25-Hydroxyvitamin D_3 3-sulphate is a major circulating form of vitamin D in man

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25-Hydroxyvitamin D_3 3β-sulphate has been identified in human plasma. The compound was isolated by anion-exchange chromatography and following hydrolysis it was characterized by high-performance liquid chromatography and gas chromatography-mass spectrometry. The mean concentration of sulphated 25-hydroxyvitamin D_3 in plasma from 60 patients was 16.7 ± 7.1 ng/ml and the levels often exceeded those of the corresponding free compound. The study also shows that unconjugated 25-hydroxvitamin D_3 is not readily sulphated by man in vivo.

Vitamin D *25-Hydroxyvitamin D* *Sterol metabolism* *Steroid conjugation* *Sulfate ester* *Human plasma*

1. INTRODUCTION

Vitamin D_3 is a prehormone in an endocrine system for calcium homeostasis in man and the metabolism of this secosteroid has been extensively investigated (see [1–4]). Several hydroxylated and biologically active metabolites have been identified, the most potent hormone being 1α,25-dihydroxyvitamin D_3. However, little is known about the further metabolism and excretion of vitamin D_3 metabolites in man. So far no conjugates of vitamin D_3 or its metabolites have been detected in human blood, although sulphated forms have been found in milk from lactating women [5–8]. Here I report that 25-hydroxyvitamin D_3 3β-sulphate is a major circulating form of vitamin D_3 in man. The concentration of the sulphate often exceeds that of free 25-hydroxyvitamin D_3, previously recognized as the quantitatively major metabolite in blood [1–4]. The biosynthesis of the sulphate has not yet been elucidated. However, as demonstrated here, the parent compound is not readily conjugated in vivo, indicating formation from a sulphated precursor. The results suggest an important role of sulphates in the metabolism of vitamin D_3 in man.

2. MATERIALS AND METHODS

2.1. *Reference compounds*

Unlabelled 25-hydroxyvitamin D_3 was a kind gift from Roche-Producter AB, Sweden and 25-[23,24-^{3}H(n)]hydroxyvitamin D_3 (90 Ci/mmol) was from the Radiochemical Centre (Amersham, England). ^{3}H-labelled 25-hydroxyvitamin D_3 monosulphate was prepared by reacting the radioactive free compound with pyridine-sulphur trioxide in pyridine for 2 min at room temperature [9]. The product was purified by chromatography on TEAP-LH-20 (see below). Radioactivity was determined in an LKB/Wallac 1215 Rackbeta liquid scintillation counter with Instagel as scintillation liquid.

2.2. *Plasma samples*

Blood from human subjects was collected in heparinized tubes and centrifuged immediately. The plasma was separated and stored at -20°C until analyzed.

2.3. *Analytical procedure*

Potential conjugated metabolites of vitamin D_3 were extracted from plasma (5 ml, diluted with

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0.5 M aqueous triethylamine sulphate) [10] on a column (1 × 0.8 cm) of octadecylsilane (ODS)-bonded silica (preparative C_{18}, Waters Associates) at 64°C [10–12]. Steroid conjugates were eluted with 10 ml of 65% aqueous methanol prior to elution of unconjugated metabolites of vitamin D_3 with hexane/chloroform [12]. The conjugates were then separated into glucuronides, and mono- and disulphates on a column (4 × 0.4 cm) of the lipophilic anion exchanger, TEAP-LH-20 (HCO_3^-) [11,13]. The monosulphate fraction was desalted on a small ODS-silica column [11] and steroid sulphates were hydrolyzed by solvolysis in acidified tetrahydrofuran [11,13]. Liberated compounds were purified by rechromatography on columns of TEAP-LH-20 (HCO_3^-) [11,13] and ODS-silica [12] prior to analysis by high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS).

2.4. *HPLC and GC-MS analysis*

HPLC was carried out on a column (250 × 4.5 mm) of LiChrosorb (Hibar, Si 100, 10 μm, Merck, Darmstadt) with hexane/isopropanol as the mobile phase. The pump (Constametric III) and the fixed wavelength (254 nm) detector were from LDC/Milton Roy, Riviera Beach, FL [12]. Repetitive magnetic scanning GC-MS was performed on a Finnigan 1020 instrument housing a fused silica column (25 m × 0.3 mm), coated with SE-30 [12]. The oven temperature was programmed from 190 to 285°C at a rate of 5°C/min.

3. RESULTS AND DISCUSSION

3.1. *Identification of 25-hydroxyvitamin D_3 3-sulphate*

HPLC analysis of the solvolyzed monosulphate fraction from plasma revealed the presence of a UV-absorbing compound with the same retention time as authentic 25-hydroxyvitamin D_3 (fig.1). The compound was isolated, converted into a trimethylsilyl (TSM) ether derivative [12] and definitively identified by GC-MS [12]. The formation and relative amounts of 2 isomers (pyro and isopyro forms), their retention times on an open-tubular column coated with SE-30 (32.65 and 35.25 methylene units, respectively) [12], and their mass spectra were identical to those of the TMS ether of the reference compound, 25-hydroxyvitamin D_3. The mass spectrometric fragmentation of this compound has been discussed [14]. When solvolysis of the sulphate fraction from plasma was omitted, no compound corresponding to free 25-hydroxyvitamin D_3 was detected by HPLC or GC-MS. The possibility that the conjugate was an artefact of the isolation procedure was excluded after studies of the chromatographic behaviour of ^{3}H-labelled 25-hydroxyvitamin D_3 added to plasma.

The mobility on TEAP-LH-20 of the compound in plasma and hydrolysis by a mild solvolytic procedure strongly suggested that 25-hydroxyvitamin D_3 was conjugated with sulphuric acid. Further evidence was obtained by rechromatography of the sulphate fraction from plasma on a column of Sephadex LH-20 using methanol-chloroform (1 : 1) containing 0.01 M NaCl as mobile phase [15]. This separation system has been widely employed for isolation of steroid sulphates [15–17]. About 85% of the compound appeared in the monosulphate fraction, as determined by HPLC after solvolysis.

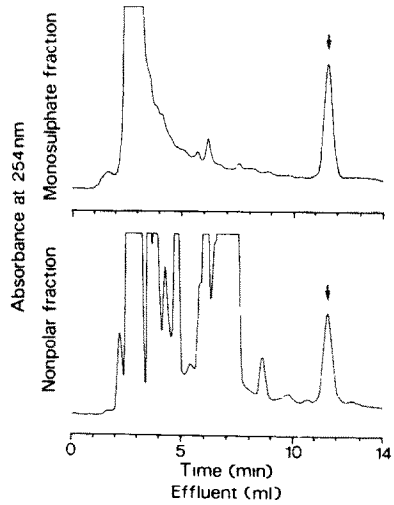

Fig.1. HPLC analysis of 25-hydroxyvitamin D_3 in the solvolyzed monosulphate fraction isolated from plasma of a healthy man. For comparison, the analysis of the unconjugated compound in the nonpolar fraction from the same plasma sample is shown. The arrows indicate the retention time of the reference compound. The equivalent of about 0.3 ml plasma was injected and the peaks represent about 10 and 8 ng, respectively. A column of LiChrosorb connected to a UV detector was used with hexane/isopropanol (97:3) as mobile phase.

The synthesis of ^{3}H-labelled 25-hydroxyvitamin D_3 monosulphate permitted a comparison of retention times with the conjugated compound in plasma on an HPLC column. As seen in fig.2, the intact conjugate from plasma and the ^{3}H-labelled sulphate co-eluted, providing strong evidence that the conjugated form of 25-hydroxyvitamin D_3 in plasma is a monosulphate. The identity of the HPLC peak was ascertained following solvolysis.

Since 25-hydroxyvitamin D_3 has 2 hydroxyl groups, the position of the sulphate group was investigated. When free 25-hydroxyvitamin D_3 was reacted with acetic anhydride/pyridine (1:1) for 3 h at room temperature, only the 3-hydroxy group was acetylated as demonstrated by HPLC and GC-MS [18]. When the reaction was carried out with the monosulphate fraction from plasma followed by solvolysis (which does not hydrolyse acetate groups) more than 90% of the originally sulphated 25-hydroxyvitamin D_3 in plasma was recovered as the free compound. Unconjugated ^{3}H-labelled 25-hydroxyvitamin D_3 added to the sulphate fraction prior to acetylation was detected as the 3-acetate (retention time 0.43 relative to that of underivatized 25-hydroxyvitamin D_3 by straight-phase HPLC, fig.1). Thus, 25-hydroxyvitamin D_3 is sulphated in the 3-position.

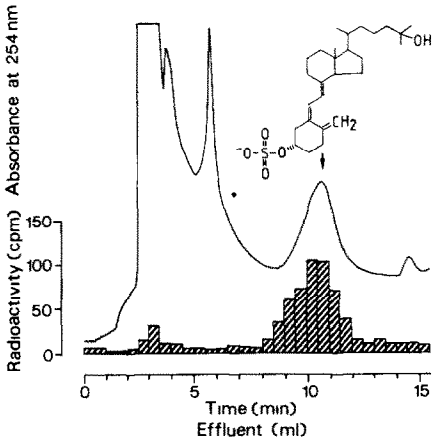

Fig.2. HPLC analysis of intact 25-hydroxyvitamin D_3 sulphate isolated from human plasma. Synthesized ^{3}H-labelled 25-hydroxyvitamin D_3 sulphate was added to the monosulphate fraction prior to injection and radioactivity in 0.5-ml fractions of the effluent was determined. The column and UV detector were those in fig.1, and the mobile phase was hexane/isopropanol (1:1).

3.2. *Concentration in human plasma*

The concentrations of 25-hydroxyvitamin D_3 3-sulphate and of the corresponding free compound in plasma from 10 apparently healthy human subjects were determined by HPLC (fig.3). Addition of ^{3}H-labelled free reference compound following separation of conjugates served to correct for degradation and losses during solvolysis and further purification. No correction for losses prior to solvolysis was made, however, yields of the ^{3}H-labelled sulphate added to plasma were essentially quantitative. During winter (February) the mean concentration of sulphated 25-hydroxyvitamin D_3 in plasma was 21.1 ± 9.2 ng/ml, which was similar to that of the corresponding free compound (mean 19.8 ± 10.1 ng/ml). During summer (August) the concentration of the free compound was increased in 8 subjects (mean 29.9 ± 9.9 ng/ml), and exceeded the sulphate levels in all subjects studied (mean 21.9 ± 7.2 ng/ml). Thus, the seasonal variation of plasma levels of free 25-hydroxyvitamin D_3 previously demonstrated [19] and also seen here was not observed for the sulphated compound, indicating that its formation does not directly reflect the levels of the free compound. However, statistical evaluation of levels of the free and sulphated compound in plasma from 60 patients (mean 17.1 ± 7.2 ng/ml and 16.7 ±

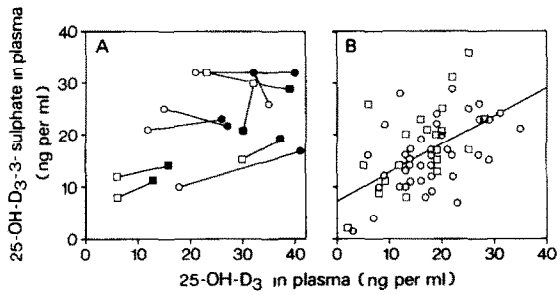

Fig.3. Concentration (ng/ml) of unconjugated and sulphated 25-hydroxyvitamin D_3 in plasma from (A) 10 apparently healthy human subjects (age 26–35 years) and (B) from 60 patients (age 18–82 years). Plasma samples were collected from men (squares) and women (circles) during the winter (unfilled symbols) and summer (filled symbols) season. Correlation coefficient was 0.52 for both groups and $y = 7.48 \pm 0.54x$ [20].

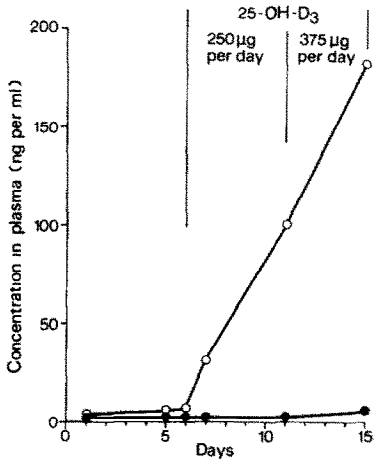

Fig.4. Concentration (ng/ml) of unconjugated (open circles) and sulphated (filled circles) 25-hydroxyvitamin D_3 in plasma of a woman given orally 250–375 μg of 25-hydroxyvitamin D_3 daily.

7.0 ng/ml, respectively) provided strong evidence for a relationship between the 2 compounds (fig.3). Great differences in concentrations of the 2 compounds have been observed in plasma from a few subjects, e.g. the levels of the free and sulphated 25-hydroxyvitamin D_3 in plasma from a 4-month-old child (given vitamin D_2 as supplement) were 0.7 and 12.6 ng/ml, respectively. The significance of this result is not yet understood.

3.3. *Possible origin in man*

The biosynthesis of 25-hydroxyvitamin D_3 3-sulphate in man is not known. In analogy with other steroid metabolites, sulphation of 25-hydroxyvitamin D_3 in the liver might be expected. Sulphation of vitamin D_2 has been demonstrated following incubation with rat liver homogenates [21]. However, when 25-hydroxyvitamin D_3 was administered orally to 2 women with hypocalcemia no significant increase of concentrations of the sulphated compound in plasma was observed (fig.4). These results show that 25-hydroxyvitamin D_3 is not readily sulphated in vivo and indicate that 25-hydroxyvitamin D_3 sulphate may be formed from a sulphated precursor. Cholesterol sulphated in the 3-position is widely distributed in the body [22], and may be considered as a potential precursor [22]. Formation from this compound could possibly explain the previous failure of detecting the sulphate following administration of radiolabelled vitamin D_3. A dietary source of the compound or its precursor cannot be excluded, and vitamin D_3 sulphate has previously been detected in significant amounts in dairy milk [5,6].

Vitamin D_3 sulphate has also been found in milk from lactating women [5–8] but its proposed role in preventing rickets in breast-fed infants has been questioned [23,24]. Recent studies have shown that vitamin D_3 (and D_2) sulphate possesses little or no biological activity [25–27]. In an early study, 25-hydroxyvitamin D_3 sulphate was detected as a minor component in milk from women [6]. When reanalyzed by the same procedure as described here for plasma, levels in milk collected from 4 women a few days after delivery were much lower than in plasma and below the detection limit (<1 ng/ml) in all samples. Therefore, it does not seem likely that 25-hydroxyvitamin D_3 sulphate is the 'antirachitic agent' in human milk.

This study has shown that the sulphate of 25-hydroxyvitamin D_3 is a quantitatively important form of secosteroids in man. Whether it possesses biological activity, reflects the presence of a new biosynthetic pathway to hydroxylated vitamin D_3, or is an inactive excretion product remains to be established.

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