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SPECIFIC MASS FRAGMENTOGRAPHIC ASSAY FOR 25,26-DIHYDROXYVITAMIN D IN HUMAN PLASMA USING A DEUTERATED INTERNAL STANDARD

RUTH D. COLDWELL, D.J.H. TRAFFORD and H.L.J. MAKIN*

The Steroid Laboratory, Department of Chemical Pathology, The London Hospital Medical College, Turner Street, London E1 2AD (U.K.)

M.J. VARLEY

The Steroid Laboratory, Department of Chemical Pathology, The London Hospital Medical College, Turner Street, London E1 2AD (U.K.) and The Chemistry Department, Westfield College, Kidderpore Avenue, London NW3 7ST (U.K.)

and

D.N. KIRK

The Chemistry Department, Westfield College, Kidderpore Avenue, London NW3 7ST (U.K.)

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SUMMARY

A specific mass fragmentographic assay for the measurement of 25,26-dihydroxyvitamin D_3 [$25,26(OH)_2D_3$] in human plasma, using a stable isotope labelled internal standard {[$26,27\text{-}^2H_5$]$25,26(OH)_2D_3$}, is described.

Plasma samples (5 ml) were extracted with acetonitrile and applied to a C_{18} Sep-Pak cartridge, from which the vitamin D metabolites were eluted with methanol. The metabolites were then applied to a Sep-Pak SIL cartridge and three fractions were collected. The most polar fraction, containing the polyhydroxylated metabolites, was further purified by high-performance liquid chromatography on Zorbax SIL. The eluent containing $25,26(OH)_2D_3$ was collected, and the 25,26-*n*-butylboronate cyclic ester 3-trimethylsilyl ether derivative was formed. Gas chromatography—mass spectrometry was carried out, monitoring the intensities of the ions at *m/z* 449 and *m/z* 454 (for the internal standard). These ions represent the loss of a methyl group and the 3-silanol group, $(M - 90 - 15)^+$.

The minimum limit of detection of the assay was estimated to be approximately 0.05 μg/l.

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Inter-assay (3.7%) and intra-assay (8.0%) precision was acceptable and added $25,26(OH)_2D_3$, over the concentration range 0.5—1.5 μg/l, was recovered quantitatively. The plasma $25,26(OH)_2D_3$ level was estimated in 26 healthy volunteers and ranged from 0.05 to 1.30 μg/l, with a mean value of 0.54 μg/l.

INTRODUCTION

25,26-Dihydroxyvitamin D_3*, first isolated in 1970 by Suda et al. [1], is one of the polyhydroxylated vitamin D metabolites formed in the kidney [2] and possibly in extra-renal sites [3] by 26-hydroxylation of $25(OH)D_3$. Its precise role is unknown but it is probably a catabolic product formed, like $24,25(OH)_2D_3$, as an alternative to the active calcium homeostatic hormone $1,25(OH)_2D_3$. Although $25,26(OH)_2D_3$ circulates in normal human plasma in low nanomolar concentrations, it has not been widely studied, probably because of the lack of specific methods for assay. It is usually measured by competitive protein binding (CPB) assay using vitamin D binding globulin [4, 5]. This protein is relatively non-specific and therefore extensive purification is necessary prior to assay. A radioimmunoassay using an antiserum for $1,25(OH)_2D_3$ has also been described [6]. Because of the presence of increasing numbers of metabolites of vitamin D which are being described [7], the majority of which involve side-chain modifications, it is becoming more and more difficult to ensure that a sample prepared for assay is free of interfering metabolites. More specific assay techniques for $25,26(OH)_2D$ need to be developed in order to establish whether it possesses any physiological significance. Gas chromatography—mass spectrometry (GC—MS) offers a highly specific physicochemical means of assay and could be used as a definitive reference method against which other assays could be evaluated. This paper is the first report of the use of a method other than protein binding assay or immuno assay for the measurement of $25,26(OH)_2D$ in human plasma. A preliminary report of this procedure has already been published [8].

MATERIALS AND METHODS

Materials

25,26-Dihydroxy[26,27-$^2H_5$]vitamin D_3 was synthesised from [26,27-$^2H_6$]cholest-5-ene-3β,25-diol [9] by the route outlined in Fig. 1. Non-labelled standard $25,26(OH)_2D_3$ was also synthesised by the same route using non-deuterated cholest-5-ene-3β,25-diol. Both deuterated and non-deuterated standards were mixtures of the 25*R* and 25*S* isomers. The $25S,26(OH)_2D_3$ was a generous gift from Dr. M. Uskokovic (Hofmann-LaRoche, Nutley, NJ, U.S.A.). Labelled and unlabelled standards were purified by high-performance liquid chro-

*Systematic and trivial names of vitamin D and its metabolites used in this paper are as follows: vitamin D_2 (9,10-seco-ergosta-5,7,10(19),22-tetraen-3β-ol): D_2, vitamin D_3 (9,10-seco-cholesta-5,7,10(19)-trien-3β-ol): D_3, 25-hydroxyvitamin D: 25(OH)D, 24,25-dihydroxyvitamin D: $24,25(OH)_2D$, 25,26-dihydroxyvitamin D: $25,26(OH)_2D$, 1α,25-dihydroxyvitamin D: $1,25(OH)_2D$. The term D is used when there is no need to distinguish between D_2 and D_3.

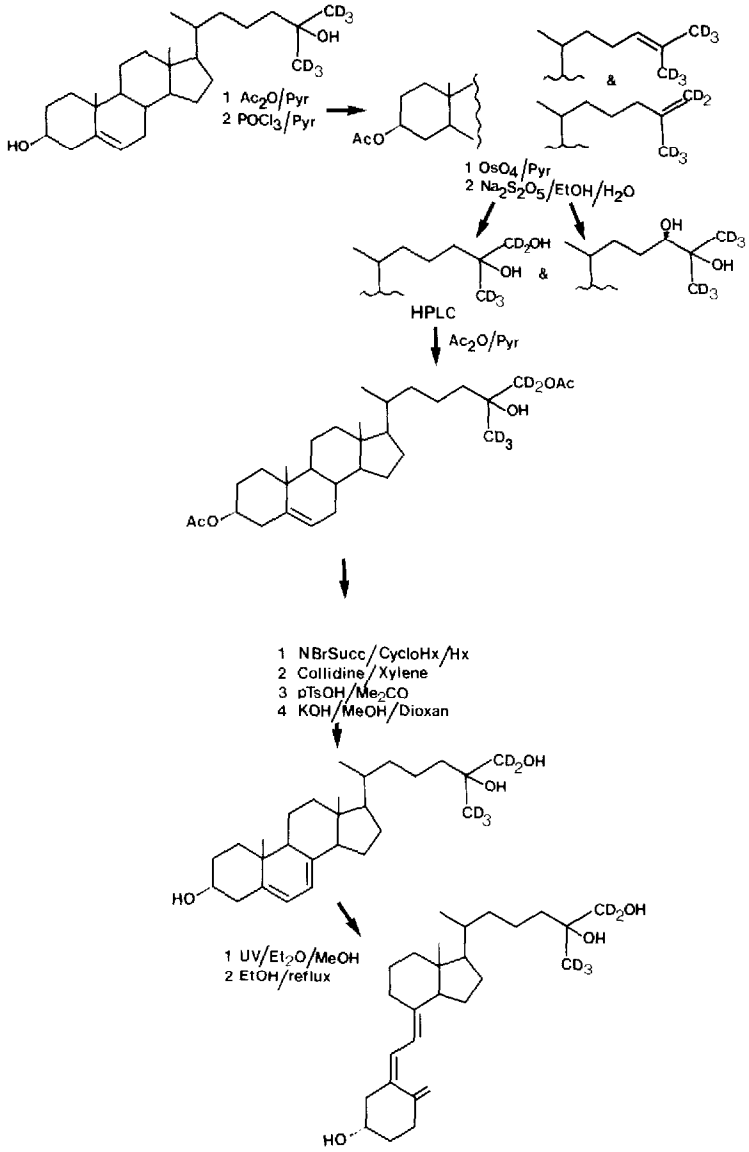

Fig. 1. Outline of the synthesis of $[^2H_5]25,26(OH)_2D_3$ for use as an internal standard. Non-standard abbreviations used are: Ac_2O = acetic anhydride; Pyr = pyridine; EtOH = ethanol; MeOH = methanol; NBrSucc = N-bromosuccinimide; CycloHx = cyclohexane; Hx = hexane; pTsOH = *p*-toluenesulphonic acid, Me_2CO = acetone, UV = ultraviolet light.

matography (HPLC) on receipt and repurified monthly thereafter. Concentrations of 5,7-diene steroids in solution were determined by UV absorbance at 264 nm, assuming a molar extinction coefficient at this wavelength of 18,300 [10]. Extraction solvents (AR grade wherever possible, from BDH , Poole, U.K.) and HPLC solvents (Rathburn Chemicals, Walkerburn, U.K.) were redistilled before use unless otherwise specified. Acetonitrile was shaken with activated charcoal (Sigma London, Poole, U.K.) and filtered immediately before use. Sep-Pak C_{18} and Sep-Pak SIL cartridges were purchased from Waters Assoc. (Northwich,

U.K.) and were used as described previously [11]. *n*-Butyl-, methyl-, and phenylboronic acids and bis(trimethylsilyl)trifluoracetamide (BSTFA) (Pierce and Warriner, Chester, U.K.) were used without further purification.

High-performance liquid chromatography (HPLC) was carried out as described previously [12] using a straight-phase Zorbax SIL column, eluted with isopropanol—methanol—hexane solvent, as described below.

Mass fragmentography was carried out as previously described [13] using a Model 2091 gas chromatograph—mass spectrometer (LKB Instruments, Croydon, U.K.).

All glassware was silanised by soaking overnight in 1% (v/v) dimethyldichlorosilane in toluene and washed with methanol. Blood from apparently healthy volunteer workers was collected into heparinised containers and the plasma separated. Unless analysed immediately, plasma was stored at −20°C under nitrogen.

Sample extraction and purification (Fig. 2)

Approximately 50 ng of [$^2H_5$]25,26(OH)$_2$D$_3$ were added to 5 ml plasma and equilibrated at room temperature for 10 min. An equal amount of freshly charcoal-washed acetonitrile was added and the samples were left to stand for a further 60 min, with occasional vortex-mixing. The precipitate formed was removed by centrifugation (7000 *g*, 15 min) and the supernatant added to 2.5 ml of 0.2 *M* acetate buffer, pH 5.6. This extract was applied to a C_{18} Sep-Pak cartridge which had been pre-washed first with 20 ml methanol and then 10 ml water. After application of the extract, the cartridge was washed with 3 ml methanol—water (60:40, v/v), and the vitamin D metabolites were eluted with 6 ml methanol.

The methanol extract was evaporated to dryness, redissolved in 300 µl isopropanol—hexane (1:99, v/v) and loaded at 4°C onto a Sep-Pak SIL cartridge prewashed with 10 ml methanol and 10 ml isopropanol—hexane (1:99, v/v). The SIL cartridge was washed with 13 ml of the same solvent. Then 10 ml isopropanol—hexane (3:97, v/v) were added to elute 25(OH)D. The polyhydroxylated metabolites, including 25,26(OH)$_2$D$_3$, were eluted with 10 ml isopropanol—hexane (30:70, v/v).

The solvent was evaporated and the residue was redissolved in 100 µl methanol—isopropanol—hexane (3:7:90, v/v/v) and injected onto a Zorbax SIL HPLC column. 24,25(OH)$_2$D$_3$, 25,26(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$ were completely separated in this system. The relative retention time of 25,26(OH)$_2$D$_3$ was approximately 11 min. The fractions containing 25,26(OH)$_2$D$_3$ (10—12 min) were collected, evaporated to dryness and either stored in 1 ml ethanol at −20°C or immediately prepared for GC—MS.

Derivatisation and assay

The dried extracts and a series of standards (50 ng deuterated 25,26(OH)$_2$D$_3$ + 0, 3, 5, 8 and 10 ng 25,26(OH)$_2$D$_3$), were converted into *n*-butylboronate (nBBA) cyclic ester 3-trimethylsilyl (TMSi) ether derivatives, by incubating with 30 µl *n*-butyl boronic acid in tetrahydrofuran (1 mg/ml) at room temperature for 30 min. The solvent was removed and the residue taken up in 30 µl BSTFA. After 30 min, 10-µl aliquots were injected onto the GC—MS

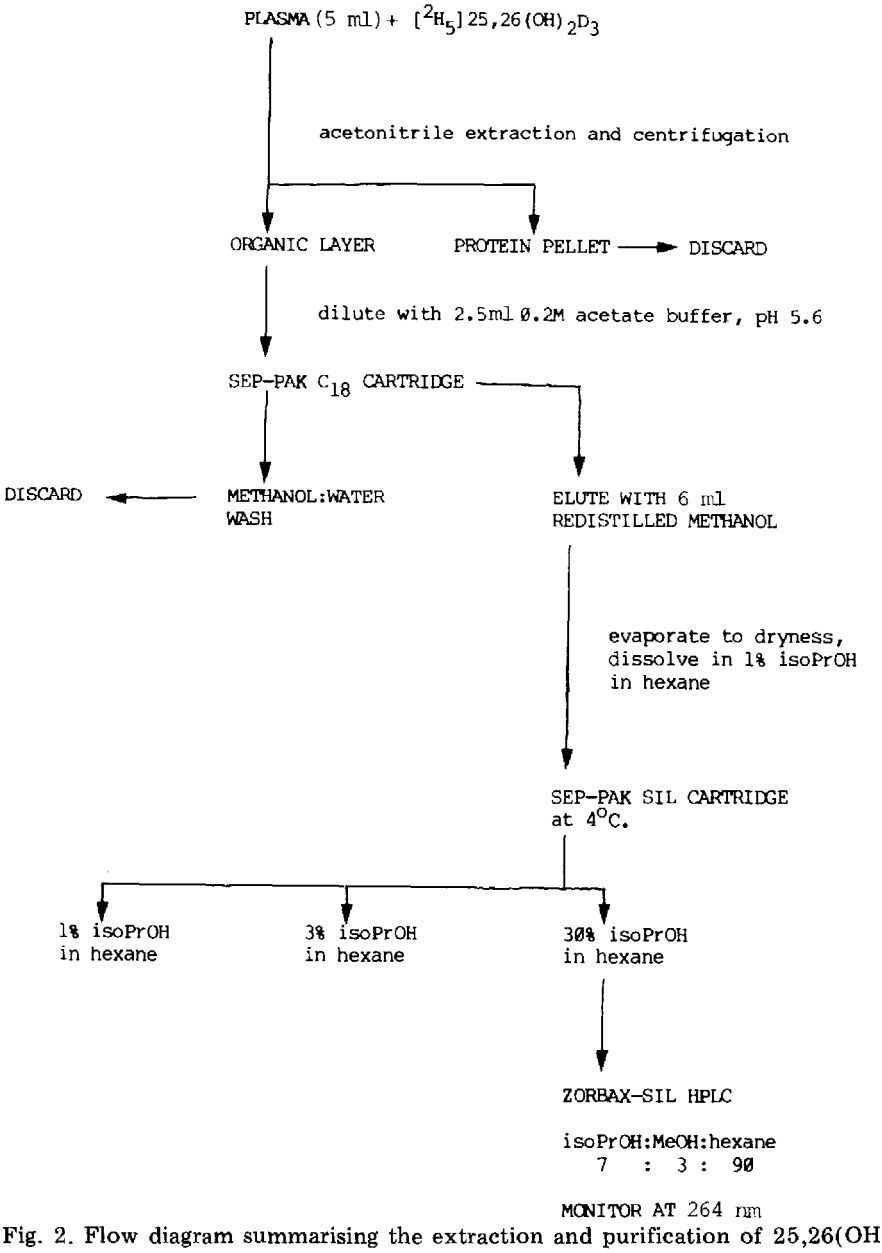

Fig. 2. Flow diagram summarising the extraction and purification of 25,26(OH)$_2$D prior to GC—MS. The HPLC fraction containing 25,26(OH)$_2$D (10—12 min after injection) was collected and analysed by GC—MS as described in the text. IsoPrOH = isopropanol; MeOH = methanol.

system. These derivatives do not appear to be very stable and therefore GC—MS was carried out immediately after formation. The pyro-isomer of the 25,26-*n*-butylboronate cyclic ester 3-trimethylsilyl ether derivative of 25,26(OH)$_2$D$_3$ (25,26(OH)$_2$D$_3$-nBBA-3-TMSi) gave a 20-eV mass spectrum (Fig. 3) with a base peak at *m/z* 449 (*m/z* 454 for the deuterated standard). These ions, $(M - 90 - 15)^+$, are formed from the molecular ion by the loss of a methyl group and the 3-silanol group. The ions at *m/z* 449 and *m/z* 454 were

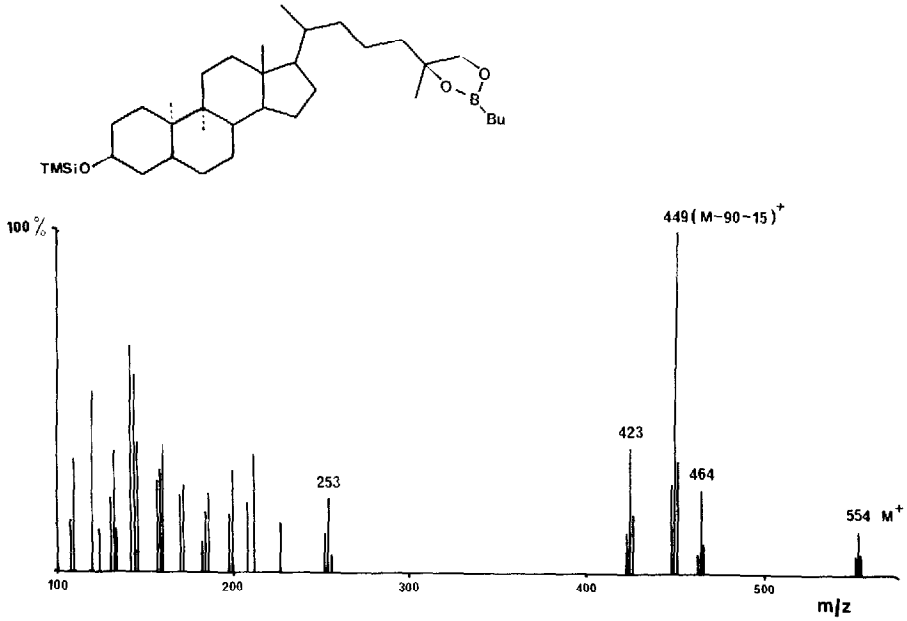

Fig. 3. The 24-eV mass spectrum of the pyro isomer of 25,26$(OH)_2D_3$-nBBA-3-TMSi using the LKB 2091 gas chromatograph—mass spectrometer. The derivative was analysed by GC—MS as described in the text and a mass spectrum (*m/z* 100—600) was obtained at the maximum of the emergent peak. Peak heights have been normalised with respect to the base peak (*m/z* 449, 100%). Peaks of less than 5% are not recorded.

monitored and the peak heights of the pyro-isomers of 25,26$(OH)_2D_3$ and the internal standard were measured. After correction for channel amplification, the ratio, peak height of ion at *m/z* 449/peak height of ion at *m/z* 454 for the series of standards and the plasma samples was calculated. A standard curve, relating this ratio to the amount of standard 25,26$(OH)_2D_3$, was plotted, from which the amount of 25,26$(OH)_2D_3$ in each sample could be determined.

Derivatisation

In a previous publication [13] the GC—MS of isotachysterol isomers of some vitamin D metabolites derivatised as trimethylsilyl ethers was described. These derivatives fragment to give a relatively high-intensity molecular ion and preliminary mass fragmentographic studies were carried out on 24,25$(OH)_2D_3$. At the time no standard 25,26$(OH)_2D_3$ was available and it was suggested that these two metabolites separated in the GC system used. When standard 25,26$(OH)_2D_3$ became available, it was discovered that 24,25$(OH)_2D_3$ and 25,26$(OH)_2D_3$ had identical retention times when applied to the gas chromatograph as tri-trimethylsilyl ethers (tri-TMSi). However, these two metabolites were well separated as *n*-butylboronate cyclic ester 3-trimethylsilyl ethers [14]. The use of these derivatives also increased specificity as cyclical boronates will only form across hydroxyls in close proximity, although not necessarily only on adjacent carbons [15].

Preparation of the trimethylsilyl ether, methylboronate and phenylboronate derivatives of 24,25$(OH)_2D_3$ and 25,26$(OH)_2D_3$ was also attempted. The

TABLE I

GAS CHROMATOGRAPHIC RETENTION TIMES OF METHYL-, *n*-BUTYL- AND PHENYLBORONATE-3-TMSi DERIVATIVES OF $24,25(OH)_2D_3$ AND $25,26(OH)_2D_3$

Derivative*	Retention time**	
	Pyro isomer	Isopyro isomer
$24,25(OH)_2D_3$		
Methylboronate-3-TMSi	1.6137	2.3357
n-Butylboronate-3-TMSi	2.6250	3.5625
Phenylboronate-3-TMSi	5.9403	8.0450
$25,26(OH)_2D_3$		
Methylboronate-3-TMSi	Not formed	
n-Butylboronate-3-TMSi	3.1797	4.2656
Phenylboronate-3-TMSi	6.6522	8.8551

*The appropriate 24,25- or 25,26-cyclic boronate ester 3-trimethylsilyl ether was prepared as described in the text and analysed by GC using an OV-1 column at 275°C (for methylboronate derivatives) or 300°C.
**Relative to the retention time of pyro-D_2-TMSi (1.7 min at 300°C and 7 min at 275°C).

methylboronate cyclic ester 3-trimethylsilyl ether derivative of $25,26(OH)_2D_3$, however, could not be formed. The mass spectrum obtained from $25,26(OH)_2D_3$ after reaction with methylboronic acid and BSTFA was the same as that obtained from $25,26(OH)_2D_3$ reacted with BSTFA alone, suggesting an inability to form a methylboronate cyclic ester across the hydroxyls on carbons 25 and 26, or subsequent removal by reaction with BSTFA [16]. The phenylboronate derivatives of both metabolites had very long retention times in GC system used (Table I). The mass spectra of methyl- and phenylboronate TMSi derivatives showed similar fragmentations to those shown in Figs. 3 and 4 and no improvement in the intensities of high-mass ions were observed. *n*-Butylboronate-3-TMSi derivatives of $25,26(OH)_2D_3$ were therefore used.

Mass spectrometry

The use of isotachysterol isomers of vitamin D metabolites increases sensitivity due to the formation of a single peak on GC, and, in the case of the tri-TMSi derivatives, by increasing the intensity of the molecular ion, in comparison to that of the pyro isomer [17]. However, using the LKB 2091 gas chromatograph—mass spectrometer, the isotachysterol isomer of $25,26(OH)_2D_3$-nBBA-3-TMSi gave a normalised 24-eV mass spectrum with a base peak at *m/z* 253 and greatly reduced intensities of ions of high mass (Fig. 4). The ion at *m/z* 253 is formed by the loss of the 3-silanol group and the entire C_8 side-chain. This high-intensity ion, however, could not be used for mass fragmentography because the deuterium label on the side-chain was lost during this fragmentation. The pyro isomer was therefore used, monitoring the ion at *m/z* 449 (*m/z* 454 for the internal standard), which gave enhanced sensitivity over that of the molecular ion (*m/z* 632) of $25,26(OH)_2$-isotachysterol$_3$-triTMSi.

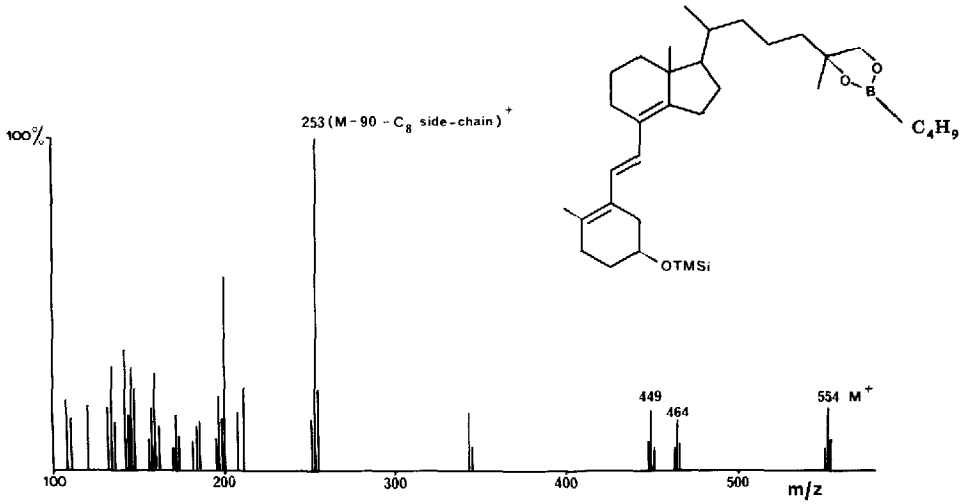

Fig. 4. Normalised mass spectrum of 25,26(OH)$_2$-isotachysterol$_3$-nBBA-3-TMSi obtained at 24 eV using the LKB 2091 gas chromatograph—mass spectrometer. Details are as given in legend to Fig. 3.

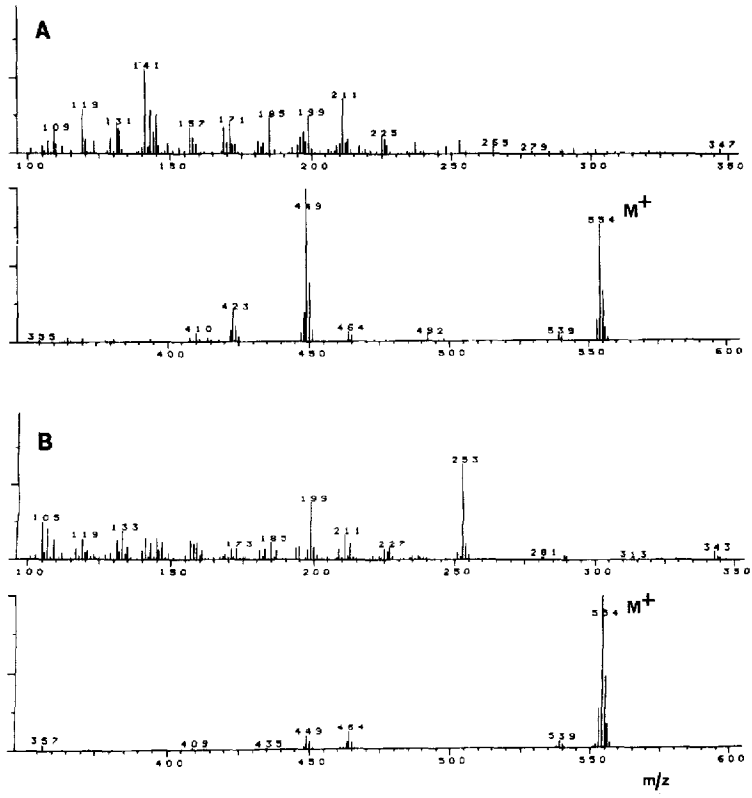

Fig. 5. Normalised mass spectrum of (A) pyro-25,26(OH)$_2$D$_3$-nBBA-3-TMSi and (B) 25,26(OH)$_2$-isotachysterol$_3$-nBBA-3-TMSi obtained at 20 eV using a Nermag R10-10C gas chromatograph—mass spectrometer. Mass spectra were obtained in a similar fashion to those illustrated in Figs. 3 and 4. The peak heights have been normalised with respect to the base peaks (*m/z* 449, 100% for A; *m/z* 554, 100% for B). The fragmentation is qualitatively, but not quantitatively, similar to those obtained in Figs. 3 and 4.

During an evaluation of other mass spectrometers, spectra for $25,26(OH)_2D_3$-nBBA-3-TMSi were obtained on two different quadrupole instruments (Model R10-10C, Nermag SN, Rueil-Malmaison, France and Model 12250, VG Analytical, Manchester, U.K.). Fig. 5 shows the normalised 20-eV mass spectra of pyro and isotachysterol isomers of $25,26(OH)_2D_3$-nBBA-3-TMSi obtained using the Nermag R10-10C. The VG 12250 produced similar mass spectra at 24 eV to those obtained on the LKB 2091 (Figs. 3 and 4), although the pyro isomer showed a molecular ion of greater intensity. At 70 eV, mass spectra produced by the Nermag R10-10C were the same as the 24-eV spectra produced by the LKB 2091 and the VG 12250 (Figs. 3 and 4). On the Nermag R10-10C, the base peak of the 20-eV mass spectrum of the $25,26(OH)_2$-isotachysterol$_3$-nBBA-3-TMSi derivative was the molecular ion m/z 554. It has not yet been possible to assess the relative intensities of the ions produced in the different mass spectrometers. However, it would appear that the Nermag R10-10C offers some advantages since it would allow the use of $25,26(OH)_2$-isotachysterol-nBBA-3-TMSi derivatives, thus avoiding the production of two peaks on the gas chromatograph. Similar differences between the mass spectra obtained on these three instruments were obtained for $24,25(OH)_2D_3$-nBBA-3-TMSi derivatives. Such differences in fragmentation are unexpected and may be due to differences in ion source geometry.

RESULTS

Normal values

The standard curve relating peak height ratio to quantity of $25,26(OH)_2D$ injected was always linear over the range 0—10 ng (e.g. y (peak height ratio) = $0.018x$ (ng of $25,26(OH)_2D$) + 0.0008, correlation coefficient was 1.0017 ($P < 0.001$). Examples of traces obtained from a water blank taken through the procedure, and two plasma samples are illustrated in Fig. 6. Radiolabelled $25,26(OH)_2D$ was not available to us and therefore it was not possible to assess overall recovery with any accuracy. However, rough assessment of the recovery of added deuterated $25,26(OH)_2D_3$ indicated that recoveries were very similar to those previously obtained for $24,25(OH)_2D_3$ [14] at around 60%. The minimum limit of detection of the assay was estimated to be approximately 0.05 μg/l. Plasma concentrations of $25,26(OH)_2D_3$ were measured in fifteen healthy volunteers in the U.K. — a mean value of 0.39 μg/l with a range from 0.05 to 0.79 μg/l was obtained. Samples were also obtained from eleven sun-baked Australians (by courtesy of Professor S. Posen, Miss Dianne Lissner and Mrs Angelike Trube, Royal North Shore Hospital, N.S.W., Australia) and the mean value obtained was 0.76 μg/l with a range from 0.30 to 1.30 μg/l. Estimations of the level of 25(OH)D were also carried out on these plasma samples and linear regression analysis showed a close correlation between the levels of $24,25(OH)_2D_3$, $25(OH)D_3$ and $25,26(OH)_2D_3$ [y ($25,26D_3$) = $0.013x$ ($25D_3$) + 0.137, r = 0.8777; y ($25,26D_3$) = $0.196x$ ($24,25D_3$) + 0.137, r = 0.9234].

Specificity

The extraction procedure prior to derivative formation and GC—MS includes

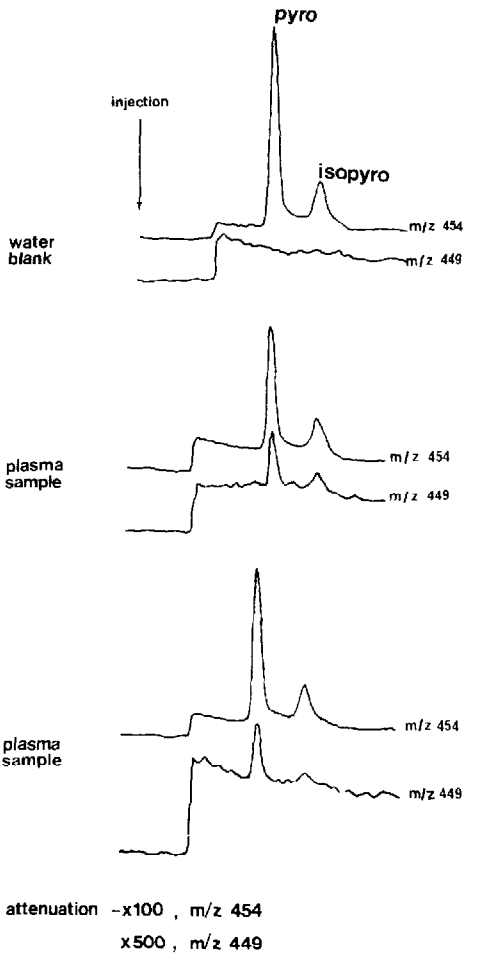

Fig. 6. Representative mass fragmentograms from a water blank and two plasma samples processed as described in the text. No non-deuterated $25,26(OH)_2D_3$-nBBA-3-TMSi was detected in the *m/z* 449 channel of the water blank (upper trace). The plasma samples illustrated had $25,26(OH)_2D_3$ concentrations of 0.81 μg/l (middle trace) and 0.78 μg/l (bottom trace). Amplification settings for each channel are given at the bottom of the Fig. The retention time of the pyro peak was approximately 6 min.

an HPLC step. This is included because this method has been developed as part of a profile procedure in which all the major metabolites of vitamin D can be separated and assayed individually. However, extracts of plasma samples were found to be clean enough after fractionation by Sep-Pak SIL for the $25,26(OH)_2 D_3$ levels to be measured at this stage, together with $24,25(OH)_2D_3$. Some potential contaminants which may interfere in CPB assay also elute from the HPLC in a similar position to $25,26(OH)_2D_3$, but it is unlikely that any would form cyclic boronate esters and would run in the same position on GC and fragment to give similar ions on GC—MS. Absolute specificity cannot however be assured until standards for all the possibly interfering metabolites become available.

An indirect method was used to assess the effect of any $25,26(OH)_2D_2$

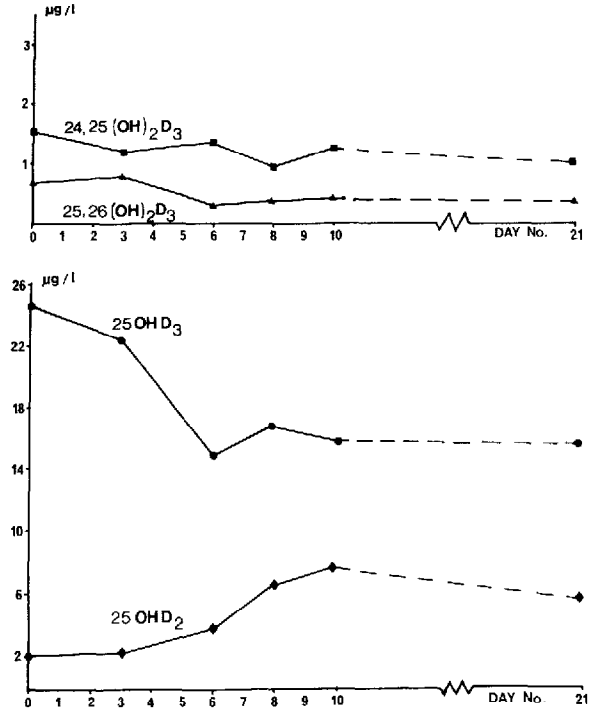

Fig. 7. Plasma concentrations of $25(OH)D_2$, $25(OH)D_3$, $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$ in a normal person taking oral D_2 (day 1 to day 9, 12,000 I.U. per day).

which might be present in the sample, because no standard $25,26(OH)_2D_2$ was available to us. A normal volunteer was dosed with 12,000 I.U. vitamin D_2 per day for nine days, and the varying plasma levels of the vitamin D metabolites were measured during and after this time. The appropriate $(M - 90 - 15)^+$ ion for $25,26(OH)_2D_2$ was monitored, but no peak was detected. Equally, no significant rise in the level of $25,26(OH)_2D_3$ was observed, indicating that if metabolites of D_2 were formed under these conditions, they did not interfere with the assay (Fig. 7).

Reproducibility

Within- and between-batch reproducibility studies were carried out and the results obtained are given in Table II. Plasma samples used in the between-batch study were stored under nitrogen at −20° C and assayed over a period of seven weeks.

TABLE II

REPRODUCIBILITY STUDIES

Two samples were assayed six times. For inter-assay studies the plasma sample was stored at −20° C and analysed over a period of seven weeks.

	Mean value (μg/l)	Standard deviation	Coefficient of variation (%)
Within-batch (intra-assay) precision	0.28	0.01	3.7
Between-batch (inter-assay) precision	0.24	0.02	8.0

TABLE III

RECOVERIES OF STANDARD 25,26(OH)$_2$D$_3$ ADDED TO PLASMA

Values recorded are those from four experiments. Standard 25,26(OH)$_2$D$_3$ was added to 5 ml plasma to obtain the concentrations indicated. The plasma was incubated for 60 min at room temperature and assayed as described in the text.

Concentration (μg/l)		Percentage recovered (mean ± S.D.)
Added	Recovered (mean ± S.D.)	
0.5	0.52 ± 0.04	103.6 ± 7.7
1.0	1.02 ± 0.04	101.8 ± 3.9
1.5	1.55 ± 0.09	103.6 ± 5.6

Recovery experiments

Standard 25,26(OH)$_2$D$_3$ was added to plasma at concentrations of 0.5, 1.0 and 1.5 μg/l and assayed as described above. The recovery of added 25,26(OH)$_2$D$_3$ was calculated and the results are given in Table III.

DISCUSSION

This paper reports the development and evaluation of the first specific assay for 25,26(OH)$_2$D in human plasma which does not utilise relatively non-specific binding proteins or antibodies and does not rely on competitive binding assay. It thus provides the means for the evaluation of the specificity of existing methodology or alternatively offers the opportunity to measure specifically the levels of this metabolite in human plasma under various physiological and pathological conditions. Although the method describes the estimation of 25,26(OH)$_2$D$_3$, it can also be used to measure 25,26(OH)$_2$D$_2$ providing standards are available. Both these compounds can be measured in a single GC run by also monitoring the intensity of the appropriate mass fragment from 25,26(OH)$_2$D$_2$. It has been demonstrated that with a mass spectrometer with sufficient multiple-ion detection channels, it is possible to measure 24,25(OH)$_2$D and 25,26(OH)$_2$D simultaneously immediately after the separation on Sep-Pak SIL without the need for an HPLC step. If a mass spectrometer with many ion monitoring channels is used, it is possible not only to measure the levels of these metabolites in plasma extracts but also to verify the specificity of the peak being measured. If a number of different ions are monitored simultaneously, a pure peak uncontaminated with interfering material will give peak height ratios which are the same as those calculated from the mass spectrum of the pure compound. The more peaks which are monitored and thus the more ratios which agree with those found in the pure compound, the greater the demonstrated specificity. In addition the mass spectrometer can be used in the less sensitive mass chromatography mode and complete mass spectra can be collected at regular intervals throughout the chromatographic run. Peaks with the correct retention time can therefore be checked to ensure that they are homogeneous and that the mass spectrum at the peak corresponds with that of the pure standard. Thus GC—MS is a very

powerful method for the specific measurement of biological compounds and can be used as a definitive method against which other possibly less-specific procedures can be evaluated. A similar method using GC—MS has been described for the measurement of plasma levels of $24,25(OH)_2D$ [14].

It is not clear whether $25,26(OH)_2D$ has any physiological function or whether it represents another catabolic pathway from 25(OH)D. Although $25,26(OH)_2D$ was reported to stimulate the retention of calcium by the intestine [1], it is considerably less effective in this respect than the active calcium homeostatic hormone, $1,25(OH)_2D$. Other physiological roles for $25,26(OH)_2D$ have been suggested [18, 19] but no firm evidence has been provided. The possibility of measuring this metabolite specifically offers the opportunity to establish whether it has any important physiological role or not.

Naturally occurring $25,26(OH)_2D$ has been variously identified as 25*R* [20], 25*S* [21] and as an epimeric mixture of 25*R* and 25*S* isomers [22]. In the GC—MS procedure described here, the deuterated internal standard is a mixture of 25*R* and 25*S* isomers, but this has caused no problems since this isomeric mixture does not separate in any of the chromatographic systems used. The use of the 25*S* isomer or a mixture of 25*S* and 25*R* isomers as standards has given identical results.

The GC—MS results obtained from normal plasma samples are very similar to those reported by Markestad [23] but slightly higher than the values reported using radioimmunoassay [6]. Interestingly, the correlation between the levels of $25(OH)D_3$ and $25,26(OH)_2D_3$ found here is almost exactly the same as that reported by Fraher et al. [6]. Correlation between $24,25(OH)_2D$ and $25,26(OH)_2D$ has also been reported previously [6, 23] but the correlation coefficients are closer to 1.000 in the present study. The minimum detection limit using 5 ml of plasma using radioimmunoassay [6] was 0.04 μg/l which is very similar to that reported here (0.05 μg/l). However, as expected, the precision achieved by GC—MS was substantially better than that of radioimmunoassay. Although absolute specificity has not been demonstrated here, it is clear that GC—MS can provide sensitive and highly specific methods for the assay of $25,26(OH)_2D$ and other metabolites of vitamin D in a variety of body fluids. The use of modern computerised mass spectrometers with improved detection systems, and different methods of ionisation offers the possibility of greatly improved sensitivity without any sacrifice in specificity. Use of high-resolution mass spectrometry can, by reducing the signal-to-noise ratio, also improve sensitivity and a method for the measurement of oestradiol-17β in 2 ml of plasma with a minimum detectable limit of 0.005 μg/l, ten times lower than that described here, has been reported [24].

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REFERENCES

1 T. Suda, H.F. DeLuca, H.K. Schnoes, Y. Tanaka and M.F. Holick, Biochemistry, 9 (1970) 4776.
2 J.L. Napoli, R.T. Okita, B.S. Masters and R.L. Horst, Biochemistry, 20 (1981) 5865.
3 R.M. Shepard, R.L. Horst, A.J. Hamstra and H.F. DeLuca, Biochem. J., 182 (1979) 55.
4 R.L. Horst, in R. Kumar (Editor), Vitamin D, Basic and Clinical Aspects, Martinus Nijhoff, Boston, MA, 1984, pp. 423.
5 D.A. Seamark, D.J.H. Trafford and H.L.J. Makin, J. Steroid Biochem., 14 (1981) 111.
6 L.J. Fraher, T.L. Clemens, S.E. Papapolous, J. Redel and J.L.H. O'Riordan, Clin. Sci., 59 (1980) 257.
7 J.L. Napoli and R.L. Horst, in R. Kumar (Editor), Vitamin D, Basic and Clinical Aspects, Martinus Nijhoff, Boston, MA, 1984, p. 91.
8 R.D. Coldwell, D.J.H. Trafford, H.L.J. Makin, M.J. Varley and D.N. Kirk, Abstracts of the Fifth International Symposium on Mass Spectrometry in the Life Sciences, Ghent, May 1984, p. 12.
9 M.J. Varley, D.N. Kirk, D.J.H. Trafford and H.L.J. Makin. J. Chem. Soc., Perkin Trans. I, (1963) 2563.
10 A.W. Norman, Vitamin D, The Calcium Homeostatic Hormone, Academic Press, New York, 1979, p. 50.
11 C.J. Rhodes, P.A. Claridge, D.J.H. Trafford and H.L.J. Makin, J. Steroid Biochem., 19 (1983) 1349.
12 D.A. Seamark, D.J.H. Trafford, P.G. Hiscocks and H.L.J. Makin, J. Chromatogr., 197 (1980) 271.
13 D.A. Seamark, D.J.H. Trafford and H.L.J. Makin, Clin. Chim. Acta, 106 (1980) 51.
14 R.D. Coldwell, D.J.H. Trafford, H.L.J. Makin, M.J. Varley and D.N. Kirk, Clin. Chem., 30 (1984) 1193.
15 C.J.W. Brooks, W.J. Cole, T.D.V. Lawrie, J. MacLachlan, J.H. Borthwick and G.M. Barrett, J. Steroid Biochem., 19 (1983) 189.
16 W.C. Kossa, in R.W. Frei and J.F. Lawrence (Editors), Chemical Derivatisation in Analytical Chemistry, Vol. 1, Chromatography, Plenum Press, New York, 1981, p. 99.
17 H.L.J. Makin and D.J.H. Trafford, in R. Kumar (Editor), Vitamin D, Basic and Clinical Aspects, Martinus Nijhoff, Boston, MA, 1984, p. 497.
18 A.D. Care, D.W. Pickard, S.E. Papapolous, J.L.H. O'Riordan and J. Redel, J. Endocrinol., 78 (1978) 303.
19 J.A. Kanis, T. Cundy, R. Smith, G. Heynen, G.T. Warner, J. Lorains and R.G. Russell, Contr. Nephrol., 18 (1980) 192.
20 J. Redel, N. Bazely, Y. Tanaka and H.F. DeLuca, FEBS Lett., 94 (1978) 228.
21 J.J. Partridge, S-J. Shiuey, N.K. Chadha, E.G. Baggiolini, J.F. Blount and M.R. Uskokovic, J. Amer. Chem. Soc., 103 (1981) 1253.
22 N. Ikekawa, N. Koizumi, E. Oshima, S. Ishizuka, T. Takeshita, Y. Tanaka and H.F. DeLuca, Proc. Nat. Acad. Sci. U.S., 80 (1983) 5286.
23 T. Markestad, J. Clin. Endocr. Metab., 57 (1983) 755.
24 S.J. Gaskell and B.G. Brownsey, Clin. Chem., 29 (1983) 677.