Aqueous solubilisation of vitamin D₃ in normal man

M RAUTUREAU AND J C RAMBAUD

with the technical assistance of Mrs A Bisalli, and M Rongier

From the Unité de Recherches sur la Physiopathologie de la Digestion, INSERM U. 54, Hôpital Saint-Lazare, Paris, France

SUMMARY Jejunal aqueous solubilisation of vitamin D₃ was assessed in eight normal subjects, after ingestion of a standard liquid test meal. Percentages and total concentrations of vitamin D₃ in the aqueous phase were significantly higher in the first post-prandial 30 minutes than during the following two hours, as were bile salts, total lipids, and free fatty acids. As shown by partial correlation analysis, a statistically significant relationship was found between aqueous concentrations of free fatty acids and of vitamin D₃ in the jejunal content during all the 2½ hours of the study. From these data it is concluded that, in healthy man, vitamin D₃ is solubilised *in vivo* in mixed micelles only, and is governed in the aqueous phase.

Intestinal absorption of vitamin D₃ includes three steps: the intraluminal aqueous phase, the enterocyte phase, and the entry into mesenteric lymph. Most studies have been devoted to the second and the third steps, but information regarding the first step is not available. Some indirect evidence has been reported¹ that dietary fats may enhance vitamin D₃ absorption and one of the possible mechanisms of this would be the formation of mixed micelles in the intestinal lumen, which would increase the aqueous solubility of vitamin D₃.²

Vitamin D₃, like cholesterol, belongs to the class I insoluble non-swelling polar lipids, which are poorly solubilised in bile salt micelle solutions.³ However, the actual aqueous solubilisation, *in vivo*, of vitamin D₃ has not been studied in man. The purpose of the present study was to determine, in healthy subjects receiving a standard homogenised liquid test meal, whether aqueous solubilisation of vitamin D₃ in the jejunal lumen depends primarily on bile salt or lipid in the aqueous phase.

Methods

SUBJECTS AND EXPERIMENTAL PROCEDURE

Eight subjects (21-64 years old, six males and two females) were studied who were without gastrointestinal, pancreatic, hepatic, or bile duct disease. Their daily diet contained 60-100 g fat. All were inpatients, and all gave informed consent. The evening before the study, a radio-opaque double

lumen tube was passed and the subject fasted until the next morning. The tips of the tube were then positioned under fluoroscopic control in the stomach and in the first jejunal loop respectively.

A standard test meal consisting of 30 g corn oil, 25 g milk protein powder, 50 g D-glucose, 12 µCi = 0.78 nmol synthetic (1α, 2α (n) 3H) vitamin D₃ (³H-D₃) (98% purity, Amersham, England) 20 nmol unlabelled vitamin D₃ and adjusted to a total volume of 400 ml with water, was emulsified in a blender and quickly infused into the stomach. Jejunal contents were collected by siphonage every two minutes. An aliquot of each two minute sample was heated at 70°C for 10 minutes to inactivate lipase, and then maintained at 37°C. When vitamin D₃ is heated to 80°C, 78% remains in the same molecular form, and 22% is isomerised to previtamin D₃.⁴ The 15 two minute inactivated aliquots obtained in each ½ hour period were pooled. Collections were continued for 2½ hours. Samples of each ½ hour period were centrifuged at 100 000 g for 18 hours at 37°C to separate the aqueous phase. The entire aqueous phase was recovered by aspiration through the side wall of the polycarbonate centrifuge tube, near the sediment. Other samples of each ½ hour period, taken after thorough mixing, were not centrifuged and represented the total phase of the jejunal content. Assays of bile salts, free fatty acids, total lipids, and ³H-D₃ were performed in the aqueous and total phases.

TECHNIQUES

Lipids were extracted using the system of Blanken-

Table Concentrations of bile salts, lipids, free fatty acids, and vitamin 3H-D₃ in total and aqueous phases

Periods of sampling (min)	Bile salts (mmol/l)		Lipids (g/l)		Fatty acids (mmol/l)		Vitamin ³ H-D ₃ (mmol/l)	
	Total	Aqueous	Total	Aqueous	Total free	Aqueous	Total	Aqueous
1 0-30	16.18±3.08*	14.90±3.27*	8.32±1.7	4.75±1.09*	13.84±2.42†	8.08±1.81	13.22±2.64	8.67±1.82*
2 30-60	7.77±1.12	6.45±1.06	4.34±1.51	1.9 ±0.59	7.95±2.78	3.12±1.01	6.73±1.95	3.63±1.07
3 60-90	5.88±1.09	4.97±0.89	2.18±0.7	0.75±0.18	4.37±1.14	1.33±0.30	3.21±0.75	1.74±0.43
4 90-120	6.67±0.94	5.45±1.06	4.41±2.11	1.15±0.54	7.16±2.94	1.87±0.89	6.33±3.0	2.37±0.95
5 120-150	6.6 ±0.63	5.15±0.41	6.02±3.03	1.33±0.53	8.67±3.87	2.21±0.91	9.8 ±6.07	2.86±1.29
Mean n=40	8.62±2.06	7.38±2.08	5.05±0.89	1.98±0.36	8.40±1.26	3.32±0.61	7.86±1.53	3.85±0.64
Analysis of variance	F=7.69 P<0.001	F=8.44 P<0.001	F=2.02 NS	F=10.68 P<0.001	F=3.02 P<0.05	F=12.91 P<0.001	F=2.10 NS	F=12.43 P<0.001

Newman-Keuls test * > 1, 2, 3, 4, 5 P < 0.01.

† > 1, 3 P < 0.05.

Results are given as mean ± SEM (n = 13).

horn and Ahrens⁵ and measured gravimetrically. Free fatty acids were estimated by Dole's technique⁶; Bile salts were separated by thin layer chromatography after successive passages in three solvent systems.⁷⁻⁹ The four main fractions isolated: taurocholic acid (TC), taurochenodeoxycholic acid (TCD)+taurodeoxycholic acid (TDC), glycocholic acid (GC); glycochenodeoxycholic acid (GCD)+glycodeoxycholic acid (GDC) were eluted with methanol and estimated fluorimetrically¹⁰ with 3 α -hydroxysteroid-dehydrogenase¹¹ by comparison with a standard sample obtained in the same technical conditions. In two subjects, monoglycerides were separated by thin-layer chromatography⁹ and glycerol was measured fluorimetrically.¹²

Tritiated vitamin D₃ radioactivity was measured by liquid scintillation counting in Bray's fluid using a Tricarb-Packard spectrometer.

Oil phase and sediment volumes were neglected for the calculation of bile salt, lipid, free fatty acid, and vitamin D₃ concentration in the aqueous phase. All results were expressed as mean ± SEM. Inter-relationships between the different variables were assessed by partial correlation analysis. Analysis of variance, which takes into account the block effect, was carried out to study differences between the periods, and the Newman-Keuls test was used for comparisons of the means.

Results

The concentrations of bile salts, total lipids, free fatty acids, and ³H-D₃ in the total and aqueous phases are shown in the Table. For all periods mixed, the bile salt concentration in the total phase was 8.62 ± 0.92 mmol/l (n=40). The values for TC, TCD+TDC, GC, GCD+GDC, were 0.92 ± 0.1, 1.47 ± 0.15, 2.65 ± 0.28, 3.58 ± 0.43 mmol/l respectively.

The percentage of bile salts present in the aqueous phase was 83.7 ± 2.3%. This percentage was 87.9 ±

3.8 for TC, 83.8 ± 2.5 for TCD+TDC, 87.8 ± 1.8 for GC, and 78.3 ± 2.9 for GCD+GDC.

Analysis of variance with the block method followed by the Newman-Keuls test showed that bile salt concentration in the total phase was significantly higher in the first 30 minute period than in the following ones, which did not differ significantly from each other (Table). The concentrations of total lipids and ³H-D₃ and of free fatty acids in the total phase showed small variations during the whole study, although statistical analysis showed significant differences between two periods for free fatty acids (Table).

In the aqueous phase, significant differences were seen in the five periods, between bile salt concentrations and between free fatty acid and vitamin D₃ concentrations. The concentrations of these three compounds were significantly higher in the first period than during the other ones, which did not

Fig. 1 Percentages of bile salts, lipids, free fatty acids, and vitamin ³H-D₃ present in the aqueous phase.

Bile salt ●. Lipids ■. Free fatty acids ○.

Vitamin ³H-D₃ □. Vertical bars = means ± SEM

(n=13): means significantly higher (by Newman-Keuls test) are shown as: a (1 > 2, 3, 4, 5, P < 0.01).

b (1 > 2, 3, 5, P < 0.05).

Fig. 2 Correlation between concentrations in the aqueous phase of bile salts and of vitamin $^3\text{H-D}_3$.

Fig. 3 Correlation between concentrations in the aqueous phase of free fatty acids and of vitamin $^3\text{H-D}_3$.

differ significantly from each other. The percentages of bile salts and $^3\text{H-D}_3$ present in the aqueous phase did not differ significantly between the periods, whereas the percentages of total lipids and free fatty acids followed the same pattern as in their respective aqueous concentrations, and were significantly higher in the first period than during the following ones (Fig. 1).

The composition of the aqueous and total phases expressed as percentages of the molar sum of free fatty acids (FFA) monoglycerides (MG), diglycerides (DG), and triglycerides (TG) was determined in two subjects. In the total phase, the mean values for FFA, MG, DG, TG were respectively 70.3 ± 2.4 , 16.7 ± 1.4 , 8.6 ± 1.2 , $4.4 \pm 0.9\%$, and in the aqueous phase: 76.2 ± 1.8 , 18.2 ± 1.4 , 4.5 ± 1 , $1.1 \pm 0.4\%$.

For all periods mixed, significant correlations were found between aqueous bile salt concentrations and aqueous free fatty acid, lipid, or $^3\text{H-D}_3$ concentrations (Fig. 2). There was also a significant correlation between aqueous free fatty acid (Fig. 3) monoglyceride ($r=0.66$, $P<0.05$) or total lipid concentrations and aqueous $^3\text{H-D}_3$ concentrations. Calculation of partial correlation coefficients (Fig. 4) showed that, for a constant concentration of aqueous free fatty acids or lipids, aqueous concentration of bile salts and $^3\text{H-D}_3$ were not correlated, whereas, for a constant concentration of aqueous bile salts, there was a statistically significant correlation between aqueous concentration of $^3\text{H-D}_3$ and aqueous free fatty acids or total lipid concentrations.

Discussion

Indirect evidence suggests that dietary fats enhance vitamin D absorption in man.¹ The means of this enhancement are complex. Thompson *et al.*² and Hollander *et al.*¹³ have studied, with conflicting results, the effects of aqueous swelling lipids on the entry of vitamin D₃ into the small intestinal mucosa by the method of intraluminal infusion: decreased entry of vitamin D₃ was found in one study¹³ while

Fig. 4 Correlations between aqueous bile salts (BS), aqueous vitamin D₃ (D₃), aqueous free fatty acids (FFA, right), or aqueous lipids (TL, left). Straight lines: correlations, curved lines: partial correlations. ● NS. *P < 0.001.

no significant effect compared with simple taurocholate micelles was observed in the other.² Moreover, Thompson *et al.*² have found that swelling lipids increased transport of vitamin D₃ out of the intestinal wall into the lymph. Our results show that dietary lipids also govern the aqueous solubilisation of vitamin D₃. With the test meal we used, the aqueous phase of the intestinal content contained mixed micelles, which were chiefly composed of free fatty acids, monoglycerides, and bile salts. Biliary lecithin was probably also present in a significant amount during the first 30 minute period, but was thereafter hydrolysed in lysolecithin, which is readily absorbed.^{14,15} Monoglycerides and 'fatty acids soaps' (combination of anion and protonated free fatty acid) behave as insoluble swelling amphiphiles which expand the size of the micelle when added to bile salt solutions; this micelle expansion is necessary to solubilise vitamin D₃.³

There was a significant partial correlation between aqueous free fatty acids and vitamin D₃, but not between aqueous bile salt and vitamin D₃. A partial correlation between monoglycerides and vitamin D₃ could not be determined, as monoglycerides were assayed in only two subjects. There was, however, a significant correlation between aqueous monoglycerides and vitamin D₃. Moreover, as aqueous free fatty acid concentrations were more than three times those of monoglycerides, their role in the aqueous solubilisation of vitamin D₃ is probably more important. These findings offer an explanation of why, in normal subjects, appreciable quantities of vitamin D₃ are absorbed when fed with triglycerides, hydrolysis of which produces free fatty acids and monoglycerides, whereas, when vitamin D₃ only is fed, it is scarcely absorbed.¹⁶ Similar conclusions have been drawn for cholesterol, another insoluble non-swelling polar lipid.^{3,17}

We have found that the mean percentage of total bile salts present in the aqueous phase, is close to that published by Simmons *et al.*,¹⁸ but is lower than that reported by other authors.^{18a-21} In order to minimise the errors produced by the ultracentrifugation,^{22,23} it was performed at a high speed, over a prolonged period of time, and with polycarbonate tubes, and the aqueous phase was recovered *in toto* to avoid concentration gradients. The other factor which determines the concentration of bile salts in the aqueous phase is the amount of bile salts present in the total phase.

The fact that aqueous solubilisation of vitamin D₃, an absolute prerequisite for its absorption, is dependent upon mixed micelle formation has pathophysiological implications. In pancreatic insufficiency, the usual malabsorption of vitamin D₃ demonstrated by tritiated vitamin D₃ oral test^{24,25},

may be explained by the impaired hydrolysis of triglycerides into monoglycerides and free fatty acids. In adult coeliac disease, osteomalacia and malabsorption of vitamin D₃ may be observed in spite of minimal steatorrhoea.²⁵ This could be explained by the low ability of the ileum—which is usually normal in these cases of coeliac disease—to absorb vitamin D₃, whereas the fatty acid absorption rate, although lower than in the jejunum, is still noticeable. However, another possibility is that the secretory state prevailing in the proximal small intestine²⁶ and the delayed contraction of the gall-bladder²⁷ lower the intraluminal bile salt concentration. This, in turn, would produce a decrease in aqueous lipids leading to vitamin D₃ malabsorption.

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