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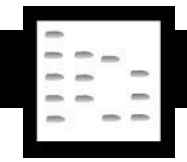
STEROIDS



Gas Chromatography

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Introduction

This review aims to summarize the application of gas chromatography (GC) to the analysis of steroids. The review concentrates mainly on hyphenated GC–mass spectrometry technology as the use of GC linked to detectors other than mass spectrometry (MS) is now decreasing. A survey of literature using MEDLINE indicated that in the period 1990 to date, more than 90% of around 400 references used GC–MS, as might be expected as the mass spectrometer is now the most effective detector for GC and simple, cheap and sensitive bench-top GC–MS systems are now widely available. Use of MS can often compensate for poor GC resolution or peak shape, but use of GC–MS still requires that attention is paid to optimization of both GC and MS behaviour, if maximum sensitivity is required. The MS, of course, has the added advantage that it can provide structural data and can be used to confirm that a GC peak is indeed a steroid. By comparing the mass spectrum obtained with those in a library can often identify the steroid. Retention time data, on their own, are not a satisfactory criterion for identification but can be considerable value when combined with MS data.

Steroids range from the C_{18} oestrogens to C_{27} sterols such as cholecalciferol (vitamin D) and include androgens, progestagens, corticosteroids and bile acids as well as a large number of synthetic steroids, some of which may be used therapeutically. The formulae of some of these steroid types are illustrated in Figure 1 in III/STEROIDS/Liquid Chromatography and Thin-Layer (Planar) Chromatography. Alternatively readers can consult the *Dictionary of Steroids*, which lists some 10 000 steroids together with their formulae, trivial and systematic names and other useful information.

Derivatization

Most steroids have melting points in excess of 150°C (estradiol-17β, the female sex hormone, for example, has a melting point of 176°C). It is therefore often necessary to derivatize steroids of interest in order to optimize their GC performance. Derivatization improves volatility, a necessary characteristic as the analyte in GC must be in the vapour phase. High injection (around 350–400°C) and column temperatures (up to 350°C) may also be necessary to achieve

separation, especially of higher molecular weight steroids and their derivatives. The raised temperature necessary to achieve satisfactory separation, also brings with it the problem of analyte decomposition, although decomposition should not be taken to mean destruction. It is, for example, possible to separate oestrogens and androgens and some progestagens without derivatization, but 17-hydroxylated C_{21} steroids (such as cortisol) undergo thermal side-chain cleavage and cholecalciferol (vitamin D_3) and its metabolites all undergo B-ring closures, giving rise to two isomers, even when derivatized. Such characteristic reactions may often have useful diagnostic features. It is also possible to enhance particular thermal reactions such as dehydration by the use of catalysts in order to obtain quantitative conversion in the injection port to dehydration products, which may have improved MS characteristics. 25-Hydroxyvitamin D_3 can be analysed in this way using aluminium powder in the injection port and the dehydration product has intense high mass ions which improve sensitivity of MS detection and of course time-consuming derivatization is avoided. **Figure 1** illustrates this particular example.

Derivatization also improves GC peak shape as the presence of hydroxyls increases adsorption during chromatography and at very low concentrations this adsorption may give rise to a nonlinear response. In addition, if GC–MS is to be used, the appropriate choice of derivative may also have a profound influence on sensitivity and/or specificity of detection. An example of this is given in **Figure 2**, where the mass spectrum of the 3,17β-di(trimethylsilyl) ether of 19-nor-androsterone is compared with the spectrum from the 17β-trimethylsilyl (TMS) ether. In this example (this is the urinary metabolite which is measured in order to confirm abuse of the anabolic steroid nandrolone), it can be seen that the intensity of the two high mass ions in the di-TMS ether (which is a 3-enol ether) are considerably greater than those for the mono-TMS ether, allowing greater sensitivity and specificity of measurement. **Table 1** lists some of the common methods of derivatization, which protect against adsorption and decomposition and at the same time improve MS characteristics. Negative-ion chemical ionization (CI) techniques, which use soft ionization and yield predominately the molecular or pseudo-molecular ion, can provide very sensitive assay methods but require the presence of electron-capturing moieties. Most steroids do not possess these and derivatization is often used in this context to provide steroid derivatives containing the necessary chlorine, iodine or bromine atoms (e.g. perfluoroacyl or chloro- or iodomethyldimethylsilyl ether derivatives). For the higher energy electron impact ionization (EI), halogenated derivatives are not necessary and hydroxyl groups are usually derivatized as TMS ethers and oxo groups as O-methyloximes (or enolized to give enol-TMS ethers). Mixed derivatives are also used (e.g. O-methyloxime-TMS derivatives) and in this example the oxime is formed first and protects the oxo group against subsequent enolization by the silylating reagent.

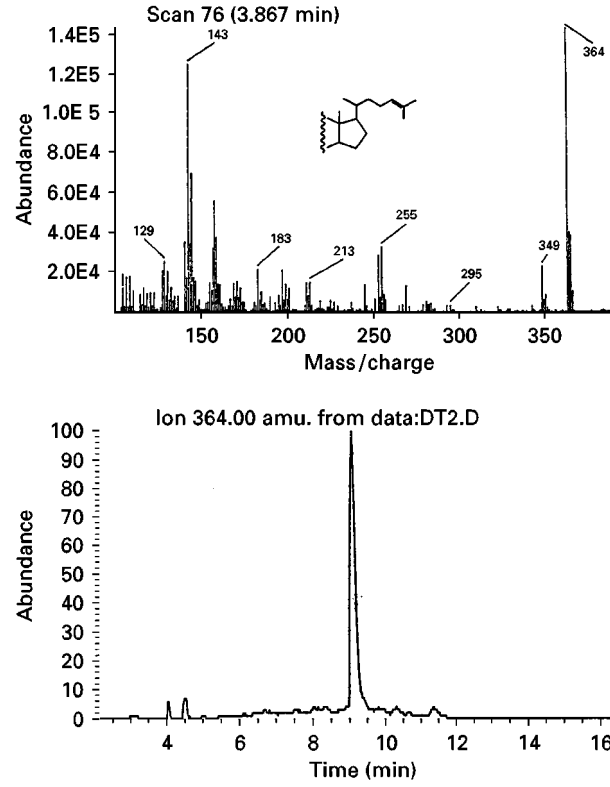

Figure 1 On-column quantitative dehydration of underivatized 25-hydroxyvitamin D_3 using aluminium powder in the injection liner. (Upper panel) EI(+) mass spectrum of the dehydration product(s) – there are at least two dehydration products, which do not separate, but only one is illustrated. Note the greatly increased intensity of the molecular ion – m/z 364. (Lower panel) Single ion monitoring of m/z 364, indicates only a single peak. (From G Jones *et al.* In: *Modern Chromatographic Analysis of Vitamins* (eds A DeLeenheer, WE Lambert and HJ Nellis), 2nd edn. New York: Marcel Dekker, 1992, with permission of authors and publisher.)

17-Hydroxylated C_{21} steroids are thermostable when derivatized as 17-TMS-ethers-20-oximes and can thus be analysed without degradation. Steroid carboxylic acids (e.g. bile acids) will not run in GC systems except as aliphatic esters (usually this means formation of methyl esters as otherwise molecular weight and thus retention time increases). Other esters have, however, been used for GC of faecal extracts to separate the bile acids from the neutral sterols, which are insufficiently resolved as methyl-TMS ethers. Use of *n*-butyl-TMS ethers increases the

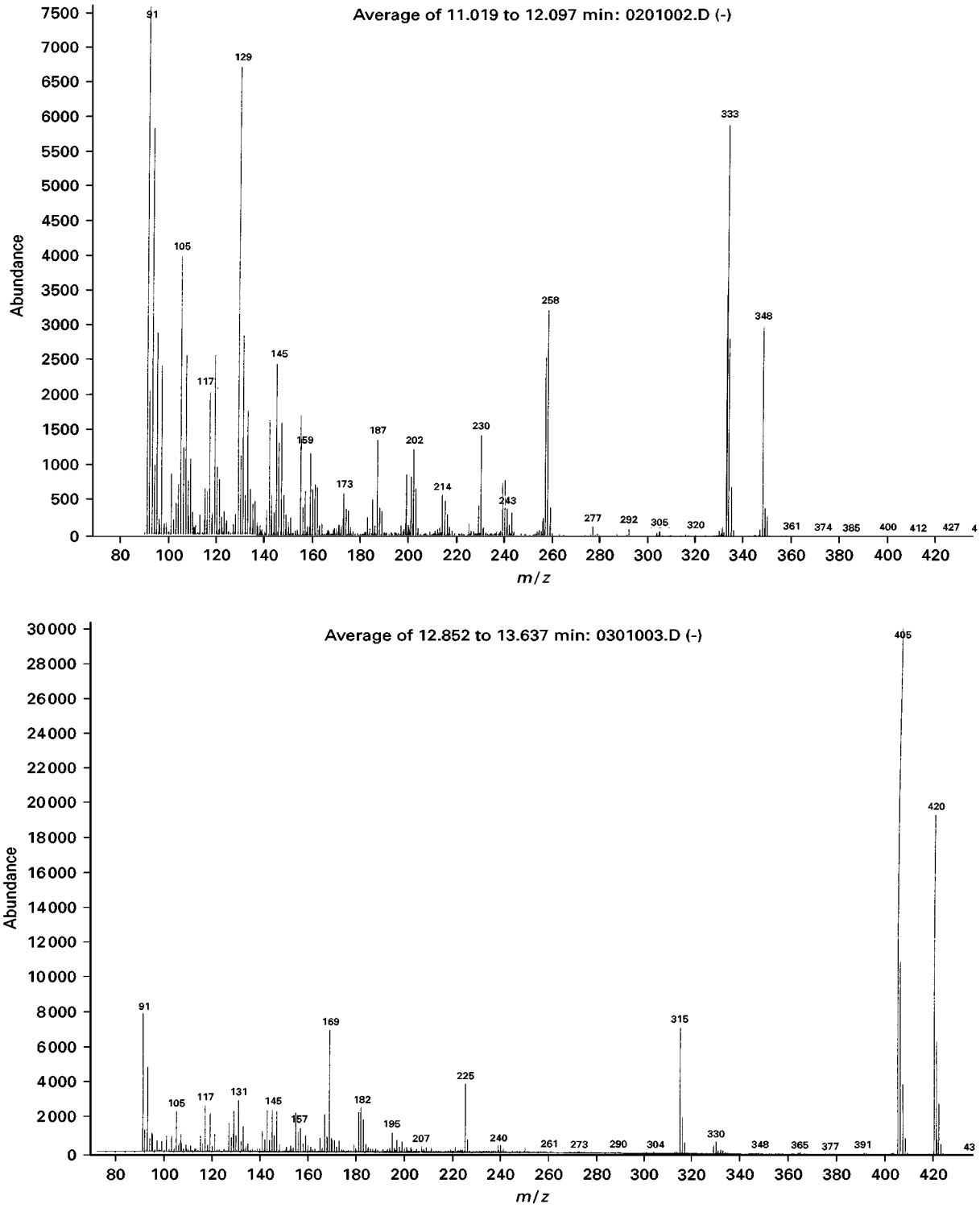

Figure 2 Enhanced sensitivity of detection of the anabolic steroid, nandrolone, by formation of different derivatives. (Top) EI(+) mass spectrum of the 17β-trimethylsilyl ether and (bottom) EI(+) mass spectrum of the 3-enol,17β-di(trimethylsilyl) ether. It can easily be seen that the two ions at m/z 405 and m/z 420 of the di-TMSI carry more of the total ion current than the corresponding ions (m/z 333 and m/z 348) of the mono-TMS. These mass spectra were produced using equal amounts of nandrolone and the ion at m/z 91 offers a useful index for comparison. (With permission of Mrs J Nolan.)

retention time of the bile acids sufficiently to separate them from the sterol-TMS ethers. This is illustrated in **Figure 3**. Other derivatives have also been used which are selective for particular parts of the steroid structure, such as formation of cyclic boronates across vicinal hydroxyls. Such derivatives being selective

Table 1 Some derivatization procedures used for the GC and GC–MS analysis of steroids. This list is not comprehensive but includes the majority of the most popular derivatives

Steroid group	*Derivative*	*Formula**
Hydroxyl	Trimethylsilyl ether (TMS)	$(CH_3)_3Si–O–St$
	t-Butyldimethylsilyl ether (TBDMS)	$(CH_3)(CH_3)_2Si–O–St$
	Chloromethyldimethylsilyl ether	$(CH_2Cl)(CH_3)_2Si–O–St$
	Dimethylethylsilyl ether	$(CH_3CH_2)(CH_3)_2Si–O–St$
	Pentafluorophenyldimethylsilyl ether	$(C_6F_5)(CH_3)_2Si–O–St$
	Acetate ester	$CH_3CO–O–St$
	Formate ester	$HCO–O–St$
	Hepta- and pentafluorobutyrate ester	$CF_3CF_2CH_2CO–O–St$
		$CF_3CF_2CF_2CO–O–St$
	Dimethylisopropylsilyl ether	$(CH_3)_2(CH_3CHCH_3)Si–O–St$
Vicinal hydroxyls	*n*-Butylboronate ester	$CH_3(CH_2)_3B–(O)_2–St$
Oxo groups	O-Methyloxime	$(St–C)=N–O–CH_3$
	Enol-TMS ether	$(St–C=C)–O–Si(CH_3)$
	O-perfluorobenzyloxime	$(St–C)=N–O–C_6F_5$
Carboxylic acids	Methyl ester	$(St–CO)OCH_3$
	Isobutyl ester	$(St–CO)O(CH_2)CH(CH_3)_2$
	n-Butyl ester	$(St–CO)O(CH_2)_3CH_3$

*St = steroid.

are diagnostic of structure and may also have the advantage of improving sensitivity and specificity of measurement.

Column Performance

For good GC performance, the intention is to obtain symmetrical peaks with retention times as short as necessary to achieve the desired separation. In the past considerable attention was paid to the development of different stationary phases in order to optimize resolution but the advent of capillary column and their linkage to MS systems has reduced the need for new stationary phases. Although capillary columns with a variety of bonded stationary phases are available, most GC–MS systems for steroids use non-selective (nonpolar) methylsilicone phases (e.g. HP1 columns from Hewlett-Packard), although more polar phases may be necessary for particular separations (i.e. C_{20} steroid carboxylic acids). Columns are usually around 15–30-m long (i.d. 0.2–0.4 mm with film thickness from around 0.1 μm upwards) and carrier gas flow rates are 1–2 mL min^{-1}, allowing direct insertion of the column exit into the ion source of the mass spectrometer. There are numerous means of sample injection but we have found the easiest to be direct on-column splitless injection using a syringe. For optimum chromatographic performance, we have found that the injection temperature is best kept at 400°C and that the choice of solvent can also have influence. This high temperature causes considerable problems in that most injection port septa are not suitable and breakdown products cause interference. This has been overcome by use of a septumless injection system (JADE injector) in which the syringe needle injects onto the column through two stainless-steel ball-bearings, which form the back-pressure seal. Other injection procedures have found favour in the steroids field, all of which strive to inject as much of the extract as possible. These systems include a dropping glass needle in which the sample is loaded into small glass capillaries which can be automatically loaded sequentially into the heated zone of the injector. Cold trapping splitless injection has also proved useful in that it allows for the on-column injection of relatively large volumes of solvent into silanized glass liners. Injection systems which load the whole of the extract onto the top of the column necessarily shorten column life and for quantitative work, column deterioration must be monitored to achieve consistent and high sensitivity. When deterioration is detected, the column can be regenerated by removal of the top 10 cm or so but this may lead to alteration in retention characteristics of steroid derivatives.

Prior to GC analysis, steroids must be extracted and purified, the degree of purification depending upon the specificity of the detector system employed. Specific GC–MS systems require less pre-purification but the possible contamination of the MS ion source must always be considered. Extended column life and increased periods between ion source cleaning are

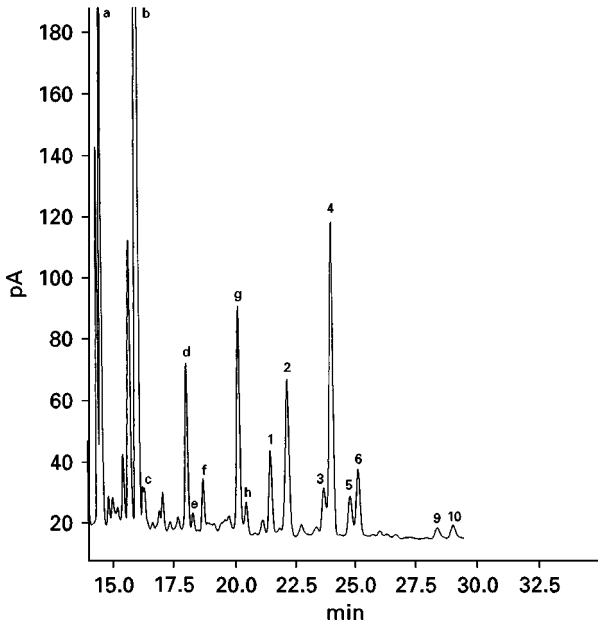

Figure 3 GC chromatogram of sterols and bile acids present in stool from a healthy control. 10 mg of freeze-dried stool containing 20 μg nor-cholic acid was subjected to derivatization. After dissolving in 200 μL hexane, 1 μL was injected into the GC column. Chromatographic and derivatization details can be found by consulting the original paper. Peak identification: 1, nor-cholic acid; 2, lithocholic acid; 3, iso-deoxycholic acid; 4, deoxycholic acid; 5, chenodeoxycholic acid; 6, cholic acid; 9, 3-oxo,12α-hydroxy-5β-cholanoic acid; 10, 12-oxo-lithocholic acid; a, coprostanol; b, cholesterol; c, 24-methyl-coprostanol; d, campesterol; e, 24-ethylcoprostanol; f, stigmasterol; g, sitosterol; h, sitostanol. (From AK Batta *et al* (1999) *Journal of Lipid Research* 40: 1148–1154, with permission of authors and FASEB.)

obtained if attention is paid to pre-column purification. Silylating reagents should also be removed prior to injection by use of small Lipidex 5000 columns, unless they are sufficiently volatile not to cause a problem. Trimethylsilylimidazole, a valuable reagent for the formation of TMS ethers on sterically hindered hydroxyls (e.g. at positions C11β, C17α, C25), must be removed before GC–MS, whereas N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) can be injected directly. Steroid glucuronides and sulfates must be hydrolysed prior to GC as they do not run in GC systems unless special derivatization methods are adopted. While we have found trimethylsilyl ethers to be stable, others have not. It is advisable therefore to store and inject steroid TMS ethers in MSTFA.

Mass Spectrometry and Other Detectors

The GC of steroids can be carried out with a variety of detectors, flame ionization (FID) being the most widely used today. Electron-capture detectors (ECD) which were commonly used in the past to improve sensitivity of detection, have now largely been replaced with negative ion chemical ionization (CI) mass spectrometry. Selective detection of steroid oximes can be accomplished using nitrogen-phosphorus detectors. Today, however, the mass spectrometer in various forms offers the most versatile detection system for GC, providing improved selectivity and sensitivity in comparison to other detectors. Because of the successful development of immunoassays for most of the clinically important steroids, GC has not in recent times found much application for individual steroid analysis, although occasional publications can still be found. However, the advent of capillary columns with immense resolving power suggested the possibility of utilizing GC as a means of examining in a quick and simple way, the complex patterns of steroids in human urine and how they change in disease states. In the late 1970s Shackleton, utilizing the pioneering work of Gardiner and Horning of ten years before, introduced the concept of urinary steroid profiling. Urinary steroid extracts (with or without β-glucuronidase hydrolysis) were derivatized to form steroid O-methyloxime-TMS ether derivatives and analysed using capillary open-tubular columns monitoring the analytes by flame ionization detection. Use of two internal standards allowed the quantification of 23 different steroids in children with various steroid abnormalities. These robust techniques are still in use today and provide valuable information to assist clinical diagnosis and monitoring of treatment and modern data handling technology has greatly eased the task of interpreting these complex profiles. The urinary profiling technique also allows identification of unknown peaks in the extract, when the original flame ionization detector is replaced with a mass spectrometer. An example of this methodology is illustrated in **Figure 4**. Further information about this valuable approach to urinary steroid analysis by GC–MS and its application in the diagnosis of steroid related disorders can be found in Shackleton's article in the Further Reading section.

The necessary process of purification and derivatization means that for quantitative work, suitable internal standards must be used. For GC–MS the best internal standards are of course stable isotope (deuterium or carbon-13) labelled analogues of the analyte. In these situation at least three isotopic atoms must be incorporated and the percentage of the triply labelled standard (i.e. in the case of deuterium labelled, d_3) should be greater than 99%. Deuterium labels are usually introduced by acid-catalysed deuterium exchange and thus the label may not be

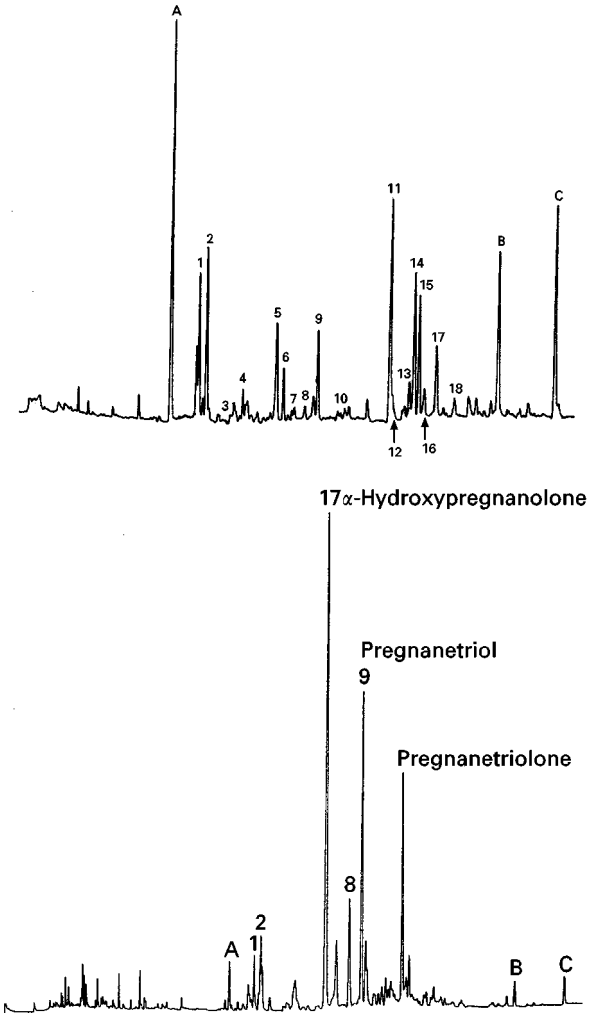

Figure 4 Steroid profiles by gas–liquid chromatography obtained from urine samples from (upper trace) a normal adult and (lower trace) a 16-year-old male with congenital adrenal hyperplasia (21-hydroxylase deficiency). Steroids were extracted with Sep-Pak C18 cartridges and after hydrolysis of glucuronide and sulfate conjugates, re-extracted and O-methyloxime-trimethylsilyl ether derivatives were formed. These were analysed by GLC using an OV1 capillary column. The major metabolites of 17-hydroxyprogesterone (the substrate of the 21-hydroxylase enzyme) are named in the lower trace. Other peaks are as follows: A, B and C: internal standards, androstanediol, stigmasterol and cholesteryl butyrate; 1: androsterone; 2: aetiocholanolone; 3: dehydroepiandrosterone (DHEA); 4: 11-oxo-androsterone; 5: 11β-hydroxy-androsterone; 6: 11β-hydroxy-aetiocholanolone; 7: 16α-hydroxy-DHEA; 8: pregnanediol; 9: pregnanetriol; 10: androstenetriol; 11: tetrahydrocortisone; 12: tetrahydro-11-dehydrocorticosterone; 13: tetrahydrocorticosterone; 14: *allo*-tetrahydrocorticosterone; 14: tetrahydrocortisol; 15: *allo*-tetrahydrocortisol; 16: α-cortolone; 17: β-cortolone + β-cortol; 18: α-cortol. (Kindly provided by Dr Norman Taylor, King's College School of Medicine and Dentistry.)

stable in acid conditions. Ideally ^{13}C-labelled standards are to be preferred but this requires incorporation into the nucleus of the steroid which can only be achieved by extensive synthetic chemistry. All steroids are analysed by GC–MS in the same way and the criteria used to ensure specificity/accuracy are those adopted by the Substance Abuse and Mental Health Services Administration (SAMHSA) for drug confirmation in employee drug-screening programmes – two, but preferably three, specific ions (with as high a mass : charge ratio as possible) must be monitored and the results derived from each ion must not deviate by more than 10% from the mean. **Figure 5** illustrates the chromatograms obtained by multiple ion detection, monitoring two of the relevant ions of the analyte (25-hydroxyvitamin D_3) and the equivalent two ions from the hexadeuterated internal standard present in a plasma sample extract. In this example the standard curve relating peak height ratio (analyte : internal standard against mass of standard analyte) was linear and the intercept was not significantly different from zero.

Isotope dilution GC–MS is widely acknowledged as the gold standard of steroid analysis and is used as a means of providing target values for external quality-assurance schemes and for the confirmation of immunoassay screening procedures for drugs of abuse. **Table 2** gives brief details of the application of this methodology to the analysis of steroids in body fluids, which are taken from papers in the literature published between 1998–1999 and use both stable isotope-labelled and unlabelled internal standards. The availability of accurate and precise methods of steroid analysis by GC–MS is becoming of increasing public interest as the number of sportsmen and women in whose urine metabolites of anabolic steroids are found, continues to increase. It is clear that it is important for steroid (and other) drug testing that proper methodology for both qualitative detection and quantitation is available and this methodology can withstand public scrutiny. GC–MS provides precisely this. Excellent and up to date reviews of the application of GC and GC–MS to the analysis of steroids can be found in the Further Reading.

Mass Spectrometry for Structural Analysis

The other important aspect of GC–MS, apart from providing a high specificity method of steroid assay, is the role of MS as a means of identifying both known and unknown steroids. The use of the GC in this context is simply a means of delivering a relatively purified steroid derivative to the MS. The present author and his colleagues have successfully used GC–MS as a means of studying the metabolism of calcitriol analogues in target tissues and while the illustrations given are derived from these studies, they have wider application and the methodology used can

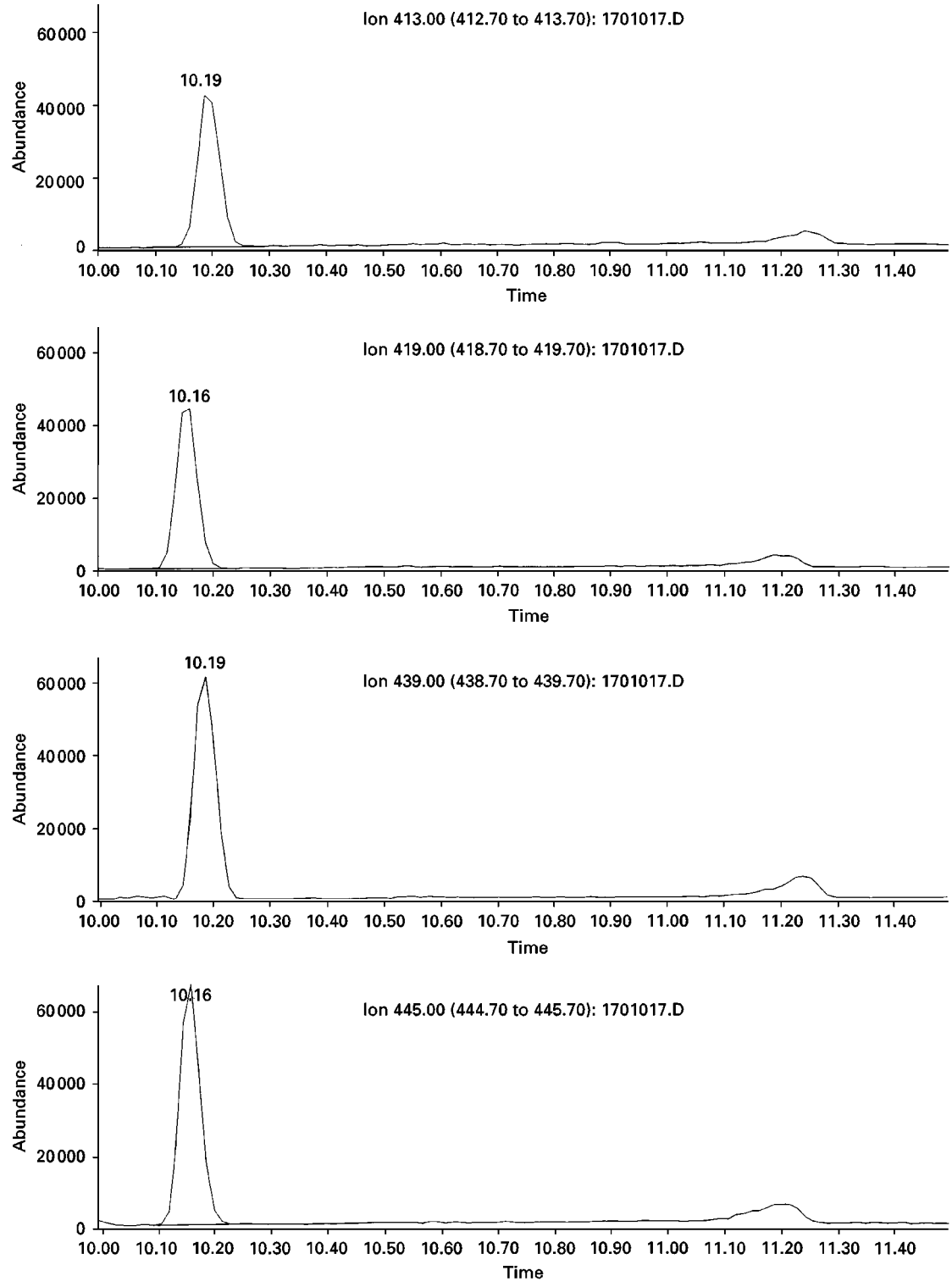

Figure 5 Isotope dilution mass fragmentography of 25-hydroxyvitamin D_3. GC was carried out after formation of per-trimethylsilyl ether derivatives using a non-selective OV1 column. An internal standard, [25,26-$^2H_6$]25-hydroxyvitamin D_3, was added to the plasma sample prior to extraction and purification. The GC column was inserted into the ion source of the mass spectrometer and four ions were monitored (*m*/*z* 413 and 439 from the analyte and the corresponding ions, *m*/*z* 419 and *m*/*z* 445, from the internal standard). It will be noted that the hexadeuterated internal standard runs slightly earlier than the non-deuterated analyte. In this case the ratio of peak areas of analyte to internal standard gave a straight-line response which went through zero. Only two ions were monitored in this example whereas increased specificity can be obtained if three are monitored. The major peaks, the pyro-isomer and the isopyro-isomer, can be seen at approximately 11.20 min. Both peaks have a cyclized B-ring.

Table 2 Some examples of methods for the measurement of steroids by gas–liquid chromatography, published in 1998–1999

Subject	*Column details*	*Derivatives used*	*Internal standard*	*Detection*
Urinary steroid metabolite analysis	Non-selective methylsiloxane and 5% phenylmethylsiloxane 17 and 25 m × 0.2 mm	O-methyloxime-trimethylsilyl ethers	Androstanediol, stigmasterol and cholesteryl butyrate	Flame ionization detection* or mass spectrometry (EI+)
Ovarian steroids in the catfish	15 m DB1 column	O-methyloxime-trimethylsilyl ethers	None given	Mass spectrometry (EI+)
3α-Reduced neuroactive steroids in human plasma	30 m × 0.25 mm with 0.2 μm film thickness – HP5	O-methyloxime-heptafluorobutyrate esters	None given	Mass spectrometry (EI+)
Anabolic steroid metabolites in urine	30 m × 0.2 mm with 0.33 μm film thickness. 5% phenylmethylsiloxane (ultra-2)	Pentafluoropropionates	[1,2-$^{2}H_2$]-Testosterone	Mass spectrometry (CI−) using methane as reagent gas)
Detection of exogenous testosterone administration	DB7 (50% phenylmethylsiloxane). 30 m × 0.25 mm with 0.15 μm film thickness	Acetates	Not relevant as only $^{13}C/^{12}C$ ratios being measured	Mass spectrometry – combustion isotope ratio
Endogenous 19-nor-androsterone and aeticholanolone in human urine	HP1 30 m × 0.25 mm with 0.25 μm and HP5 (5% phenylmethylsiloxane) 25 m × 0.2 mm with 0.33 μm	Trimethylsilyl ethers (enols) and t-butyl-dimethylsilyl ethers	Trideuterated 19-nor-aetiocholanolone	Mass spectrometry (EI+)
Serum DHA and DHA sulfate	DB5 30 m × 0.25 mm with 0.25 μm film thickness	No derivatization	Androst-5-en-3β-ol-16-one methyl ester	Ion trap mass spectrometry (EI+)
Testosterone: epitestosterone in equine urine	17 m × 0.2 mm with 0.11 μm film thickness (5% phenyl-methylsiloxane)	3-Trimethylsilyl ether-17-pentafluoro-phenyldimethylsilyl ether	Not applicable as ratio being measured	Mass spectrometry (EI+)
Testosterone in hair	Optima 1 25 m × 0.2 mm with 0.1 μm film thickness	Heptafluorobutyrate	Trideuterated testosterone	Mass spectrometry (EI+)
Urinary 3-oxo-Δ^4-bile acids	30 m × 0.2 mm methylsiloxane	Carboxylic acid methyl ester-dimethylethylsilyl ether and O-methyloximes	3α,7α-Dihydroxy-24-nor-5β-cholanic acid	Mass spectrometry (EI+)
Biliary elimination of endogenous 19-nortestosterone	None given	Heptafluorobutyrates	Trideuterated 19-nortestosterone	High-resolution mass spectrometry (EI+)

*See Figure 4 which illustrates the application of GC–FID for urinary steroid analyses.
DHA, dehydroepiandrosterone.

be applied to all steroids and their metabolites. **Figure 6** shows the GC trace of calcitriol (1,25-dihydroxyvitamin D_3) as the per-trimethylsilyl derivative. Two peaks are always seen, as B-ring cyclization which occurs at the high temperature of the injection port quantitatively produces pyro- and isopyro-isomers which are always formed in the same ratio. Thus for every vitamin D-like compound, two GC peaks are observed. It is the pyro-peak which predominates and Figure 6 also shows the EI(+) mass spectrum derived at the apex of the pyro-peak after background subtraction. For the purpose of structural analysis, EI(+) spectra are preferable to CI spectra as CI is a much softer technique giving less useful fragmentation – a similar objection applies to LC–MS, which also uses soft ionization. It is of course also possible to obtain a spectrum of the underivatized, albeit cyclized, calcitriol by ignoring the GC and inserting the calcitriol into the ion source of the MS by direct probe. This gives a molecular ion (M^+) of 416. Examination of the mass spectrum of the per-TMS derivative shown in Figure 6 indicates a molecular ion of 732. Knowing that each TMS formed increases the molecular weight by 72 amu, it is possible to calculate the number of hydroxyl groups in an unknown compound ($632 - 416 = 216$ and $216/72 = 3$). Metabolism of calcitriol and its analogues usually involves cytochrome P450-catalysed

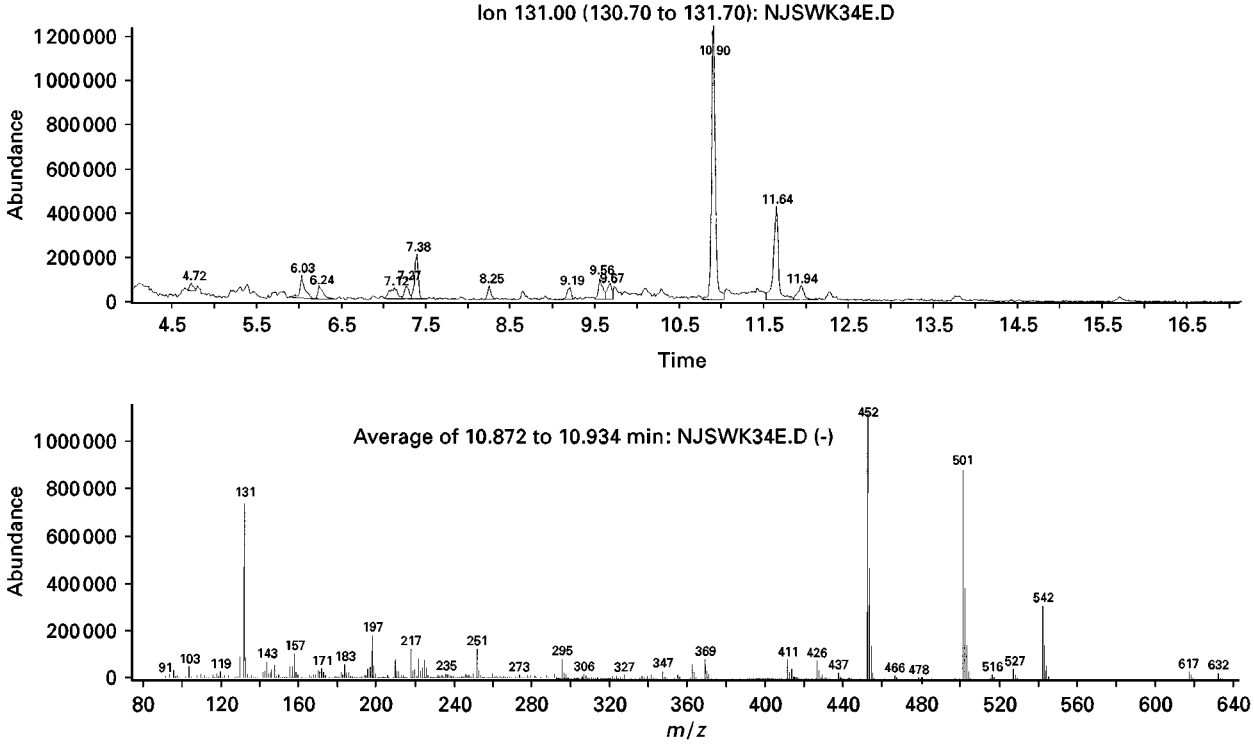

Figure 6 EI(+) mass spectrum of 1,25-dihydroxyvitamin D_3. The total ion current is shown in the upper panel indicating the two cyclized isomers (pyro- at 10.90 min and isopyro- at 11.64 min) which are formed. In the lower panel is the mass spectrum of the per-trimethylsilyl ether of the pyro-isomer.

hydroxylation(s). The number of hydroxylations can be determined by the same procedure described above and if the MS of the per-TMS of the substrate is known, direct probe MS is not necessary. However further interpretation of the MS becomes necessary in order to decide where on the steroid molecule the hydroxylation has occurred. It is clearly also possible to deduce the presence of an oxo group as this increases the molecular ion of the substrate by 14 amu but again knowledge of the presence of this group does not determine its position. To carry out these calculations, it is necessary to be able to determine the molecular ion value. It is not always possible to do this directly as the mass spectra of the steroids usually have very low intensity molecular ions. However, as can be seen in Figure 6, all these cyclized steroid-TMS ethers have a prominent $(M\text{-}131)^+$ ion, which is usually derived from A-ring cleavage, as well as $(M\text{-}90)^+$ ions, derived by successive loss of silanols. It is therefore possible even in the absence of discernible M^+ ions in the spectrum, to determine the m/z value of the molecular ion.

For the identification of the position of extra hydroxyls and oxo groups or even truncation, where cytochrome P450 lyases have cleaved the side chain, GC retention time data can prove extremely useful. Hydroxylation increases retention time but the further out along the side chain (distal) the hydroxylation is, the longer the retention time. Truncation, by reducing molecular weight, clearly decreases retention time. Retention time, although useful is not sufficient on its own and further study of the fragmentation data has to be made. Further examples can be obtained by consultation of the texts listed in the Further Reading section. Consideration should also be given to the use of chemical reactions which modify the molecule under investigation. Reduction of oxo groups with sodium borohydride and subsequent derivatization as TMS ethers and GC–MS provides further evidence of structure. Cleavage of carbon–carbon bonds between vicinal hydroxyl groups with periodate can also provide valuable information about the site of hydroxylation if the reaction product is subsequently derivatized and subjected to GC–MS.

A very good example of the interpretation of mass spectra obtained from GC–MS of per-TMS derivatives is given in **Figure 7**. The metabolites illustrated here are all mono-hydroxylated metabolites of 1α-hydroxyvitamin D_3 and thus give the same value of 632 amu for their molecular ion. All four metabolites show the characteristic $(M\text{-}131)^+$ ion at m/z 501 as well as $(M\text{-}90)^+$, 542 and $(M\text{-}90\text{-}90)^+$, 452. The abundance of the M^+ ion is, as usual, very low but it can easily be confirmed as being the ion at m/z 632 by

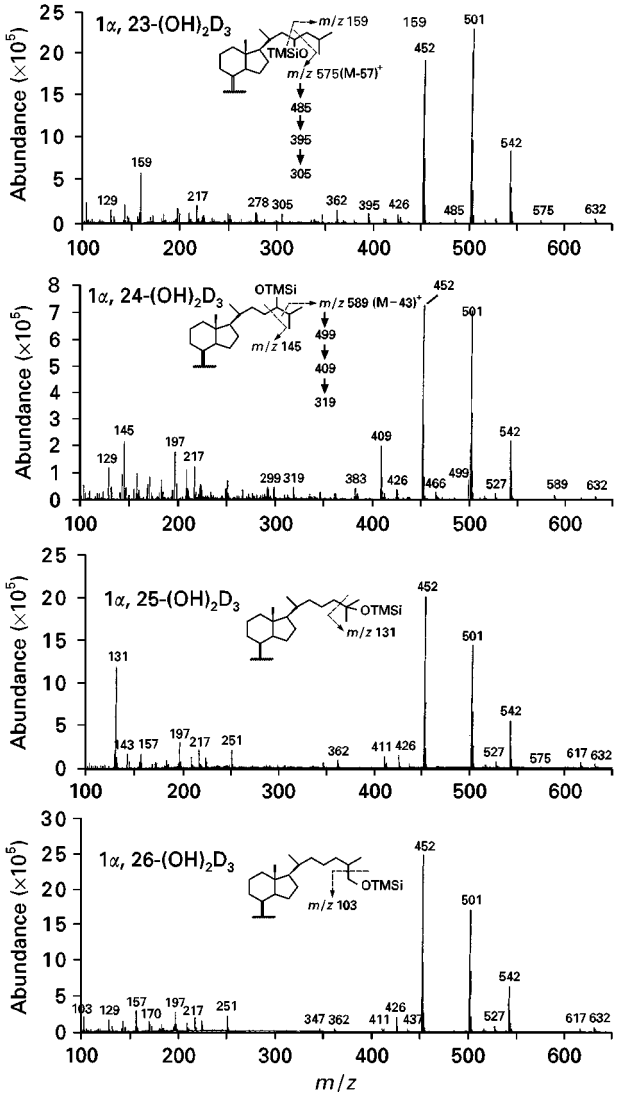

Figure 7 The EI(+) mass spectra of metabolites of 1α-hydroxyvitamin D_3 (1α-OHD_3). GC–MS was carried out after derivatization to form the per-trimethylsilyl ethers. Both pyro- and isopyro-isomers of each metabolite were observed but the mass spectrum of the pyro-isomer (the major peak) is shown in each case. The major ions (*m/z* 632 (M^+), *m/z* 542, 432 and 362 (not highlighted) (M^+ losing successive silanols) and *m/z* 501 (M^+ losing 131 by A-ring cleavage) are the same in all the spectra. *m/z* 217 is the characteristic ion always seen in these 1,25-dihydroxylated steroids and *m/z* 251 (not highlighted) arises by side-chain cleavage and subsequent loss of three silanols. It is however possible to distinguish each isomer from the characteristic fragmentation patterns illustrated for each above the appropriate spectrum. (From G Jones and HLJ Makin (2000) In: *Modern Chromatographic Analysis of Vitamins* (eds A DeLeenheer, WE Lambert and HJ Nellis), 3rd edn. New York, Marcel Dekker, to be published, with permission of authors and publisher.)

consideration of the origin of the more abundant ions. Although not shown here, the retention times increase as the hydroxylation position moves distally along the side chain. It is the presence of other less abundant ions of lower *m/z* value, which are diagnostic for the position of the hydroxyl on the side chain and the derivation of these ions is shown in the fragmentation patterns illustrated in Figure 7. Many other examples of this sort of elucidation of seco-steroid structure can be given, all of which rely on the same sort of approach.

Routine steroid analysis at ng mL^{-1} concentrations by GC–MS utilizes low-resolution mass spectrometry but there are occasions when increased sensitivity is required for the detection/measurement of steroids at concentrations in the pg mL^{-1} range. This can be achieved by using high-resolution (double-focusing)

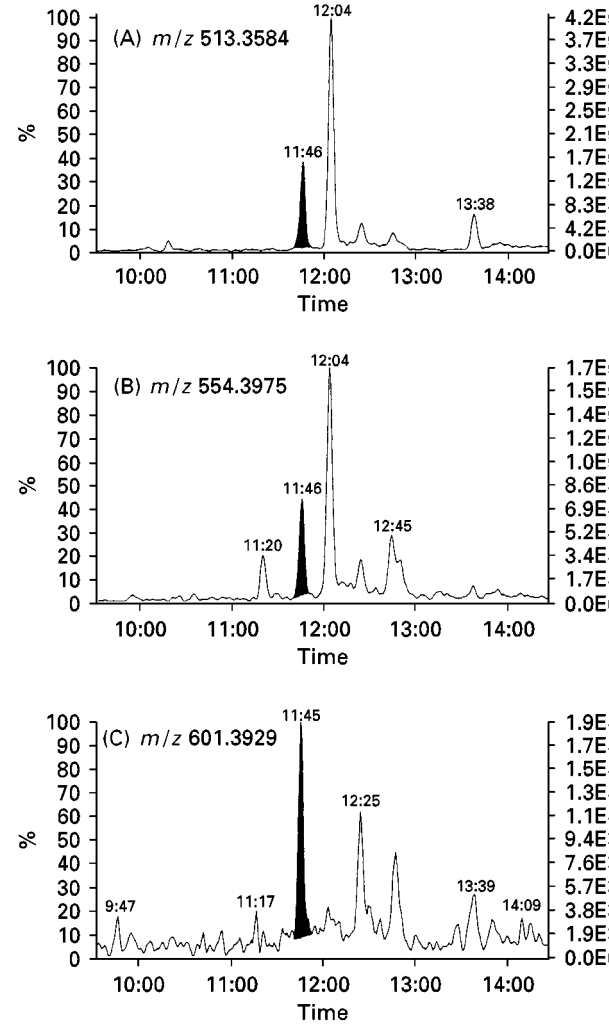

Figure 8 High-resolution mass fragmentography of an extract of serum from a patient taking vitamin D_2, showing ion chromatograms of per-trimethylsilylated (TMS) ether of putative 1α,24-$(OH)_2D_2$, monitoring three separate ions, *m/z* 513.3584 (A), *m/z* 554.3975 (B), and *m/z* 601.3929 (C), showing the trace between 9 and 14 min. The peaks from the pyro-isomer of 1α,24-$(OH)_2D_2$-TMS are shaded. The ion ratios in this extract are the same as those in the mass spectrum of the authentic compound. (From EB Mawer *et al.* (1998) *Journal of Clinical Endocrinology and Metabolism* 83: 2156–2166, with permission of authors and publisher.)

instruments which, although they increase specificity, reduce overall sensitivity but paradoxically allow increased sensitivity of measurement by increasing the signal : noise ratio. GC–HRMS has successfully been used for the measurement of a calcitriol analogue, hexafluorocalcitriol, with a minimum detectable limit of 2 pg mL^{-1}, which gives this assay the sensitivity to measure plasma calcitriol itself, which circulates at concentrations around 30 pg mL^{-1}. This principle is, of course, generally applicable and most steroids can be detected at lower concentrations by the use of GC–HRMS. This technique has been used mainly by laboratories interested in the detection of anabolic steroids in athletes' urine (e.g. metandienone, stanozolol and clostebol) as a means of detection of drug abuse in sport but also as a means of detecting illicit steroid administration to cattle (4-chlorotestosterone). It has occasionally been suggested, as in the case of nandrolone, that metabolites observed have arisen *de novo* by *in vivo* metabolism from other steroids rather than from exogenous sources. Use of GC–combustion-MS (isotope ratio mass spectrometry) has been shown, by measuring the $^{12}C : {}^{13}C$ ratios, to have considerable potential as a means of distinguishing between exogenous and endogenous sources. **Figure 8** gives a further example of the sensitivity of GC–HRMS which was used to demonstrate the presence of 1α,24-dihydroxyvitamin D_2 in human plasma by focusing on three specific ions and demonstrating that they had a retention time the same as the standard and were present in the same ratio and as they were in the MS of the pure standard. Similar studies with low-resolution MS detection were unable to demonstrate the presence of this steroid.

Conclusion

GC–FID of steroids is today primarily confined to the analysis of urinary steroid profiles, a technique introduced in the 1980s but, as a brief examination of the recent literature will show, still produces valuable clinical information today. Much improved data are obtained when the GC is interfaced with the a mass spectrometer, allowing greater sensitivity and specificity of detection with the added benefit of structural information about unknown steroids. It is interesting to note that C_{21} steroids are usually analysed by immunoassay or LC–MS whereas GC–MS is still widely used for the specific analysis of oestrogens and androgens, particularly in the sports area where the definitive detection of anabolic steroids is required. GC–MS, particularly when high-resolution MS is used, is still more sensitive than LC–MS for steroid assay and EI(+) ionization methodology provides more useful structural information than can be achieved with LC–MS or even LC–MS–MS. It will be interesting to see whether GC–MS will hold its own against LC–MS over the next ten years.

See also: **II/Chromatography: Gas:** Derivatization; Detectors: Mass Spectrometry; High Temperature Gas Chromatography. **III/Steroids:** Liquid Chromatography and Thin-Layer (Planar) Chromatography; Supercritical Fluid Chromatography.

Further Reading

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Liquid Chromatography and Thin-Layer (Planar) Chromatography

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Introduction

This review aims to summarize the application of liquid chromatography (LC) in all its forms, including thin-layer chromatography (TLC), for the analysis of steroids. As LC relies on either adsorption or partition, extraction of the analyte from the matrix, a similar process, has been considered, as has the necessary final step of LC-quantitation. Readers who seek further information are encouraged to use the texts given in the Further Reading section, which are valuable sources of information from which original research references can be obtained as well as information about alternative means of steroid analysis.