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Results of Spin Immunoassay for Simultaneous Measurement of Phenytoin and Phenobarbital in Serum Compared with Those of Liquid Chromatography[1)]

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We describe a spin immunoassay (SIA) in which the two antiepileptic agents, phenytoin (PHT) and phenobarbital (PB), are determined simultaneously in a single measurement. The method involves labeling the two with ^{15}N- and ^{14}N-nitroxide, respectively, whose electron spin resonance peaks do not overlap each other. With 2 μl of serum, the smallest detectable concentration for either drug was 1.25 mg/l. PHT and PB levels in Q-PAK therapeutic drug-monitoring control sera were determined by SIA. The values obtained by SIA were in good agreement with those presented by the manufacturer. Sera from rabbits after administration of PHT and/or PB were analyzed by SIA and high-performance liquid chromatography. There was a good correlation between results obtained by the two methods.

Keywords——spin immunoassay; electron spin resonance; phenytoin; phenobarbital; dual assay; drug monitoring; deuterated spin label; ^{15}N-spin label; HPLC

Monitoring of the circulating concentrations of drugs used for prophylactic treatment of epilepsy is useful in determining the proper dosage. The antiepileptic agents are frequently prescribed in combination, and hence multiple assays may be required. Gas-liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) have been used for this purpose.[2)]

GLC methods require an excessive amount of time, as well as a chemical derivatization step, and extraction to clean the sample. HPLC methods also have a number of disadvantages which include the requirement of a long operating time, coelution of the hydroxylated metabolites of anticonvulsants, and the frequent loss of column performance due to build up of impurities on top of the column bed.

We describe here a novel method for concurrently determining phenytoin (PHT) and phenobarbital (PB) in human and rabbit sera by spin immunoassay (SIA) and compare the results with those obtained from a HPLC method. Since the nuclear spin of the ^{15}N isotope is 1/2, substitution of the ^{15}N isotope for the naturally occurring ^{14}N isotope in the spin-label causes a marked shift in the electron spin resonance (ESR) peaks, and the ESR peaks of the ^{15}N-spin-label do not overlap those of the ^{14}N-spin-label. Therefore, if PHT and PB are labeled with ^{15}N- and ^{14}N-nitroxide, respectively, it is possible to determine PHT and PB simultaneously with a single ESR scan. The use of spin-labels in a dual assay has the advantages of stability and ease of differential measurement, which are not found with the use of two radio-isotopes or two enzyme labels.

Experimental

Reagent——4-Amino-1-oxyl-2,2,6,6-tetramethyl-^{15}N-piperidine-d_{17} (**1**) was prepared essentially by the method of Bates *et al.*[3)] [^{15}N]Ammonia was liberated from $^{15}NH_4Cl$ (CEA, France) in D_2O and CaO, and then introduced to

a well-stirred mixture of acetone-d_6 and $CaCl_2$. ^{15}N-2,2,6,6-Tetramethyl-4-piperidone-d_{17} thus obtained was oxidized and then reductively aminated by the method of Rosen.[4] **1**: red oil, MS *m/e*: 189 (M^+). 5.5-Diphenylhydantoin-3-butyric acid (PHT-BUA) was prepared essentially according to the method of Deleide *et al.*[5] and recrystallized from hexane–ethyl acetate: white needles, mp 151—152 °C. *Anal.* Calcd for $C_{19}H_{18}N_2O_4$: C, 67.44; H, 5.36; N, 8.28. Found: C, 67.54; H, 5.31; N, 8.22. MS *m/e*: 338 (M^+). 5-Ethyl-5-phenylbarbituryl-1-butyric acid (PB-BUA) and 1-(2,2,6,6-tetramethylpiperidine-1-oxyl-d_{17}-4-carbamoylpropyl)-5-ethyl-5-phenylbarbituric acid (PB-SL) were prepared as described previously.[6] 3-(2,2,6,6-Tetramethyl-^{15}N-piperidine-1-oxyl-d_{17}-4-carbamoylpropyl)-5,5-diphenylhydantoin (PHT-SL) was prepared from PHT-BUA and **1** by the same method as applied for the preparation of the cortisol spin-label.[7] Orange powder, mp 221—222 °C. IR ν_{max}^{KBr} cm^{-1}: 3420 (NH), 2245 (CD_3), 2125 (CD_2), 1720 (C=O), 1655 (CONH). MS *m/e*: 509 (M^+).

Antibody——A PHT-BUT/bovine serum albumin (BSA) conjugate was prepared by the method of Erlanger *et al.*[8] Young male New Zealand white rabbits were immunized by the method of Furuyama *et al.*[9] Blood was drawn from the rabbits after 4 months. The immunoglobulin fraction was isolated as described previously.[7] 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 pg of 5,5-[phenyl-4-^{3}H(N)]-diphenylhydantoin at a final dilution of 1 : 4000 in 0.06 M phosphate buffer (pH 7.4) containing 0.01 M ethylenediaminetetraacetic acid (EDTA)–2Na and 0.5% BSA. The antibody for PB was prepared as described previously.[6] 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——ESR spectra were recorded as described previously.[6] MS were obtained on a Hitachi M-60 spectrometer at an ionizing voltage of 20 eV. HPLC was carried out with a Waters Associates model-510 solvent delivery system, U6K universal LC injector, Z-module radial compression separation system with an 8NV C-18 (5 μ) cartridge, and a Uvidec-100 IV UV detector (Japan Spectroscopic Co.).

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 standard solutions or the assay buffer) were added to a plastic microcentrifuge tube together with 50 μl of the antibody-spin label mixture containing PHT-antibody, PB-antibody, PHT-SL, and PB-SL. The final concentrations of PHT-SL and PB-SL in the assay mixture were both 3×10^{-7} M. The final volume of the mixture in the tube was 250 μl. The mixture was stirred and allowed to stand for 1 h at room temperature, then aspirated into a capillary. The capillary was introduced into the ESR cavity and the amplitudes of the high-field peaks of ^{15}N- (A_N=23.8 G, g=2.0055) and ^{14}N-spin labels (A_N=17.0 G, g=2.0055) were determined (peaks A and B in Fig. 1 of reference 1). It takes 5 min to record the two peaks. The method requires no further procedures. The PHT-SL and PB-SL solutions (2×10^{-6} M, respectively) were stable on storage for more than one year in a refrigerator.

Treatment of Rabbits——PHT sodium salt was dissolved in sodium hydroxide solution (pH 11.5). The concentration of PHT in the solution was 5—20 mg/l. PB sodium salt was dissolved in water (5—20 mg/l as PB). One ml of the PHT solution and/or one ml of the PB solution were injected intramuscularly into male matured rabbits. Venous blood samples were collected from the marginal ear vein and centrifuged to obtain serum fractions. Aliquots of 10—20 μl of the sera were used for SIA without further procedures. Another aliquot of 100 μl of the sera in a tapered tube was deproteinized by addition of 200 μl of acetonitrile containing hexobarbital (12 μg) as an internal standard. The contents of the tube were mixed thoroughly on a vortex mixer and then centrifuged for 3 min. The supernatant was filtered with a Millipore SJHV004NS filter (0.45 μm) and aliquots of 50 μl of the filtrate were injected into the HPLC instrument and eluted with a mobile phase of 0.01 M potassium biphosphate (pH 4.5)–methanol–acetonitrile (65 : 21 : 14) at a flow rate of 1.5 ml/min. Anticonvulsants were monitored at 215 nm.

Results and Discussion

Standard Curves of Spin Immunoassay

Standard curves for the PHT and PB dual assay are shown in Fig. 1. The Δpeak-to-peak represents the high-field peak minus the blank resonance. The standard curve for PHT was not influenced by the presence of up to 200 ng/tube of PB or *vice versa*. The minimum detectable concentration of the assay was arbitrarily defined as the minimum concentration of drug which could be distinguished with 99% confidence from both double that concentration and the zero calibrator. The values obtained were 2.5 ng/tube for both drugs, that is, with a 2 μl sample, both compounds can be detected at concentrations as low as 1.25 mg/l. Since the therapeutic serum levels of PHT and PB are 10 to 20 and 10 to 40 mg/l, respectively,[2a] the dual assay appears to be well suited for routine measurements of PHT and PB.

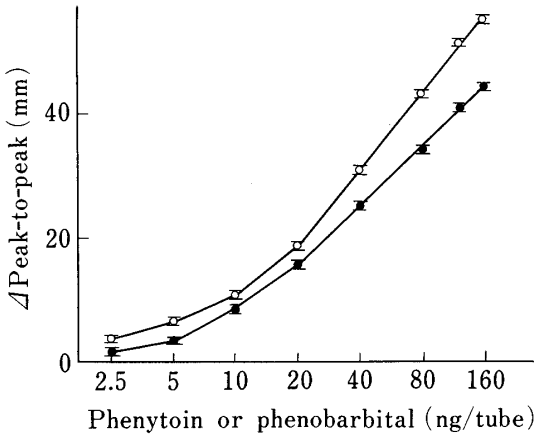

Fig. 1. Standard Curves for the SIA Dual Assay

○, PHT; ●, PB.
The points represent the means of 5 replicates. The vertical bars indicate 2 S.D. on either side of the mean.

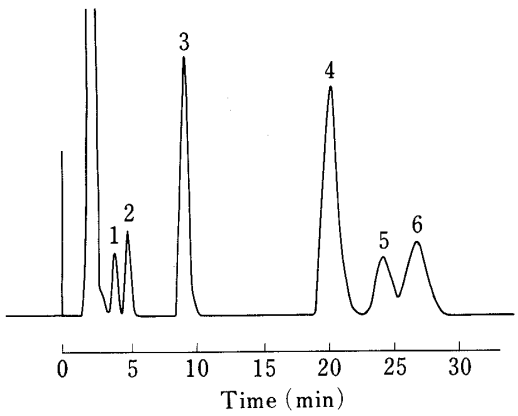

Fig. 2. Liquid Chromatograms of Some Antiepileptic Drugs

1, ethosuximide; 2, primidone; 3, phenobarbital; 4, hexobarbital; 5, phenytoin; 6, carbamazepine.

TABLE I. Relative Cross Reactivities of Several Antiepileptic Drugs and Metabolites of PHT and PB

	PHT	PB	PHT-OH	PB-OH	Primidone	Carbamazepine	Theophylline	Ethosuximide
PHT	100	0.06	5.1	0.08	0.06	0.2	0.04	0.05
PB	0.2	100	0.1	2.0	0.5	0.3	0.08	0.08

PHT-OH, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin; PB-OH, *p*-hydroxyphenobarbital.

TABLE II. Comparison of the Serum Phenytoin and Phenobarbital Levels Determined by the SIA Dual Assay with Those Presented by the Manufacturer

Method	Phenytoin (mg/l)		Phenobarbital (mg/l)	
	Level I	Level II	Level I	Level II
SIA	13.2±0.64	20.8±0.65	20.2±1.1	47.8± 1.8
Abbott TDX®	14.4±2.3	22.5±3.5	20.2±3.1	48.4± 8.6
Ames TDA®	13.5±3.2	21.0±6.3	20.1±3.0	48.7± 8.1
EMIT®	14.0±2.0	21.8±3.4	20.4±2.7	49.1± 6.0
GLC	12.1±2.7	20.4±3.6	22.2±4.0	48.8±10.2
HPLC	13.8±2.0	22.4±3.7	19.9±3.5	50.0± 8.6
RIA	11.9±2.6	21.1±3.9	19.2±4.8	45.1±10.5

SIA: mean value±standard deviation (S.D.), $n=8$. Other methods: mean value±interlab expected range. RIA, radioimmunoassay.

Cross Reactivity

The cross reactivities of the antibodies with several compounds that are frequently used with PHT or PB as antiepileptic drugs were checked by SIA. The control serum containing 25 mg/l PHT or PB was used as the reference standard. The results are shown in Table I. Only 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (PHT-OH) and *p*-hydroxyphenobarbital (PB-OH) cross reacted to some extent in the PHT and the PB assay, respectively. However, the value for PB-OH was rather small compared with those reported previously.[10] This is because the PB

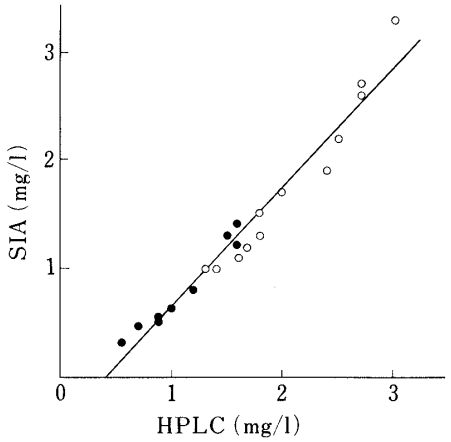

Fig. 3. Comparison of Results for Phenytoin in Rabbit Serum Obtained by SIA and HPLC

●, administration of PHT; ○, administration of PHT and PB.
$y = -0.44 + 1.10x$, $r = 0.978$, $n = 23$.

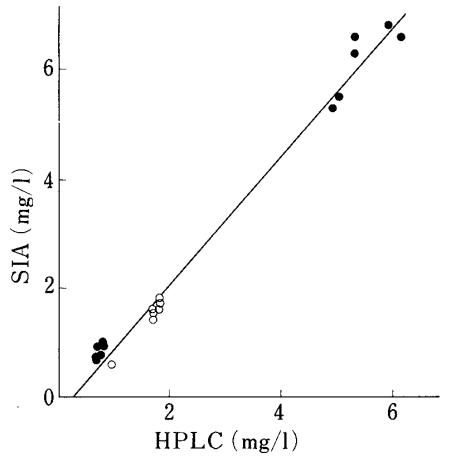

Fig. 4. Comparison of Results for Phenobarbital in Rabbit Serum Obtained by SIA and HPLC

●, administration of PB; ○, administration of PHT and PB.
$y = -0.28 + 1.18x$, $r = 0.993$, $n = 24$.

antibody was elicited with BSA-conjugate which was linked at the 1-position of PB through a relatively long alkyl bond. Primidone, carbamazepine, and PHT, which are commonly used in conjunction with PB for seizure control, did not cross react significantly.

Intra- and Inter-assay Precision and Accuracy

The PHT and PB concentrations in Q-PAK therapeutic drug monitoring control sera-anticonvulsants were determined by the SIA dual assay. The results are summarized in Table II. Intra-assay variation was determined by eight measurements with each of two different samples. The coefficients of variation were 4.8 (level I) and 3.2% (level II) for PHT, and 5.4 (level I) and 3.9% (level II) for PB, respectively. Inter-assay variation was estimated by assaying the same samples on six different occasions. The coefficients of variation were 6.0 (level I) and 3.6% (level II) for PHT, and 6.7 (level I) and 5.2% (level II) for PB, respectively. The PHT and PB levels in the two samples determined by the SIA dual assay were in good agreement with those presented by the manufacturer.

HPLC Method

A typical chromatogram of the five anticonvulsants in the Q-PAK monitoring sera is shown in Fig. 2. Although the peaks of the five anticonvulsants did not overlap each other, addition of PB-OH to the sera increased the peak height of ethosuximide, which indicated that the peak of PB-OH overlapped with that of ethosuximide. The peak of PHT-OH did not overlap with those of the five anticonvulsants. In the present study, since only PHT and PB were given to rabbits, this HPLC method was used to evaluate the results of SIA.

Assay of PHT and PB in Rabbit Sera

To compare the results of SIA with those of HPLC we analyzed 11 serum samples from a rabbit treated with PHT, 12 samples from a rabbit treated with PB, and 12 samples from a rabbit treated with both PHT and PB by both methods. Since the concentrations of PHT and PB in rabbit sera were fairly low compared with those in human sera, 10—20 μl aliquots of the rabbit sera were used for SIA, and hence the incubation time required to obtain a steady, reproducible ESR signal was extended to 3 h. This longer period of incubation is necessary because of the competitive interaction of the spin-labeled and free drugs, not only for the antibody but also for nonspecific binding sites on the serum proteins. The results are shown in Figs. 3 and 4. The coefficients of correlation, slopes, and x-intercepts were 0.978, 1.10 and

0.402, and 0.993, 1.18 and 0.238 mg/l for PHT and PB, respectively. There was no significant difference between the results obtained from administration of a single drug and those obtained from administration of both drugs.

Although the correlation between the results obtained by the two methods was fairly good, SIA gave somewhat higher values at high levels of both drugs. On the other hand, at low concentrations of PHT, SIA gave smaller values. The latter result may be ascribed to the PHT-antibody, which requires a much longer period of incubation to attain the maximum ESR intensity, since the amount of rabbit sera used for SIA was doubled at lower concentrations of PHT. As to the assay of human sera, the concentrations of PHT and PB are much larger than those in rabbit sera, and hence 2 μl of sera is sufficient for the assay. Therefore, there should be no such difficulty for the assay of human sera.

Evaluation of SIA

Recently Dean *et al.* described a simultaneous determination of PHT and PB by substrate-labeled fluorescence immunoassay.[11] Although the sensitivity and accuracy of their method are comparable to ours, their method requires two separate measurements of fluorescence at different wavelengths 20 and 35 min after the reaction is started. Although the shelf life of the enzyme solution was not described, that of the spin-labels should be much longer. HPLC can analyze more than two drugs simultaneously. However, coelution of hydroxylated metabolites of drugs frequently prevents multiple assays. Furthermore, HPLC necessitates much longer periods of measurement and pretreatment of samples. The dual assay described here can be more easily adapted to an automated instrument. The principle of the SIA dual assay would appear to be generally applicable and it should be possible to develop other useful SIA dual assays.

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