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Gas chromatography–mass spectrometry in the investigation of on-column dehydration of steroid hormones during gas–liquid chromatography*

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Abstract: Some underivatized steroids when injected onto conventional packed columns for gas–liquid chromatography underwent varying degrees of dehydration. This problem was traced to the presence of small pieces of broken glass on the top of the column at the point of injection. This observation provoked an examination of the effect of pre-column dehydration on a number of different types of steroids. Powdered aluminium was placed in the injection liner of a Hewlett–Packard gas chromatograph fitted with an HP1 capillary column connected to a mass selective detector, and injections were made using a new high temperature septumless injection system at temperatures between 200 and 400°C. 5α-androstan-3α-ol, a simple monofunctional C19 steroid chosen as a model to establish optimum conditions, underwent dehydration at injection temperatures greater than 250°C and the product reached a maximum at 400°C when no unchanged steroid was present. Monohydroxylated androgens and oestrogens underwent dehydration at 400°C producing products whose mass spectra indicated they were monenes, although the position of the double bond could not be assigned. Polyfunctional androgens and oestrogens and corticosteroids underwent complex changes producing a number of products some of whose structures could not be determined. The dehydration products had the advantage that they had relatively intense high mass ions and for suitable steroids this might provide enhanced sensitivity of detection during mass fragmentography. In such cases dehydration was reproducible and straight line standard curves were obtained. C27 and C28 secosteroids (vitamins D_2 and D_3) and some of their metabolites (e.g. 25-hydroxyvitamin D) underwent efficient dehydration, again producing products with intense molecular ions. In the case of 24,25-dihydroxyvitamin D_3 and 25,26-dihydroxyvitamin D_3, dehydration produced different products which were easily resolved in the chromatographic system used. Dehydration of vitamin D metabolites eliminates the need for derivatization and gives enhanced sensitivity of measurement by gas chromatography–mass spectrometry.

Keywords: *Pre-column dehydration; gas–liquid chromatography–mass spectrometry; steroid hormones; vitamin D; androgens; oestrogens; C21 steroids.*

Introduction

Steroid hormones such as the 17-hydroxylated C21 steroids (e.g. hydrocortisone) are usually derivatized before analysis by gas–liquid chromatography (GLC) since they may be thermally labile and/or contain polar hydroxyl groups which may be adsorbed to the 'inert' support of the column or to other parts of the GLC system. The formation of trimethylsilyl ethers and *O*-methyl oximes is widely used for the analysis of steroid hormone metabolites in urine [1]. While the gas chromatography of steroids, popular in the past, has been largely replaced for routine clinical use by immunoassay, the increasing availability of cheap benchtop mass spectrometers and the need for target values for quality control [2], have revived interest in gas chromagraphy–mass spectrometry. The necessity for the formation of derivatives prior to GLC increases the complexity of, and time required for, analysis.

During the investigation of the GLC of some vitamin D metabolites, dehydration was observed to occur from time to time when using conventional packed columns. This was subsequently shown to be due to the presence of broken glass on the top of the column [3]. From the point of view of subsequent mass spectrometry, this dehydration conferred the considerable advantage that intense high mass ions were observed in the mass spectrum of the product. This paper describes an investigation into the behaviour of a variety of steroid hormones, from C18 oestrogens to C27 secosteroids, using aluminium powder placed in the

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injection liner of the gas chromatograph which is then heated to high temperatures. The degree of conversion to dehydrated product, the formation of single or multiple products, and the mass spectrometric characteristics of the products with a view to subsequent mass fragmentography, were the principal attributes that were studied.

Materials and Methods

Chemicals and reagents

Steroids were obtained from the MRC Steroid Reference Collection or from commercial sources (Steraloids Inc., Wilton, NH, USA and Sigma Chemical Co., Poole, Dorset, UK). C27 secosteroids were a generous gift from Roche Products UK Ltd (Welwyn Garden City, Herts, UK). Reagents for derivatization were obtained from Pierce & Warriner Ltd, (Chester, Cheshire, UK) and were used as supplied. Aluminium powder (coarse) was purchased from BDH Chemicals Ltd (Poole, Dorset, UK).

Apparatus

Gas chromatography was carried out using HP1 (a WCOT non-selective methylsilicone, 12.5 m long × 0.3 mm i.d.) supplied by Hewlett–Packard (Bracknell, Berkshire, UK). This column was inserted into an HP5890 gas chromatograph and the end of the column placed directly in the ion source of a mass selective detector (HP5970). Since the injection temperature used in this investigation was 400°C and the upper separation limit for the HP1 capillary column is 350°C, the top 3 cm of the column used was stripped of the methylsilicone phase by pre-heating prior to insertion into the injector. The top part of the stripped column was silanized by dipping into 2% dimethyldichlorosilane in toluene. The carrier gas was helium at a flow rate of 1 ml min^{-1} and steroids dissolved, unless otherwise specified, in methanol were automatically injected on to the column using an HP 7673 automatic injection system in the splitless mode. Injection temperature was varied depending on whether the derivatized steroids were being determined directly (250°C) or whether dehydration was being carried out (400°C). The injection liner of the HP system was modified for dehydration purposes by inserting approximately 0.1 g of aluminium powder into the split–splitless injection liner supplied by Hewlett–Packard using a depth of approximately 1 cm. Figure 1 gives a diagrammatic representation of this liner. For high temperature injections (greater than 300°C) the Hewlett–Packard injection septum was replaced with a septumless Jade injector (SGE, Milton Keynes, UK). The initial oven temperature was 75°C which was held for 2.5 min and raised thereafter at a rate of 10° min^{-1} to a final temperature of 300°C.

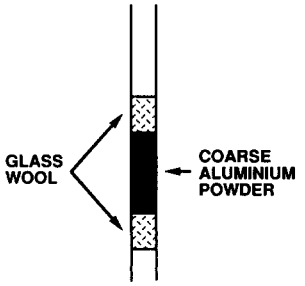

Figure 1
Diagrammatic representation of the dehydration injection liner inserted into the Hewlett–Packard gas chromatograph.

The mass spectra (EI+ at 70 eV) were obtained for each peak and the background signals were subtracted when appropriate. This manipulation was carried out using the HP ChemStation data system supplied with the HP5970. Solvents were analytical reagents from BDH Chemicals and were used without further purification.

Results

Injection temperature for efficient dehydration

5α-androstan-3α-ol was injected into the GC system (including the aluminium powder) described above and the injection temperature was varied over the range ambient to 400°C. Over this range two effects were observed. Firstly, steroids did not emerge from the column at injection temperatures below 250°C, presumably due to either inadequate volatilization and/or to adsorption/decomposition in the injection system. The second effect was the efficiency of dehydration. Figure 2 illustrates these effects. Dehydration appears to be at its most efficient at an injection temperature of 400°C. It was not possible to raise the injection temperature to levels above 400°C with the HP system and thus it has not been possible to investigate the effect of higher injection temperatures. However, it can be seen from Fig. 3

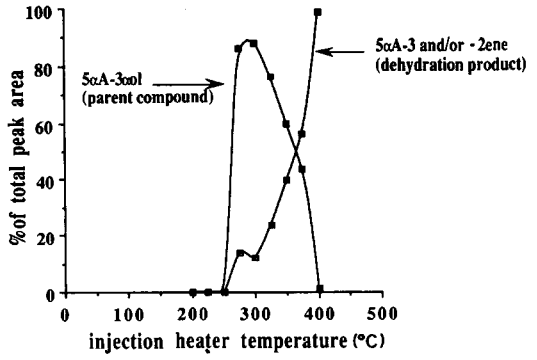

Figure 2
Effect of temperature on the dehydration of 5α-androstan-3α-ol (5αA-3αol). The injection heater temperature was raised from ambient to the maximum (400°C) and 5αA-3αol was injected. Peak areas were integrated and the peak area of the parent compound and dehydration product, either the 5α-2-ene or 5α-3-ene, were expressed as a percentage of the total.

that at an injection temperature of 400°C a single peak was observed and no trace of the original androstanol (less than 3%) could be seen. For obvious reasons it has also not been possible to estimate the overall recovery of the dehydrated product formed in the system described.

However, as will be described below, the dehydration does appear to be reproducible and linear curves relating peak area of dehydrated product to the amount of precursor injected have been obtained for some of the steroids investigated. All subsequent dehydration was carried out at 400°C although it is recognized that this may not be the optimum injection temperature for steroids other than the androstanol investigated.

C18 oestrogens

A number of oestrogens were injected into the dehydration system described above. Oestrien-3-ol, containing the single phenolic 3-hydroxyl group was the first oestrogen examined and injection of this compound gave rise to a single peak whose mass spectrum was consistent with that of the injected precursor

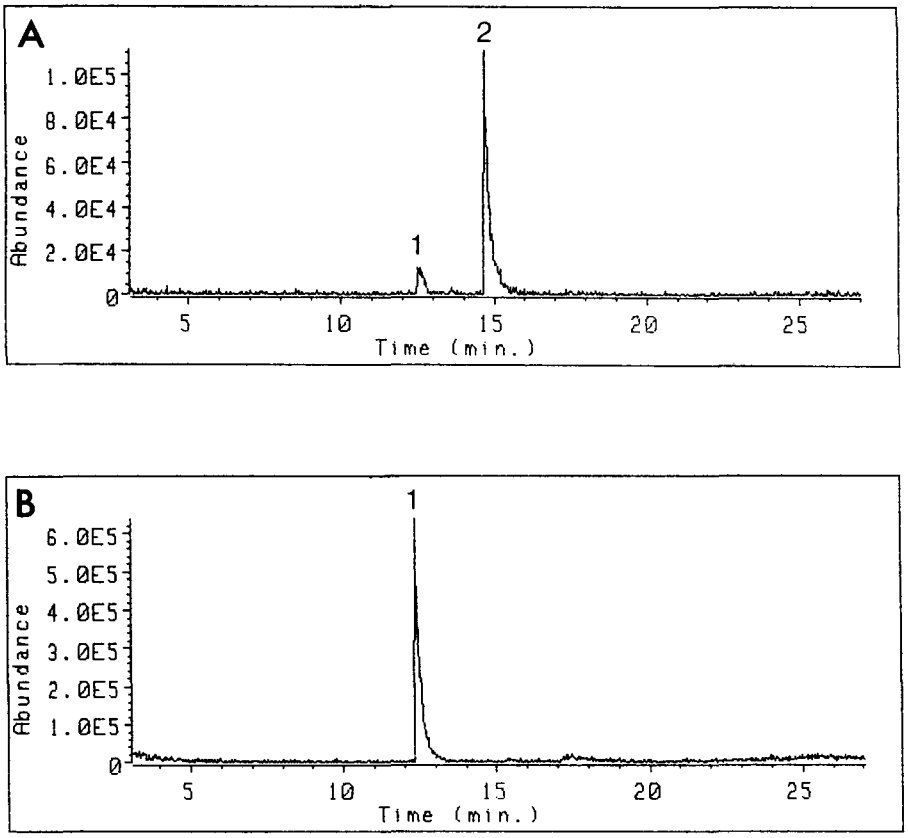

Figure 3
Total ion chromatograms of 5α-androstan-3α-ol injected at a temperature of (A) 275° and (B) 400°C. At an injection temperature of 400°C no trace of the parent compound can be seen. Peak 2 represents the unchanged 5αA-3αol and Peak 1 the dehydration product.

and thus it is concluded that, as expected, dehydration of this compound does not occur. Three other oestrogens were examined, namely oestrone, oestradiol and oestriol. In all cases, dehydration was highly inefficient, producing from oestrone and oestriol less than 20% of the expected product, and both the precursor and product gave very poor peak shapes. Oestradiol gave a slightly higher concentration of dehydration product than the other two oestrogens (approximately 30%). Dehydration of oestrone and oestradiol gave apparently the same product: the retention times were the same and both products had very similar mass spectra with intense molecular ions (base peaks of *m/z* 254). This ion represents the loss of 16 mass units from oestrone, as is also the molecular ion of the *delta*-16 product which would be produced by dehydration at the 17-hydroxyl position of oestradiol. Oestriol gives rise to a dehydration product with a molecular ion of *m/z* 270.

C19 androgens

A number of androgens were examined using the injection system described above. In general terms the peak shapes of both the precursor and products were far superior to those observed with the oestrogens and dehydration appeared to be more efficient than with oestrogens. Table 1 lists the androgens that were examined and gives the major ions observed in the mass spectra of the dehydration product(s). As has been previously described, androstanol (5α-androstan-3α-ol) gave rise to a single peak with a molecular ion at *m/z* 258 (48% of the base peak *m/z* 189). This fragment represents loss of a single molecule of water producing presumably the expected *delta*-2 or *delta*-3 product. Testosterone and androstenedione once again gave low yields of the dehydration product, androstenedione giving a molecular ion (base peak of *m/z* 286) which represents the unchanged precursor. Injection of 5β-androstan-16-ene-3α-ol and 5α-androstan-17β-ol both gave rise to small yields of the expected dehydration products (molecular ions representing loss of 18 mass units from the precursor compound). 5α-androstan-17β-ol was also injected in toluene rather than ethanol and it was observed that increased amounts of the dehydration products were formed. However the proportion of dehydration product did not exceed 50%.

The other two androgens which were examined in this investigation, androsterone and testosterone gave rise to more complex dehydration products, the structures of which are unknown. Androsterone was almost completely dehydrated with only a very small proportion of the unchanged precursor being observed. The major product had a mass spectrum with an intense ion at *m/z* 272 which represents the loss of water from the precursor compound. Injection of testosterone gave rise to the most complicated picture of all the androgens studied. Figure 4 shows the total ion current obtained when testosterone was injected into the dehydration liner as described. The reaction which occurs in the liner is extremely complex and three distinct products can be seen. Each product seems to occur as a doublet and the mass spectrum of each component of the doublet is essentially identical. The first pair of peaks (Peaks 1 and 1a, Fig. 4) have the same mass spectra as that observed for unchanged testosterone with molecular ions at *m/z* 288. The second pair of peaks (Peaks 2 and 2a, Fig. 4) have a molecular ion at *m/z* 272 representing a loss of 16 mass units from the parent compound. The third pair of peaks (Peaks 3 and 3a, Fig. 4) have a molecular ion at *m/z* 270, which represents the loss of 18 mass units from the parent compound and is presumably due to dehydration at C17. The final pair (Peaks 4 and 4a, Fig. 4), present in low concentrations, have a molecular ion at *m/z* 254, which represents the loss of 34 mass units (possibly 16 + 18 mass units).

It is clear that the dehydration of C19 androgens which contain *delta*-4-3-oxo groups is complex and that there is no clear analytical advantage of using this dehydration procedure. Saturated androstanols can be induced to give the expected dehydration product(s) with varying yields. The dehydration reactions which occur in the injection system are very interesting and further studies will be undertaken in attempts to establish exactly what is happening.

C21 steroids

The simplest C21 steroid hormone, progesterone, which like testosterone contains the *delta*-4-3-oxo group, does not give rise to dehydration products but does, like testosterone, give rise to a doublet. Both of the peaks in the doublet give a molecular ion at *m/z* 314, which is the molecular weight of

Table 1
Dehydration of androgens. A summary of the mass spectra of the major products

Androgen (molecular weight)		Suggested molecular ion of major products (when more than one peak is seen both peaks are listed in order of elution from GC)	Other significant fragments of *m/z* 100 and above (% of base peak)
Androsterone	*(290)*	*256 (100%)*	*241 (48), 202 (50), 148 (32), 147 (36), 107 (45), 106 (36), 105 (39)*
		272 (100%)	275 (22), 218 (93), 190 (37), 161 (48), 108 (31), 107 (32), 105 (40)
5α-Androstan-3α-ol	(276)	258 (48%)	243 (32), 204 (61), 189 (100)
5α-Androstan-17β-ol	(276)	258 (100%)	243 (54), 149 (59), 148 (72)
5α-Androstan-17-one	(274)	274 (100%)*	241 (39), 230 (53), 148 (39), 109 (67), 108 (45), 107 (30)
5β-Androst-16-en-3α-ol	(274)	256 (92%)	241 (100), 147 (44), 146 (30), 133 (33), 107 (32), 105 (45)
Dehydroepiandrosterone	(288)	270 (52%)	145 (33), 143 (32), 131 (30), 121 (100), 107 (47), 105 (60)
Androst-4-ene-3,17-dione	(286)	286 (100%)	244 (49), 124 (69), 109 (37), 107 (42)
Testosterone	(288)	(4) 254 (100)†	159 (43), 147 (32), 146 (31), 145 (49), 143 (41), 133 (37), 105 (37)
		(3) 270 (100%)†	No other ions >20%
		(2a) 272 (100%)†	145 (31), 143 (37), 131 (30), 121 (65), 119 (32), 117 (31), 107 (31), 105 (75)
		(2) 272 (100%)†	257 (35), 121 (50), 107 (40), 105 (50).

*This represents unchanged 5α-androstan-17-one.

†Each of these peaks is a doublet and the number in parenthesis before the molecular ion refers to the number of the peak (i.e. 2 and 2a, 3 and 3a, 4 and 4a, see Fig. 4).

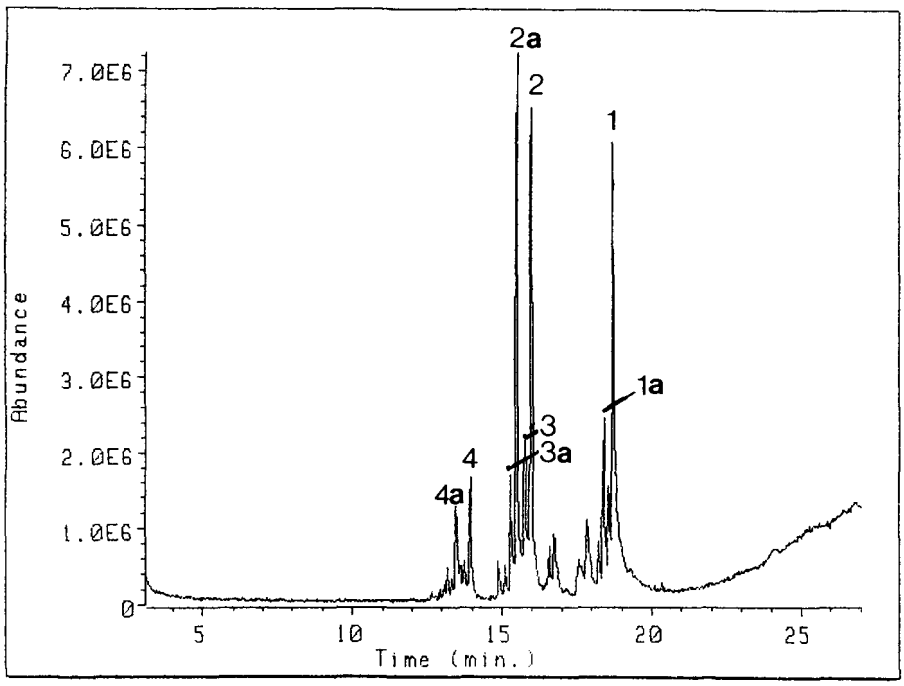

Figure 4
Total ion current chromatogram obtained when testosterone was injected into the dehydration system. The numbering of the peaks is discussed in the text and in Table 1.

unchanged progesterone. The early eluting peak gave a base peak at *m/z* 314 with intense ions at *m/z* 124, 272, 229 (in decreasing order of abundance), whereas the late eluting peak gave a base peak at *m/z* 244 and the peaks at *m/z* 124, 229 and 272 were less than 30% of the base peak. It is clear that some rearrangement is taking place in the injection system but the exact nature of this is unknown, although one possibility is the production of the 3-enol. The late eluting peak is clearly the product formed in the injection system since the mass spectrum of the early eluting peak is identical to that produced by progesterone introduced in the direct probe. This conversion takes place with an efficiency of around 70%. The introduction of a 17α-hydroxyl group into progesterone to produce 17-hydroxyprogesterone seems to render the molecule completely unstable to the dehydration system since it has not been possible to obtain any peaks after injection of 17-hydroxyprogesterone. Cortisol gave rise to a major peak which represented loss of side chain and two minor peaks resulting from loss of side chain plus a further 18 mass units in one peak and 16 mass units in another. Other corticosteroids did not give rise to single peaks of any abundance and complex patterns were observed.

No clear advantage, and, in fact, positive disadvantages, resulted from the use of the dehydration injection system for corticosteroids. Gas–liquid chromatography of C21 steroids is best achieved, as has been reported on many occasions previously, by the formation of *O*-methyloxime-trimethylsilyl ether derivatives which give single peaks, although occasionally *syn*- and *anti*-isomers of the oximes separate [1].

C27 and C28 secosteroids (vitamins D_2 and D_3 and metabolites/derivatives)

These secosteroids are very unstable, the C28 series being more so than the C27 series, and undergo thermal rearrangement when injected into a gas chromatograph forming *pyro*- and *isopyro*-isomers in the ratio 2:1. It is also necessary to form derivatives to prevent adsorption occurring during the chromatographic process. The mass spectra of the derivatives commonly used for 25-hydroxyvitamin D (trimethylsilyl ether) give mass spectra with relatively low abundance of high mass ions (Fig. 5). This decreases the sensitivity of mass fragmentographic measurements unless specificity is sacrificed by utilizing low mass ions such as *m/z* 131. Surprisingly, when vitamin D and 25-hydroxyvitamin D were

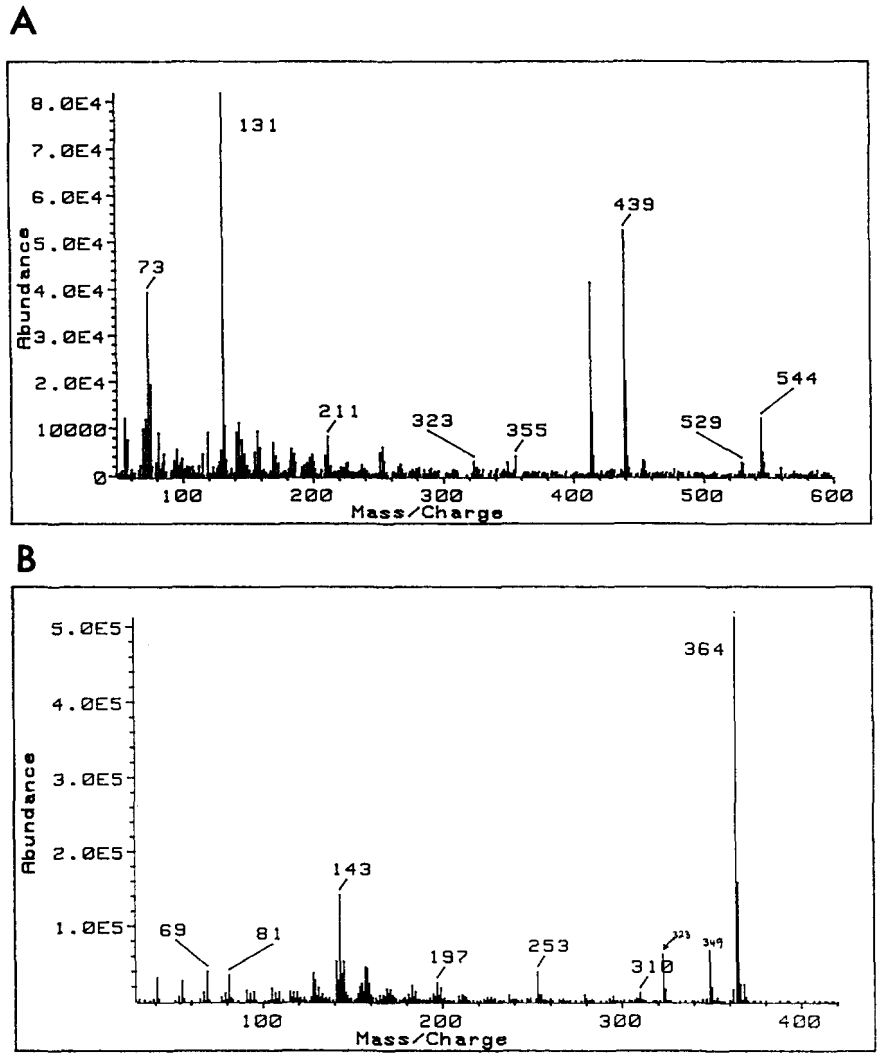

Figure 5
EI (+) mass spectra of (A) 25-hydroxyvitamin D_3-pertrimethylsilyl ether and (B) underivatized 25-hydroxyvitamin D_3 injected in the dehydration system.

injected into the dehydration system, single peaks were obtained both for the D_2 and D_3 series and intense high mass ions are obtained (see Fig. 5). It is clear that the thermal rearrangement will probably still occur but the abundance of the peak of the *isopyro*-isomer is greatly reduced, probably not in absolute terms but, because of the reduced efficiency of ionization in the ion source of the mass spectrometer, the total ion current appears to be less than for the peak of the *pyro*-isomer. For 25-hydroxyvitamin D further investigation showed that what had initially seemed to be a single peak was in fact a combination of four separate isomers which represent complete dehydration at C3 and C25 each producing two different isomers [C3 dehydration producing *delta*-2 and *delta*-3, and C25 producing *delta*-24 and *delta*-25 (26 or 27 but these are indistinguishable from each other)]. With 25-hydroxyvitamins D_2 and D_3 (25-OHD_3) this dehydration seems to be reproducible and straight line calibration curves can be obtained over a concentration range of 1–10 ng injected [y (peak height ratio) = $0.0106x$(ng 25-OHD_3) + 0.0047; correlation coefficient 0.995 with an intercept that is not significantly different than zero]. Clearly this process can be used for the development of a sensitive quantitative procedure for the measurement of 25-hydroxyvitamin D in human plasma and this will be the subject of further investigation. 25-hydroxyvitamin D is further hydroxylated in the kidney producing a number of metabolites, the most

important of which in physiological terms is 1α, 25-dihydroxyvitamin D. 24,25-dihydroxyvitamin D and 25,26-dihydroxyvitamin D are also formed but appear to have no major physiological role. These secosteroids also underwent dehydration when injected into the system described. Figure 6 illustrates the separation of 24,25- and 25,26-dihydroxyvitamin D_3 and their associated mass spectra. 24,25-dihydroxyvitamin D_3 can be dehydrated at C3, C24, and C25 whereas 25,26-dihydroxyvitamin D_3 can be dehydrated only at C3 and C25. Thus dehydrated 25,26-dihydroxyvitamin D_3 has a considerably longer retention time since it contains a polar hydroxyl group. This is an advantage since the trimethylsilyl ethers of these two vitamin D metabolites do not usually separate on non-selective GC phases [4]. 1α,25-dihydroxyvitamin D_3, on the other hand, undergoes more complex dehydration producing a number of different compounds which separate in the GC system used. Figure 7 shows the mass spectra of the two major peaks seen after injection of 1α,25-dihydroxyvitamin D_3 which are qualitatively similar but these two peaks are well separated in the GC system used. The ion *m/z* 362 represents the loss of 54 mass units (probably three molecules of water). Formation of *n*-alkyl boronates across the vicinal hydroxyl groups in 24,25- and 25,26-dihydroxyvitamin D protects against dehydration on the side-chain and when these derivatives are injected, dehydration occurs only at C3. The mass spectra of these com-

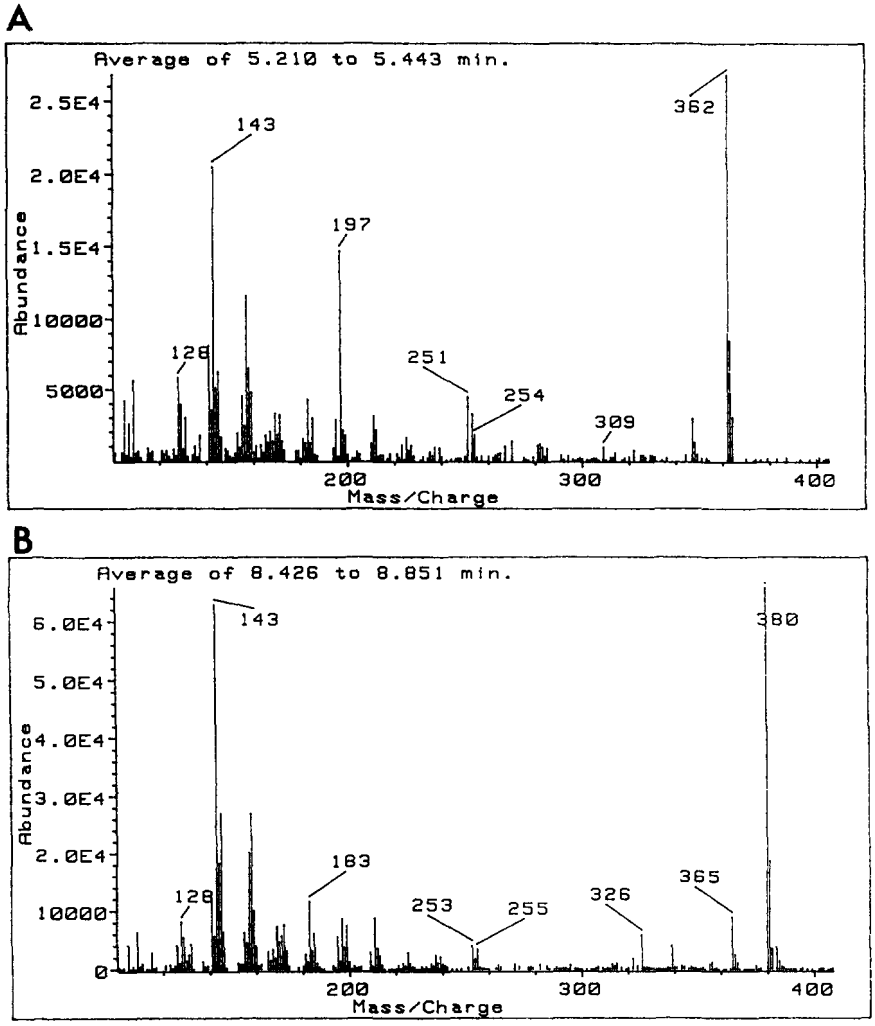

Figure 6
EI (+) mass spectra of (A) 24,25-dihydroxyvitamin D_3 and (B) 25,26-dihydroxyvitamin D_3 injected in the dehydration system. Note that the retention time (given in upper left of each mass spectrum) of 24,25-dihydroxyvitam D_3 dehydration product is substantially less than that of 25,26-dihydroxyvitamin D_3 dehydration product.

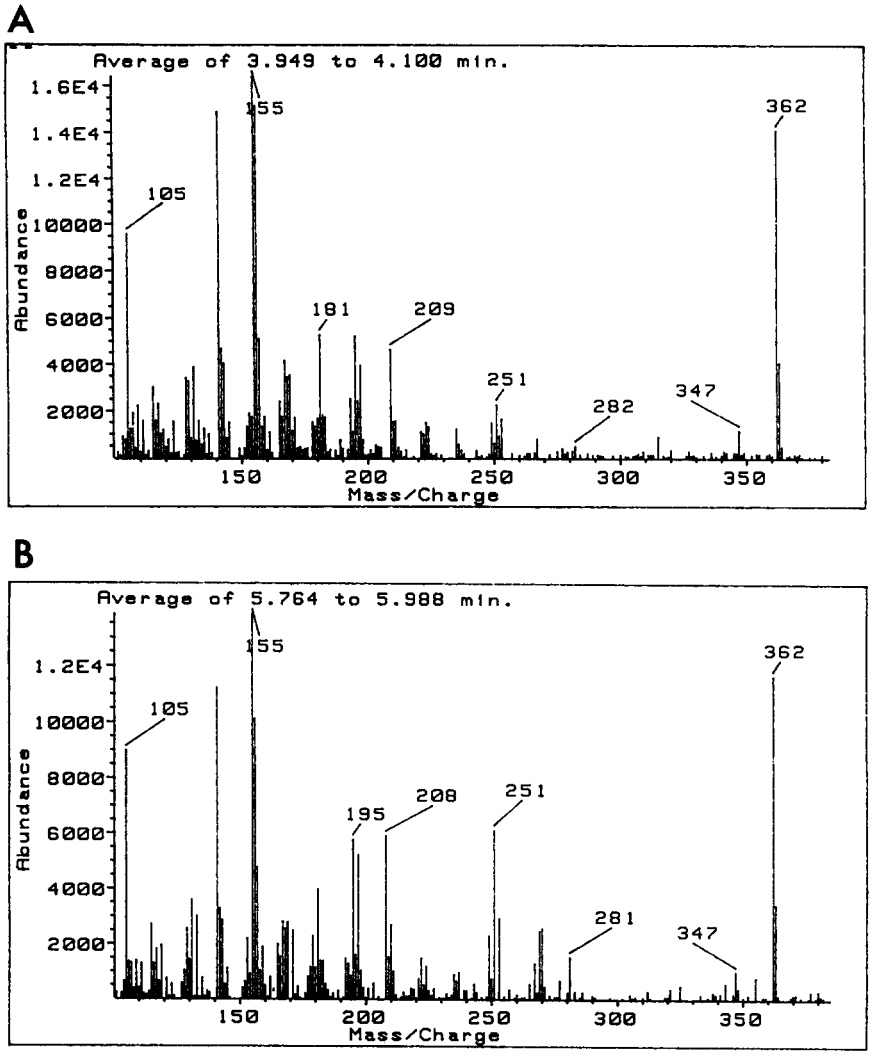

Figure 7
EI (+) mass spectra of the two major peaks obtained from 1,25-dihydroxyvitamin D_3 when injected in the dehydration system. Although the spectra are similar, the retention times (given in upper left of each mass spectrum) are very different.

pounds showed considerable improvement in that they have intense high mass ions which offer the possibility of improved sensitivity and/or specificity of measurement of these metabolites (see Fig. 8).

Discussion

The use of aluminium powder in the injection liner of the Hewlett–Packard GC allows high temperature dehydration of underivatized steroids prior to GC. Dehydration of vitamin D and its metabolites had previously been observed using powdered glass in the injection system [3]. The use of glass gave non-reproducible results and a number of other possible dehydrating agents were investigated finally leading to the use of aluminium powder, a purely empirical choice which seems to work efficiently. The results indicate that the use of this dehydration system for saturated hydroxylated C19 steroids may have advantages. However, most of the steroid hormones examined underwent complex dehydration reactions in the injection system and as a result gave multiple peaks. On the other hand, use of the dehydration system for the C27 and C28 secosteroids (vitamins D_2 and D_3) gave rise to single peaks with intense high mass ions and the dehydration was reproducible. The use of *n*-alkyl boronates protected the side-chain

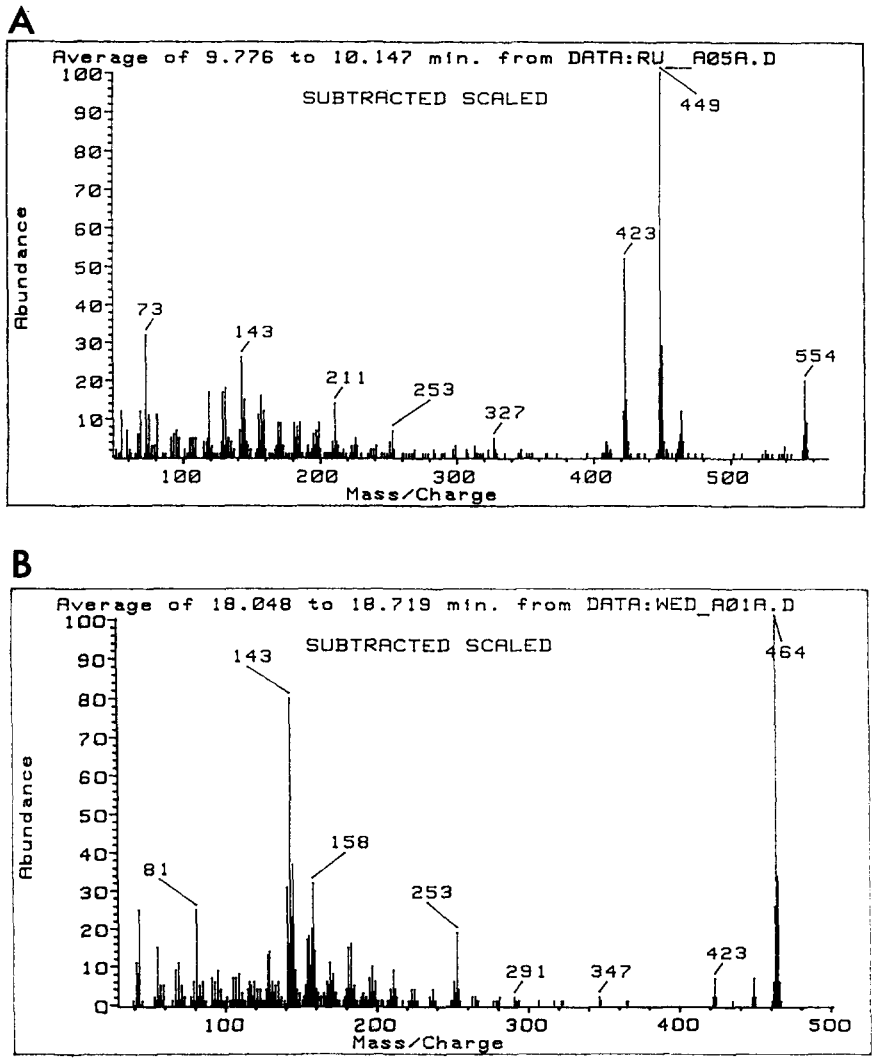

Figure 8
EI (+) mass spectra of (A) 24,25-dihydroxyvitamin D_3 as the 24,25-*n*-butyl boronate-3-trimethylsilyl ether and (B) 24,25-dihydroxyvitamin D_3-24,25-*n*-butyl boronate injected in the dehydration system.

hydroxyl groups, allowing dehydration only at C3. Dehydration has opened the possibility of developing GC–MS methods for the measurement of these two metabolites in human plasma with greatly increased sensitivity but without the need for complex derivatization prior to GC–MS. Similarly the use of pre-column dehydration has allowed the development of GC–MS methods for the measurement of 25-hydroxyvitamins D_2 and D_3, which can be automated and, using a simple benchtop mass spectrometer, can perhaps be used as a routine procedure, replacing some of the less precise competitive binding assay or saturation analysis procedures.

The reactions which occur in the dehydration liner of the GC are clearly of interest to the organic chemist but it has been difficult to interpret the mass spectra of many of the dehydrated products. Extensive investigations into the introduction of double bonds into steroids have been undertaken previously but it is believed that this paper reports the first investigation of on-column dehydration prior to gas chromatographic analysis. It has been possible to raise the injection temperature to 400°C without complications from septum decomposition because of the use of the Jade septumless injector. It has not been possible to raise the injection temperature beyond 400°C because the GC used in this investigation does not have such a facility. It may be that higher injection temperatures would give rise to enhanced dehydration of some of the steroids

which have been examined here. Other factors may be worthy of investigation, such as the effect of solvents in which the compounds are injected, an aspect that has received only minor attention in this paper. Only aluminium powder has been examined here but it is possible that other materials may offer advantages.

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