Journal of Steroid Biochemistry. 1975. Vol. 6, pp. 227–232. Pergamon Press. Printed in Great Britain

ANALYSIS OF STEROIDS BY LIQUID-GEL CHROMATOGRAPHY AND COMPUTERIZED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Work carried out in this laboratory aiming at the development of generally applicable methods for analysis of steroids in biological materials is reviewed. Isolation and separation of groups of steroids is achieved by chromatography in miscible solvent systems on neutral or ion exchanging lipophilic derivatives of Sephadex. Reversed or straight-phase systems can be designed to obtain columns which act as filters to remove unwanted material.

The hydrolyzed steroids are converted to O-methyloxime-trimethylsilyl ether derivatives which are purified by liquid-gel chromatography and analyzed by computerized gas chromatography–mass spectrometry. Repetitive magnetic scanning of the entire mass range or repetitive accelerating voltage scanning of a limited mass range is used to monitor individual fragment ion currents. Spectra are recorded on magnetic tape, and peaks in fragment ion current chromatograms are located by the computer which performs a preliminary search for potential molecular ions in spectra taken at the located peaks. Quantitative determinations are based on areas of peaks with correct retention time in chromatograms based on the current of specific fragment ions.

Similar methods are used to determine the number and abundance of heavy atoms in steroids formed from precursors labelled with stable isotopes and in metabolites formed from steroids labelled with stable isotopes in specific positions.

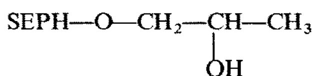
The aim of the work reviewed in this paper is to develop generally applicable methods for unbiased analysis of steroids in biological materials. Because of its high specificity gas chromatography–mass spectrometry (GC–MS) has been chosen as the final step in the analyses. Efficient use of GC–MS for routine analysis of large numbers of samples requires computerization of data acquisition and evaluation.

The analytical procedure may be divided into three stages

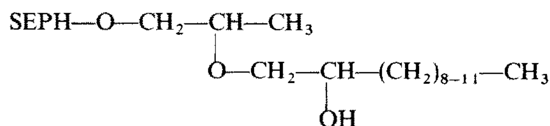
1. Extraction, purification and derivative formation; 2. GC–MS-analysis; 3. computer evaluation of data. Methods for the first stage should be selected to meet the requirements of the GC–MS-computer analysis. Therefore, isolation procedures devised for use with other analytical methods may not be suitable.

PREPARATION OF SAMPLES FOR GC–MS ANALYSIS

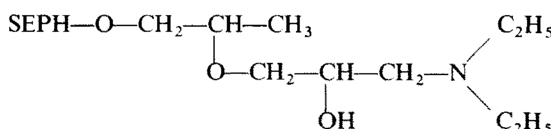
The system for isolation and purification of steroids developed in this laboratory is based on the synthesis of lipophilic-hydrophobic Sephadex derivatives which contain neutral or charged substituents [1–5]. These derivatives can be used with miscible organic solvent systems to give columns for straight-phase or reversed-phase partition (or adsorption) chromatography. Commonly used derivatives may be exemplified by the following schematic structures:



(Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden), for straight-phase chromatography)



(Lipidex-1000 containing 10% and Lipidex-5000 containing 50% (w/w) hydroxyalkyl groups (Packard-Becker, Groningen, Holland) for straight- or reversed-phase chromatography)



(DEAP-Sephadex, for anion exchange and straight- or reversed-phase chromatography).

The use of DEAP-Sephadex permits rapid group separations of different types of acidic steroids, *e.g.* glucuronides, mono and disulphates as exemplified in Fig. 1 [8]. For quantitative binding of acidic steroids to this ion exchanger it is necessary first to remove organic cations by filtration of the extract through a strong cation exchanger [9]. Compared to neutral steroids phenolic steroids are retarded on DEAP-Sephadex columns. However, they can be eluted with neutral solvents without addition of electrolytes.

Since most steroids cannot be directly subjected to gas chromatography it is necessary to protect hydroxyl and oxo groups prior to GC–MS analysis. If mixtures containing a variety of steroid structures are to be analyzed, reactions with a wide range of applicability. giving single derivatives with good mass

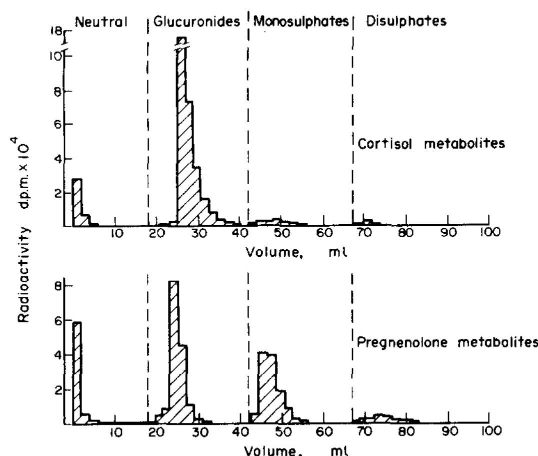


Fig. 1. Separation of radioactive metabolites in urine from male subjects given ^3H labelled cortisol (upper curve) and 3β-hydroxy-5-pregnen-20-one (lower curve). Column: 0·6 g of DEAP-Sephadex, 130 × 4 mm. Solvents: 72% aq. ethanol (0–18 ml, non-acidic compounds), 0·25 M formic acid in 72% ethanol (18–42 ml, glucuronides), 0·3 M potassium acetate pH 6·1 in 72% ethanol (42–67 ml, monosulphates) and 0·5 M potassium acetate pH 10 in 72% ethanol (67–90 ml, disulphates) (from Setchell, Almé, Axelson and Sjövall, to be published).

spectrometric properties, should be chosen. At present the most versatile derivative appears to be the fully reacted O-methyloxime-trimethylsilyl (MO–TMS) ether, prepared as described by Thenot and Horning[7].

By appropriate combinations of solumn systems based on Lipidex and other Sephadex derivatives and by selecting a suitable steroid derivative, it is possible to design methods for purification of groups of steroids in extracts of tissues or body fluids. For example, a sample suitable for GC–MS analysis and containing neutral steroids with polarities between those of pregnanedione and pregnanepentol may be prepared from plasma in the following way [6]: 1. Extraction of diluted plasma by Amberlite XAD-2 at 64°C. 2. Filtration through DEAP-Sephadex in methanol–water–chloroform, 9:1:2 by vol., to remove acidic material (*e.g.* conjugated steroids). 3. Filtration through Lipidex-5000 in the same solvent to remove nonpolar lipids. 4. Formation of fully protected MO–TMS derivatives. 5. Filtration of reaction mixture through Lipidex-5000 in hexane–pyridine–hexamethyldisilazane, 98:1:1 by vol., to remove nonvolatile reagents and polar contaminants. This procedure can be completed in about one day. A very clean sample is obtained and an amount corresponding to 5 ml of plasma can be injected into packed columns without overloading or damage to the column. The low weight of the extract and the absence of nonvolatile reagents also permits the use of solid injection systems for glass capillary columns.

Prior to GC–MS analysis, steroid mixtures may be subfractionated according to number of hydroxyl and oxo groups by chromatography on Sephadex LH-20 [3, 10] or Lipidex [4, 11].

GC–MS ANALYSIS

The use of a mass spectrometer as detector in gas chromatography involves monitoring of the variation with time of currents of fragment ions of specified mass. This can be done in different ways: 1. Repetitive scanning of the entire mass range. This permits construction of as many fragment ion current (FIC) chromatograms (mass chromatograms [12]) as the number of *m/e* values covered by the scan [12, 13]. 2. Repetitive magnetic or accelerating voltage scanning over a limited mass range. The number of data points per mass spectrometric peak is increased, and digital filtration can be used to increase signal–noise ratio. The sensitivity of this method in steroid analysis is 100–300 times higher than that obtained by scanning entire mass spectra [13, 14]. 3. Repetitive accelerating voltage switching between a few selected *m/e* values (multiple ion detection) and recording of "mass fragmentograms" of these ions [15, 16]. This method permits the use of long time constants, and high sensitivity is obtained.

The method of single [17] or multiple ion detection has been applied to the analysis of a number of steroids [18–27]. Multiple ion detection offers high sensitivity, especially when combined with the use of a deuterium labelled internal standard [20, 28, 29]. However, the method is limited to analysis of one or a few steroids at a time and it is not suitable for analysis of unknown steroid mixtures since the *m/e* values to be monitored must be selected prior to the start of the analysis.

Repetitive scanning methods have been less frequently used in steroid analysis. This is probably because of the need for computers for acquisition and evaluation of the massive amount of data produced. However, these methods are more versatile than multiple ion detection. In the system used in this laboratory the spectra are recorded on magnetic tape. Thus, the tape will contain FIC chromatograms of all *m/e* values covered by the scan. When complex mixtures are analyzed, all components may be revealed by repeated processing of the tape and selection of *m/e* values characteristic of specific steroid structures [13, 31]. The sensitivity obtained by repetitive scanning of a limited mass range (*e.g.* 3–20 mass units) is comparable to that reported for single and multiple ion detection [13, 20–28, 30]. The scan methods offer better possibilities for analysis of complex patterns of labelling with stable isotopes [32].

COMPUTER EVALUATION OF GC–MS ANALYSES

The data are recorded on a magnetic tape which is processed on an IBM 1800 computer with 24-K core storage, two 500-K disk storages, two 9-track magnetic tape units, a card read punch, a line printer, a plotter and a CRT display. Computer programs have been developed for evaluation of GC–MS data obtained by repetitive scanning of a complete or limited mass range, for determination of stable isotopes, and for kinetic analysis of metabolic experiments employing stable isotopes. Details on these programs are given in Refs. [13, 31, 32].

The process of evaluation of a GC–MS analysis may be divided into three parts: 1. location of compounds eluted singly or in mixtures, and determina-

tion of the retention times, 2. interpretation of mass spectra taken at located peak maxima of individual compounds, and 3. quantitative determination of identified steroids.

Peak locating program, PLOCP

This is based on a search for peaks in individual FIC chromatograms of all *m/e* values covered by the scan (usually 500–700 chromatograms). When criteria for the detection of a compound are fulfilled, information is printed about retention time and about mass and intensity of major ions given by the compound. The use of FIC chromatograms instead of the total ion current (TIC) chromatogram to monitor the appearance of compounds in the mass spectrometer makes it possible to detect the elution of overlapping compound zones and makes the search more sensitive. The procedure requires about 1·5 s per spectrum, *i.e.* about 3 min for a routine GC–MS analysis.

Interpretation of mass spectra

This is partly computerized partly manual. When the peak of a compound has been located, a subprogram of PLOCP, TMSID, is used in a search for potential molecular ions of steroids in the spectrum taken at the apex of the peak. Structural limits for the search are given by the investigator (*e.g.* limit the search to C_{19} and C_{21} steroids containing 2–5 oxygen substituents) and empirical fragmentation rules are applied to define potential molecular ions. The mass of a potential molecular ion is translated to a structure within the limits given. When the search is completed, it may be automatically repeated with the peak-front and peak-tail difference spectra. This is of particular value in the interpretation of spectra due to mixtures of steroids.

As an aid in further manual interpretation of a spectrum, a program, FRAGC, is used which searches for previous spectra (*e.g.* in library files) showing a given set of fragment ions or fragment losses or a combination of these [31]. If the specified set of ions is found in a spectrum, the name of the compound giving this spectrum is printed (or identification number of the spectrum if the compound is unknown). This is an aid in relating fragments to steroid structure and corresponds to the use of key ions and key fragments as described by Spiteller's group [33]. Programs which compare unknown with known spectra are often less useful, since the spectra obtained in GC–MS analyses of steroid profiles in biological materials are frequently due to mixtures of compounds.

The peak locating program is intended to give a reasonably complete detection of steroids in GC–MS analyses of biological materials. The search for potential molecular ions of naturally occurring steroids, which is part of this program, is mainly intended as an aid in the manual interpretation of complex spectra. In addition, selected ions which are characteristic of special steroids or steroid structures may be searched for [31]. Such a method has also been described by Baty and Wade[34] and this is a useful procedure in routine analyses. The located specified compound may then be quantified from peak areas in specific FIC chromatograms [32]. To simplify this type of directed analysis, a program, MINIG, is used which constructs and plots FIC chromatograms of the selected *m/e* values. Twenty overlapping FIC chromatograms can be plotted together and amplification or reduction factors may be used. In a separate part of this program, FENCE, peaks in the chromatograms are located [35]. Examples of FIC chromatograms obtained in analyses of corticosteroids in urine, $C_{21}O_2$ steroids in plasma from pregnant women and neutral steroids in bovine corpus luteum are shown in Figs. 2–4.

Quantitative determination of steroids

The methods for quantitative determination are based on peak areas in specific FIC chromatograms. Two programs have been written which differ mainly by the degree of automation of the analysis.

For program AREAS, a known amount of reference compound(s) and the unknown samples are injected as solutions containing a constant concentration of an internal standard. The internal standard is used to correct for differences in volumes of reference and sample solutions injected, and makes possible a direct comparison between peak areas given by reference compound and compounds in the sample. The investigator inspects FIC chromatograms of appropriate *m/e* values and selects tape sectors containing peaks and background. The computer calculates a peak area corrected for background contribu-

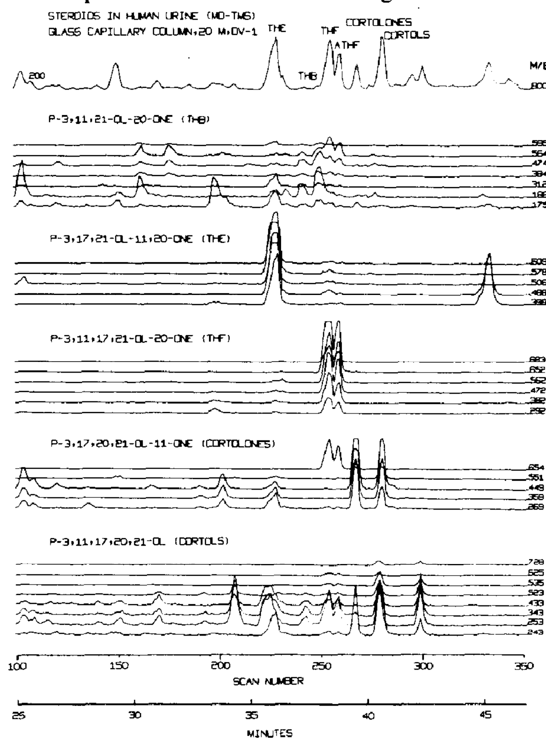


Fig. 2. FIC chromatograms constructed by the computer in a GC–MS analysis of steroids in urine from a healthy man. The upper curve is the partial ion current (PIC) chromatogram (total intensity of ions between *m/e* 200 and 800), the other curves are FIC chromatograms of *m/e* values characteristic of MO–TMS derivatives of different corticosteroid metabolites. The column was a 20 m × 0·3 mm glass capillary coated with OV-1 [36] (from ref. 32 with permission).

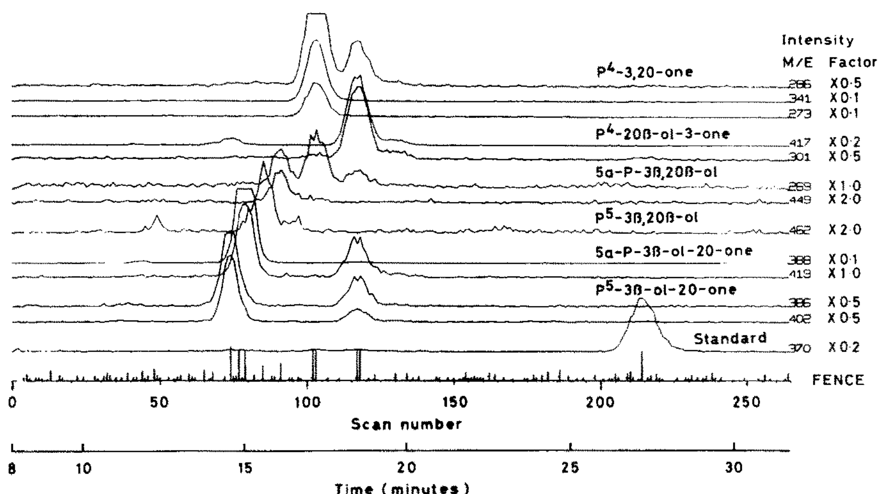


Fig. 3. FIC chromatograms specific for MO-TMS derivatives of steroids in bovine corpus luteum. For purposes of illustration the intensities of the ion currents were multiplied by the factors shown to the right of the *m/e* values. The sample was from a corpus luteum taken at the 17th day of the oestrous cycle (from ref. 37 with permission).

tion, and area is manually converted to weight. When tested in the quantitative analysis of a synthetic mixture of steroids occurring in bovine corpus luteum the precision of this method was ±3–10% (S.D.) in 12 experiments. An example of FIC chromatograms used for quantitative analysis of steroids in a sample from bovine corpus luteum is shown in Fig. 3 [37].

With the equipment used in this laboratory the technique permits a quantitative analysis of 5–500 ng amounts of steroid using packed columns and repetitive scanning of the entire mass range. Figure 4 shows an example of an analysis of unconjugated neutral steroids in plasma from a woman in the 29th week of pregnancy. With the exception of progesterone (10·4 min, 60 ng/ml) several of the steroids detected are present in a concentration range near the lower limit for determination by repetitive scanning of complete spectra (3α-hydroxy-5ξ-pregnan-20-one, 7·5 min, 15 ng/ml; 20α-hydroxy-4-pregnen-3-one, 11·7 min, 4·7 ng/ml; 5α-pregnane-3,20-dione, 9·5 min, 3·8 ng/ml; and 3β-hydroxy-5α-pregnan-20-one, 9 min, 1·8 ng/ml). However, the important difference between this and other methods is that the steroids can be detected even if the analysis did not primarily aim at their determination. This may be of decisive value when samples from patients with unknown defects in steroid metabolism are analyzed.

When higher sensitivity is needed, repetitive accelerating voltage scanning of a narrow mass range must be used. In this case the minimum detectable level depends mainly on the degree of loss of steroid in the gas chromatographic column and in the interface to the ion source, and on the intensity of the column and sample background bleed. In the ideal case the sensitivity is 100–300 times higher than when entire spectra are scanned. This method is also useful when deuterated internal standards are employed and when deuterium analyses have to be made on small amounts of sample or when the ions of interest have a low relative intensity [32].

The other program for quantitative analysis, QUANT, does not require inspection of FIC chromatograms. It operates with internal or external standards. The information required by the program is: 1. number of the GC–MS analysis of external standard (the GC–MS analyses on the tape are given consecutive numbers and are separated by a file gap); 2. amount of external standard; 3. numbers of the GC–MS analyses of samples; 4. names of the compounds to be determined and relative retention time ranges within which each compound is expected to appear; 5. ten (or less) *m/e* values typical for each compound to be determined. The computer finds the peaks in individual FIC chromatograms, notes retention times and calculates peak area. A baseline is constructed (horizontal or linear slope) and background is subtracted. The peak area in individual FIC chromatograms is converted to the equivalent total ion current peak area. This can be done from knowledge of the percentage contribution of the specific fragment ion to the total ionisation of the compound. The reason for the conversion is that it permits comparisons to be made between weight and total ionisation for any compound, and "response factors" between compounds can be established. Finally, peak area is con-

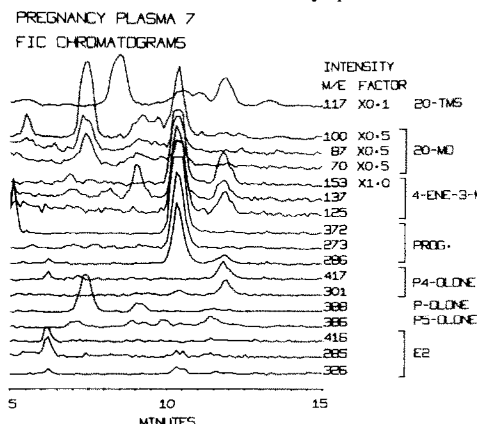


Fig. 4. FIC chromatograms constructed by the computer in an analysis of steroids in plasma from a woman in the 29th week of prgnancy. *m/e* Values characteristic of structures in progesterone and some of its potential precursors and metabolites were selected (from ref. 6 with permission).

verted to weight. In analyses of bile acid derivatives using 7–9 different *m/e* values the precision of determinations from a single FIC chromatogram was ±5–9% [32].

Determination of stable isotopes

An important application of stable isotopes in conjunction with GC–MS analysis is the use of internal standards labelled with stable isotope atoms for quantitative analysis of steroids in the subnanogram range (see above). The labelled standard serves two purposes: 1. since it can be added at the beginning of the analysis it suffers the same losses in the isolation procedure as the identical (with the exception of the stable isotope atoms) endogenous steroid, and 2. since losses of steroids on the g.l.c. column and in the interface to the ion source are usually nonlinear and inversely related to the amount of steroid analyzed, addition of a large amount of labelled reference steroid decreases the losses of endogenous steroid in the GC–MS system and increases the sensitivity of the analysis. When stable isotopes are used in this way for purely analytical purposes it is obvious that the steroid to be analyzed has to be known and must be selected prior to the start of the analysis.

When GC–MS analyses can be evaluated by computer methods, it may often be more practical to use stable rather than radioactive isotopes in metabolic studies. Tedious determinations of specific radioactivity are avoided since heavy isotope excess is directly measured. The position of a heavy atom may sometimes be directly determined from the isotope content of appropriate fragment ions. Molecules differing by the number of heavy atoms incorporated can readily be distinguished which is very difficult and requires chemical degradation if radioactive isotopes are used. Furthermore, there are several situations where radioactive isotopes cannot be employed, *e.g.* for studies in infants and pregnant women [38].

To avoid errors due to isotope separation effects, to increase accuracy and sensitivity and to permit analysis of many fragment ions with differing numbers of heavy atoms it is necessary to use repetitive scanning. A program, ISOTN, has been written which calculates isotopic composition by comparing the averaged mass spectrum (or partial mass spectrum) obtained by repetitive scanning over the peak of the labelled compound with the averaged spectrum of the unlabelled compound. Usually a sample taken before the injection of labelled steroid can provide this unlabelled reference compound. ISOTN has been extensively used in studies of the metabolic relationships between oxidation of [1,1-$^{2}H_{2}$]- and [2,2,2-$^{2}H_{3}$]-ethanol and biosynthesis and reduction of steroids and glycerolipids in the intact rat [39, 40]. It permits calculation of the abundance of a maximum of 19 ^{2}H or ^{13}C atoms per molecule (or fragment ion). When the compounds consist of molecules with 0–15 heavy atoms and repetitive scanning of entire mass spectra is used, sample sizes of 0·1–1 μg are required for measurements on major fragment ions. A 100-fold increase of sensitivity is obtained by repetitive accelerating voltage scanning over a limited mass range [32]. The presence of contaminating compounds and column bleed is an important factor in determining sensitivity and accuracy of the determinations.

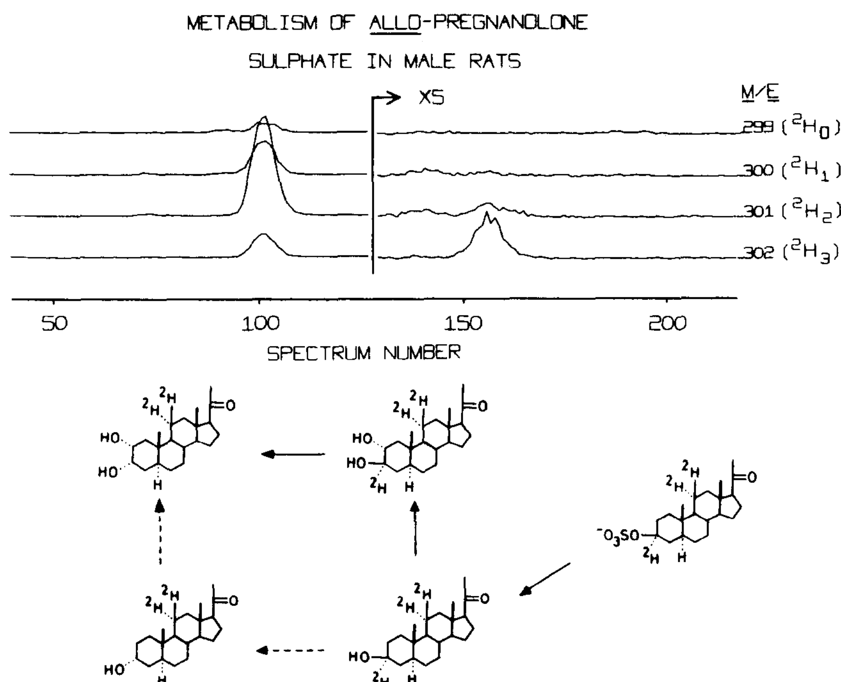


Fig. 5. FIC chromatograms of *m/e* values characteristic of TMS ethers of 2,3-dihydroxy-5α-pregnan-20-ones. The 2α,3α (spectra 94–106) and 2α,3β (spectra 147–164) isomers were isolated from the glucuronide fraction of bile from male rats given 3β-hydroxy-5α[3α,11,11-$^{2}H_{3}$]pregnan-20-one sulphate. The *m/e* values represent molecules containing zero, one, two and three ^{2}H atoms. No corrections for natural abundance of ^{13}C, ^{2}H or ^{18}O have been made. Solid arrows indicate probable major metabolic pathway (from Baillie, Eriksson, Herz and Sjövall, to be published).

It is obvious that pool sizes, turnover times and metabolic conversions *in vivo* may be studied with the aid of steroids labelled with stable isotopes. A special method, the twin ion technique [41], may be particularly useful in studies of the metabolism of steroidal drugs. This method involves the administration of unlabelled and labelled steroid mixed in such a proportion that fragment ions in the mass spectrum of the mixture appear as twins of about equal intensity. If the label is in a sufficiently chemically and metabolically inert position, metabolites of the steroid can be detected by the typical twin ions in their mass spectra.

A related method is being used in this laboratory for studies of the turnover and metabolism of endogenous steroids. The steroid to be investigated is labelled with two deuterium atoms at a position remote from sites of expected biological attack. These atoms serve as markers for the steroid skeleton and make it possible to detect metabolites by mass spectrometry, and to determine turnover time of the steroid skeleton. When, in addition a further deuterium atom is introduced at each centre bearing a hydroxyl group a means is provided whereby the extent of oxidoreduction at these sites may be simultaneously determined. When testing this method in a study of the metabolism of 3β-hydroxy-5α-pregnan-20-one sulphate in male rats, the probable metabolic sequence outlined in Fig. 5 could be suggested [42]. Investigations of turnover and metabolism of steroid sulphates in pregnant women using this technique are presently being carried out.

Acknowledgements—Part of this work was supported by the Swedish Medical Research Council (grant No. 13X-219) and by the World Health Organization.

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