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ELECTROCHEMICAL IMMUNOASSAY COMBINED WITH COLUMN LIQUID CHROMATOGRAPHY: DETERMINATION OF PHENYTOIN IN HUMAN SERUM

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SUMMARY

A new electrochemical immunoassay combined with column liquid chromatography has been developed for the determination of phenytoin in human serum. Phenytoin was labelled with the electrochemically active nitroxide, and the separation of the free labelled antigen from other electrochemically active compounds in serum was accomplished by the use of gel chromatography. Serum samples were mixed with the antibody and the labelled antigen, incubated for 90 min, and then a 100-μl aliquot of the mixture was directly injected to the column, which was equipped with an electrochemical detector. With 10 μl of serum, the smallest detectable concentration of phenytoin was 2 μg/ml.

INTRODUCTION

In order to avoid the drawbacks associated with the use of radioisotope labels, much effort has been devoted in recent years to the development of non-isotopic immunoassays [1]. Thus far, enzyme labels are the most successful, and both homogeneous and heterogeneous assays are commercially available [2]. The application of modern electrochemical techniques, such as liquid chromatography and flow injection analysis with electrochemical detection (ED), differential pulse anodic stripping voltammetry and square-wave voltammetry, to enzyme immunoassay has also been investigated by a number of groups [3–6]. Weber and Purdy [7] described a homogeneous voltammetric assay in which the free electrochemically tagged morphine was measured at a glassy carbon anode. Although the current signal for the oxidation of the tagged morphine was considerably decreased on binding of the antigen, application of this assay system to real samples was blocked by the ubiquity of naturally occurring electrochemically

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active molecules, and some chemical sample pretreatment was required.

We have previously reported applications of spin immunoassay (SIA) [8–13]. By the use of ^{15}N-spin-labelled phenytoin (PHT) and ^{14}N-spin-labelled phenobarbital (PB), it was possible to determine PHT and PB simultaneously with a single electron spin resonance (ESR) scan. However, ESR spectrometers are scarce in clinical laboratories, and hence it is hard to make SIA a routine method for therapeutic drug monitoring. Recently, column liquid chromatography (LC) with ED has been popular in clinical laboratories. Therefore, we have attempted to develop a new electrochemical immunoassay (ECIA), which is simple and easily adapted to an automated instrument. PHT was labelled with the electrochemically active nitroxide, and the separation of the free labelled antigen from other electrochemically active compounds in human serum was accomplished by the use of gel chromatography. This method is more sensitive and simple than the direct column LC method [13] and is suitable for the routine determination of PHT in human serum.

EXPERIMENTAL

Reagents

5,5-Diphenylhydantoin-3-butyric acid (PHT-BUA) was prepared as described previously [13]. 3-(2,2,5,5-Tetramethylpyrrolidine-1-oxyl-3-carbamoylpropyl)-5,5-diphenylhydantoin (PHT-NO) was prepared from PHT-BUA and 3-amino-2,2,5,5-tetramethylpyrrolidine-1-oxyl (Eastman Kodak, Rochester, NY, U.S.A.) by the same method as applied for the preparation of the cortisol spin label [9]. Data for PHT-NO: yellow powder, m.p. ca. 110°C; calculated for $C_{27}H_{33}N_4O_4$: C=67.90; H=6.97; N=11.73; found: C=67.10; H=7.02; N=11.61; IR $\upsilon_{max}^{KBr}(cm^{-1})$: 3340 (NH), 1775 (C=O), 1715 (C=O), 1660 (CONH). MS *m/e*: 477 (M^+), 447 ($M^+ - NO$). The antibody for PHT was prepared as described previously [13]. Q-PAK chemistry control serum I and therapeutic drug monitoring control sera–anticonvulsants were obtained from Hyland Diagnostics, (Bannockburn, IL, U.S.A.). The assay buffer was 0.1 *M* potassium dihydrogen phosphate–disodium hydrogen phosphate (pH 7.2) containing 1 m*M* Na_2EDTA and 0.01% sodium 1-hexanesulphonate. Water was purified with a Millipore MILLI-R/Q system. Ethanol was mixed with lead acetate and potassium hydroxide and then distilled before use. The antibody and PHT-NO stock solutions and the assay buffer were filtered with a Millipore SJHV004NS filter. All other chemicals used were of reagent grade.

Apparatus

Column chromatography was carried out with an M-45 solvent delivery system, U6K universal LC injector, Protein PAK 60 column (100×7.8 mm I.D., Waters Assoc., Milford, MA, U.S.A.), ERC-3320 degasser (Erma Optical Works, Japan) and E-502 electrochemical detector (Irica Kogyo, Kyoto, Japan). IR and mass spectra were obtained on Hitachi 260-30 and M-60 spectrometers, respectively.

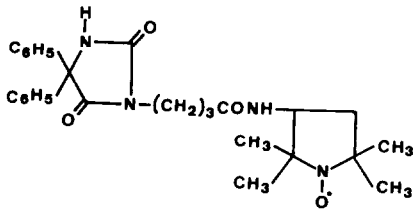

Fig. 1. Structure of the electrochemically labelled phenytoin.

Assay procedure

A 10-μl aliquot of serum samples and a 440-μl aliquot of the assay buffer were added to a plastic tube together with 50 μl of the antibody–PHT-NO mixture. The final concentration of PHT-NO in the assay mixture was $8 \cdot 10^{-7} M$. The concentration of the antibody was fixed in such a way that an addition of the antibody to the free PHT-NO reduced its peak current to 35% of the original value. The mixture was stirred and allowed to stand for 90 min at room temperature ($21 \pm 1°C$), and then a 100-μl aliquot of the mixture was injected into the chromatograph and eluted with a mobile phase of 50 mM potassium dihydrogen phosphate–disodium hydrogen phosphate (pH 7.2) containing 0.1 mM Na_2 EDTA, 0.05% sodium 1-hexanesulphonate and 2% ethanol at a flow-rate of 1.5 ml/min and an applied potential of +0.6 V versus silver/silver chloride. All separations were done at ambient temperature ($21 \pm 1°C$). The mobile phase was filtered through a 0.45-μm Millipore filter (Type HV) immediately before use.

RESULTS AND DISCUSSION

Electrochemically labelled antigen

Although ferrocene derivatives can be used as a label for LC with ED [14], they are photosensitive, and hence they are not suitable for ECIA, which requires a long incubation time before the injection of test solutions. On the other hand, nitroxides are oxidized at ca. +0.7 V versus silver/silver chloride [15], and their aqueous solutions are not photosensitive and they can be stored for more than two years in a refrigerator [8–13]. Therefore, a nitroxide derivative of PHT, PHT-NO (Fig. 1), was used as the electrochemically tagged antigen. The dependence of the detector response on the applied potential is shown in Fig. 2. Since the peak response of PHT-NO approached the limiting value at +0.6 V versus silver/silver chloride, and the background current increased steeply beyond +0.6 V, the applied potential was set at +0.6 V. The effectiveness of PHT-NO as antigen has already been established in SIA [13].

Column liquid chromatography

Although the binding of PHT-NO to the antibody caused the disappearance of electrochemical activity and PHT itself was electrochemically inactive, there are many electrochemically active molecules, such as catechols, present in human serum. Therefore, chromatographic separation of PHT-NO from other interfering substances was necessary. In order to inject serum samples directly into col-

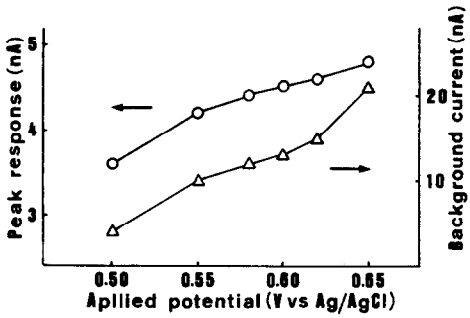

Fig. 2. The dependence of peak response (○) and background current (△) on applied potential. Conditions: sample, $8 \cdot 10^{-7}$ *M* PHT-NO; injection volume, 100 μl; mobile phase, 50 m*M* potassium dihydrogen phosphate–disodium hydrogen phosphate (pH 7.2) containing 0.1 m*M* Na_2EDTA, 2% ethanol and 0.05% sodium 1-hexanesulphonate; flow-rate, 1.5 ml/min.

umn, a gel chromatography column, Protein PAK 60, was adopted. Since the separation of proteins is not required, the column was reduced to 10 cm. The interaction between PHT-NO and the column was very strong, and PHT-NO was not eluted within 30 min with 50 m*M* phosphate buffer containing 0.1 m*M* Na_2EDTA (pH 7.2) as an eluent. Therefore, ethanol (2%) and sodium 1-hexanesulphonate (0.05%) was added to the phosphate buffer. Fig. 3 shows typical chromatograms of the blank serum and the assay mixture.

Standard curve of electrochemical immunoassay

A standard curve for the PHT assay was obtained by analysing spiked controlled serum. A 10-μl aliquot of serum sample was used for the assay. Serum

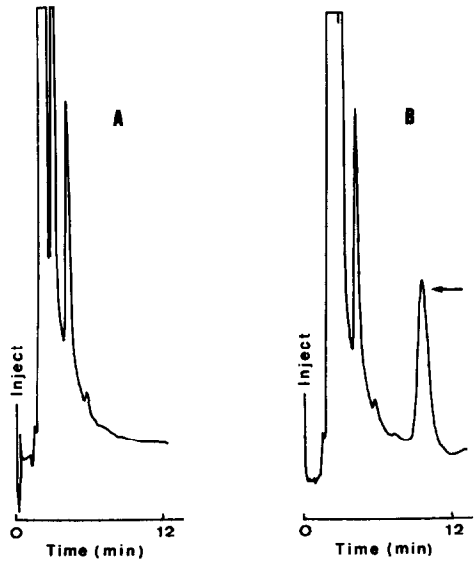

Fig. 3. Typical chromatograms for the blank serum (A) and the assay of phenytoin spiked in human serum (B). The arrow indicates the peak of PHT-NO. Conditions are the same as in Fig. 2.

TABLE I

COMPARISON OF THE SERUM PHENYTOIN LEVELS DETERMINED BY ECIA WITH THOSE LISTED BY THE MAUFACTURER

ECIA: mean value ± standard deviation (S.D.), $n=7$. Other methods: mean ± interlab. expected range.

Method	Phenytoin (mg/l)	
	Level I	Level II
This assay (ECIA)	12.4 ± 0.95	22.9 ± 1.48
Ames TDA®	13.5 ± 3.2	21.0 ± 6.3
Abbott TDX®	14.4 ± 2.3	22.5 ± 3.5
HPLC	13.8 ± 2.0	22.4 ± 3.7
GC	12.1 ± 2.7	20.4 ± 3.6

samples were diluted and mixed with the antibody–PHT-NO mixture and incubated for 90 min at room temperature (21 ± 1°C), and a 100-μl aliquot of the assay mixture was injected into the chromatograph. When the concentration of PHT (C) is in the range 2–200 μg/ml, a plot of the peak area versus log C gives a straight line ($r=0.9979$). Fouling of the glassy carbon electrode was not significant under these conditions, and no polishing of the electrode was required until at least 200 samples had been measured. The minimum detectable concentration of the assay was arbitrarily defined as the minimum concentration of PHT that could be distinguished with 95% confidence from double that concentration and the zero calibrator. The value obtained was 2 μg/ml. The standard deviation of the assay ($n=5$) is somewhat large at high concentrations of PHT (100–200 μg/ml). However, since the therapeutic serum level of PHT is 10–20 μg/ml [16], the assay appears to be well suited for routine measurements of PHT.

Cross-reactivity

The cross-reactivities of the antibody with several compounds that are used with PHT as antiepileptic drugs were checked. The control serum containing 5 μg/ml PHT was used as the reference standard. The values obtained were: PB, 0.09%; primidone, 0.06%; ethosuximide, 0.04%; and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (PHT-OH), 3.0%. Since the PHT-OH concentration is usually less than 2 μg/ml [17,18], the major metabolite of PHT, PHT-OH, and other drugs at the concentrations expected in serum have no effect on the assay.

Intra- and inter-assay precision and accuracy

The PHT concentration in Q-PAK therapeutic drug monitoring control sera (anticonvulsants) were determined by ECIA. The results are summarized in Table I. Intra-assay variation was determined on the basis of seven measurements of two different samples. The coefficients of variation (C.V.) were 7.7% (level I) and 6.5% (level II). Inter-assay variation was estimated by assaying the same samples on six different occasions. The C.V. values were 9.8% (level I) and 6.5% (level II). The PHT levels in the two samples determined by ECIA were in good agreement with those listed by the manufacturer.

Evaluation of ECIA

Although the C.V. values are somewhat large compared with those obtained by high-performance liquid chromatographic (HPLC) methods [19,20], they lie in the range expected for immunoassays [21,22]. The ECIA described here is fast and easy to perform and requires only 10 μl of serum sample per test and no sample pretreatment. The shelf-life and cost of the electrochemically tagged antigen are much longer and cheaper than those of radioisotopes and enzymes. The antiepileptic agents are frequently prescribed in combination, and hence multiple assays may be required. HPLC can determine more than two drugs simultaneously, whereas ordinary immunoassays cannot. Since ECIA combined with HPLC involves a separation procedure, it can differentiate between two electrochemically tagged antigens. Therefore, this system offers excellent prospects for the development of dual assays.

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