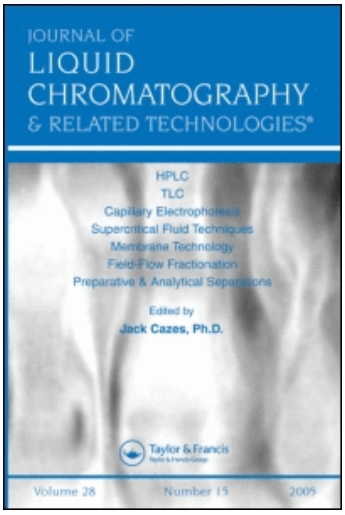


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# CHARACTERISTICS OF INTERACTION BETWEEN BARBITURATE DERIVATIVES AND VARIOUS SORBENTS ON LIQUID CHROMATOGRAPHY AND DETERMINATION OF PHENOBARBITAL IN JAPANESEHUMAN BREAST MILK

Ritsuko Shimoyama[a]; Tadashi Ohkubo[a]; Kazunobu Sugawara[a]
[a] Department of Pharmacy, Hirosaki University Hospital, Hirosaki, Japan

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# CHARACTERISTICS OF INTERACTION BETWEEN BARBITURATE DERIVATIVES AND VARIOUS SORBENTS ON LIQUID CHROMATOGRAPHY AND DETERMINATION OF PHENOBARBITAL IN JAPANESE HUMAN BREAST MILK

Ritsuko Shimoyama, Tadashi Ohkubo,* Kazunobu Sugawara

Department of Pharmacy
Hirosaki University Hospital
Hirosaki, 036 - 8563 Japan



## ABSTRACT

A high performance liquid chromatographic (HPLC) method was developed for the determination of phenobarbital in human breast milk and plasma. The chromatographic behaviors of phenobarbital and other barbiturate derivatives on various sorbents, including octadecyl ($C_{18}$), octyl ($C_8$), methyl ($C_1$), cyanopropyl (CN), phenyl (Ph), and dimethyl tert-butyl (tert-But), were investigated as stationary phases, and then chromatographic separation was achieved by $C_8$ analytical column using a potassium dihydrogenphosphate buffer (pH 3.5 for milk, pH 6.5 for plasma) / acetonitrile (70 : 30, v/v) as the mobile phase because interference peaks affected differently in each specimen. Phenobarbital and 5-methyl-phenobarbital as an internal standard were detected by ultraviolet detection method at 230 nm. Phenobarbital was extracted by a rapid and simple procedure based on $C_{18}$ bonded-solid phase extraction in breast milk and plasma.

Determination of phenobarbital in human breast milk and plasma was possible in the concentration range of 0.05 - 30μg/mL. The recoveries of phenobarbital added to human breast milk and plasma were 83.8 - 100.6 % and 95.6 - 102.0 %, respectively, with coefficient of variation of less than 6.6 % and 8.4 %.

This method was used for drug-level monitoring in human breast milk and plasma from patients who were being treated with phenobarbital. The mean concentration of phenobarbital in breast milk and plasma was 6.05 ± 1.2 μg/mL and 14.0 ± 9.0 μg/mL, respectively. The average ratio between the breast milk concentration versus plasma concentration (M/P ratio) was 0.35 ± 0.1.



## INTRODUCTION

Phenobarbital is frequently used as an antiepileptic drug in epileptic patients. Several studies have reported a high performance liquid chromatographic method for the determination of phenobarbital in plasma.[1-4] Previously reported HPLC methods for phenobarbital analysis[1-5] have used the octadecyl analytical column; however, we think that the octadecyl analytical column is not always suitable for analysis of phenobarbital because of the analytical time required and the lack of separation efficiency.

Similarly, octadecyl phase was used for analysis of other barbiturate derivatives by HPLC.[6-9] We could not find the HPLC analysis method of barbiturate derivatives using other columns. B. Slater et al.[10] discussed the correlation between partition coefficients by various measurement methods and HPLC methods, which were obtained from log k' values according to the following equation (log p = a log k'+ b).[10] It is suggested that the k' values of analytes were correlated with partition coefficient, which was the force of interaction between analytes and octadecyl stationary phase. However, there was no data about interaction of barbiturate derivatives with several other stationary phases.

We think that the cleared mechanism of barbiturate interaction with several stationary phases in HPLC column were determined to be a suitable condition in HPLC analysis of several barbiturate derivatives.

We are describing in this paper the use of this method. Characterization of barbiturate derivatives on the 6 kinds of stationary phases (namely, $C_{18}$, $C_8$, $C_1$, CN, Ph and tert-But) was examined by the effect of mobile phase pH on the k' values; then, we established a better chromatographic system than the previous method. We also discuss mechanisms of interaction between barbiturate derivatives and column stationary phases.

On the other hand, it is well known that antiepileptic drugs passing to infants via the breast milk of epileptic mothers can frequently cause undesirable effects.[11] However, these patients often must continue to take antiepileptic drugs during lactation for seizure control. It must be considered, therefore, when treating breast-feeding mothers for epilepsy, that the uptake of antiepileptic drugs by maternal milk may produce adverse effects on the infant, because the drug metabolic enzyme system of infants is not as developed as compared to that of adults.[11] Therefore, clinically relevant advice is needed about the risks and safety of breast-feeding by lactating mothers who are being treated with antiepileptic drugs.[11] Thus, the monitoring of drug concentrations in breast-feeding mothers is very important.

Reports concerning phenobarbital transition to the breast milk,[12,13] have shown that nursing by epileptic mothers being treated with phenobarbital can have a distinct influence on neonatal behavior, such as sedation or withdrawal symptoms. Therefore, therapeutic drug-level monitoring of phenobarbital in breast milk is required. However, there are very few studies in literature that have reported HPLC determination methods for phenobarbital in human breast milk.[13] Previous HPLC methods in plasma phenobarbital were time-consuming and require a tedious liquid-liquid extraction step of phenobarbital from plasma,[2] and involved sample preparation that requires protein precipitation by acetonitrile.[1,3,4] These protein-precipitation methods cause problems in column damage. Moriyama et al. have reported a solid phase extraction method of phenobarbital from plasma.[5] Previous HPLC methods for the determination of phenobarbital in plasma have not been applied to analysis of human breast milk.

In our previous paper, we described solid phase extraction methods for the determination of several drugs in human breast milk.[14-17] In the present paper, we describe a HPLC technique based on obtained our chromatographic system and using a solid phase extraction method for phenobarbital in human breast milk and plasma.

## EXPERIMENTAL

### Materials

Phenobarbital, 5-methyl-phenobarbital, hexobarbital, barbital, pento-barbital, and amobarbital were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The chemical structures of these compounds are shown in Figure 1. The Sep-Pak® $C_{18}$ cartridge was purchased from Waters Co. (Milford, Mass, USA). All solvents used were of HPLC grade (Wako Pure Chemical Industries, Osaka, Japan). All other reagents and chemicals were purchased from Wako Pure Chemical Industries or Nakarai Tesque (Kyoto, Japan).

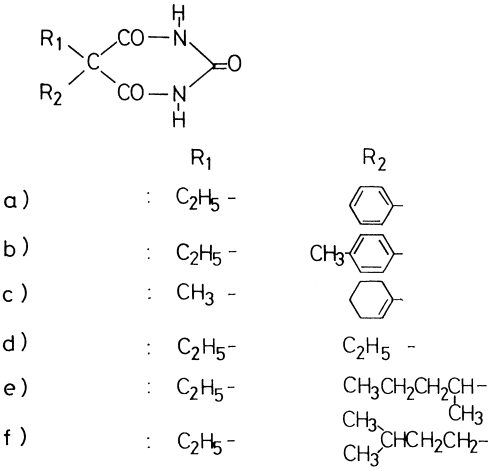


**Figure 1**. Chemical structures of barbiturate derivatives; a) phenobarbital, b) 5- methyl phenobarbital, c) hexobarbital, d) barbital, e) pentobarbital, f) amobarbital.



## Apparatus

The apparatus used for HPLC was a Model PU 4010 chromatographic pump (Philips, Pye Unicam Ltd., Cambridge, UK) equipped with a Pye Unicam PU 4020 ultraviolet detector. The wavelength was set at 230nm. Test samples were introduced using a Rheodyne Model 7125 injector (Reodyne, Inc., Cotati, CA, USA) with an effective volume of 100 µL. The HPLC column used Develosil $C_{18}$-5, Develosil $C_8$-5, Develosil Ph-5, and Develosil CN-5 stationary phases (5 µm, 150mm x 4.6mm I.D. Nomura Chemical, Seto, Japan); dimethyl tert-But and $C_1$ stationary phases were synthesized in our laboratories using Fine SIL100-5 (5 µm, JASCO). The analytical columns (150 mm x 4.6 mm I.D.) of two synthesized stationary phases were packed in these laboratories by a conventional high pressure slurry packing procedure. The mobile phase consisted of 0.5 % $KH_2PO_4$ - acetonitrile (70: 30, v/v). Before mixing, the pH of the mobile phase was adjusted with 50 % phosphoric acid and 2M potassium hydroxide. After mixing, it was degassed ultrasonically.

## Extraction Method

### *Breast Milk*

5-Methyl phenobarbital (300 ng for low range and 2 µg for high range) in methanol (10 µL) was added to the breast milk sample (1 mL) as internal stan-

dard, then the milk sample was diluted with 5 mL of 50 mM $KH_2PO_4$ (pH 3.0) and the solution was briefly mixed. The mixture was applied to a Sep-pak $C_{18}$ cartridge that had previously been activated with 5 mL of methanol and water. The cartridge was then washed with 5 mL of water and 5 mL of 20 % methanol in water. The desired fraction was eluted with 5 mL of 40 % methanol in water. The eluate was evaporated to dryness in vacuum at 60°C by a rotary evaporator (Iwaki, Tokyo, Japan). The residue was dissolved in 200 µL of mobile phase. The samples (100 µL) were injected into the HPLC apparatus.

***Plasma***

5-Methyl phenobarbital (300 ng for low range and 2 µg for high range) in methanol (10 µL) was added to the plasma sample (1 mL) as an internal standard, then the plasma samples were diluted with 5 mL of 1N hydrochloride and the solution was briefly mixed. The mixture was applied to a Sep-pak $C_{18}$ cartridge that had previously been activated as described above. The cartridge was then washed with 5 mL of water and 5 mL of 10 % methanol in water. The rest of the procedure was the same as described for breast milk.



**Phenobarbital Determination in Breast Milk and Plasma Samples**

The determinations of phenobarbital extracted from breast milk and plasma were carried out by Develosil $C_8$ analytical column using of 0.5 % $KH_2PO_4$ (pH 3.5) - acetonitrile (70: 30, v/v) and 0.5 % $KH_2PO_4$ (pH 6.5) - acetonitrile (70: 30, v/v), respectively. HPLC apparatus was set at 230 nm. A flow-rate of 1mL/min was used.

**Calibration Graphs**

Known amounts of phenobarbital in the range of 0.05 - 1 µg/mL at low concentration and 1- 10 µg /mL at high concentration were added to blank breast milk samples. Known amounts of phenobarbital in the range of 0.05 - 1 µg /mL at low concentration and 1 - 30 µg /mL at high concentration were added to blank plasma samples. These samples were treated according to the described procedure. Graphs were constructed of the peak-height ratio of phenobarbital to 5-methyl-phenobarbital as an internal standard, and plotted against the concentration of phenobarbital.

**Recovery and Assay Validation**

The spiked samples were prepared by adding 0.05, 0.2, 0.6, 2.0, 4.0, and 8.0 µg /mL phenobarbital to blank milk, and 0.05, 0.2, 0.6, 7.5, 12.5, and 25.0 µg /mL phenobarbital to blank plasma. Sample extraction and the subse-

quent HPLC technique were carried out as described above. Recovery was determined by comparing the peak heights from extracted samples with those obtained from evaporated drug in methanol without extraction. Within-day and between-day variability was assessed by extracting six spiked samples in the same day, or, repeatedly over several days in order to assures reproducibility of results.

### Sampling from Phenobarbital Treated Patients

Six breast milk samples and eight plasma samples were obtained from four patients who were being treated with phenobarbital. Blood samples were taken 2 - 3 hours after drug administration and breast milk samples were taken immediately before or after blood sampling. All of the women gave informed consent for the clinical examinations.



## RESULTS

The effects of the pH of the mobile phase on the capacity factors (k') of phenobarbital, 5-methyl-phenobarbital, hexobarbital, barbital, pentobarbital and amobarbital were studied using six kinds of stationary phase (Figure 2). In all these barbiturate derivatives, the k' values were not changed with pH in the range of 2.5 - 6.5 with 0.5 % $KH_2PO_4$: acetonitrile (70: 30, v/v). However, the k' values decreased at pH 7.5. Sufficient resolution between phenobarbital and the other five barbiturate derivatives was obtained on each of the six stationary phases. 5-Methyl-phenobarbital, hexobarbital, amobarbital, and pentobarbital separations were obtained but not satisfactorily. Low k' values and insufficient resolution were seen on the $C_1$, CN, Ph, and tert-But stationary phases for the six barbiturate derivatives. High k' values and sufficient resolution were obtained on the $C_8$ and $C_{18}$ stationary phase.

Sharper chromatogram peaks and better chromatogram quality were obtained on $C_8$ stationary phase than on $C_{18}$ stationary phase (Figure 3). Broader peaks were obtained on the chromatogram when $C_{18}$ stationary phase was used (Figure 3). Therefore, $C_8$ analytical column was used for analysis of phenobarbital and 5-methyl-phenobarbital as an internal standard in breast milk and plasma.

Typical chromatograms of blank breast milk and plasma and of the samples spiked with phenobarbital and 5-methyl-phenobarbital as an internal standard are shown in Figure 4. The separation of phenobarbital and internal standard obtained were satisfactory, and then the isolation of analysis peaks from endogenous interfering substance was established by our extraction method and chromatographic system using mobile phase of 0.5 % KH2PO4

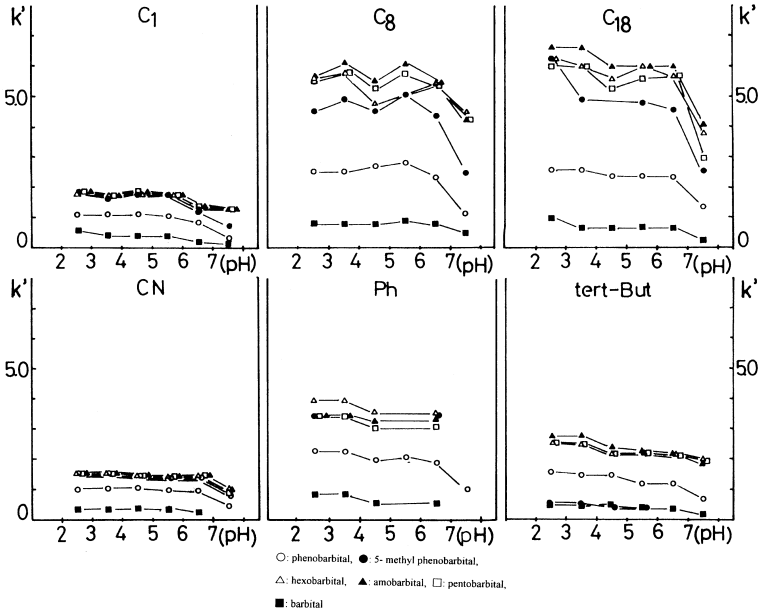


**Figure 2**. Effects of mobile phase pH on the k' values of barbiturate derivatives on six kinds of stationary phases.

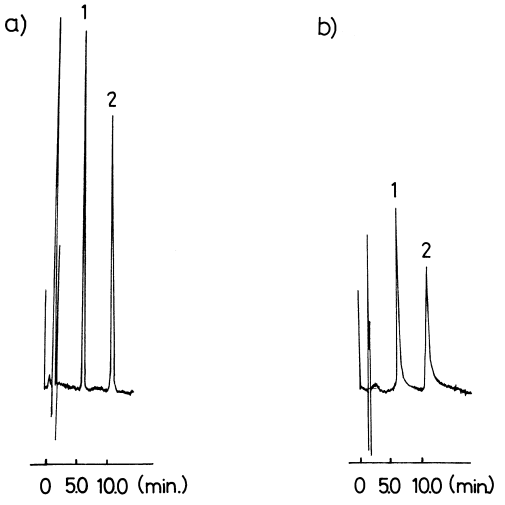


**Figure 3**. The typical chromatograms of phenobarbital and I.S. using of a a) C8 stationary phase and b) C18 stationary phase. peak 1: phenobarbital, peak 2: I.S. (5-methyl phenobarbital) mobile phase: 0.5% KH2PO4 (pH 6.5): CH3CN = 70: 30 (v/v).

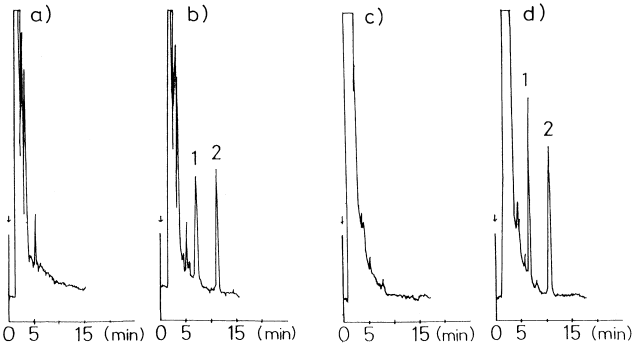


**Figure 4**. The typical chromatograms of a) blank breast milk, b) blank breast milk spiked phenobarbital and I.S., c) blank plasma, d) blank plasma spiked phenobarbital and I.S.peak 1: phenobarbital, peak 2: I.S. (5-methyl phenobarbital).



(pH 6.5): acetonitrile (70: 30, v/v) in breast milk and 0.5 % KH2PO4 (pH 3.5): acetonitrile (70: 30, v/v) in plasma.

Calibration graphs for phenobarbital in human breast milk and plasma were linear in the range of 0.05 - 10 μg /mL and 0.05 - 30 μg /mL, respectively. The limit of detection for phenobarbital was 0.01 μg /mL (signal- to- noise =5). The results of recovery are shown in Table 1. The recovery of phenobarbital was determined by adding the six known concentrations of 0.05, 0.2, 0.6, 2.0, 4.0, and 8.0 μg /mL to blank milk, and 0.05, 0.2, 0.6, 7.5, 12.5, and 25.0 μg /mL to blank plasma, respectively. The recovery values for phenobarbital were 83.8 - 100.6 % at concentration range of 0.05 - 8.0 μg/mL in breast milk and 95.6 - 102.0 % at concentration range of 0.05 - 25.0 μg/mL in plasma. Within-run and between-run assay variations of phenobarbital were less than 6.6 % in breast milk and less than 8.4 % in plasma. The accuracy and precision of the proposed method were defined from these results.

The concentration of phenobarbital in the breast milk and plasma samples from the patients being treated with phenobarbital were measured using our present method. The phenobarbital dosage and sampling times for the patients are shown in Table 2. The levels of phenobarbital in breast milk and plasma in the patients, who received 30 - 150 mg phenobarbital at three time in a day, were 6.05 ± 1.2 μg /mL (mean ± S.D., range of 4.5 - 7.6 μg /mL) and 14.0 - 9.0 μg /mL (mean ± S.D., range of 4.3 - 29.6 μg /mL), respectively. The cord blood plasma level was 3.4 μg /mL (Table 2). The milk versus plasma concentration ratios (M / P ratio) of phenobarbital were 0.35 - 0.1 (mean ± S.D., range of 0.23 ± 0.44). The level of phenobarbital in breast milk was lower than in plasma.

**Table 1**

**Accuracy and Precision of Determination of Phenobarbital in Breast Milk and Human Plasma***

| Added | Found (Mean ± SD) | Recovery (%) | CV(%) Within Assay | CV(%) Between Assay |
|---|---|---|---|---|
| **Human Breast Milk (μg/mL)** | | | | |
| 0.05 | 0.05 ± 0.003 | 99.6 | 2.4 | 5.7 |
| 0.2 | 0.17 ± 0.005 | 83.8 | 1.6 | 3.0 |
| 0.6 | 0.52 ± 0.023 | 85.8 | 1.6 | 4.4 |
| 2.0 | 1.98 ± 0.13 | 99.1 | 5.1 | 6.6 |
| 4.0 | 4.02 ± 0.24 | 100.6 | 1.6 | 6.1 |
| 8.0 | 7.60 ± 0.35 | 95.0 | 3.4 | 4.6 |
| **Human Plasma (μg/mL)** | | | | |
| 0.05 | 0.05 ± 0.003 | 102.0 | 4.6 | 6.6 |
| 0.2 | 0.19 ± 0.009 | 97.2 | 4.2 | 4.7 |
| 0.6 | 0.57 ± 0.029 | 95.6 | 3.2 | 4.9 |
| 7.5 | 7.19 ± 0.60 | 95.8 | 3.2 | 8.4 |
| 12.5 | 12.41 ± 0.70 | 99.3 | 3.3 | 5.6 |
| 25.0 | 24.79 ± 1.21 | 99.2 | 1.6 | 4.8 |

* (n = 6).



## DISCUSSION

Initially, our effort was directed towards developing an efficient chromatographic system for the analysis of barbiturate derivatives including phenobarbital. Suitable conditions for the efficient chromatography were examined in detail with six kinds of stationary phase which were $C_{18}$, $C_8$, $C_1$, CN, Ph, and tert-But analytical columns. The results of obtained k' values on several columns (Figure 2) suggest that the interaction between barbiturate derivatives and stationary phases mainly originated from the side chain moiety of the barbiturate derivatives. This is due to the fact that the k' values on $C_1$ and tert-But stationary phase were lower than $C_8$ and $C_{18}$. However, k' values of $C_8$ and $C_{18}$ columns were not directly proportional to carbon chain length on the stationary phase. The k' values were saturated on $C_8$ stationary phase. The peaks of bar-

**Table 2**

**Human Breast Milk and Human Plasma Levels of Phenobarbital in Patients**

| Case No, | Daily Dose (mg) | Day After Delivery (Day) | Milk (μg/mL) | Plasma (μg/mL) | Ratio (M/P) |
|---|---|---|---|---|---|
| 1 | 30 | --- | --- | 3.4 (cord blood) | --- |
| 1 | 30 | 1 | --- | 4.3 | --- |
| 1 | 30 | 2 | 4.5 | 10.3 | 0.44 |
| 1 | 30 | 5 | --- | 12.5 | --- |
| 2 | 150 | 2 | 7.6 | 29.6 | 0.26 |
| 2 | 150 | 5 | 5.6 | 24.0 | 0.23 |
| 3 | 150 | 3 | 7.0 | --- | --- |
| 4 | 120 | 3 | 5.2 | 12.8 | 0.41 |
| 4 | 120 | 7 | 6.4 | 15.1 | 0.42 |
| Mean ± S.D. | | | 6.05 ± 1.2 | 14.0 ± 9.0 | 0.35 ± 0.1 |



biturate derivatives were broadened on $C_{18}$ stationary phase, because the lipophilic interaction of barbiturate side chain may occur more strongly on $C_{18}$ stationary phase than $C_8$. The electrostatic dipoles of the CN moiety and π-electrons on the phenyl moiety of the stationary phase may disturb the lipophilic interaction of barbiturate side chains on the stationary phase. Thus, the low k' value of CN and Ph stationary phases were obtained in our present experiments.

The described results in previous papers[6-10] presented our results. The same results were obtained on octadecyl stationary phase in previous papers.[6-10] There was structural correlation between the driving force of interaction with the stationary phase and barbiturate structure depending on the lipophilic property of the side chain. For example, higher partition coefficients by the HPLC measurement method were shown in order of quinal-barbitone > pentobarbitone > mylobarbittone > butobarbitone > phenobarbitone.[10] These results suggest that the most important contribution factor for interaction with the stationary phase is the length and bulkiness of the side chain moiety in barbiturates. It can be generally considered that the driving force of interaction was increased according to the increasing of carbon number in the barbiturates side chain. However, branches, bulkiness and π electron rich unsaturated structure in side chain were decreased interaction force between barbiturate and stationary phase.

Several papers have described the HPLC method for the determination of phenobarbital in plasma samples using a $C_{18}$ stationary phase.[1-5] However, the analysis of barbiturate derivatives using a $C_{18}$ column causes the broadening of the peaks on the chromatogram (Figure 3). We think, from our present results, that the $C_{18}$ stationary phase is not necessarily suitable for the analysis of phenobarbital. The results of our present study suggested that a $C_8$ stationary phase was suitable for analysis of phenobarbital by HPLC, because sharper peaks of barbiturate derivatives were obtained on a $C_8$ stationary phase than on a $C_{18}$ phase (Figure 3). Therefore, a $C_8$ stationary phase was selected for analysis of phenobarbital in breast milk and plasma in our present study. The pH of mobile phase was maintained at pH 3.5 in breast milk and at pH 6.5 in plasma, because the interference peak appeared at the retention time of phenobarbital in the case of breast milk. Notably, many serious interference peaks appeared from breast milk when the pH of the mobile phase was higher than pH 3.5. Therefore, the effect of pH of the mobile phase on the k' values of the interference peaks was examined.

Interference peaks exhibited remarkably decreased k' values with de creasing pH value in the range 2.5 - 3.5 (data not shown). Fortunately, the k' value of barbiturate derivatives were not affected by the pH of the mobile phase, according to the results from our present examination. Therefore, we were able to established separation of the phenobarbital peak from interference peaks with a mobile phase of 0.5% $KH_2PO_4$ (pH 3.5): acetonitrile (70: 30, v/v) (Figure 4).

Previous reports of HPLC methods for phenobarbital determination have included several purification methods of plasma samples.[1-4] However, acetonitrile precipitation of plasma protein was used for sample clean up in these method.[1,3,4] These methods cause column damage, which originate from endogenous biological substances. Lu-Steffes et al.[2] described a liquid-liquid extraction method to clean up the samples. However, the two methods described require an additional procedure due to the high fat content of milk, relative to other biological fluids. If the samples precipitated or extracted by these methods were injected into the HPLC, the lipid globules would be partitioned strongly into the stationary phase and would need to be periodically flushed out with an organic solvent to prevent deterioration of the chromatography column. Therefore, there are very few analytical methods using a direct injection method for the assay of phenobarbital in breast milk.[18]

The HPLC determination of barbiturate derivatives in plasma by a $C_{18}$ solid phase extraction technique has been described in a previous paper.[5] On the other hand, in previous papers, we described an extraction method for several drugs in milk[14-17] using solid phases, which were $C_{18}$, $C_8$ and CN reversed stationary phase. In the present study, therefore, we established a solid phase extraction method using reversed stationary phase for the determination of phe-

nobarbital in human breast milk. By doing this, we achieved a highly efficient extraction in which there were no interference peaks from endogenous substances on the chromatogram.

The present assay method could accurately measure the concentration of phenobarbital in human breast milk over the range of 0.05 - 10.0 μg/mL and in plasma over the range of 0.05 - 30 μg/mL. The minimum quantifiable concentration was set at lower levels than are required in adults because we may need to measure lower drug concentrations from smaller amounts of plasma sample from infants of epileptic mothers in the near future. We have found that the present method for the determination of phenobarbital is rapid, reproducible, specific, and sensitive enough to allow simultaneous quantitation of phenobarbital in breast milk and plasma. We would, therefore, propose that this method is suitable for routine clinical monitoring of phenobarbital therapy during lactation.



The results of this study demonstrated that the M/P ratio of phenobarbital is nearly equal to that reported in previous papers.[12,13] It is suggested that our HPLC method has dependable efficiency for clinical therapeutic monitoring of phenobarbital in breast feeding patients. It is obvious that the alteration of M/P ratio needs to be taken into consideration when monitoring phenobarbital in milk postpartum. Likewise, the acceptable amount of milk, which would be safe for nursing infants who are being fed by phenobarbital treated mothers, should be decided by determining the phenobarbital level in each patient. According to our results, with regard to phenobarbital exposure during lactation, when an infant fed a normal lactation diet from a mother receiving standard phenobarbital treatment in Japan, a 4 kg infant taking 0.5 liter of milk per day would ingest approximately 2.25 - 3.80 mg (0.56 - 0.95 mg/kg) per day. It is considered that this amount of drug exposure probably does not have significant pharmacological effects on the infants, because the dose is lower than the therapeutic level (approximately 4 mg/kg/day). Since we establish a baseline for the acceptable amount of drug that an infant can safely ingest via milk during lactation, however, we should be monitoring the drug level in milk when phenobarbital is given to the patient during lactation. Clinical monitoring of drug concentrations in breast milk and the knowledge about the secretion and accumulation of drug in breast milk are very important for the prevention of adverse drug events in infants though intake of milk.

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