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New techniques in quantitative mass spectrometry

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Introduction

Mass spectrometry has been, for more than 15 years, an essential aid in the structure determination of organic compounds. The technique is attractive because of the large amount of structural information that can be obtained from nanogram quantities of the sample. Furthermore, the mass spectral fragmentation patterns are often sensitive to subtle structural changes (Budzikiewicz, Djerassi & Williams, 1967).

The first applications of quantitative mass spectrometry as an assay technique were published after the introduction of combined gas chromatography–mass spectrometry (Hammar, Holmstedt & Ryhage, 1968). In this method, the powerful resolving power of a gas chromatograph is employed as an on-line purification procedure, while the spectrometer is operated as a highly sensitive and specific detector by tuning the instrument to monitor the intensities of characteristic ions due to the compounds under investigation. By this procedure, picomolar concentrations of the drug chlorpromazine and its major metabolites were assayed in extracts of human plasma (Hammar *et al.*, 1968).

This technique, which is variously referred to as multiple-ion detection (MID), mass fragmentography (MF) or, better, selected-ion detection (SID), has become the method of choice for the analysis of drugs and their metabolites in body fluids. Several groups of workers have also employed the method for the measurement of endogenous steroid concentrations (Adlercreutz & Hunneman, 1973; Bournot, Maume & Padieu, 1974; Dehennin, Reiffsteck & Scholler, 1974), but only when the steroid levels were unusually high and extensive purification procedures were applied before the analysis.

Although the inherent sensitivity of mass spectrometers is high, frequently allowing the detection of picogram and even femtogram quantities of pure compounds, SID is usually unreliable or inapplicable for the measurement of subnanogram amounts. All the organic material entering the mass spectrometer is ionized, producing characteristic fragment ions. There are, therefore, always contributions to the ion intensity at each mass unit which arise from contaminating compounds, mostly eluting from the gas chromatograph column. These contributions critically influence the 'signal-to-noise' ratio and, consequently, the true detection limit for a particular compound.

The problems of interfering peaks in SID can be partly overcome by providing further purification steps before the analysis, but at the risk of losing much of the material to be assayed. A simpler and more reliable approach, however, is to increase the specificity of SID by employing a mass spectrometer with high resolving power and tuning it to the exact masses of characteristic ions. By this method, the salient ions are discriminated from others at the same integer masses arising from contaminating compounds, because of their differing elemental compositions. In addition, the signal-to-noise ratio is enhanced, permitting lower detection limits than are feasible using low-resolution SID.

The procedure of high-resolution SID for quantitative steroid analysis was pioneered at the Tenovus Institute, Cardiff (Millington, 1975), and is now employed routinely when other methods are impracticable. This report describes the development of the technique and is illustrated with examples of its application.

Materials and Methods

The techniques for high-resolution selected-ion detection and for the extraction of steroids from various tissues have been published in detail (Millington, 1975; Millington, Buoy, Brooks, Harper & Griffiths, 1975; Pierrepoint, Davies, Millington & John, 1975; Millington *et al.*, 1976).

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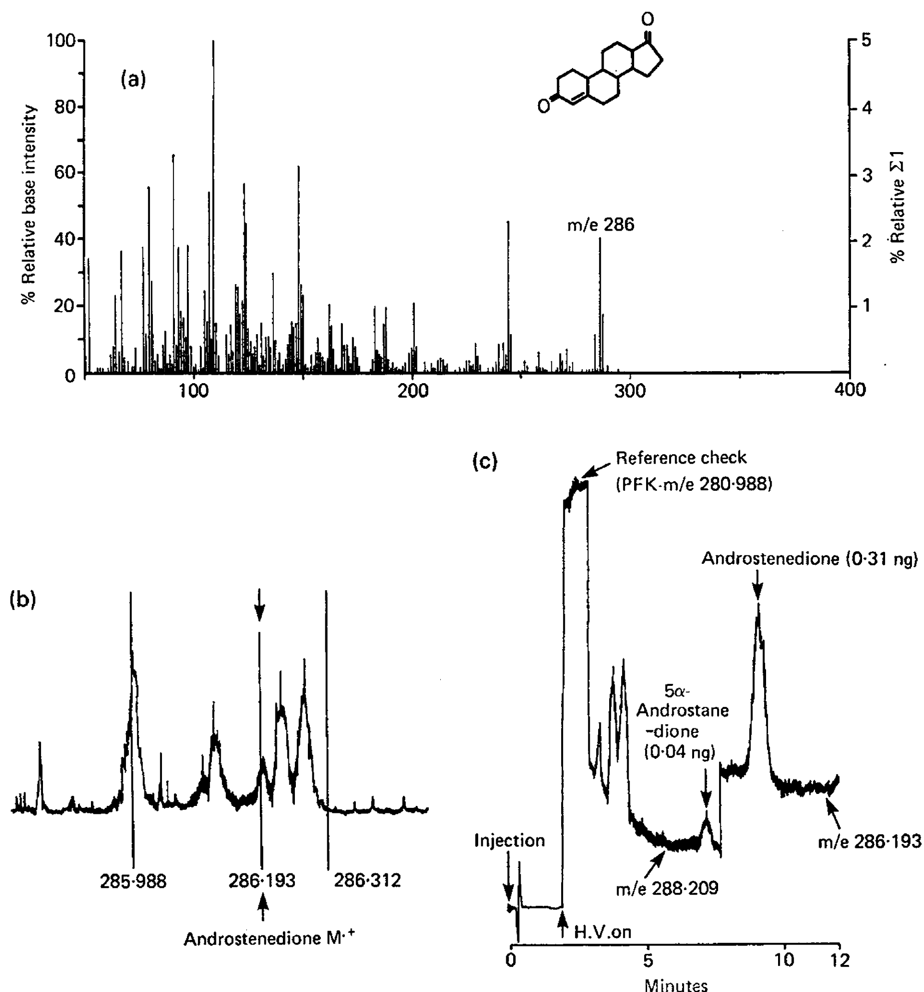

Text-fig. 1. The detection of androstenedione in a crude extract of canine testicular vein plasma. (a) Mass spectrum taken during combined gas chromatography–mass spectrometry. (b) Diagrammatic illustration of other ions of integer mass 286 associated with androstenedione in the mass spectrometer. High resolution analysis at *m/e* 286·193 ensures their separation from the signal from the monitored ion. (c) High-resolution mass fragmentograms, monitoring at *m/e* 286·193, from the crude extract of canine testicular vein plasma.

For high-resolution SID, a Varian 2040 gas chromatograph fitted with a 2 m × 2 mm int. diam., 3% OV-17 packed column was interfaced to a Varian-MAT 731 double focusing mass spectrometer via a two stage Watson–Biemann-type molecular separator. The flow rate of helium was 25 ml min^{-1} and the gas chromatograph oven and injector temperatures were chosen to suit the steroid being assayed.

*Use of deuterated oestradiol-*17β *to assay recovery of steroid extraction*

To 2·0 ml homogenized breast tumour tissue (20% w/v in tris buffer) were added 100 μl of a standard solution of 2,4-dideutero-oestradiol-17β (20 pg/μl ethanol). After incubation at 4°C for 16 h, the suspension was homogenized with 10 ml acetone in a Silverson mixer and the supernatant was decanted after centrifugation. The residue was again extracted with acetone (10 ml) and the combined supernatants were concentrated *in vacuo*. To the residue was added 2 ml 2 N-sodium hydroxide, and

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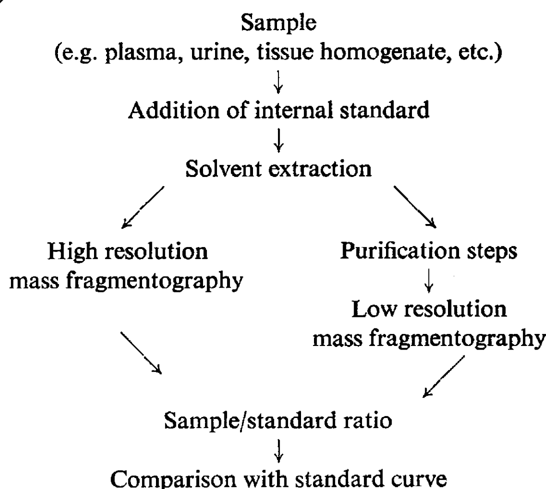
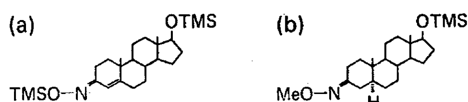
the solution was extracted twice with ether (2 ml) and the extracts discarded. The pH of the aqueous phase was then adjusted to 8·5–9·0, 2 ml of a bicarbonate buffer solution (0·1 M-$KHCO_3$ + 0·1 M-K_2CO_3, 9 : 1 v/v) were added and the solution was re-extracted with ether (2 × 2 ml). The combined extracts were washed with 2 ml 0·1 N-HCl, then with 2 ml distilled water and dried in nitrogen. At this stage, a second steroid standard, 2 ng dihydroequilenin, was added and the mixture transferred to a small tube (30 × 2 mm). After drying, the final residue was desiccated over P_2O_5 for 1 h *in vacuo* and treated with bis-(trimethylsilyl) acetamide (15 μl) for at least 6 h at 20°C before assay of the oestradiol-17β in the breast tumour tissue.

Results and Discussion

The typical procedure for steroid assay by using the gas–liquid chromatography–selected-ion detection technique is outlined below. One of the main advantages over other assay methods is the ability to add a compound which is chemically closely related to that under investigation as an internal standard before extraction. Ideally, the measurement of concentration is assessed from the ratio of sample to internal standard (determined from relative characteristic peak heights) and needs no correction for losses during extraction and purification. This implies a greater confidence in the accuracy of SID compared with other existing assay techniques. As indicated, purification stages after the solvent extraction can usually be by-passed when high-resolution SID is employed, because of its greater specificity.



The inherent advantage of specificity in the high-resolution SID method is strikingly demonstrated in Text-fig. 1. The mass spectrum of the pure steroid (Text-fig. 1a) is typically complex, but it is necessary only to detect the ions at mass 286·193, representing the intact molecule $C_{19}H_{26}O_2$, for characterization in the SID mode. The result of continuous monitoring of mass 286·193 after injection of an aliquot of the crude plasma extract onto the gas chromatograph column is shown in Text-fig. 1(c), and emphasizes the importance of a clean baseline for a good signal-to-noise ratio. The analysis was uncomplicated by the presence of other ions of integer mass 286 (Text-fig. 1b) which were also present in the spectrometer at the elution time of androstenedione. At low resolution, these ions would have merged into a single peak, making it impossible to identify 300 pg of the steroid positively.



Text-fig. 2. The derivatives formed for analysis of testosterone and 5α-dihydrotestosterone: (a) the 3-trimethylsiloxyimino-17-trimethylsiloxy compound, (b) the 3-methoximino-17-trimethylsiloxy compound.

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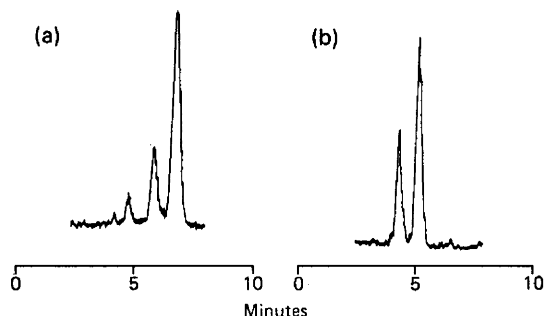
By careful application of the external calibration procedures described previously (Millington, 1975), the concentrations of androstenedione and several other C_{19}-steroids were measured in testicular, deferential and peripheral venous plasma from individual dogs. These were the first recorded measurements of androgen levels in the deferential vein and evidence was thus provided that this vessel acts as a local androgen transport system in the dog (Pierrepoint *et al.*, 1975).

To improve the conditions for SID, the polar functional groups in C_{18}- and C_{19}-steroids can be converted into less polar derivatives by simple chemical procedures. This important step both improves the transfer of steroids through the gas chromatograph and increases the stability of the molecules in the mass spectrometer. A simple procedure has been described for conversion of hydroxysteroids into trimethylsilyl (TMS) derivatives (Millington, 1975) and the gas chromatograph–mass spectrometric characteristics and detection limits in high-resolution SID of several C_{18}- and C_{19}-steroid–TMS derivatives were reported. Methyloximes were found to be good derivatives of the carbonyl groups in ketosteroids (Millington *et al.*, 1975), but complications can arise when the gas chromatograph column is capable of resolving the two isomers (*syn* and *anti*) which result from formation of an oxime derivative.

Suitable derivatives for analysis of testosterone and 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) on the same OV-17-coated packed gas chromatograph column are the 3-trimethylsiloxyimino-17-trimethylsiloxy and 3-methoximino-17-trimethylsiloxy compounds respectively (Text-fig. 2). These derivatives are homogeneous on gas chromatography and were used to measure testosterone and 5α-dihydrotestosterone in human hyperplastic prostate tissue (Millington *et al.*, 1975). In this case, several purification steps, including thin-layer chromatography, were required before application of high-resolution SID and the addition of internal standards before work-up of the homogenized tissue was essential. Since pilot experiments showed no detectable trace of 17α-hydroxysteroids in the tissue, the 17α-epimers of testosterone and 5α-dihydrotestosterone were employed as internal standards. The high-resolution SID traces shown in Text-fig. 3 demonstrate the separation by gas chromatography of the 17α- and 17β-steroid epimers, which facilitated measurement of the ratio of their peak heights. Concentrations of each steroid in the original tissue were then determined from calibration curves such as that shown in Text-fig. 4 for 5α-dihydrotestosterone. The concentrations of the two C_{19}-steroids, measured in several different prostatic tissue samples (Millington *et al.*, 1975), compare well with those determined previously by a double-isotope dilution technique (Siiteri & Wilson, 1970).



Text-fig. 3. Determination of (a) 5α-dihydrotestosterone and (b) testosterone in human prostatic tissue by high-resolution selected-ion detection using a 3% OV-17 column at 265°C and 270°C respectively. The mass spectrometer was tuned to monitor the signal intensity at *m/e* 391·291 for (a) and *m/e* 447·299, for (b). The smaller peaks represent the internal standards, 5α-epidihydrotestosterone (a) and epitestosterone (b).

The internal standards in this case were chosen to favour the particular circumstances in which the endogenous steroids occurred and would not necessarily be applicable for measurement of the same steroids in other tissues, In general, the most desirable standard is a stable isotope derivative of the substance to be assayed, because the two should be nearly identical in their chemical and biochemical behaviour but easily distinguished by mass spectrometry. Such labelled steroids are,

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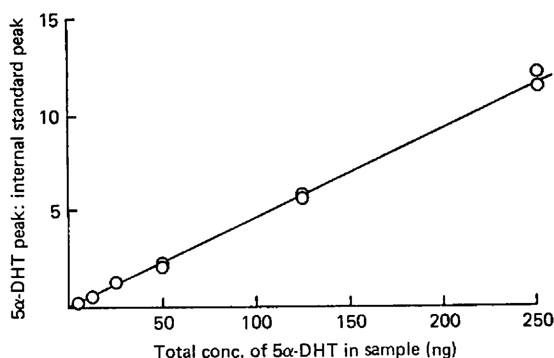
however, not yet commercially available on a scale comparable with that of their radioactively-labelled counterparts and usually must be specially synthesized.

An assay procedure for oestradiol-17β, using 2,4 dideutero-oestradiol-17β as the internal standard, was devised with the primary objective of measuring the concentration of this hormone in human carcinomatous breast tissue. A preliminary study using an external calibration procedure indicated a wide variety of oestradiol levels in different tumours, most being in the range of 0·5 to 10 pg/mg tissue wet weight (Millington, Jenner, Jones & Griffiths, 1974; Millington, 1975). The assay described earlier was designed to cover this range of values in a total of 200 mg tissue sample. A slightly modified procedure has also been used to determine the oestradiol concentration of plasma pools used in the U.K. Supraregional Assay Service quality-control study (D. W. Wilson, B. M. John, G. V. Groom, C. G. Pierrepoint & K. Griffiths, unpublished). The values for oestradiol determined by radioimmunoassay and by selected-ion monitoring compared well, but the mass spectrometric technique generally indicated a lower base level, reflecting the greater specificity of the method and increasing confidence in its application to the study of breast tissue homogenates.

Replicate analyses (7) of a sample of homogenized breast tissue pooled from 20 individual tumours showed a range of 1·6–2·1 pg/mg and a mean value of 1·9 ± 0·2 pg/mg.



Text-fig. 4. Calibration graph for the determination of 5α-dihydrotestosterone by using 5α-dihydro-epitestosterone as internal standard.

Another opportunity to compare the selectivity of high-resolution SID with that of radioimmunoassay was afforded during studies of the steroid synthesis *in vitro* by a feminizing adrenocortical tumour (Millington *et al.*, 1976). Steroids released into the medium from cultured explants of the tumour tissue were measured by radioimmunoassay and selected-ion monitoring (Table 1). High-

Table 1. Comparison of concentrations of steroids (pg/mg wet wt of tissue) secreted by a feminizing adrenocortical tumour into the culture medium after incubation with various hormones for 72 h and measured by radioimmunoassay (RIA) or high resolution fragmentography (MF)

	RIA		MF		
	Oestradiol-17β	Androstene-dione	Oestradiol-17β	Oestrone	Androstene-dione
Control	44	590	9	330	180
LH (5 μg)	54	—	—	—	—
LH (50 μg)	33	2320	15	692	2200
GH (2·5 μg)	57	2910	25	650	2300
ACTH (1·0 μg)	204	1730	150	660	1540
Prolactin (5·0 μg)	134	1310	50	1170	530
Insulin (0·08 mU) + prolactin (1·0 μg)	303	—	260	1120	—

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resolution SID gave consistently lower values for oestradiol and androstenedione and indicated high quantities of oestrone, which gave a cross-reactivity of only 1% in the radioimmunoassay for oestradiol.

In conclusion, combined gas chromatography and high-resolution selected-ion detection is a powerful new technique for steroid assay. Its advantages over existing techniques of comparable sensitivity are greater specificity and accuracy, especially when internal standards are employed, a visual recording of every measurement which enables the operator to assess the contribution, if any, from interfering compounds, and greater versatility, allowing the determination of specific steroids in various tissues and body fluids without difficulty. These advantages are, of course, offset by the high capital cost of the equipment and the limited number of assays which can be carried out in a working day. However, the technique should become useful as a definitive method by which other assays can be assessed. It is also ideal for specific problems, such as the study of the rare adrenocortical tumour described above, in which accurate screening for several steroids is required.

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