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Letter to the Editor

Simultaneous determination of phenytoin and phenobarbital in human serum by electrochemical immunoassay combined with high-performance liquid chromatography

Sir,

Phenytoin (PHT) and phenobarbital (PB) are widely used antiepileptic drugs with a narrow therapeutic range. So that their doses may be adjusted, the drug levels in the serum must be monitored. Since PHT and PB are frequently prescribed in combination, multiple assays with high sensitivity and selectivity are required for the routine measurement of antiepileptic drugs in serum. In a previous paper [1] we described an electrochemical immunoassay (ECIA) combined with high-performance liquid chromatography (HPLC) for the determination of PHT in human serum. Since this method involves a separation procedure, it can differentiate between two electrochemically tagged antigens. We describe here a new ECIA, which can determine PHT and PB simultaneously without pretreatment of serum samples.

EXPERIMENTAL

Reagents

1-(2,2,6,6-Tetramethylpiperidine-1-oxyl-4-carbamoylpropyl)-5-ethyl-5-phenylbarbituric acid (PB-NO) was prepared from 5-ethyl-5-phenyl-1-butyric acid [2] and 4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine by the same method as applied to the preparation of the cortisol spin label [3]. 3-(2,2,5,5-Tetramethylpyrrolidine-1-oxyl-3-carbamoylpropyl)-5,5-diphenylhydantoin (PHT-NO) [1] and the antibodies for PHT [4] and PB [2], anti-PHT and anti-PB, were prepared as described previously. The assay buffer was 0.1 *M* potassium dihydrogenphosphate–sodium hydrogenphosphate (pH 6.8) containing 1 m*M* Na_2EDTA and 0.02% sodium 1-octanesulphonate.

Assay procedure

A 5-μl aliquot of serum samples and a 418-μl aliquot of the assay buffer were added to a plastic tube with 77 μl of a mixture of anti-PHT, anti-PB, PHT-NO and PB-NO. The final concentrations of PHT-NO and PB-NO were $6 \cdot 10^{-7}$ and $3 \cdot 10^{-7}$ *M*, respectively. The mixture was stirred and allowed to stand for 90 min

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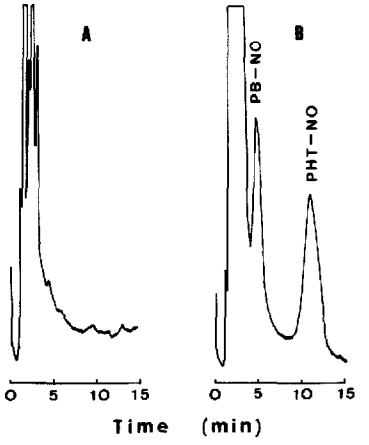

Fig. 1. Typical chromatograms for the blank serum (A) and the assay of human serum spiked with 50 μg/ml PHT and 100 μg/ml PB (B). Injection volume, 100 μl; sensitivity, 2 nA/FS; other conditions as described in text.

at room temperature (21 ± 1°C), and then a 100-μl aliquot of the mixture was injected into the HPLC instrument. HPLC was carried out as described previously. The mobile phase was 50 m*M* potassium dihydrogenphosphate–sodium hydrogenphosphate buffer (pH 6.8) containing 0.1 m*M* Na_2EDTA, 0.01% sodium 1-octanesulphonate and 1.5% ethanol. The flow-rate was 2 ml/min. The applied potential of the electrochemical detection was set at +0.60 V vs. Ag/AgCl.

RESULTS AND DISCUSSION

In order to inject serum samples directly into the HPLC apparatus, a gel filtration column, Protein Pak 60, was adopted. Since the interaction between PHT-NO and the column was very strong, the column length was reduced to 10 cm, and ethanol (1.5%) and sodium 1-octanesulphonate (0.01%) were added to the phosphate buffer. Fig. 1 shows typical chromatograms of the blank serum and the assay mixture. Standard curves for the PHT and PB dual assay are obtained by analysing spiked controlled serum. The standard curves for PHT were not influenced by the presence of up to 50 μg/ml PB or vice versa. The minimum detectable concentrations of PHT and PB were 2 and 10 μg/ml, respectively. Since the therapeutic serum levels of PHT and PB are 10–20 and 10–40 μg/ml, respectively, the dual assay appears to be well suited for routine measurements 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. Only 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (3.3%) and *p*-hydroxyphenobarbital (1.3%) cross-reacted to any extent in the PHT and the PB assay, respectively. Primidone, carbamazepine and ethosuximide did not cross-react significantly.

The PHT and PB concentrations in Q-PAK therapeutic drug-monitoring control sera anticonvulsants were determined by the dual assay. Intra-assay varia-

tions ($n=8$) were 5.9 and 6.7% for PHT and PB, respectively. Inter-assay variations ($n=9$) were 7.3 and 7.1% for PHT and PB. The PHT and PB levels in the sample determined by the dual assay were in good agreement with those presented by the manufacturer.

In summary, the dual assay described here is fast and easy to perform and requires only 5 μl of serum sample per test and no sample pretreatment.

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