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# Studies on the measurement of 25-hydroxy vitamin D in human saliva

BY ANGELA FAIRNEY AND P. W. SAPHIER

*Department of Chemical Pathology, St. Mary's Hospital Medical School, London W2 1PG*

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1. A competitive protein-binding assay for 25-hydroxy vitamin D (25-OHD) in saliva has been established by adaptation of that previously described for 25-OHD in serum (Fairney *et al.* 1979).

2. Random values of salivary 25-OHD in patients attending hospital for venesection showed a wide range of results (105–1000 pg/ml, $n$ 55). These values corresponded to 1·2% of the total serum values with which they showed a significant relation ($r$ 0·45, $P < 0·001$).

3. There was no relation between salivary 25-OHD and measured serum free 25-OHD in eighteen pairs of saliva and serum studied.

4. Studies in two individuals showed that salivary 25-OHD values varied throughout the day and that a vitamin D load (19·5 $\mu$g), given as pickled herrings at lunch, produced a marked rise in 25-OHD values 5–8 h later.

5. Diurnal profile studies of salivary 25-OHD in Caucasian and Asian 11-year-old male schoolchildren showed lower values in Asian children eating a vegetarian diet, and a significant variation with time and ethnic group ($P < 0·001$).

6. It is concluded that 25-OHD is present in saliva and that the values vary throughout the day. The values obtained may relate to dietary intake of vitamin D and the subject's ethnic origin.

Cholecalciferol (vitamin $D_3$) is hydroxylated in the liver to form 25-hydroxycholecalciferol (25-$OHD_3$). This is the major circulating form of vitamin D (Haussler & McCain, 1977). Measurements of this metabolite are now widely used in the assessment of vitamin D status in patients (Avioli & Haddad, 1984) but the estimations are usually undertaken on random samples of blood and represent protein-bound values of the metabolite. The measurement of steroid hormones in saliva has attracted attention as it often correlates with the free hormone in serum. However, little attention has been paid to the use of measurements of the free and protein-bound fractions of 25-$OHD_3$ in clinical practice. We have therefore adapted our serum assay for 25-hydroxy vitamin D (25-OHD) (Fairney *et al.* 1979) for use on saliva, as an attempt to provide a system for the measurement of free 25-OHD in patients. The present communication describes a highly sensitive, competitive, protein-binding assay suitable for measurement of 25-OHD in saliva. The assay has been used to study diurnal variations of 25-OHD in saliva.

## METHODS

### *Collection of samples*

Unstimulated samples of whole saliva (approximately 2 ml) were collected directly into glass bottles from normal volunteer subjects or patients attending for phlebotomy. Serum was collected simultaneously and all samples were stored in glass bottles at −20° until thawed before assay. Permission to collect these samples was obtained from the Local Ethical Committee.

The saliva samples contained varying amounts of particulate matter to which 25-$OHD_3$ bound on storage. To obtain a homogenous mixture for assay, the thawed samples were sonicated at amplitude 6 for 30 s before use, using a MSE Soniprep 150 (MSE Scientific Instruments, Crawley, Sussex).

*Assay of 25-OHD in saliva*

*General reagents.* AnalaR (analytical grade) ethanol (EtOH) and methanol (MeOH), and standard laboratory grade dichloromethane ($CH_2Cl_2$) were freshly distilled before use. Sodium phosphate buffer (pH 7·5), barbitone buffer (pH 8·0), Triton X100 and dextran-coated charcoal were prepared as previously described (Fairney *et al.* 1979). Radioactivity was counted using RIA LUMA scintillant (LKB Instruments Ltd, South Croydon, Surrey).

*Steroids.* Unlabelled 25-$OHD_3$ was supplied by Hoffmann–La Roche Inc., Nutley, N.J., USA. 25-hydroxy-[23,24(n)−$^3$H]cholecalciferol (TRK 558) ([$^3$H]25-$OHD_3$) was obtained from Amersham International plc, Amersham, Bucks.

*Extraction and assay procedure* (*see Table 1*). Sonicated saliva (250 $\mu$l) was extracted with 5 ml $CH_2Cl_2$–MeOH (92:8 v/v) and estimations performed in duplicate as previously described (Fairney *et al.* 1979) with the following modifications. Tubes containing standard amounts of 25-$OHD_3$ (6·25, 12·5, 25, 50 and 100 pg) were prepared in 50 $\mu$l EtOH. Portions (1·5 ml) of the $CH_2Cl_2$–MeOH sample extracts were used for assay and, after evaporation under nitrogen, 20 $\mu$l [$^3$H]25-$OHD_3$ (8·7 pg) were added to each tube. Normal rat-kidney binding protein was used at 1:4500 w/v in phosphate buffer.

*Identification of 25-$OHD_3$ in saliva*

Unstimulated saliva (100 ml) was collected over a period of 2–3 h. This was divided into two portions in 500-ml round-bottomed, Quickfit flasks for lyophilization using an Edwards Modulyo freeze-drier (Edwards High Vacuum, Crawley, Sussex). After lyophilization, the residue was redissolved in 20 ml distilled water. This solution was then extracted three times each with 200 ml $CH_2Cl_2$–MeOH (92:8, v/v), the extracts combined and the solvent evaporated under oxygen-free $N_2$ in a water-bath at 40°.

This extract was then purified on a 100 × 10 mm column of Sephadex LH20 (Pharmacia (GB) Ltd, Hounslow, Middlesex), presoaked in hexane–chloroform–MeOH (9:1:1, by vol.). The column was calibrated with [$^3$H]25-$OHD_3$ and the extract from the 100 ml saliva was applied to the column and the 25-$OHD_3$ fractions collected. These were dried and further purified by high-pressure liquid chromatography using a reverse-phase Hypersil-ODS 250 mm column (Shandon Southern Products Ltd, Runcorn, Chesire), eluting with MeOH–water (92:8, v/v) at 1 ml/min. The 25-$OHD_3$ fraction was collected, dried under $N_2$ and then rechromatographed on a straight-phase Hypersil 250 mm column (Shandon), eluting with hexane–isopropyl alcohol (98:2,v/v). The 25-$OHD_3$ fraction was collected and used for gas chromatography–mass spectrometry (GS–MS) analysis.

Initially derivatives of a standard sample of 25-$OHD_3$ were prepared by treatment with hexamethyldisilazane–trimethyl chlorosilane – pyridine (2:1:3, by vol.) (Whitney *et al.* 1979) and analysed by GC–MS. The pyro and isopyro derivatives obtained were run on a 25 m glass capillary OV-1 column, fitted with a solid injection system, at 255°, column temperature 240° programmed at 1°/min. Mass spectroscopy was performed using a Varian MAT 731 mass spectrometer, source temperature 250°, 8 kV accelerating voltage, 70 eV ionization potential and resolving power 1000. The spectra of the pyro form are shown in Fig. 1 and were obtained from approximately 80 ng 25-$OHD_3$ injected. Under the conditions used, about 20 ng were necessary to obtain a readily recognizable, full spectrum. In addition, selected ion monitoring was also undertaken permitting detection of the compound at the 1 ng level.

Subsequently four portions of the salivary extract were treated with the trimethysilylation reagent as described previously. Each sample was examined by selected ion monitoring of ions m/z 454, 439, and 413. These were observed in the correct intensity ratios at the expected retention time. The remaining sample of the extract was then also trimethylsilylated

Table 1. *Assay procedure for salivary 25-hydroxy vitamin D*

| Total activity tubes | Non-specific binding tubes | Specific binding tubes |
|---|---|---|
| — | — | Standard or sample extract (1·5 ml) |
| — | — | Dry under $N_2$ |
| Add [$^3$H]25-OHD$_3$ (2000 counts/min in 20 $\mu$l ethanol) | Add [$^3$H]25-OHD$_3$ (2000 counts/min in 20 $\mu$l ethanol) | Add [$^3$H]25-OHD$_3$ (2000 counts/min in 20 $\mu$l ethanol) |
| Add phosphate buffer (500 $\mu$l) | Add phosphate buffer (500 $\mu$l) | Add binding protein (1:4500 w/v) in phosphate buffer (500 $\mu$l) |
| Mix; incubate 1 h at room temperature | Mix; incubate 1 h at room temperature | Mix; incubate 1 h at room temperature |
| Incubate in ice water-bath 10 min; add Triton X100 (1:20 w/v; 20 $\mu$l); mix | Incubate in ice water-bath 10 min; add Triton X100 (1:20 w/v; 20 $\mu$l); mix | Incubate in ice water-bath 10 min; add Triton X100 (1:20 w/v; 20 $\mu$l), mix |
| Add dextran solution (250 $\mu$l); mix | Add dextran-coated charcoal (250 $\mu$l); mix | Add dextran-coated charcoal (250 $\mu$l); mix |
| Centrifuge (20 min, 4°, 830 ***g***) | Centrifuge (20 min, 4°, 830 ***g***) | Centrifuge (20 min, 4°, 830 ***g***) |
| Pour off liquid and count in $\beta$-counter | Pour off supernatant fraction and count in $\beta$-counter | Pour off supernatant fraction and count in $\beta$-counter |

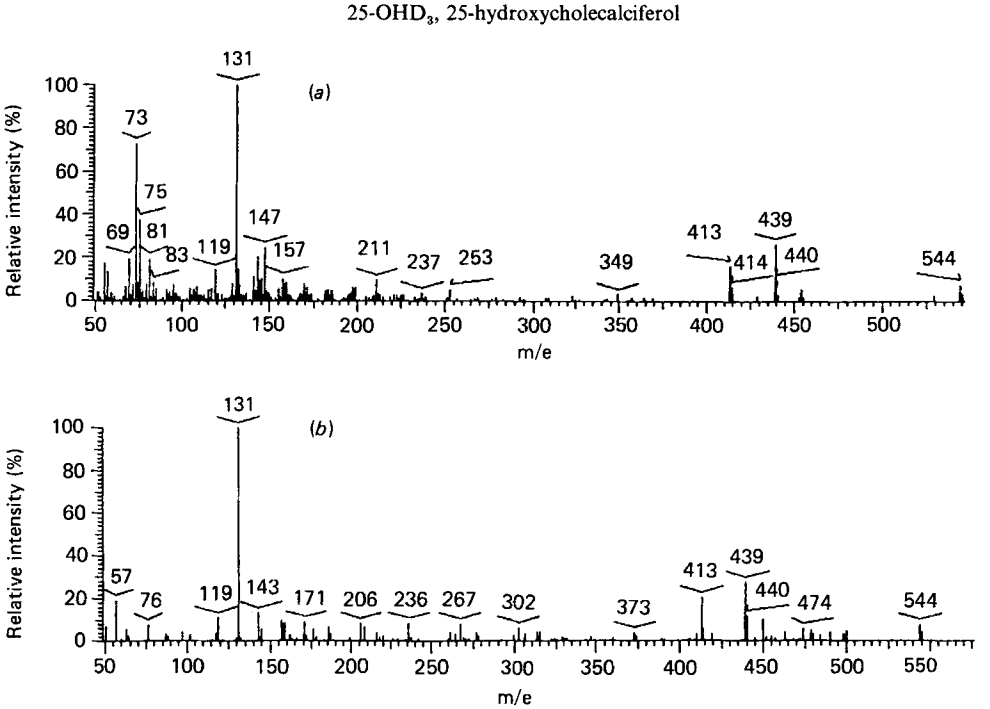


Fig. 1. Spectra of the pyro form of 25-hydroxy vitamin $D_3$ (*a*) compared with that from human saliva (*b*). m/e, mass/electron.

and injected into the GC–MS and a spectrum of pyro-25-OHD$_3$ TMS-2 obtained (Fig. 1). Care was taken to avoid any 'wash-off' from the GC column from previously injected standard samples of 25-OHD$_3$.

*Measurement of free 25-OHD in serum*

Attempts to measure free 25-OHD by a dialysis method were unsuccessful as 25-OHD bound to the dialysis membranes used. An alternative ultracentrifugation method was therefore devised.

Serum (1·5 ml) was diluted with an equal volume of saline (9 g sodium chloride/l) in plastic ultracentrifugation tubes, 20 $\mu$l EtOH containing [$^3$H]25-OHD$_3$ (0·04 $\mu$Ci) added and the tubes mixed by inversion. The tubes were then incubated at 4° for 5 h followed by centrifugation in a Spinco model L ultracentrifuge (Beckman RIIC Ltd, High Wycombe, Bucks) using a SW50L rotor head at 197000 ***g*** for 18 h at 4°. Fractions (250 $\mu$l) were then carefully removed sequentially from the top of the tube and 25-OHD, vitamin D-binding protein and radioactivity measured. Vitamin D-binding protein was undetectable in the top four fractions and the percentage free 25-OHD was calculated (Beisel *et al.* 1964).

*Other assays*

Measurements of 25-OHD in serum were performed as previously described (Fairney *et al.* 1979) and vitamin D-binding protein was measured by a commercially supplied radial immunodiffusion method for $G_c$ globulin (Hoechst Pharmaceuticals, Hounslow, Middlesex).

*Subjects*

Studies were undertaken on different groups of subjects to determine: (*a*) the random values of 25-OHD in saliva and their relation to the total amount of 25-OHD in serum (*b*) the relation of 25-OHD in saliva to free 25-OHD in serum, (*c*) whether or not the values of 25-OHD in saliva remained constant throughout the day or did they show any form of physiological variation.

Details of the subjects studied in each of the groups described are as follows: (*a*) random paired samples of saliva and serum were obtained from patients with miscellaneous diagnoses attending the department for phlebotomy; (*b*) as part of another study to assess the vitamin D status of 12-year-old local schoolchildren, there was sufficient spare serum from eighteen children to measure total 25-OHD, free 25-OHD by ultracentrifugation and vitamin D-binding protein of each sample. Specimens of saliva obtained at the time of phlebotomy were also available from subjects. A comparison of total 25-OHD in serum, free 25-OHD in serum, calculated free 25-OHD in serum using the total 25-OHD and vitamin D-binding-protein values (Bouillon *et al.* 1977), and salivary 25-OHD values was therefore undertaken on these children; (*c*) the effect of dietary vitamin D on salivary 25-OHD values throughout the day was initially studied in two, male, normal volunteers. Serial saliva samples were collected throughout the day. Two daily salivary 25-OHD profiles were obtained in one of the volunteers: the 1st day when the subject took no food and only black coffee and the subsequent day when the usual diet containing 1·725 $\mu$g vitamin D was taken. The second volunteer provided samples throughout the day having taken lunch which included a vitamin D load (19·5 $\mu$g) taken in the form of pickled herrings (roll mops). Subsequently saliva was collected at different times throughout the day from three groups of 11-year-old male schoolchildren. The children were grouped according to race and diet so that the three groups studied were: Caucasian children on an English diet (group 1), Asian children on an English diet (group 2) and Asian children on a vegetarian diet (group 3). The values obtained were log transformed before unbalanced analysis of variance.

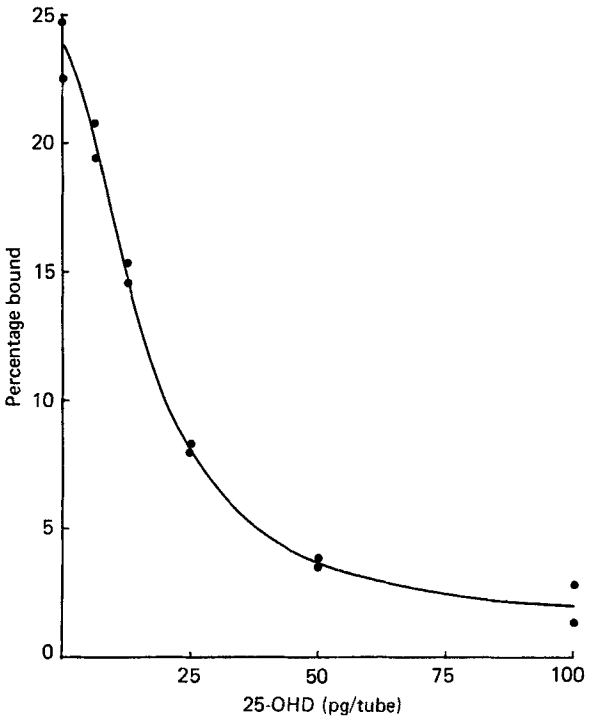


Fig. 2. Standard curve for assay of 25-hydroxy vitamin D (25-OHD) in human saliva.

Table 2. *Precision of assay for 25-hydroxy vitamin D (25-OHD)*

| Variation | No. of replicates | 25-OHD (pg/ml) Mean | SD | Coefficient of variation (%) |
|---|---|---|---|---|
| Intra-assay | 9 | 602 | 54 | 9 |
| | 9 | 167 | 16 | 8·7 |
| Interassay | 21 | 605 | 69 | 11·4 |

## RESULTS

### *Assay of salivary 25-OHD*

The standard curve is shown in Fig. 2 and the lower limit of sensitivity was 5 pg/tube. The intra- and interassay coefficients of variation are shown in Table 2. Extraction recoveries were 100·6% (SD 2·8, *n* 63) and recoveries of unlabelled 25-$OHD_3$ added to saliva samples of known 25-OHD content were 107·5 and 96·7% when 100 and 300 pg respectively were added to 1 ml saliva 30 min before assay. The values obtained on dilution of a single saliva sample are shown in Fig. 3.

### *Identification of 25-$OHD_3$ in saliva*

The presence of 25-$OHD_3$ in saliva was detected by selected ion monitoring in all four portions of the salivary extract examined. The spectrum of pyro-25-$OHD_3$ TMS-2 obtained

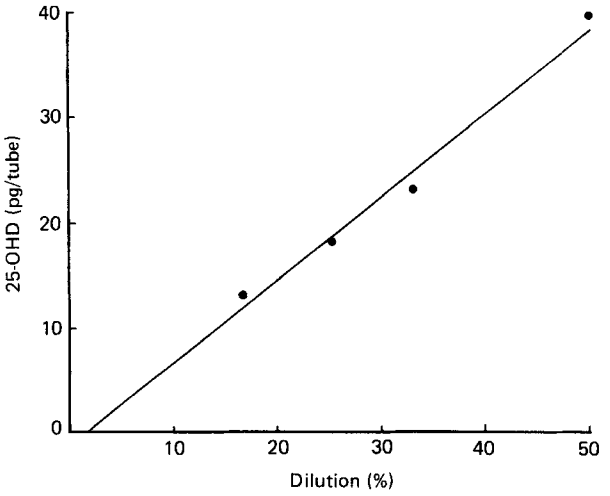


Fig. 3. Values for 25-hydroxy vitamin D (25-OHD) obtained on dilution of a single human saliva sample.

after trimethylsilylation of the saliva extract is shown in Fig. 1(*b*). Although the spectrum is not as intense as would be desirable and contains a co-eluting contaminant, it firmly supports the presence of 25-$OHD_3$ in saliva. The sample was estimated to contain about 20 ng 25-$OHD_3$. Therefore, as it was derived from 100 ml saliva and the recovery of [$^3$H]25-$OHD_3$ using a similar preparatory procedure before GS–MS was 56%, the concentration obtained in the sample examined was approximately 357 pg/ml or 954 pmol/l.

*Random values of 25-OHD in saliva*

The random values of salivary 25-OHD in patients attending for phlebotomy showed a very wide range (105–1000 pg/ml, $n$ 55; see Fig. 4). The saliva results represented 1·2% of the serum results, and correlation analysis after log transformation of the values showed that there was a significant relation between the salivary and serum 25-OHD ($r$ 0·45, $P < 0·001$; see Fig. 5).

*Relations of salivary 25-OHD to free and serum 25-OHD*

The results of the comparison of total serum 25-OHD, free 25-OHD in serum, calculated free and salivary 25-OHD are shown in Fig. 6. The measured free 25-OHD represented 0·66% of the total serum 25-OHD, the salivary 25-OHD represented 1·04% of the total serum 25-OHD and the calculated free 25-OHD 0·005% of the total amount in serum. After log transformation of the values, correlation analysis showed no relation between salivary 25-OHD and measured free 25-OHD in serum. However, there was a relation between salivary 25-OHD and the calculated amount of free 25-OHD in serum ($r$ 0·5, $P < 0·05$).

*Changes in salivary 25-OHD throughout the day*

The studies of the two individual volunteers showed a flatter profile on the day that the first subject only drank black coffee compared with the following day when he took his normal diet (see Fig. 7). The second volunteer showed a marked evening rise in salivary 25-OHD, the peak response occurring 5–8 h after the load (see Fig. 8).

The results of the salivary 25-OHD profiles in the three different groups of children are seen in Fig. 9. Analysis of the individual groups showed that in both groups of children

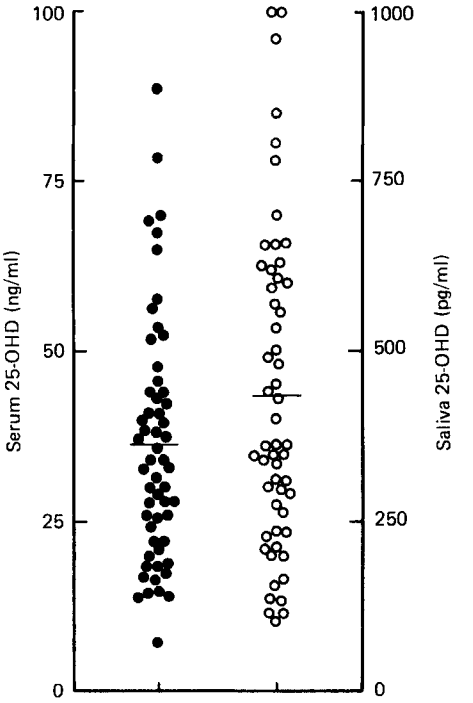


Fig. 4. Random values of 25-hydroxy vitamin D (25-OHD) in fifty-five pairs of serum (●) (mean 36·4 ng/ml) and saliva (○) (mean 434 pg/ml).

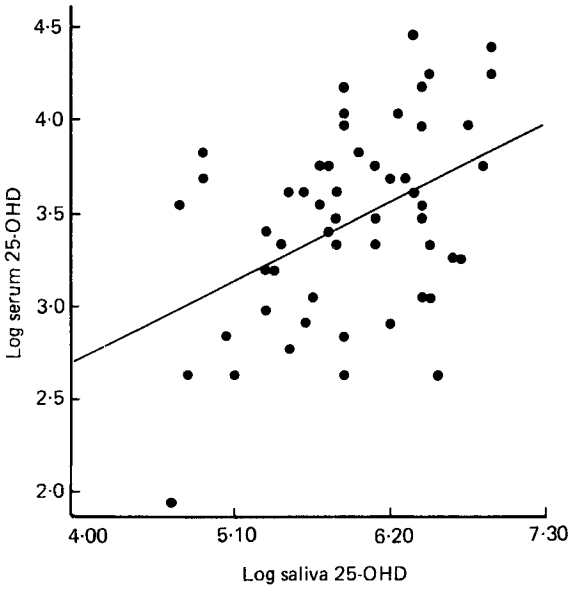


Fig. 5. Relation between 25-hydroxy vitamin D (25-OHD) in serum and that in human saliva. $R$ 0·44, $P < 0·001$.

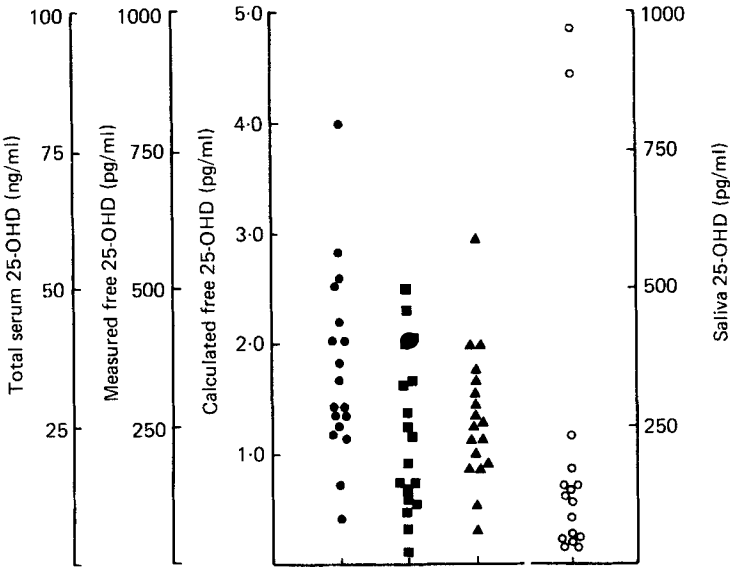


Fig. 6. Comparison of salivary 25-hydroxy vitamin D (25-OHD) (○) with total serum 25-OHD (●), measured free serum 25-OHD (■) and calculated free serum 25-OHD (▲) in human patients.

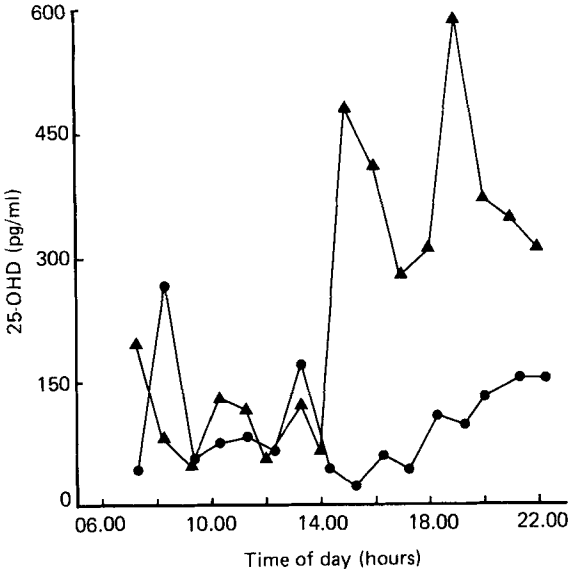


Fig. 7. Salivary 25-hydroxy vitamin D (25-OHD) profiles in a normal human subject taken on 1 d when eating his normal diet of 1·725 μg vitamin D (▲) compared with 1 d when only black coffee was consumed (●).

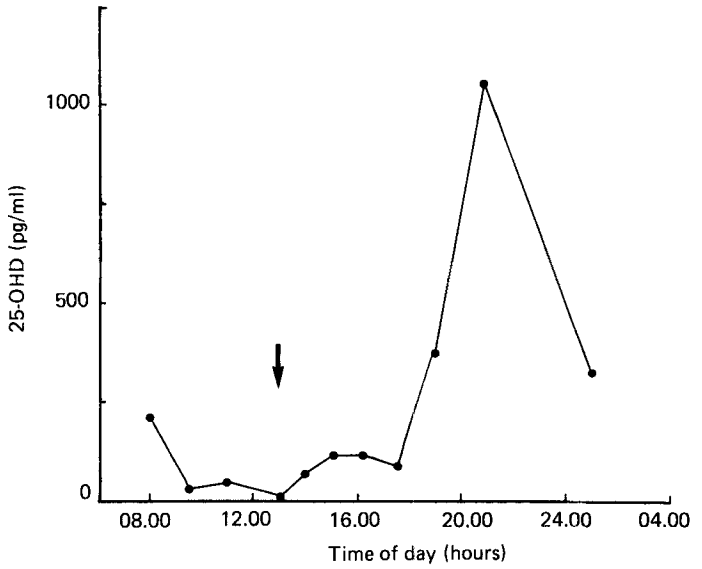


Fig. 8. Salivary 25-hydroxy vitamin D (25-OHD) profile in normal human subject showing response to vitamin D load of 19·5 $\mu$g given as pickled herring (↓).

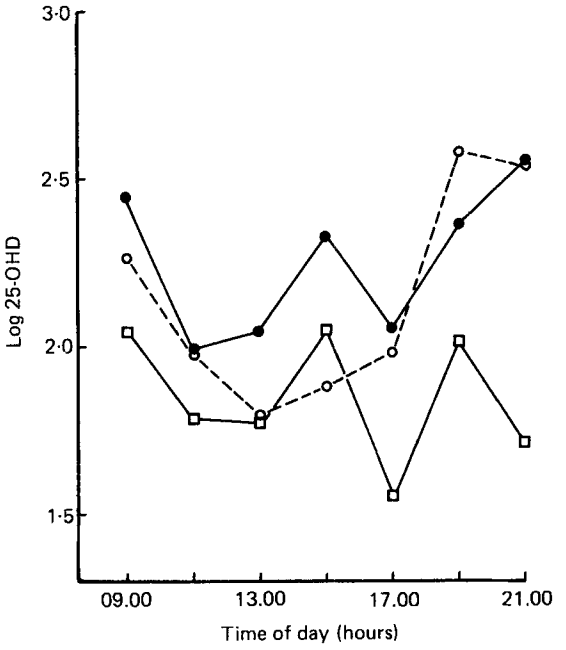


Fig. 9. Salivary profiles of 25-hydroxy vitamin D (25-OHD) in three groups of school children. (●), Caucasian boys on an English diet (*n* 9); (○), Asian boys on an English diet (*n* 11); (□), Asian boys on a vegetarian diet (*n* 12).

eating an English diet (groups 1 and 2) there was a significant variation with time throughout the day ($P < 0{\cdot}001$). This was not seen in the group of Asian vegetarians (group 3) alone. However, when all three groups (1–3) were analysed together there was a significant effect with time and type of child ($P < 0{\cdot}001$).

## DISCUSSION

In the present study we have found that 25-OHD is present in saliva, the values fluctuate throughout the day and quantitatively may be related to dietary vitamin D intake, and ethnic origin.

At present the most widely used biochemical index of vitamin D status is the measurement of circulating 25-OHD (Avioli & Haddad, 1984). The assays available measure the total protein-bound metabolite and have ignored the possible importance of free 25-OHD metabolites in blood. The determination of free vitamin D metabolites is important because it is still unclear whether the free or the total concentration of these metabolites is the physiologically important concentration. For example, values of 1,25-dihydroxy vitamin D and vitamin D-binding protein are increased during pregnancy (Bouillon *et al.* 1981) but pregnant women do not become hypercalcaemic. It is, therefore, unknown whether these changes are wholly related to the increase in binding protein due to pregnancy or represent a physiological homeostatic mechanism for maintaining normal calcium balance in the fetus. Consequently an accurate determination of free values of the vitamin D metabolites would be of great interest in this situation.

An attempt to overcome the lack of free metabolite measurements has been made by calculation of the free values from the total 25-OHD and vitamin D-binding protein results in serum (Bouillon *et al.* 1977). These calculations used previously determined values of the binding-affinity constant of vitamin D-binding protein for the vitamin D metabolite of interest. Unfortunately, estimates of the binding-affinity constant of human vitamin D-binding protein for 25-$OHD_3$ vary, so that considerable uncertainty about the validity of these calculations has arisen.

Direct measurement of the free fraction of the metabolite in blood has been difficult because of the very strong binding of 25-OHD to its transport protein, as well as technical problems. Estimation of the free fraction of other similar substances in plasma, e.g. testosterone, has been overcome by the estimation of the substance in saliva, and measurement of steroid hormones in saliva has been shown to reflect the unbound plasma concentration (Baxendale *et al.* 1982). Salivary assays are non-invasive, and collection of samples is simple, providing an opportunity to look at variations of the substance measured throughout the day. The concentrations are very low, and therefore require specific and highly sensitive assays. Unconjugated steroids enter saliva by diffusing through the cells of the salivary glands and their concentration does not depend on the rate of saliva production. However, conjugated steroids enter saliva via ultrafiltration through the junction between the acinar cells and their concentration in saliva is highly flow-rate dependent (Vining *et al.* 1983).

Our studies have shown it is relatively easy to measure 25-OHD in saliva by adjusting the concentration of binding protein and tracer to make a highly sensitive assay. The concentrations of 25-OHD that we found in saliva did not reflect the free values measured in serum, but the method for measuring the free fraction in blood was technically difficult to perform. An ultracentrifugation method had to be used as the more traditional dialysis method was unsuitable because 25-$OHD_3$ adhered to the different dialysis membranes used. Using the results from the correlation of saliva and total serum values of 25-OHD, the percentage of 25-OHD in saliva was 1·04%, compared with 0·66% for the free metabolite

measured, and 0·005% for the calculated free value. The percentage of metabolite present in saliva is similar to that obtained for testosterone (James, 1982).

Our studies have confirmed the presence of 25-$OHD_3$ in saliva by mass spectrometry using an extract of 100 ml saliva. The detection limit of the technique used is such that at least 200 pg/ml were present in the sample analysed even if calculations were not made for losses during preparation of the sample. This value is within the range of 105–1000 pg/ml for 25-OHD found in random samples of mixed saliva. The mass spectrometry value is likely to be lower than that obtained with mixed saliva using the simple 25-OHD competitive protein-binding assay, as this uses normal rat-kidney binding protein. This protein is known to cross-react with 25-$OHD_3$, 25-hydroxy vitamin $D_2$, 24,25-dihydroxy vitamin $D_3$ (Fairney *et al.* 1979) and possibly 25-$OHD_3$ 26,23-lactone. In addition, a diurnal fluctuation was found in our studies and as the sample analysed was collected in the late morning, and not when values were expected to be maximal in response to lunch, the result is within the part of the random range expected for the morning.

Studies on the presence of 25-OHD in human saliva by Trafford & Makin (1983) have, however, shown different results. Using a specific high-performance liquid chromatographic procedure in which they claim a lower limit of sensitivity of 50 pg/ml (130 pmol/l), no 25-OHD could be detected in saliva. Also using GC–MS a peak, possibly derived from 25-$OHD_3$, was detected which was not in excess of 65 pmol/l (25 pg/ml). It is not obvious why these workers could not detect higher amounts of 25-$OHD_3$ more comparable to our own findings. In the mass spectrometry part of Trafford & Makin's (1983) study the times of saliva collection are not specified so that it is possible that samples were obtained at times when the values would be expected to be very low in relation to the diurnal fluctuation. Also, Trafford & Makin (1983) used a different method of derivative preparation before mass spectrometry. Their method involved the formation of isotachysterol isomers which requires very carefully evaluated and controlled conditions to avoid destruction of the sample. It is possible that some of the 25-$OHD_3$ in their sample was destroyed before analysis.

The wide variation of random values of salivary 25-OHD obviously prevents the use of this type of measurement as a replacement for random serum 25-OHD estimations. However, its variation throughout the day raises interesting questions. It is assumed that 25-OHD is present in an unconjugated form as for other steroid hormones. Therefore it should be relatively unaffected by salivary flow (Vining *et al.* 1983). The effect of oral vitamin D loading to enhance the evening rise in salivary 25-OHD supports the view that the diurnal changes are related to diet. Examination of the profiles from the schoolchildren shows a small rise in salivary 25-OHD in the early afternoon in the Caucasian boys. It may be related to eating patterns throughout the day as the dietary questionnaires revealed that some of the children ate vitamin D in the form of fortified cereals for breakfast. Although seasonal variations in serum 25-OHD concentration are well established (McLaughlin *et al.* 1974), diurnal variations of serum 25-OHD have not been observed (Juttmann *et al.* 1981). The differences between seasonal and diurnal rhythms of serum 25-OHD may be explained by Fraser's (1981) hypothesis that vitamin D formed in the skin and that obtained in the diet are handled by different mechanisms. The seasonal pattern in serum 25-OHD follows the solar. u.v. light in temperate regions and reflects the physiological cutaneous source of vitamin D. This source of vitamin D associates with vitamin D-binding protein so that the body is protected against sudden changes in overproduction of 25-OHD. Dietary vitamin D bypasses this mechanism, being absorbed in association with fat and being rapidly cleared by the liver. The absorption of vitamin D from the intestine is followed by a rapid secretion of 25-OHD from the liver. This might then be reflected in the saliva as observed in our studies after vitamin D loading. It is possible that studying diurnal changes in salivary

25-OHD may be a way to differentiate between dietary and actinic sources of vitamin D.

The variation in salivary 25-OHD observed in the children studied may be related to the circadian rhythm that is now accepted as a basic element of human physiology (Moore-Ede *et al.* 1983). The well-established circadian changes in cortisol secretion seem to anticipate an environmental change so that a peak is reached just before waking. The shape of the salivary 25-OHD profiles is, if anything, the reverse of this and more reminiscent of the growth hormone peak during the beginning of sleep. However, the highest salivary 25-OHD values occurred in the evening before sleep.

Vitamin D deficiency has been extensively documented in Asian subjects in Britain. Asian immigrants are known to have low plasma 25-OHD values (Preece *et al.* 1973) and to have a low dietary intake of vitamin D (Hodgkin *et al.* 1973). The Asian vegetarian children that we studied had a lower dietary vitamin D intake than the Caucasian children. This may account for the lower salivary 25-OHD values in the Asian children.

The studies described in the present paper show the presence of 25-OHD in saliva and that it varies throughout the day. It most probably reflects the dietary intake of vitamin D and diurnal profiling of this metabolite might be very useful as the clinical test to assess the dietary source of this nutrient. In addition, the purpose of 25-OHD in saliva is interesting to speculate. In rats there are receptors for vitamin D in the parotid glands (Aoki *et al.* 1982; Peterfy & Tenenhouse 1982) and in the developing incisor teeth (Kim *et al.* 1983). Microradiographs of rat incisor dentine show a regular alternation of highly and lowly mineralized bands. Dentinogenesis progresses at a rate of 20 $\mu$m/d (Simmons, 1981). As a result circadian rhythms are seen as an important component of tooth growth in the rat. Diurnal variations of salivary vitamin D may have a physiological role in the mineralization process of the developing tooth. Further studies relating salivary 25-OHD profiles to age, sleep disorders, dietary deficiency of vitamin D and the morphology of teeth and bone are now required.

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