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Enzyme Immunoassay for Phenobarbital[1]

KIMIKO AOKI, NAMIKA TERASAWA, and YUKIO KUROIWA

Faculty of Pharmaceutical Sciences, Showa University[2]

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A competitive enzyme immunoassay for phenobarbital with alkaline phosphatase as a label is described.

p-Aminophenobarbital was used to prepare the immunogen and the alkaline phosphatase conjugate. The IgG fraction prepared from anti-phenobarbital antiserum was bound to Sepharose 4B or adsorbed on polystyrene tubes. The solid phase antibody could not discriminate *p*-hydroxyphenobarbital from phenobarbital, but had almost no cross-reactivity with metharbital, barbituric acid, thiobarbiturates and other antiepileptic drugs.

The ranges of phenobarbital concentration measurable by enzyme immunoassay with Sepharose-IgG (EIA-I) and with polystyrene tube-IgG (EIA-II) were 10 to 100 ng/tube and 100 to 1000 ng/tube, respectively.

The results obtained by radioimmunoassay correlated well with those obtained by EIA-I ($r=0.995$) and EIA-II ($r=0.943$).

Intra- and inter-assay variations of EIA-II were determined by replicate assays of sera containing 20 μg/ml and 80 μg/ml phenobarbital. The intra-assay coefficients of variation were 3.6% and 5.8%. The inter-assay coefficients of variation were 11.2% and 9.2%.

Because of the convenience of the EIA-II procedure, the method is suitable for the routine assay of serum phenobarbital in small clinical laboratories.

Keywords——enzyme immunoassay; phenobarbital; alkaline phosphatase; *p*-aminophenobarbital; Sepharose 4B; polystyrene tube

Phenobarbital is one of the most commonly used antiepileptic drugs. Its dosage regimen must be adjusted by monitoring the drug level in the serum. Therefore, a convenient analytical method with high sensitivity and specificity is required for routine measurement of phenobarbital in serum.

As an alternative to radioimmunoassay,[3] the enzyme immunoassay has been developed. A homogeneous enzyme immunoassay using glucose-6-phosphate dehydrogenase is commercially available as EMTI® kits (Syva Co., Palo Alto, U.S.A.). A competitive enzyme immunoassay with β-D-galactosidase and antibodies bound to cell walls has also been reported.[4] Compared with homogeneous enzyme immunoassay, competitive enzyme immunoassay is time-consuming, but has the advantages that the equipment required is relatively cheap and widely available.

In this paper, we report a competitive enzyme immunoassay for phenobarbital with alkaline phosphatase and solid phase antibody using Sepharose 4B or polystyrene tubes.

Materials

5-Ethyl-5-phenyl-barbituric-2-^{14}C-acid (^{14}C-phenobarbital; 3.13 mCi/mmol) was obtained from New England Nuclear, Boston, U.S.A. Alkaline phosphatase from calf intestine (grade I) was purchased from

1) Part of this work was reported at the 52nd General Meeting of the Japanese Pharmacological Society, Tokyo, Japan, March, 1979.
2) Location: *1-5-8, Hatanodai, Shinagawa-ku, Tokyo, 142, Japan.*
3) *a*) H. Satoh, Y. Kuroiwa, and A. Hamada, *J. Biochem.*, **73**, 1115 (1973); *b*) H. Satoh, Y. Kuroiwa, A. Hamada, and T. Uematsu, *J. Biochem.*, **75**, 1301 (1974); *c*) S. Spector and E.J. Flynn, *Science*, **174**, 1036 (1971); E.J. Flynn and S. Spector, *J. Pharmacol. Exp. Ther.*, **181**, 547 (1972); A. Chung, S.Y. Kim, L.T. Cheng, and A. Castro, *Experientia*, **29**, 820 (1973).
4) S. Kurooka and K. Sunahara, Japan. Patent 65886 (1978) [*C.A.* 173930 (1978)].

Boehringer Mannheim, Mannheim, Germany. Bovine serum albumin (BSA) was obtained from ICN Pharmaceuticals, Inc., Cleveland, U.S.A. and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC) was from E. Merck, Darmstadt, Germany.

m-, *p*-Aminophenobarbital and *m*-, *p*-hydroxyphenobarbital were synthesized from phenobarbital as described previously.[5] 3′-Ketocyclobarbital and 3′-hydroxycyclobarbital were synthesized by the method of Tsukamoto and Kuroiwa.[6]

Carbamazepine was a kind gift from Ciba Geigy (Japan) Ltd., Osaka.

Sephadex G-25, G-200 and CNBr-activated Sepharose 4B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden and diethylaminoethyl (DEAE)-cellulose (DE 52) was from Whatman Biochemicals Ltd., Kent, England.

Polystyrene tubes (F701; 11×80 mm) were obtained from Eiken Instruments Company, Tokyo.

Methods

Protein was determined by the method of Lowry *et al.*[7] The amount of phenobarbital incorporated into the protein was determined by the radioimmunoassay described below.

Measurement of Alkaline Phosphatase Activity[8]——The substrate solution—6 mM *p*-nitrophenylphosphate in 0.1 M glycine buffer, pH 10.5, containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ (Buffer A) -was incubated with alkaline phosphatase at room temperature. The reaction was stopped by adding 0.5 ml of 1 N NaOH to 3 ml of the substrate solution. The amount of *p*-nitrophenol liberated was measured by following the absorbance at 405 nm with a Shimadzu UV 150-02 spectrophotometer.

Preparation of Immunogen——*p*-Aminophenobarbital was coupled to BSA by condensation with glutaraldehyde followed by reduction with $NaBH_4$.[9]

A mixture of *p*-aminophenobarbital (15 mg) in dimethylformamide (20 μl), 0.1 M phosphate buffer, pH 7.0 (6 ml) and BSA (45 mg) was treated dropwise with a 1% aqueous solution of glutaraldehyde (0.5 ml), with gentle stirring. The reaction mixture was allowed to stand for 2 hr. After dialysis against 0.1 M phosphate buffer, pH 8.0, at 4° for 8 hr, $NaBH_4$ (2 mg) was added. The reduction was allowed to proceed at 4° overnight. The mixture was dialyzed against physiological saline, pH 7.0, and finally chromatographed on Sephadex G-25 equilibrated with the saline. The fractions containing phenobarbital-BSA conjugate were stored in a deepfreeze until use. The phenobarbital: BSA molar ratio of the conjugate was about 21.

Immunization——The immunization procedure used was that of Landon and Moffat.[10] The immunogen (0.5 mg) in saline (0.25 ml) emulsified in complete Freund's adjuvant (0.75 ml: Iatron Laboratories) was injected intramuscularly into each limb and intradermally in multiple sites on the back of a rabbit. After 6 weeks, booster injections containing 0.25 mg of the immunogen were given every two weeks. The blood was drawn from the ear vein 13 days after each injection and the serum was separated. The antibody titers were monitored by the radioimmunoassay procedure.

Preparation of Solid Phase Antibody——The IgG fraction of the antiserum was prepared by precipitation with Na_2SO_4 and DEAE-cellulose chromatography.[11] After dialysis against water at 4°, the IgG fraction was lyophilized.

i) Sepharose-IgG: The IgG (24 mg) was coupled with CNBr-activated Sepharose 4B (1 g) in the usual way.[12] The Sepharose-IgG was washed thoroughly with 0.1 M Tris-HCl buffer, pH 7.6, and suspended in 3.5 ml of the buffer with 0.1% NaN_3.

ii) Polystyrene Tube-IgG: The polystyrene tubes were each coated with 1 ml of 1.27 mg/ml IgG in 0.01 M phosphate-buffered saline containing 0.1% NaN_3 at 4° overnight or for a longer time. The coating solution was used repeatedly. Before assay, the coated tubes were incubated with 1 ml of 0.1% BSA in 0.05 M phosphate buffer, pH 8.0 (buffer B), for 1 hr and washed twice with buffer B.

Preparation of Phenobarbital-Alkaline Phosphatase Conjugate (Enzyme Conjugate)——*p*-Aminophenobarbital-alkaline phosphatase conjugate was prepared by the method described by Ogihara *et al.*[13] except for the use of EDC.

5) T.C. Butler, *J. Pharmacol. Exp. Ther.*, **116**, 326 (1956); A.E. Pierce and M.M. Rising, *J. Am. Chem. Soc.*, **58**, 1361 (1936).
6) H. Tsukamoto and Y. Kuroiwa, *Chem. Pharm. Bull.*, **7**, 731 (1959).
7) O.H. Lowry, N.J. Rosebrough, A.L. Fare, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
8) "Biochemical Information," Vol. II, Boehringer Mannheim GmbH, Biochemica, Mannheim, 1975, p. 115.
9) E. Engvall and P. Perlmann, *Immunochemistry*, **8**, 871 (1971); M. Numazawa, T. Ohkubo, and T. Nambara, *FEBS Letters*, **79**, 215 (1977).
10) J. Landon and A.C. Moffat, *Analyst* (London), **101**, 225 (1976).
11) K. Kato and E. Ishikawa, *Kagaku To Seibutsu*, **14**, 737 (1976).
12) "Affinity Chromatography-Principles and Methods," Pharmacia Fine Chemicals, Uppsala, p. 9.
13) T. Ogihara, K. Miyai, K. Nishi, K. Ishibashi, and Y. Kumahara, *J. Clin. Endocrinol. Metab.*, **44**, 91 (1977).

A suspension of alkaline phosphatase (1 mg/200 μl) was centrifuged. The precipitate was dissolved in water (0.7 ml) and the solution was mixed with EDC (10 mg) and *p*-aminophenobarbital dimethyl formamide solution (12 mg/20 μl). The mixture was gently stirred overnight and applied to a Sephadex G-200 column (1.5×47 cm) equilibrated with Buffer B. The fractions (0.5 ml each) of eluate with high enzyme activity were combined and adjusted to 10 ml with buffer B.

The protein content of the solution was 76 μg/ml. The phenobarbital: enzyme molar ratio of the conjugate was estimated to be about 4.3, taking the molecular weight of the enzyme as 100000. The specific activity of the conjugate was 234.2 U/mg-protein.

The solution could be kept for at least 2 years with little loss of activity at 4° and was diluted with buffer B containing 0.5% normal rabbit serum and 0.25% BSA just before use.

Enzyme Immunoassay Procedure——i) Enzyme Immunoassay with Sepharose-IgG (EIA-I): Either 0.1 ml of sample (diluted serum) or 0.1 ml of standard phenobarbital solution, 0.5 ml of diluted Sepharose-IgG suspension (1: 120) and 0.1 ml of diluted enzyme conjugate solution (1: 200) were added to a polystyrene tube and incubated for 1 hr in an immunorotator. After the addition of 3 ml of buffer A, the mixture was centrifuged at 3000 rpm for 5 min and the supernatant (3.4 ml) was aspirated off. The precipitated Sepharose-IgG was washed with 3 ml of buffer A and the procedure was repeated. The enzyme bound to Sepharose-IgG was incubated with 2.7 ml of the substrate solution for 30 min in an immunorotator. After the addition of 1 N NaOH and centrifugation, the absorbance of the supernatant was measured.

Under these conditions, Sepharose-IgG exhibited 50% binding of the enzyme conjugate.

ii) Enzyme Immunoassay with Polystyrene Tube-IgG (EIA-II): Either 0.1 ml of sample (diluted serum) or 0.1 ml of standard phenobarbital solution, 0.7 ml of buffer B, 0.1 ml of diluted normal rabbit serum (1: 10) and 0.1 ml of diluted enzyme conjugate solution (1: 750) were added to the coated tubes. The tubes were left to stand overnight and washed twice with Buffer A. The enzyme bound to polystyrene tube-IgG was incubated with 1.5 ml of the substrate solution in an immunorotator. After 1.5 hr, the absorbance was measured as described above.

Polystyrene tube-IgG could bind 60% of the enzyme conjugate under these conditions.

Radioimmunoassay Procedure——The procedure was the same as that described by Satoh *et al.*[3b] The mixture of 0.1 ml each of ^{14}C-phenobarbital (3000 cpm), diluted antiserum (1: 8), diluted normal rabbit serum (1: 10), 0.1 M Tris-HCl buffer, pH 7.6, and standard phenobarbital solution or sample was incubated for 1 hr. After the addition of 0.5 ml of saturated $(NH_4)_2SO_4$ solution, the mixture was centrifuged. The precipitate was washed once with 50% saturated $(NH_4)_2SO_4$ solution and dissolved in 1 ml of H_2O. The solution was added to 10 ml of toluene-Triton scintillator and the radioactivity was counted.

Results and Discussion

The anti-phenobarbital antiserum was produced in rabbits by immunization with *p*-aminophenobarbital-BSA conjugate. Figure 1 shows a typical pattern of antibody production. The binding capacity of the antiserum for phenobarbital increased until nearly the 20th week, but decreased thereafter. The antiserum had a higher titer than those raised against *p*-azo-phenobarbital-BSA conjugate prepared by the method described by Satoh *et al.*[3a] This could be ascribed in part to the nature of the bond between hapten and carrier protein. *p*-Aminophenobarbital was linked the ε-amino group of lysine *via* five methylene groups, while *p*-azophenobarbital was bound to tyrosine or histidine.

Figure 2 shows the standard curve for EIA-I. The percentage of enzyme conjugate bound was plotted against the logarithm of the amount of phenobarbital. With this curve, phenobarbital could be determined in the range of 10 to 100 ng/tube. The sensitivity of this method is comparable to that of radioimmunoassay.

Phenobarbital levels in rat sera were assayed by EIA-I and radioimmunoassay. The rats were fed phenobarbital admixed with powdered food, and blood was drawn from the orbital veniolex by means of a glass capillary. As shown in Fig. 3, a satisfactory correlation was found between the results obtained by two methods.

With the standard curve for EIA-II, phenobarbital could be determined in the range of 100 to 1000 ng/tube (Fig. 4).

The amount of IgG adsorbed on a polystyrene tube was estimated to be about one-fourth of that bound to Sepharose used in EIA-I by measurement of the amount of enzyme conjugate bound to solid phase antibody. Nevertheless, a much larger amount of phenobarbital was required to compete with the enzyme conjugate for polystyrene tube-IgG. This result indi-

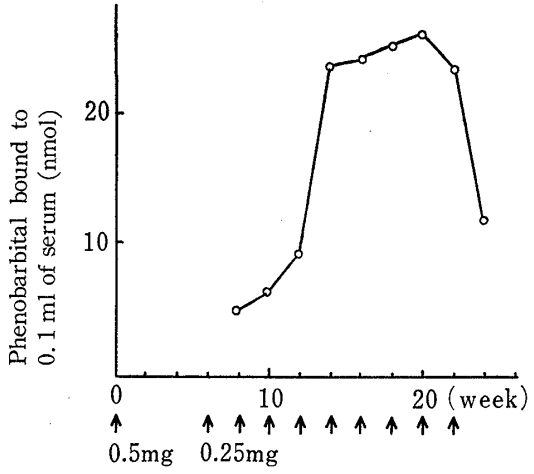

Fig. 1. Anti-phenobarbital Antibody Production Pattern

To calculate the amount of phenobarbital bound to 0.1 ml of undiluted antiserum, the radioactivity of ^{14}C-phenobarbital bound to 0.1 ml of serially diluted antiserum was counted according to the radioimmunoassay procedure described in "Methods."

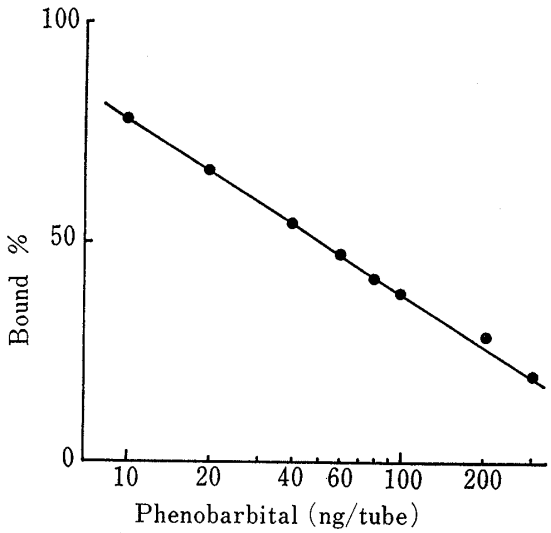

Fig. 2. Standard Curve for Phenobarbital by EIA-I

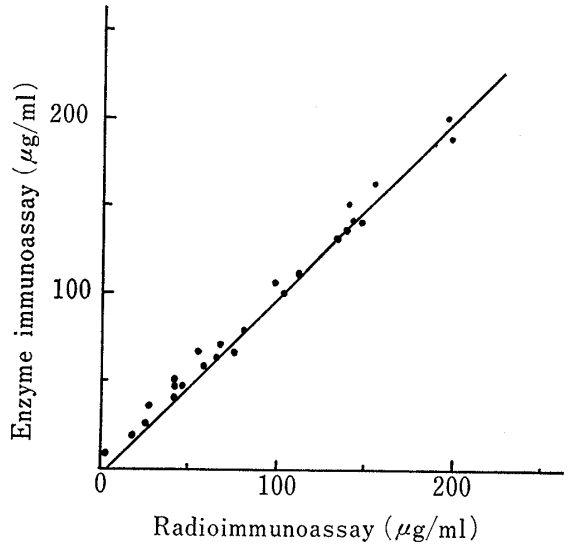

Fig. 3. Correlation of Phenobarbital Level measured by Radioimmunoassay and by EIA-I

The rat sera were diluted 200-fold with buffer B. $y=1.07x-4.84$, $r=0.995$, $n=25$.

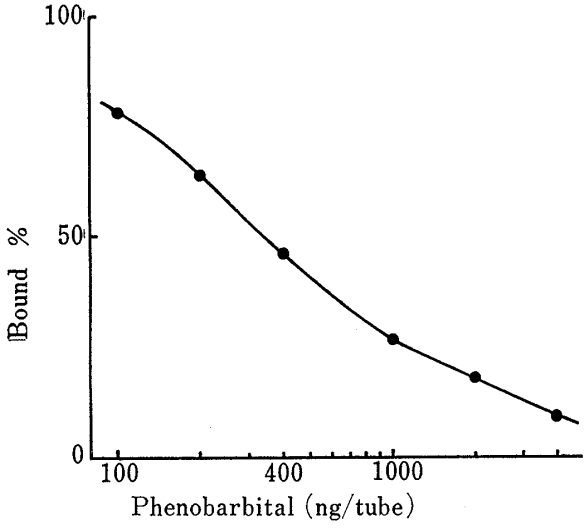

Fig. 4. Standard Curve for Phenobarbital by EIA-II

cated that polystyrene tube-IgG had a stronger affinity for the enzyme conjugate than Sepharose-IgG.

Figure 5 shows the correlation between the phenobarbital levels of the rat sera measured by EIA-II and radioimmunoassay.

Table I shows cross-reactivities with barbiturates and antiepileptic drugs assayed by EIA-I and EIA-II. Almost no cross reactivity was found with metharbital, barbituric acid, thiobarbiturates and antiepileptic drugs. However, the antibody could not discriminate phenobarbital from the metabolite, *p*-hydroxyphenobarbital.

The precision of EIA-II was examined by carrying out replicate assays of authentic phenobarbital added to normal human serum. Intra-assay variation was determined by ten mea-

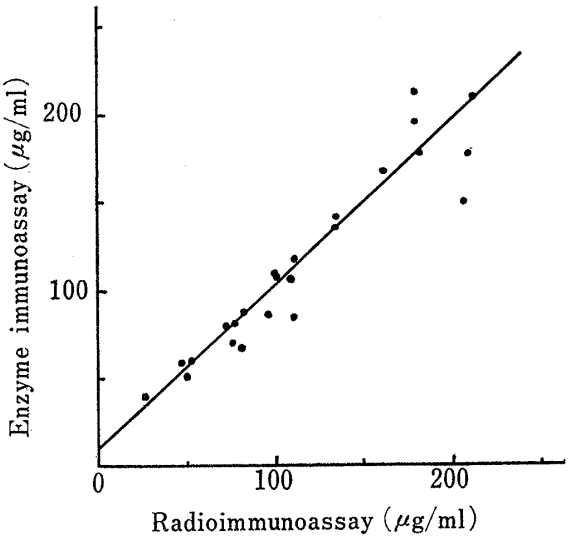

Fig. 5. Correlation of Phenobarbital Level measured by Radioimmunoassay and by EIA-II

The rat sera were diluted 200-fold for radioimmunoassay and 29-fold for EIA-II with buffer B.
$y=0.89x+11.2$, $r=0.943$, $n=24$.

Table I. Per Cent Cross-reaction of Anti-phenobarbital Antiserum with Barbiturates and Antiepileptic Drugs

	% cross-reactivity (50%)	
	EIA-I	EIA-II
Phenobarbital	100	100
p-Hydroxyphenobarbital	133	275
m-Hydroxyphenobarbital	33	92
p-Aminophenobarbital	111	191
m-Aminophenobarbital	67	70
Cyclobarbital	50	30
3′-Ketocyclobarbital	11	4
3′-Hydroxycyclobarbital	9	6
Pentobarbital	33	13
Secobarbital	6	1
Barbital	13	3
Metharbital	<0.1	<1
Barbituric acid	<0.1	<1
Thiopental	<0.1	<1
Thiobarbituric acid	<0.1	<1
Primidone	<0.1	[b] (16 μg/ml)
Glutethimide[a]	<0.1	[b] (16 μg/ml)
Phenytoin	<0.1	[b] (10 μg/ml)
Carbamazepine[a]	<0.5	[b] (2 μg/ml)

a) The drug was dissolved in normal human serum.
b) No cross reactivity was found at the drug levels in parentheses. Because serum diluted at least 10-fold was assayed by EIA-II, the drug level corresponds to more than twice the toxic blood level reported by Winek.[14]

surements of two different samples, 20 μg/ml and 80 μg/ml. The coefficients of variation were 3.6% and 5.8%, respectively. Inter-assay variation was estimated by assaying the same samples on 12 different occasions. The coefficients of variation were 11.2% and 9.2%. The precision is sufficient.

The procedure of EIA-II is simpler than that of EIA-I, since it does not involve centrifugation for washing the solid phase. The instruments required are a spectrophotometer and an immunorotator. The amount of serum required for duplicate assays by EIA-II is less than 30 μl, because the therapeutic serum level of phenobarbital is 15 to 40 μg/ml. EIA-II appears to be well suited for the routine measurement of phenobarbital in small clinical laboratories.

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14) C.L. Winek, *Clin. Chem.*, **22**, 832 (1976).