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Review

Clinical applications of gas chromatography and gas chromatography–mass spectrometry of steroids

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Abstract

This review article underlines the importance of gas chromatography–mass spectrometry (GC–MS) for determination of steroids in man. The use of steroids labelled with stable isotopes as internal standard and subsequent analysis by GC–MS yields up to now the only reliable measurement of steroids in serum. Isotope dilution GC–MS is the reference method for evaluation of routine analysis of serum steroid hormones. GC–MS is an important tool for detection of steroid hormone doping and combined with a combustion furnace and an isotope ratio mass spectrometer the misuse of testosterone by athletes can be discovered. Finally the so called urinary steroid profile by GC and GC–MS is the method of choice for detection of steroid metabolites in health and disease.

Keywords: Reviews; Steroids; Hormones

Contents

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1. Introduction

Steroids are lipid compounds with a perhydro-1,2 cyclopentanophenanthrene ring system, which can be classified into six groups according to the number of C-atoms. C_{17} *gonanes*; C_{18} *oestranes*, as estradiol and estrone; C_{19} *androstanes*, as testosterone and androstenedione; C_{21} *pregnanes*, as progesterone and cortisol; C_{24} *cholanes*, as the bile acids cholic acid and desoxycholic acid; and C_{27} *cholestanes*, as cholesterol. The forementioned compounds are natural hormones or precursors with exception of the cholanes. According to their function steroid hormones can be divided into estrogens, androgens, and glucocorticoids and mineralocorticoids. The systematic names of steroids were defined by the IUPAC commission on the Nomenclature of Organic Chemistry and the IUPAC–IUB Commission on Biochemical Nomenclature, as published in 1969 and definitively in 1972 [1,2].

Since the introduction of gas–liquid chromatography (GC) by James and Martin in 1952 [3] it took eight years before the first analysis of apolar steroids by GC was described by Vanden Heuvel et al. [4]. Using the technique of derivatizing the (hydr)oxy groups of the steroid molecules, polar steroids could also be separated and determined by GC. For this purpose oxo groups were methoximated, followed by silylation of the hydroxy groups [5].

In 1964, Ryhage was the first investigator to use a mass spectrometer as a detector of GC effluents [6]. This paved the way to determine unknown steroids or to confirm the structure of known steroid compounds. Since then the GC system, with or without coupling to mass spectrometry (MS), enabled the analysis of all possible steroids, as described by Thenot and Horning [7] and Sjövall and Axelson [8].

In 1957, Golay developed open tubular glass capillary columns for high-resolution separation of compounds [9], which was another milestone for analysis of a wide range of steroids [10,11], especially since the introduction of the fused silica capillary columns [12].

In this paper the authors will restrict themselves to review the determination by GC of steroids, which originate from the gonads and adrenals in man, i.e. the endogenous compounds as well as the medically applied synthetic compounds, or to their structural analogues, like the anabolic and or androgenic steroids. No attention will be given to other steroids, such as sterols, bile acids and bile alcohols, and vitamin D and its analogues. The main subjects of gas chromatographic analysis of steroids in this manuscript are (1) the use of GC–MS as reference method in the field of routine analysis of steroid hormones, (2) anabolic and androgenic steroids (AASs) and (3) urinary steroid profiling in health and disease. See Section 5 for a list of used trivial names of steroids, abbreviations and corresponding systematic names.

2. The use of isotope dilution GC–MS as reference method for routine analysis of steroid hormones

2.1. Poor standardization of routine steroid hormone analysis requires the use of isotope dilution GC–MS as reference method

Steroid hormone analysis plays an important role in clinical laboratories in the investigation of endocrinological disorders, related to adrenal or gonadal function. Since many years concentrations of functional steroid hormones or their precursors are determined mainly in plasma or serum samples of patients, occasionally in saliva or tissues, or as their metabolites, in urine or amniotic fluid. Steroid hormones assayed in plasma are primarily cortisol, aldosterone, testosterone, estradiol and progesterone, while many laboratories have also facilities for determining 17-hydroxyprogesterone, dehydroepiandrosterone-sulphate and androstenedione. Only a few laboratories perform analyses of corticosterone, 11-deoxycortisol, cortisone, 18-hydroxycorticosterone et cetera. The method of choice in routine analysis of steroid hormones in serum is immunoassay. While in the seventh and eighth decade radioimmunoassay (RIA) dominated the market of steroid hormone analysis, nowadays the majority of laboratories apply immunoassays making use of non-radioactive-labelled material. For this purpose a choice of automated immunoassay analyzers with appropriate reagent kits for various steroids is available, enabling every laboratory to perform analyses easily with reasonable costs.

Although the daily workload can thus be handled very conveniently, the quality of the analytical results in terms of precision and accuracy is not guaranteed and should be regularly monitored. The precision of a test can easily be checked within the laboratory by so called internal quality control assessment, in which one or more serum pools are repeatedly assayed on one particular day and the standard deviations of the means are determined (called within-day precision or repeatability). Comparison with quality control runs on other days similarly yields the between-day precision or reproducibility. Accuracy of a test – the ability to produce the true result – could in principle be investigated by analyzing standard solutions of known concentrations of the steroid of interest, but as a result of so called matrix effects, caused by the presence of various components in the analyzed serum, correct values obtained for standards do not ensure correct values for analyses in native serum. This is a general problem in clinical chemistry for all kinds of assays apart from immunoassays, and relates to specificity and robustness – the ability to measure the true value of the analyte concentration irrespective of the composition of the matrix under varying circumstances. Immunoassays suffer more or less from cross reactivity – i.e. substances closely related to the analyte may also interact with the antibody molecules due to lack of their specificity in binding and thus influence the result. Moreover other components, not having any structural resemblance with the analyte, can disturb the measuring principle.

Of course all manufacturers of immunoassay analyzers aim at a high performance of all their available immunoassay tests, but in practice – certainly for steroid hormone assays – this is still not the case. This becomes overt when results of external quality assessment (EQA) schemes for steroid hormones are contemplated. In such quality control schemes a national or international organisation sends serum samples to the participating laboratories, which then determine steroid hormone concentrations, each laboratory using its own routine method. The obtained results are collected, evaluated and reported to the participants. As an example we put forward a recent paper of Middle [13], describing results obtained in the UK National External Quality Assessment Scheme (UK NEQAS) for estradiol for two different pool serum samples of low and high concentration, dispatched on January 1997. Fourteen different assay techniques, employed by more than 150 participants, were practised. The All-Laboratory Trimmed Mean (ALTM) for a low pool was 167 pmol/l, values ranging from 73 to 347 pmol/l. The mean values calculated for the analytical methods ranged from 106 to 254 pmol/l. For a high pool the ALTM was 603 pmol/l, results ranging from 340 to 1001 pmol/l and method means from 475 to 799 pmol/l. Several reports involving also other steroids (e.g. [14–16]) clearly demonstrate the poor standardization of steroid hormone immunoassays. It is not justified to assume that ALTM values in EQA schemes represent the true values, as the bias of none of the analytical methods is exactly known.

Therefore reference methods have to be available to judge the accuracy of immunoassay methods. A reference method can be defined as one which, after exhaustive investigation, has been shown to have negligible inaccuracy in comparison with its imprecision [17]. The application of reference methods makes it possible to establish target values in samples used in EQA schemes, so that the performance of current routine methods can be judged. Manufacturers of diagnostic kits can make use of them to validate their products.

Isotope dilution gas chromatography–mass spectrometry (GC–ID-MS) has proven to be a technique which fulfills the criteria that have to be imposed on reference methods for a number of substances, including steroid hormones. The methodological aspects of this technique are outlined in the IFCC document 1985/2 by Lawson et al. [18]. An extensive review, outlining the relevant backgrounds and applications of isotope dilution mass spectrometry in clinical chemistry, was presented by De Leenheer et al. [19]. The compound of interest in a sample is determined by adding a known amount of the same compound, labelled with one or more, preferably stable, isotopes, to a certain (known) volume of the sample. This isotopically labelled compound serves as internal standard. After equilibration, subsequently extraction, purification and derivatization steps are carried out, before injecting the sample into a GC–MS combination. During this cleaning-up process the ratio between the labelled and unlabelled molecules remains essentially the same, because of their practi-

cally identical physical and chemical properties. The GC–MS combination then enables to measure both fractions on the basis of their difference in molecular mass. Along the gas chromatographic separation the two fractions remain together, entering the ion source of the mass spectrometer in purified form. After ionization characteristic ion fragments temporarily arise as Gaussian chromatographic peaks, which can subsequently be measured by the MS detector. Mass fragmentography is applied, in which the mass spectrometer is programmed to measure (quasi) simultaneously the abundance of two characteristic fragment ions, one originating from the labelled and the other from the unlabelled compound. During the GC–MS run thus two peaks emerge. Occasionally four fragments are selected, two from the unlabelled and two from the labelled species. The ratio between the heights or areas of the peaks, which originate from the mass fragments of unlabelled and labelled compound, enables one to calculate the concentration of the unlabelled compound by comparison with a calibration curve of standard mixtures of unlabelled and labelled material. Mass fragmentography is not only a very sensitive technique (detection limits in the order of the picogram range) but also very specific as it (after a suitable work-up procedure) combines gas chromatographic separation with measurement of ions, which are characteristic for the measured compound, while the use of the labelled analogue as internal standard permits the calculation of accurate results, because no correction for losses during work-up is necessary. GC–ID-MS is not suitable as routine procedure because of the time consuming analytical process, but owing to its analytical merits very useful for the development of reference methods.

2.2. Isotope dilution GC–MS used as reference method in steroid hormone analysis: necessary conditions

In the GC–ID-MS technique applied for reference methodology concerning steroid analysis in serum, five subsequent steps can be distinguished, which all influence the total quality of the end result. These include (1) choice of standard and internal standard, (2) serum extraction, (3) further purification, (4) derivatization, (5) GC–MS analysis and calculation of the result, and will be critically discussed in the following part.

Step 1: Use of standard and internal standard. In order to obtain accurate values for the steroid concentration, the used standard should be as pure as possible (near 100%) and anyhow the amount of steroid present in a certain quantity of the standard material should be exactly known. For this purpose certified reference material is often applied throughout many years, as e.g. in the case of cortisol, where standard reference material SRM No. 921, certified by the National Institute for Standards and Technology (Gaithersburg, MD, USA), containing $98.9 \pm 0.2\%$ cortisol is available [20–23]. The choice of the internal standard, the labelled counterpart of the steroid to be analyzed, is of key importance. During the analytical procedure, its identity should be fully preserved, which means that not any of its isotopic labels is permitted to exchange for some part. In the first reports mostly a ^{14}C-label was employed (located in the steroid skeleton), which has the advantage, that no label exchange occurs, but the disadvantage of being radioactive. Another disadvantage of ^{14}C-labelled steroids is the general inclusion of appreciable amounts of unlabelled material. For instance Björkhem et al. [24] mentioned a composition of 89% actually ^{14}C-labelled testosterone and 11% unlabelled testosterone for the internal standard used in their investigation. Such high amounts of unlabelled material present in the internal standard impede high analytical precision, as will be discussed later. Both arguments more or less apply also to the use of ^{3}H-labelled steroids. Therefore, at present most workers employ stable isotopically labelled steroids, in which some (at least two) hydrogen atoms are replaced for deuterium ones at exchange free positions or in which (at least two) ^{12}C atoms are replaced for ^{13}C. Generally, besides being devoid of radioactivity, these internal standards contain only a few percent unlabelled steroid.

Of crucial importance is the exactness in mixing the unlabelled with labelled steroid, as this eventually determines the accuracy and precision of the end result. The used analytical balance and pipetting devices (necessary for preparing the standard calibration mixtures and combining serum and internal standard solution) should be carefully calibrated and

their tolerances known. In all relevant papers much attention has been paid to this subject. In the hands of experienced personnel this stage in the analytical procedure can be carried out in such a way that it contributes no more than 0.1–0.2% to the total error in the final result.

The internal standard should be added as first analytical step, mostly as an alcoholic solution; it is important that good equilibrium is reached before extraction and that the amount of alcoholic solution added does not result in precipitation of serum proteins.

Step 2: Extraction. After equilibration, serum extraction with e.g. dichloromethane or hexane has not necessarily to be quantitative, as the extraction process does not affect the ratio of unlabelled and labelled steroid. However, whether or not at the time of extraction full equilibrium is already attained cannot be checked. In practice, no difficulties have ever been mentioned in the literature as to this point. In some publications instead of extraction immunoadsorption as second step has been applied, e.g. for cortisol [22], progesterone [25] and estradiol [26,27]. In this procedure, serum is passed through a column filled with cellulose-like material (e.g. Sepharose), to which antibody raised against the steroid to be analyzed has been coupled. This steroid is then selectively adsorbed to the column via the antibody and after washing with water eluted from the column by addition of methanol. As no further purification is needed the eluate can be directly derivatized (step 4). The antibody material can be regenerated and used successfully again several times. Nevertheless, after the mentioned publications (the latest at 1988) this procedure has not been applied anymore.

Step 3: Clean-up of the extract. Mostly the serum extract requires further clean-up. Concerning the determination of cortisol in some papers (e.g. [22,28,29]) this step was not included, whereas in other reports (e.g. [20,23]) it was. Interestingly, in those laboratories where step 3 was included as well as omitted (e.g. [21,22]), no differences were found, indicating that for cortisol step 3 is superfluous indeed. A multitude of procedures is available. In the early work of Björkhem et al. [24,30] purification by means of thin layer chromatography was used, which later on was never repeated by others. Still very popular to day is Sephadex LH-20 or occasionally Lipidex-5000 or 1000 gel chromatography, first introduced by Siekmann [31]. In short, this procedure runs as follows. After evaporation of the solvent the residue of the serum extract is redissolved in another solvent (mixture) as eluent, next applied to a glass column (usually 30×0.4 cm) filled with the equilibrated gel, finally eluted with further solvent and the small fraction, known to contain the wanted steroid, is isolated. Patterson et al. [21] for the first time used reversed-phase HPLC, in which the residue, dissolved in HPLC eluent, is injected and the portion of the eluate, containing the wanted steroid, is isolated by fraction collection. More examples are described by Dikkeschei et al. [16] for cortisol, estradiol, testosterone, progesterone and Thienpont et al. for estradiol [27]. Finally Thienpont et al. [32] used cyclodextrins for purifying serum extracts in the GC–MS determination of progesterone and testosterone. The procedure consists of shaking the extract with a solution of hydroxypropyl-β-cyclodextrin in water resulting in back-extraction of the steroids in aqueous solution. After washing with hexane, progesterone is extracted from the cyclodextrin solution with toluene and testosterone with dichloromethane.

Sometimes more than a one single prepurification step, in particular for those steroids present in very low concentration, has been reported as e.g. for aldosterone by Siekmann et al. [33], who used both Sephadex LH-20 and Lipidex 5000 and by Stöckl et al. [34], who used Sephadex LH-20 combined with HPLC fractionation.

The use of Sepharose-coupled antibodies as prepurification step has already been dealt with previously.

Step 4: Derivatization. A derivatization reagent should be chosen which produces in high yield only one (if possible) derivative, stable up to the moment of analysis and with good mass spectrometric properties. The last property includes a number of features. First of all, in the MS ionization mode chosen, one or more fragment ions in the mass spectrum should be present with m/z values at least greater than 400 and with an abundance enabling precise mass fragmentographic measurement in the

lower picogram range. Secondly, after selection of a pair of fragments for steroid and internal standard, best results in terms of accuracy and precision are obtained (see below: step 5) when the unlabelled steroid does not considerably contribute to the mass fragment chosen for the labelled steroid (two or three mass units higher than that of the unlabelled steroid), as a result of the natural isotopic abundance of the derivative. This contribution can never be avoided completely because of the presence of 1.1% ^{13}C in natural carbon along with the number of carbon atoms present in the measured steroid derivatives (more than twenty). It can be calculated and actually visualized by performing mass fragmentography of pure unlabelled steroid, that at the fragment two mass units higher than the main one with ^{13}C-atoms only the unlabelled steroid still demonstrates an intensity in the order of 4%. Silylated derivatives of steroids have to avoided as much as possible, due to the fairly high abundance of ^{29}Si and ^{30}Si in natural silicon (composed of 92.2% ^{28}Si, 4.7% ^{29}Si and 3.1% ^{30}Si), unless an Si-free fragment is chosen. For a long time only the methoxime-trimethylsilyl, $(MO)_2$-$(TMS)_3$, derivative was the best available substance for mass fragmentographic analysis of cortisol [16,20–22,28,29]. However, it can be calculated that relative to the main intensity (m/z 605) the fragment $(M-31)^+$ of cortisol contributes 23.6% and 7.08% to m/z 607 and m/z 608, respectively. Recently it appeared possible to employ a fluoroacyl derivative with better properties than the former one [23] and with only contribution of ^{13}C to the isotopic variation. In fact, up to now most used derivatives are fluoroacyl derivatives, prepared by reaction with trifluoroacetic anhydride (TFA) as e.g. for estriol [35], or pentafluoropropionic anhydride (PFPA), as e.g. for testosterone [36] or heptafluorobutyric anhydride (HFBA, giving HFB derivatives), as e.g. for progesterone [16,25,30,32], testosterone [16,31,32,37], estradiol [16,26,27,35,38], cortisol [23] and aldosterone [33,34]. However as for estradiol, Dehennin [39] advocates the use of a mixed TMS-HFB derivative because of higher stability with respect to the di-HFB derivative and thus higher sensitivity during MS-measurement. Using trideuterated estradiol as internal standard a precision with a coefficient of variation of 1.71% was achieved.

Step 5: Mass fragmentography and calculation of the result. Provided that the formerly mentioned conditions (see steps 1–4) are fulfilled, the GC–MS measurement itself has to answer rigorous conditions in order to be considered as reference work with a total deviation from the true value less than 2%. A detailed discussion as to this aspect of the technique can be found in the review paper of De Leenheer et al. [19]. The first part of the GC–MS analysis is the gas chromatographic separation. The GC system should not show a so-called memory ('ghosting') effect, a very treacherous aspect sometimes occurring and to be ascribed to shortcomings of the injection port, the GC column or the interface between the GC and MS, resulting in 'carry-over'. This can be checked by repeated injection of different standard mixtures of unlabelled and labelled material and blanks, followed by seeing whether or not significantly different results for the same samples are observed. This problem was more serious in the early period of GC–ID-MS work, where packed columns were employed and in order to split off some of the carrier gas separation devices between GC and MS were necessary. As nowadays fused silica capillary columns (see Table 1) directly coupled to the ion source mostly combined with splitless injection are used, many shortcomings formerly encountered have disappeared, but one should nevertheless keep in mind possible memory effects. Further improvements refer to better performance of the GC–MS instrumentation in terms of sensitivity, reproducibility, accuracy and data handling. Whereas before 1980 only expensive magnet sector MS instrumentation could provide the necessary conditions, later on cheaper quadrupole MS instrumentation could perform adequately as well. In the majority of cases electron impact (EI), but occasionally also positive (CI) or negative chemical ionization (NCI), is used as ionization technique.

The reproducibility of the result is dependent on the amount of analyte (i.e. steroid and internal standard) injected and the sensitivity of the MS instrument and in order to obtain results with a reproducibility of less than 0.5%, one should inject an amount of analyte giving a signal-to-noise ratio >200. These aspects should be sorted out before actual reference work can be started. The quality of

Table 1
Chemically bound stationary phase of the capillary column in the GC and GC–MS applications

Manufacturer's code	% Diphenyl	Polarity	Ref.
CP-Sil 5CB[a]	0	nonpolar	
HP-1, Ultra 1[b]			[75,91,98,99]
DB-1[c]			[72,73,85]
SPB-1[d]			
OV1[e]			[75]
CP-Sil 8CB	5	nonpolar	
HP-5			[72,74]
DB-5			[73]
SPB-5			
BPX-5[e]			[73,97]
XLB (DB-12)[c]	12	low polar	[73]
BPX-35	35	intermediate	[73]
DB-17	50	intermediate	[84,85]

[a–e] The lengths of the used columns range from 17 to 30 m, the diameters from 0.2 to 0.32 mm while the film thicknesses range from 0.1 to 0.33 μm. The stationary phases consist mostly of polydimethylsiloxane with 0–50% diphenyl groups. (a) Chrompack; (b) Hewlett Packard; (c) J & W Scientific; (d) Supelco; (e) SGE.

the measurement can be judged from the peak shapes of the monitored mass fragments (horizontal base-lines, no peak tailing, and no visible base-line noise) and can be proven by reproducibility studies. Mostly peak area ratios of unlabelled and labelled material are measured, although measurement of the peak height ratio can also be sufficiently accurate.

Finally, a very important aspect is the lower and upper limit of the measured ratio of unlabelled and labelled steroid. This has been extensively reviewed by De Leenheer et al. who fully discussed the mathematical details [19]. In short, this subject is related to the already mentioned fact that the unlabelled steroid contributes to the signal of the labelled steroid and vice versa. When this mutual overlap would be absent and different mixtures of unlabelled and labelled steroid would be measured we would find a straight calibration line $y=x$. In reality however, the calibration line shows a minimal value (intercept with y-axis) equal to the measured ratio of the two isotopically different fragments in the pure internal standard and is curved, with a maximum y value equal to the similarly measured ratio of the two different fragments in the pure unlabelled steroid. Striking examples of curved calibration lines can be found in the work of Björkhem et al. [24,28,30,40] and although amendments for overlap can be applied resulting in 'corrected' straight calibration lines, the adherent problems related to this fact remain present, as has been acknowledged in a later review paper [41]. Namely, in mixtures with a low relative amount of unlabelled steroid a result will be calculated with relatively high error, owing to the small difference in measured ratio between this sample and that of the pure internal standard in combination with a relatively high signal to noise ratio of the lower mass peak. On the other hand, mixtures with a high relative amount of unlabelled steroid cannot be measured with acceptable precision, because a relatively high increment in the amount of unlabelled steroid results in a relatively small increment in measured ratio. Therefore, depending on the actual concentration of the steroid in serum, variable analytical precision is obtained, optimal at 'intermediate' concentrations and minimal at very 'low' or 'high' concentrations. This is the reason why in ID-MS reference methods the so called bracketing method, in combination with a mutual mass overlap of only a few percent, is applied by all workers, aiming at the smallest possible error. As calibration points, this method works with only three standard mixtures of unlabelled and labelled material (with always the same amount of labelled material and varying amounts of unlabelled material), yielding measured peak area ratios of about 0.8, 1.0 and 1.2 (measured with high precision). The peak area ratio, obtained for the steroid present in serum samples, should fall within this region, which is realized by a previous 'rough' measurement of the steroid concentration by e.g. immunoassay or by GC–ID-MS using a broader range of calibration mixtures. The volume of serum, containing an amount of steroid in between the lowest and highest 'bracketing standard' and to which also exactly the same amount of internal standard should be added, can then be calculated for each individual sample and be combined with internal standard by pipetting. From the measured peak area ratio the precise steroid concentration can be definitely established by linear interpolation on the basis of the peak area ratios obtained for the calibration mixtures.

2.3. Development of analytical methods and validation of the accuracy of isotope dilution GC–MS in steroid hormone analysis

The applicability of ID-MS as reference methodology was already recognized, when RIA was just starting to become of significance in the field of steroid hormone analysis. In Germany at the University in Bonn, Siekmann et al. [42] published in 1970 the first report of a GC–ID-MS method for estrone in plasma of pregnant women, soon followed by aldosterone [33]. In 1979 Siekmann [31] had developed GC–ID-MS methods for estradiol, estriol, testosterone, progesterone, aldosterone and cortisol. As internal standards 4-^{14}C-labelled analogues were applied, of which fixed amounts dissolved in ethanol were added; after equilibration, serum samples were extracted with dichloromethane and for further purification subjected to Sephadex LH-20 chromatography. (In the cases of estradiol and estriol, before extraction, also naphtol was added as carrier substance to minimize adsorption of these steroids to the test tubes). Estradiol, testosterone and progesterone were converted into HFB esters. Estriol was derivatized to its tripentafluoropropionic (PFP) ester. Cortisol was reacted first with methoxamine–HCl, followed by trimethylbromosilane to give the 3,20-dimethoxime,11β,17,21-tri-trimethylsilyl ether (MO-TMS derivative). For aldosterone, a more elaborate analytical procedure was followed: after extraction and LH-20 chromatography, the eluate was treated overnight with 3.5 mol/l hydrochloric acid to form the 18,21-acetal, followed by column chromatography on Lipidex-5000 and finally derivatization to the 18,21-acetal-3-enol-HFB-ester. Mass fragmentography was carried out on a LKB 9000 GC–MS combination. For cortisol and aldosterone packed GC-columns were employed, for the other steroids 10 m glass capillary columns. Relative standard deviations ranged from 1–3.5%.

Meanwhile in Sweden, Björkhem and coworkers published a series of papers, describing GC–MS determinations of cortisol [28], testosterone [24], (urinary) estriol [40] and progesterone [30].Relative standard deviations ranged from 2.7% to 6.2%. For cortisol, testosterone and progesterone also 4-^{14}C-labelled internal standards were used, for estriol [2,4-$^{2}H_{2}$]estriol. After adding the appropriate internal standard, serum extraction for cortisol took place with dichloromethane, for testosterone with diethylether and for progesterone with hexane. The extracts obtained for progesterone and testosterone were further purified by thin-layer chromatography, whereas the extract obtained for cortisol was no further cleaned up. Progesterone was converted into the 3-enol-HFB-ester and cortisol into the MO-TMS derivative, while testosterone was converted into the 3-enol,17β-di-TMS ether. Urine samples for estriol were subjected to acid hydrolysis (for deconjugation), followed by addition of internal standard, ether extraction and alkaline washing of the extract. After solvent evaporation the tri-trimethylsilyl derivative was prepared. Mass fragmentography was also carried out on a LKB 9000 GC–MS combination using packed columns.

The early work of Siekmann and Björkhem and their coworkers cited above paved the way for actual application of GC–ID-MS as reference methodology in clinical chemistry, not only with regard to steroid hormone analysis, but also to other analytes of interest, such as glucose, creatinine, urea. Later achievements include improvements in methodology, resulting in an eventual accuracy and precision in the range 1–2%.

In spite of the mentioned merits of this technique, the presence of interfering components cannot definitely be excluded and therefore the final results cannot blindly be trusted, unless it has been ‘exhaustively’ investigated that the candidate reference method indeed gives accurate results in various circumstances. Many investigations have addressed this issue. First of all, within one single laboratory, employing GC–MS, one or more of the steps in the analytical process can be varied (e.g. the internal standard, the extraction, the purification, the derivatization, the monitored fragments), followed by investigating whether these changes affect the results observed for the same samples. Furthermore an often used, simple check is the omittance of the internal standard and inspecting whether the observed ratio for a serum sample is exactly the same as that for the standard (unlabelled) steroid, thus proving that at least no interference at the fragment, monitored for the internal standard, is present. Then the consequences of addition of possible interferents to the serum can be studied. Next, recovery experiments,

adding a known amount of steroid to the sample and measuring whether the expected calculated raise in concentration occurs, is also a common feature in most studies. Another simple check consists of monitoring at the same time two mass fragments for both unlabelled and labelled compound and comparing the results calculated from the two found ratios, which should be the same, if interference is indeed absent. It would be rather unlikely that the interfering substance(s) would affect both ratios in the same manner. As an example, Siekmann and Breuer [20] monitored in the determination of plasma cortisol peak area ratios with m/z=605/607 and 636/638 (being respectively the M^+-31 fragments and M^+ fragments of cortisol and ^{14}C-cortisol internal standard of the MO-TMS derivative) and calculated for both ratios the same results. Although these measures are helpful, even more convincing are those studies in which numerous circumstances were varied, as e.g. in the study of Patterson et al. [21], who carried out GC–MS determinations of cortisol in a lyophilized, fortified (with cortisol) pool serum in five different ways, using [9,12,12-$^2H_3$]cortisol as internal standard and MO-TMS derivatization. Different ion ratios were measured (*m/z* 605/608 and *m/z* 636/639 in the EI mode and *m/z* 459/462 in the NCI mode) in combination with dichloromethane extraction. Then, instead of solvent extraction, cortisol was also isolated by means of solid-phase extraction (using reversed-phase C_8-cartridges) and as a last method solvent extraction was followed by reversed-phase HPLC fractionation. The means of the obtained analytical results for these various methods were not significantly different and the imprecisions less than 1%. Finally it was investigated whether mass fragments of cortisol degradation products could have effects, which appeared not to be the case. Gaskell et al. [22] employed three different analytical procedures for plasma cortisol: solvent extraction, immunoadsorption and extraction on Lipidex 1000 and found excellent agreement between them. Thienpont et al. [23] established that twenty-four structurally related steroids did not show any interference in their plasma cortisol assay, in which HFBA was the derivatization agent. In the determination of estradiol in plasma Dehennin [39] used differently labelled internal standards and various derivatives (TMS, HFB and mixed TMS-HFB), and noted no significant differences in a single pool serum. Likewise in this connection, Thienpont et al. found only small differences by comparing HPLC and immunoaffinity chromatography as purification method for estradiol [27]. Secondly, most convincing evidence for the accuracy of a test is the finding that analysis of the same samples in different laboratories (each with their own procedure) produces statistically the same results. Also here, numerous examples can be given. Gaskell and Siekmann [43], working in Cardiff and Bonn respectively, exchanged a number of different pool sera for determining cortisol by means of GC–ID-MS. In spite of differences in methodology the measured concentrations in Bonn and Cardiff were virtually the same (differences less than 4%, except in two cases equal to 7%, which in comparison with routine immunoassay methods is quite acceptable). Another striking example is the work described by Thienpont et al. [44] in 1991, financed by the European Union. Here, the Community Bureau of Reference (BCR), recently called Standards, Measurements and Testing Department of the European Union (S, M & T) is existent with the task to achieve standardization in Europe. In the above mentioned example, on instruction of BCR four pool sera were composed, three natural ones and one fortified with cortisol, which were subsequently lyophilized. Then a number of European laboratories, located in Belgium, the Netherlands, Germany, France, the UK and Italy with a certain reputation in the field of GC–ID-MS analysis of steroid hormones, were asked to determine the concentration of cortisol in two of these pools (later on designated as CRM 192 and 193) and that of progesterone in the other two (CRM 347 and 348) pools (in reality some laboratories did both, whereas some only participated in the cortisol project and others only in that of progesterone). Once determined, these lyophilized pools could serve as Certified Reference Material (CRM), i.e. material with known concentration of a certain substance, validated by an accompanying certificate and to be used to validate routine methods or as control material for reference methods. In summary, discrepancies between laboratories ranged from only 1.48% to 2.16%. The calculated uncertainties at the 95% confidence interval (for CRM 192, 193: 272.7±5.5 nmol/l and 763.4±13.6 nmol/l cortisol, respectively, and for CRM 347, 348:

10.13±0.21 nmol/l and 40.3±1.0 nmol/l progesterone, respectively) were considered compatible with the intended use as reference material. Some other examples of inter-laboratory comparison of GC–ID-MS comparison are described in papers by Thienpont et al. [45] for aldosterone, cortisol, progesterone, estradiol and testosterone with mean relative standard deviations always <2%, by Thienpont et al. [23] for cortisol with a total error less than 2.05%, and by Stöckl et al. [34] for aldosterone (total error about 2%). In these last three studies two different laboratories were involved, one located in Ghent, Belgium and the other in Düsseldorf, Germany.

2.4. Application of isotope dilution GC–MS methods for steroids in the evaluation of routine methods

One of the first reports in this field came from Björkhem et al. [46], in which the determination of cortisol (and other compounds) in a frozen pool serum was described, both by GC–MS and routine methods in Sweden. As for cortisol, 18 laboratories participated, performing different routine methods like RIA or fluorometry. Whereas the mean GC–MS value was found to be 436 nmol/l, the overall mean value for routine methods amounted to 563 nmol/l, obviously too high. In 1983 Röhle et al. [15] reported results of external quality control schemes ('Ringversuche') for steroid hormone determinations, mainly executed in German clinical laboratories and organized by the 'Deutsche Gesellschaft fur Klinische Chemie' (German Society for Clinical Chemistry) during 1977–1981, including aldosterone, cortisol, estradiol, estriol, progesterone and testosterone. In this period 14 times two serum or plasma samples (each time different) were distributed, after having determined GC–ID-MS reference values. In the first two years only 'stripped' plasma samples (i.e pool sera, which were first treated with charcoal to remove all endogenous steroids, subsequently again added in known amounts) were taken, later on mainly native serum or plasma pools. Large differences in results were found, ranging from 20% for cortisol to 57% for estradiol. Whereas for cortisol and aldosterone in stripped samples mean values near the GC–MS reference values were observed, in native samples too high values were measured, with differences ranging from over 100% for aldosterone to approximately 20% for cortisol. Finally routine methods for estradiol generally scored too high for all samples, stripped or not, while for the remaining steroids (progesterone, testosterone and estriol) means corresponding to the reference values were observed. In the already quoted review paper of Middle [13], describing results of serum estradiol assays obtained in UK National External Quality Assessment Schemes (UK NEQUAS), the samples were also analyzed by GC–ID-MS and the obtained target values compared with the all-laboratories trimmed means (ALTM). Although good correlation between the reference method and the overall mean of routine methods was observed, the slope of the linear regression line was less than one, with a positive intercept. Indeed, a difference plot, in which the observed differences (ALTM minus GC–MS) were plotted as a function of the corresponding averaged value of ALTM and GC–MS, revealed that at lower estradiol concentrations the routine methods yielded too high results, whereas at higher concentrations too low values were scored. This effect was even more pronounced for manipulated sera, namely those with low endogenous concentration enriched with additional estradiol (called 'recovery samples'). Furthermore, mean values, obtained for each of the used routine methods, were compared with GC–MS values, most of them showing slope and intercept significantly deviating from the ideal values of one and zero respectively. This was the case for both natural as for recovery samples. Dikkeschei et al. [16] compared results obtained with routine methods in the Wellcome Quality Control Program (an international, commercial external quality assessment program of the Wellcome Foundation) during a certain period with GC–MS results, concerning cortisol, estradiol, testosterone and progesterone. Also these authors concluded that the observed range of immunoassay results was disturbingly high. Compared with GC–MS target values the overall means of the routine methods mostly disagreed. Practically all routine methods yielded too high values for cortisol, resulting in a too high overall mean. Gaskell et al. [22] provided GC–MS target values for plasma cortisol in the UK NEQUAS during the period 1981–1982, in which routine methods based on fluorometry, competitive protein

binding and radioimmunoassay were practised. In contrast to GC–MS, routine methods demonstrated large fluctuations and practically all exhibited a positive bias. When compared by regression analysis, a linear relationship between GC–MS and most routine methods was observed, with positive intercepts and slopes close to unity. In a 'stripped' sample supplemented with cortisol however, the fluorometric method produced an accurate result, whereas the immuno- and competitive protein binding assays yielded too low values, which fact gave rise to the remark that assessment of routine steroid assays must be primarily based on the use of samples with a fully intact matrix. Gaskell et al. [25] compared radioimmunoassays for progesterone with GC–ID-MS. In some of the used RIA methods sera were directly analyzed, whereas in others sera first underwent a solvent extraction process as purification before actual analysis. All RIA methods showed a positive bias, which was however greater for the 'direct' methods. A good correlation between immunoassays and GC–MS was observed. Gosling et al. [14] reported on standardization of immunoassays for serum cortisol, presenting experiences within the UK NEQUAS (in 1992) and German external quality control schemes during 1985–1992. As usual, in the latter program GC–MS was incorporated. In 1985 the overall mean values of routine methods were about 15% higher than the corresponding GC–MS values, gradually decreasing to about 10% higher in 1992. The reported immunoassay values persisted in showing a tolerance range of about 30% throughout the years, but most laboratories managed to report values well within the upper tolerance limits (GC–MS target values ±33%). In the UK program comparable variances were found; in addition it was observed that in native samples to which exogenous cortisol was added, recoveries were generally less than 100%. De Brabandere et al. [47] compared three immunoassay kits for cortisol with a GC–MS reference method (similar to the method described in [23]), using a panel of 15 patient sera covering a representative (wide) range of cortisol concentrations. Here, the best immunoassay kit showed a mean deviation of only +9.7% from the GC–MS target values, varying from +2.3% in the midnormal to pathological range (370–1600 nmol/l) to +21% in the range below 370 nmol/l. However, the second and third kit showed an overall mean positive bias of 47 and 49% respectively, while in the low concentration range mean deviations of even 83% and 91% respectively were seen. The authors concluded from this fairly recent study that 'at present, a lack of accuracy and compatibility of the investigated cortisol methods also exists with patients specimens'. Stöckl et al. [48] confirmed this finding by showing that 4 different immunoassay kits, applied in the analysis of cortisol in four single-donation frozen sera with known GC–MS target values, yielded values deviating 20–30% from the target values. Fitzgerald et al. [49] developed a GC–MS method for serum testosterone and compared it with an immunoassay kit for testosterone. For male sera (covering the clinically significant range of 0–40 nmol/l), sufficient correlation between immunoassay (y) and GC–MS (x) was observed ($y=1.07x+0.19$ nmol/l; $r^2=0.98$). However, for female sera, covering the clinically significant range of 0–4 nmol/l, the correlation was very bad ($y=0.72x+1.2$ nmol/l; $r^2=0.31$). This investigation clearly showed that this immunoassay method was excellent for investigating male serum testosterone concentrations, but more or less failed in the analysis of female sera.

2.5. Future developments: implementation of reference methods in steroid hormone analysis, leading to international standardization of routine assay methods

At present GC–ID-MS reference methods exist for commonly assayed steroids (cortisol, aldosterone, estradiol, testosterone and progesterone) in plasma/serum with an inaccuracy no more than 2% [45]. Looking at EQA schemes, carried out in several countries during the past twenty years, it is still obvious that immunoassay methods produce overall mean values, which may be significantly different from the true values as determined by reference methods. In most EQA schemes the true values of the samples are unknown, because no GC–ID-MS measurements have been performed at all and it is then wrongly assumed that the overall mean values approximate the true values. At the moment only in the EQA scheme, organized by the German Society for Clinical Chemistry, samples are also analyzed by

GC–ID-MS and the results from the participants judged by comparison with the reference values, whereas in the majority of other EQA programs scores are derived from comparison with laboratories using the same assay method. The German EQA program bears another aspect, namely 'proficiency testing', which means that it can be considered as a kind of examination, i.e.when a laboratory regularly fails to produce satisfactory results, it may lose the licence entitling payment for its services by the health authorities or insurance companies. These two aspects, quality control based on comparison with reference methods and proficiency testing, explain why in Germany the results of EQA programs, although capable of improvement, contrast favourably with those obtained elsewhere.

This situation is highly unsatisfactory, as from these results medical diagnoses and treatments have to be derived. Medical decision making is impeded due to often inaccurate measurements of immunoassay analyzers. Evaluations of EQA schemes, like done by Middle [13] and Gosling et al. [14] revealed that biases from the true value often are dependent on the measured level, e.g. positive in the low level range and negative at the high level range. Apparently up to date most of the currently used routine methods were not thoroughly tested by manufacturers by means of comparison with reference methods, before bringing them on the market. There are several reasons for this distressing fact. First of all in practically no country governmental rules have yet been issued requiring that manufacturers should deliver instrumentation and accessory reagents for clinical assays, which should produce accurate results. Stöckl et al. [48] enumerate necessary requirements for 'accuracy-based' analytical methods: (1) the availability of reference methods and certified reference materials (CRMs), (2) calibration and validation of routine methods by reference methods and (3) availability of internal and external quality control materials with reference target values. This package of tools enables results of test kits to become traceable to true values and to no longer differ from each other. At present the above mentioned conditions for improving routine methods for steroid analysis are more or less available, but not yet implemented. Industry argues that validation of routine methods by GC–ID-MS is very expensive (leading to increased costs of test kits) and moreover needs official approval by national and/or international authorities. Thienpont et al. [50] appreciate these arguments, and propose that validation of routine methods by GC–ID-MS should be mandatory for various countries. This is more or less in line with the forthcoming 'Directive for in vitro medical devices' from the Commission of the European Union, requiring that results of routine methods, performed in the member states, should be traceable to the true value. In order to reach this goal, in the first place in Europe but if possible also world wide, a number of objectives have yet to be defined, such as outlined in papers published by members of International Working Groups [51–54]. Siekmann et al. [51] and Thienpont et al. [52] advocate the establishment of Networks of European Reference Laboratories (NERLs) for a number of clinical chemical analytes for which reference methods are available. Each network would comprise a number of laboratories, carrying out reference methods with regard to a certain class of substances of clinical chemical interest, such as steroid hormones, electrolytes or organic substances classified as 'substrates' (e.g. glucose, cholesterol). One of the networks would be the NESRL: Network of European Steroid Reference Laboratories, under the coordination of Thienpont [13,51]. The existing Standards, Measurements and Testing (S, M & T) Department of the European Union could provide the basis for such networks [51]. Alternatively they could be institutionalized by establishment of the 'European Reference System for the Medical Laboratory' (ERSML), in which body industry, medical societies and national/European governments should participate [52]. The tasks of the networks would be fourfold: (1) certification of reference materials (CRMs), (2) target setting in EQA schemes, (3) validation of diagnostic kits, including their calibration procedures, (4) providing assistance as to specific problems encountered in individual laboratories. Candidate laboratories for setting-up NERLs would be European laboratories with expertise in the field of reference methods, selected on the basis of stringent criteria, outlined in a number of papers [52,53,55]. The required accuracy and precision of a diagnostic test is related to the within-subject variation and the between-subject variation, which for some parame-

ters (e.g. serum sodium) are very small but for others (e.g. serum cortisol) much greater and in turn permit one to discriminate between a normal and diseased state. For instance it can be derived that in order to establish correct performance and thus clinical effectiveness, it is desirable that serum sodium results in EQA schemes do not deviate more than 0.9% from the true value, whereas for cortisol in pooled sera this figure is 28%. In order that in EQA schemes no participants are unjustly disqualified because their results are out of the expected range it can be statistically shown that reference methods should have a maximal tolerable error of at least one fifth of the maximally tolerated error of routine methods [52–54]. In practice this means that reference methods should demonstrate a maximal allowable error (from the true value) of no more than approximately 3.5%, which is, as has been reviewed elsewhere in this paper, within reach of qualified reference laboratories. For testing existing or newly developed routine methods panels of pure (i.e. non-manipulated, as e.g. 'stripped') single donation patient samples covering a wide range of low, normal and high values of the different steroid hormones should be available, of which reference values have been determined. An example of such an approach was recently given by Thienpont and De Leenheer [56] for estradiol. Finally the services rendered by reference networks should be incorporated within EQA schemes, for which guidelines and objectives have been set out [54].

If standardization of steroid hormone analysis has been accomplished, the performance of such assays will considerably improve and thus its use in health care. Let us hope that the words of the title of Thienpont's recent paper [57] 'Standardization of steroid hormone assays – in theory an easy task' will be prophetic.

2.6. Isotope dilution GC–MS methods for less commonly determined steroids or in special circumstances

Apart from the already discussed steroid hormones (cortisol, aldosterone, testosterone, progesterone, estradiol, estriol) and their respective GC–ID-MS reference methods, in the literature GC–ID-MS methods for more steroids of clinical interest, of which mostly immunoassay methods are available, are described. Whereas in past years much knowledge has been gathered in judging accuracy and precision of the more common tests, for less often determined steroids performance of the employed methods, especially immunoassays, is still scarcely mapped and most of these steroids do not form part of EQA programs. Here, GC–ID-MS has to play a more important role in establishing accurate levels of those steroids in all kinds of relevant clinical situations. It is even imaginable that in certain circumstances immunoassay methods can not sufficiently be improved and that in such cases GC–MS remains the only method ensuring accurate results. A special field of interest is the determination of steroids, including the more commonly determined ones, during the newborn period, which so far is poorly investigated by means of reference methodology. In the newborn period in the first months of life, the foetal zone of the adrenal gland produces large amounts of 3β-hydroxy-5-ene steroids and their sulphates, e.g. C21-steroids like 16-OH-pregnenolone, 17-OH-pregnenolone and C19-steroids like 16-OH-DHEA, which constitute a very unusual matrix in comparison with older children and adults. Hence, cross reactivity of such steroids in immunoassay kits for various steroids is likely to occur and in fact, many examples has been reported so far. For instance in a paper of Fuqua et al. [58] it is shown that direct assay of serum testosterone in infants less than three weeks old, using an ICN RIA kit, yields values which are on the average 3.8-fold higher than values obtained in the same samples after extraction with CCl_4 and subsequent Sephadex LH-20 chromatography. This large difference disappeared at the age of about 3 months, but correlation between the direct and extraction method was then rather poor. It can be concluded that the testosterone immunoassay is unreliable for the newborn period. Therefore, the establishment of the correct diagnosis of gonadal disorders, like ambiguous genitalia or micropenis, in newborns should not be made on the basis of routine analysis of testosterone. This applies also to the determination of serum 17-hydroxyprogesterone for demonstrating 21-hydroxylase deficiency (see section 4.3). Honour and Rumby [59] mentioned the risk that during the newborn period cross reaction with 3β-hydroxy-5-ene steroids occurs, which to a certain

extent can be reduced by extraction and moreover by use of more specific immunoassay kits [60]. Wong et al. [61] by using GC–MS identified a number of steroids disturbing radioimmunoassays of 17-hydroxyprogesterone in neonatal plasma, 17-hydroxypregnenolone being the most significant interferent.

In order to evaluate hyperandrogenic conditions in women and children with more accuracy than possible with immunoassay methods, Wudy et al. [62] have developed an GC–ID-MS method for the simultaneous determination of six plasma steroids: testosterone, 4-androstenedione, 17-hydroxyprogesterone, 5α-androstane-3α,17β-diol, 5α-dihydrotestosterone and dehydroepiandrosterone. For each steroid, a deuterium labelled internal standard was added for quantification and thus 12 different ion fragments had to be monitored to determine the concentrations of the six steroids. Purification of the samples was performed by extraction and Sephadex LH-20 chromatography and derivatization was done with HFBA. The detection limit of the steroids was approximately 10 pg and by applying this assay in hirsute girls and women easier delineation of disorders than with existing RIA methods can be expected.

Furuta et al. [63] described a GC–ID-MS method for the simultaneous determination of plasma androstenedione and testosterone, using for both steroids trideuterated internal standards. Solid-phase extraction with Sep-Pak C_{18} cartridges and thin-layer chromatography were used as purification, followed by derivatization with HFBA. Detection limits were in the lower picogram range and acceptable accuracy was achieved.

Dikkeschei et al. [64] developed a GC–ID-MS method for free androstenediol and androstenediol-3-sulphate in plasma and urine using a deuterated internal standard and HFBA derivatization. For free androstenediol plasma was purified by extraction and HPLC fractionation, whereas urine was only extracted. For the sulphate, the polar phase of the above mentioned extraction mixture was deproteinized with acetone, the protein removed by centrifugation and the solvent evaporated. After redissolving in buffered saline, extraction with ethyl acetate, addition of HCl and solvolysis for 1 h at 60° Celsius, samples were further processed as described for free androstenediol. Reproducibility was in the order of 10% and recoveries were near 100%, indicating sufficient accuracy.

Hubbard et al. [65] reported on the simultaneous determination of cortisone and cortisol in human nasal and bronchoalveolar lavage fluids and in plasma using GC–ID-MS, in which MS in the NCI-mode with methane as reaction gas was applied. Commercially obtained tetradeuterated cortisol and trideuterated cortisone were the internal standards. The biological fluids were acidified, applied to Sep-Pak C_{18} cartridges, which were subsequently eluted with methanol. Both steroids were converted into (new) pentafluorobenzylcarboxymethoxime-trimethylsilyl derivatives, with excellent mass spectrometric properties in the NCI mode of ionization. Reproducibility was good (correlation coefficients less than 10%), taking into consideration that no bracketing was applied. Detection limits were less than 1 pg. Thus it was possible to measure a cortisol and cortisone concentration in nasal lavage fluid at levels of 25 and 47 pg/ml.

Noteworthy is the report of GC–MS measurements of cortisol, cortisone, 6β-hydroxycortisol and 18-hydroxycortisol simultaneously, each with their own deuterated internal standard, in urine [66]. After addition of a cocktail of the four internal standards, urine was purified via solid-phase extraction with Sep-Pak C_{18} cartridges. The cartridges were eluted with methanol and the steroids present in the extracts were converted into MO-TMS derivatives. This procedure enabled to measure the four steroids in the free state. By addition of Helix pomatia juice before extraction, conjugates of these steroids, present in urine, can be hydrolyzed and add to the already present free steroids. Therefore in this study the four steroids in the free state as well as their conjugates were measured, by treating urine both without and with Helix pomatia. Reliable normal values of the urinary excretion of these steroids in males, females and children aged 7–16 could be obtained. In the free state 6β-hydroxycortisol and 18-hydroxycortisol are excreted in significantly higher amounts than cortisol and cortisone and values of men exceed those of women, especially for 18-hydroxycortisol. Cortisol and cortisone occur predominantly in conjugated form, whereas the hydroxylated cortisols are mainly present in the free state. Taking the free and

conjugated excretions together the four steroids show a comparable concentration (in the order of 100 μg/l). This assay is very useful for clinical purposes and can be applied for the diagnosis of Cushing's disease (cortisol and 6β-hydroxycortisol highly raised), for the diagnosis of apparent mineralocorticoid excess syndrome (AME), an inborn error caused by 11β-hydroxysteroid dehydrogenase deficiency (very high cortisol/cortisone ratio) and for the diagnosis of glucocorticoid remediable aldosteronism (GRA), an inborn error caused by the presence of a chimeric 11β-hydroxylase/18 hydroxylase gene in affected patients (huge overproduction of 18-hydroxycortisol).

3. Anabolic and androgenic steroids

The natural male hormone testosterone (T) is involved in prenatal sexual differentiation, the growth stimulus of the body, especially in the young male during pubescence, and in the sexual maturation and persistence of fertility throughout life of the adult male. Testosterone can be regarded as the endogenous steroid with anabolic properties. It is administered to male patients who suffer from hypogonadism or from insufficient sensitivity to testosterone, or have to recover from catabolic states [67]. However, if taken orally it is very rapidly metabolized and therefore inappropriate as such. For this reason chemical analogues of testosterone were synthesised to strongly retard its metabolism without loss of hormonal activity. Methyltestosterone, 17β-hydroxy-17α-methylandrost-4-en-3-one, was the first synthesised anabolic steroid [68]. Between 1935 and 1965, especially in the fifties, a large number of anabolic steroids appeared, the metabolism of which has been reviewed recently [69]. The main features of androstane metabolism (see Fig. 1) are (1) the reduction of the double bond at C4, leading to 5α- and/or 5β-isomers; (2) the reduction of the 3-oxo group to 3α-hydroxy or 3β-hydroxysteroids;(3) the oxidation of the 17β-hydroxy group to a 17-carbonyl group if a 17α-methyl group is absent; (4) hydroxylation at e.g. C6, C12, C16; epimerization of the 17β-hydroxy group into a 17α-hydroxy one, or 17α-methyl, 17β-hydroxysteroids into 17β-methyl, 17α-hydroxysteroids; (5) glucuronidation and/or sulphation of e.g. the 3-hydroxy group. More details of steroid metabolism can be read in the above mentioned review [69]. Due to the large and actually unfair misuse of these so-called anabolic androgenic steroids (AASs) and other agents by top athletes all over the world the International Olympic Committee (IOC) as well as the International and National Sport Federations banned the use of these compounds since 1974. Moreover, apart from muscle growth promotion long term use of AASs leads to medically adverse effects, like vascular diseases, increased risk of prostatic disease in men and virilization in women, and psychological disorders [67]. Specialised laboratories accredited by the IOC are qualified to detect AAS metabolites in the urine of the athletes (see Table 2). As a consequence of the slowed down metabolism of the AASs the metabolites of these compounds can be demonstrated in the urine of the users rather long after ending the administration of the forbidden steroids. Doping in sport has been the

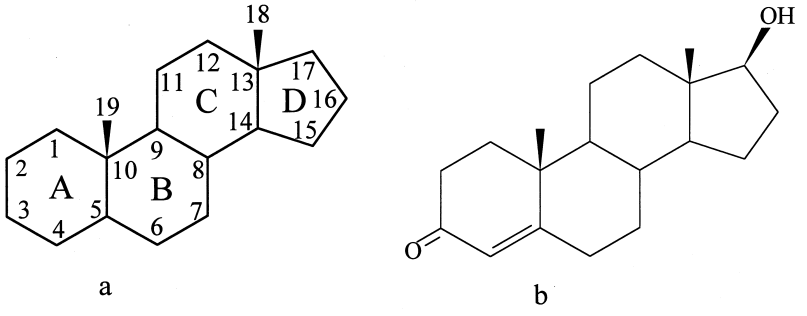

Fig. 1. The general structure of a C_{19} steroid (a), where the numbers refer to the carbon atoms of the androstane skeleton, and the structure of testosterone (b).

Table 2
Anabolic androgenic steroids (AASs) and metabolites [69,72–74,84,85,88,91,97,98]

Trivial name	Systematic name
Bolasterone	*7α, 17α-dimethyl-17β-hydroxy-4-androsten-3-one*
	7α, 17α-dimethyl-5β-androstane-3α,17β-diol
	7α, 17β-dimethyl-5β-androstane-3α,17α-diol
	7α, 17α-dimethyl-5β-androstane-3α,17β-diol-17-sulphate
	7α, 17,17-trimethyl-5β-androst-13-en-3α-ol
Boldenone	*17β-hydroxy-androst-1,4-dien-3-one*
	17β-hydroxy-5β-androst-1-en-3-one
	5β-androst-1-ene-3α,17β-diol
	3α-hydroxy-5β-androst-1-en-17-one
Calusterone	*7β, 17α-dimethyl-17β-hydroxy-4-androsten-3-one*
	7β, 17α-dimethyl-5β-androstane-3α,17β-diol
	7β, 17α-dimethyl-5α-androstane-3α,17β-diol
	7β, 17β-dimethyl-5β-androstane-3α,17α-diol
4-Chloro-Met(h)andienone	*4-chloro-1,2-dehydro-17α-methyltestosterone*/
Dehydrochlorotestosterone	*4-chloro-17β-hydroxy-17α-methylandrosta-1,4-dien-3-one*
	6β-hydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone
	6β,16β-dihydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone
	6β,12ξ-dihydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone
	4ξ-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-ene-16-one
(proposed structure)	4-chloro-3ξ,12ξ,17β-trihydroxy-17α-methylandrosta-1,4-dien-3-one
(proposed structure)	4-chloro-6β,12ξ,17β-trihydroxy-17α-methylandrosta-1,4-dien-3-one
Clostebol	*4-chloro-17β-hydroxyandrost-4-en-3-one*
	3α-hydroxy-4-chloro-androst-4-en-17-one
	4ξ-chloro-3α-hydroxy-5β-androstan-17-one
	4ξ-chloro-3α-hydroxy-5α-androstan-17-one
	4ξ-chloro-3β-hydroxy-5α-androstan-17-one
	4ξ-chloro-3α,16ξ-dihydroxy-5ξ-androstan-17-one
Danazol	*17α-ethynyl-17β-hydroxy-(2,3-d)-isoxazol-pregna-2,4-diene*
Dehydroepiandrosterone	*3β-hydroxy-androst-5-en-17-one*
	3β-hydroxy-5α-androstan-17-one
	androst-5-ene-3β,17β-diol
5α-dihydrotestosterone (Stanolone)	*17β-hydroxy-5α-androstan-3-one* (5αDHT)
	5α-androstane-3α,17β-diol (5αAD)
	5α-androstane-3α,17α-diol (17α,5αAD)[a]
Drostanolone	*17β-hydroxy-2α-methyl-5α-androstan-3-one*
	2α-methyl-5α-androstane-3α,17β-diol
	3α-hydroxy-2α-Methyl-5α-androstan-17-one
Epitestosterone	*17α-hydroxy-androst-4-en-3-one*
	5α-androstan-3α,17α-diol (17α,5αAD)[a]
	5β-androstan-3α,17α-diol (17α,5βAD)
Ethylestrenol	*19-nor-17α-pregn-4-en-17-ol*; or *17α-ethyl-17β-hydroxy-estr-4-ene*
	17α-ethyl-5α-estrane-3α,17β-diol
	17α-ethyl-5β-estrane-3α,17β-diol
	17α-ethyl-5ξ-estrane-3α,17β,21-triol
Fluoxymesterone	*9α-fluoro-11β,17β-dihydroxy-17α-methyl-androst-4-en-3-one*
	9α-fluoro-6β,11β,17β-trihydroxy-17α-methyl-androst-4-en-3-one
	9α-fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetrol
	9α-fluoro-11β,17β-dihydroxy-17α-methyl-androst-4-en-3-one-17-sulphate
	9α-fluoro-11β,17α-dihydroxy-17β-methyl-androst-4-en-3-one
	9α-fluoro-11β-hydroxy-18-nor-17,17-dimethylandrosta-4,13-dien-3-one
Formebolone	*2-formyl-11α,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one*
	2-hydroxymethyl-11α,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one

Table 2 (*continued*)

Furazabol	*17β-hydroxy-17α-methyl-5α-androstano[2,3-c]-furazan*
	16ξ,17β-hydroxy-17α-methyl-5α-androstano[2,3-*c*]-furazan
Mestanolone	*17β-hydroxy-17α-methyl-5α-androstan-3-one*
	17α-methyl-5α-androstane-3α,17β-diol
	17α-methyl-5α-androstane-3α,17β-diol-17-sulphate
	17β-methyl-5α-androstane-3α,17α-diol
	18-nor-17,17-dimethyl-5α-androst-13-en-3α-ol
Mesterolone	*17β-hydroxy-1α-methyl-5α-androstan-3-one*
	1α-methyl-5α-androstane-3α,17β-diol
	3α-hydroxy-1α-methyl-5α-androstan-17-one
Metandienone (Dianabol)	*17β-hydroxy-17α-methylandrosta-1,4-dien-3-one*
	6β,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one
	17β-hydroxy-17α-methylandrosta-1,4-dien-3-one-17-sulphate
	18-nor-17,17-dimethylandrosta-1,4,13-trien-3-one
	17α-hydroxy-17β-methylandrosta-1,4-dien-3-one
	17β-hydroxy-17α-methyl-5β-androst-1-en-3-one
	17α-methyl-5β-androst-1-ene-3α,17β-diol
	17β-methyl-5β-androst-1-ene-3α,17α-diol
	18-nor-17,17-dimethyl-5β-androsta-1,13-dien-3α-ol
	17α-methyl-5β-androstane-3α,17β-diol[b]
Methenolone	*17β-hydroxy-1-methyl-5α-androst-1-en-3-one*
	1-methyl-5α-androst-1-ene-3α,17β-diol
	3α-hydroxy-1-methylen-5α-androstan-17-one
Methandriol	*17α-methylandrost-5-ene-3β,17β-diol*
	17α-methyl-5β-androstane-3α,17β-diol[b]
Methyltestosterone	*17β-hydroxy-17α-methylandrost-4-en-3-one*
	17α-methyl-5α-androstane-3α,17β-diol[c]
	17α-methyl-5β-androstane-3α,17β-diol[b]
Mibolerone	*17β-hydroxy-7α,17α-dimethylandrost-4-en-3-one*
	7α,17α-dimethyl-5β-androstane-3α,17β-diol
Nandrolone (19-nortestosterone)	*17β-hydroxyestr-4-en-3-one*
	3α-hydroxy-5β-estran-17-one
	3α-hydroxy-5α-estran-17-one]
	3β-hydroxy-5α-estran-17-one
Norclostebol	*4-chloro-17β-hydroxyestr-4-en-3-one*
	4-chloro-3α-hydroxyestr-4-en-17-one
	4ξ-chloro-3α-hydroxy-5β-estran-17-one
	4ξ-chloro-3α-hydroxy-5α-estran-17-one
	4ξ-chloro-3α,16ξ-dihydroxy-5ξ-estran-17-one
Norethandrolone	*17β-hydroxy-17α-ethylestr-4-en-3-one*
	17α-ethyl-5α-estrane-3α,17β-diol
	17α-ethyl-5β-estrane-3α,17β-diol
	17α-ethyl-5β-estrane-3α,17β,21-triol
Oxandrolone	*17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one*
	16ξ,17β-dihydroxy-17α-methyl-2-oxa-5α-androstan-3-one
	17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one-17-sulphate
	17α-hydroxy-17β-methyl-2-oxa-5α-androstan-3-one
	18-nor-17,17-dimethyl-2oxa-5α-androst-13-en-3-one
Oxymesterone	*4,17β-dihydroxy-17α-methylandrost-4-en-3-one*
Oxymetholone	*17β-hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one*
	3α, 17β-dihydroxy-17α-methyl-5α-androstane-2ξ-carboxylic acid
	17α-methyl-5α-androstane-3α,17β-diol***

(*continued on next page*)

Table 2 (*continued*)

Quinbolone	*17β-(1-cyclopenten-1-yloxy)-androsta-1,4-dien-3-one*
	17β-(1-cyclopenten-1-yloxy)-5β-androst-1-en-3-one
	17β-(1-cyclopenten-1-yloxy)- 5β-androst-1-en-3α-ol
Stanozolol	*17β-hydroxy-17α-methyl-5α-androst-2-eno[3,2-c]pyrazole*
	3',17β-dihydroxy-17α-methyl-5α-androst-2-eno[3,2-*c*]pyrazole
	16β,17β-dihydroxy-17α-methyl-5α-androst-2-eno[3,2-*c*]pyrazole
	4β,17β-dihydroxy-17α-methyl-5α-androst-2-eno[3,2-*c*]pyrazole
	3′,17α-dihydroxy-17β-methyl-5α-androst-2-eno[3,2-*c*]pyrazole
Stenbolone	*17β-hydroxy-2-methyl-5α-androst-1-en-3-one*
	3α-hydroxy-2-methyl-5α-androst-1-en-17-one
	3α-hydroxy-2ξ-methyl-5α-androstan-17-one
	16ξ,17β-dihydroxy-2-methyl-5α-androst-1-en-3-one
	3ξ,16ξ-dihydroxy-2-methyl-5α-androst-1-en-17-one
	16ξ-hydroxy-2-methyl-5α-androst-1-en-17-one
Steranabol	*3ξ,4ξ-androst-5ξ-an-17-one*
Testosterone	*17β-hydroxy-androst-4-en-3-one*
	3α-hydroxy-5α-androstan-17-one (androsterone)
	3α-hydroxy-5β-androstan-17-one (etiocholanolone)
	3α,17β-dihydroxy-5α-androstane (5αAD)
	3α,17β-dihydroxy-5β-androstane (5βAD)
	5α-androstane-3,17-dione]
Trenbolone[d]	*17β-hydroxyestra-4,9,11-trien-3-one*
	17α-hydroxyestra-4,9,11-trien-3-one

[a] 5α-androstan-3α,17α-diol is a common metabolite of epitestosterone and 5α-dihydrotestosterone [84].
[b] 17α-methyl-5β-androstane-3α,17β-diol is a common metabolite of metandienone, methandriol and methyltestosterone.
[c] 17α-methyl-5α-androstane-3α,17β-diol is a common metabolite of methyltestosterone and oxymetholone.
[d] Used as a veterinary compound.

subject of many investigations as shown e.g. in a report in 1993 on international cooperation in analytical chemistry, where the outcome of all sorts of banned compounds, used and detected after the XI Pan American Games in Havana 1991, was given [70]. Another example is the remarkable report in 1997 on hormonal doping and androgenization of (female) athletes of the former German Democratic Republic till 1989 [71]. GC and GC–MS parameters of AAS metabolites [69], such as methylene units or relative retention times, mass spectra and or important ion fragments were recently (1996) published in this journal [72–75]. Examples of frequently applied androgenic steroids (see Table 2) apart from testosterone (testosterone enanthate or testosterone propionate) are dianabol [69–71,75] and stanozolol [69,70,75]. Others are norethandrolone [70]; 4-chloro-met(h)andienone mestanolone; nandrolone, applied as the phenylpropionate ester (durabolin) or the decanoate ester (decadurabolin) [71], and furazabol [69,74].

The detection of these xenobiotic anabolic steroids and their metabolites in urine by means of GC–MS can be conducted either by overall screening procedures affording the measurement of any of them including also the metabolites, or by measurements of only some specific compounds, if their presence is suspected. Sample preparation includes a number of analytical steps: solid phase extraction, deconjugation and derivatization. Nowadays urinary steroids are primarily isolated by means of solid phase extraction (SPE) using minicolumns containing the neutral polystyrene resin Amberlite XAD-2 or the stronger reversed phase bounded silica particles as in the Sep-Pak C_{18} cartridges, originally described in 1968 [76] and 1980 [77], respectively. The former columns have a low flow rate (0.2 ml min^{-1} cm^{-2}) and a low capacity (0.5 g resin/ml), while the more expensive cartridges have a high capacity (3 mg/ml) and a rather high flow rate (10 ml/min) [78]. Details of the extraction procedure and column treatment can be found in the literature [79,80]. After elution with

methanol the conjugated steroids are hydrolysed, mostly by the enzymes β-glucuronidase and sulphatase from the snail *Helix pomatia* in acetate buffer at pH 4.5–5.0 or by β-glucuronidase from *Escherichia coli* in phosphate buffer at pH 6.8–7.0 [81]. The deconjugated steroids are reextracted as described above and are mostly derivatized to methoxime-trimethylsilyl ethers using methoxylamine–HCl in pyridine and next trimethylsilylimididazole (TMSI), the latter in case of sterically hindered 17α-hydroxylated compounds [7,82,83]. AASs however are mostly converted into only N-TMS- (if primary or secondary N-atoms are present) and O-TMS derivatives (the OH groups), using a strong silylation mixture, in which moreover carbonyl groups are converted into enol-TMS ether moieties. Experimental details for these and other derivatives can be read elsewhere [72,75,80,84]. In overall screening procedures the derivatized steroids can then be detected by mass fragmentography, using quadrupole mass spectrometers in the EI mode, in which in a large number of time windows each time several different specific mass fragments are chosen in such a way that they correspond with those of the steroids expected to emerge in that time window. An example of such a procedure is described by Ayotte et al. [72], who in one run monitored 110 different mass fragments, spread over eleven time windows (10 different ions in each window). This method is not specific and sensitive enough to measure AASs at very low concentrations, as for instance in cases where the use of these drugs has been suspended for several days. More sensitivity can be obtained by only monitoring a few mass fragments and measuring only one anabolic steroid and/or its metabolites by quadrupole GC–MS, as e.g. has been done by Kim et al. [74] for furazabol. Sometimes such methods still lack the required specificity to prove the presence of the forbidden substance at very low concentrations and then highly sophisticated techniques have to be applied. A striking example is the report of Schänzer et al. [75], who by the use of double focusing magnet sector MS instrumentation employed high-resolution GC–MS (with a resolution of 3000) and were able to detect abuse of metandienone and stanozolol by specifically measuring these compounds and their metabolites in urine at very low levels in combination with additional clean-up procedures such as HPLC fractionation or immuno affinity chromatography (performed in a similar way as mentioned above in the section on reference methodology). Moreover they showed that, when the more general approach (low-resolution GC–MS without special pretreatment) was applied, the presence of these drugs in the investigated samples escaped any notice due to the abundant presence of spurious peaks in these circumstances. Another way to improve sensitivity and selectivity is the use of GC–MS–MS techniques as offered by triple quadrupole instruments or the presently available quadrupole ion traps. In the mass analyzer of the latter instrument after ionization a specific (for the substance of interest) mass fragment can be selectively trapped and subsequently be subjected to fragmentation again by CID (collision induced dissociation). The resulting secondary mass spectrum, if similar to the reference compound, is unequivocal proof of the presence of the expected substance and generally is hardly obscured by interfering substances having the same retention time, as practically all mass fragments of the interferents have already been filtered out. Bowers et al. [73] clearly demonstrate the strength of this highly selective technique in conjunction with large volume injection for tracing an impressive number of certain anabolic steroids and metabolites at the lower picogram level. It can thus be expected that the use of ion trap GC–MS instrumentation, which is considerably less expensive than high resolution sector instruments, will find broad application in future AAS screening.

The possibility to be found positive on AAS screening at official sport games led the athletes and their helpers to use synthetic testosterone, which if intramuscularly administered as a depot of, for instance, 250 mg testosterone enanthate causes supraphysiological levels in blood for at least a week [85]. In 1983 Donike et al. proposed to use the ratio of testosterone to its endogenously produced nonhormonal epimer epitestosterone (17α-hydroxy-androst-4-en-3-one) for screening of misuse of testosterone [86]. If the ratio *T*/*epiT* in the urinary glucuronide fraction was found to be larger than 6, the athlete had misused testosterone, at least to the authors [86]. Determination of *T*/*epiT* in a large number of

urinary samples from sportsmen show that the skewed distribution of *T/epiT* with a maximum around 1 has an upper limit at about 6, indeed [70,87]. Only few men have a ratio larger than 6 [88], while on the other hand with a low number of athletes or male volunteers the depot dosing of testosterone does increase the *T/epiT* but not exceeding 6 [85,88]. Therefore, if post-competition analyses indicate that *T/epiT* in a urinary sample >6, the IOC recommends extra but unannounced testing of the suspected athletes to decide whether the first test should be considered to be positive or not [89]. Dehennin showed that if instead of the ratio *T/epiT* of the two glucuronidated steroid epimers, *TG/epiTG*, that of *TG/(epiTG+epiTS)*, ([testosterone glucuronide]/[epitestosterone glucuronide+epitestosterone sulphate]), is calculated, the distinction between physiologically high ratios ($6<T/epiT<10$) and pharmacologically high ratios ($T/epiT>6$) is significantly better [90]. For this purpose one part of the urine is used for enzymatic hydrolysis of *TG* while the steroids in a second part are methanolysed to get total *epiT* [91]. The method of measuring the *T/epiT* ratio does not exclude the masking action of epitestosterone if being used to lower the expected increase of the ratio.

Therefore, performing of supplemental tests in the same urine specimen is desirable, such as the testosterone to luteinizing hormone ratio, *T/LH*, which will be apparently increased [88,92,93] if due to negative feedback of constantly high plasma testosterone concentrations the secretion of LH is diminished. Another test has been described recently by Shackleton et al. [85]. In 1990 Southan and coworkers showed that by using gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) the ^{13}C content of synthetic testosterone differed from that of endogenous testosterone [94]. For this purpose the relative difference between the $^{13}C/^{12}C$ isotope ratio of testosterone (R_T) and that of an international carbonate standard PDB (R_{PDB}) is used: $\delta^{13}C‰=[(R_T-R_{PDB})/R_{PDB}]\cdot 1000$. Comparing $\delta^{13}C‰$ of urinary testosterone, analysed as testosterone acetate, with $\delta^{13}C‰$ of urinary cholesterol (acetate), Becchi and co-workers could demonstrate that the former $\delta^{13}C‰$ decreased if synthetic testosterone has been misused [95–97]. Recently, Shackleton et al. improved the sensitivity of the method by using the more abundant urinary metabolites (4%) of testosterone, namely 5α-androstanediol (5αAD, 3α,17β-dihydroxy-5α-androstane) and 5β-androstanediol (5βAD, 3α,17β-dihydroxy-5β-androstane) instead of the urinary testosterone itself [85]. Upon dosing of testosterone enanthate to 8 Chinese male subjects, for five of them, $\delta^{13}C‰$ of 5αAD and 5βAD, analysed as diacetates, was compared during 17 days (2 days before dosing) to that of the urinary pregnanediol (PD), also analysed as the diacetate ester [85]. The $\delta^{13}C‰$ of testosterone enanthate, analysed as testosterone acetate, was −30.41, which value almost approximated the lowest value obtained for the androstanediol diacetate measurements in this study [85]. Before dosing $\delta^{13}C‰$ of the androstanediols ranged from −26 to −28 with a minimum of −28.3. After administration of the hormone the value decreased to about −29 to −30. For the reference steroid PD $\delta^{13}C‰$ remained at −25 to −27. Clearly, the decrease of $\delta^{13}C‰$ in the urinary androstanediols could be irrefutably demonstrated for over 8 days after administration of testosterone enanthate [85]. The authors suggest that if the ratio of $\delta^{13}C‰$ (AD) to $\delta^{13}C‰$ (PD) is 1.10 or higher the misuse of testosterone is evident. For only 3 out of the 8 Chinese men the *T/epiT* ratio exceeded 6 on more than one day, showing a high rate of false negative values. Furthermore the study shows that the used method is valuable only if the period between doping and analysis does not exceed 8 days [85]. The same group expanded their tests to detect also the administration of exogenous dihydrotestosterone (5αDHT, stanolone, 17β-hydroxy-5α-androstan-3-one), epiT, dehydroepiandrosterone (DHEA) and pregnenolone [84]. For 2 days 50 mg/d of these steroids (T, epiT, 5αDHT, DHEA, and pregnenolone) was administered to one male volunteer, where each of the steroids was given in separate experiments with an interval of 2 weeks. Urines voided between 3 and 6 h after the second administration were used to isolate the corresponding diols, 5αAD and 5βAD from T, 17α,5αAD (5α-androstan-3α,17α-diol) and 17α,5βAD (5β-androstan-3α,17α-diol) from epiT, 5αAD and unexpectedly also 17α,5αAD from 5αDHT, androstenediol (androst-5-ene-3β,17β-diol) from DHEA, and pre-

gnenediol (3β,20α-dihydroxy-pregn-5-ene) from pregnenolone. For all steroids $\delta^{13}C‰$ fell from −26 to −27 before administration to −29 to −30 three to six h thereafter. The use of *epiT*, if administrated to athletes to cover the misuse of synthetic testosterone by decrease of the otherwise increased *T*/*epiT* ratio, can be detected by measuring the decrease of $\delta^{13}C‰$ of 17α,5αAD+17α,5βAD next to that of 5αAD and 5βAD from testosterone. The authors suggest that their method, i.e. calculation of $\delta^{13}C‰$ using GC–C–IRMS analysis of metabolites of forbidden steroids, could be a general basis for verifying misuse of these compounds at official games [84]. However, it should be taken into account that detection of testosterone/epitestosterone misuse by GC–IRMS could be obstructed by administration of a mixture of synthetic testosterone and ^{13}C labelled testosterone resulting in a $\delta^{13}C‰$ ratio of about −27, together with a sufficient amount of a similar mixture of epitestosterone and ^{13}C labelled epitestosterone. Such a procedure then can only be discovered either by measuring the increased mass ratio of the combined ADs to PD, and or the T/LH ratio.

In nearly all these recently reported studies the gas chromatographic column was one of the essential parts of the used instruments. A capillary column was used, coated with several types of bonded phases as given in Table 1. Bowers and Borts showed that the polysilphenylene–polydimethylsiloxane bonded phase capillary columns bleed much less than the normally used polysiloxane capillaries [73]. Moreover, the presence of the former columns increases the possibility to separate special epimer pairs of AAS metabolites, in comparison to the latter ones [73,85].

4. Urinary steroid profiling in health and disease

4.1. Introduction

GC and GC–MS have furthermore been proven to be very suitable for the analysis of urinary steroids in cases of endocrinological disorders since many years. So called urinary steroid profiles have been used and are still used to find and define the corresponding defects and to guard the correctness of medication. Early work on urinary steroid profiling started with Horning and coworkers [100]. Afterwards Shackleton became one of the outstanding pioneers in this field and from his hand, many papers have been published, followed by comprehensive and excellent reviews on this subject in the course of the years [101,102]. In cooperation with Taylor and Honour an atlas showing urinary steroid profiles for various endocrinological disorders was produced [103]. By consulting these works the interested reader acquires a fairly complete knowledge of the technique of urinary steroid profiling and its clinical applications, which has lost nothing of its value up to the present time. When these mentioned reviews were written (mid eighties), most of the possible disorders, which could be explored by applying steroid profiling, had already been described. In addition a few years ago two expert reviews from Honour and Brook have been published, in which also more recent developments have been described [104,105]. Therefore we were inclined to refrain from reviewing urinary steroid profiling, but we decided nevertheless to devote some pages to this subject, since a relatively small number of later interesting developments, extending the applicability of this kind of assays, were not (yet) covered in the above mentioned review papers. These will be briefly overviewed after a short summary of the technique and its applications.

Urinary steroid profiling is not essentially different from analysis of urinary anabolic steroids, reviewed in the preceding section. The collected urine sample is similarly worked up, mostly by solid phase extraction, followed by enzymatic deconjugation of the steroid sulphates and glucuronides and subsequent derivatization, practically always methoximation of the carbonyl groups combined with silylation of the hydroxy groups. The derivatized steroids are separated by capillary gas chromatography and analyzed by flame ionization detection or mass spectrometric detection using GC–MS. In urine the pattern of the metabolites arising from the active steroid hormones and their precursors, originating from adrenals and gonads, often reveals the nature of the disease and/or is useful in evaluating treatment.

The main applications concern the establishment of adrenal tumours and of enzyme defects in steroid hormone synthesis with respects to adrenal and gonads such as described in one of the mentioned review papers [101].

In the following part some applications of steroid profiling, appearing after publishing of this review will be discussed, while referring also to earlier developments, if opportune.

4.2. Determination of reference values

One of the problems of steroid profiling is the difficulty to characterize the resulting chromatogram as being normal or not, since the 24 h excretions of the steroid metabolites strongly depend on age and sex of the patient. Moreover, they are dependent on the analytical method employed to a certain extent. For this reason only provisional values were available for a long time. Therefore, several laboratories in the Netherlands, after a completion of a standardization program, took part in a project to determine reference values for children, men and women in hundreds of urine samples of normal persons of different ages, which were published by Weykamp et al. [106]. Honour et al. [107] studied urinary steroid excretion in samples of 127 normal boys (aged 7.5–15.6 years) during 3 years and of 14 pubertal girls during 2 years. Moreover data were collected from 115 young, hospitalized patients with normal adrenal function. The excretion of cortisol metabolites was constant for body size, whereas androgen metabolites rose sharply at the onset of puberty. These two studies are very supportive in the interpretation of steroid profiles of children and adults.

4.3. Congenital adrenal hyperplasia

For the adrenal cortex, the inborn errors of steroid synthesis on the way to the glucocorticosteroid cortisol and the mineralocorticosteroid aldosterone are known as hydroxylase deficiencies and indicated by *congenital adrenal hyperplasia*. The most frequently occurring form of congenital adrenal hyperplasa (CAH) is the *21-hydroxylase deficiency*, which leads to the decreased secretion of cortisol, the increased secretion of the intermediate 17-hydroxyprogesterone and increased excretion in the urine of pregnanediolone (PDL), pregnanetriol (PT) and pregnanetriolone (PT-one) in children and adults [101]. Immediately after birth, newborns affected by this disorder excrete PDL [108] and also other marker compounds, as androstanetriolone [109] and 15β-hydroxy-PDL [110]. These patients also have a very high ratio of 16α-hydroxypregnenolone to 16α-hydroxy-DHEA [110,111]. Some weeks after birth the babies show a mixed pattern of PT, PT-one, PDL, 15β-hydroxy-PDL and 5α-hydroxylated isomers of e.g. PDL, 15β-hydroxy-PDL, and PT [112]. The less frequently occurring variants of CAH are 17-hydroxylase deficiency, 11β-hydroxylase deficiency and 3β-hydroxysteroid dehydrogenase deficiency. In case of *17α-hydroxylase deficiency*, the abnormally high secretion of 11-deoxycorticosterone (DOC), which causes hypertension, and of corticosterone (compound B) is evident, and the production of androgens and estrogens by the adrenals and gonads is virtually absent. The urinary steroid profile is marked by the presence of relatively high amounts of 11-deoxy-tetrahydrocorticosterone (THDOC), 11-dehydrotetrahydrocorticosterone (THA), tetrahydrocorticosterone (THB and aTHB) and their hexahydro analogues [113–115]. Moreover the presence of 6α-hydroxy derivatives of THA [115] and THB [102,115], as detected by GC–MS, has been confirmed by the synthesis and characterization of the corresponding steroids [116]. In the urine of neonates with CAH due to 17α-hydroxylase deficiency, an elevated ratio of 16α-hydroxypregnenolone to 16α-hydroxy-DHEA as well as an increased excretion of 6α-hydroxy-THA [116] can be noticed [101]. CAH due to *11β-hydroxylase deficiency* is characterized by the increased secretion of DOC, 11-deoxycortisol (Compound S) as well as adrenal androgens. In the urine the excretion of tetrahydroCompound S (THS), hexahydroCompound S, androsterone and etiocholanolone is increased [101,102,117]. We also found in newborns the urinary steroid 6α-hydroxy-THS [117], as characterized by us recently [116]. This steroid is one of the main urinary compounds excreted by neonates with this disorder [102]. CAH

due to *3β-hydroxysteroid dehydrogenase deficiency* gives rise to elevated excretion of all 3β-hydroxy-5-ene compounds in the urine, a condition which also exists in the urine of healthy newborns due to the presence of the large foetal zone of the adrenal cortex [101,102]. Nevertheless it is possible to detect this disease in the newborn period by steroid profiling [118,119]. The diagnosis can be established, apart from the clinical symptoms (salt loss, ambiguous genitalia), on the basis of highly elevated levels of 16α-hydroxy-DHEA and 16α-hydroxypregnenolone, while at the same time PDL, PT (like in the case of 21-hydroxylase deficiency) can be identified by GC–MS, which steroids are normally absent in newborn urine samples. The presence of PDL and PT, both metabolites of 17-hydroxyprogesterone, which is one of the products of the 3β-hydroxysteroid dehydrogenase enzyme, seems paradoxical, but can be ascribed to peripheral conversion of 17-hydroxypregnenolone, which accumulates in this disease [118]. In older children and adults the disease can easily be traced by means of urinary steroid profiling on the basis of high excretion or by means of elevated excretion of 3β-hydroxy-5-ene steroids, especially 5-pregnenetriol, but the diagnosis can sometimes be missed, when these steroids, all of them present as 3-sulphates, are incompletely deconjugated, as can occur with batches of *Helix pomatia* digestive juice with insufficient sulphatase activity. Therefore additional solvolysis (hydrolysis of steroid sulphates in an acidified organic solvent, e.g. HCl in ethyl acetate) is a prerequisite to obtain quantitatively the 3β-hydroxy-5-ene steroids with certainty [120].

4.4. Disorders in mineralocorticoid metabolism

Pseudohypoaldosteronism is an endocrinological disorder, presenting in early childhood. The occurring salt loss (hyperkalemia and hyponatremia) suggests insufficient secretion of aldosterone. However, instead the production of aldosterone is highly increased because of an insufficient number of receptors of aldosterone in the target organ cells (kidney tubuli), which results in salt loss in spite of the increased production rate of aldosterone [121]. Therefore, the patient can be treated only by an extra salt (NaCl) gift. In a newborn with this disease the urinary steroid profile apparently suggested the existence of 21-hydroxylase- or 18-hydroxylase deficiency, because of the presence of 15β-hydroxy-PDL and a high level of THA, respectively [122]. Therefore, the correct diagnosis and subsequent medication was possible only after an additional quantitative measurement of aldosterone and its urinary metabolite tetrahydroaldosterone [122,123].

11β-hydroxysteroid dehydrogenase (11βHSD) deficiency is an inborn error in steroid metabolism, characterized by hypertension, low plasma renin activity (PRA) and hypokalemia, which are typical symptoms also observed in primary hyperaldosteronism. Nowadays about 30 patients have been reported, of whom only one was an adult. Probably many patients die in early childhood escaping correct diagnosis. However therapy is effective and similar to the case of primary hyperaldosteronism, namely administration of the aldosterone antagonist spironolactone and potassium suppletion. The antagonist prevents occupation of the receptor by cortisol in the kidney. In contrast to primary hyperaldosteronism, plasma aldosterone in 11βHSD deficiency is virtually absent and therefore this disease often is named ‘apparent mineralocorticoid excess (AME)-syndrome’. Although the disease has been known since 1974 [124], its cause remained obscure for many years. Ulick et al. [125] for the first time discovered a defect in the interconversion of biological cortisol into inactive cortisone, which was reflected in a highly reduced level of urinary cortisone metabolites compared to cortisol metabolites. The aberration can be typified by summing up the excretion of the two main metabolites of cortisol, i.e. tetrahydrocortisol (THF) and allotetrahydrocortisol (aTHF) and dividing it by the excretion of the main metabolite of cortisone, i.e tetrahydrocortisone (THE); in this disorder this ratio (THF+aTHF)/THE, normally being approximately 1, exceeds 20. In addition, plasma cortisol half life is increased, resulting in a decreased cortisol production rate and consequently a decreased excretion of cortisol (cortisone) metabolites, while the ratio cortisol metabolites/free cortisol in urine metabolites, (THF+

aTHF)/F (A-ring reduction parameter), is decreased. Finally the ratio aTHF/THF is raised, reflecting 5α-reductase versus 5β-reductase activity. Later investigations fully disclosed the nature of the disease, reviewed in detail by White et al. [126]. The disease is caused by congenital deficiency of the isoenzyme form of 11β-hydroxysteroid dehydrogenase called 11-HSD2 and normally abundantly present in the kidneys. This enzyme is able only to convert cortisol into cortisone. On the other hand another isoenzyme, 11-HSD1, mainly present in the liver, is able to oxidize and reduce cortisol into cortisone and the reverse. In practice a constant equilibrium exists between cortisol and cortisone. The 11-HSD2 reaction is necessary because cortisol has equal binding capacity as well as action towards the mineralocorticoid receptors in the kidney as aldosterone. In order to prevent that cortisol exerts any mineralocorticoid activity, the kidney efficiently oxidizes cortisol to the inactive cortisone. This process however is impaired in this disease due to 11-HSD2 deficiency resulting in 'pseudohyperaldosteronism'. As secondary effect the metabolism of cortisol is slowed down. Since a variant of this disease exists with the same symptoms but another urinary steroid excretion pattern, the above mentioned disorder is often designated as 11βHSD deficiency, type 1. The variant, called *11βHSD deficiency, type 2*, can also be investigated by urinary steroid profiling. Like in the type 1 variant, hypertension, hypokalemia, low aldosterone and low PRA are present, as well as prolonged cortisol half life. Treatment is similar as in the type 1 variety. Unlike in type 1 patients, the ratio (THF+aTHF)/THE is normal, but also here the excretion of cortisol metabolites and urinary (THF+aTHF)/F are decreased [127]. The exact nature of this puzzling disease has not yet been elucidated, but is certainly connected with 11-HSD, as has been argued [128]. *Licorice intoxication* produces effects which are exactly the same as in AME type 1 and type 2, and urinary steroid profiles are obtained, which are in between those seen in the two AME types: raised (THF+aTHF)/THE, but considerably less than in type 1, and decreased (THF+aTHF)/F. The explanation for this phenomenon is that the active component of licorice, glycyrrhetinic acid, is a strong inhibitor of HSD-2 and thus produces the same disturbance as occurs in AME type 1 [129,130]. A quite different enzymatic defect, also causing hypertension and hypokalemia, to be treated by oral dexamethazone, is the disease known as glucocorticoid-remediable aldosteronism (GRA). The disease is characterized by measurement of grossly enhanced excretion of 18-oxocortisol, 18-hydroxycortisol (showing mineralocorticoid effects) and their tetrahydro metabolites, which can be completely suppressed by dexamethazone administration [131]. The nature of this defect has been elucidated [132]. In the adrenal glands a number of cytochrome P450 oxidases are present, resulting in the zone glomerulosa in the production of aldosterone and in the zone fasciculata in cortisol. For the last three steps in aldosterone synthesis, 11β-hydroxylase-, 18-hydroxylase and 18-dehydrogenase activity [105], the same enzyme (CYP11B2) is responsible, whereas a highly similar enzyme (CYP11B1) in the zone fasciculata only performs 11β-hydroxylation. In case of GRA a chimeric gene is produced, due to crossing-over, with the ACTH regulatory elements of CYP11B1 and 18-hydroxylase activity of CYP11B2, resulting in the excretion of 18-oxygenated cortisol by the zone fasciculata, stimulated by ACTH.

A disorder not causing disturbance of mineralocorticoid regulation, but mentioned here because it represents the reversed situation as encountered in AME type 1, is the so called 11-oxo reductase deficiency, marked by a highly decreased urinary ratio (THF+alloTHF)/THE (about 0.04) and a very high metabolic clearance of cortisol [133,134]. Symptoms of the patients (two pairs of sisters) were hirsutism and oligomenorrhea. These patients apparently are able to convert cortisol into cortisone, but cannot accomplish the reduction of cortisone into cortisol, being the reverse situation in comparison with AME type 1. Patients can be treated by the glucocorticoid dexamethasone, which suppresses the cortisol secretion and is more slowly metabolized than cortisol.

5. Steroid nomenclature: trivial names, systematic names and some abbreviations of the mentioned steroids[2]

Aldosterone	18,11-hemiacetal of 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al
Androstanediol, AD 5αAD	5α-androstane-3α,17β-diol
5βAD	5β-androstane-3α,17β-diol;
17α,5αAD,	5α-androstane-3α,17α-diol;
17α,5βAD,	5β-androstane-3α,17α-diol);
Androstenediol	androst-5-ene-3β,17β-diol;
Androstenedione	androst-4-ene-3,17-dione;
Androsterone	3α-hydroxy-5α-androstan-17-one
Corticosterone, compound B	11β,21-dihydroxy-pregn-4-ene-3-20-dione;
Cortisone, compound E	17,21-dihydroxy-4-pregnene-3,11,20-trione
Cortisol, compound F	11β,17,21-trihydroxy-4-pregnene-3,20-dione
11-Dehydrotetrahydrocorticosterone or tetrahydroCompound A, THA	3α,21-dihydroxy-5β-pregnane-11,20-dione
Dehydroepiandrosterone, DHEA	3β-hydroxy-androst-5-en-17-one;
Dexamethasone	9α-fluoro-16α-methyl-11β,17,21-trihydroxy-pregna-1,4-diene-3,20-dione
11-deoxycorticosterone, DOC	21-hydroxy-pregn-4-ene-3-20-dione
11-Deoxycortisol, compound S	17,21-dihydroxy-4-pregnene-3,20-dione
11-Deoxytetahydrocorticosterone, THDOC	3α,21-dihydroxy-5β-pregnan-20-one;
Epitestosterone, epiT	17α-hydroxy-androst-4-en-3-one
Estradiol	1,3,5(10)-estratriene-3,17β-diol
Estriol	1,3,5(10)-estratriene-3,16α,17β-triol
Estrone	3-hydroxy-1,3,5(10)-estratrien-17-one
Etiocholanolone	3α-hydroxy-5β-androstan-17-one
HexahydroCompound S, HHS,	5β-pregnane-3α,17,20α,21-tetrol
18-Hydroxycorticosterone, 18OH-B	11β,18,21-trihydroxy-4-pregnene-3,20-dione
6β-Hydroxycortisol, 6βOH-F	6β,11β,17,21-tetrahydroxy-4-pregnene-3,20-dione
18-Hydroxycortisol, 18OH-F	11β,17,18,21-tetrahydroxy-4-pregnene-3,20-dione
16α-Hydroxy-DHEA, 16αOH-DHEA	3β,16α-dihydroxyandrost-5-en-17-one
16α-Hydroxypregnenolone	3β,16α-dihydroxypregn-5-en-20-one
17-hydroxypregnenolone	3β,17-dihydroxypregn-5-en-20-one
15β-Hydroxypregnanediolone, 15βOH-PDL	3α,15β,17-trihydroxy-5β-pregnan-20-one
17-Hydroxyprogesterone	17-hydroxy-4-pregnene-3,20-dione
6α-HydroxytetrahydroCompound A, 6αOH-THA	3α,6α,21-trihydroxy-5β-pregnane-11,20-dione
6α-HydroxytetrahydroCompound B, 6αOH-THB	3α,6α,11β,21-tetrahydroxy-5β-pregnan-20-one
18-Oxocortisol	11β,17,21-trihydroxy-3,20-dioxo-4-pregnen-18-al
Pregnanediol, PD	5β-pregnane-3α,20α-diol
Pregnanediolone, PDL	3α,17-dihydroxy-5β-pregnan-20-one
Pregnanetriol, PT	5β-pregnane-3α,17,20α-triol
Pregnanetriolone, PT=one	3α,17,20α-trihydroxy-5β-pregnan-11-one
Pregnenediol	5-pregnene-3β,20α-diol
Pregnenetriol	5-pregnene-3β,17,20α-triol
Pregnenolone	3β-hydroxy-pregn-5-en-20-one
Progesterone	4-pregnene-3,20 dione
Testosterone	17β-hydroxy-androst-4-en-3-one
Tetrahydrocorticosterone, THB	3α,11β,21-trihydroxy-5β-pregnan-20-one
Allotetrahydrocorticosterone, aTHB	3α,11β,21-trihydroxy-5α-pregnan-20-one
TetrahydroCompound S, THS	3α,17,21-trihydroxy-5β-pregnan-20-one
Tetrahydrocortisol, THF	3α,11β,17,21-tetrahydroxy-5β-pregnan-20-one
Allotetrahydrocortisol, aTHF	3α,11β,17,21-tetrahydroxy-5α-pregnan-20-one
Tetrahydrocortisone, THE	3α,17,21-trihydroxy-5β-pregnan-11,20-dione

[2]See Table 2 for the exogenously applied anabolic and/or androgenic compounds and their metabolites.

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