Plasma Levels of 25-Hydroxyvitamin D₂ and 25-Hydroxyvitamin D₃ in Maternal, Cord and Neonatal Blood¹

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Summary A high-performance liquid chromatographic method for simultaneous assay of 25-hydroxyvitamin D₂ (25-OH-D₂) and 25-hydroxyvitamin D₃ (25-OH-D₃) proposed in a previous paper (1) was applied to determine the plasma levels of the metabolites in perinatal and postnatal periods. The plasma samples of maternal, cord and neonatal blood were collected in summer and winter seasons. 25-Hydroxyvitamin D₃ was detected in all the samples. The plasma levels of the metabolite in mothers, cords and newborn infants (at life within 24 hr) in summer were 33.9 ± 12.5 , 18.9 ± 8.4 and 16.6 ± 6.4 (mean $\pm$ S.D.) ng/ml, respectively, while those in winter were 15.8 ± 6.6 , 8.8 ± 3.4 and 7.7 ± 3.2 ng/ml, respectively. The data in summer were significantly higher than the respective data in winter and there were high significant correlations between mothers and cords and between mothers and newborns. In both seasons, the plasma levels of mothers were about two times higher than the respective data of cords and newborns which were nearly identical with one another. The similar tendency was always observed in the individual data of mothers-cords-newborns pair samples. In this study, many plasma samples from mothers, cords and newborns were examined, but 25-OH-D₂ was detected in only few samples (6/41 for mothers, 3/36 for cords and 2/34 for newborns). However, the metabolite began to appear in all the samples during nursing with vitamin D₂-fortified dry milk to show 4.6 ± 1.3 and 4.8 ± 1.2 ng/ml in the summer and winter samples of neonates

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¹ Following abbreviations are used: 25-OH-D₂/D₃, 25-hydroxyvitamin D₂/D₃ (25-hydroxy-ergocalciferol/-cholecalciferol); HPLC, high-performance liquid chromatography; CPBA, competitive protein-binding assay.

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at life of 5–6 days, respectively. When the variation of plasma 25-OH-D₂ and 25-OH-D₃ levels was examined in postnatal periods until one month, the levels of exogenous 25-OH-D₂ was increased while those of endogenous 25-OH-D₃ was decreased. The sum of vitamin D₂ intake from fortified dry milk was highly significantly correlated with the plasma levels of 25-OH-D₂, which indicates that daily intake of exogenous vitamin D₂ is very important in nutrition during postnatal periods of bottle-fed infants.

Key Words cord, high-performance liquid chromatography, 25-hydroxyvitamin D₂, 25-hydroxyvitamin D₃, mother, neonate, plasma levels, perinatal period, postnatal period, vitamin D₂, vitamin D₃

It has been documented that newborn infants usually show low calcium plasma concentrations during the first 48 hr of life that gradually recover thereafter to show normal levels after about 10 days (1). Some authors (2–6) have reported that the vitamin D status of newborn infants directly depends on that of their mothers. Recently, the major circulating metabolites of vitamin D in blood have been ascertained to be 25-OH-D and its plasma concentration directly reflects the repletion status of vitamin D (7). These facts suggest that the vitamin D metabolism is one of the most important factors for calcium homeostasis in the early period of neonates. Therefore, it must be very important to determine the plasma levels of 25-OH-D in maternal, cord and neonatal blood for the studies of calcium homeostasis in early periods of neonates.

Hillman *et al.* (2) and Shimotsuji *et al.* (4) showed that there was a significant correlation between the plasma levels of 25-OH-D in maternal and cord blood by using CPBA methods for the determination of the metabolite. However, since their CPBA methods used were incapable of separating 25-OH-D₂ and 25-OH-D₃, their results were merely shown as the total amounts of 25-OH-D. Vitamin D₂ which shows practically the same physiological effects as vitamin D₃ with humans has been predominantly added to commercial drugs (*e.g.*, multivitamins preparations) and enriched foods (*e.g.*, fortified dry milk) because of its lower price than vitamin D₃ in Japan. Therefore, if such commercial drugs are given to pregnant females, both exogenous 25-OH-D₂ and endogenous 25-OH-D₃ derived from skin with sunlight exposure may usually be detected in their plasma and the mother's vitamin D status may directly reflect that of newborns. On the other hand, if pregnant females only receive sunlight exposure without administration of such drugs or foods, only endogenous 25-OH-D₃ may be detected in the plasma of mothers and newborns. Furthermore, if the newborns are nursed with commercial dry milk enriched by vitamin D₂, the exogenous 25-OH-D₂ from the milk may appear in their plasma with the endogenous 25-OH-D₃. Therefore, when separative assay of 25-OH-D₂ and 25-OH-D₃ is applied to the plasma in perinatal and postnatal periods, very interesting results must be obtained. From these viewpoints, our previously proposed method (8) was applied to examine the plasma levels of the metabolites in the respective periods.

EXPERIMENTAL

1. *Materials and reagents*

The materials and reagents described in the previous paper (8) were used.

2. *Plasma samples*

Plasma samples of maternal, cord and neonatal blood were collected from delivered mothers and their neonates. Permission for the study was obtained from the mothers.

Plasma samples of 17 pairs of mothers-cords-neonates collected in a summer season (1979.7–1979.9) and those of 12 pairs collected in a winter season (1978.12–1979.2) were studied. In addition, plasma samples which were not paired with each other were collected from another 7 mothers and 8 neonates in the summer season while from another 5 mothers and 10 neonates in the winter seasons. These samples were also studied. Maternal peripheral venous blood was collected in 2–24 hr before delivery. Plasma samples of cord venous blood were collected at the time of delivery. Neonatal peripheral venous blood was collected at life within 24 hr, at life of 5–6 days and at life till one month. All the gestational ages at birth were between 38 and 42 weeks. All the mothers and neonates were healthy and the body weight of the neonates was more than 2,500 g at birth. The neonates were nursed either with mother's breast milk together with fortified dry milk or with fortified dry milk alone. The plasma was separated and stored at -20°C until assayed.

3. *Determination of 25-OH-D₂ and 25-OH-D₃ in plasma*

Exactly 0.5 ml of plasma was placed in a test tube with a stopper, and then 25-OH-D₂ and 25-OH-D₃ were determined according to the procedure described in the previous paper (1).

4. *Determination of calcium in plasma*

Plasma was diluted with 0.2% strontium chloride solution to give a suitable concentration and then levels of calcium were determined with a Hitachi 508 atomic absorption spectrometer.

RESULTS AND DISCUSSIONS

1. *Profiles of the analytical HPLC on plasma samples of maternal, cord and neonatal blood*

Plasma samples obtained from maternal, cord and neonatal blood in a pair were subjected to the assay procedure of 25-OH-D₂ and 25-OH-D₃ according to EXPERIMENTAL. Figure 1 shows the typical profiles of the analytical HPLC on the 25-OH-D fractions isolated from the preparative HPLC of the plasma samples. The peak corresponding to 25-OH-D₃ was observed in all the chromatograms (Fig. 1a–1d), but the peak corresponding to 25-OH-D₂ was observed only in the chromatogram of the neonate at life of 5 days (Fig. 1d). Since the neonate was nursed with both mother's breast milk and fortified dry milk after birth, the peak of

Fig. 1. Typical profiles. Profiles of the analytical HPLC on the 25-OH-D fractions isolated from the preparative HPLC of the mother-cord-neonate pair plasma samples collected in summer. (a) Mother, (b) cord, (c) neonate (newborn infant at life within 24 hr), (d) neonate (at life of 5 days). *, 25-OH-D₂; **, 25-OH-D₃.

25-OH-D₂ might be derived from the metabolism of the vitamin D₂ added to the dry milk.

2. Assayed values of the plasma levels of 25-OH-D₂, 25-OH-D₃ and calcium in maternal, cord and neonatal blood

Plasma samples of maternal, cord and neonatal blood were collected in summer and winter seasons and then the levels of 25-OH-D₂, 25-OH-D₃ and calcium in the plasma samples were determined according to the procedures described in EXPERIMENTAL. 25-OH-D₃ was detected in all the samples, whereas 25-OH-D₂ was detected in few samples of mothers (6 in 41 samples), cords (3 in 36 samples) and newborns at life within 24 hr (2 in 34 samples). However, 25-OH-D₂ appeared in all the samples of neonates at life of 5–6 days, which suggested that the metabolites might be derived from fortified dry milk given during nursing.

Figure 2 shows the plasma levels of 25-OH-D₃ in all the samples and those of 25-OH-D₂ in the samples of neonates (5–6 days). The plasma levels of 25-OH-D₃ in mothers, cords and neonates (<24 hr) in summer were 33.9 ± 12.5 , 18.9 ± 8.4 and 16.6 ± 6.4 (mean $\pm$ S.D.) ng/ml, respectively, while those in winter were 15.8 ± 6.6 , 8.8 ± 3.4 and 7.7 ± 3.2 ng/ml, respectively. The data in summer were significantly higher than the respective data in winter ($p < 0.005$). These results suggested that abundant supply of sunlight in summer might enhance the photo-biogenesis of vitamin D₃ in mother's skin and that the mother's vitamin D status might directly reflect that of newborns (<24 hr). In both seasons, the plasma levels of 25-OH-D₃ of mothers were about two times higher than the respective data of cords and

Fig. 2. Plasma levels of 25-OH-D₂ and 25-OH-D₃ in maternal, cord and neonatal (at life within 24 hr and at life of 5-6 days) blood.

newborns (<24 hr) which were nearly identical with one another.

Tables 1 and 2 show the plasma levels of 25-OH-D₃, 25-OH-D₂ and calcium determined from 17 pair samples of mothers-cords-neonates in summer and 12 pairs in winter, respectively. When the individual plasma levels of 25-OH-D₃ of mothers in the pair samples were compared with the respective data of cords and newborns (<24 hr), the former were always higher than the latter as shown in Tables 1 and 2.

Shimotsuji *et al.* (4) also determined the plasma levels of 25-OH-D in pair samples of mothers-cords-neonates by using a CPBA method and reported that the mother's levels were higher than those of cords and newborns which were nearly identical with one another. Although the ratio (70-80%) of the averaged levels of cords and newborns to that of mothers was a little higher than our given ratio (45-55%) and some exceptions in the individual data of pair samples were observed, the tendency that the mother's levels were higher than those of cords and newborns was similar to our results. Hillman *et al.* (2) also reported the plasma levels of 25-OH-D in mothers-cords pairs and mentioned that the averaged ratio of cord levels to maternal levels was about 80% at maternal levels of 15-30 ng/ml while about 108% at maternal levels below 15 ng/ml. Although their results were different from our results at the low levels (The reason is not clear to us), a similar tendency to ours was observed at high levels.

Table 1. Assayed values of 25-OH-D₃, 25-OH-D₂ and calcium in plasma samples of maternal, cord and neonatal blood in pairs collected in a summer season (1979.7–1979.9).

Subject no.	Mother			Cord			Neonate (within 24 hr)			Neonate (5–6 days)		
	25-OH-D ₃ (ng/ml)	25-OH-D ₂ (ng/ml)	Ca (mg/dl)	25-OH-D ₃ (ng/ml)	25-OH-D ₂ (ng/ml)	Ca (mg/dl)	25-OH-D ₃ (ng/ml)	25-OH-D ₂ (ng/ml)	Ca (mg/dl)	25-OH-D ₃ (ng/ml)	25-OH-D ₂ (ng/ml)	Ca (mg/dl)
1	20.5	n.d.	9.3	14.5	n.d.	11.4	14.0	n.d.	8.8	13.3	5.5	8.8
2	48.2	n.d.	8.3	24.3	n.d.	8.5	22.5	n.d.	9.0	14.6	4.6	8.2
3	14.0	n.d.	7.9	10.7	n.d.	9.8	8.8	n.d.	8.1	7.4	5.5	8.5
4	40.0	n.d.	7.7	13.8	n.d.	9.4	13.6	n.d.	8.9	9.0	4.8	5.8
5	23.6	n.d.	7.7	10.7	n.d.	9.3	10.5	n.d.	9.5	4.5	3.3	6.6
6	27.9	n.d.	10.1	11.4	n.d.	10.2	11.9	n.d.	—	6.4	2.9	8.3
7	25.7	n.d.	10.2	11.4	n.d.	9.6	8.3	n.d.	—	6.2	3.1	8.9
8	40.9	n.d.	7.8	18.6	n.d.	9.2	21.9	n.d.	7.9	19.8	5.0	8.3
9	22.9	n.d.	7.8	13.3	n.d.	8.7	11.4	n.d.	7.8	10.5	3.6	7.7
10	50.7	n.d.	6.9	34.3	n.d.	8.8	32.4	n.d.	7.8	23.1	4.3	8.0
11	38.2	n.d.	7.8	23.6	n.d.	9.3	20.7	n.d.	8.4	21.0	3.6	8.1
12	25.7	n.d.	8.9	16.7	n.d.	11.4	14.8	n.d.	9.4	11.7	5.0	10.0
13	38.6	n.d.	9.8	25.5	n.d.	7.3	23.8	n.d.	—	16.7	6.2	—
14	36.8	n.d.	9.6	22.4	n.d.	10.9	21.2	n.d.	9.1	15.7	3.6	8.6
15	26.8	n.d.	9.0	21.1	n.d.	10.6	17.6	n.d.	10.6	13.8	5.5	9.5
16	34.6	n.d.	10.5	18.9	n.d.	11.0	13.6	n.d.	8.2	13.1	6.2	7.3
17	50.3	n.d.	8.5	25.7	n.d.	9.1	21.4	n.d.	7.8	17.6	6.2	7.3
M ± S.D.	33.3 ± 10.9	n.d.	8.7 ± 1.1	18.2 ± 6.7	n.d.	9.7 ± 1.1	16.5 ± 6.4	n.d.	8.7 ± 0.8	13.0 ± 5.5	4.5 ± 1.1	8.1 ± 1.0

n.d. = not detected (< 1 ng/ml). M ± S.D. = mean ± standard deviation. Note: The bars in the table mean that the calcium levels could not be assayed due to lack of samples.

Table 2. Assayed values of 25-OH-D₃, 25-OH-D₂ and calcium in plasma samples of maternal, cord and neonatal blood in pairs collected in a winter season (1978.12-1979.2).

Subject no.	Mother		Cord		Neonate (within 24 hr)			Neonate (5-6 days)		
	25-OH-D ₃ (ng/ml)	25-OH-D ₂ (ng/ml)	Ca (mg/dl)	25-OH-D ₃ (ng/ml)	25-OH-D ₂ (ng/ml)	Ca (mg/dl)	25-OH-D ₃ (ng/ml)	25-OH-D ₂ (ng/ml)	Ca (mg/dl)	25-OH-D ₃ (ng/ml)
1	15.8	n.d.	8.2	6.0	n.d.	10.4	6.6	n.d.	8.1	5.0
2	12.1	n.d.	8.3	7.0	n.d.	11.4	7.2	n.d.	9.4	4.9
3	31.8	n.d.	9.3	13.1	n.d.	10.9	11.1	n.d.	8.6	7.7
4	20.4	n.d.	10.0	7.5	n.d.	11.9	6.2	n.d.	9.0	4.5
5	22.8	n.d.	8.5	11.3	n.d.	11.1	10.0	n.d.	10.6	7.5
6	15.8	n.d.	9.0	7.7	n.d.	11.2	6.8	n.d.	—	5.1
7	8.2	n.d.	8.8	4.8	n.d.	8.4	4.4	n.d.	—	4.3
8	13.6	n.d.	5.5	6.4	n.d.	9.0	6.0	n.d.	—	5.5
9	9.5	n.d.	9.3	6.4	n.d.	10.5	3.3	n.d.	—	4.0
10	20.9	n.d.	9.4	13.9	n.d.	10.9	16.5	n.d.	9.0	12.8
M±S.D.	17.1 ±7.1	n.d.	8.6 ±1.2	8.4 ±3.2	n.d.	10.6 ±1.1	7.8 ±3.8	n.d.	9.1 ±0.8	6.1 ±2.7
										4.6 ±1.0
11	8.4	3.0	9.5	4.4	2.3	—	4.6	2.6	9.5	4.6
12	24.0	5.7	9.0	12.0	1.8	10.6	11.3	1.8	8.9	12.2
M	16.2	4.4	9.3	8.2	2.1	10.6	8.0	2.2	9.2	8.4
										3.9
										10.5

n.d. = not detected (<1 ng/ml). M = mean. S.D. = standard deviation. Note: The bars in the table mean that the calcium levels could not be assayed due to lack of samples.

3. Correlations of plasma 25-OH-D₃ levels between mothers and cords and between mothers and newborns

When all data of plasma 25-OH-D₃ levels in mothers were compared with those in cords and newborns (<24 hr), highly significant correlations were observed between mothers and cords and between mothers and newborns as shown in Figs. 3a and 3b, respectively. Hillman *et al.* (2) and Shimotsuji *et al.* (4) also reported similar correlations.

There was no significant correlation between the plasma levels of 25-OH-D₃ and calcium, in agreement with Shimotsuji *et al.* (4).

Fig. 3. Correlation between the 25-OH-D₃ plasma levels of mothers and cords and between those of mothers and newborn infants (at life within 24 hr). (a) Mothers vs. cords, (b) mothers vs. newborn infants.

4. Variation of plasma levels of 25-OH-D₂ and 25-OH-D₃ in postnatal periods

The fact that the proposed method is capable of separative assay of 25-OH-D₂ and 25-OH-D₃ is very convenient for this study. As mentioned in INTRODUCTION, when both 25-OH-D₂ and 25-OH-D₃ are detected in plasma samples of mothers, the former metabolite is thought to be derived from the exogenous vitamin D₂ in commercial drugs or fortified foods received in pregnant periods, while the latter metabolite is thought to be derived from the endogenous vitamin D₃ biosynthesized in their skin. Such vitamin D status of mothers may directly reflect that of newborn infants (<24 hr). As shown in Tables 1 and 2, the individual data on the pair samples of mothers-cords-newborns (<24 hr) clearly supported the considerations mentioned above. 25-OH-D₃ was detected in all the pair samples, while 25-OH-D₂ was detected only in the two pair samples (subject no. 11 and 12 in Table 2). As observed in the two pair samples, when 25-OH-D₂ was detected in the plasma of mothers, the metabolite was also detected in the plasma of the respective cords and newborns (<24 hr). The plasma levels of the metabolite in mothers were higher than the respective data in cords and newborns which were nearly identical with one another. The tendency that the former's levels were higher than the latter's was

Fig. 4. Variation of plasma levels of 25-OH-D₂ and 25-OH-D₃ in postnatal periods until the elapse of one month. The clear region and the shaded region in each bar show the levels of 25-OH-D₃ and 25-OH-D₂, respectively. The heights of the bars are the sum of 25-OH-D₃ and 25-OH-D₂ levels.

similar to that of 25-OH-D₃.

At the start of nursing with vitamin D₂-fortified dry milk, 25-OH-D₂ appeared in all the plasma samples of neonates (5–6 days) as shown in Tables 1, 2 and Fig. 2. These results strongly suggested that the newly appeared 25-OH-D₂ was the metabolite of the exogenous vitamin D₂ derived from the fortified dry milk. Then, we continued the observation on the variation of plasma levels of 25-OH-D₂ and 25-OH-D₃ in the postnatal periods until the elapse of one month. The results were divided into the summer and winter seasons as shown in Fig. 4. In both seasons, the levels of 25-OH-D₂ gradually increased while those of 25-OH-D₃ gradually decreased. The sum of vitamin D₂ intake from the fortified dry milk during nursing was highly significantly correlated with the plasma levels of 25-OH-D₂ as shown in Fig. 5. These results strongly suggest that daily intake of exogenous vitamin D₂ is very important in the vitamin D nutrition during postnatal periods of bottle-fed infants.

On the other hand, the plasma levels of 25-OH-D₃ were gradually decreased as shown in Fig. 4. Since the neonates were nursed in the hospital rooms and received little sunlight exposure during the nursing periods, the photo-biogenesis of endogenous vitamin D₃ in their own skin might have not yet begun at the nursing periods, and the decrease of 25-OH-D₃ levels might be due to gradual consumption of the endogenous vitamin D₃ and 25-OH-D₃ initially obtained from their mothers. However, most of the neonates in this study were nursed with fortified dry milk together with mother's breast milk which is known to contain mainly vitamin D₃ rather than vitamin D₂, and therefore the levels of 25-OH-D might be partly derived

Fig. 5. Correlation between sum of vitamin D₂ intake from fortified dry milk and plasma levels of 25-OH-D₂ in postnatal periods.

from the breast milk. The results shown in Fig. 4 suggested that the plasma levels of 25-OH-D might be more predominantly affected by large amounts of fortified dry milk than small amounts of breast milk taken by the neonates. In order to evaluate vitamin D nutrition on mother's breast milk more exactly, a similar study to this should be performed on neonates nursed with breast milk alone. At the same time, identification and determination of vitamin D, 25-OH-D, 1 α ,25-dihydroxyvitamin D and other metabolites in mother's breast milk should be also carried out. These aspects are now under investigation and will be reported in the future.

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