Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 685–688. Pergamon Press. Printed in Great Britain

MASS FRAGMENTOGRAPHY AS REFERENCE METHOD IN CLINICAL STEROID ASSAY

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SUMMARY

Mass fragmentography may serve as a powerful tool in the fields of steroid biochemistry and clinical chemistry; it provides a simple and highly specific method for the determination of steroids in body fluids. Isotopically labelled steroids, corresponding to the natural occurring steroids, may be used as internal standards in order to correct for procedural losses as well as adsorption effects on the gas chromatographic columns. Since the mass spectrometer is capable of separating isotopes, quantitative determinations may be performed by comparing the peak areas of the unlabelled and the isotopically labelled steroids; the peaks are recorded simultaneously by monitoring two *m/e*-values (multiple ion detection). With respect to sensitivity and specificity, the method may be considerably improved by the use of special derivatives, *e.g.* heptafluorobutyrates. Because of their high specificity, determinations by mass fragmentography will be increasingly used as reference methods in the field of steroid hormones. In the present paper the procedure has been applied to the specific determination of oestrogens, testosterone, 5α-dihydrotestosterone, cortisol and aldosterone in human plasma.

INTRODUCTION

During recent years, numerous methods have been described for the quantitative determination of steroids in body fluids. However, the criteria of reliability were not met adequately by a number of methods. Therefore, normal ranges for steroid hormones frequently changed, particularly when new and more reliable methods were introduced. Even today it can be stated that quantitative figures about steroid hormones can vary considerably from one method to the other and from one laboratory to the next. This indicates that many analytical results deviate more or less from the true value. Obviously, precise and accurate results can only be obtained if suitable reference methods are available.

Since many years, combined gas chromatography–mass spectrometry has been successfully used as a qualitative analytical method for the identification of steroid hormones. More recently, mass fragmentography, as a special technique in the field of mass spectrometry, has been applied to the quantitative determination of steroids following gas chromatography [1–8]. The method is highly specific and very sensitive. With mass fragmentography, the magnetic field of the instrument is fixed to an *m/e* value either of the molecular ion or a fragment ion of the steroid. This selected *m/e* value should be characteristic for the steroid to be detected; no signals are obtained from substances which do not form ions of the same *m/e* value. In practice, the substance to be investigated is injected into the inlet of the gas chromatograph and separated by the column. After having reached the ion source of the mass spectrometer, the substance is ionised, fragmented and accelerated by high voltage. As a result of the fixed *m/e* value, a fragmentogram is recorded which looks like a gas chromatogram; this signal originates from one single mass. The mass spectrometer serves as a detector for gas chromatography with adjustable specificity. The application of mass fragmentography as reference method for the determination of steroid hormones in body fluids will be described in the present paper.

RESULTS AND DISCUSSION

When very small amounts of steroids are to be quantitated, difficulties may arise which are due to two main reasons: [1] There is no possibility of increasing the sensitivity of the instrumentation (and thus of the detector) by simple means; [2] irreversible adsorption of steroids on gas chromatographic column may become significant in the picogram range. As we shall see later, the sensitivity of mass fragmentography can be considerably increased by introducing special steroid derivatives.

The problem of adsorption may be overcome by using authentic substances as internal standards. Compounds, suitable as internal standards in mass fragmentography, are isotopically labelled steroids which correspond to the naturally occurring steroids. The simultaneous recording of isotopically labelled steroids together with the non-labelled substances requires a multiple ion monitoring system. As described for single ion monitoring, the mass spectrometer is adjusted to a defined *m/e* value, using the magnetic field. By using an accelerating voltage alternator, it is possible to adjust the accelerating voltage to several values which correspond to several masses. During analysis, the so-called multiple ion detector switches continuously between the preadjusted accelerating voltages, thus yielding several gas chromatograms which correspond to the adjusted *m/e* values. This technique will be demonstated by the determination of *oestradiol* in plasma of pregnant

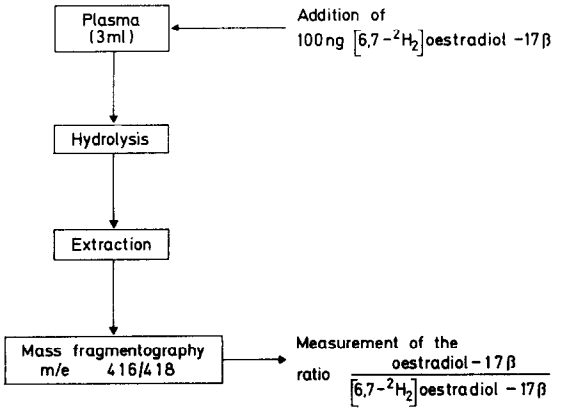

Fig. 1. Procedure for the determination of oestradiol-17β in the plasma of pregnant females.

women (Fig. 1). 100 ng of deuterated oestradiol is added to a sample of 3·0 ml of plasma. This deuterated material serves as internal standard and is carried through the analysis together with the oestradiol from the plasma sample. The analytical procedure results in two gas chromatograms which are recorded simultaneously (Fig. 2), one representing the unlabelled oestradiol and the other the deuterated standard. In the total ion current chromatogram, no definite peak at a retention time of the oestradiol derivative can be seen. The content of unlabelled oestradiol in the sample can be easily calculated from a comparison of the peak areas and the known amount of added deuterated oestradiol.

A further example for using internal standards is given by the determination of *cortisol* in plasma. To 0·1 ml serum is added 0·5 μCi of tritiated cortisol of high specific activity. After extraction with dichloromethane, the sample is treated with methoxyamine hydrochloride. The 3,20-dimethoxime of cortisol is formed which is then reacted with trimethyl bromosilane, thus yielding the persilylated methoxime derivative [9]. Quantitative determination is performed by

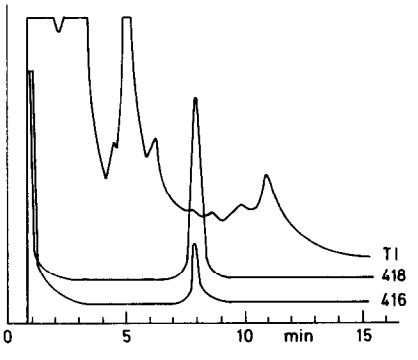

Fig. 2. Mass fragmentogram of the TMS ether of oestradiol-17β (*m/e* 416) and [6,7-$^2H_2$]oestradiol-17β (*m/e* 418) after processing a plasma sample (3 ml) from a pregnant female subject; TI: total ion current chromatogram. Gas chromatograph/mass spectrometer: LKB 9000; g.l.c. column: 180 × 0·25 cm., 3% OV-1, 220°C; separator: 220°C; ion source: 250°C. 60 μA, 23 eV.

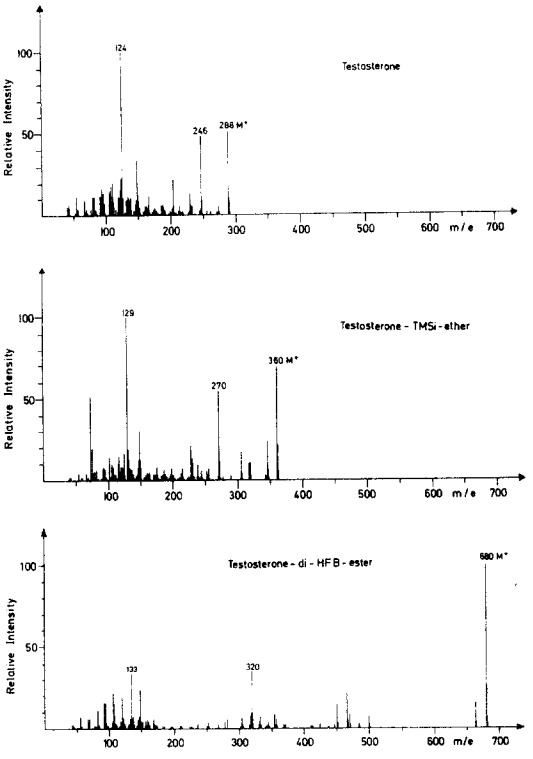

Fig. 3. Mass spectra of testosterone, testosterone TMS ether and testosterone 3-enol, 17β-diheptafluorobutyrate; mass spectrometer: LKB 9000; direct inlet system: 40–60°C; ion source: 60 μA, 35 eV, 230°C.

mass fragmentography, recording *m/e* 605 and 609. The lower limit of detection is at 0·5 ng cortisol.

As already indicated, the sensitivity of mass fragmentography can be significantly increased by using special derivatives of steroids. Heptafluorobutyric esters were found particularly suitable for the sensitive and specific determination of steroids by the mass spectrometer. The high sensitivity may be explained by the electron capturing behaviour of the halogen atoms and also by the fact that one single ion shows a particularly high abundance as compared to the total amount of ionised fragments. This may be seen from a comparison of the mass spectra of free testosterone, the trimethylsilyl ether of testosterone and the diheptafluorobutyric ester of testosterone (Fig. 3). The diheptafluorobutyric ester exhibits a molecular ion at *m/e* 680 with a very high relative intensity, whereas the spectra of free testosterone and the corresponding trimethylsilyl ether show considerable fragmentation, resulting in numerous fragment ions. Using heptafluorobutyrates, a high specificity is achieved by the marked increase in molecular weight with 196 mass units per heptafluorobutyric group. It can be stated that, at high *m/e* values, only very few ions of accompanying impurities from biological material or from the continuous bleeding of the stationary phase may interfere with the determination. Therefore, a procedure for the determination of testosterone in male as well as in female plasma, including the formation of heptafluorobutyrates, was developed. It

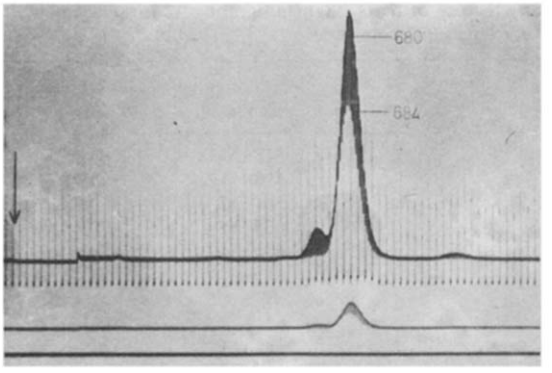

Fig. 4. Mass fragmentogram of the 3-enol, 17β-diheptafluorobutyrates of testosterone (*m/e* 680) and [1,2-$^3H_2$]-testosterone (*m/e* 684) after processing a plasma sample (2 ml) from a female subject; the arrow indicates the time of injection. Gas chromatograph/mass spectrometer: LKB 9000; g.l.c. column: 130 × 0·25 cm., 3% OV-101 235°C; separator: 240°C, ion source 250°C, 60 μA, 26 eV.

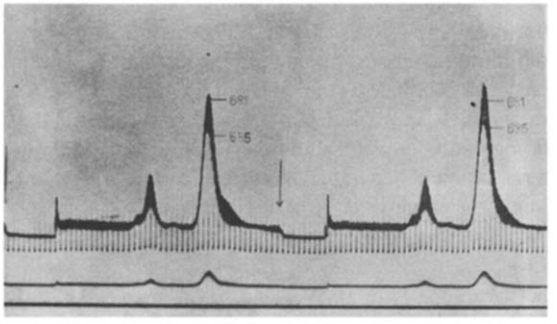

Fig. 5. Mass fragmentograms of 5α-dihydrotestosterone (*m/e* 681) and [1,2-$^3H_2$]-5α-dihydrotestosterone (*m/e* 685) (heptafluorobutyrated methoxime derivatives) after processing duplicates of a plasma sample (2 ml) from a female subject; the arrows indicate the time of injection.
Gas chromatograph/mass spectrometer: LKB 9000; g.l.c. column: 130 × 0·25 cm., 3% OV-101 245°C; separator 240°C; ion source: 250°C, 60 μA, 26 eV.

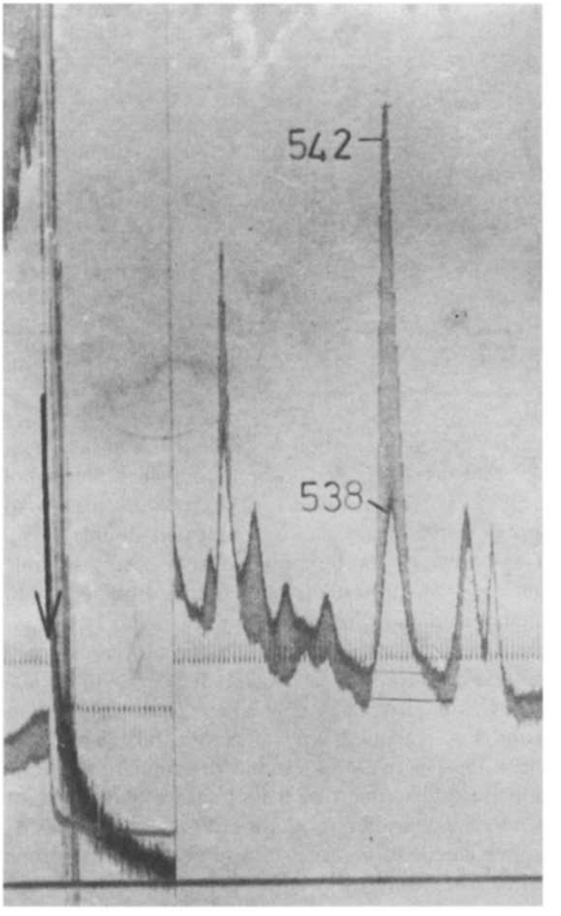

Fig. 7. Mass fragmentogram of 3 enol-heptafluorobutyric ester derivatives of the 18,21-acetal of aldosterone (*m/e* 538) and [1,2-$^3H_2$]-aldosterone (*m/e* 542) after processing a plasma sample (5 ml); the arrow indicates the time of injection.
Gas chromatograph/mass spectrometer: LKB 9000; g.l.c. column: 130 × 0·25 cm., 3% OV-101 250°C; separator: 240°C; ion source: 250°C, 60 μA, 26 eV.

consists of the addition of tritiated testosterone to the sample of 1 ml of male or 2 ml of female plasma, respectively, extraction by dichloromethane, thin layer chromatography and formation of the diheptafluorobutyrates of testosterone. Quantitation was performed by monitoring *m/e* 680 and 684, corresponding to testosterone as well as to the tritiated standard steroid (Fig. 4). The fragmentogram was obtained after processing a plasma sample from a female subject. The isotope ratios, from which the results are calculated, show good agreement. The great excess of impurities present in the material injected into the gas chromatograph did not interfere with the mass specific detection of the selected ions.

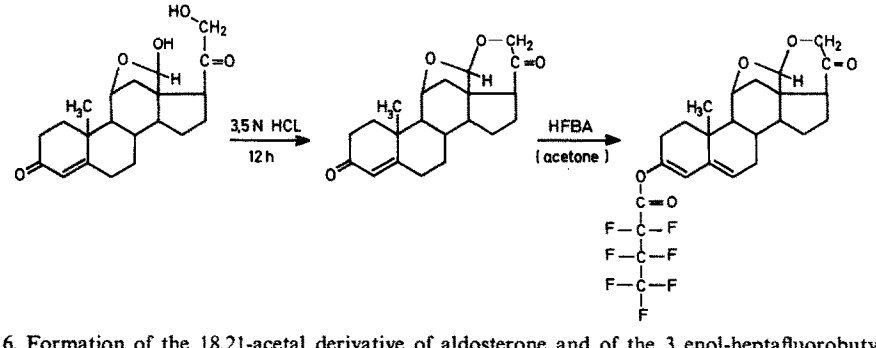

Fig. 6. Formation of the 18,21-acetal derivative of aldosterone and of the 3 enol-heptafluorobutyrate of the 18,21-acetal of aldosterone.

Table 1. Lower limit of detection of various steroid derivatives by the use of mass fragmentography TMSi = Trimethylsilyl; MO = Methoxime; HFB = Heptafluorobutyric

Steroid	Derivative	*m/e*	Lower limit of detection
Oestrone	TMSi-ether	342 (M^+)	0·05 ng
Oestradiol-17β	TMSi-ether	416 (M^+)	0·05 ng
Oestriol	TMSi-ether	504 (M^+)	0·10 ng
Cortisol	3,20-MO-11β,17α,21-TMSi-ether	605 (M − 31)	0·25 ng
Testosterone	3-Enol,17β-di-HFB-ester	680 (M^+)	0·005 ng
5α-Dihydrotestosterone	Product after reaction with MO hydrochloride and HFB anhydride	681	0·01 ng
Aldosterone	18,21-Acetal-3-enol-HFB-ester	538 (M^+)	0·05 ng
3α,5β-Tetrahydroaldosterone	18,21-Acetal-3-HFB-ester	542 (M^+)	0·25 ng

For the determination of *5α-dihydrotestosterone*, a different derivative had to be found, since the 3-enol heptafluorobutyrate does not form with the 3-oxo group in the absence of a conjugated double bond. The following procedure proved to be a very suitable one. 5α-Dihydrotestosterone is first treated with methoxyamine hydrochloride to give the corresponding 3-methoxime. Subsequent addition of heptafuorobutyric acid leads to a product with an ion at *m/e* 681; this is identical with the base peak of the spectrum. For reasons discussed before, this ion allows the very sensitive and specific determination of 5α-dihydrotestosterone. The mass fragmentograms which were obtained after processing duplicates of plasma from a female subject are shown in Fig. 5. The isotope ratios, from which the results are calculated, show good agreement.

Based on the experience gained with the method for the determination of testosterone, a similar procedure was developed for the specific quantitation of *aldosterone* in plasma. A new derivative was prepared as follows (Fig. 6). Aldosterone was treated with hydrochloric acid to form the (11, 18) (18, 21)-acetal in quantitative yield. By the use of heptofluorobutyric acid anhydride in acetone, the acetal reacts to the 3-enol heptafluoro butyrate. The procedure for the determination of aldosterone in human plasma comprises addition of 150 nCi tritiated aldosterone to the plasma sample, column chromatography on Amberlite XAD-4, formation of the acetal of aldosterone, thin layer chromatography on silica gel and formation of the heptafluoro butyrate. Quantitative determination is carried out by mass fragmentography, comparing the peak areas of the aldosterone derivative and its tritiated standard as shown in Fig. 7.

Table 1 summarises the steroids which so far have been determined by mass fragmentography. Depending on the nature of the derivative and on the pattern of fragmentation, the lower limits of detection vary between 5 and 100 picogram. The high specificity of mass fragmentography makes this technique particularly suitable for the development of reference methods. It appears that, at present, no other methodology equally well fulfills the requirements and criteria of high accuracy and specificity in the determination of steroids in biological fluids.

Acknowledgements—The present investigation was supported by the Deutsche Forschungsgemeinschaft and the Stiftung Volkswagenwerk.

REFERENCES

1. Siekmann L., Hoppen H. O. and Breuer H.: *Z. Anal. Chem.* **255** (1970) 294–298.
2. Kelly R. W.: *J. Chromatogr.* **54** (1971) 345–355.
3. Brooks C. J. W. and Middleditch B. S.: *Clin. chim. Acta* **34** (1971) 145–157.
4. Siekmann L., Spiegelhalder B. and Breuer H.: *Z. Anal. Chem.* **261** (1972) 377–381.
5. Siekmann L., Martin S. and Breuer H.: *Scand. J. clin. Lab. Invest.* **29,** Suppl. 126 (1972) 8.8.
6. Chapman J. R. and Bailey E.: *J. Chromatogr.* **89** (1974) 215–224.
7. Aldercreutz H. and Hunnemann D. H.: *J. steroid Biochem.* **4** (1973) 233–237.
8. Siekmann L.: *J. steroid Biochem.* **5** (1974) 727–732.
9. Aringer L., Eneroth P. and Gustafsson I. A.: *Steroids* **17** (1971) 377–398.