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Capillary electrochemical enzyme immunoassay (CEEI) for phenobarbital in serum

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Abstract

A competitive heterogeneous capillary enzyme immunoassay with electrochemical detection has been developed for phenobarbital in serum. The oxidized primary antibody was attached covalently to the modified interior surface of a microcapillary (22 μl). The competition between analyte phenobarbital and alkaline phosphatase labeled phenobarbital for a limited number of antibody binding sites was complete in 1.5 h. The enzymatic product (*p*-aminophenol) from the catalytic conversion of the substrate (*p*-aminophenyl phosphate) was detected by amperometric flow injection analysis. The calibration curve for phenobarbital had a detection limit of 30 μg l^{-1} (2.8 pmoles or 0.65 ng) and a range of 30–3000 μg l^{-1}. The assay could be used to determine the phenobarbital serum concentration in a 4 μl clinical serum sample without pretreatment.

Keywords: Phenobarbital; Capillary enzyme immunoassay; Electrochemical detection; Serum

1. Introduction

Therapeutic drug monitoring of a variety of antiepileptic drugs is used routinely as a guide to individualizing the drug treatment for patients with epilepsy. Phenobarbital (PB) has been the most commonly used of these worldwide since it was introduced into clinical practice in 1912 [1]. Individual differences in drug metabolism result in wide variations in serum drug concentrations achieved with any given dosage. Thus, by monitoring the serum PB concentration, the dose administered can be adjusted to the optimal level for effective therapeutic control but with minimal side effects such as neurological toxicity. The efficacy of antiepileptic drug treatment of neonatal seizures is difficult to assess with respect to the prevention of brain damage. As PB is the drug of choice for neonatal seizures, close monitoring of PB blood levels is mandatory in order to achieve rapid seizure control without neurotoxicity [2–4]. Neonates in intensive care present special monitoring problems. The very small size of neonates demands that the sample size taken for monitoring should be as small as possible in order to avoid anemia and the risk of blood transfusion related diseases. The therapeutic range of 10–40 μg ml^{-1} (43–172 μmol l^{-1}) is usually associated with optimal seizure control. The potentially toxic

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concentration is above 40 μg ml^{-1} exceeding 60 μg ml^{-1} (259 μmol l^{-1}) usually produces serious toxicity [4–6]. Therapeutic PB monitoring often includes the subtherapeutic range of 1–10 μg ml^{-1} [7–10]. Thus, the monitoring method in neonatal care in addition to being precise and accurate, should emphasize sensitivity, wide measuring range, speed, and most important, only require a small sample volume.

Historically, there are several methods for determining PB concentration in serum. Well established techniques that rely on extraction, or derivatization and chromatographic separation such as gas chromatography, gas chromatography-mass spectrometry, and high performance liquid chromatography are available. However, as a result of the increase in the number of determinations done for antiepileptic drugs, immunoassay has become the most common method for routine therapeutic drug monitoring because of its simplicity, reliability and speed [11,12]. As well as radioimmunoassay, there are two popular nonradioactive immunoassays for routine therapeutic PB monitoring: EMIT enzyme immunoassay (Syva, Palo Alto, CA), a homogeneous enzyme immunoassay with spectrophotometric detection, and fluorescence polarization immunoassay (TD × TM; Abbott Laboratories Diagnostics Division, Irving, TX) [12–14].

Capillary electrochemical enzyme immunoassay (CEEI) uses a modified microcapillary as an immunoreactor for a heterogeneous enzyme immunoassay with electrochemical detection, and has been developed successfully in our research group. Typical applications have been a sandwich assay for mouse IgG, a sequential saturation assay for digoxin, and a competitive assay for atrazine [15–25]. Enzyme immunoassay, combining the specificity of an antigen–antibody reaction with the sensitivity of an enzyme label amplification, is a powerful technique for the determination of clinically important compounds in a variety of matrices. In the final detection step of such an immunoassay, it is the concentration of the enzymatic product that is measured, and this depends upon the volume of the reactor. The advantages of the microcapillary immunoreactor (10 cm × 0.53 mm i.d., 22 μl) are then obvious: the small sample volume and large surface area-to-volume ratio result in a more sensitive assay, and efficient mass transport due to the reduced diffusional path length yields a faster assay [26]. Amperometric detection is used because it is more sensitive than spectrophotometric methods, and is ideally suited for such small-volume immunoassays [27–30]. The combination of the capillary enzyme immunoassay with electrochemical detection thus meets the specific requirements of neonatal analysis.

The objective here was to develop a competitive heterogeneous CEEI for PB in serum, with particular consideration of the needs of neonatal analysis (Fig. 1) [31].

We anticipate that the same methodology could be used for essentially any analyte for which a selective antibody exists and an appropriate enzyme conjugate can be made. For example, gentamicin and caffeine, which are commonly used in neonatal care and require monitoring, should also be amenable to this technique.

2. Experimental

2.1. Apparatus

The FIAEC system used for the assay consists of a BAS pump (PM-60), a BAS LC-4B amperometric detector and a CC-5 thin-layer flow cell (3.5 μl volume) with dual glassy carbon working electrodes, a Ag/AgCl reference electrode, and a stainless steel auxiliary electrode. *p*-Aminophenol (PAP) was measured at 300 mV versus Ag/AgCl. The six-port injection valve has a sample loop of 5 μl. The flow rate was set at 1.0 ml min^{-1}. The data were collected by a D5217-5AQ strip chart recorder (Houston Instrument, Austin, TX). A syringe pump (infusion pump 22, Harvard Apparatus, South Natick, MA) was used for capillary modification.

The UV detection and flow cell used in the size exclusion chromatography were a Beckman model 153 detector and a Beckman analytical optical unit (Beckman Instruments, Fullerton, CA). The UV spectrophotometer was a Hewlett-Packard 8452D diode array spectrophotometer (Hewlett-Packard, Naperville, IL).

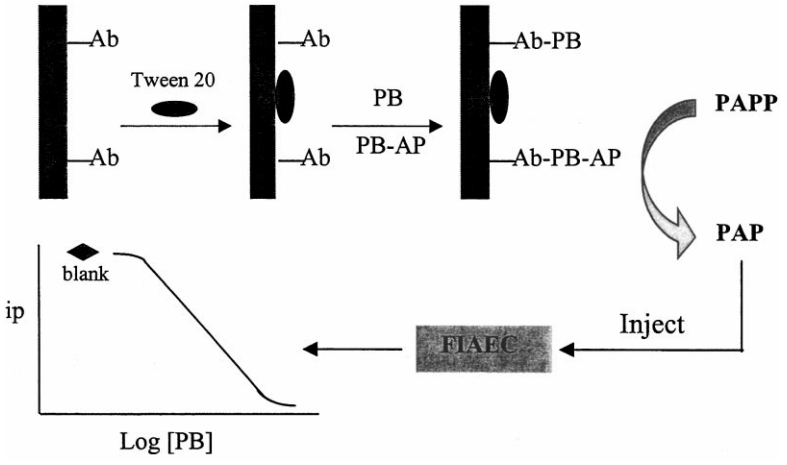

Fig. 1. Schematic representation of the competitive CEEI assay. Ab was attached covalently to the inner wall of a modified fused-silica capillary. Tween 20 was a blocking reagent to minimize nonspecific adsorption. AP was the label. The PB-AP competed with PB for a limited number of Ab binding sites, and catalyzed the hydrolysis of PAPP, yielding electroactive PAP, which was detected by flow injection analysis with amperometric detection. The blank signal represents the background current observed at a zero dose response of PB.

2.2. Materials and reagents

Protein A-purified monoclonal PB antibody (P01-99-81 M) and alkaline phosphatase–PB conjugate (PB-AP) (P91-99-81A) were from Biostride (Palo Alto, CA). PB standard (P 3643), bovine serum (S 1507), and Sephadex G-25 were from Sigma (St. Louis, MO). *p*-Nitrophenyl phosphate (PNPP) was from Boehringer Mannheim (Indianapolis, IN), and *p*-aminophenyl phosphate (PAPP) was synthesized as reported [32] and stored at −20°C. The undeactivated fused-silica capillary (Lot No. 61016) was from Alltech Associates (Deerfield, IL).

2.3. Buffer compositions

Buffer A: the antibody immobilization buffer was 0.1 M sodium acetate, 0.15 M sodium chloride and 0.02% sodium azide (NaN_3), pH 4.5. Buffer B: the PB/PB-AP incubation buffer was 0.1 M Tris, 0.02% NaN_3 and 0.05% Tween 20, pH 7.8. Buffer C: the substrate development buffer was 0.1 M Tris, 1 g l^{-1} magnesium chloride and 0.02% NaN_3, pH 9.0.

2.4. Procedures

2.4.1. Antibody oxidation

Hydroxyl groups on the glycan chains of the monoclonal Ab for PB (Ab-OH) were oxidized into the aldehyde group (Ab-CHO) [24,25,33] for further Ab immobilization on the modified interior surface of the capillary. Here, Ab-OH (2.06 mg ml^{-1}, 0.5 ml) was diluted with 400 μl buffer A (pH 5.5), and 30 μl of 0.4 M sodium periodate were added for the selective periodate oxidation of Ab-OH. The concentration of the oxidized antibody solution was 10^{-6} M by UV-absorbance.

2.4.2. Capillary modification

A hydrazide-activated spacer of polyethylene glycol for the aldehyde group of oxidized antibody to bind to was prepared and conjugated to the interior surface of a capillary (10 m) according to a method previously described [25]. Solutions were introduced into the capillary with a syringe pump at 2.5 ml min^{-1}.

2.4.3. Assay procedure

The assay essentially followed a previously described procedure [25]. A separate piece of capillary was used for each standard calibrator of PB. PB serum calibrators were prepared by spiking PB with PB-AP into the bovine serum matrix diluted with incubation buffer. Each calibrator was assayed in a timed sequence of 3 min intervals.

3. Results and discussion

3.1. Substrate incubation

The optimal substrate incubation time depends on the activity of the AP label, and is related to the diffusion rate of substrate to the AP label, which is bound to the interior surface of the capillary for enzyme catalysis. It can be affected by the stabilities of PAPP and PAP.

Tris buffer (pH 9.0) was selected since it has been found optimal for PAPP [34]. The stability of PAP in air-saturated Tris buffer was investigated under assay operating conditions with a solution of 5.0 μM PAP. An aliquot was injected into the FIAEC every 6 min, and the resulting peak current values, i_p, were plotted versus time (Fig. 2). The decomposition of PAP caused by its oxidation into quinone by dissolved oxygen is evident after 8 min.

The stability of PAPP against hydrolysis to PAP was studied with a 4 mM substrate PAPP solution prepared in the absence of the enzyme and monitored for formation of PAP by the same FIAEC method. The amperometric response versus time is plotted in Fig. 3. The low initial background signal of 66 nA, which corresponds to the amount of PAP contamination in the PAPP solid, decayed rather than increased, indicating that the oxidation of this small amount of PAP was proceeding at a faster rate than the hydrolysis of PAPP. Thus, the hydrolysis of PAPP was not detectable in the absence of the enzyme.

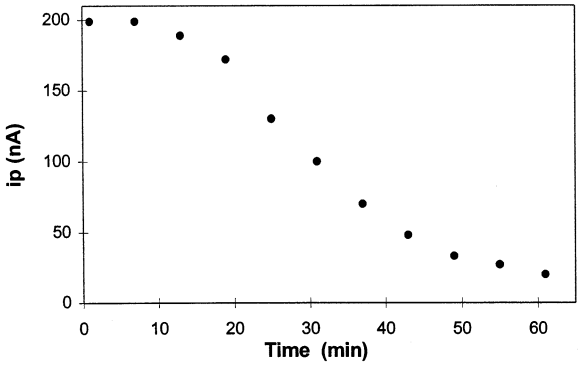

Fig. 2. Stability of PAP: 5 μM PAP in air-saturated Tris buffer, pH 9.0.

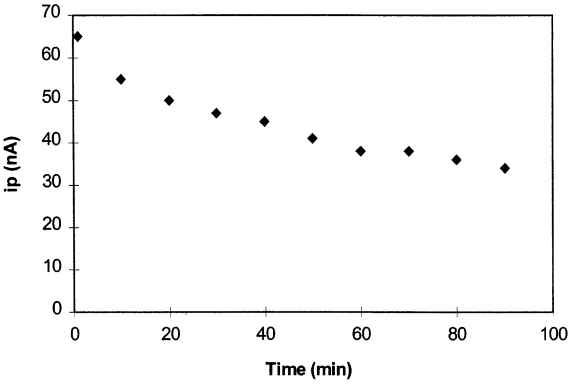

Fig. 3. Nonenzymatic hydrolysis of PAPP: 4 mM PAPP in air-saturated Tris buffer, pH 9.0.

Progress curves for the enzyme-catalyzed hydrolysis of PAPP that were obtained by measuring PAP in a mixture of PAPP and PB-AP in Tris buffer are shown in Fig. 4 for two dilutions of PB-AP. The shapes of these curves reflect the resultant of the production of the PAP by enzyme catalysis and its decomposition in air-saturated

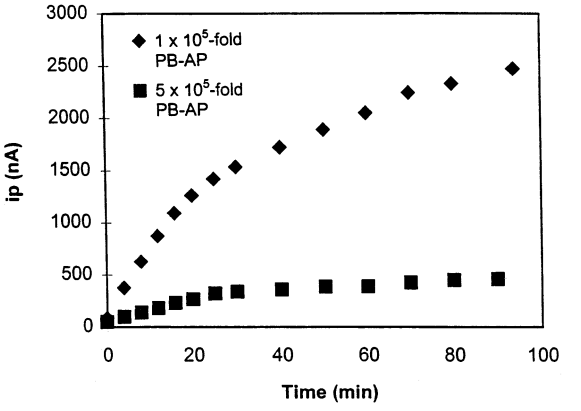

Fig. 4. Progress curve for the hydrolysis of 4 mM PAPP catalyzed by AP in Tris buffer, pH 9.0. Dilution of PB-AP indicated on graph.

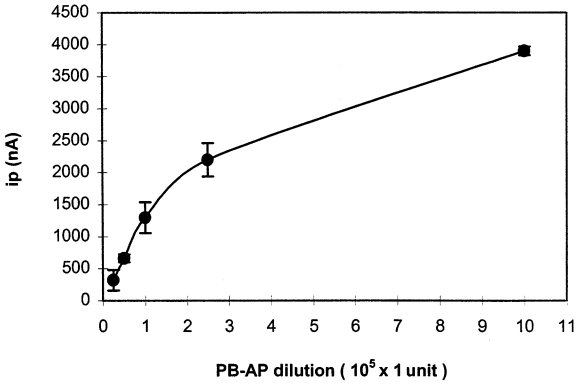

Fig. 5. PB-AP dilution curve: 0.7×10^{-9} M Ab, 12 h incubation, pH 4.5 for Ab coating; PB-AP 30 min incubation, pH 7.8; 4 mM PAPP 30 min incubation in Tris buffer, pH 9.0. Duplicate measurements; the error bar gives the range of the two measurements and the point is the average value.

buffer. It is clear, however, that the enzyme activity of the AP label was high enough to generate detectable PAP despite air oxidation. Even though the substrate development time of 20 min gave an easily detectable signal at shorter assay times, a substrate incubation time of 30 min was used in order to reduce measurement errors in the manually timed procedure used for assay development.

3.2. Amount of PB-AP

Based on the competitive binding design used for PB analysis, the sensitivity of the assay in terms of both the slope of the dose–response curve and the detection limit are governed by the equilibrium constant of the antibody–antigen binding reaction [35]. Since PB-AP competes with PB for a limited number of antibody binding sites, the concentration of immobilized Ab and the amount of PB-AP must be optimized for the assay range and detection limit desired [25,35]. The amount of PB-AP should be high enough to give the maximum competition with PB that will give the widest detectable range, but an excess amount of PB-AP would result in an unbalanced competition for PB [36]. The appropriate amount of PB-AP can be determined from the PB-AP dilution curve in the absence of PB as shown in Fig. 5. The portion of the curve showing the greatest sensitivity provides a rough working dilution range for PB-AP, which is 0.2–1.25 (10^5 U) (5×10^5–8×10^4-fold) PB-AP dilution, for the subsequent optimization studies.

3.3. Ab concentration

The Ab dilution curves of Fig. 6 illustrate a wide range of Ab dilution factors for three different PB-AP dilution factors in the buffer system. The lowest i_p signals below 0.5×10^{-9} M Ab at different concentrations of PB-AP are indistinguishable. Therefore, 0.5×10^{-9} M Ab is the minimum working concentration. Obvious differences in i_p signal corresponding to different PB-AP levels are observed above 1×10^{-9} M Ab. The higher Ab concentration provides a larger chance for PB-AP to bind in competition with PB, producing a more sensitive response, but a higher detection limit. Since a low detection limit is a priority of the PB assay development, the analytical sensitivity of the assay has to be compromised as a result of the lower concentration of antibody used to optimize detection limit. As seen in Fig. 6, the amount of Ab immobilized from 10^{-9} M is adequate to show current signal differences for different amounts of PB-AP. Thus, this concentration provides enough Ab binding sites for ef-

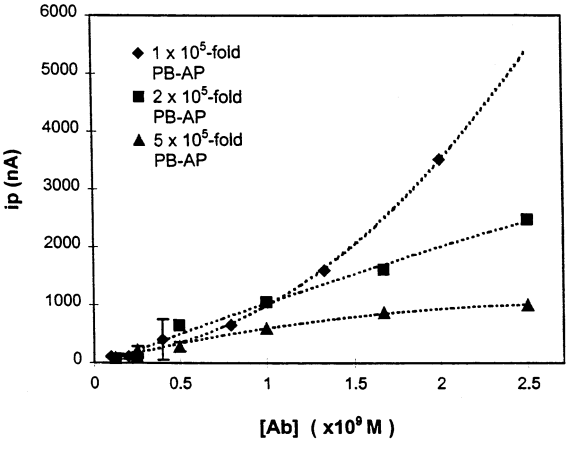

Fig. 6. Ab dilution curves: Ab incubation 12 h, pH 4.5; PB-AP incubation 20 min, pH 7.8; 4 mM PAPP 30 min incubation in Tris buffer, pH 9.0. Duplicate measurements.

fective competition to occur. The highest PB-AP dilution (5×10^5-fold) is too large to provide an adequate competitive range, but the 2×10^5-fold PB-AP dilution provides a wider range with an adequately high i_p signal. Therefore, the 10^{-9} M Ab and 2×10^5-fold PB-AP dilution were chosen for further investigation with PB.

3.4. Optimizing the trend of calibration curves

The calibration curve (i_p signal versus log concentration) in a competitive binding assay is sigmoidal, flattening at low and high concentrations of analyte. As the concentration of PB increases, more PB-AP is displaced from the Ab binding sites. Effective competition occurs in the pseudo linear portion of the curve, where the response to a further addition of PB has the greatest slope. In order to find the optimal sensitivity of the assay efficiently, only the lowest and the highest concentrations of PB in a wide concentration range were chosen (0.1 μg l^{-1} and 100 mg l^{-1} corresponding to the highest and the lowest current signal, respectively). The goal here is to provide adequate sensitivity, then, while keeping [PB-AP] as low as possible, to minimize nonspecific binding. These conditions were met for PB-AP dilutions between 2 and 1.25×10^5-fold for Ab immobilized from 10^{-9} M.

3.5. Serum matrix effect

A serum matrix is necessary as a control sample to be used as a calibrator in the assay development, since therapeutic drug monitoring typically involves the quantitation of drugs in serum. Quality control serum spiked with PB standards was analyzed to investigate the feasibility of the assay [37–41]. However, due to the potential biohazards of using human serum, bovine serum was chosen as a substitute matrix. There is no significant difference between the results determined in human and bovine serum spiked with PB [39].

A series of standard blank calibrators was run to investigate the effect of the serum matrix on the assay background. These were prepared by adding different volumes of bovine serum to the incubation buffer prior to assay (Fig. 7). The i_p decreases rapidly as the serum concentration increases, and then levels off. The 30-fold serum dilution was chosen as the matrix used for the assay calibration in order to reduce possible analytical error in the sample dilution procedure.

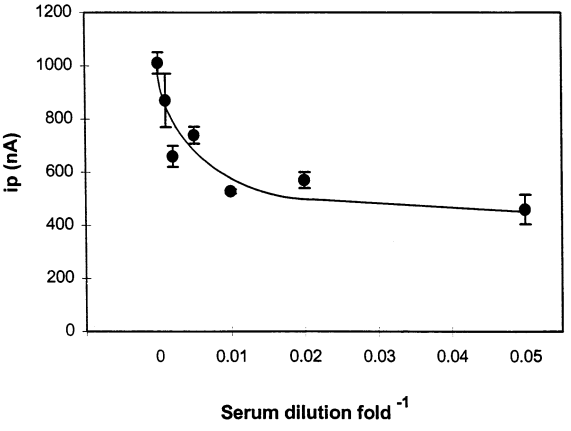

Fig. 7. Serum matrix effect on blank signal: 1×10^{-9} M Ab, 12 h incubation, pH 4.5; 2×10^5-fold PB-AP 30 min incubation, pH 7.8; 4 mM PAPP 30 min incubation in Tris buffer, pH 9.0. Duplicate measurements.

3.6. Standard calibration curve

PB serum calibrators were prepared by adding PB to PB-free bovine serum diluted 30-fold with the incubation buffer. The monitoring range of serum samples according to this dilution is given in Table 1. Concentrations of PB were chosen to cover the subtherapeutic to toxic levels taking into account the 30-fold dilution. A similar set of PB calibrators was prepared in buffer for comparison. Both series of eight calibrators were assayed using a 2×10^5-fold PB-AP dilution and 10^{-9} M Ab. The resulting calibration curves for PB in the serum and buffer are shown in Fig. 8, which illustrates the effect of the serum on the PB assay.

The higher PB-AP level (1.25×10^5-fold dilution) was tested as a means to compensate for the inhibition by serum on the sensitivity of the assay, other conditions being unchanged. The results are shown in Fig. 9. The most sensitive part of the calibration curve lies between 30–3000 μg l^{-1} as shown in Fig. 10. This covers the entire PB monitoring range according to the 30-fold sample dilu-

Table 1
Serum sample dilution

Clinical level ($\mu g\ l^{-1}$)	Low PB in neonatal care ($\mu g\ l^{-1}$)	Therapeutic range		High toxic level ($\mu g\ l^{-1}$)
		Lower limit PB ($\mu g\ l^{-1}$)	Higher limit PB ($\mu g\ l^{-1}$)	
PB	1000	10 000	40 000	>60 000
After 30-fold dilution	33	333	1333	>2000
Detection limit	30			

tion: 33–333 $\mu g\ l^{-1}$ corresponds to the subtherapeutic level (1–10 $\mu g\ ml^{-1}$), 334–1333 $\mu g\ l^{-1}$ corresponds to the therapeutic level (10–40 $\mu g\ ml^{-1}$), and 1334–3000 $\mu g\ l^{-1}$ corresponds to the toxic level (40–90 $\mu g\ ml^{-1}$).

It is worth noting that, using the 22 μl capillary immunoreactor for each sample, the detection limit of the assay is 129 nmol l^{-1} (2.8 pmoles, 0.65 ng), and only 4 μl of serum sample are needed for each assay. This high sensitivity and small sample size not only meets the specific objective in neonatal drug monitoring, but also confers the important advantage of being able to dilute the sample enough to avoid the matrix effect of serum.

4. Conclusions

Compared with the EMIT PB Assay [6], the CEEI assay has a somewhat lower detection limit (1 versus 5 $\mu g\ ml^{-1}$), a similar range (1–90 versus 5–80 $\mu g\ ml^{-1}$), but a much smaller sample size (4 versus 50 μl). No special sample pretreatment is needed. The TDEX assay for PB is comparable to EMIT, but it requires a yet larger sample size (150 μl) for each assay [12,42]. At this point, the CEEI assay meets the needs of neonatal care and could be adapted as a beneficial alternative in neonatal PB analysis.

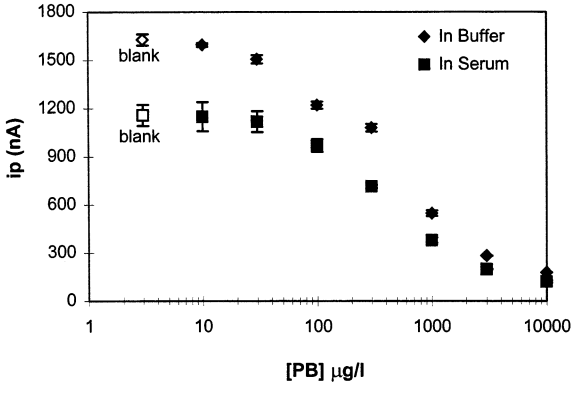

Fig. 8. Comparison of calibration curves for PB in serum and buffer: 10^{-9} M Ab, 12 h incubation, pH 4.5; 2×10^5-fold dilution PB-AP 30 min incubation, pH 7.8; 30-fold dilution serum; 4 mM PAPP 30 min incubation in Tris buffer, pH 9.0. Duplicate measurements.

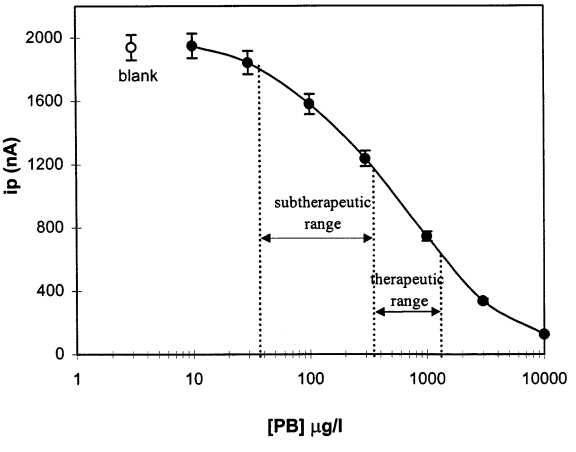

Fig. 9. Calibration curve for PB in serum: 10^{-9} M Ab 12 h incubation, pH 4.5; 1.25×10^5-fold dilution PB-AP 30 min incubation, pH 7.8; 30-fold dilution serum; 4 mM PAPP 30 min incubation in Tris buffer, pH 9.0. Duplicate measurements.

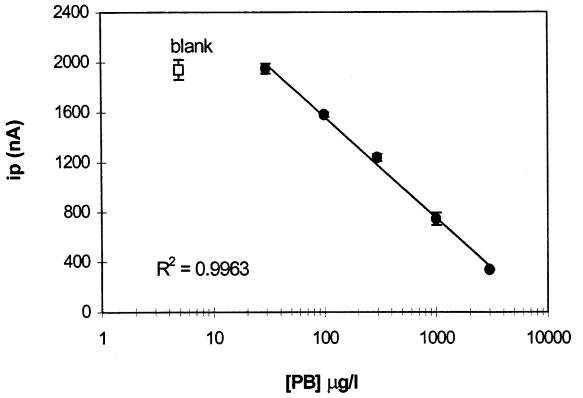

Fig. 10. Calibration curve of effective assay range for PB in serum from Fig. 9: 10^{-9} M Ab, 12 h incubation, pH 4.5; 1.25×10^5-fold dilution PB-AP 30 min incubation, pH 7.8; 30-fold dilution serum; 4 mM PAPP 30 min incubation in Tris buffer, pH 9.0. Duplicate measurements.

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