Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 239–245. Pergamon Press. Printed in Great Britain

DETERMINATION OF HORMONAL STEROID CONCENTRATIONS IN BIOLOGICAL EXTRACTS BY HIGH RESOLUTION MASS FRAGMENTOGRAPHY

D. S. MILLINGTON

Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath Park, Cardiff, CF4 4XX, Wales

SUMMARY

In the measurement of steroid concentrations in crude biological extracts, particularly from human tissue, present analytical techniques are stretched to or beyond their limits of sensitivity and specificity. In this report, high resolution mass fragmentography of steroid trimethylsilyl ethers, a technique having great specificity and sensitivity, is described. The method entailed molecular ion monitoring of steroid derivatives during combined gas chromatography–mass spectrometry at a resolution of 10,000 (10% valley definition) and enabled unequivocal identification and quantitation of the particular steroids of interest in crude tissue and plasma extracts, without recourse to prior purification.

The method was successfully applied to assay classical oestrogens and some C_{19}-steroids in human primary breast tumours. In addition, a range of C_{19}-steroids were determined in plasma from the radial and testicular veins of a dog. The method was less sensitive for C_{19}-steroids than for oestrogens by more than one order of magnitude and an approach to the enhancement of sensitivity for testosterone and 5α-dihydrotestosterone, by utilising t-butyl dimethylsilyl ethers, is outlined.

INTRODUCTION

Most ultrasensitive methods now in use for steroid assays in clinical studies depend for their successful application on preliminary extraction and purification procedures. These are often tedious and multistaged, necessarily designed to achieve a high degree of specificity particularly in previously uninvestigated systems, but inevitably incurring sample losses which may be intolerably high for some steroids. Mass fragmentography (MF) [1], despite incorporation of the powerful gas chromatographic technique, must generally be included in this category when the mass spectrometer is used at low resolution (around 1000, 10% valley definition). The specificity of MF is however greatly enhanced by increasing the resolution of the mass spectrometer. On a modern, double focussing instrument such as the Varian-MAT 731, resolution can be increased to the point where specific ion identification is unequivocal owing to the filtering out of other ions having the same integer mass arising from contaminating GC components or from column "bleed". A resolution of 10,000, for example, enables mass identification with a precision of ±2 mamu (milli-atomic mass units), while the concomitant loss in sensitivity is compensated by an enhanced signal to noise ratio, compared with low resolution MF.

This paper describes in detail the technique of high resolution mass fragmentography (HRMF) of steroid derivatives and indicates its advantages over other methods of steroid assay, particularly when applied to new problems. Examples of its use in measuring endogenous C_{18}- and C_{19}-steroid concentrations in human primary breast carcinomata and in crude plasma extracts are given.

EXPERIMENTAL

Materials

All solvents were distilled analytical grade. Bis(N,O) trimethylsilyl acetamide was obtained from Jones Chromatography Ltd., Llanbradach, Glam, U.K. in samples of 1 ml contained in sealed glass ampoules. When required, the contents of one ampoule were transferred to a screw cap vial (2 ml capacity) and stored at 4°C inside a desiccator containing activated silica gel (self indicating). Precoated gas chromatography packing materials were also supplied by Jones Chromatography. Tertiary-butyldimethylchlorosilane was obtained from PCR Inc., Gainesville, Florida, U.S.A. and the standard steroids were supplied by Sigma Chemicals, St Louis, Missouri.

Instruments

A Silverson tissue grinder was used to homogenise tissue. The gas chromatograph employed was a Varian 2740 incorporating two similar helical pyrex columns (2 m × 2 mm, i.d.) packed with 3% OV-17 on Gaschrom Q (100–120 mesh). One column was connected to a FID detector and used for assessment of the GC characteristics of standard steroid derivatives and the other was linked to the mass spectrometer *via* a two-stage Watson–Biemann helium separator and a glass lined probe. These high performance columns were stable indefinitely when used only for steroid trimethylsilyl (TMS) derivatives. It was important also that the separator not be exposed to unsilylated material, otherwise its efficiency for the elution of the TMS compounds was impaired.

The Varian-MAT 731 mass spectrometer employed in these studies was operated in tandem with a computer (Spectrosystem 100) to obtain complete mass spectra during GC–MS. The relevant spectra were recorded on a Statos 21 electrostatic plotter after automatic background ion subtraction and correction for changes in total ion current during the scans. When used for HRMF, the spectrometer was tuned to a resolution of 10,000, which led to a drop in sensitivity of about 90% compared with normal sensitivity at a resolution of 1000. The output from the signal amplifier was displayed on a Varian A-25 chart recorder and two positions in the mass scale within a ratio of 10% could be monitored sequentially (but not simultaneously) using the standard accelerating voltage alternator.

Extraction of steroids from tissue

Details of the extraction procedure for C_{18}- and C_{19}-steroids from human breast tissue used in our laboratory have been published [2], therefore a brief account only will be given here. A known weight of tissue was homogenised in acetone, centrifuged and the supernatant decanted. After two further extractions with acetone, the combined extracts were evaporated, the residue dissolved in methanol–water (4:1 v/v) and the solution extracted with petroleum ether, b.p. 60–80°C. The aqueous solution was evaporated, the residue dissolved in ether and after extraction with water, the ether solution was again evaporated and the final residue dissolved in a known volume of ethanol. Solutions were stored at −20°C prior to assay.

Preparation of the samples for mass fragmentography

Usually about six tissue samples were extracted at the same time during one day, by the method described in the preceding section. Analysis of up to seven steroids in each extract was then accomplished on the day following derivatisation.

An aliquot was taken from each sample solution at 20°C corresponding to 0·5 g wet wt. of tissue or 2·5 ml plasma and each aliquot evaporated to dryness in a clean pyrex tube (3 ml). The residues were carefully transferred in ethanol (3 × 50 μl) into smaller tubes (30 mm × 4 mm) each fitted with a ground glass stopper. After drying down the samples and leaving them for 1 h in a vacuum desiccator over silica gel at 0·1 mm Hg, bis(N,O) trimethylsilyl acetamide (15 μl) and petroleum ether, b.p. 60–80°C (10 μl) were added to each tube and the stoppers replaced with a light smear of silicone grease around the top 3–5 mm. This ensured an airtight seal and facilitated removal of the stoppers later on without contaminating the solutions. At the same time, aliquots taken from the standard solutions of the steroids to be assayed were transferred to similar small tubes, dried and the residues dissolved in the reagent/pet. ether mixture such that final concentrations of 10, 100 and 1000 pg/μl of oestrogens and 100 and 1000 pg/μl of the C_{19}-steroids were obtained. The solutions were allowed to stand for at least 16 h at 20°C before analysis. Standard solutions containing 25 μl or more were stable for over 3 months at 20°C provided that the tube remained airtight and not more than 30% of the contents were removed as 1 μl aliquots. Although desirable, it was not critical to assay the samples immediately after derivatisation. However, in this report all samples were analysed within 3 days of complete derivatisation and the standards used were always freshly prepared at the same time as the samples.

Preparation of t-butyldimethylsilyl ethers [4] of testosterone and 5α-dihydrotestosterone

Pure steroid (100 μg) was transferred to a dry 10 ml tube and 2M t-butyldichloromethylsilane in dimethylformamide (100 μl) followed by 5 M imidazole in dimethylformamide (100 μl) were added. The well-stoppered tube was allowed to stand at 20°C for 18 h, then saturated sodium chloride solution (1 ml) and diethyl ether (0·5 ml) were added to the reaction mixture and the tube vigorously shaken. After centrifugation, the ether layer was decanted and the aqueous solution extracted twice more with ether (0·5 ml). The combined ether extracts were dried over anhydrous sodium sulphate and evaporated. The residue was dissolved in dry benzene (200 μl) and the solution stored in a 2 ml screw cap vial.

Combined gas chromatography–mass spectrometry

The gas chromatography conditions employed during GC–MS paralleled those established beforehand on the duplicate system coupled to a FID detector. The helium flow rate was 20 ml/min, while the temperatures of the injector block, detector oven, helium separator, probe and ion source were maintained at 275, 275, 260, 255 and 150°C respectively. The GC oven temperature was adjusted for each steroid derivative to allow an analysis time of about 6–10 min. The total ion current (TIC) was recorded during GC–MS on a potentiometric recorder for visual display and simultaneously by the computer to enable automatic ion intensity correction for changes in TIC during the MS scans. During analysis a mass spectrum was recorded just before the emergence of the GC peak and used to subtract background ions from the sample spectrum, which was recorded at or near maximum concentration as shown by the TIC trace.

High resolution mass fragmentography

As described in the foregoing section, the mass spectrometer coupled to a computer can be used to analyse the effluent from a gas chromatograph, providing identification of or useful structural information on the major GC components. However, when the salient components are present in concentrations below 50 ng/μl, especially when contaminated by other compounds having similar GC characteristics, the MS must be used in the more sensitive and specific mode of single or multiple ion detection (mass fragmentography) [1] to permit quantitative analyses. Several research groups have used MF in biological

steroid analysis [5–7] but it is clear that the method cannot be applied to crude extracts where the steroid concentration is low, unless purification procedures which may result in intolerably high sample losses are adopted. In such cases, an alternative and more desirable approach is to improve the specificity of MF by increasing the resolution of the MS.

When the resolution of the MS was adjusted to around 10000 instead of the usual 1000, the position in the mass scale of a particular ion was pinpointed to within an accuracy of ± 2 mamu, using the internal reference compound perfluorokerosene (PFK), thereby eliminating interference from compounds other than isomers that gave rise to mass spectral ions having the same integer mass. Thus, in order to assay oestradiol, the MS was tuned to m/e 416·257, corresponding to the molecular weight of oestradiol bis(trimethylsilyl) ether, using the ion at m/e 404·976075 in PFK as a reference. While continuously monitoring the signal intensity at m/e 416·257 on a potentiometric recorder, 1 μl of the standard oestradiol-17β TMS ether solution containing 100 pg/μl was injected onto the GC–MS system with the GC oven temperature set at 265°C. After passage of the solvent and reagent peaks, the reference mass position was checked and adjusted if necessary using the fine control of the magnet current regulator. In general, no adjustments were necessary after an initial stabilising period of about 10 min. After the reference ion check, the mass fragmentogram for oestradiol-17β was obtained and the maximum height of the signal was recorded in arbitrary units. The standard was injected twice more and the mean value for 100 pg of steroid was calculated. A blank solution of reagents containing no steroids was then injected to check that no absorption of the previous sample had occurred, then three consecutive injections of the first sample to be analysed were made and the recorder responses noted. Measurements were then continued by running another blank followed by the next sample, and so on, until all oestradiol-17β measurements were completed. A standard was included after every fourth sample measurement as a check on stability. In this manner, it was possible to make 3–4 measurements per hour, since the retention time for oestradiol-17β was about 6 min under the conditions described. The mass spectrometer was then tuned to the molecular ion peak of another steroid of interest, the process taking only 2–3 min, and after allowing 10 min for stabilisation of the magnet current and new GC oven temperature, measurements were restarted. The sensitivity, reliability and specificity of the HRMF procedure are described in the following section.

RESULTS AND DISCUSSION

Sensitivity, precision and calculation of the results

The detection limit (LD), or sensitivity of HRMF for each steroid derivative was dependent on physical properties which affected their mass spectrometric behaviour. For example, the classical oestrogens oestradiol-17β, oestrone and oestriol as their TMS derivatives each exhibited a molecular ion peak which was the most intense ion in its mass spectrum and carried about 10% of the total ionization. The LD for these compounds was approximately 3 pg (10 fmol) per μl in a standard solution. It is however more meaningful to use the term lower limit for reliable measurement (LRM) when referring to biological samples. This corresponded to 10 pg/μl for the oestrogens, since recorder response varied linearly with concentration in the range 10 pg–100 ng/μl, but the linearity did not hold below 10 pg/μl.

For the TMS ethers of the C_{19}-steroids androsterone (3α-hydroxy-5α-androstan-17-one), epiandrosterone (3β-hydroxy-5α-androst-17-one), 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), dehydroepiandrosterone (3β-hydroxyandrost-4-ene-17-one), testosterone, androst-4-ene-3,17-dione, 5α-androstan-3,17-dione and the 5α-androstan-3,17-diols, the LD and LRM were at least one order of magnitude higher than for the classical oestrogens. One factor contributing to this difference is illustrated in Fig. 1, which compares the mass spectra of oestradiol-17β and testosterone as their TMS ethers. Although the molecular ion is the most intense peak in both spectra, fragmentation of the C_{19}-steroid nucleus is more extensive than that of the more stable oestrogen, so that the percentage of total ionization carried by the molecular ion of testosterone TMS ether is the lower of the two, resulting in reduced sensitivity. The situation can be improved for the C_{19}-steroids, especially testosterone and 5α-dihydrotestosterone, by forming tertiary-butyldimethylsilyl (tBDMS) ethers. As was shown recently by Kelly and Taylor [8], tBDMS derivatives of steroids and prostaglandins exhibit intense ions at 57 amu less than their corresponding molecular weights that carry a high proportion of the total ionization. Figure 2 compares the mass spectra of 5α-dihydrotestosterone TMS and tBDMS ethers and clearly indicates that monitoring $(M\text{-}57)^+$ ions in the spectra of C_{19}-hydroxysteroid tBDMS derivatives will lead to enhanced sensitivity in these assays.

Summarised in Table 1 are the LD and LRM values for all the steroids assayed by HRMF in this study. In addition, the GC oven temperature and corresponding retention time and exact molecular weight of each steroid assayed are tabulated.

The precision of the method is independent of the sample concentration since it is largely determined by the error in estimation of the sample volume injected onto the GC–MS. A 5 μl syringe was used to inject 1 μl aliquots and could be read to an accuracy of $\pm 5\%$. The standard deviation for a series of 10 measurements of one steroid was also about $\pm 5\%$ and since external standards were employed, the error in the final results was of the order of $\pm 10\%$.

The calculation of steroid concentration in a sample was effected by comparing the mean sample peak height measured on the recorder for three injections with that of a standard. Figure 3(a) shows mass fragmentograms of oestradiol-17β in extracts of two

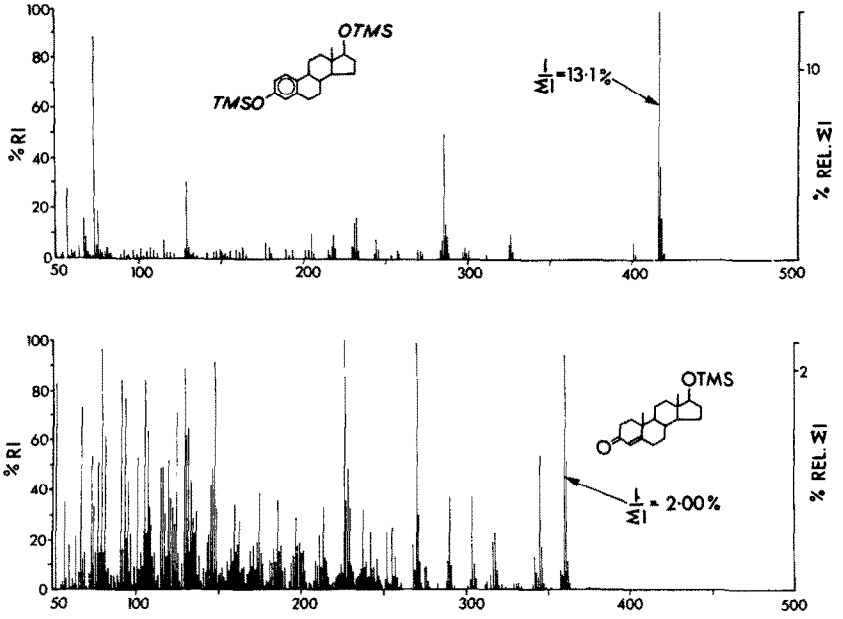

Fig. 1. Comparison of the mass spectra of oestradiol-17β *bis* (trimethylsilyl) ether (upper) and testosterone trimethylsilyl ether (lower), obtained during combined GC–MS.

breast tumours compared with a standard (upper trace). The high quality of the traces reflects the great specificity and good signal/noise ratio achieved by HRMF. The presence of oestradiol-17α in one of the samples was confirmed by using a standard; this example also illustrates the importance of ensuring that the GC column is capable of resolving isomeric derivatives. Mass fragmentograms showing simultaneous determination of dehydroepiandrosterone and testosterone in dog plasma, obtained by monitoring at m/e 360·248, are given in Fig. 3(b). The four isomeric 5α-androstan-3,17-diols were also assayed simultaneously since they were fully resolved on the GC column as bis(TMS) ethers.

Steroid concentrations in human breast tumour tissue

Apart from two brief communications from our laboratory [2,3] there is no literature concerning the measurement of endogenous steroid concentrations in human breast tissue. The data obtained in this study are summarised in Table 2. Concentrations of oestradiol-17β, oestrone and oestriol in each of ten primary

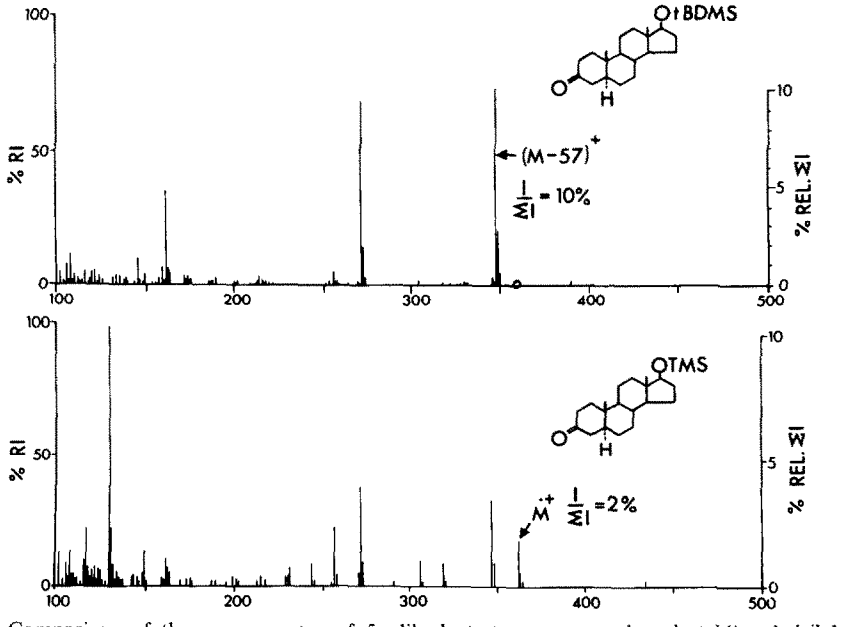

Fig. 2. Comparison of the mass spectra of 5α-dihydrotestosterone as the t-butyldimethylsilyl ether (upper) and trimethylsilyl ether (lower) obtained during combined GC–MS.

Table 1. Physical data relevant to HRMF for TMS derivatives of some C_{18}- and C_{19}-steroids

Steroid	DL (pg/μl)	RML (pg/μl)	RT (min)	Temp. (°C)	M^+ (a.m.u.)
E_2	3	10	5·5	265	416·257
E_1	3	10	7·5	265	342·202
E_3	3	10	9·0	265	504·291
A	25	50	5·3	255	362·264
EA	25	50	7·1	255	362·264
DHT	100	200	7·5	255	362·264
A-α,α-D	30	50	4·0	250	436·319
A-α,β-D	25	50	4·9	250	436·319
A-β,α-D	25	50	5·7	250	436·319
A-β,β-D	25	50	6·5	250	436·319
T	75	200	9·8	255	360·248
ET	50	100	8·3	255	360·248
DHA	25	50	7·0	255	360·248
5αA	10	20	6·3	275	288·209
AD	20	40	8·0	275	286·193

Abbreviations: DL, detection limit for pure compound; RML, limit of reliable measurement in biological samples; RT, GC retention time at the temperature given; M^+, precise molecular weight in atomic mass units (a.m.u.) and the MS ion used for HRMF; E_2, oestradiol-17β; E_1, oestrone; E_3, oestriol; A, androsterone; EA, 3-epiandrosterone; DHT, 5α-dihydrotestosterone; A-α,α-D, 5α-androstan-3α,17α-diol; A-α,β-D, 5α-androstan-3α,17β-diol; A-β,α-D, 5α-androstan-3β,17α-diol; A-β,β-D, 5α-androstan-3β,17β-diol; T, testosterone; ET, 17-epitestosterone; DHA, dehydroepiandrosterone; 5αA, 5α-androstan-3, 17-dione; AD, androst-4-ene-3, 17-dione.

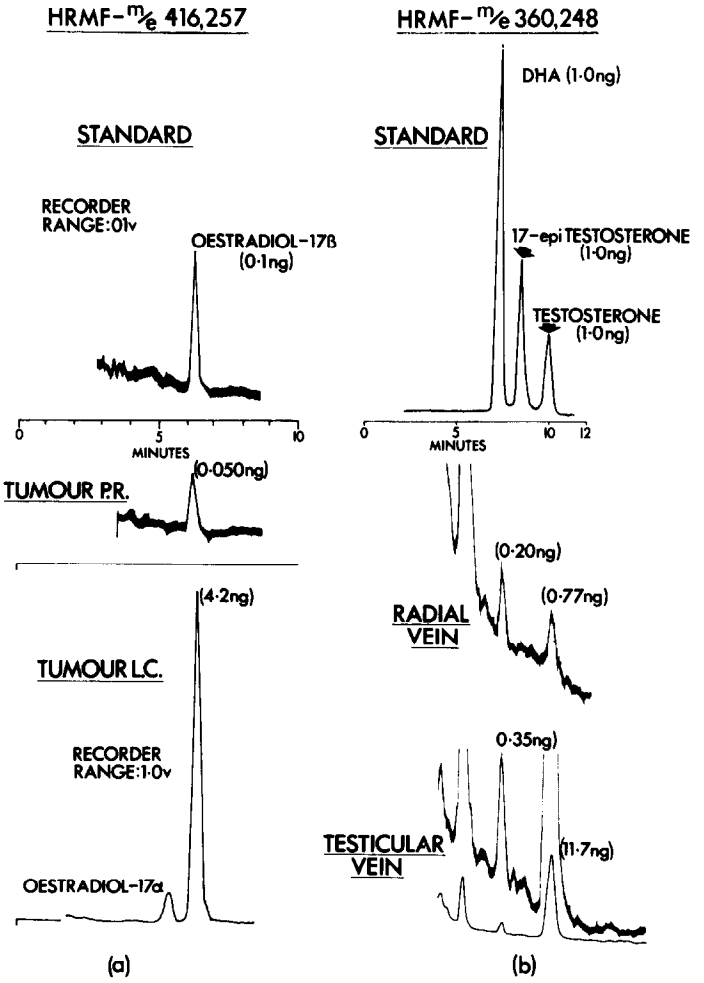

Fig. 3. High resolution mass fragmentograms showing (a) oestradiol in a standard solution (upper) and in two extracts of primary breast tumours (middle and lower) and (b) simultaneous determinations of DHA, epi-testosterone and testosterone in a standard (upper) and in extracts of blood from the radial (middle) and testicular (lower) veins of a dog.

Table 2. Steroid concentrations in human primary breast tumours determined by HRMF, expressed in ng/g wet wt. Abbreviations: DHA, dehydroepiandrosterone; T, testosterone; A, androsterone; EA, 3-epiandrosterone; E_1, oestrone; E_2, oestradiol-17β; E_3, oestriol. Figures in parenthesis are approximate and those prefixed < are below the detection limit. The values are not corrected for losses incurred during the extraction procedure

Tumour	DHA	T	A	EA	E_1	E_2	E_3
R.L.	15	25	10	10	3	16	<1
M.B.	35	55	75	38	8	500	<1
M.H.	30	100	55	60	20	15,000	22
E.D.	25	25	50	5	17	50	3
C.C.	19	<1	—	—	<1	10	<1
R.M.	50	<5	—	—	<1	51	<1
E.C.	16	<1	—	—	5·5	175	22
—	28	<1	—	—	<0·2	4·0	<0·2
L.E.	2	<1	—	—	<0·2	(1)	<0·2
R.K.	30	<1	—	—	<0·2	(1)	<0·2
C.M.	25	<1	—	—	—	2·7	—
G.W.	198	<1	—	—	—	(0·5)	—
E.J.	25	8·3	—	—	—	<0·2	—
A.L.	15	13	—	—	—	(0·5)	—
T.A.	14	<1	—	—	—	3·4	—
P.R.	2·4	<1	—	—	—	4·4	—
M.P.	—	—	—	—	—	2·5	—
S.B.	—	—	—	—	—	2·5	—

breast carcinomata are presented at the head of the list and show clearly that oestradiol-17β is the major classical oestrogen in these tumours. Although there is a wide variation of oestradiol values between individuals, most figures were in the range 0·5–10 ng/g tissue (wet weight). Testosterone concentrations were generally below the detection limit of the method but dehydroepiandrosterone was found in measurable concentration in every tumour assayed for this steroid. Oestradiol-17β is assayed routinely by HRMF on all breast tumours received by the Institute.

C_{19}-steroids in peripheral, testicular and deferential venous plasma from a dog

The present study was undertaken to compare concentrations of a range of C_{19}-steroids in peripheral, testicular and deferential venous plasma from one dog. The results, summarised in Table 3, confirm that high levels of androgens, particularly testosterone and androstenedione, are carried by both the testicular and deferential veins of the dog and show a striking similarity between the steroid concentrations in these two veins. These and other data provide evidence that the deferential vein acts as a local androgen transport system in the dog [9].

CONCLUSION

High resolution mass fragmentography is a very specific and sensitive method for the quantitative determination of steroid concentrations in crude biological extracts. The technique is ideally suited to the detailed study of hormonal steroids in previously uninvestigated biological samples.

Errors involved in the procedure described in this paper can largely be eliminated by the use of internal standards, preferably deuterium labelled analogues of the steroids to be measured.

Acknowledgements—The author wishes to thank Miss M. E. Buoy for technical assistance, and the Tenovus Organisation for generous financial support.

Table 3. Steroid concentrations in radial, testicular and deferential venous plasma of a dog, determined by HRMF, expressed as ng/ml plasma. The values are not corrected for losses incurred during the extraction procedure

	Radial	Testicular	Deferential
Testosterone	7·7	195·0	185·0
DHA	2·0	3·5	9·0
5α-Androstane-3,17-dione	<0·1	0·35	0·4
Androst-4-ene-3,17-dione	<0·1	3·0	4·0
5α-Androstane-3α,17α-diol	—	<0·1	<0·1
5α-Androstane-3α,17β-diol	—	0·3	0·5
5α-Androstane-3β,17α-diol	—	<0·1	0·3
5α-Androstane-3β,17β-diol	—	0·5	1·0
Oestradiol-17β	<0·05	0·05	—

REFERENCES

1. Hammar C. G., Holmstedt B. and Rhyhage R.: *Analyt. Biochem.* **25** (1968) 532–547.
2. Millington D. S., Jenner D. A., Jones T. and Griffiths K.: *Biochem. J.* **139** (1974) 473–475.
3. Millington D. S., Jones T., Jenner D. A. and Griffiths K.: *J. Endocr.* **61** (1974) liv–lv.
4. Kelly R. W.: personal communication.
5. Bournot P., Maume B. F. and Padieu P.: *Biomed. Mass Spectrom.* **1** (1974) 29–39.
6. Adlercreutz H. and Hunneman D. H.: *J. steroid Biochem.* **4** (1973) 233–237.
7. Dehennin L., Reiffsteck A. and Scholler R.: *J. steroid Biochem.* **5** (1974) 81–86.
8. Kelly R. W. and Taylor P. L.: Abstracts of the Second International Symposium on Mass Spectrometry in Biochemistry and Medicine, Instituto di Richerche Farmacologiche Mario Negri, Milan (1974) 51.
9. Pierrepoint C. G., Davies P., Millington D. S. and John B. M.: *J. Reprod. Fert.* (1975), in press.