Communications to the Editor

[Chem. Pharm. Bull. 32(4)1675—1678(1984)]

SIMULTANEOUS DETERMINATION OF PHENYTOIN AND PHENOBARBITAL IN SERUM BY SPIN IMMUNOASSAY

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We describe a spin immunoassay in which the two antiepileptic agents, phenytoin and phenobarbital, are measured simultaneously in a single run. The method involves labeling the two respectively with ^{15}N- and ^{14}N-nitroxide whose ESR peaks do not overlap each other. With 2 μl of serum, the smallest measurable concentration for either drug is 1.25 mg/l. Results of this dual assay compare well with those of conventional assays.

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

Monitoring 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. Liquid- and gas-liquid chromatographic systems have been used for this purpose.[1] However, these procedures are rather tedious and time-consuming, making them unsatisfactory for routine use.

We report here a novel method for concurrently determining phenytoin (PHT) and phenobarbital (PB) in serum by spin immunoassay (SIA). Since the nuclear spin of the ^{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 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 feasible to determine PHT and PB simultaneously with a single ESR measurement. 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 radioisotopes or two enzyme labels.

4-Amino-1-oxyl-2,2,6,6-tetramethyl-^{15}N-piperidine-d_{17} was prepared essentially by the method of Bates *et al.*[2] 5,5-Diphenylhydantoin-3-butyric acid (PHT-BUA) and 5-ethyl-5-phenylbarbituryl-1-butyric acid (PB-BUA) were prepared essentially by the method of Deleide *et al.*[3] A PHT-BUA/bovine serum albumin conjugate and a PB-BUA/bovine serum albumin conjugate were prepared by the method of Erlanger *et al.*[4] Antisera to PHT and PB were raised in rabbits and immunoglobulin fractions were isolated as described previously.[5] 3-(2,2,6,6-Tetramethyl-^{15}N-piperidine-1-oxyl-d_{17}-4-carbamoylpropyl)-5,5-diphenylhydantoin (PHT-SL) and 1-(2,2,6,6-tetra-

methylpiperidine-1-oxyl-d_{17}-4-carbamoylpropyl)-5-ethyl-5-phenylbarbituric acid (PB-SL) were prepared as described previously.[5] ESR spectra were recorded at room temperature (24 ± 1°C) on a JEOL FE-1X spectrometer equipped with 100 kHz field modulation. The ESR settings were: microwave power 50 mW; receiver gain 10×1000; time constant 10 s; scan rate 128 min/100 G.

Assay Procedures —— 160 µl of the assay buffer (0.06 M sodium phosphate buffer containing 0.01 M EDTA-2Na and 0.002% Triton X-100, pH 7.4) and a 40 µl aliquot of the control or monitoring serum (previously diluted 20-fold in the assay buffer) was 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 3×10^{-7} M, respectively. 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). It takes 5 min to record the two peaks. The method requires no further procedures.

Standard curves for the PHT and PB dual assay are shown in Fig. 2. ΔPeak-to-peak (ΔP-P) represents the high-field peak minus the blank resonance. The

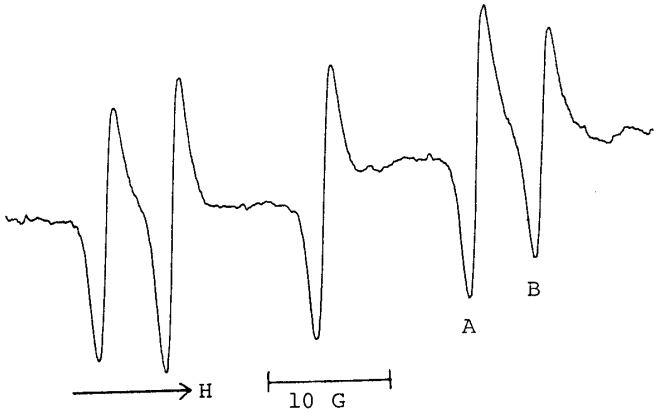

Fig. 1. ESR Spectrum of the Mixture of PHT-SL and PB-SL

A: high-field peak of PHT-SL.
B: high-field peak of PB-SL.

Only peaks A and B need to be recorded for the SIA dual assay.

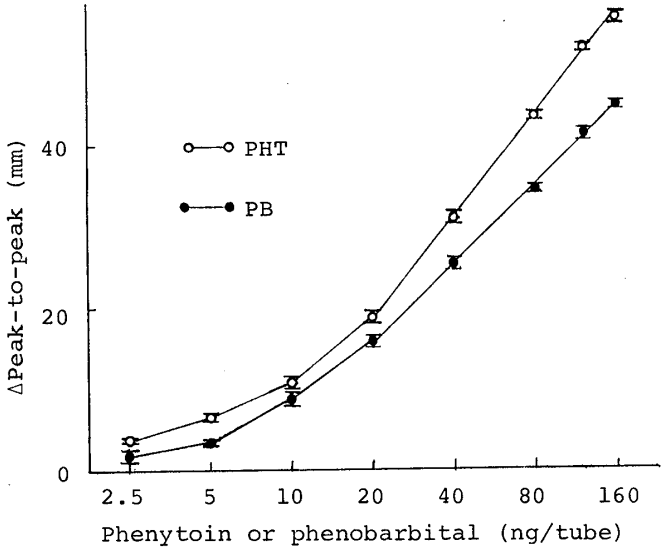

Fig. 2. Standard Curves for the SIA Dual Assay

ΔPeak-to-peak represents the high-field peak height minus the blank resonance. The points represent the means of 5 replicates. The vertical bars indicate 2 SD (standard deviation) on either side of the mean.

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

	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

	Phenytoin (mg/l)		Phenobarbital (mg/l)	
Method	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 (SD), n = 8;
other methods: mean value ± interlab expected range.

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 in 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,[1a)] the dual assay appears to be well suited for the routine measurement of PHT and PB.

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.

The PHT and PB concentrations in Q-PAK therapeutic drug monitoring control sera-anticonvulsants (Hyland Diagnostics, U.S.A.) were determined by the SIA dual assay. The results are summarized in Table II. Intra-assay variation was determined by eight measurements at 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.

Recently Dean *et al.* described a simultaneous determination of PHT and PB by substrate-labeled fluorescent immunoassay.[6] Although the sensitivity and accuracy of their method is comparable to ours, their method requires two separate measurements of fluorescence at different wavelengths 20 and 35 min after the reaction is started. Therefore, 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 should enable any two substances to be measured simultaneously.

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(Received February 27, 1984)