Current topic

Vitamin D and its metabolites in human breast milk

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It is rare for Western breast fed babies of mothers with adequate dietary vitamin D* to get rickets,¹ although there have been reports of decreased bone mineralisation² and a few cases of clinical rickets³ in breast fed infants without vitamin D supplementation. The relation between maternal vitamin D status and the concentration of vitamin D and its metabolites in the neonate at parturition has been shown with respect to 25-hydroxyvitamin D₃ (25-OHD₃) in humans,⁴ and sheep, and more recently in cows⁵ with respect to 25-OHD₃, 24, 25-dihydroxyvitamin D₃ (24,25(OH)₂D₃), and 25, 26-dihydroxyvitamin D₃ (25,26(OH)₂D₃). A direct relation has been shown between 25-OHD concentrations in maternal plasma and concentrations in the plasma of suckling infants, implying that metabolites of vitamin D can be transferred from mother to child in breast milk.⁶

Since maternal breast milk is a major source of vitamin D in unsupplemented breast fed infants, a number of studies have been carried out on the antirachitic activity of human breast milk (HBM). Attempts have also been made to correlate this activity with the concentration of vitamin D and its known metabolites. Early estimates of the concentration of vitamin D in HBM varied widely (4.2-90 IU/l (105-2250 ng/l)) and were based on the use of non-specific bioassays or colorimetric assays after saponification or organic solvent extraction, or both. Since HBM does not apparently contain adequate quantities of vitamin D, supplementation of HBM with 400 IU (10 000 ng) vitamin D/day has been recommended.⁷

By analogy with some steroid hormones that are found in urine conjugated to sulphuric acid, it has been suggested⁸ that vitamin D sulphate may be present in HBM. The sulphate conjugate which is

more water soluble is less likely to have been found in organic extracts and may therefore have been ignored in previous assays of vitamin D in HBM. In addition vitamin D sulphate has been synthesised⁹ and found in the urine of rats given large doses of ergocalciferol⁹ and cholecalciferol.¹⁰ In these studies HBM was extracted with ethanol, the 'sulphate' was precipitated with barium hydroxide, and saponified, after which the vitamin D was estimated by an antimony trichloride colour reaction, indicating a concentration of 950 IU/l (23 750 ng/l). Unesterified vitamin D in the lipid fraction was estimated as 15.7 IU/l (393 ng/l).⁸ In a subsequent investigation by the same group,¹¹ 'vitamin D sulphate' was measured by bioassay using a vitamin D deficient rat tibia healing method,¹² and a concentration of 780 IU/l (19 500 ng/l) was reported. Using the non-specific quantitation method and relying solely on barium hydroxide precipitation for the isolation of 'vitamin D sulphate' was unreliable since this method did not provide definitive identification of what was being measured.

Vitamin D sulphate was synthesised⁹ and was shown to separate clearly from vitamin D during thin layer chromatography (TLC)¹³ and on LH 20 columns.¹⁴ After ethanol extraction, and on this occasion insertion of a TLC separation of vitamin D sulphate, concentrations of 25 000 ng/l of vitamin D sulphate were reported in HBM.¹³ Characterisation of the vitamin D formed after solvolysis of the isolated vitamin D sulphate was carried out using gas liquid chromatography (GLC). The vitamin D sulphate was quantitated using a non-specific methylene blue colorimetric reaction used for the estimation of sulphates. These observations were subsequently independently confirmed,¹ but concentrations of the water soluble conjugate of vitamin D were lower than previously reported (10 000-17 000 ng/l (400-680 IU/l)), although the method was the same as that previously described

*When it is not required to distinguish between ergocalciferol (D₂) and cholecalciferol (D₃) the term vitamin D is used.

(using the antimony trichloride colour reaction for quantitation).⁸ This group¹ also reported that concentrations of vitamin D sulphate were highest in HBM collected within 3–5 days from the start of lactation and fell thereafter to a value that remained constant for up to 42 days of lactation.

Subsequent experiments by LeBoulch *et al.* using the LH 20 separation method¹⁴ and radioactively labelled vitamin D and vitamin D sulphate suggested that mammary glands of lactating rats contained vitamin D sulphate which could be hydrolysed by newborn suckling pups.^{15–17} Despite the wealth of published material suggesting an important role for vitamin D sulphate, what is clear from a modern analytical point of view is that all the work on 'vitamin D sulphate' up to this point in 1980 relied on relatively non-specific colorimetric reactions and simple separation techniques. In none of the published work had the so called 'vitamin D sulphate' been unequivocally characterised and it was far from clear that the more polar peak that separated from vitamin D on TLC or LH 20 was actually vitamin D sulphate at all.

In recent years with the application of more sophisticated separation techniques and specific methods of quantitation, the previously reported work on the presence of large amounts of vitamin D sulphate in HBM has not been confirmed. A preliminary report¹⁸ using high pressure liquid chromatography (HPLC), subsequently confirmed in a full report,¹⁹ failed to detect vitamin D sulphate in whey from HBM. Added tritiated vitamin D sulphate put through the isolation procedure was recovered (77.6%) after the final HPLC stage. Added unlabelled vitamin D sulphate could be detected but no vitamin D sulphate was found in concentrations greater than 1000 ng/l (40 IU/l) in the milk whey from HBM collected from 6 women, 1–8 days postpartum (that is, the time according to previous reports that vitamin D sulphate was at its

maximum concentration¹). In addition Leerbeck and Sondergaard²⁰ failed to show any vitamin D activity after saponification of the water soluble fractions prepared as described previously,^{8,13} although the saponification procedure used may have been destructive. Other workers, however, (Makin *et al.* unpublished data) using HPLC, chemical modification, and gas chromatography mass spectrometry have also failed to detect vitamin D sulphate at values in excess of 500 ng/l (20 IU/l) in any of the samples of HBM examined.

LeBoulch *et al.*²¹ recently published a reappraisal of their previous work using HPLC showing that there was vitamin D sulphate in a pooled extract from 3 litres of HBM. Unfortunately no quantitative data was given. A published report using HPLC²² had given values for vitamin D sulphate of 720–880 IU/l (18 000–22 000 ng/l), but this method used a reverse phase HPLC system for separation in which the vitamin D sulphate was eluted from the HPLC column at a retention time of approximately 1 m after injection—a point where impurities from the solvent injection and non-specific polar material from the extract also elute. In addition no evidence was given for the identity of the peak used for quantitation. It is therefore becoming clear, on the evidence so far published, that vitamin D sulphate is not present in HBM in concentrations above 500 ng/l (20 IU/l) and that previous reports of the presence of large quantities of a water soluble conjugate of vitamin D in HBM were erroneous.

Vitamin D sulphate has been synthesised by 2 independent groups^{23,24} by 2 different chemical procedures. After careful characterisation by mass spectrometry, nuclear magnetic resonance, infrared and ultraviolet spectroscopy, and HPLC it was shown, using a number of biological assays—intestinal calcium transport, bone calcium mobilisation, and calcification of epiphyseal plates—that vitamin D sulphate had no antirachitic activity in

Table Vitamin D metabolite concentrations in human breast milk (ng/l)

Metabolite	Greer <i>et al.</i> ²⁵	Hollis <i>et al.</i> ^{26*}	Reeve <i>et al.</i> ²⁷	Hollis ^{28**}	
Ergocalciferol		40	41	51	43
Cholecalciferol			338	115	2800
25-OHD2	ND(<500)	311	ND(<20)*	101	131
25-OHD3			163	151	203
24,25(OH)2D		52	ND(<21)		
25,26(OH)2D		30			
1,25(OH)2D	ND(<3)	5.2	ND(<0.6)		

*Mean of values reported for whole milk and milk whey; **mean concentration before (left column) and after (right column) ultraviolet irradiation of mother.

ND = not detectable above concentration (ng/l) given in parenthesis.

25-OHD2 = 25-hydroxyvitamin D2; 25-OHD3 = 25 hydroxyvitamin D3; 24,25(OH)2D = 24,25 dihydroxyvitamin D; 25,26(OH)2D = 25,26 dihydroxyvitamin D; 1,25(OH)2D = 1 α -25 dihydroxyvitamin D.

rats. Large oral doses of vitamin D sulphate given to vitamin D deficient rats caused only a small increment in the values of 25-OHD.²⁴

Analysis of HBM for the presence of non-conjugated metabolites has produced equivocal results. The Table summarises the recent values reported by 3 groups.²⁵⁻²⁸ From the results of Hollis *et al.*,²⁶ Reeve *et al.*²⁷ and Hollis²⁸ it is clear that even though the concentrations of vitamin D do not exceed 15 IU/l, the values of 25-OHD, which is a more potent antirachitic agent than vitamin D itself, are sufficiently high to provide an extra 30-60 IU/l, giving a total antirachitic activity for these 2 metabolites alone of some 45-75 IU/l, depending upon the antirachitic value ascribed to 25-OHD.²⁷ As may be expected, the concentration of 1 α , 25-dihydroxyvitamin D (1,25(OH)₂D) in HBM is shown to be very low²⁶ or undetectable.^{25, 28} Hollis²⁸ has recently published an improved analytical procedure for the estimation of ergocalciferol, cholecalciferol, 25-OHD₂, and 25-OHD₃ in HBM and has shown that oral administration of ergocalciferol to, or ultraviolet irradiation of the mother increases the concentration of the appropriate vitamin D and its 25-hydroxy metabolite in the milk. Ultraviolet irradiation increased the mean cholecalciferol concentration by a factor of 200 (see Table).

Some points, however, remain to be made. Although it has been shown that synthetic vitamin D sulphate has no biological activity when fed to vitamin D deficient rats, it has not been shown that a similar situation pertains in humans. It is also possible that breast fed infants do not require vitamin D to absorb calcium in a similar fashion to that described in suckling rat pups,²⁹ where intestinal 1,25(OH)₂D receptors were absent until weaning. It must also be noted that vitamin D sulphate is unstable and it may therefore be necessary to examine fresh HBM to detect this conjugate. Neither Hollis *et al.*^{18, 19} nor Makin, Seamark and Trafford (unpublished data) were able to detect vitamin D sulphate in HBM in concentrations in excess of 1 ng/ml and 0.5 ng/ml respectively. Assuming these reports are correct and assuming a recovery of 50%, the extraction of 3 litres HBM²¹ would yield around 500 ng of vitamin D sulphate which could easily be detected in the HPLC and ultraviolet systems used.

Paediatricians should therefore be aware that the suggestion that large quantities of vitamin D sulphate are present in HBM is largely a myth³⁰ and that while vitamin D sulphate may be present in HBM in small concentrations, unless hydrolysed to vitamin D it probably contributes nothing to the total antirachitic activity of HBM. There is no evidence to

support the presence of appreciable amounts of water soluble vitamin D in HBM and thus the question of whether or not HBM needs to be supplemented so that breast fed children received the recommended 400 IU of vitamin D per day still needs to be considered.

Further work needs to be carried out to obtain more data on the precise concentrations of conjugated and unconjugated vitamin D metabolites present in HBM, to confirm the absence of any hitherto unknown or ignored antirachitic factor, and thus to provide the information necessary to decide whether HBM alone provides sufficient antirachitic activity to prevent rickets in all or some breast fed infants. If the data given in the Table are correct and no further antirachitic activity can be ascribed to HBM, infants must consume around 8 l of HBM per day (7-14 times the normal daily intake) to reach the recommended daily intake of 400 IU. It has been suggested that the incidence of nutritional rickets is higher than has been supposed,³¹ and Finberg³² has recommended daily supplementation of HBM with 400 IU of vitamin D, observing that this level of supplementation is relatively inexpensive, without risk, and convenient. The question of whether or not to supplement HBM is still controversial and the proof that vitamin D sulphate is not present in appreciable amounts in HBM has not resolved this controversy. It is probably fair to say that term infants of mothers with adequate dietary vitamin D can survive on unsupplemented HBM without considerable deficiency in bone mineralisation. There may, however, be a case for giving vitamin D supplements to children of mothers without adequate dietary vitamin D, preterm infants, infants with pigmented skin, and those from deprived social backgrounds.

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References

- 1 Lakdawala DR, Widdowson EM. Vitamin-D in human milk. *Lancet* 1977;i:167-8.
- 2 Greer FR, Searcy JE, Levin RS, Steichen JJ, Steichen-Asche PS, Tsang RC. Bone mineral content and serum 25-hydroxyvitamin D concentrations in breast-fed infants with and without supplemental vitamin D: one-year follow-up. *J Pediatr* 1982;100:919-22.
- 3 Little JA. Water-soluble vitamin D in human milk (letter). *Pediatrics* 1982;70:499.
- 4 Hillman LS, Haddad JG. Human perinatal vitamin D metabolism. I. 25-hydroxyvitamin D in maternal and cord blood. *J Pediatr* 1974;84:742-9.
- 5 Goff JP, Horst RL, Littledike ET. Effect of the maternal vitamin D status at parturition on the vitamin D status of the neonatal calf. *J Nutr* 1982;112:1387-93.

- ⁶ Rothberg AD, Pettifor JM, Cohen DF, Sonnen-decker EWW, Ross FP. Maternal-infant vitamin D relationships during breast-feeding. *J Pediatr* 1982;**101**: 500-3.
- ⁷ Committee on Nutrition; American Academy of Pediatrics Committee. Nutrition and lactation. *Pediatrics* 1981;**68**:435-43.
- ⁸ Sahashi Y, Suzuki T, Higaki M, Asano T. Metabolism of vitamin D in animals. V. Isolation of vitamin D sulfate from mammalian milk. *J Vitaminol* 1967;**13**:33-6.
- ⁹ Higaki M, Takahashi M, Suzuki T, Sahashi Y. Metabolic activities of vitamin D in animals. III. Biogenesis of vitamin D sulfate in animal tissues. *J Vitaminol* 1965;**11**: 261-5.
- ¹⁰ Le Boulch N, Marnay-Gulat C. Elimination urinaire du cholecalciferol sous forme d'ester sulfurique chez le rat. *Biochimie* 1971;**53**:1219-20.
- ¹¹ Sahashi Y, Suzuki T, Higaki M, Asano T. Antirachitic potency of vitamin D sulfate in human milk. *J Vitaminol* 1969;**15**:78-82.
- ¹² Bourdillon RB, Bruce HM, Fischmann C, Webster TA. *The quantitative estimation of vitamin D by radiography*. Special Report Series No 158. London: Medical Research Council, 1931.
- ¹³ Le Boulch N, Gulat-Marnay C, Raoul Y. Dérivés de la vitamine D3 des laits de femme et de vache: ester sulfate de cholecalciférol et hydroxy-25 cholecalciférol. *Int J Vitam Nutr Res* 1974;**44**:167-79.
- ¹⁴ Sorgue Y, Miravet L. Chromatographic separation of vitamin D3 sulfate and vitamin D3. *Steroids* 1978;**31**: 653-60.
- ¹⁵ Le Boulch N, Marnay-Gulat C, Laromiguiere M, Miravet L, Raoul Y. Répartition et évolution de la vitamine D3 sulfoconjuguée chez la ratte gestante ou allaitante. *C R Acad Sci Paris* 1979;**288**:409-12.
- ¹⁶ Le Boulch N, Laromiguiere M, Marnay-Gulat C, Miravet L, Raoul Y. Vitamin D3 sulfate in lactating normally fed rats and suckling pups after maternal administration of 3H vitamin D3 or 35S vitamin D3 sulfate. In: Norman AW, Schaefer K, Herrath DV, et al., eds. *Vitamin D basic research and its clinical application*. Berlin: De Gruyter, 1979: 85-8.
- ¹⁷ Le Boulch N, Gulat-Marnay C, Miravet L, Laromiguiere M, Raoul Y. Vitamin D3 sulfoconjugate in pregnant and lactating mother rats after dosing with 3H vitamin D3. *Steroids* 1980;**36**:21-6.
- ¹⁸ Hollis BW, Lambert PW, Draper RR. On the occurrence of vitamin D sulfate in milk. *J Nutr* 1980;**109**:24.
- ¹⁹ Hollis BW, Roos BA, Draper HH, Lambert PW. Occurrence of vitamin D sulfate in human milk whey. *J Nutr* 1981;**111**:384-90.
- ²⁰ Leerbeck E, Sondergaard H. The total content of vitamin D in human milk and cows' milk. *Br J Nutr* 1980;**44**:7-12.
- ²¹ Le Boulch N, Cancela L, Miravet L. Cholecalciferol sulfate identification in human milk by HPLC. *Steroids* 1982;**39**:391-7.
- ²² Asano T, Hasegawa T, Suzuki K, Masushige S, Nose T, Suzuki T. Determination of vitamin D3-sulfate in milk by high performance liquid chromatography. *Nutr Rep Int* 1981;**24**:451-7.
- ²³ Reeve LE, DeLuca HF, Schnoes HK. Synthesis and biological activity of vitamin D3-sulfate. *J Biol Chem* 1981;**256**:823-6.
- ²⁴ Nagubandi S, Londowski JM, Bollman S, Tietz P, Kumar R. Synthesis and biological activity of vitamin D3 3 β -sulfate. *J Biol Chem* 1981;**256**:5536-9.
- ²⁵ Greer FR, Ho M, Dodson D, Tsang RC. Lack of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D in human milk. *J Pediatr* 1981;**99**:233-5.
- ²⁶ Hollis BW, Roos BA, Draper HH, Lambert PW. Vitamin D and its metabolites in human and bovine milk. *J Nutr* 1981;**111**:1240-8.
- ²⁷ Reeve LE, Chesney RW, DeLuca HF. Vitamin D of human milk: identification of biologically active forms. *Am J Clin Nutr* 1982;**36**:122-6.
- ²⁸ Hollis B. Improved quantitation of vitamin D2, vitamin D3, 25-hydroxyvitamin D2, and 25-hydroxyvitamin D3 in human milk. *Analyt Biochem* 1983;**131**:211-19.
- ²⁹ DeLuca HF, Franceschi RT, Halloran BP, Massaro ER. Molecular events involved in 1,25-dihydroxyvitamin D3 stimulation of intestinal calcium transport. *Fed Proc* 1982;**41**:66-71.
- ³⁰ Greer FR, Reeve LE, Chesney RW, DeLuca HF. Water-soluble vitamin D in human milk: a myth. *Pediatrics* 1982;**69**:238.
- ³¹ Rudolf M, Arulanantham K, Greenstein RM. Un-suspected nutritional rickets. *Pediatrics* 1980;**66**:72-6.
- ³² Finberg L. Human feeding and vitamin D supplementation—1981. *J Pediatr* 1981;**99**:228-9.

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