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Mass Fragmentographic Assay for 25-Hydroxyvitamin D in Plasma Without Derivatization: Enhanced Sensitivity for Metabolites of Vitamins D_2 and D_3 After Pre-column Dehydration

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A mass fragmentographic method for the measurement in plasma of underivatized, dehydrated 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 was developed. Quantitative dehydration of vitamin D metabolites was achieved prior to gas chromatography using a new high-temperature injection system and a small precolumn packed with aluminium powder in the injection port, followed by mass fragmentography on an inexpensive bench-top mass spectrometer. Plasma (2 ml) was incubated with hexadeuteriated 25-hydroxyvitamin D_3 prior to acetonitrile extraction and purification using cartridges pre-packed with microparticulate silica using reversed- and normal-phase solvent systems. After purification, capillary gas chromatography mass spectrometry was carried out following dehydration of the secosteroids on the aluminium powder pre-column at 400 °C. High-intensity dehydrated molecular ions were produced which were used for selected ion monitoring. The assay sensitivity for 25-hydroxyvitamin D was approximately 1 ng ml^{-1}. The intra-assay variation was less than 7% and the recovery of added standard was quantitative. It is suggested that this method may be used to provide target values for much needed quality control schemes to monitor assays routinely used in the estimation of 25-hydroxyvitamin D. The behaviour of a number of other hydroxylated vitamin D metabolites, when injected without derivatization on to a gas chromatographic column, was also investigated.

INTRODUCTION

Vitamin D‡ and its metabolites are measured routinely by radioimmunoassay, competitive protein binding or radioreceptor assay, after varying degrees of extraction and purification.[1,2] However, many of these methods, although sometimes giving good intra-laboratory precision, have given very poor inter-laboratory figures, particularly for 25-hydroxyvitamin D.[3,4] In many cases accuracy has not been properly assessed and it has only been possible to examine the accuracy of many of the assays currently available since the introduction of gas chromatography-mass spectrometry (GC-MS) methods for the vitamin D analyte under consideration. Two surveys[3,5] of the methods used for the measurement of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D have demonstrated extremely poor inter-laboratory variation and indeed in some laboratories the precision was such that physiological variations in the analyte could not be determined.[5] The introduction of quality assurance for a wide variety of steriod hormone assays has proved to be extremely valuable in improving precision and accuracy. Assays for vitamin D and its metabolites could likewise be improved if proper quality assurance schemes were in place using target values provided by GC-MS.[6]

Methods have been developed, the majority by two groups in Europe, for the measurement of several vitamin D metabolites in human plasma by mass fragmentography, all using conventional magnetic sector mass spectrometers.[7] Although such techniques provide definitive methods which may be used to assess less rigorous assays, the equipment involved is expensive and hence not generally available for routine use. However, the advent of highly efficient, small-scale, bench-top mass spectrometers allows access to this most valuable analytical technique. GC-MS systems such as the Hewlett-Packard mass-selective detector, used in the present work, are moderately priced, simple to use and are linked to computer software capable of extensive data handling.

Vitamin D and its metabolites are secosteroids containing one or more hydroxyl groups in their structure. Gas chromatography of underivatized compounds may lead to adsorption of the secosteriods on the inert support of the column, resulting in poor peak shape or

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‡ Vitamin D_2 (ergocalciferol, 9,10-seco-5,7,10(19),22-ergostatetraen-3β-ol), D_2; vitamin D_3 (cholecalciferol, 9,10-seco-5,7,10(19),22-cholestatrien-3β-ol), D_3; 25-hydroxyvitamin D_2, 25-OHD_2; 25-hydroxyvitamin D_3, 25-OHD_3; 24,25-dihydroxyvitamin D_2,24,25-$(OH)_2D_2$; 24,25-dihydroxyvitamin D_3, 24,25-$(OH)_2D_3$; 25,26-dihydroxyvitamin D_2, 25,26-$(OH)_2D_2$; 25,26-dihydroxyvitamin D_3, 25,26-$(OH)_2D_3$; 1,25-dihydroxyvitamin D_3, 1,25-$(OH)_2D_3$. Unless it is required to distinguish between vitamin D_2 and vitamin D_3, the term vitamin D is used.

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destruction. Because of this, all previously published GC-MS methods involve the formation of derivatives prior to injection on to the column. These derivatives include pertrimethylsilyl ethers,[8] *tert*-butyl dimethylsilyl ethers[9] and *n*-butyl boronate ester–trimethylsilyl ethers.[10,11] The formation of derivatives, particularly mixed derivatives, can be time consuming and may lead to loss of the analyte. The choice of derivative for successful GC separation is not always the best choice for MS as derivatives which provide successful GC separation may not have the high-mass ions in their spectrum with the necessary high abundance for sensitive assay by single ion monitoring.

During previous studies on vitamin D metabolites by GC-MS using conventional packed columns, dehydration occurred from time to time. This was found to be due to the presence of broken glass on the top of the packing material.[12] This observation was investigated further using capillary GC and has led to the development of a dehydrating pre-column[13] which removes the need for sample derivatization prior to assay. Dehydration produces stable products for both the vitamin D_2 and vitamin D_3 series and mass spectra that are ideally suited for mass fragmentography. The dehydration method for 25-hydroxyvatimin D described here incorporates both dehydration and the use of a simple bench-top instrument.

EXPERIMENTAL

Materials

Standard 25-hydroxyvitamin D_2 (25-OHD_2) was isolated from patients taking large oral doses of vitamin D_2, extensively purified and its identity determined by mass spectrometry. 25,26-Dihydroxyvitamin D_2 was a generous gift from Dr E. B. Mawer (Department of Medicine, University of Manchester, UK). All other metabolites, including hexadeuteriated standards, were chemically synthesized as described previously.[14] Standards were purified by normal-phase high-performance liquid chromatography (HPLC) or solid-phase extraction using Sep-Pak SIL cartridges, dissolved in ethanol and their concentration determined by measuring the UV absorbance at 264 nm, using the molar extinction, coefficient of 18 200. Bond-Elut C18 extraction columns (200 mg) (Analytchem International) were obtained from Anachem (Luton, Beds., UK) and Sep-Pak SIL cartridges were purchased from Millipore (UK) (Watford, Herts., UK). Capillary gas chromatography mass spectrometry was carried out using a Hewlett-Packard HP 5890 gas chromatography fitted with an HP1 capillary column (WCOT non-selective silicone, 12.5 m × 0.3 mm i.d.) and an HP 5970 mass-selective detector attached to a ChemStation computer system capable of extensive data handling. Helium was used as the carrier gas at a flow-rate of 1 ml min^{-1}. All spectra were obtained by electron impact (EI) ionization monitoring the positive ions produced at an ionization energy of 70 eV. The ion source temperature was 200 °C. Standards and samples, prepared in sealed glass microvials (100 µl) (Hewlett-Packard, Winnersh, Berks., UK) were injected in the splitless mode, via an HP 7673 autoinjection system and a Jade septumless injector (SGE, Milton Keynes, Beds., UK). The derivatizing agent *n*-trimethylsilylimidazole (TMSI) was purchased from Pierce and Warriner (UK) (Chester, Cheshire, UK). HPLC-grade hexane and propan-2-ol were obtained from Rathburn Chemicals (Walkerburn, Scotland). All other extraction solvents were of analytical-reagent grade, from BDH (Poole, Dorset, UK) or May and Baker (Dagenham, Essex, UK). Absolute ethanol (98.86%, v/v) was purchased from Hayman (Witham, Essex, UK) and was redistilled before use. Glassware to be used during the drying of sample extracts or standards was silanized by soaking overnight in 1% (v/v) dimethyldichlorosilane (BDH) in toluene and rinsed thoroughly with methanol before use. Silanized glass-wool was prepared similarly, and was thoroughly dried in a vacuum oven at 37 °C before storage.

Method

Sample extraction. The method used for sample extraction is summarized in Fig. 1. Approximately 100 ng of [$^2H_6$]-25-hydroxyvitamin D_3 were added to 2 ml of plasma or serum, smaller samples being diluted to 2 ml with distilled water. After equilibration at room temperature for 30 min, the samples were extracted with an

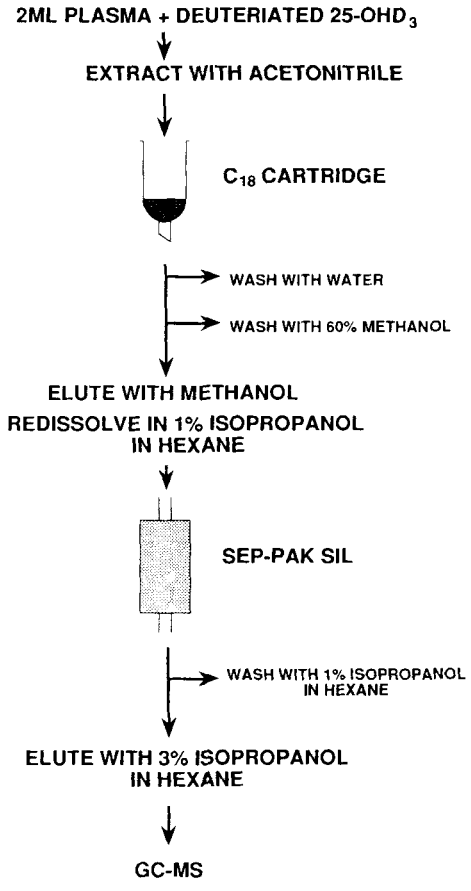

Figure 1. Flow diagram of sample extraction procedure.

equal volume of acetonitrile, vortex mixed and centrifuged at 4000 *g* for 10 min. The supernatant was made 65% aqueous with 0.4 M phosphate buffer pH 10.5 and applied to a Bond-Elut C18 cartridge which had been pre-washed with methanol and water. The cartridge was sequentially washed with 3 ml of water and 3 ml of methanol–water (60:40 v/v), and vitamin D and its metabolites were eluted with 3 ml of methanol. The methanol eluate was evaporated to dryness in a vacuum oven at 37 °C and the residue was dissolved in approximately 300 µl of propan-2-ol–hexane (1:99, v/v), then applied to a Sep-Pak silica cartridge which had been washed with both methanol and propan-2-ol–hexane (1:99, v/v). The Sep-Pak cartridge was washed with a further 10 ml of propan-2-ol–hexane (1:99, v/v) to remove vitamin D, cholesterol and other contaminants. 25-Hydroxyvitamin D was then eluted with 10 ml of propan-2-ol–hexane (3:97, v/v). More polar metabolites of vitamin D could be eluted with propan-2-ol–hexane (30:70, v/v).

Gas chromatography-mass spectrometry. Sample extracts were dried in a vacuum oven at 37 °C, then transferred in redistilled ethanol into silanized microvials prior to GC-MS. A series of standard solutions containing 100 ng of $[^2H_6]$-25-hydroxyvitamin D_3 and various amounts of 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 was also prepared. Ethanol was removed in the heated vacuum oven and all standards and samples were reconstituted in small volumes of redistilled ethanol (typically 15–50 µl). The microvials were sealed and 3 µl aliquots were auto injected in the splitless mode on to the GC column via a Jade septumless injector and dehydration liner, which were heated to 400 °C (Fig. 2). The dehydration liner was made up of a pre-column of silanized borosilicate glass packed with a small quantity of coarse aluminium powder held in place by silanized glass-wool plugs, which served to dehydrate the standards and samples prior to chromatography. The top 3 cm of the capillary column was stripped of its methylsilicone phase and protected by silanization before use, as the high-temperature limit of the original coating was less than the 400 °C required for effective dehydration. The standards and samples were cold-trapped on the top of the capillary column at 75 °C, and the temperature was raised to 240 °C at 30 °C min^{-1}, held at 240 °C for 1 min and then increased to 270 °C at 5 min^{-1}. Standards and samples had eluted from the column by 12 min, but the column temperature was finally raised to 300 °C for 3.5 min in order to purge the column prior to the next injection. The dehydrated molecular ions of $[^2H_6]$-25-hydroxyvitamin D_3 (*m/z* 370), 25-hydroxyvitamin D_3 (*m/z* 364) and 25-hydroxyvitamin D_2 (*m/z* 376) were monitored (dwell time on each mass 300 ms), and the heights of the pyro peaks produced were measured. Peak-height ratios of 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 to the hexadeuteriated internal standard were determined, and used to construct calibration graphs from which the plasma and serum levels of 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 were calculated.

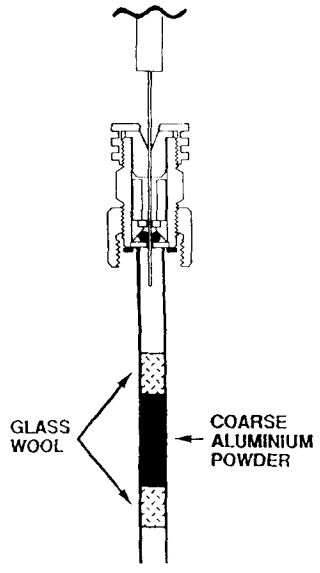

Figure 2. Diagrammatic representation of the septumless injector and dehydration liner (not to scale).

RESULTS

Samples prepared as described above produced clean, unambiguous traces when subjected to GC-MS with dehydration. Typical mass fragmentograms of both standards and sample extracts are shown in Fig. 3 (25-OHD_3) and Fig. 4 (25-OHD_2). Statistical validation of the assay was carried out and the results are given in Table 1. No inter-assay data are given in Table 1 as this method has not been in operation for a sufficient time to gather meaningful figures.

Development of the method

Choice of temperature for dehydration. Previous investigations, using 5α-androstan-3α-ol, a simple monofunctional C_{19} steroid, had indicated that at 400 °C dehydration was complete and no unchanged steroid could be detected.[13] All vitamin D metabolites examined followed this pattern and therefore all dehydration was carried out at 400 °C. Figure 5 shows peak-height ratios (25-OHD_3/ 5α-cholestane (monitoring *m/z* 372)) in a number of situations: (1) 25-hydroxyvitamin D_3-per-TMSI without dehydration at an injection temperature of 250 °C, monitoring the most abundant high-mass ion at *m/z* 439 $[M - 90 - 15]^+$, (2) 25-hydroxyvitamin D_3-per-TMSI without dehydration at an injection temperature of 400 °C, again monitoring the ion at *m/z* 439 $[M - 90 - 15]^+$ and (3) 25-hydroxyvitamin D_3 using the dehydration system at 400 °C without derivatization, monitoring the molecular ion at *m/z* 364. A common internal standard containing no hydroxyl groups, 5α-cholestane, was used in this experiment so that absolute comparisons could be made in order to establish the sensitivity of the technique. It can be seen that raising the temperature to 400 °C

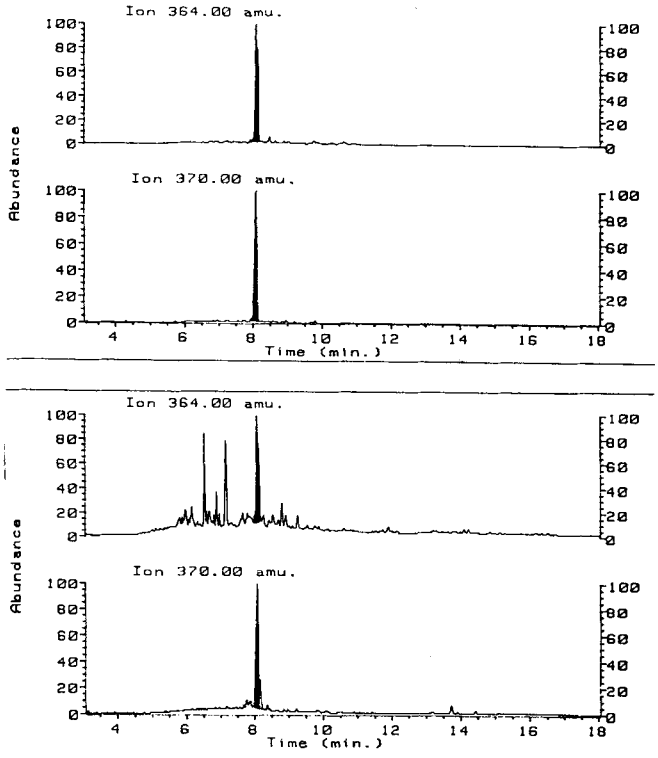

Figure 3. Typical mass fragmentograms of standards and sample extract from the assay of 25-hydroxyvitamin D_3. Upper trace: standard 25-hydroxyvitamin D_3 (*m/z* 364, 80 ng standard as shown in Fig. 7(A)) and hexadeuteriated 25-hydroxyvitamin D_3 (*m/z* 370). Lower trace : plasma extract (16.3 ng ml^{-1}).

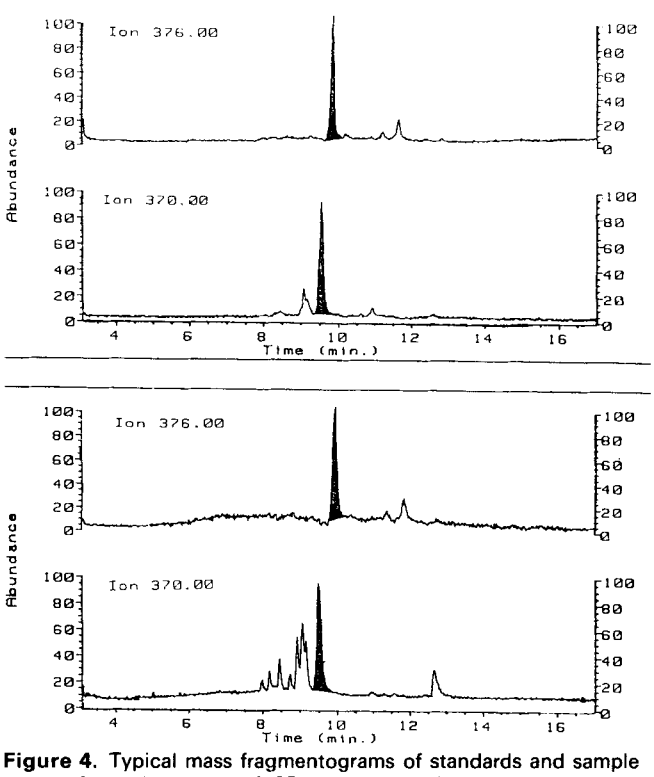

Figure 4. Typical mass fragmentograms of standards and sample extract from the assay of 25-hydroxyvitamin D_2. Upper trace: standard 25-hydroxyvitamin D_2 (*m/z* 376, 80 ng standard as shown in Fig. 7(A)) and hexadeuteriated 25-hydroxyvitamin D_3 (*m/z* 370). Lower trace : plasma extract (9.7 ng ml^{-1}).

increased the sensitivity of detection even when dehydration did not occur. Dehydration however, monitoring the dehydrated molecular ion at *m/z* 364, improved the sensitivity even further. The slopes of the three lines illustrated in Fig. 5 are in the ratio 1:4.5:7.4. It will also be noted that the line using dehydration is the only one where the intercept on both *x* and *y* axes is not significantly different from zero.

Dehydration of 25-hydroxyvitamin D. Mass spectra of standard 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2 and [$^2H_6$]-25-hydroxyvitamin D_3 were obtained by monitoring the total ion current produced after dehydration and GC as described above. Averaged background-subtracted spectra were generated and are shown in Fig. 6. The spectra contained high abundance dehydrated molecular ions and showed little other fragmentation. In contrast, the mass spectrum of the pertrimethylsilyl ether of 25-hydroxyvitamin D_3, a commonly used derivative in the GC-MS of vitamin D metabolites, also given in Fig. 6, shows considerable fragmentation and a relatively weak molecular ion. Mass spectra of the pertrimethylsilylated derivatives of the vitamin D_2 series showed extensive fragmentation and a molecular ion peak of negligible intensity.

In previous methods for the mass fragmentographic measurement of several hydroxylated metabolites of vitamin D_2, it has been observed that these secosteriods are more labile than metabolites of vitamin D_3 and that the sensitivity of mass fragmentography is not as good as that achieved for vitamin D_3 metabolites. The use of dehydration, however, avoided the inevitable losses

Table 1. Statistical validation of the assay

Recovery of added standard
25-Hydroxyvitamin D_2
119% (*n* = 10), 13.2 ng added to 2 ml of plasma, 15.8 ng recovered
25-Hydroxyvitamin D_3
112% (*n* = 7), 32 ng added to 2 ml of plasma, 35.8 ng recovered
Intra-assay variation
25-Hydroxyvitamin D_2
6.9% (*n* = 10), mean concentration = 15.8 ng ml^{-1}
25-Hydroxyvitamin D_3
4.7% (*n* = 9), mean concentration = 20.4 ng ml^{-1}

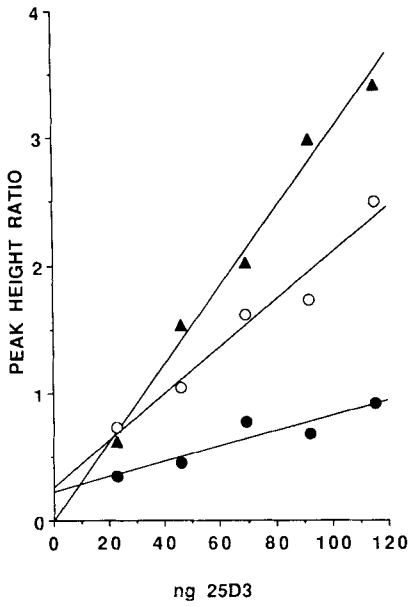

Figure 5. Comparison of the calibration graphs for derivatized and dehydrated 25-hydroxyvitamin D_3, injected at different temperatures, using 5α-cholestane as internal standard. Peak-height ratios of 25-OHD_3 to 5α-cholestane (monitoring *m/z* 372) are plotted thus: (● 25-hydroxyvitamin D_3-per-TMSI at 250 °C, monitoring *m/z* 439 ($[M-90-15^+]$) ($y = 0.2182 + 5.9510 \times 10^{-3}\ x$, $R^2 = 0.865$); (○ 25-hydroxyvitamin D_3-per-TMSI at 400 °C, monitoring *m/z* 439 ($[M-90-15]^+$) ($y = 0.2533 + 1.8317 \times 10^{-2}\ x$, $R^2 = 0.959$); ▲ 25-hydroxyvitamin D_3 after dehydration at 400 °C, monitoring *m/z* 364 ($y = 6.8560 \times 10^{-3} + 3.0612 \times 10^{-2}\ x$, $R^2 = 0.986$).

which occurred during derivatization and gave mass spectra with high-mass ions of roughly equal abundance to those obtained with vitamin D_3 metabolites, a considerable improvement on any other derivative for the D_2 series of metabolites that have been examined so far. This is illustrated in Fig. 7, where it can be seen that the calibration graphs for $25D_3$ and $25D_2$ share a common slope. The peak-height ratio plotted is that calculated using dehydrated hexadeuteriated $25D_3$ as the internal standard. Also illustrated in Fig. 7 is a comparison of the calibration graphs for 25-OHD_3 and 25-OHD_2 as pertrimethylsilyl ether (per-TMSI) derivatives, with peak-height ratios calculated using the per-TMSI derivative of hexadeuteriated 25-OHD_3. The sevenfold increase in sensitivity after dehydration has already been demonstrated in Fig. 5.

Careful inspection of Fig. 3 indicates that injection of underivatized 25-OHD_3 on to the GC column gave rise to two peaks which were largely unresolved. It is suggested that these are two isomers which arise from dehydration at C-25 and use of 25,26-hexadeuteriated 25-hydroxyvitamin D_3 confirmed this view, although theoretically isomers could occur at both C-3 and C-25. Only a single peak was observed for hexadeuteriated 25-hydroxyvitamin D_3 when monitoring the molecular ion at *m/z* 370. The reason for this can be seen by inspection of the mass spectrum shown in Fig. 6(C), which shows the presence of two molecular ions of roughly equal abundance at *m/z* 369 and 370. These can

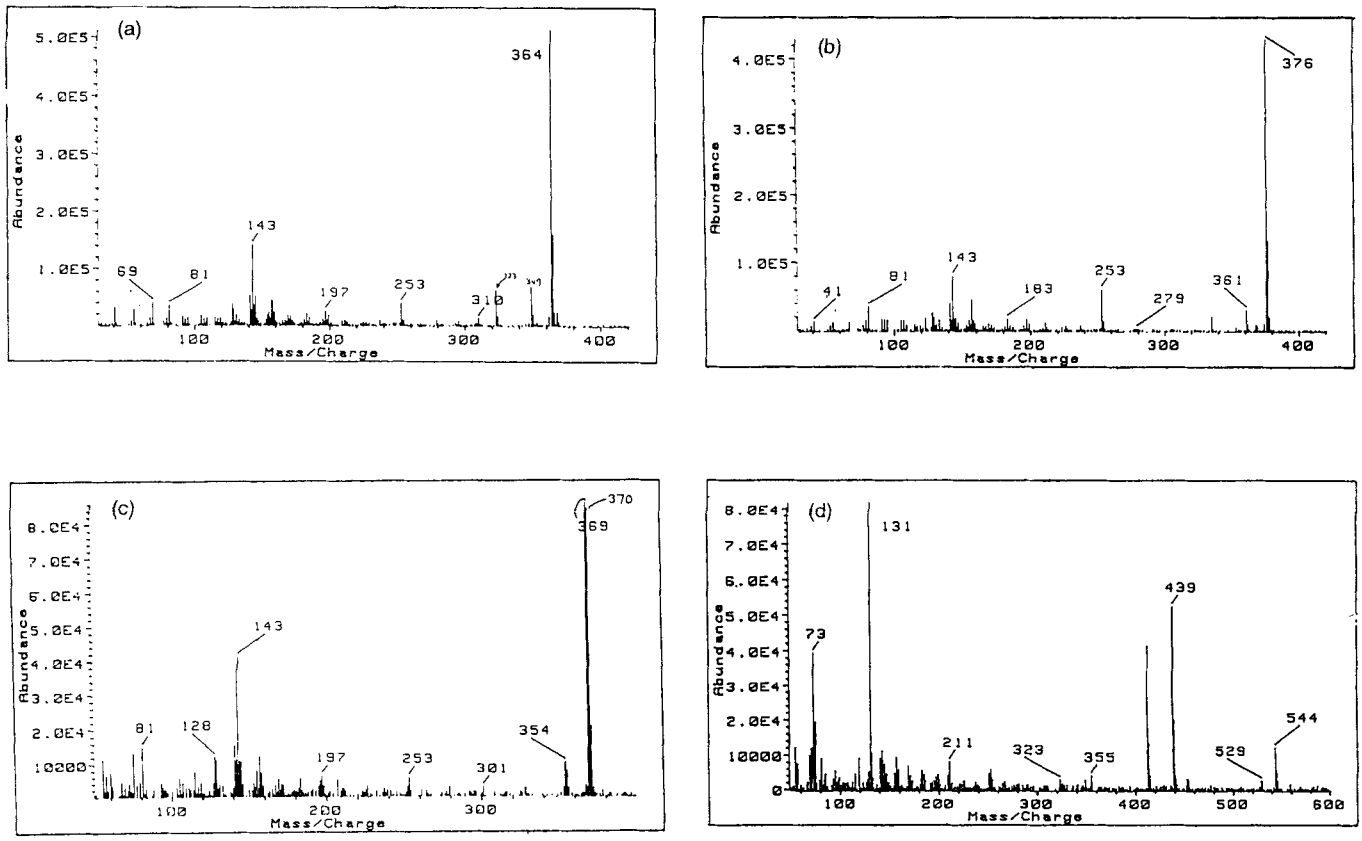

Figure 6. Averaged, background-subtracted spectra (EI, positive ion 70 eV) of (A) dehydrated 25-hydroxyvitamin D_3, (B) dehydrated 25-hydroxyvitamin D_2, (C) dehydrated [$^2H_6$]-25-hydroxyvitamin D_3 and (D) the pertrimethylsilyl ether derivative of 25-hydroxyvitamin D_3 (pyro peak).

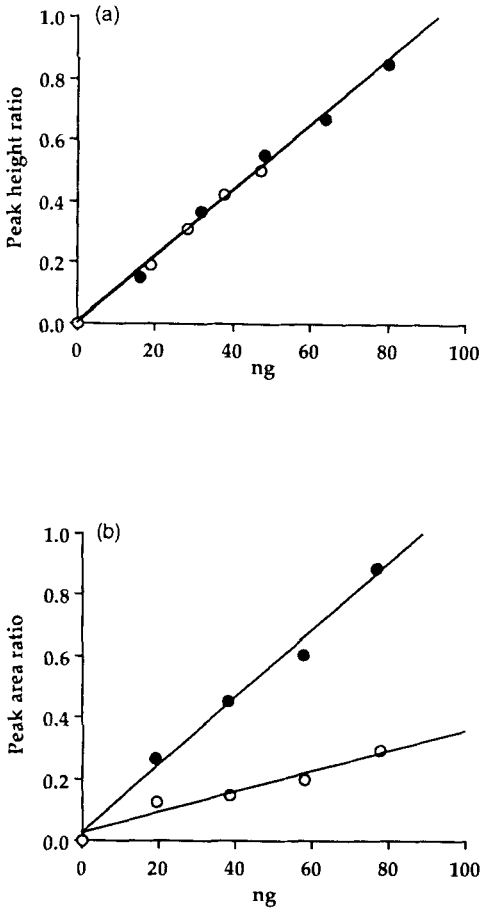

Figure 7. Calibration graphs for (● 25-hydroxyvitamin and (○) 25-hydroxyvitamin D_2 using [$^2H_6$]-25-hydroxyvitamin D_3 as internal standard and (A) the dehydration method described in the text, monitoring molecular ions at *m/z* 364 (25-hydroxyvitamin D_3), 376 (25-hydroxyvitamin D_2) and 370 ([$^2H_6$])-25-hydroxyvitamin D_3) (peak-height ratios of analyte to internal standard) or (B) pertrimethylsilyl ether derivative formation and selected ion monitoring of $[M - 90 - 15]^+$ (*m/z* 439 (25-hydroxyvitamin D_3), 451 (25-hydroxyvitamin D_2) and 445 ([$^2H_6$]-25-hydroxyvitamin D_3) (peak-area ratios of analyte to internal standard).

be resolved by mass chromatography, monitoring the two ions separately (Fig. 8). As the internal standard is deuteriated at C-25 and C-26, dehydration of the C-25 hydroxyl caused loss of deuterium in approximately 50% of the product to form the two molecular ions observed in the spectrum (Fig. 9). Injection of 25-hydroxyvitamin D_2, on the other hand, appeared to give rise to a single peak with a dehydrated molecular ion at *m/z* 376. An explanation for this may be that only one isomer is preferentially formed owing to the presence of a methyl group on C-28 and a conjugated double bond in this molecule. No deuteriated 25-hydroxyvitamin D_2 is available to investigate this further. During its use in the determination of 25-hydroxyvitamin D only one of the dehydrated molecular ions from the internal standard (*m/z* 370) needed to be monitored. Attempts to use the data-handling software to provide integrated areas were not entirely successful since the split peak of 25-OHD_3 gave rise to a number of inconsistencies in the determination of peak area. The use of peak heights, however, gave reproducible results, using the peak height of the first peak for quantification. This method gave values in normal plasma which were identical with those using a previously described mass fragmentographic procedure.[8]

All vitamin D metabolites when injected on to a GC column undergo B-ring cyclization, producing two isomers, pyro and isopyro. These are usually formed with a pyro:isopyro ratio of 2:1. In the case of the dehydrated compounds, only one major peak is observed. This is a common finding during GC-MS of many vitamin D metabolite derivatives and the explanation for this observation is unclear. Presumably the isopyro isomer is not ionized to the same degree as the pyro isomer. The presence of single peaks when using dehydration is a further advantage of this method of mass fragmentography.

Other vitamin D metabolites. The studies described here were directed towards the production of a simple mass fragmentographic assay for 25-hydroxyvitamins D_2 and D_3, but the possibility of extending this method to include the dihydroxylated metabolites has also been considered. Table 2 lists the retention times of all the metabolites of vitamins D_2 and D_3 which have been studied as dehydration products. It can be seen that all the metabolites examined are completely resolved from each other when subjected to GC-MS. Table 3 summarizes the EI positive-ion mass spectra of the dehydration products which were obtained, listing the major

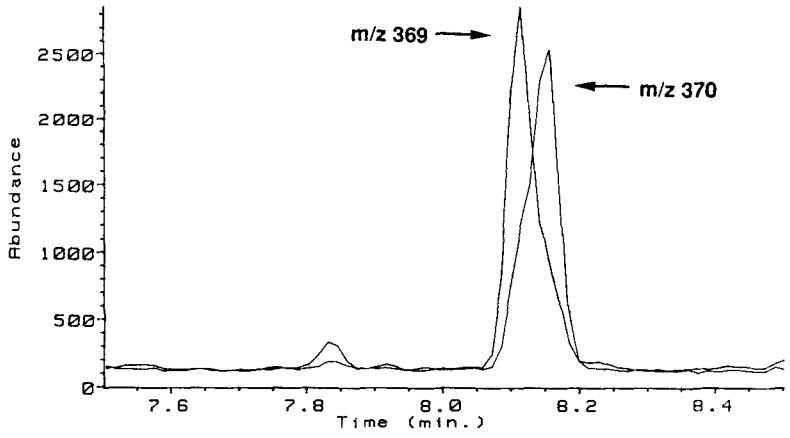

Figure 8. Mass chromatography of the two ions produced by dehydration of [25,26-$^2H_6$]-25-hydroxyvitamin D_3.

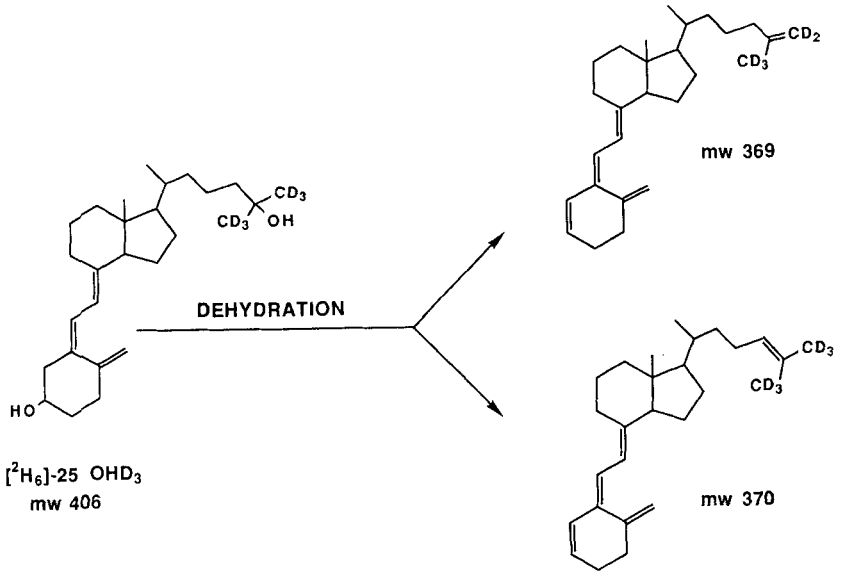

Figure 9. Suggested mechanism for the formation of the two isomers observed after dehydration of [25,26-$^2H_6$]-25-hydroxyvitamin D$_3$. The position of the double bond in the A-ring is not known and is illustrated here, for convenience, as being in the 2,3-position, but it could equally well be in the 3,4-position.

ions and the relative intensity of the base peak as a proportion of the total ion current.

Studies were carried out to establish whether it was possible to protect the side-chain from dehydration by the use of cyclic boronate derivatives and whether or not these derivatives gave enhanced chromatographic or mass spectral characteristics which could be utilized for the development of more sensitive methods for their analysis. For example, Fig. 10 compares two mass spectra obtained from 25,26-dihydroxyvitamin D_3 one fully dehydrated and one as the 25,26-*n*-butyl boronate ester, dehydrated only at C-3. Both are clean spectra with high-intensity molecular ion peaks, making both ideal for selected ion monitoring. The formation of a boronate derivative across C-25 and C-26, however increases the retention time considerably. Boronate formation is very simple and causes negligible loss of analyte. The formation of such a selective and sensitive derivative may be useful when investigating complex extracts, and allow them to be analysed after a minimum of purification.

The physiologically important dihydroxylated metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3, has also been studied in the dehydration system described. From the retention times shown in Table 2 it can be seen that two well separated peaks are formed when this metabolite is injected into the gas chromatograph. When the mass spectra of these two peaks were examined the

Table 2. Retention times of vitamin D_2, vitamin D_3 and metabolites that were studied as dehydration products

Dehydration product from	Relative retention time[a]	As *n*-BBA derivatives	Relative retention time[a]
Vitamin D_3	2.37	24,25-$(OH)_2D_3$	3.76
25-OH-vitamin D_3	2.45	25,26-$(OH)_2D_3$	4.13
24,25-$(OH)_2D_3$	2.51	[$^2H_6$]-24,25-$(OH)_2D_3$	3.73
25,26-$(OH)_2D_3$	3.00	[$^2H_5$]-25,26-$(OH)_2D_3$	4.04
1,25-$(OH)_2D_3$	2.63[b]	24,25-$(OH)_2D_2$	3.70/3.75[c]
Vitamin D_2	2.46	25,26-$(OH)_2D_2$	4.13/4.19[c]
25-OH-vitamin D_2	2.44	[$^2H_3$]-24,25-$(OH)_2D_2$	3.71
24,25-$(OH)_2D_2$	2.92		
[$^2H_6$] vitamin D_3	2.35		
[$^2H_6$]-OH- vitamin D_3	2.44		
[$^2H_6$]-24,25-$(OH)_2D_3$	2.50		
[$^2H_5$]-25,26-$(OH)_2D_3$	2.99		
[$^2H_3$]-24,25-$(OH)_2D_2$	2.89		

[a] Relative to that of 5α-androstane (retention time 4.2 min).
[b] Dehydrated 1,25-$(OH)_2D_3$ gives two peaks on GC/MS.[13] Data for the first peak only given.
[c] Two peaks of roughly equal height were observed for these two steroids. The mass spectra of both peaks were identical.

Table 3. Summary of mass spectra produced by dehydration products of vitamin D_2, vitamin D_3 and metabolites, listing major ions and the relative intensity of the base peak as a proportion of the total ion current

Dehydration production from	Main ions in mass spectrum (*m/z* [% abundance relative to base peak])	% TIC[a]
Vitamin D_3	376 [30.3], 366 (M^+)[100], 158 [26.3], 143 [62.6]	21.0
25-OH-vitamin D_3	365 [29.8], 364 (M^+) [100], 158 [19.6], 143 [47.4]	25.8
24,25-$(OH)_2D_3$	363 [33.1], 362 (M^+) [100], 143 [41.4],	21.0
25,26-$(OH)_2D_3$	381 [31.0], 380 (M^+) [100], 158 [22.3], 143 [59.9]	18.9
1,25-$(OH)_2D_3$	363 [24.0], 362 (M^+) [100], 156 [27.2], 155 [35.1], 141 [25.0], 105 [33.5]	22.0
Vitamin D_2	379 [31.2], 378 (M^+) [100], 253 [27.2], 157 [26.3], 143 [41.5]	19.9
25-OH-vitamin D_2	377 [34.2], 376 (M^+) [100], 253 [30.0], 157 [28.0], 143 [38.8]	18.51
24,25-$(OH)_2D_2$	393 [30.3], 392 (M^+) [100], 253 [11.5], 143 [22.7]	29.7
[$^2H_6$]vitamin D_3	373 [34.9], 372 (M^+) [100], 158 [27.6], 143 [65.8]	19.54
[$^2H_6$-25-OH-vitamin D_3	371 [31.5], 370 (M^+) [100], 369 [89.3], 253 [19.5], 157 [26.8], 141 [91.8]	10.5
[$^2H_6$-24,25-$OH)_2D_3$	368 [32.6], 367 (M^+) [100], 366 [53.3], 143 [68.5], 141 [28.6]	9.3
[$^2H_5$]-25,26-$OH)_2D_3$	384 (M^+) [100], 158 [59.0], 145 [61.0], 144 [47.0], 143 [99.0]	7.9
[$^2H_3$]24,25(OH_2D_2	396 [29.3], 395 (M^+) [100], 157 [26.0], 143 [43.0], 81 [30.1]	15.2
As *n*-BBA derivatives		
24,25-$(OH)_2D_3$	454 [32.5], 464 (M^+) [100], 463 [24.3], 158 [22.3], 143 [43.7]	14.5
25,26-$(OH)_2D_3$	465 [32.0], 464 (M^+) [100], 463 [24.1], 158 [19.7], 143 [38.0]	20.0
[$^2H_6$]-24,25-$(OH)_2D_3$	471 [36.5], 470 (M^+) [100], 158 [35.7], 145 [33.2], 143 [66.2]	14.0
[$^2H_5$]-25,26-$(OH)_2D_3$	470 [41.2], 469 (M^+) [100], 468 [26.2], 158 [44.9], 143 [83.6]	8.7
24,25-$(OH)_2D_2$	477 [346], 476 (M^+) [100], 157 [30.7], 143 [43.8], 81 [31.6]	11.6
25,26-$(OH)_2D_2$	476 (M^+) [52.7], 157 [18.4], 143 [22.6], 141 [100], 81 [30.3]	16.4
[$^2H_3$]-24,25-$(OH)_2D_2$	480 [33.9], 479 (M^+) [100], 252 [26.6], 157 [33.0], 143 [51.1]	12.5

[a] % Total ion current (TIC) of base peak.

appeared to be qualitatively identical, although there were differences in the abundances of some ions (notably those at *m/z* 209, 208 and 251). The molecular ion in both cases occurred at *m/z* 362, indicating the loss of three hydroxyls. The spectra of these compounds have been published elsewhere.[10]

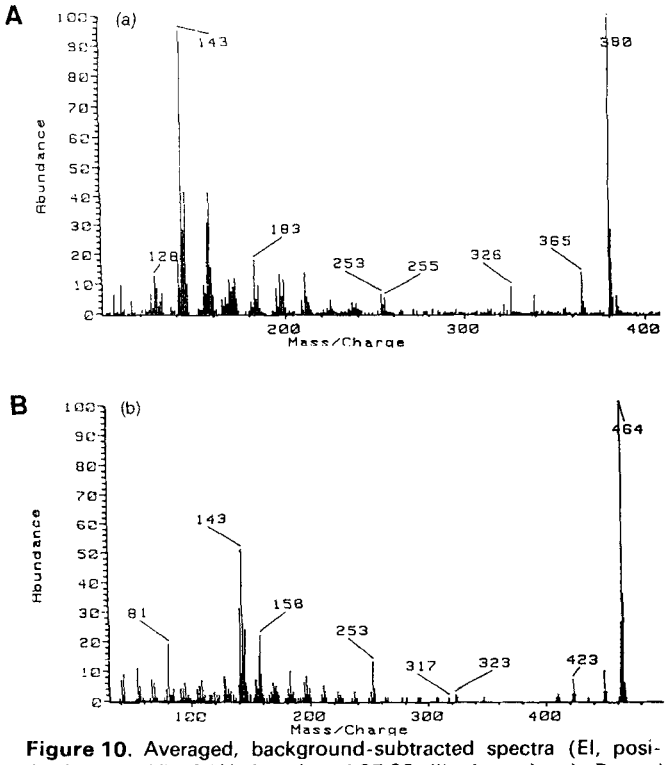

Figure 10. Averaged, background-subtracted spectra (EI, positive ion, 70 eV) of (A) dehydrated 25,26-dihydroxyvitamin D_3 and (B) the 25,26-*n*-butyl boronate ester 3-dehydrated derivative of 25,26-dihydroxyvitamin D_3.

DISCUSSION

A method has been developed for the accurate determination of 25-hydroxyvitamin D_3 and 25-hydro-xyvitamin D_2 in plasma or serum by the use of highly selective mass fragmentography. Sample preparation is simple but rigorous. Time-consuming derivatization prior to gas chromatography, previously necessary for secosteroids, has been replaced by on-column dehydration, which provides mass spectra with high-abundance dehydrated molecular ions ideal for selected ion monitoring. The use of autoinjection allows extracts to be analysed overnight and a batch of 20 samples can be processed in 24 h. In addition, data-editing software can be written to produce computer-calculated results at the end of each analysis.

The quality of the mass spectra produced after dehydration, for the purposes of mass fragmentographic quantification, is a great improvement on that obtained from pertrimethylsilyl ether derivatives. This is especially true for the vitamin D_2 series of metabolites, which in our hands show extensive fragmentation as trimethylsilyl ethers and have molecular ions of negligible abundance. Polyhydroxylated vitamin D_2 metabolites (24,25-dihydroxyvitamin D_2 and 25,26-dihydroxyvitamin D_2) appear to be particularly unstable when prepared as either pertrimethylsilyl ethers or as the *n*-butyl boronate ester–25-trimethylsilyl ether mixed derivative. Although mass fragmentography is possible, sensitivity is reduced and ions other than the molecular ion have

to be monitored. Such problems may be overcome using on-column dehydration of underivatizated compounds.

Inter-laboratory comparisons of methods used for the measurement of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, the most commonly investigated metabolites, have highlighted the need for quality control schemes to be developed in this area. GC-MS is generally accepted as the definitive technique in the determination of plasma metabolites. The method described here could be used to provide target values for a quality control scheme for 25-hydroxyvitamin D. The cost of implementing this method is only moderate, as a bench-top gas chromatography mass spectrometer need cost no more than a comprehensive HPLC system.

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