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MASS FRAGMENTOGRAPHY OF STEROID HORMONES

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SUMMARY

It has recently been shown that mass fragmentography may serve as a powerful tool in the field of steroid biochemistry. This is demonstrated by the following examples. When [4-^{14}C]oestriol was incubated with liver slices of wild mice, about 50 ng of a polar radioactive metabolite were isolated. Despite the small amount of steroid and impurities present in the extract, the unknown metabolite was identified as 6-hydroxyoestriol by the technique of single ion monitoring, using g.l.c. columns of different polarity. Furthermore, mass fragmentography provides a simple and highly specific method for the determination of steroid hormones in body fluids. Using the single ion technique, the amount of steroid is calculated by comparing the peak areas of the sample and of the standard. This method can be considerably improved by the use of steroids labelled with stable or radioactive isotopes as internal standards. The determination is performed by comparing the peak areas of the steroid to be quantitated and of the isotopically labelled steroid; measurement of the peaks is achieved by monitoring two *m/e* values simultaneously (multiple ion detection). This procedure has been applied to the specific determination of oestrogens, testosterone, aldosterone and tetrahydroaldosterone in plasma and/or urine. The sensitivity of mass fragmentography can be increased by using the heptafluorobutyric esters of the various steroids.

INTRODUCTION

For many years the combined gas chromatography mass spectrometry has served as a powerful analytical tool in the field of steroid biochemistry. Recently, a special method has been developed which is called mass fragmentography. Hitherto, the application of the new technique, which is in some respect superior to the conventional gas chromatography/mass spectrometry, has been limited mainly to psychoactive drugs [1, 2], neurotransmitting substances[3–5] and prostaglandins[6–8]. In the present paper, the value of mass fragmentography for the identification and for the quantitative determination of steroids will be demonstrated by a few examples from our laboratory.

EXPERIMENTAL

The instrumentation consists of a combined gas chromatograph/mass spectrometer. The substances to be investigated are injected into the inlet of the gas chromatograph and separated by the column. A molecule separator serves as an interface between the gas chromatograph and the mass spectrometer in order to remove the carrier gas. The substances reach the ion source of the mass spectrometer via the separator; they are ionized, fragmented and accelerated by high voltage. Using the conventional technique, complete mass spectra are obtained by scanning the magnetic field and recording the signals of the electron multiplier simultaneously.

In contrast, with mass fragmentography the magnetic field is fixed to an *m/e* value either of the molecular ion or a fragment ion of the steroid. The precise adjustment is performed by introducing a sample of the authentic steroid into the ion source by means of the direct inlet system and by tuning the magnetic field to the peak maximum of the selected ion. Thereupon, the probe with the authentic steroid is removed from the ion source, and the sample to be investigated by mass fragmentography is injected into the gas chromatograph. As a result of the procedure, a fragmentogram is recorded, which looks like a gas chromatogram; however, the signal only originates from one single mass.

The high specificity of the technique is due to the fact that the mass spectrometer is focussed to a fixed *m/e* value, which is characteristic for the steroid to be detected. No signal can be obtained from all other substances which do not form ions of the same *m/e* value. From this point of view the mass spectrometer serves as a detector for gas chromatography with adjustable specificity. The technique of single ion monitoring has been described first by Henneberg[9]. Later Adlercreutz mentioned the detection of picogram amounts of pregnanediol by the use of a gas chromatograph mass/spectrometer[10].

Sweeley developed the accelerating voltage alternator which makes it possible to monitor several *m/e* values simultaneously[11]. As described for single ion

monitoring, the mass spectrometer is adjusted to a defined *m/e* value using the magnetic field. By changing the accelerating voltage, it is possible to adjust the instrument to several masses. During fragmentography, the so-called multiple ion detector switches continuously between the original and the changed accelerating voltages with a suitable switching frequency. This results in several gas chromatograms which are recorded at the same time and which correspond to the adjusted *m/e* values. Meanwhile the technique has been considerably improved by Hammar[12], and a few studies on steroid determinations have been reported[13, 14].

RESULTS AND DISCUSSION

When radioactive oestriol was incubated with liver slices from wild mice, about 50 ng of a polar radioactive metabolite was isolated that behaved like 6α-hydroxyoestriol on paper chromatography. Due to the large amounts of impurities which were eluted from paper chromatogram together with the low amount of radioactive metabolite, no distinct peak for 6α-hydroxyoestriol was observed on the gas chromatogram. Therefore, the magnetic field of the mass spectrometer was adjusted to *m/e* 502. This *m/e* value is particularly suitable for the detection of the TMS ether of 6α-hydroxyoestriol because of its high relative intensity. We obtained a mass fragmentogram (Fig. 1) that showed a main peak at a retention time which correlated to that of the authentic steroid when chromatographed on an OV-1 column.

This method of identifying a metabolite is almost as reliable as the conventional method of recording complete mass spectra. The important advantage of the technique is demonstrated by the fact that impurities, present in the sample, do not interfere with the identification of the steroid. Moreover, mass fragmentography is much more sensitive. Two reasons are responsible for the increase in sensitivity. Firstly, for the technique of mass fragmentography, less resolution is needed than for recording mass spectra; thus, the slits of the ion separation system may be opened considerably, which results in an increase of the intensity of the electron multiplier signal. Secondly, in mass fragmentography the signal can be damped very effectively by electronic filtering, since it changes very slowly as compared with recording complete mass spectra. This results in a higher ratio of signal to noise. Due to the suppression of the background noise level, a higher voltage can be adjusted at the electron multiplier, and lower amounts of substances can be detected.

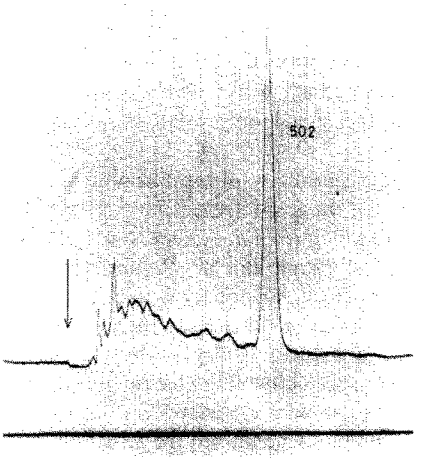

Fig. 1. Mass fragmentogram of the TMS ether derivative of 6α-hydroxy-oestriol, identified after incubation of oestriol with slices of mice liver.
Gas chromatograph/mass spectrometer: LKB 9000; g.l.c. column: 180 × 0·3 cm, 3% OV-101, 245°C; separator: 230°C; ion source: 250°C, 60 μA, 28 eV; *m/e* 502.

Mass fragmentography can also be used for quantitative determinations of steroids. Thus, the method can be applied to the estimation of testosterone in blood of male subjects. Five millilitres of plasma are extracted by ether; TMS ether derivatives are formed by adding a silylating reagent. Portions of the reaction mixture are injected into the column of the gas chromatograph/mass spectrometer which is tuned to monitor *m/e* 360, the molecular ion of the testosterone TMS ether. Whereas the total ion current chromatogram did not indicate a definite peak with a retention time of the authentic steroid, the low amount of substance could be determined quantitatively by mass fragmentography as shown in Fig. 2. The lower fragmentogram shows the recording of *m/e* 360 after processing a plasma sample; the upper curve is obtained from the authentic testosterone derivative. The lower limit of detection is at 1 ng testosterone. The linearity of the detector response is maintained over a wide range of quantities of steroids as it has been also demonstrated by Brooks [15]. It should be pointed out that this technique of single ion monitoring can be utilized without any additional device connected to a conventional mass spectrometer.

A very useful application of mass fragmentography is the simultaneous recording of isotopically labelled steroids together with the non labelled substances using the multiple ion monitoring system; the procedure allows the quantitative determination of steroids by recording the ratio of the two isotopes. This will be demonstrated by the determination of oestrone in plasma of pregnant women[16]. To a sample of 2·5 ml plasma 400 ng of deuterated oestrone is added; this internal standard is carried through the analysis together with the biological sample. At the end of the

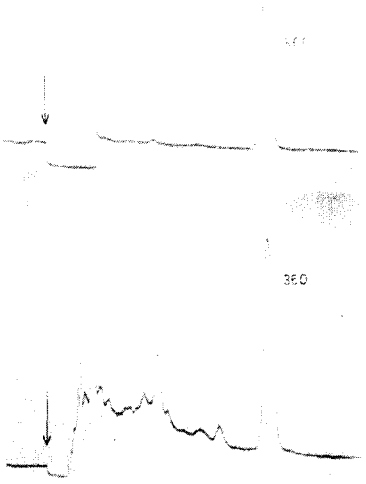

Fig. 2. Mass fragmentogram of testosterone TMS ether after processing a plasma sample (5 ml) of a male subject (lower panel); mass fragmentogram of 1 ng testosterone TMS ether (upper panel). Gas chromatograph/mass spectrometer: LKB 9000; g.l.c. column: 180 × 0·25 cm, 3% OV-3, 235°C; separator: 230°C; ion source: 250°C, 60 μA, 28 eV; *m/e* 360.

analytical procedure, two gas chromatograms are recorded simultaneously by mass fragmentography (Fig. 3), one representing the unlabelled oestrone and the other the deuterated standard. The upper curve shows the recording of the total ion current chromatogram; no definite peak at a retention time of the oestrone derivative can be seen. From a comparison of the peak areas and the known amount of added deuterated oestrone, the original content of unlabelled oestrone in the sample can be easily calculated.

Using the analytical procedure described, no difficulties arise by measuring steroids of relatively high concentration in biological material. For example in the determination of plasma cortisol, tritiated cortisol was added to the sample and after extraction the persilylated methoxime derivatives[17] were formed. Final determination was performed by mass fragmentography, recording *m/e* 605 and 609 (Fig. 4); the lower limit of detection is at 0·5 ng.

However, the question arises, whether it is possible to quantitate steroid hormones in body fluids at lower concentrations. For example in the determination of testosterone in plasma of normal women, the lower limit of detection must be in the picogram range. Since there is no possibility of increasing the sensitivity of the instrumentation by simple means, we looked for a new type of steroid derivative. Considering the similarity of the process in the electron capture detector and the electron impact in the mass spectrometer, we used heptafluorobutyric esters of steroids. As expected, these derivatives are particularly suitable for the sensitive and specific determination of steroids by the mass spectrometer. The high sensitivity may be

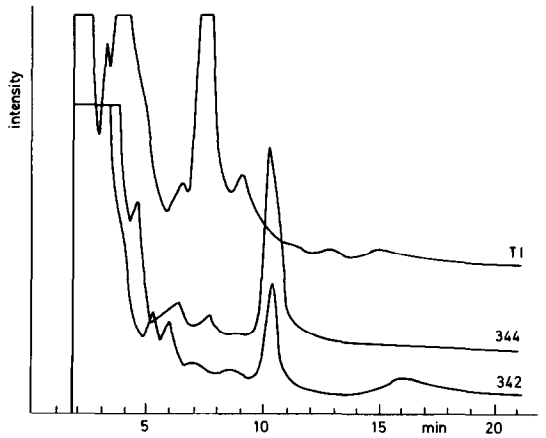

Fig. 3. Mass fragmentogram of the TMS ether of oestrone (*m/e* 342) and [6, 7-$^2H_2$] oestrone (*m/e* 344) after processing a plasma sample (2·5 ml) from a pregnant female subject: TI: total ion current chromatogram.
Gas chromatograph/mass spectrometer: LKB 9000; g.l.c. column: 180 × 0·25 cm, 3% OV-7, 240°C; separator: 220°C; ion source: 250°C, 60 μA, 23 eV.

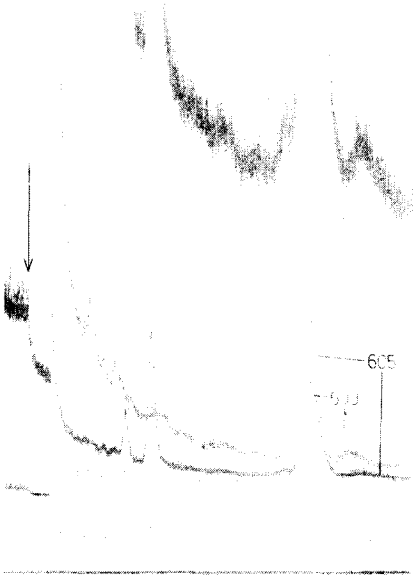

Fig. 4. Mass fragmentogram of the persilylated 3,20-*bis* (O-methyloxime) derivatives of cortisol (*m/e* 605) and [1, 2-$^3H_2$] cortisol (*m/e* 609) after processing a plasma sample (0·1 ml). Gas chromatograph/mass spectrometer: LKB 9000; g.l.c. column: 130 × 0·25 cm, 3% OV-101, 250°C; separator: 240°C; ion source: 250°C, 60 μA, 28 eV.

explained by the electroncapturing behaviour of the halogen atoms and also by the fact that one single ion shows a particularly high abundance with respect to the total amount of ionized fragments.

With respect to the specificity, the considerable increase in molecular weight with 196 mass units per heptafluorobutyric group is of great advantage. The higher the *m/e* value to be recorded, the less probable it is that the accompanying impurities from the biological material or the continuous bleeding of the stationary phase may interfere with the determination.

On the basis of these findings, a procedure for the determination of testosterone in male as well as in female plasma was developed[18]. It consists of an 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. By monitoring *m/e* 680 and 684, testosterone as well as the tritiated standard steroid are recorded (Fig. 5). The fragmentograms are obtained after processing duplicates of plasma from a female subject. Although a great excess of impurities was present in the material injected into the gas chromatograph, only testosterone and the corresponding tritiated material were seen during recording *m/e* 680 and 684. The isotope ratios, from which the results are calculated, show good agreement.

It should be mentioned that the tritiated steroids are not available as isotopically pure material. They usually contain 5–15% of unlabelled steroid. For this reason it is necessary to set up a calibration curve using four different quantities of reference testosterone. The same amount of tritiated testosterone is added to

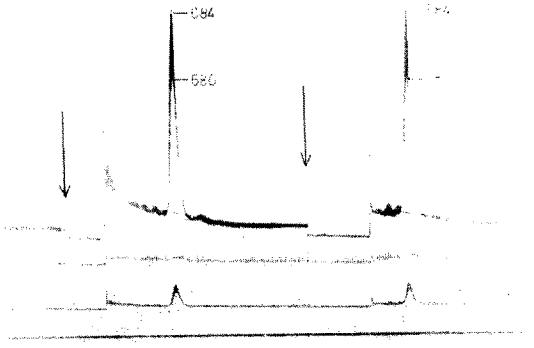

Fig. 5. Mass fragmentograms of the 3 enol, 17β-diheptafluorobutyrates of testosterone (*m/e* 680) and [1, 2-$^3H_2$] testosterone (*m/e* 684) after processing duplicates of a plasma sample (2 ml) from a female subject; the arrows indicate the time of injection.
Gas chromatograph/mass spectrometer: LKB 9000; g.l.c. column: 130 × 0·25 cm, 3% OV-101 235°C; separator: 240°C; ion source: 250°C, 60 μA, 26 eV.

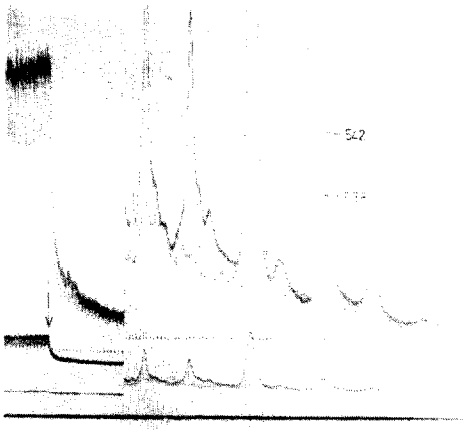

Fig. 6. Mass fragmentogram of 3 enol-heptafluorobutyric ester derivatives of the full acetal of aldosterone (*m/e* 538) and [1, 2-$^3H_2$] aldosterone (*m/e* 542) after processing a plasma sample (5 ml); the arrow indicates the time of injection.
Gas chromatograph/mass spectrometer; LKB 9000; g.l.c. column: 130 × 0·25 cm, 3% OV-101, 250°C; separator: 240°C; ion source: 250°C, 60 μA, 26 eV.

each standard as well as to the plasma samples. If the amount of testosterone in the standards is plotted against the isotope ratios measured by mass fragmentography, a standard curve is obtained which shows linearity over a wide range. When the isotope ratios of plasma samples are known from mass fragmentography, the amounts of testosterone can be taken directly from the calibration curve.

Encouraged by the good results obtained with the testosterone method, we developed a similar procedure for the determination of aldosterone in plasma[19]. For this purpose a new derivative was prepared. Aldosterone was treated overnight with 3·5 N hydrochloric acid to form the (11, 18)(18, 21)-acetal in quantitative yield. By the use of heptafluorobutyric acid anhydride in acetone, the acetal reacts to form the 3 enol-heptafluorobutyrate. This derivative proved to be particularly suitable for mass fragmentography. The procedure for determination of aldosterone in human plasma consists of the following steps: (1) Addition of 150 nCi tritiated aldosterone to the plasma sample (5 ml); (2) column chromatography on Amberlite XAD-4; (3) formation of the acetal of aldosterone; (4) thin layer chromatography on silica gel; (5) formation of the heptafluorobutyrates. Quantitative determination is performed by mass fragmentography, comparing the peak areas of the aldosterone derivative and its tritiated standard as shown in Fig. 6.

In analogy to this procedure, a simple method for the simultaneous determination of aldosterone and 3α, 5β-tetrahydroaldosterone in human urine was de-

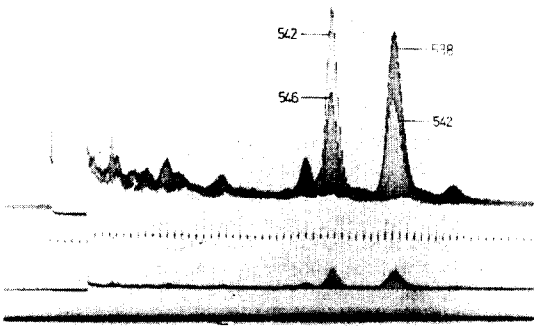

Fig. 7. Mass fragmentogram of the 3 enol-heptafluorobutyric ester derivatives of the full acetal of aldosterone (*m/e* 538) and [1, 2-$^3H_2$] aldosterone (*m/e* 542) as well as the 3α-heptafluorobutyric ester derivatives of the full acetals of tetrahydroaldosterone (*m/e* 542) and [1, 2-$^3H_2$] tetrahydroaldosterone after processing a urine sample (0·1 ml). Gas chromatograph/mass spectrometer: LKB 9000: conditions as described in Fig. 6.

veloped. To 100 μl of urine tritiated aldosterone as well as tritiated tetrahydroaldosterone are added as internal standards. The urine sample is reacted with 1 ml of 3·5 N hydrochloric acid at 60°C for 3 h. In this step the simultaneous hydrolysis of the conjugates and formation of the acetals occur. The acetals are extracted by benzene and purified by thin layer chromatography on silica gel. After formation of the heptafluorobutyrates the steroids are measured by mass fragmentography comparing the signals of the unlabelled and the corresponding isotopically labelled compounds as demonstrated in Fig. 7. For this purpose the mass spectrometer is adjusted to *m/e* 538, 542, 546. The peak with the lower retention time represents the tetrahydroaldosterone together with its tritiated standard material. The second peak originates from the two aldosterone isotopes, the unlabelled one and the tritiated.

CONCLUSIONS

As far as the future application of mass fragmentography in the field of analytical biochemistry is concerned, it should be stated that mass fragmentography has certain advantages over other methods, such as the radioimmunological determination of steroids. Mass fragmentography not only provides information about the quantity of a substance by measuring peak areas; the quality of a result can also be judged by an inspection of the peak shape. This leads to a high reliability of the analytical procedure. Because of their high specificity, mass fragmentography determinations will be increasingly used as reference methods in clinical chemistry. It may be expected that considerable technical improvements will be achieved in the future by computer-controlled fragmentography.

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DISCUSSION

Adlercreutz:

Dr. Siekmann showed very nicely how these techniques can be applied to determinations of various steroids. I would just very briefly like to show some recent developments we have made. We can now measure 11 estrogens in almost any biological fluid, also outside pregnancy.

Some data were obtained when the method was applied to samples of non-pregnancy bile and urine and also results of determinations of 11 estrogens in the unconjugated fraction of pregnancy plasma. All results will be presented in an article, in this journal. (**5** (1974) 211–217).

Siekmann:

What type of deuterated estrogens do you use?

Adlercreutz:

I use deuterated derivatives of estrogens i.e. deuterated trimethylsilyl ethers with the deuterium in the 3-methylsilyl group.

Siekmann: I think this is very different from our procedure as we introduce the internal standard material at the beginning of the analysis and you introduce it just before doing fragmentography. Is it very necessary to introduce it in order to control mass fragmentography which I think works very reliably by itself. The whole analytical procedure can lead to differing results and therefore it should be controlled by adding internal standards at the beginning of the analysis.

Adlercreutz:

Yes, it is much better to add deuterated estrogens in the beginning of the analysis if they would be available, but you cannot buy any of these compounds. If you can get them from Drs. Breuer or Siekmann then you have a chance to improve the technique, but you probably do not have all 11 estrogens available? I would like to add that the mass fragmentographic step is frequently unreliable because of adsorption to the column or of drifting of the mass marker which can occur during a day's analysis and other factors. It depends also on the mass spectrometer you are using. Some are more stable and others are not very stable.

Pasqualini:

I have a question on the last part of the presentation concerning this derivative of aldosterone. Can you give some details of the experimental conditions? What is the percentage of transformation of aldosterone to this 18–21 derivative. Can you also apply this method to other 18–OH steroids such as 18-OH-deoxycorticosterone or 18-OH-corticosterone?

Siekmann:

In answer to your first question, the conditions are very simple. As I said, 3·5 N HCl is applied to the dried sample at room temperature overnight and the derivative is formed quantitatively as we could see by the use of radioactive aldosterone. To your second question, we did not try any other 18-hydroxylated compounds until now, but I think this will be a question of time.

Ungar:

Do you get a good molecular ion for all steroids? And what is the approximate cost of the instrumentation that you are using?

Siekmann:

We use an LKB 9000 instrument and I do not know exactly the cost of this instrumentation at this time, but I think that in the future there will be some developments in the direction of cheaper instruments. I think for the same purpose, and I have checked this, you can use a quadruple mass spectrometer which will cost about half as much as this LKB 9000 instrument for example. I do think that especially these "quadruple" instruments will become cheaper. The molecular ions did not always serve as suitable peaks for mass fragmentography; therefore, sometimes we recorded fragment ions for example in the case of the determination of cortisol. On the other hand the 3-enol heptafluorobutyrates exhibit very abundant molecular ions, which proved to be particularly suitable for fragmentography.

Dehennin:

I agree with Dr. Siekmann that dienol heptafluorobutyrates are very good derivatives for the estimation of 4-ene 3-keto steroids. Concerning the explanation of these very abundant molecular ions, I think this is due to the conjugation and not to the presence of halogens. Trifluoroacetates, for example, have very poor responses in electron capture detection but give very abundant molecular ions. So people who want to have very abundant molecular ions should try to have conjugation in the molecule.

Siekmann:

Yes, we also checked the HFB-derivative of tetrahydro-aldosterone which has no conjugation in the molecule. We were able to record the molecular ion with about one fourth of the sensitivity of the aldosterone derivative.