 60.37; H, 5.70; N, 8.80. Found: C, 60.56; H, 5.74; N, 8.67. MS *m/e*: 318 (M^+), 301 ($M^+ - OH$), 273 ($M^+ - CO_2H$).

1-(2,2,6,6-Tetramethylpiperidine-1-oxyl-d_{17}-4-carbamoylpropyl)-5-ethyl-5-phenylbarbituric acid (PB-SL) was prepared from PB-BUA and 4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-d_{17} by the same method as applied to the preparation of the cortisol spin label.[1c] Orange powder. mp 205—206 °C. IR ν_{max}^{KBr} cm^{-1}: 3420 (NH), 2240 (CD_3), 2130 (CD_2), 1756 (C=O), 1720 (C=O), 1648 (CONH), 1308 (C–N), 1054 (C–O). MS *m/e*: 488 (M^+).

Antibody——A PB-BUA/bovine serum albumin (BSA) conjugate was prepared by the method of Erlanger *et al.*[5] Young male New Zealand white rabbits were immunized by the method of Furuyama *et al.*[6] Blood was drawn from the rabbits after 4 months. The immunoglobulin fraction was isolated as described previously.[1c] Sodium azide (0.1%) was added to the antibody stock solution, which was then stored in a refrigerator. The antibody stock solution bound 50% of 10 ng of pheno[2-^{14}C]barbital at a final dilution of 1 : 600 in 0.06 M phosphate buffer (pH 7.4) containing 0.01 M ethylenediaminetetracetic acid (EDTA)-2Na and 0.5% BSA.

Q-PAK Chemistry Control Serum I and Therapeutic Drug Monitoring Control Sera-Anticonvulsants were obtained from Hyland Diagnostics, U.S.A. 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——Electron spin resonance (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; scan rate 128 min/100 G.

Assay Procedure——A 160 μl aliquot of the assay buffer and a 40 μl aliquot of the control or monitoring serum (previously diluted 20-fold in the assay buffer) were added to a plastic microcentrifuge tube, and 50 μl of the antibody-spin label mixture, which had been prepared by mixing equal volumes of the diluted antibody (1 : 6) and PB-SL (2×10^{-6} M), was added to the tube. The mixture was stirred and allowed to stand for 1 h at room temperature, 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 the high-field peak was determined. The PB-SL solution (2×10^{-6} M) was stable on storage for 8 months in a refrigerator.

Results and Discussion

Standard Curves of Spin Immunoassay

Since it is preferable to measure drug concentrations without sample pretreatment or extraction, the effects of serum proteins on the ESR signal intensities were investigated. As previously reported by Montgomery *et al.*,[3b] the equilibration time required to obtain the maximum signal for the highest concentrations of PB in sera was 60 min. This longer period as compared to that required in previous papers[1] is attributable to the competitive interaction of the spin-labeled and free drug, not only for the antibody but also for the nonspecific binding sites on the serum proteins. Figure 1 shows the peak-to-peak amplitude of the high-field peak (after subtraction of the non-PB-containing serum blank), $\Delta P-P$, for various PB concentrations. When the concentration of PB (C) is 20 ng/tube or above, a plot of $\Delta P-P$ *vs.* $\log C$ gave a straight line ($r=0.999$), but the slope of the plot was less between $C=10$ and 20 ng/tube. The minimum detectable concentration of PB was arbitrarily defined as the minimum concentration of PB which could be distinguished from double that concentration with 99% confidence. The value obtained was 10 ng/tube; that is, with a 2 μl sample volume, the compound can be detected at concentrations as low as 5 μg/ml. Since the therapeutic serum level of PB is 15 to 45 μl/ml, SIA of PB appears to be well suited for the routine measurement of PB.

Cross Reactivity

The cross reactivity of the antibody with several compounds that are frequently used with PB as anticonvulsants was checked by SIA. The control serum containing 25 μg/ml PB was used as the reference standard. The per cent cross reactivities were: *p*-hydroxyphenobarbital 5.0%; primidone 0.5%; carbamazepine 0.1%; phenytoin 0.2%. Although the antibodies so far reported for immunoassays of PB could not discriminate *p*-hydroxyphenobarbital, one of the major metabolites of PB, from its parent compound,[7] the antibody used in this study showed

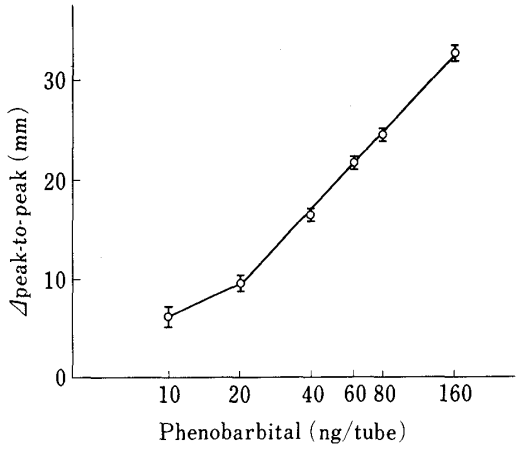

Fig. 1. Standard Curve for the Assay of Phenobarbital

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.

TABLE I. Comparison of the Serum Phenobarbital Levels Determined by SIA with Those Presented by the Manufacturer

Method	Phenobarbital level (μg/ml)	
	Level I	Level II
SIA	20.4±1.4[a)]	49.8± 3.2
Abbott TDX	20.2±3.1[b)]	48.4± 8.6
Ames TDA	20.1±3.0[b)]	48.7± 8.1
EMIT	20.4±2.7[b)]	49.1± 6.0
GLC[c)]	22.2±4.0[b)]	48.8±10.2
HPLC[d)]	19.9±3.5[b)]	50.0± 8.6
RIA[e)]	19.2±4.8[b)]	45.1±10.5

a) Mean value±standard deviation (S.D.), $n=8$.
b) Mean value±interlab expected range.
c) Gas liquid chromatography.
d) High performance liquid chromatography.
e) Radioimmunoassay.

only 5.0% cross reactivity with the former. This is because the antibody was elicited with BSA-conjugate which was linked at the 1-position of PB through a relatively long alkyl bond. Primidone, carbamazepine, and phenytoin, which are commonly used in conjunction with PB for seizure control, did not cross react significantly.

Intra- and Inter-assay Precision and Accuracy

The PB concentrations in Q-PAK therapeutic drug monitoring control sera (anticonvulsants) were determined by SIA. The results are summarized in Table I. Intra-assay variation was determined on the basis of eight measurements of two different samples, 20.4 and 49.8 μg/ml. The coefficients of variation were 6.6 and 6.4%, respectively. Inter-assay variation was estimated by assaying the same samples on six different occasions. The coefficients of variation were 8.0 and 9.0%. The PB levels in the two samples determined by SIA were in good agreement with those presented by the manufacturer. Since the amount of serum required for an assay by SIA is only 2 μl, SIA should be useful for monitoring phenobarbital levels in human serum.

The antiepileptic agents are frequently prescribed in combination, and hence multiple assays may be required. Liquid- and gas-liquid chromatographic systems are generally used for this purpose. However, these procedures are rather tedious and time-consuming, making them unsatisfactory for routine use. Since the nuclear spin of ^{15}N isotope is 1/2, substitution

of the ^{15}N isotope for the naturally occurring ^{14}N in the spin-label causes a marked shift in the ESR peaks, and the ESR peaks of the ^{15}N-spin-label do not overalp with those of the ^{14}N-spin-label. Therefore, if another antiepileptic agent, for example, phenytoin is labeled with ^{15}N-spin-label, it should be feasible to determine PB and phenytoin simultaneously in a single, simple measurement. This assay system offers excellent prospects for the development of dual assays which can be easily automated.

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