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Monitoring of phenytoin in human breast milk, maternal plasma and cord blood plasma by solid-phase extraction and liquid chromatography

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Abstract

A rapid liquid chromatographic method for the quantitation of phenytoin in human breast milk, maternal plasma and cord blood plasma was developed using a Develosil® C_8 5 μm reversed phase column and a potassium dihydrogen phosphate buffer/acetonitrile mobile phase. Phenytoin and mephenytoin as an internal standard were detected by ultraviolet absorbance at 240 nm. The sample preparation method involves a rapid and simple procedure based on solid-phase extraction using a C_{18}-bonded phase. Phenytoin could be determined in the concentration range of 0.05–3 μg ml^{-1}. The recovery of phenytoin added to human breast milk and plasma were 91.6–94.7 and 91.6–96.0%, respectively, with coefficient of variation less than 4.2 and 8.7%. The method has been used for drug level monitoring in the human breast milk, maternal plasma and cord blood plasma samples that were taken from patients treated with phenytoin. The average ratio between the breast milk concentration versus the plasma concentration was 0.28 ± 0.1, with a rather poor correlation ($r = 0.3033$). © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Phenytoin; Human breast milk; Plasma; Solid-phase extraction; HPLC

1. Introduction

Breast milk is well known as an optimal type of food for infant nutrition. The motivation of breast milk feeding has popularized in Japan. Generally, it is said that the breast milk feeding increases the mother's will to care, so the physicians encourage breast feeding. However, drug excretion in breast milk from plasma in the case where the mother is using drugs is a problem. Especially, the drugs used for chronic disease such as antiepileptic drugs should be continued throughout pregnancy and lactation [1]. There is little information in the literature about drug transfer into maternal breast milk [2]. As a result of the lower metabolic activity in infants, compared with adults, a number of negative effects have been reported in infants during lactation

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with drug containing breast milk. This means that the final dose, given to an infant, must be determined with respect to both the milk secretion rate and the disposition of the drug in the infant. Phenytoin is one of the most efficacious and widely prescribed anticonvulsants for the treatment of epilepsy. The association of phenytoin with teratological sequelae in animals and humans has been reviewed [3]. In the previous paper, methemoglobinemia in the infant causes an adverse effect on phenytoin was reported [4]. Therefore, the monitoring of phenytoin in maternal breast milk is necessary.

Phenytoin level in breast milk was reported in the previous papers [5–7]. Determination of phenytoin in Japanese breast milk is important because there is only one preliminary report [7].

Several gas-chromatographic (GC) methods have been described for the analysis of phenytoin in human breast milk [5–7]. However, high-performance liquid chromatographic determination of phenytoin in human breast milk has not been described. On the other hand, several high-performance liquid chromatographic methods have been described for the analysis of phenytoin in plasma using liquid–liquid extraction methods [8–11] and direct sample injection system [12–16]. However, these methods have not been applied to the analysis of human breast milk. Liquid–liquid extraction method is most widely used for the cleanup of drugs in milk. However, due to the high fat content of milk relative to other biological fluids, an aqueous back extraction of the organic phase is often necessary to remove the lipids. In our previous paper, we developed a solid-phase extraction method for the determination of several drugs in human breast milk [17–19]. However, there has been no previous paper describing the solid-phase extraction method of phenytoin in human milk. In this paper, we describe the development of a rapid and simple HPLC method of phenytoin in human breast milk and plasma. We then determined the level of phenytoin in breast milk and plasma samples taken from five patients. Additionally, we measured the cord blood plasma concentration of two patients.

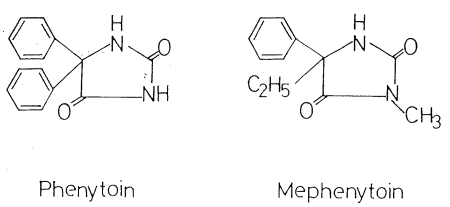

Fig. 1. Chemical structures of phenytoin and mephenytoin.

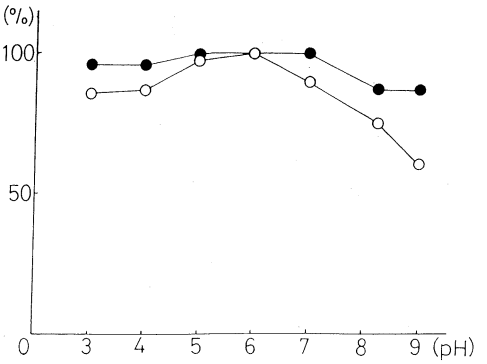

Fig. 2. Recovery profile of phenytoin and mephenytoin in human breast milk for Sep-Pak C_{18} cartridges using various pH as an applied solution. ○, Phenytoin, ●, Mephenytoin.

2. Experimental

2.1. Materials

Phenytoin (Fig. 1) was purchased from Sigma (St. Louis, MO) and mephenytoin (Fig. 1) was obtained from Sumika Chemical Analysis Service (Osaka, Japan). Sep-Pak® C_{18} cartridge was purchased from Waters (Milford, MA). The organic solvents used were of HPLC grade (Wako Pure Chemical Industries, Osaka, Japan). Other reagents and chemicals were purchased from Wako Pure Chemical or Nakarai Tesque (Kyoto, Japan).

2.2. Subjects

Five lactating women with a mean age of 28 years (range 27–31 years) and mean body weight of 59.6 ± 10.2 kg (range 50–70 kg) participated in the study. The patients received phenytoin, patients 2 and 4 received additional antipsychotic drugs during the lactation. The breast milk and

plasma samples were collected from all of the patients. All patients gave informed consents for the clinical drug monitoring. The problem of continuous sleeping was observed in the infant of patient 4 at bed time.

2.3. Drug analysis

The concentrations of phenytoin in human breast milk and plasma were measured by high-performance liquid chromatography with ultraviolet detection. The HPLC system was consisted of a Phillips, Pye Unicam PU 4010 pump with Pye Unicam PU 4020 UV detector at 254 nm (Pye Unicam, Cambridge, UK). The sample injector used was a Rheodyne model 7125 injector (Rheodyne, Cotati, CA). Phenytoin and mephenytoin (internal standard) was separated on a C_8 stationary phase column (Develosil C_8-5, 150 × 4.6 mm I.D., Nomura Chemicals, Seto, Japan). The mobile phase consisted of 0.5% potassium dihydrogen phosphate (pH 4.5)-acetonitrile (70:30, v/v). Before mixing, the pH of the mobile phase was adjusted with 50% phosphoric acid. The flow rate of the mobile phase was 1.0 ml min^{-1} at the ambient temperature.

2.4. Sample preparation

Methanolic solution of mephenytoin (800 ng 10 μl^{-1}) was added to the breast milk or plasma sample (1.0 ml) as an internal standard. The human breast milk and plasma samples were diluted with 5 ml of 0.5% potassium dihydrogenphosphate buffer (pH 6.0) and the sample solutions were briefly mixed. These mixtures were applied to a Sep-Pak® C_{18} cartridge, washed with 5 ml of water and 20% methanol. The desired fraction was eluted with 5 ml of 100% methanol. The eluate was evaporated and the residue was dissolved in 200 μl of mobile phase and then injected into HPLC.

2.5. Calibration graphs and recovery experiment

Known amounts of phenytoin 0.05–3 μg ml^{-1} were added to blank breast milk or plasma samples, respectively. These samples were treated according to the extraction procedure described above. The peak-height ratios of phenytoin to mephenytoin were measured and plotted against the concentration of analyte.

A recovery experiment was carried out using drug free breast milk or plasma spiked with 0.75, 1.50 and 2.50 ng ml^{-1} of phenytoin. These spiked samples were extracted by the above described method. The control samples were prepared from standard methanol solution and not extracted, but directly evaporated and reconstituted in mobile phase. Recoveries were determined by comparison between solid-phase extraction and not extracted control sample. The accuracy and precision of HPLC were established by the determination of within-day (intra-) and between-day (inter-) assay variance (percent of coefficient of variation, CV%).

3. Results

Fig. 2 shows the recovery of phenytoin from milk by using Sep-Pak® C_{18} cartridge for extraction and various pH values of the sample solution. The maximum extraction rate ($>90\%$) of phenytoin and mephenytoin were obtained at pH 6.0. The typical chromatograms of blank breast milk and plasma are shown in Fig. 3. The satisfactory separation of phenytoin and mephenytoin, and elimination of interfering peaks in biological matrix, were obtained. Calibration graphs for phenytoin in human breast milk and plasma were linear in the range of 0.05–3 μg ml^{-1}. The limit of detection for phenytoin was 0.025 μg ml^{-1} (signal-to-noise = 5). The results of recovery studies are shown in Table 1.

The recovery of phenytoin was determined by adding the three known concentrations of 0.75, 1.50 and 2.50 μg ml^{-1} to blank breast milk and plasma. The recovery values of phenytoin were 91.6–94.7% for breast milk and 91.6–96.0% for plasma at a concentration range of 0.75–2.5 μg ml^{-1}. Within-run and between-run assay variations of phenytoin were less than 4.2% in breast milk and less than 8.7% in plasma. The accuracy and precision of the proposed method were defined from these results.

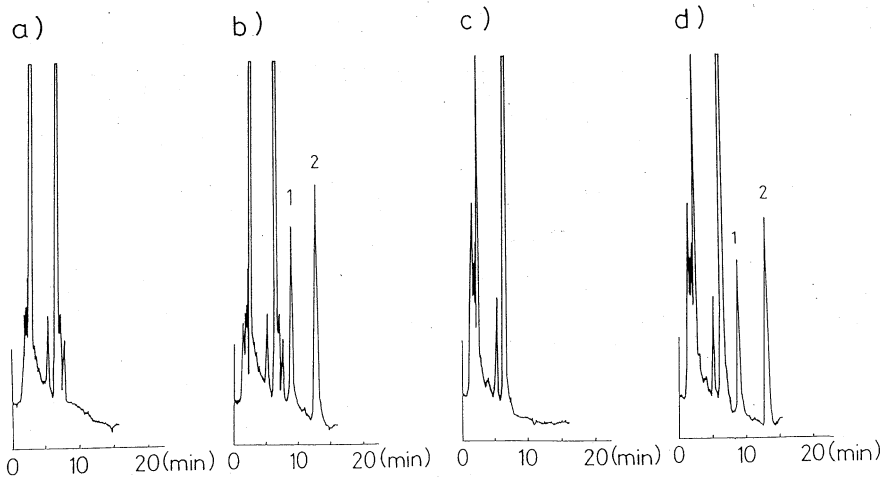

Fig. 3. Typical chromatogram of: (a) blank milk sample; (b) milk spiked with phenytoin; (c) blank plasma sample; and (d) plasma spiked with phenytoin. Peaks: 1 = phenytoin; 2 = mephenytoin.

The concentration of phenytoin in clinical breast milk and plasma samples from phenytoin treated patients were obtained. The dosing amounts of phenytoin for several patients are shown in Table 2. The levels of phenytoin are shown at a range of 0.41–1.30 μg ml^{-1} (mean ± S.D., 0.70 ± 0.28 μg ml^{-1}) in breast milk and a range of 1.60–3.40 μg ml^{-1} (mean ± S.D., 2.50 ± 0.66 μg ml^{-1}) in plasma after a dosage of 100 mg day^{-1} for patient 1 and 300 mg day^{-1} for the other patients. The cord blood plasma level was 1.12 and 2.64 μg ml^{-1} from two patients with a 300-mg day^{-1} dosage (Table 2). The milk versus plasma concentration ratios of phenytoin were obtained at range of 0.13–0.52 (mean ± S.D., 0.289 ± 0.113). The milk versus plasma ratio values were not correlated with the day after postpartum (Fig. 4). A good correlation of phenytoin level in breast milk and plasma was not obtained ($y = 0.1269X + 0.378$, $r = 0.3033$).

4. Discussion

Several investigators have clearly demonstrated that phenytoin is teratogenetic, causing cleft lip, cleft palate and other malformations. Mirkin [5] reported a high and widespread distribution of phenytoin in fetal tissues and noted congenital anomalies in three out of seven infants. The extent of the placental transfer of phenytoin is very important with respect to these effects. In the previous paper, Rane et al. [20] reported that the plasma level of phenytoin in neonates showed a rough correlation with the maternal plasma level. However, the cord blood plasma level may give a better correlation than the maternal plasma level. Therefore, the monitoring of the cord blood plasma level is useful for predicting the placental transfer of phenytoin, both cord blood levels examined were slightly lower than the maternal blood level. We also investigated an efficient extraction method for the measurement of phenytoin in human breast milk. The extraction method presented here is rapid, efficient and specific enough to eliminate interference peaks in breast milk. Previous methods of plasma phenytoin analysis by HPLC have used liquid–liquid extraction [8–11] and direct injection [12–16]. However, liquid–liquid extraction of milk requires periodical flushing with an organic solvent to prevent deterioration of the chromatography column. On the other hand, direct injection of milk into a liquid chromatography column only achieves a limited success [21]. Immunoaffinity sample clean up [12] and automated sequential trace enrichment of dialysate (ASTED) [14,16] have the same problem due to the high lipid content.

Table 1
Accuracy and precision of determination in human plasma and human breast milk ($n = 6$)

Phenytoin added (μg ml^{-1})	Found (μg ml^{-1}; Mean ± S.D.)	Recovery (%)	CV (%)	
			Between-day	Within-day
Human plasma				
0.75	0.69 ± 0.06	92.0	8.7	4.8
1.50	1.44 ± 0.08	96.0	5.6	3.0
2.50	2.29 ± 0.09	91.6	3.9	2.7
Human breast milk				
0.75	0.69 ± 0.01	92.0	1.5	1.1
1.50	1.42 ± 0.06	94.7	4.2	3.6
2.50	2.29 ± 0.09	91.6	3.9	2.0

In our previous papers [17–19], we described reversed solid-phase extraction methods for several drugs in milk. Rossi and Wright [21] commented on the advantages of our extraction methods in their review. However, reversed solid-phase extraction of phenytoin in milk was not previously reported. In the present study, we established a reversed solid-phase extraction method for phenytoin in human breast milk. Extraction was highly efficient and there were no interference peaks from endogenous substances in the chromatogram (Fig. 3).

The phenytoin concentration in the breast milk of our patients ranged from 13 to 52.0% (mean 28.9 ± 11.3%) of the corresponding maternal plasma concentration. In previous papers [6,7] the milk versus plasma ratio of phenytoin was reported to be 0.13 in six women and 0.18 in nine women. These ratios were lower than the our present result. On the other hand, Fleishaker et al. [22] found that the milk/plasma ratio of phenytoin was 0.336 in four healthy lactating women, a value that corresponds to our present data. They discussed various factors affecting the milk to plasma ratio in lactating women, but we could not explain the differences between the reported levels by their theories. We found that the correlation between milk and plasma phenytoin concentrations was not significant ($r = 0.3033$). In contrast, Steen et al. [6] described a good correlation between milk

Table 2
Human plasma and human breast milk level of phenytoin in patients

Patient no.	Dose (mg day^{-1})	Day since delivery (day)	Sampling time (h; after drug ingestion)	Plasma (μg ml^{-1})	Milk (μg ml^{-1})	Ratio
1	100.0	3	8.0	1.60	0.76	0.48
2	300.0	3	3.0	3.10	0.41	0.13
2	300.0	8	5.5	3.00	0.64	0.21
3	300.0	2	2.5	2.52	1.30	0.52
3	300.0	4	2.5	3.28	0.80	0.24
4[a]	300.0	0	5.5	2.64 (cord blood)	—	—
4	300.0	1	1.5	1.76	0.53	0.30
4	300.0	5	1.0	3.40	1.06	0.31
5	300.0	0	2.5	1.12 (cord blood)	—	—
5	300.0	3	2.5	2.12	0.54	0.25
5	300.0	6	1.5	1.66	0.52	0.31
5	300.0	14	2.5	2.28	0.41	0.18
5	300.0	30	1.5	2.76	0.68	0.25

[a] Sleeping baby.

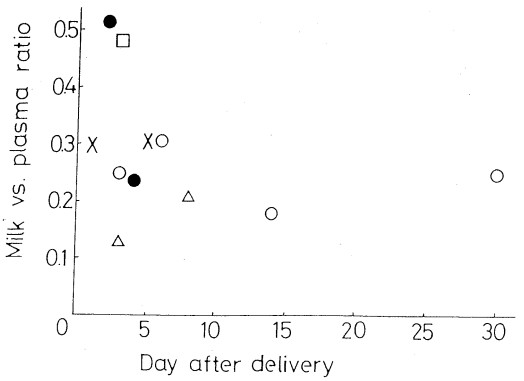

Fig. 4. The profile of milk versus plasma ratio after the day of the postpartum. △; patient 2, ●; patient 3, X; patient 4, ○; patient 5.

and plasma levels ($r = 0.97$). This difference may be based on variations in the sampling time. Our samples were obtained from hospitalized women and outpatients, so there was a variable time after drug ingestion and various periods after delivery. It is well known that the protein and fat content of milk changes rapidly in the postpartum period and this could lead to a change in the milk versus plasma ratio (Fig. 4). Syversen and Ratkje [23] have described the distribution of drugs in human milk.

The distribution of phenytoin in the lipid phase fat fraction in breast milk was not affected by pH over a range of 6.2–7.8, and phenytoin remained concentrated in the milk fat fraction. However, we could not predict the phenytoin level in breast milk, because a good correlation of the milk/plasma ratio was not obtained in our patients. Therefore, we concluded that the dose amount of phenytoin to nursed infants should be decided from the measurement of the drug level in each patients. The present study indicated that a 4-kg infant drinking 1 l of milk daily would ingest approximately 0.41–1.30 mg (0.10–0.33 mg kg^{-1}) of phenytoin when the Japanese standard dose was administered. Pharmacological effects of such drug doses are probably not significant in infants, because these doses are lower than the therapeutic dose range, However, we should monitor the drug level in milk when phenytoin is given to a patient during lactation in order to prevent adverse effects on the infant.

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