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Short communication

Determination of plasma phenobarbital concentration by high-performance liquid chromatography in rat offspring

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Abstract

Plasma phenobarbital (PB) concentrations in rat offspring were determined using a 9 μl capillary by high-performance liquid chromatography (HPLC). Capillary plasma which was put into a Bond Elut® cartridge column by using 1 ml of 0.01 *M* KH_2PO_4 was applied to the column with 50 μl of 2 μg/ml of acetanilide (internal standard, I.S.). After washing the column, PB and I.S. were eluted with methanol and injected into the HPLC system. There were excellent linear correlation between the amount of PB and length of the capillary at three different concentrations. Calibration for PB was linear in the range of 0–50 μg/ml. The coefficients of variation were 3.4–5.0% and 5.9–7.5% in the within-day and between-day assays, respectively. The extraction recovery rates were 87.5–105.4%. By this method, it was possible to measure plasma PB concentrations in rat offspring without killing. These results suggested that this method is very useful to determine the plasma PB concentration derived from mother's milk in newborn rats.

Keywords: Phenobarbital

1. Introduction

Phenobarbital (PB) is widely used as an anticonvulsant drug clinically. Although the influence of lactation on PB concentrations in epilepsy patients has been examined [1], detailed pharmacokinetic examination during pregnancy and lactation is difficult due to problems with compliance, dose/weight, and age in epilepsy patients. Previously, we examined the influence of pregnancy and lactation on plasma PB concentration in rats [2,3]. It is well known that anticonvulsant drugs are passed from the mother to the child through the placenta or mother's milk [4]. It is necessary to measure PB concentrations in many fetuses to examine the influence of this anticonvulsant drug administered to the mother on the fetus in rats [5]. Using the method reported previously, 60 μl of blood was needed to measure plasma PB concentrations [6]. However, it is difficult to collect 60 μl of blood from offspring. In the present study, we measured the plasma PB concentration using very small blood samples of only 9 μl.

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2. Experimental

2.1. Materials

PB (Sigma, St. Louis, MO, USA) was suspended in 0.5% sodium carboxymethylcellulose (CMC). Acetanilide (Waco Pure Chemical Industries, Osaka, Japan) was used as the internal standard (IS) and was dissolved in 2 μg/ml in 50% methanol. All reagents used were of reagent grade.

Heparinized capillaries (9 μl) (0.6×32 mm) were purchased from Compur-Electronic GmbH (Munich, Germany).

2.2. Animals

Five female Wistar rats (Charles River Japan, Atsugi, Japan) weighing 195–230 g at the beginning of the study were used. They were housed in plastic cages (26×36×25 cm) in a room maintained under a 12-h light-dark cycle (lights on from 7:00 to 19:00) and at 22–24°C and 60% relative humidity.

2.3. Experimental apparatus

The plasma was separated by centrifugation at 12 000 rpm for 3 min using a hematocrit centrifuge (Compur M 1100, Compur-Electronic, Munich, Germany). The plasma concentrations of PB were determined by HPLC (Pump-Type 510, Waters Associates, Tokyo, Japan), with UV detection (Type 481UV Lambda-Max) and calculated using a data module (Type 730). Specimens were injected with an automatic sample processor (Type 710B). The separation column was a stainless steel column packed with octadecyl silica (LiChroCART Superspher 100 RP-18(e), 4 μm particle size, 4×125 mm, Kanto, Tokyo, Japan) and maintained at room temperature. The mobile phase was a mixture of acetonitrile/0.01 *M* KH_2PO_4 (25/75, v/v), the flow-rate was 0.8 ml/min and absorbance was measured at 210 nm.

2.4. Correlation of amount of PB and length of 9 μl capillary

We tested whether the amount of PB can be calculated from the length of the 9 μl capillary. The stocked solution of PB was diluted with 50% methanol to obtain concentrations of 10, 15 and 30 μg/ml. These stocked solutions were trapped in 9 μl capillaries (9 μl=32 mm) at lengths of 8, 16, 32 and 64 mm, and analyzed three times as described below. The amount of PB was calculated from peak-area ratio of PB to I.S..

2.5. Pretreatment of Plasma

The plasma was separated from the blood using a hematocrit centrifuge (Compur M 1100, Compur-Electronic). After centrifugation, the blood in the 9 μl capillary was divided into plasma and blood corpuscles. Then, the amount of plasma was calculated from the length of the capillary (9 μl=32 mm). For purification of PB and I.S., a Bond Elut® C-18 solid-phase extraction column (1 ml volume, No. 1210-2001, Varian SPP, Harbor City, CA) was used. The column was successively washed twice with 1 ml of methanol and twice with 1 ml of 0.01 *M* KH_2PO_4. Capillary plasma, which was put into a Bond Elut® column by using 1 ml of 0.01 *M* KH_2PO_4 was applied to the column with 50 μl of 2 μg/ml I.S. solution. After the column was washed with 1 ml of 0.01 *M* KH_2PO_4 twice, PB and I.S. were eluted with 250 μl of methanol, which did not affect the shape of the chromatogram. Then, 30 μl of eluate was injected into the HPLC system. The chromatographic peaks of PB determined from the plasma was coincident with that of the authentic standard.

2.6. Calibration curve

A 9 μl (32 mm) amount of stocked solution of PB (5, 10, 25 and 50 μg/ml) was applied to the extraction column (Bond Elut® C-18) with drug-free plasma and extracted . The absolute quality of PB can be calculated from the length of the 9 μl capillary (9 μl=32 Mm). The concentrations of PB were calculated from the peak-area ratios of PB to I.S..

2.7. Recovery and precision

Under ether anesthesia, about 10 ml of blood was obtained from the interior vena cava of rats 0.5, 2

and 4 h after oral administration of PB at a dose of 20 mg/kg. The blood specimens were centrifuged at 3000 rpm for 10 min to obtain plasma. After centrifugation, plasma samples were stored at −20°C until the assay.

Stocked plasma samples (three different PB concentrations) were used for the recovery test. The extraction recovery rate was calculated three times by adding PB at concentrations of 5 and 10 μg/ml to plasma samples.

To investigate the precision of plasma PB measurements, stocked plasma samples containing three different concentrations of PB were analyzed 10 times continuously (within-day assay). In addition, three different stocked plasma samples were analyzed every day for 10 days (between-day assay).

2.8. Determination of the plasma PB concentration in rat offspring

Body weights of dams were measured and PB (20 mg/kg) was administered by gavage twice a day (at 7:00 and 19:00). When the plasma concentrations of PB reached the steady state (7–10 days after starting administration), female rats were mated with males for 5 days. Pregnant rats gave birth to litters of six to sixteen pups. Three dams were selected from all rats which had given birth 13–16 pups and offspring were divided into four groups of three or four from each dam. During the 12 days from delivery, blood specimens were collected according to a rotation schedule to prevent sampling from the same pup on consecutive days. Blood specimens were collected from the tail vein using 9 μl capillaries immediately after birth and every morning thereafter for 11 days. Blood specimens were collected four times for each group.

3. Results

3.1. Chromatography

As shown in Fig. 1, the retention times of I.S. and PB were 4.6 and 7.7 min, respectively. No interfering endogenous substances were present (Panel A). Panel B shows the chromatograms of the standard specimen, while panel C is a typical chromatogram of a plasma specimen collected from a rat pup 2 days after birth.

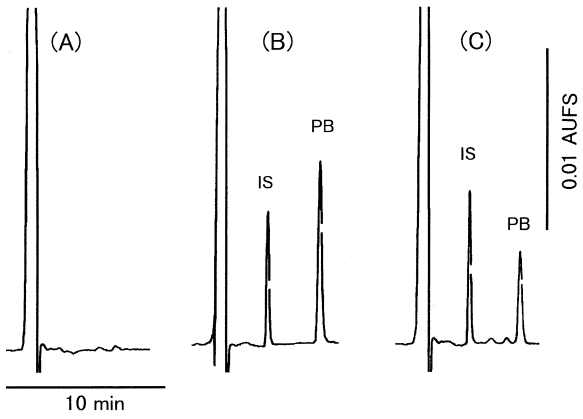

Fig. 1. Typical chromatograms for drug-free plasma specimen (A), a 25 μg/ml standard specimen (B) and a plasma specimen from a rat baby 2 days after birth (C). PB, phenobarbital; I.S., internal standard (acetanilide).

3.2. Correlation between amount of PB and length of 9 μl capillary

There were excellent linear correlation between the amount of PB and length of the capillary at three different concentrations (Fig. 2). The linear regressions of 10, 15 and 30 μg/ml were $y=3.071x+3.731$ ($r=0.999$), $y=4.677x+5.169$ ($r=0.999$) and $y=9.170x-4.201$ ($r=0.999$), respectively.

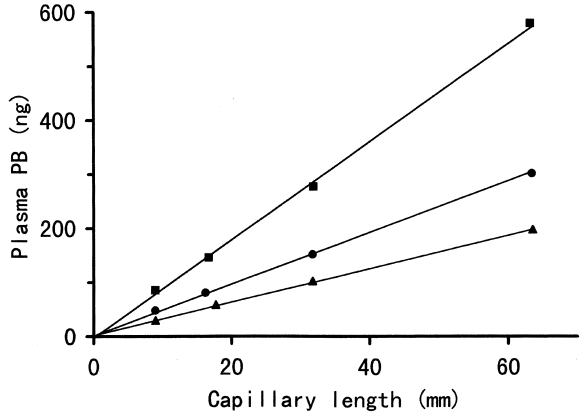

Fig. 2. Correlation of amount of PB and length of 9 μl capillary. Symbol indicate different concentrations of PB: ▲, 10 μg/ml; ●, 15 μg/ml; ■, 30 μg/ml.

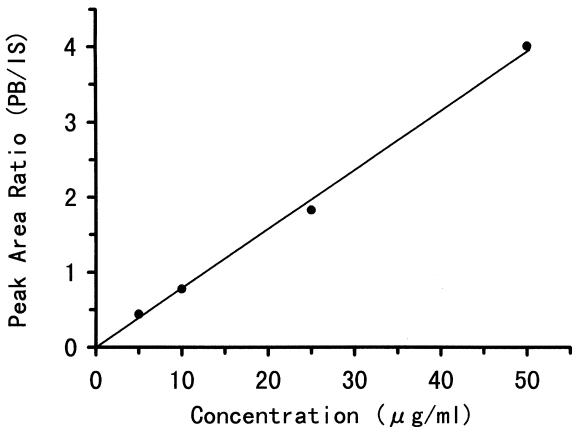

Fig. 3. Peak-area ratios of drug plasma concentration to I.S..

3.3. Calibration curve

An excellent linear correlation was obtained between the PB/IS peak-area ratio in plasma in the range 5–50 μg/ml (Fig. 3). The linear regressions of 5, 10, 25 and 50 μg/ml were $y=0.080x-0.022$ ($r=0.999$). The detection limit at a signal-to-noise ratio of 3 was 0.3 μg/ml.

3.4. Recovery rate and precision

The extraction recovery rates, calculated from the amounts of PB added at two different concentrations to the plasma, were 87.5–105.4% (Table 1). The coefficient of variation (C.V.) values in within-day assays for the three different concentrations were 3.4–5.0 (Table 2). The C.V. values in between-day assays at the three different concentrations were 5.9–7.5 (Table 2).

3.5. Plasma PB concentrations in offspring transmitted from their mothers' milk

PB concentration in plasma collected immediately after birth was 34.6±4.1 μg/ml which was the same as that in dams (data not shown). By the fourth day after birth, plasma PB concentration in offspring

Table 1
Recovery determination of PB[a]

Plasma sample (μg/ml) (A)	Added (μg/ml) (B)	Measured (μg/ml) (C)	Recovered (μg/ml) (c-A)	% Recovery (μg/ml) [(C-A)/B]X100
0	5.0	5.27	5.27	105.4
	10.0	10.05	10.05	100.5
11.62	5.0	16.43	4.81	96.2
	10.0	20.87	9.35	93.5
17.35	5.0	21.97	4.52	90.4
	10.0	27.62	10.27	102.7
30.82	5.0	35.45	4.63	92.6
	10.0	39.57	8.75	87.5

[a] Recovery rate was estimated by comparing the peak areas of added PB with those of pure standard. Each value indicates the mean value of three determinations.

Table 2
Precision (within-day and between-day assay) of PB in rat plasma[a]

Within-day assay				Between-day assay			
Sample	*n*	Concentration (mean±SD) (μg/ml)	C.V. (%)	Sample	*n*	Concentration (mean±SD) (μg/ml)	C.V. (%)
I	10	14.70±0.50	3.4	IV	10	10.75±0.81	7.5
II	10	25.13±1.28	5.0	V	10	16.46±1.15	7.0
III	10	32.91±1.25	3.8	VI	10	29.20±1.71	5.9

[a] Blood samples were obtained from the interior vena cava of rats 0.5, 2 and 4 h after oral administration of PB at a dose of 20 mg/kg. C.V., coefficient of variation.

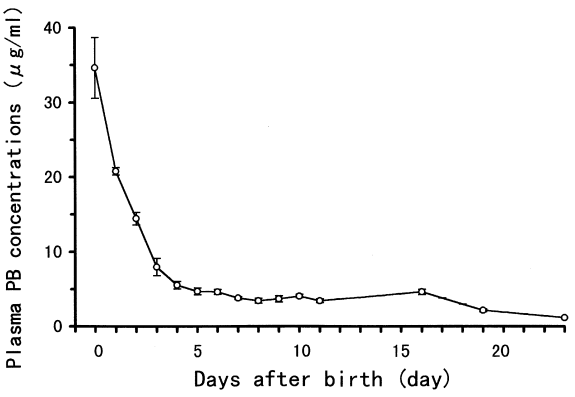

Fig. 4. Plasma PB concentration in rat offspring derived from mother's milk. Each point indicates the mean±SE of PB (n=4–6).

decreased rapidly to 5.5±0.5 μg/ml (Fig. 4). Plasma PB concentration decreased gradually to weaning. All offspring matured normally and none died during the experimental period.

4. Discussion

In the present study, we determined the concentrations of PB in a very-small volume of plasma, using heparinized 9 μl capillaries. The internal diameter of the 9 μl capillaries was very narrow (0.6 mm), so it was not possible to extract the plasma using a micro-pipette. Therefore, we calculated the amount of plasma from the length of the 9 μl capillary. There were excellent linear correlation between the amount of PB and length of the capillary at three different concentrations. Our results suggested that it was possible to calculate the plasma PB concentration from the length of the 9 μl capillary.

Clinically, PB is used in combination with other anticonvulsant drugs. In the present method using HPLC, PB was well separated from other anticonvulsant drugs. The retention times for zonisamide, phenytoin, nitrazepam, diazepam and clonazepam were 5.4, 20.0, 25.7, 30.4 and 31.9 min, respectively (data not shown). Therefore, using the present method, it was possible to determine the plasma PB concentrations in only 9 μl of blood obtained from rat offspring.

DeVane et al. [5] analyzed whole fetal PB and phenytoin concentrations using a method which required the fetuses to be killed. We measured plasma PB concentrations in rat offspring without killing. Offspring were very small (4–5 g), and 60 μl of blood (required in previous methods [6]) represents a large proportion of the total volume. Thus, sampling of very small amounts of blood is indispensable to measure drug concentrations in offspring. In the present study, offspring were divided into four groups of 3–4 pups from each dam. We collected blood specimens daily over a period of 11 days from delivery according to a rotation schedule to prevent multiple sampling from the same pup on consecutive dams. All offspring grew normally and there were no fatalities.

These results suggested that this method is very useful to determine the plasma PB concentration in newborn rat pups.

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