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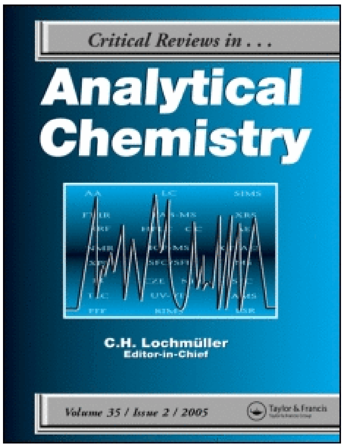

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THE ANALYSIS OF STEROIDS

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I. INTRODUCTION

As a consequence of the great importance of steroids in clinical chemistry, biochemistry, and pharmacy, a continously increasing interest has been shown towards their analysis. The number of publications from the field of steroid analysis reviewed by *Analytical Abstracts* in 1978 is about 400. Almost the same number of nonanalytical papers were published in organic chemical or biochemical journals containing important analytical data and methodology. The vast quantity of literature makes it extremely difficult to follow the rapid development of the field of steroid analyis.

The aim of this review is to give an outline of the present state of the analytical chemistry of steroids with special respect to the recent developments. The main points considered when various methods will be compared are selectivity, sensitivity, simplicity, and capability of being automated.

Steroid analysts exert their activities in three main fields:

1. Structure elucidation of steroids
2. Biological-clinical steroid analysis
3. Pharmaceutical steroid analysis

In this review, the first field is entirely omitted and the treatment of the second and third fields will be restricted to quantitative aspects, omitting the theory and practice of chromatographic separation and detection.

The material discussed in this review is classified according to the most important methods used in the steroid analysis (e.g. classical, spectroscopic, chromatographic, and radioanalytical techniques) omitting some special biochemical methods. The usefulness of these methods will be illustrated by examples taken from the field of the analysis of the most frequently occurring groups of steroids such as sex and adrenocortical hormones, sterols, D vitamins, bile acids, cardiac glycosides, and sapogenins, again omitting some minor groups (alkaloids, ecdysones, etc.).

In selecting the examples from the tremendous number of papers, the author of the present review tries to balance the biological-clinical and the pharmaceutical applications. The reader is requested to consider that although the vast majority of papers have been published from the first field, the author has much more personal experience in the second one.

To conclude the introductory remarks, the reader's attention is drawn to some monographs dealing with general,[1,2] biological-clinical,[3,4] pharmaceutical,[5] chromatographic,[6,7] colorimetric, and fluorimetic[8] aspects of steroid analysis. A few more specialized books and comprehensive chapters of general monographs are referred to in the appropriate sections.

II. METHODS FOR THE DETERMINATION OF STEROIDS

A. Classical Methods

1. Gravimetric Assays

The precipitation of progesterone as the 2,4-dinitrophenylhydrazone and of the testosterone esters as their semicarbazones for the gravimetric assay is the classical method for their determination in pharmaceutical formulations, mainly in oil-injectables.[5,9] These methods are rather time consuming, and their selectivity and sensitivity are also poor and can therefore be regarded as obsolete. It is however, worth mentioning that as a consequence of their extremely high reliability and accuracy these methods are still in use and prescribed in the latest editions of pahrmacopoeias.[10,11] Antoher field where gravimetry used to have importance is the determination of sterols with 3 β-hydroxyl group as their digitonides.[5]

2. Titration of Some Functional Groups

With the exception of bile acids and alkaloids, steroids usually do not contain acidic or basic groups serving as the basis for their direct titration. The indirect determination of keto and hydroxyl groups based on formation of oximes and acylation, respectively, do not seem to be in general use now. However, the determination of the 17-ethynyl group based on the titration with standard alkali of the nitric acid liberated by the reaction between the ethynyl steroid and silver nitrate belongs to the standard methods for the determination of bulk ethinyl steroids even now. Pharmacopoeias[10,12] prescribe a poteniometric titration in tetrahydrofuran medium, but the same precision can be attained with titration to thymolphtalein end-point in pyridine medium.[13]

Two further indirect acidimetric methods are mentioned. Allyloestrenol reacts at the Δ^4 and the allylic double bonds with mercury (II) acetate and methanol, and the two liberated moles of acetic acid can be titrated with standard sodium hydroxide.[14] Steroids containing acyloxy groups can be determined by the classical alkaline saponification followed by back-titration of the excess alkali with standard hydrochloric acid. This method cannot be applied directly to 21-esters of corticosteroids, as the free 21-hydroxy corticosteroids would lead to acidic breakdown product on heating with excess alkali. The assay can be carried out by using a sodium hydroxide - sodium borohydride saponification reagent and back-titration to a phenol-red end point; that is, sodium borohydride rapidly reduces the 20-keto group prior to the hydrolysis of the 21-ester and the 20-hydroxy derivative is no longer sensitive to treatment with alkali.[15]

Of redox titrations, the determination of Δ^5 steroids with acetous bromine[16] is worth mentioning. Using a microburette and a 0.01-N reagent enables diosgenin to be titrated after TLC separation.[17] Steroids with a 16α,17α-diol group can be titrated with lead tetraacetate in acetic acid to a potentiometric end point. The micro version of this method using a 0.001-N solution enables triamcinolone contaminant to be determined in its 16,17-acetonide.[18] In general, however, it can be stated that titrimetry does not play an important role in the determinations of steroids.

B. Polarography

In the heroic period of polarography, several attempts were made to determine steroids in biological samples and pharmaceuticals.[19] The relatively poor sensitivity and selectivity of the polarographic method were not compatible with the rapidly increasing demands in the biological field and for this reason, the use of polarography was soon almost completely restricted to the pharmaceutical field.

In spite of the fact that polarography did not show remarkable advantages compared to the standard spectrophotometric, colorimetric, etc. methods of that time, even in the analysis of steroid preparations, numerous papers were published describing the use of that technique based on the reduction at the dropping mercury electrode, of unsaturated carbonyl derivatives (Δ^4-3- and $\Delta^{1,4}$-3-ketones, cardiac glycosides) and conjugated trienes (D vitamins).

The introduction of differential pulse polarography gave a boost to the use of polarography in the pharmaceutical steroid area. Using this technique, the selectivity and, mainly, the sensitivity of the assay can greatly be increased, enabling the method to be used for the single tablet assay of low dosage formulations.[20] In these cases, the method has been claimed to have an advantage; namely,that the assay can be carried out in the presence of the tablet base (or silica gel if the assay is carried out after preliminary TLC separation[21]), and this may be useful in serial tests.

The assay of unsaturated ketosteroids is usually carried out in the mixture of water and organic solvents such as methanol, ethanol, or dimethylformamide, at various pH values, with the usual concentrations being 10^{-4} to $10^{-5}M$. In addition to tablets,[20,22,23] oil-injectables[22,24] have also been analyzed. It is interesting to note that under suitable circumstances a second peak appears in the polarogram of corticosteroids related to the reduction of the 20-keto group, both peaks being suitable for quantitation. This is illustrated in Figure 1, taken from the paper of de Boer et al.[25]

In the vitamin D field, polarography can be used for the fairly specific determination of ergosterol irradiation resins[26] and for the simultaneous determination of vitamins A and D_2 in oily solution.[27] The determination of digoxin and digitoxin can also be carried out on the basis of the reduction of the unsaturated lactone ring on the dropping mercury electrode.[28] The numerous indirect methods for the determination of saturated ketones, phenolic ring, etc. do not seem to be in use now. Of the recent achievements, two are mentioned: the determination of allyloestrenol in tablets based on methoxymercuration of the double bonds, and reduction of mercury addition compound.[14] Lynoestrenol (17α-ethinyl-4-oestren-17-ol) can be determined after transformation by treatment with 60 to 80% sulfuric acid to the polarographically active Δ^{16}-20-keto derivative.[29]

C. UV-Visible Spectrophotometry

1. Methods Based on Natural Absorption

The possibilities of the determination of steroids on the basis of their natural absorption are principally restricted to unsaturated ketosteroids (Δ^4- , $\Delta^{1,4}$- and $\Delta^{4,6}$-3-keto derivatives), phenolic steroids, and conjugated trienes such as the D vitamins. These possibilities have been completely exhausted for the assay of bulk steroids and some of their simple dosage forms.[5,9] As a consequence of the relatively short wavelength maxima of these derivative (λ_{max}, 240 to 290 nm), the direct spectrophotometric assay is by no means suitable in more complex mixtures including, in most instances, biological samples.

The applicability of spectrophotometry can be greatly improved by column or thin-layer chromatographic separation or purification of the sample. Another possibility is to use chemical reactions to eliminate or at least reduce the background interfering with the spectrophotometric assay. The principle of the difference spectrophotometric method described by Görög[30-32] for the determination of Δ^4- and $\Delta^{1,4}$-3-ketosteroids in fermentation liquor,[30] oil-injectables, and ointments,[31] as well as contraceptive pills[32]

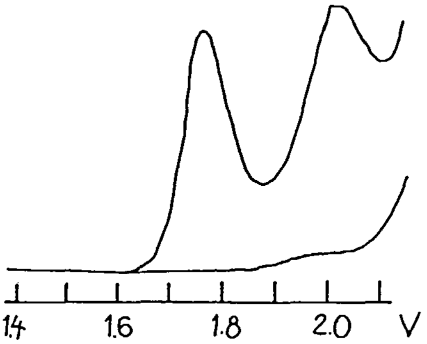


FIGURE 1. Differential pulse polarogram of 10^{-4} *M* hydrocortisone in methanol—tetramethylammonium hydroxide. (From de Boer, H. S., den Hartigh, J., Ploegmakers, H. H. J. L., and van Oort, W. J., *Anal. Chim. Acta*, 102, 141 (1978). With permission).

is the following. An aliquot of the methanolic solution of the sample is reduced by sodium borohydride to the spectrophotometrically inactive 3-hydroxy derivative, and this solution is used in the reference cell, placing the unreduced solution of the same concentration in the sample cell. Using this method, the interference originating from the light absorption of well-defined accompanying materials or ill-defined contaminants not altering their light absorption upon treatment with sodium borohydride is cancelled out. This is illustrated in Figure 2 where curve a is the overall spectrum of the extract of prednisolone ointment containing methyl *p*-hydroxybenzoate as preservative. Curve b is the difference spectrum suitable for quantitative evaluation, while curve c is the spectrum of the reduced solution (characteristic of methyl *p*-hydroxybenzoate).

Two innovations have been made to the original procedure. Chafetz et al.[33] used lithium borohydride in tetrahydrofuran as the reducing agent. This has the advantage that even $\Delta^{1,4}$-3-ketosteroids are easily reduced (these require boiling in ethanol with alkaline sodium borohydride according to the original method). Kirschbaum[34] placed propylene glycol into the reaction mixture to complex sodium metaborate (decomposition product of sodium borohydride), the presence of which (especially in aged samples of sodium borohydride) would avoid complete reduction of the 3-keto group. The Chafetz version of the method is used by Coda and Timallo[35] for the determination of fluocinolone acetonide in dermatological creams.

Estrogens with free 3-hydroxyl group (and their esters, too, after mild alkaline hydrolysis) can be determined by difference spectrophotometry based on the bathochromic shift in their spectra (from about 280 to 296 nm) when the nonionized species is ionized in alkaline medium. This principle is used by U.S.P. XIX[10] for the determination of estradiol valerate in oily solution. This method was criticized by Görög[36] for having acidic and alkaline solutions in the reference and sample cells, respectively, which were too strong and thus decreased the selectivity of the method. By using solutions of pH 9.5 and 0.2 *N* NaOH, respectively, and reducing the Δ^4-ketosteroids by sodium borohydride prior to the assay, estrogens could be determined in injectable oils even in the presence of a great excess of the latter.[36] In general it can be stated that although the above described and some other chemical methods, as well as chromatographic separations, have greatly expanded the field of application of direct UV

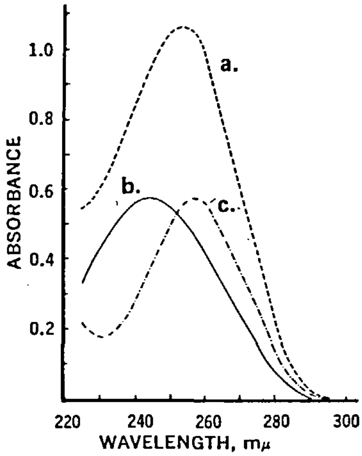


FIGURE 2. Difference UV spectrophotometric determinations of prednisolone in ointment in the presence of methyl. *p*-hydroxybenzoate. (a) Spectrum without treatment with sodium borohydride; (b) difference spectrum; (c) spectrum after treatment with sodium borohydride. (From Görög, S., *J. Pharm. Sci.*, 31, 57, 1737 (1968). Reproduced with permission of the copyright owner).

spectrophotometry to steroids, that applicability is far from being general and is slowly decreasing even in the pharmaceutical field.

2. Colorimetric Procedures Based on Chemical Reactions

The methods used at the beginnings of the determinations of steroids were colorimetric procedures. Since that time, an enormous number of papers have been published describing colorimetric methods and their application in the determination of practically all kinds of steroids both in the biological-clinical and pharmaceutical fields.

The molar absorptivities of the colored products are over 10^4, and this enables samples to be analyzed down to the microgram range. This sensitivity is enough for the solution of most pharmaceutical problems and some biological problems (mainly urinary steroids).

As most of the methods measure functional groups rather than individual compounds, the selectivity of these methods is not very high. Many of the methods, however, using reagents not containing concentrated mineral acids or metal halides and based on incompletely clarified chemical reactions, show surprising selectivity. In many cases this enables the selective assay in the presence of structurally closely related steroids. The selectivity can greatly be improved by using preliminary column or thin-layer chromatographic separations. This is thought to be the reason why the importance of the colorimetric assay of steroids has not shown a considerable decrease even in the past few years. Among the other reasons, the simplicity of the colorimetric procedures and the ease with which they can be automated also play a significant role.

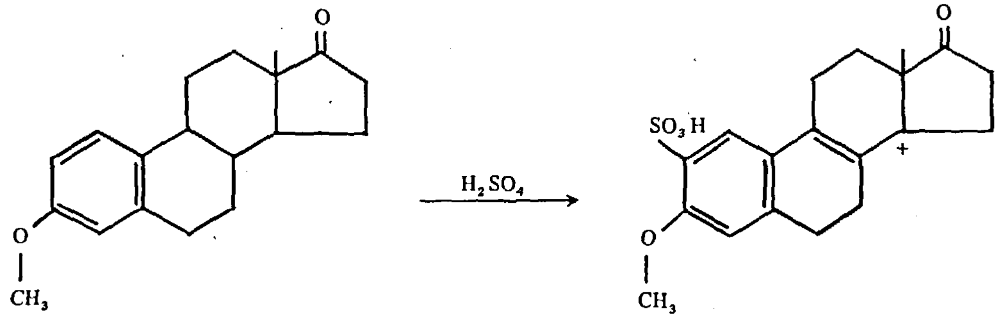
As a consequence of the extremely great number of publications, it is impossible to give even an outline on this field within the scope of the review. (Not even an excellent recently published book[8] devoted specifically to the colorimetric and fluorimetric assay of steroids can deal with all aspects of this field.)

The present generally used colorimetric methods are, in the majority of cases, more or less modernized versions of the classical procedures developed 25 to 50 years ago. Of the methods used for the assay of ketosteroids, the isonicotinoyl hydrazide method is generally used for Δ^4- and $\Delta^{1,4}$-3-ketosteroids in pharmaceutical analysis, while the use of the Zimmermann method (involving the use of 1,3-dinitrobenzene in alkaline medium) is general for 17-ketosteroids in biological samples. The 17-side chain of corticosteroids is determined by the tetrazolium method (quite generally in pharmaceutical analysis and to a somewhat lesser extent in the biological-clinical analysis). For the specific determination of glycocorticosteroids in biological samples, the Porter-Sibler method (using phenylhydrazine- sulfuric acid reagent) is still used. Sterols are usually determined by the Liebermann-Burchard method (reaction with an acetic anhydride - sulfuric acid reagent) or by the Zlatkis-Zak procedure [sulfuric acid - acetic acid - iron (III)-chloride]. The use of reagents containing antimony trichloride is quite general in steroid analysis. Their importance is the greatest in the analysis of vitamin D preparations. The most widespread methods for the determination of digitalis glycosides in plant extracts and pharmaceutical formulations are the xanthydrol method or that using periodic acid cleavage followed by treatment with thiobarbituric acid. No generally accepted colorimetric method exists for the analysis of bile acids.

The extremely widespread use of reagents containing rather concentrated sulfuric acid with or without various diluents and additives merits special mention. These methods, which can be regarded as variants of the classical Kober method, have found wide application in the analysis of most of the important groups of steroids (ketosteroids, estrogens, corticosteroids, sapogenins, etc.) and are generally used for the determination of steroids both in biological samples and pharmacutical formulation. References to the above mentioned methods can be found in standard monographs.[1,4,5,8,9]

As far as recent tendencies in the colorimetric analysis are concerned, three types of papers can be observed. First are those which provide elucidation of the mechanism of the classical methods. Of the above-listed methods, the elucidation of the mechanism of the Kober-type reactions has been the subject of thorough investigations from the very beginnings of these methods. The main steps of the very complex reaction mechanisms for estrogens are dehydration of the molecule and migration of the double bonds formed towards conjugation with phenolic ring A. During this migration the C-13 methyl group migrates to the 17-position. The sulfonation of the aromatic ring also takes place. The conjugated system thus made easily forms carbonium cation in the strongly acidic medium which is capable of resonance, and this is the basis of the color.

For example, the main reaction path of the transformation of estrone methyl ether in concentrated sulfuric acid is shown in the following structure (omitting the detailed mechanism) taken from the paper of Miura and Kimura.[37]



It must be noted that this is not the only reaction route. In the Kober-type reactions, several reaction products are formed. This is essentially the case with nonaromatic derivatives, too. In these instances, the mechanism often involves dimerization of the steroids (at the 3-position) and their trimerization (at 3- and 6-positions).[38] It is interesting that even the first investigators dealing with the mechanism of these reactions came to almost the same conclusion as described above. Recent investigations using the most up to date spectroscopic and chromatographic techniques can give only more detailed explanation and further evidences.

The reasons for the bicolor character of the blue tetrazolium reaction of corticosteroids (e.g., prednisolone) were recently investigated by Görög and Horvath.[39] It was found that an equilibrium exists between prednisolone, its oxidation product (21-aldehyde derivative), blue tetrazolium reagent, its partially reduced red monoformazan, and the totally reduced blue diformazan.

$$\begin{array}{l}\text{Prednisolone (P)} + \text{Blue tetrazolium (BT)} \rightleftharpoons \\ \text{Prednisolone-21-aldehyde (PA)} + \text{BT monoformazan (BTMF)}\end{array}$$

$$K_1 = \frac{[\text{PA}]\,[\text{BTMF}]}{[\text{P}]\,[\text{BT}]} = 3.8$$

$$\text{P} + \text{BTMF} \rightleftharpoons \text{PA} + \text{BT diformazan (BTDF)}$$

$$K_2 = \frac{[\text{PA}]\,[\text{BTDF}]}{[\text{P}]\,[\text{BTMF}]} = 0.08 \qquad (1)$$

From the equilibrium constants in Equation 1, it is evident that under the usual analytical conditions (i.e., a great excess of blue tetrazolium) the red monoformazan is almost entirely formed. The second type of papers are those which describe the improvement of the reliability, selectivity, or sensitivity of the classical methods and their automation. For example, the Zimmermann method has been made more reliable and more suitable for being automated by introducing the all-water system for the reaction.[40,41]It is interesting that for a long time it was believed that the presence of even traces of water had to be avoided in the Zimmermann reaction. On the contrary, running the reaction in water (in the presence of the detergent, Hyamine® 1622) greatly increases the stability of the colored system as the colored reaction product which would be unstable in the strongly alkaline solution is precipitated and can subsequently be safely measured after its solubilizing by dilution with aqueous Hyamine® 1622 solution or extracting with ether.

An important observation made by Graham et al.[42] on the blue tetrazolium reaction of corticosteroids is that the reaction rate can greatly be increased and the color stabilized using dichloromethane as the solvent. This method enables the sterically strongly hindered, less reactive betamethasone and its esters to be determined in pharmaceutical products at room temperature.[43] This variant of the tetrazolium method is likely to become a widely used routine method unlike the otherwise elegant method of Oteiza et al.[44] which is the reaction-rate variant of the blue tetrazolium method (analysis time is only 30 sec) requiring the use of a computer-controlled spectrophotometer equipped with a stopped-flow module.

The selectivity of the methods involving the use of high concentrations of strong acids can greatly be influenced by changing the composition of the reagent and the experimental conditions. For example, the 70:30 mixture of sulfuric acid and methanol introduced by Tsilifonis and Chafetz[45] and adopted by the USP[10] is an extremely selective and sensitive reagent for 17-ethinylated estrogens. Their molar absorptivities at

540 nm are about 3.6×10^4, while no other steroids have absorption maxima in this spectrum range. Similar observations were made by Szabó[46] with a 65:35 sulfuric acid - ethanol reagent. Both reagents enable ethinylated estrogens to be determined in dosage forms. The selectivity of the measurement can be further improved by using preliminary column chromatographic separation. In this form even the most delicate analytical problems can be solved.[47,48,124]

An example for increasing the sensitivity of the measurement is afforded by the paper of Baccou et al.[49] who introduced a sulfuric acid - anisaldehyde - ethyl acetate reagent for the determination of steroid sapogenins. As high a molar absorptivity as about 5×10^4 could be attained at 430 nm for all types of these derivatives (mono- , di- , tri- , and tetroxy derivatives, Δ^5, and 5,6-dihydro, etc. compounds).

This means that the color can be attributed to the interaction between the reagent and the spiroketal bonding system of sapogenins. This is very advantageous when the aim of the assay is the determination of the sum of sapogenins in the presence of other steroids and sugars. A great disadvantage is, however, that it is not suitable for differentiating between those sapogenins which can selectively be measured by the earlier (less sensitive) methods.

In addition to the main types of reagents discussed above, a great variety of other reagents were also introduced for the colorimetric analysis of steroids already in the classical period of steroid analysis and have also been introduced recently, although to a lesser extent. It is rather difficult to judge when the introduction of a new reagent is reasonable and when it is autotelic.

If the aim of introducing a new reagent is to determine a functional group which has not been determined before, this can by all means be regarded as an important development. This is the case, for example, with the method of Forist and Judy[50] for the determination of ketoximes based on splitting hydroxylamine, its oxidation to nitrite, diazotization of sulfanilic acid, and measurement of the azo-dye formed with *N* -1-naphtyl-ethylenediamine. Also of this type are the methods for the determination of steroid sulfates based on complex formation with pyridine and copper,[51] or with methylene blue,[52] and the determination of the 17-ethinyl group either by de-ethinylation with sodium *t* - butoxide followed by Claisen condensation with diethyl oxalate[53] or on the basis of the complex formation with Ag^+ ions and colorimetric determination of either the excess of silver[54] or bound silver[55] by the dithizone method. 21-Halo- , mesyloxy- , tosyloxy, etc. corticosteroids can be determined as the 21-quaternary pyridinium enolates.[56]

Those papers which suggest new reagents for derivatives or functional groups for the determination of which standard methods are available much more frequently in the literature. In some of these instances, the introduction of new reagents seems to be reasonable, e.g., the assay of 21-hydroxy[57] and 21-amino[58] corticosteroids as well as betamethasone- -17-benzoate[59] based on oxidation to 21-aldehyde with cupric acetate,[57,59] or mercuric chloride[58] and condensation of the product, 20-keto-21- -aldehyde, with phenylhydrazine[59] or 4,5-dimethyl- -*o* -phenylenediamine[57,58] is much more specific than the standard tetrazolium or Porter-Silber methods. In other cases the advantages of the use of new reagents to the classical ones are at least questionable, and more experience than is presented in the original papers is necessary to judge the reasonableness of the new methods [e.g., the use of 3-nitroaniline,[60] 3-methyl-2-benzothiazolinone hydrazone - Ce^{4+}, [61] nitric acid,[62] sodium hexanitrito Co (III),[63] etc. for the determination of estrogens]. The main trends in the colorimetric analysis of steroids, however, do not seem to be the introduction of new reagents, but the improvement of the reliability of the old ones and extension of their application field and, mainly, their automation. The most important recent development in this field can be regarded as the spreading of enzymatic reactions. (These are treated separately in Section F below.)

D. Fluorimetry

1. Methods Based on Natural Fluorescence

Of the various groups of steroids, only estrogens exhibit natural fluorescence suitable for quantitative measurements. However, because of the relatively short wavelength (excitation 284 nm, emission 310 to 327 nm) and low fluorescence intensity, this method suffers from interferences originating from background fluorescence or fluorescence quenching. On the other hand, these methods are very simple and less labor consuming than the fluorimetric procedures based on chemical reactions. For this reason, methods based on natural fluorescence are sometimes used in those cases when high sensitivity is not a prerequisite of the applied analytical method. Of the rather infrequently used applications, the determination of mestranol in contraceptive pills[64] and estradiol valerate in injectable oils,[65] as well as the determination of urinary estrogens[66] should be mentioned.

2. Methods Based on Chemical Reactions

Fluorimetric methods based on chemical reactions are much more sensitive than those based on natural fluorescence, and if a sufficiently selective reagent is used, the specificity of these methods is also better. In addition, these methods are not restricted to estrogens, but can be used for other sex hormones, corticosteroids, cardiac glycosides, and to a lesser extent, to bile acids, sterols, etc.

With the exception of a few cases, of which the use of dansyl derivatives for the determination of estrogens in pharmaceutical dosage forms[67] and biological samples[68,69] is the most important, most of the fluorimetric methods in steroid analysis are based on nonstoichiometric reactions involving the use of reagents containing high concentrations of strong acids. Almost all reagents of this type used in the colorimetric analysis induce fluorescence, too, and hence in many cases the methods have their colorimetric and fluorimetric variants. The former are more frequently used in pharmaceutical analysis, while in biological-clinical analysis where greater sensitivity is required, the fluorimetric variants are somewhat more often used.

The Ittrich variant[70] of the classical Kober-Brown method is the basis of most of the recent fluorimetric procedures for the determination of estrogens. In the numerous variants of this method, the chromophore or the fluorophore formed in the reaction of the estrogen with a sulfuric acid-hydroquinone reagent is extracted with various halogenated hydrocarbons containing some *p*-nitrophenol to obtain a stable solution of the chromophore or fluorophore. Although this variant of the Ittrich method has been successfully automatized[71,72] together with the phase separation step, the latter has been claimed by several authors to be rather problematic and to be avoided if possible. Another main problem is the interference from glucose and other aldehydes. Phase separation can be avoided by completely omitting organic solvents from the reaction mixture. In the method of Hammond et al.[73] the estriol standard was introduced into the solution as the aequous solution of its sodium salt. After the Kober reaction with sulfuric acid - ferrous sulfate reagent, the fluorescence was developed by diluting the reaction mixture with the aequous solution of chloral hydrate and trichloroacetic acid. The elimination of the interference of aldeyhydes is carried out in this and several other methods by their reduction with sodium borohydride prior to the reaction.

The high sensitivity of fluorimetry enables the use of this technique for the determination of estrogens in as little as 0.2 mℓ of plasma.[74] The specificity can be improved by selective extraction, e.g., estradiol and estrone can be extracted by a mixture of benzene and hexane, and the estriol that remains in the aqueous phase can be easily determined.

Of the numerous applications of sulfuric acid-induced fluorescence to the analysis of corticosteroids in urine and plasma, two papers are mentioned to illustrate the high selectivity of the fluorimetric procedures. Investigating the effect of substituents on the fluorescence intensity of corticosteroids in a 75:25 mixture of sulfuric acid and ethanol among others, the following relative values were found by Monder and Kendall[75]: hydrocortisone (cortisol) 100, aldosterone 0.21. Whigham[76] developed a method for urinary aldosterone in which its 13-aldehyde groups is oxidized to carboxilic acid using alkaline cupric sulfate - sodium citrate as the oxidizing agent. The oxidation product is extracted at pH 7.5 and washed with a buffer of pH 3.5. Finally, the fluorescence is developed by reacting the extract with an 85:15 mixture of sulfuric acid and water.

Table 1 shows the extreme specificity of this method to aldosterone (which is almost inactive in the Monder-Kendall test). The reason for this high selectivity is not only the specificity of the reaction, but removal of the other urinary steroids or their oxidation products by the above extraction steps.

As in the case of the colorimetric procedures, the introduction of enzymatic reactions affords new possibilities for the fluorimetric assay, too. These are treated in Section F below.

E. Quantitative Aspects of Other Spectroscopic Techniques

1. IR Spectroscopy

IR spectroscopy has always been an important tool for the structure elucidation of steroids, and is gradually replacing color reactions in their identification in pharmacopoeias. Attempts have been made to use the IR method for the quantitative determination of steroids since the beginnings of the use of this spectroscopic technique. The quantitative application of the IR technique, mainly in the solution phase but also in the potassium bromide pellett technique, has also been well documented[5]. This method may have some real usefulness in those cases when comensurable quantitatives of steroids in macro samples have to be determined. However, the IR method has never been a really important quantitative method for steroids, and today its importance seems to be decreasing further. The reasons for this are the following:

1. Although the selectivity of the IR method is superior to most of the UV and colorimetric procedures, the sensitivity is very poor which precludes the possibility of using it in biological-clinical analysis.
2. As the quantitative evaluation of the IR spectra is possible only by the base-line technique, the accuracy of the results is usually inferior to that of concurrent methods.

In spite of these facts, the use of IR spectroscopy is sometimes reported even in recent years, e.g., for the assay of steroid dosage forms,[77,78] Methods of this type, althought correctly evaluated and well documented, can be regarded as curiosities only. An interesting application of IR spectroscopy is the determination of total sapogenins in plant extracts on the basis of the band at 980 cm^{-1} and the determination of the ratio of the epimeric diosgenin and yamogenin on the basis of the relative intensities of the sidebands at 920 and 900 cm^{-1} mainly after column chromatographic purification.[79,80]

2. NMR Spectroscopy

Almost the same as was stated above regarding IR spectroscopy can be stated about the application of NMR spectroscopy in the quantitative analysis of steroids. The scattered data on the determination of steroid hormones in dosage forms[81] and the purity control of bulk steroids[82] by NMR are interesting papers, but can by no means be regarded as real competitors to the generally accepted methods in this field.

In those laboratories, however, where a suitable NMR spectrometer is available for quantitative purposes, several special problems can be solved by this method more easily than by other techniques. For example, the simultaneous determination of vitamin D_2 and seven related derivatives is possible if the NMR spectrum of their acetates is scanned before and after the addition of europium — dipivaloylmethane shift reagent, and this is of value in the analysis of ergosterol irradiation resins.[83] While in this case the assay can also be carried out using the most advanced chromatographic techniques, gas chromatography alone was not able to solve the simulanteous determination of the constituents of the product of the hydrogenation of 3-methoxy-1,3,5(10),8(9)-estratetraen-17 β-ol where, in addition to some starting material and its Δ^6 derivative, three isomers of 3-methoxy-1,3,5(10)-estratrien-17β-ol(8β, 9α ; 8α, 9β; 8β, 9β) are present. The 13-methyl groups of the five components give four well-resolved peaks, and hence the simultaneous determination of all the five components is possible by joint application of NMR spectrometry and gas chromatography.[84].

3. ORD Spectroscopy

The determination of the optical rotatory power of steroids with the conventional sodium or mercury lamps serves for their identification and purity control only. Scanning the ORD spectra, however, and using the maxima and minima of the Cotton curves for quantitative purposes affords new possibilities for the simulanteous determination of structually closely related pairs of steroids. Several examples of this type were already presented in the classical book of Djerassi.[85]

Quantitative spectropolarimetry enables various steroid hormones containing ketone groups to be determined in oily solutions without any diluation.[86] It also allows the direct determination of as little as 0.1 % estrone in ethinyloestradiol.[87]

4. Mass Spectrometry

Mass spectrometry is the most powerful technique of the structure elucidation of steroids. In the quantitative gas chromatographic analysis of complex steroid samples of biological origin, mass spectrometry coupled with gas chromatrography (GC-MS) is often applied as a supplementary method to identify those steroids which are quantitated by gas chromatrography. The above methods will not be treated here and the discussion is restricted to those techniques where the mass spectrometer itself is used for the quantitation.

If the total ion current is used as the basis of the quantitation in GC-MS work, the mass spectrometer can be regarded as a very sensitive detector for the gas chromatograph. The selectivity of the measurement can greatly be increased by using mass fragmentography where the multiple ion detecting capability of modern GC-MS instruments is utilized. This technique is the most effective tool for the determination of extremely low concentrations of steroids (down to the 10 pg range) and their metabolites in complex biological samples.

The mass fragmentographic methods usually involve the following steps: addition of a suitable internal standard to the sample, extraction, thin-layer, or column chromatographic cleanup of the sample, derivatization, and GC-MS scan at selected mass numbers.

In most cases the internal standard is an isotopically labeled derivative of the compound to be determined which is eluted in the same gas chromatographic peak as the unlabeled material. At least two m/e values are selected for simultaneous monitoring corresponding to the labeled and unlabeled materials, respectively. These are usually, but not necessarily, the molecular ions. The labeling can be performed using deuterated, tritiated, or ^{14}C derivatives.

Of the numerous examples, a few characteristic ones will be shown. Estradiol was determined in serum using tetradeuterated (2,4,16,16) estradiol as the internal standard in the form of bis-trimethylsilyl (TMSi) derivative using the molecular ions at m/e 416 and 420 to scan the gas chromatographic fractions.[88] In the determination of testosterone in human plasma, the internal standard was 1,2-ditritiated testosterone, and chromatography was performed in the bis-heptafluorobutyrate form using the molecular ions at m/e 680 and 684, respectively. In the same study, 5 α-dihydrotestosterone was determined using a derivative labeled at C-4 by ^{14}C as the internal standard and chromatographed as the 3-heptafluorobutyrimido-17-heptafluorobutyrate derivatives at m/e 681 and 683.[89] Progesterone was determined in tissues in underivatized form by the chemical ionization GC-MS technique using ^{14}C-labeled progesterone as the internal standard and selecting the M + 1 peaks at 315 and 317 and the recombination M + 29 peaks at 343 and 345 for scanning the chromatograms. The ratios of the peak heights at 343 to 315 and 345 to 317 were used for identification purposes, and the ratio 315 to 317 was used for the quantitation of progesterone.[90]

Of the nonhormonal steroids for the determination of serum cholesterol, both its trideuterated[91] and ^{14}C labeled[92] derivatives have been used as the internal standards. The molecular ions were selected in both referenced cases for scanning the chromatogram. The molecular ions were used in the determination of vitamin D_3 in plasma, too, after being transformed to heptafluorobutyryl isotachysterol$_3$. The internal standard in this method was not an isotopically labeled derivative, but dihydrotachysteryl$_2$ heptafluorobutyrate.[93]

Two interesting papers are mentioned from the bile acid field. Bile acids in bile were determined as the methyl ester trimethylsilyl ether derivatives using deuterated bile acids as internal standards. In this case not only the molecular ions, but those derivable by the loss of 1,2, or 3 trimethylsilanol and/or the C-17 side chain and the C-19 methyl group were monitored.[94] If a 1:1 mixture of methanol and perdeuterated methanol is used for the preparation of the methyl esters, bile acids appear in the mass spectra as equal intensity doublets differing by 3 mass units, which facilitates their identification and quantitation.[95]

As can be seen from these examples (as well as from several others not referred to here, most of which have been published in the last few years), the importance of quantitative mass spectrometry is rapidly increasing in the field of steroid analysis. At present, however, the high price of suitable instruments precludes the possibility of using this technique in the routine clinical analysis. On the other hand, this method is already often used even in clinical laboratories as a reference method to check the reliability of the routine RIA, GLC, etc. techniques.

F. Enzymatic Methods

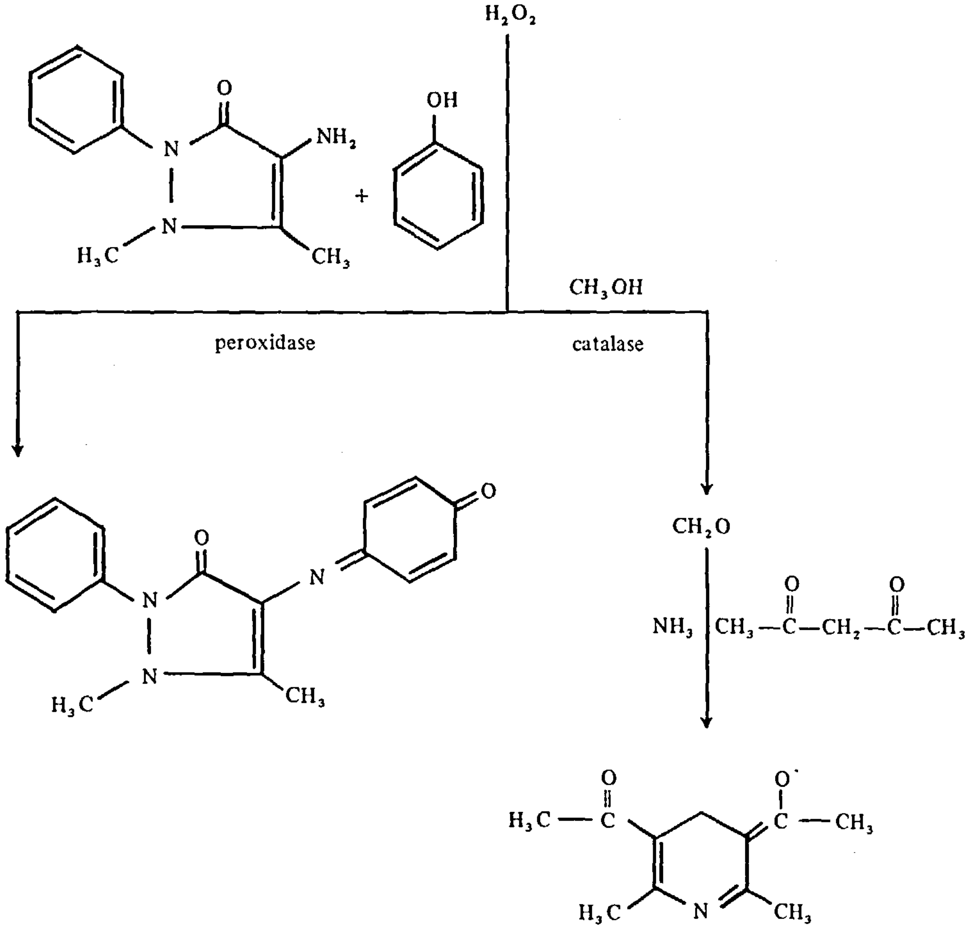
1. Methods Based on the Enzymatic Oxidation-Reduction of Steroids

One of the most important achievements in the analysis of steroids in the last decade has been the introduction and extremely rapid spreading of enzymatic methods. These are colorimetric, fluorimetric, or electroanalytical procedures where the analytical signal depends on the concentration of the product (or, very often, by-product) of the transformation of the steroid to be determined by one or, often, two or three successive enzymatic reactions. The explanation of the rapidly growing popularity of these methods is their extremely high selectivity assured by the selectivity of the enzymes used. This enables low concentrations of various steroids to be determined in the presence of complex biological matrices and other steroids which are closely related structurally. This high selectivity and the fair sensitivity of the monitoring reactions make the enzymatic methods extremely suitable for being automated and used in routine clinical work.

If the conventional colorimetric or fluorometric methods are used for the same purposes, lengthy preliminary extraction and/or chromatographic cleanup steps have to be used, while in the case of enzymatic procedures, rather small samples of urine, serum, plasma, bile, etc. can be directly introduced into the reaction mixture.

One of the most important reactions in the enzymatic analysis of steroids is the enzymatic oxidation of Δ^5-3β-hydroxy steroids to the corresponding Δ^4-3-ketones accompanied by the formation of a stoichiometric amount of hydrogen perioxide. The colorimetric or fluorimetric determination of the latter creates good possibilities for the determination of, for example, cholesterol.

$$\text{cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{cholesterol}} \text{4-cholesten-3-one} + H_2O_2$$



As seen in the structure above, the autoxidation of cholesterol is catalyzed by cholesterol oxidase. There are two main types of colorimetric methods based on forming hydrogen peroxide. In the most widespread one, it oxidizes a 4-aminoantipyrine-phenol reagent with the aid of peroxidase enzyme to a colored quinoneimine dye with an absorbance measurable at 500 to 520 nm.[96,97] In this form the method obviously measures free cholesterol only. If the total cholesterol content also has to be determined, a preliminary hydrolysis step must be added to the procedure. This can be done by alkaline treatment, but more generally enzymatic hydrolysis using cholesterol esterase is used. Although the three enzymatic reactions take place successively, the enzymes and the reagents are introduced simultaneously, and the measurement of the absorbance can be done after a short incubation time. For this reason this method is easy to automate, and practically all of the many papers reporting on the use of this method describe automated procedures. It is important to note that it is necessary to add suitable detergents to the aqueous reaction mixture in order to disrupt lipoprotein complexes and hold cholesterol in micellar solution.

Several authors claim that bilirubin interferes with the above method. The other method shown in the preceding structure is based on the enzymatic oxidation of methanol. The colorimetric,[98] or fluorimetric[99] determination of the formaldehyde product as the dihydrodiacetyl lutidine (absorbance or excitation at 405 to 415 nm; emission at 485 nm) does not suffer from this interference. A difficulty of this method is, however, that the reaction is too slow. For this reason, a rate method variant of the procedure is preferred in automatic analyzers[100,101] where the concentration of cholesterol is calculated from the initial change of the absorbance.

The enzyme cholesterol oxidase is highly specific to cholesterol and related sterols. Other Δ^5-3β-hydroxy steroids are only very slowly oxidized.[102] Changing the enzyme to 3β-hydroxysteroid oxidase, Yamaguchi et al.[103] determined urinary dehydroepiandrosterone by the quinoneimine method.

The enzyme-catalyzed autoxidation of cholesterol can also be exploited for automated routine analytical purposes using electroanalytical techniques. One of the two main trends in this field is the amperometric measurement of the consumption of oxygen that was dissolved in the reaction mixture, using an oxygen electrode to monitor the change. The oxygen concentration is proportional to the concentration of cholesterol (see the preceding structure). As seen in Figure 3a taken from the paper of Kumar and Christian,[104] the reaction is complete in less than 1 min; and the difference between the initial and final plateau in the oxygen current vs. time curve corresponds to the concentration of cholesterol. Even more suitable for the quantitation is the measurement of the peak height of the differnetial curve (see Figure 3b) which can be recorded simultaneously. Noma and Nakayama[105] developed a similar method where, after the reaction reached the second plateau, cholesterol esterase is added to the solution. As the cholesterol liberated from its esters consumes a second portion of oxygen, a second step results, the height of which allows the concentration of cholesterol esters to be calculated.

In another type of measurement, immobilized enzyme(s) is used; i.e., "cholesterol electrodes" are constructed. In the system developed by the Guilbault group[106] cholesterol esterase and oxidase are immobilized on the surface of a glutaraldehyde derivatized alkylamine glass and placed into a porous rotating cell which is rotated magnetically. The hydrogen peroxide product is monitored continously with a polarographic system consisting of a Pt electrode vs. a calomel reference electrode. The calculation of the total cholesterol concentration can be based either on the total current change or the initial rate. The selective determination of free and esterified cholesterol is also possible if cells containing only a single enzyme and the other enzyme in solution are used. Another type of "cholesterol electrode" introduced by Satoh et al.[107] consists of an oxygen electrode combined with a collagen membrane containing cholesterol oxidase for the determination of free cholesterol.

A great advantage of the amperometric methods is that one analysis can be performed within 1 to 2 min. The analysis time is somewhat longer with the "cholesterol electrodes." Their advantage is, however, that the immobilized enzymes can be used several hundred times. It is easy to predict that the importance of electrodes with the

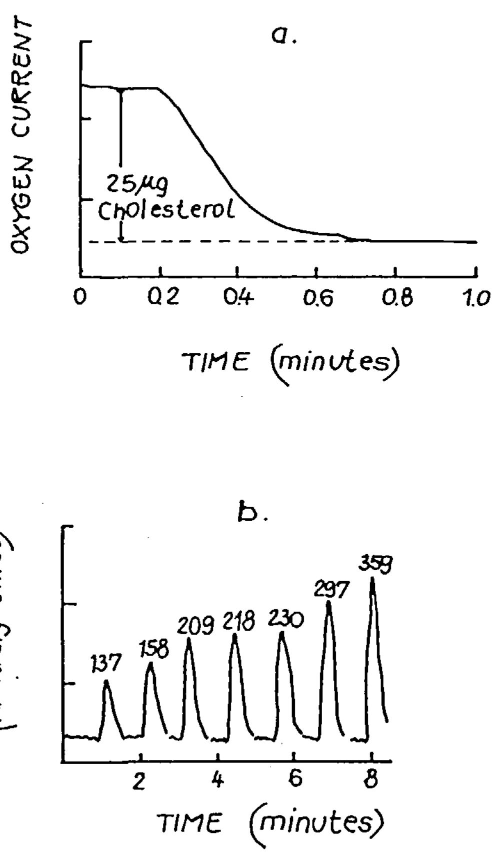


FIGURE 3. Enzymatic-amperometric determination of serum cholesterol. (a) Plot of oxygen consumption of 50 u*l* of saponified serum containing 25 µg of cholesterol.(b) Oxygen depletion differential rate curves of 25 µ*l*-serum samples. The number on the peaks represents cholesterol concentration in mg/d*l*. (From Kumar, A. and Christian, G. D., *Clin. Chim. Acta* 1, 74, 101, 1977). With permission).

immobilized enzymes will certainly increase in the analysis of cholesterol and, possibly, other steroids as well.

The other main reaction used in the enzymatic analysis of steroids (mainly in the bile acid field) is the oxidation of hydroxysteroids to the corresponding ketones by nicotinamide adenine-dinucleotide (NAD). The analytical measurement is based on the spectrophotometric[108] or fluorimetric[109] determination of the reduced form of the oxidizing agent (NADH), which has an absorption maximum at 340 nm. As the sensitivity of the spectrophotometric method is rather poor ($\varepsilon = 6300$) the fluorimetric variant (emission at 450 nm) is more widespread.

The reaction is catalyzed by 3α-hydroxysteroid dehydrogenase (3α-HSDH), by which all the important bile acids can be determined. Some selectivity can be obtained using 7α-HSDH, and this is of importance in the simultaneous determination of the chromatographically poorly separable conjugates of desoxycholic acids.[110]

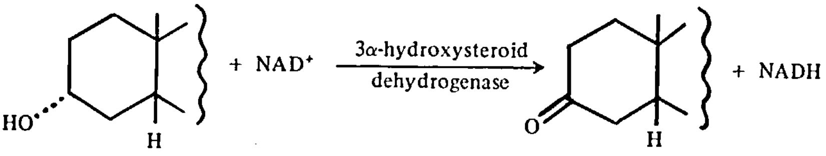
The selectivity of the method can be further increased by adding another enzymatic

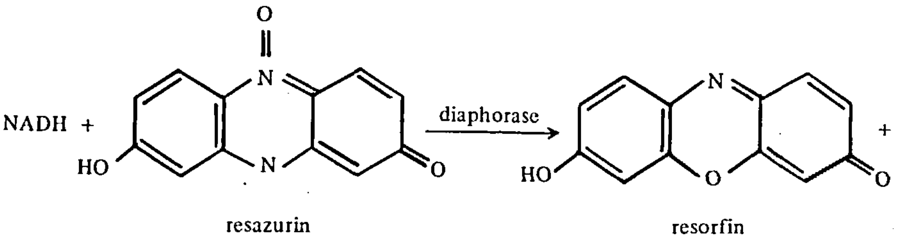
Table 1
RELATIVE FLUORESCENCES OF STERIODS MEASURED WITH THE METHOD OF WHIGHAM

Steroid	Fluorescence
Aldosterone	100
Cortisol	0.18
Cortisone	0.00
Corticosterone	1.40
11-Deoxycorticosterone	0.10
11-Deoxycortisol	0.33
Tetrahydrocortisol	0.02
Tetrahydrocortisone	0.00
Tetrahydro-11-deoxycortisol	0.02
β-Cortol	0.02
β-Cortolone	0.06
Spironolactone	0.00

Courtesy of Whigham, W. R., *Clin. Chem.* 22, 369 (1976). With permission.

reaction to the procedure. NADH formed in the first reaction reduces resazurin in the presence of diaphorase enzyme to the fluorimetrically active resorfine (excitation 565 nm; emission 580 nm).[111,112]





The enzymatic methods discussed above are generally used in clinical analysis for the determination of bile acids. Using suitable enzymes, the NAD-NADH system is suitable for the determination of various other hydroxysteroids and, moreover (using NADH as the reducing agent), ketosteroids as well.[113,114]

2. *Enzyme-Immunoassay*

Enzyme-immunoassay is the latest development in the ultramicroanalysis of steroids in biological samples. Within the last 3 to 4 years, several papers have been published on this matter. This method seems to be a potential competitor of the somewhat earlier introduced radioimmunoassay method, the principle of which is closely related to this technique. (See Section J.3 below.)

Of the varous techniques in enzyme-immunoassay, the so called "Enzyme Linked Immuno Sorbent Assay" (ELISA method) seems to be best applicable to steroids. In this method, the anitserum is immobilized by linking it to cellulose, Sepharose 4B, *p*-arylamine glass particles, or even the wall of the test tube used in the immunoreaction.

Enzyme-labeled antigens are prepared by reacting suitable derivatives of the steroids (e.g., their hemisuccinates, *O*-carboxymethyloximes, etc.) with enzymes (mainly peroxidase) to form covalently bound stable conjugates where the enzymes completely or almost completely reserve their catalytic activity. The catalytic activity is blocked or remains constant and sometimes even increases, if this conjugate is allowed to react with an excess of the specific antiserum. If, however, the steroid to be determined is introduced as a complex with a protein (usually bovine serum albumine— BSA) a competitive equilibrium will exist between this complex and the enzyme-steroid conjugate towards the antiserum. As a result , a proportional part of the enzyme-steroid conjugate (the amount of which depends on the concentration of the steroid to be determined) will remain in the solution phase. After the separation of the phases, the enzyme activity can be directly determined in the solution phase or in the precipitate with the aid of a suitable indicator reaction. From the catalytic activity, the concentration of the steroid to be determined can be calculated with the aid of a calibration curve.

As an example, the method of Joyce et al.[115] for the determination of progesterone in human plasma is shown. The antiserum raised against the 11-hydroxyprogesterone hemisuccinate-BSA conjugate is immobilized on Sepharose 4B. The equilibration takes place at pH 7. The immobilized enzyme is first incubated with the extract of the plasma and BSA, followed by the addition of and equilibration with the conjugate of peroxidase-11-hydroxyprogesterone hemisuccinate. After centrifugation, the activity of the enzyme bound to the antiserum is determined by spectrophotometeric measurement at 492 nm of the chromophore formed from the *o*-phenylenediamine-hydrogen peroxide substrate. (As the measurement is based on the determination of the enzyme activity of the precipitate, the absorbance is inversely related to the concentration of progesterone.) The method is sensitive (sensitivity 93 pg/m*l*) and specific (of the other steroids occurring in plasma, only the 4,5-dihydro metabolite of progesterone shows remarkable cross-reactivity).

Rajkowski et al.[116] described the determination of 50 pg to 1.5 ng testosterone based on similar principles. In this case the steroid is coupled with BSA and the peroxidase enzyme by means of its 3-(carboxymethyl) oxime derivative, and the enzyme activity of the solution determined with the aid of the 5-aminosalicylic acid-hydrogen peroxide indicator reaction measuring the reaction product at 490 nm.

Generally speaking, the specificity of the antisera used in enzymeimmunoassays is comparable with those of the radioimmunoassays, but one has to be very careful as the enzymes of the biological sample may interfere. The sensitivity is somewhat lower than that of radioimmunoassay, but it is sufficient to solve most of the practical problems. A disadvantage of the method is that it is rather slow: 2 to 6 hr are necessary to perform the assay. A great advantage is, however, that no radioactive materials have to be handled.

G. Chromatographic Separations

1. Column Chromatography

Column chromatography has always been an important tool for the separation and purification of steroids in synthetic and biochemical steroid research and also in their analysis. In this review only those methods are outlined whose aim is the isolation, separation, or purification of steroids for subsequent quantitative analysis. As for the literature of this very broad field, in addition to the general chromatographic books referred in Section I,[6,7] a comprehensive chapter in the fundamental book *Liquid Column Chromatography* is mentioned.[117]

Being compounds of medium polarity, almost all kinds of adsorbents and partition systems have been successfully used for the separation of steroids. The following main types of problems have been solved by column chromatography in quantitative steroid analysis.

First is the isolation of steroids from biological samples. Good examples for this task can be taken from the field of the isolation of bile acids and conjugates from blood, urine, and bile with the aid of ion-exchange chromatography which replaces solvent-solvent extraction. Of the various anion exchangers, Amberlite® XAD-2 is most frequently used. For example, Baylocq et al.[118] separated bile acids and their conjugates from bile by directly passing bile diluted with 0.1 *N* sodium hydroxide to a tenfold volume through the column. After the column was washed to neutrality with water, bile acids and conjugates were eluted by methanol. If necessary, this separation step can be repeated to get sufficiently pure sample for the subsequent analysis (which in the referenced case, was HPLC separation and quantitation of bile acids and conjugates).

Various kinds of column chromatography are very often used for the clean-up of extracts, i.e., removal of accompanying impurities or well-defined materials which would interfere with the assay. Examples are seen in the vitamin D field. In the method described by Mulder and de Vries[119] for the determination of D vitamins in multivitamin preparations, as many as three successive column chromatographic steps are prescribed prior to the antimony trichloride colorimetry: a phosphate- treated alumina column separates vitamin E, antioxidants, anhydro vitamin A, carotenes, etc. and vitamin A is separated on the partition column Celite 545/polyethyleneglycol 600 which the traces of vitamin A decomposition products and polyethyleneglycol 600 washed off the Celite column are removed by a Florex column. The addition of column chromatographic step(s) to this and innumerable other methods both in the biomedical and pharmaceutical fields makes these methods rather laborious and time consuming, but at the same time it greatly increases their reliability. The balance between economy and reliability of the method has to be estimated separately in each case.

There is one way to decrease the necessity of preliminary chromatographic purification without loss in selectivity. That is to increase the selectivity of the final quantitative analysis. Gas chromatographic (mainly capillar gas chromatographic), HPLC, radioanalytical, and similar techniques usually require less preliminary chromatographic steps than the less selective colorimetric, fluorimetric, etc. methods. In several instances, however, it has been clearly documented, even in the case of these selective methods, that column chromatography favorably decreases interfering background effects. As a characteristic example, the paper of Skinner and Wills[120] is mentioned. They found that the 25-hydroxyvitamin D_3 level in human serum was apparently considerably higher when the silica gel or Sephadex® LH-20 chromatography prior to the competitive protein-binding assay was omitted (see Figure 4).

The separation of individual steroids or even more frequently, groups of steroids from each other is also an important task for column chromatography. For this purpose in the recent years, various polidextran gels (Sephadex®) are used almost exclusively in biomedical analysis. The hydrophyllic DEAE-Sephadex® A-25 is used for the separation and purification of the most polar steroids such as conjugates, spirolactones,[121] etc. The lipophyllic Sephadex® LH-20 is of the most general use; e.g., for estrogens,[122] corticosteroids,[123] etc. The least polar steroids are best separated on the most lipophyllic hydroxyalkoxypropyl-Sephadex® (Lipidex) column. These preliminary separation steps greatly increase the selectivity of the subsequent analysis. These are, in general, used even in the case of such selective methods as radioimmunoassay if specific enough antisera are not available for the determination of an individual steroid in the presence of related steroids exerting cross reactivity.

The use of column chromatography is so far not as widely used in pharmaceutical steroid analysis for fractionation or separation as it is in biomedical analysis. Of the still numerous examples, the paper of Wu[124] is mentioned. He separated ethynodiol diacetate from mestranol by partition column chromatography using a column of formamide-impregnated diatomaceous earth and heptane as the eluent prior to the colorimetric assay of the two components of the contraceptive pill. Graham and Kenner[125] used a diatomaceous earth — acetonitrile column with heptane and chloroform as the eluents in the determination of various steroids in various dosage forms. Although these and other methods of the same type are extremely reliable, one can easily predict that as a consequence of the spreading of directly applicable selective methods, the importance of preliminary column chromatographic separation in the field of pharmaceutical steroid analysis will certainly decrease.

2. *Thin-Layer Chromatography*

Before discussing the quantitative analytical aspects of thin-layer chromatography, it has to be mentioned that paper chromatography which used to play an important role in the 1950s and 1960s in the fields of both qualitative and quantitative analysis of steroids[1,3,4,6] has been almost completely superseded by thin-layer chromatography. Although this technique still seems to be in use in some laboratories, mainly in the analysis of most polar steroids (cardiac glycosides, conjugates, etc.), and a few papers have been published in recent years on this matter, in general, paper chromatography in the steroid field can be regarded as being obsolete and therefore will not be treated in this review.

a. Spot-Elution TLC Techniques

The quantitative application of thin-layer chromatography in the steroid analysis is almost as old as the TLC technique itself. Methods based on spot elution are still in use for all classes of steroids for the solution of all types of problems such as the purification of steroid samples from accompanying contaminants and separation of individual steroids or steroid fractions for the subsequent analysis in the pharmaceutical, biological-clinical, plant, food, etc. analyses. After the elution of the spots, practically all kinds of analytical techniques used in steroid analysis are applied for the quantitation: spectrophotometry, colorimetry, fluorimetry, polarography, enzymatic methods, radioimmunoassay and related techniques, as well as gas chromatography.

As a consequence of this extremely wide application, a very high proportion of even recently published methods contain TLC steps. Of the numerous applications in the following, only a few characteristic examples are shown.

Hakl[126] demonstrated than even as old a colorimetric technique as the 2,4-dinitrophenylhydrazine method can be successfully used for the determination of dehydroepiandrosterone in urine if the urine extract is submitted to reaction with the reagent and the 2,4-dinitrophenyl-hydrazones are separated by TLC, followed by elution from the spot of the component with methanol and reading the absorbance at 366 nm.

Two TLC steps are used in the fluorimetric method of Fehér et al.[127] for the determination of urinary and biliary bile acids. After the hydrolysis of the conjugates and extraction of the bile acids, the latter are first fractionated by TLC, and the appropriate fraction acetylated and subjected to TLC again before sulfuric acid fluorimetry (excitation 436 nm; emission 510 nm). The reaction can be carried out in the presence

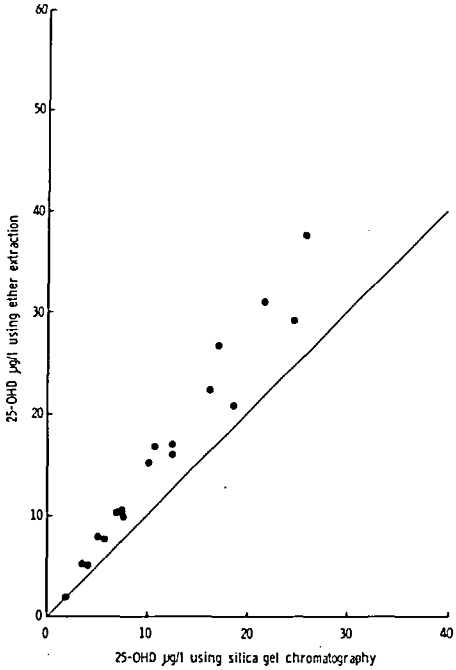


FIGURE 4. Determination of 25-OH-vitamin D in serum by competitive protein binding method. Comparison of the results obtained with and without silica gel column chromatography. The line shown represents the line of equivalence of unit slope. (From Skinner, R. K. and Wills, M. R., *Clin. Chim. Acta* 80, 543 (1977). With permission).

of the silica gel scraped off from the plate. It must be removed only before the fluorescence reading. If polarography is used for the quantitation of ketosteroids separated by TLC, separation from the silica gel is not required.[27] The moderate selectivity of the enzymatic-colorimetric NAD/NADH$^+$ method catalysed by 3α-hydroxysteroid dehydrogenase has been greatly improved by separating individual bile acids and conjugates by TLC and subjecting the eluates of the spots to enzymatic assay.[128]

Thin-layer chromatography has been successfully used to further increase the selectivity of those methods, too, which are highly selective themselves, such as radioimmunoassay and gas chromatography. For example, among others, Corpechot et al.[129] determined testosterone and dihydrotestosterone in plasma by radioimmunoassay after preliminary TLC separation. Without this separation, the RIA method would not be applicable to the simultaneous determination of the two derivatives, as the appropriate antisera show cross-reactivity. The reliability of the gas chromatographic assay of D vitamins in multivitamine preparations can be improved by using TLC to remove vitamins A and E before the assay.[130]

The main advantage of and the reason for the popularity of the spot-elution technique in the quantitative TLC analysis of steroids is its simplicity. No expensive instrumentation is necessary. By adding the high-resolving power of thin-layer chromatography to the selectivity and sensitivity of the method chosen for the quantitation, a great number of the most delicate analytical problems can be solved even in small laboratories equipped only with a basic TLC set-up and spectrophotometer or fluorimeter. However, it must be stated that although these methods are rather simple, the addition of the TLC step to the analytical procedure increases the number of potential sources of error. Hence, reliable results can only be obtained by very careful work, the application of appropriate blanks containing adsorbent scraped from an empty part of the chromatogram, and standards run on the same plate as the unknown samples.

The greatest problem with the method is that it is rather time and labor consuming and although the manufacturers of chromatographic instrumentation have introduced several kinds of semiautomated spot application and elution devices, the complete online automation of methods using TLC is practically impossible.

As a result of these factors, one can state that TLC separation and spot elution steps are still inevitable in many complex steroid analytical methods. Such methods will be used for a long time yet, mainly in laboratories with little instrumentation and where no great series of steroid samples are analyzed. As a result of the spreading of highly selective methods and TLC *in situ* techniques, the importance of the spot-elution techniques can be predicted to slowly decrease in the future.

b. In situ TLC Techniques

Direct analytical methods requiring no elution of steroid spots from the chromatogram have been long since used in steroid analysis. In the first period of use, however, the level of instrumentation available for quantitation did not permit reliable methods to be developed. In that time, spot-elution techniques were preferred. The recent great development in the field of instrumentation has, however, led to the publication of several papers dealing mainly with reflectance spectrophotometric, colorimetric, or fluorimetric quantitation of steroids without any spot elution.

In the case of UV-spectrophotometrically active steroids, not only the spot elution but also the visualization of the spots can be omitted by scanning the chromatograms in the reflectance mode at appropriate wavelengths. For example, ergosterol could be determined in mold mycelium by scanning the chromatogram of the unsaponifiable fraction at 282 nm.[131] When modern chromatogram spectrophotometers are used, even derivatives with rather low wavelength UV maxima can be detected and determined; e.g., 50 ng of cardiac glycosides with their UV maximum at 225 nm can be determined smoothly if the HPTLC technique is applied.[132]

More generally used are the methods based on staining the chromatogram with suitable reagents and scanning the chromatograms either in the reflectance or in the absorbance mode in the visible spectral range where simple and inexpensive instruments with filter photometers can also be used. For example, cholesterol can be separated by TLC from other serum lipids and visualized by staining with a phosphomolybdic acid-perchloric acid reagent and quantitated after scanning the chromatogram using a filter of 645 nm.[133] The direct densitometric method is suitable also for the estimation of steroid profiles. For example, Hara et al.[134] determined bile acids in bile after spraying the chromatogram with sulfuric acid.

The direct fluorimetric evaluation of the chromatograms after treatment with a suitable fluorogenic reagent offers the most sensitive method. For example, as little as 1 to 20 ng of digoxin can be separated and determined after treatment with hydrochloric acid vapors followed by irradiation with a UV lamp and fluorimetric scanning of the chromatogram (excitation 365 nm; emission 536 nm). This sensitivity is sufficient to use the method for the determination of digitoxin in human serum.[135] Similar sensitivity can be obtained for cholesterol and testosterone after treating the chromatogram at 120°C for 3 hr with ammonia liberated by heating ammonium hydrogencarbonate.[136] Another possibility for direct fluorimetric evaluation is to prepare the fluorescent dansyl derivatives prior to the TLC separation. Among others, this method enabled estrogens to be determined in urine in the nanogram range.[137]

If silica gel-coated glass rods are used to replace the TLC plates, the steroids separated can be directly scanned using a flame-ionization detector. This elegant technique introduced by Japanese workers[138,139] does not seem to be in general use.

The *in situ* techniques are naturally less labor consuming than the methods based on spot elution. The instrumentation required to obtain really reliable results and versatile applicability is rather expensive, and hence this technique has to be compared with gas- and high-performance liquid chromatography rather than with spot-elution thin-layer chromatography. Its great advantage compared to gas chromatography is that no derivatization is usually necessary and the least volatile and thermally unstable steroids can also be analyzed (these are naturally not advantages in comparison with high-performance liquid chromatography). Scanning of the chromatograms requires less time than in the case of GLC and HPLC, but the preparation of the sample is much more time and labor consuming. Hence, the introduction of this technique seems to be expedient in those laboratories only where great series of similar samples have to be analyzed. Otherwise, it is a method of choice only after that of GLC and HPLC, and its importance does not seem to match that of the latter techniques even with future developments.

H. Gas Chromatography

Since its introduction into the area of steroid analysis in 1960, gas chromatography has become one of the most important — if not *the* most important — method in both pharmaceutical and biomedical steroid analysis. The reasons for this are the following:

1. With the exception of a few derivatives all steroids can be separated and quantitated with or without preliminary derivatization.
2. The separation power of gas chromatography (especially of its most modern forms) is the highest among the chromatographic techniques, enabling 30 to 40 steroids to be separated and quantitated in a single chromatographic run.
3. The generally used flame ionization detector enables a few nanograms of steroids to be determined. This level can be decreased to the picogram range using an electron capture detector.
4. If gas chromatography (especially capillary gas chromatography) is used as the final step of the assay of complex mixtures, fewer cleanup procedures are usually necessary than in the case of other quantitative methods.
5. The capability of gas chromatography when coupled with mass spectrometry provides an extremely powerful method to steroid analysts. The identification and quantitation of the components of complex mixtures can be carried out using a single analytical technique.

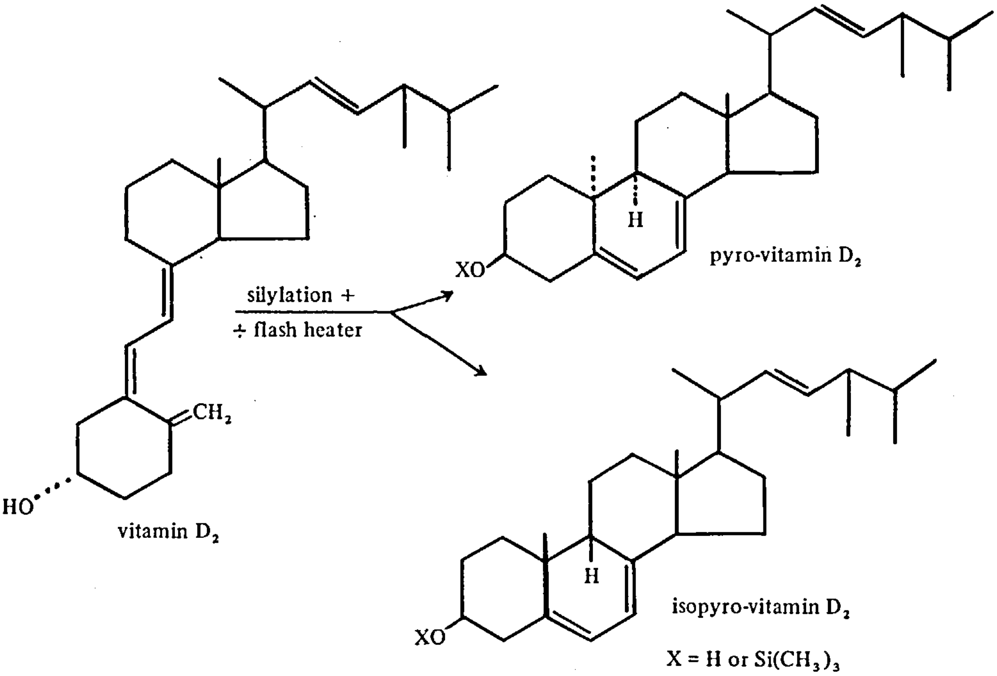
In addition to numerous articles, a fair number of books[140-145] and chapters[2,3,5-7] of books have been devoted to the gas chromatographic analysis of steroids and contain the fundamental knowledge on this field. Hence, the treatment of this matter in this review will be restricted to new developments in derivatization, capillary technique, and practical applications.

1. Derivatization Techniques

a. General Considerations

Many steroids can be chromatographed without derivatization. In spite of this, derivatization is still used in the majority of cases as the aim of derivatization is not only to increase the volatility and thermal stability of steroids, but also to introduce groups into the molecule which improve separation and enable an electron capture detector to be used. Another important point is to decrease irreversible adsorption on the column by derivatization. This does not cause serious problems in the case of microgram samples (pharmaceutical analysis), but does cause serious losses (such as total disappearance of certain peaks) when nanogram — picogram samples are chromatographed (biological-clinical analysis).

In certain cases, some thermally unstable steroids can be chromatographed without derivatization as long as the changes they undergo in the flash heater are unidirectional and quantitative. For example, Görög and Laukó[146] have found that 3-methoxy-2,5(10)-dienes quantitatively rearrange to the stable 3-methoxy-3,5-dienes and can be chromatographed in this form. D vitamins undergo thermal cyclization in the flash heater leading to the isomeric pyro and isopyro D vitamins. The measurement of the sum of the peak areas or the larger peak area is the basis of several gas chromatographic methods for the determination of vitamins D in various biological samples and pharmaceutical products.[147]



Changing the chromatographic conditions leads to the exclusive formation of the pyro derivative which has recently been described.[148] As a counter example, the problem of chromatography of corticosteroids with a 17,21-dihydroxy-20-keto side chain in the 17-position can be mentioned. These thermally unstable compounds are decomposed in the flash heater to 17-ketosteroids under given conditions, and this was claimed to be the basis of their determination.[142a] Later on, however, it was found[143a,144a] that no reliable methods could be based on this reaction as its conversion was only 20 to 50%. (The other decomposition products were likely to be more or less irreversibly adsorbed on the column).

b. Carboxyl Group

The carboxyl group occurring in bile acids, glucuronides, and etianic acids is the only functional group in steroids which must be derivatized prior to gas chromatographic assay. In the vast majority of cases, methyl ester formation using diazomethane as the esterifying agent has been applied even in routine clinical analysis. Recently, however, it was found by Shaw and Elliott[149] that using this derivatization technique, side reactions occurred at the hydroxyl groups. For example, in the case of chenodesoxycholic acid about 1% of 7α-O-CH_3 and 4% of 3α-O-CH_3 derivatives of the methyl ester are also formed. To avoid this, a 2,2-dimethoxypropane — hydrochloric acid reagent has been recommended by these and other workers, which in turn has the disadvantage that the reaction is much slower than with diazomethane.

c. Hydroxyl Groups

Steroids with one or, at the most, two hydroxyl groups can be chromatographed without treatment because of the reasons discussed above. However, derivatization is usually carried out in these cases as well, and this is almost obligatory in the case of tri- or polyhydroxy derivatives. If the primary aim of the derivatization is to improve the chromatographic properties of hydroxysteroids, then trimethylsilyl derivatives are formed almost exclusively. Depending on the silylating agent, solvent, and experimental conditions, the degree of silylation of polyfunctional derivatives can be controlled. When the aim is the persilylation of the molecule, then the most drastic reagents can be used. At present *bis*-trimethylsilyl acetamide (BSA), *bis*-trimethylsilyl trifluoroacetamide (BSTFA) and *N*-trimethylsilyl imidazole (TMSIM) can be regarded to be the most active silylating agents, especially if they are used together with trimethylchlorosilane (TMCS). A typical reaction adopted by Meunier[150] for the determination of urinary profiles is to react the mixture at 80°C overnight with a 3:3:2 mixture of TMSIM, BSA, and TMCS. If TMCS is replaced by trimethylbromosilane as the catalyst, even less reactive silylating agents, such as hexamethyldisilazane, ensures complete silylation of the least reactive hydroxyl groups.[151] A common problem, however, with the most active reagents is that they partially convert keto groups to their enol-trimethylsilyl derivatives. Thus, the keto groups have either to be protected, or the reaction made complete (see subsequent section).

In many cases, however, persilylation of the steroid molecule is not advantageous. Moreover, the selectivity of the method can be improved by making the silylation reaction selective to certain hydroxyl groups. As seen in Table 2 taken from the paper of Campbell et al.,[152] the use of BSTFA as the silylating agent without any solvent at 37°C overnight ensures silylation selective at 3α, 6α, and 7β positions and is more advantageous than the more drastic silylating agent (HMDS-TMCS-pyridine 3:1:9 for 30 min)[153] that produces persilylation. Using selective silylation, even hyodeoxycholic acid gives a well-resolved peak separated from deoxycholic and chenodeoxycholic acids, which is very difficult to achieve by the usual chromatographic systems.

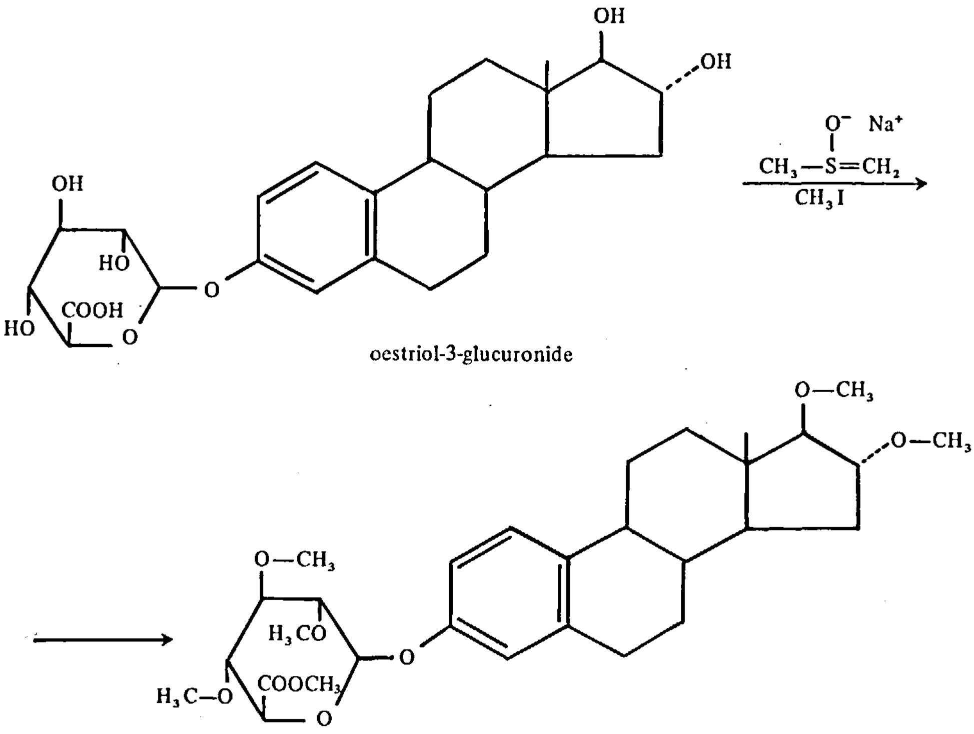
Other derivatization techniques employed to improve chromatographic properties are acetylation and methylation. The latter technique usually aims at producing methyl ethers selectively at the 3-phenolic hydroxyl group of estrogens. In addition to the classical methylation with dimethyl sulfate,[154] modern methylation uses techniques such as the extremely convenient on-column methylation with tetramethyl ammoniumhydroxide[155] and ion-pair phase-transfer methylation using methyl iodide as the methylating reagent in dichloromethane with tetrahexylammonium hydroxide as the phase-transfer catalyst.[156] The use of methylsulfinylmethide carbanion as the methylating agent merits special attention, as with this reagent permethylation of polyhydroxy steroids (both phenolic and alcoholic hydroxyls) can be attained, enabling polar derivatives such as the estrogen glucuronides to be chromatographed[157,158] (see the following structure). This is one of the rare occasions when steroid conjugates are gas chromatographed directly without preliminary enzymatic or chemical hydrolysis.

Table 2
RELATIVE RETENTION TIMES ON 3% QF-1 OF DIFFERENT BILE ACID METHYL ESTERS AS TRIMETHYLSILYL DERIVATIVES FORMED WITH DIFFERENT SILYLATING AGENTS[a]

Bile acid methyl ester	Hydroxyl position	Relative retention times HMDS-TMCS-pyridine	 BSTFA
Litocholic	3α	3.28	3.27
Hyodeoxycholic	3α, 6α	3.94	3.95
Deoxycholic	3α, 12α	3.54	6.29
Chenodeoxycholic	3α, 7α	3.77	7.04
12-Ketolitocholic	3α	10.79	10.78
Cholic	3α, 7α, 12α	3.72	13.87
-Muricholic	3α, 6β, 7β	4.89	7.90

Retention times relative to 5α-cholestane at carrier gas flow-rate of 20 ml/min.

From Campbell, R. L., Gantt, J. S., and Nigro, N. D., *J. Chromatogr.*, 155, 427 (1978). With permission.



Similarly, persilylation of both the steroid nucleus and the sugar moiety of cardiac glycosides enables these highly polar compounds of relatively high molecular weight to be chromatographed.[159] This is certainly one of the greatest achievements of steroid gas chromatography, but it remains — at least at present — without any practical importance since these derivatives can be separated and quantitated much easier by high-performance liquid chromatography.

If the aim of the derivatization of hydroxyl group is to make them suitable for being detected by the electron-capture detector then, usually, esterification with halogen-containing carboxylic acids is carried out. Of these derivatives, fluoro carboxylic acids have the most favorable chromatographic properties, but the electron-capturing properties of derivatives containing heavier halogens are much better. To improve the sensitivity towards the electron-capture detector, the number of fluorine atoms in the molecule has been increased up to eicosafluoroundecanoyl,[160] but the most frequently used derivatives in this field are the heptafluorobutyrates. Of the other derivatives, trifluoroacetates are also often used, and even the classical monochloroacetylation technique has found new application.[161]

Noncarboxylic derivatives are also used, although to a lesser extent, to introduce electron-attracting groups into the steroid molecule. Halomethyldimethylsilyl, especially iodomethyldimethylsilyl,[162] and pentafluorophenyldimethylsilyl[163] have been mentioned.

In the case of GC-MS, analysis one of the aims of derivatization is to improve the mass spectrometric properties of the materials to be separated and investigated. For this special purpose, the same derivatization is not necessarily as suitable as it would be for gas chromatography alone. For example, trimethylsilyl derivatives have acceptable characteristics, but the *tert*-butyldimethylsilyl derivatives, which are better and more stable towards hydrolysis, produce very intense M-57 peaks in their mass spectra which are extremely suitable for mass fragmentographic analysis.[164]

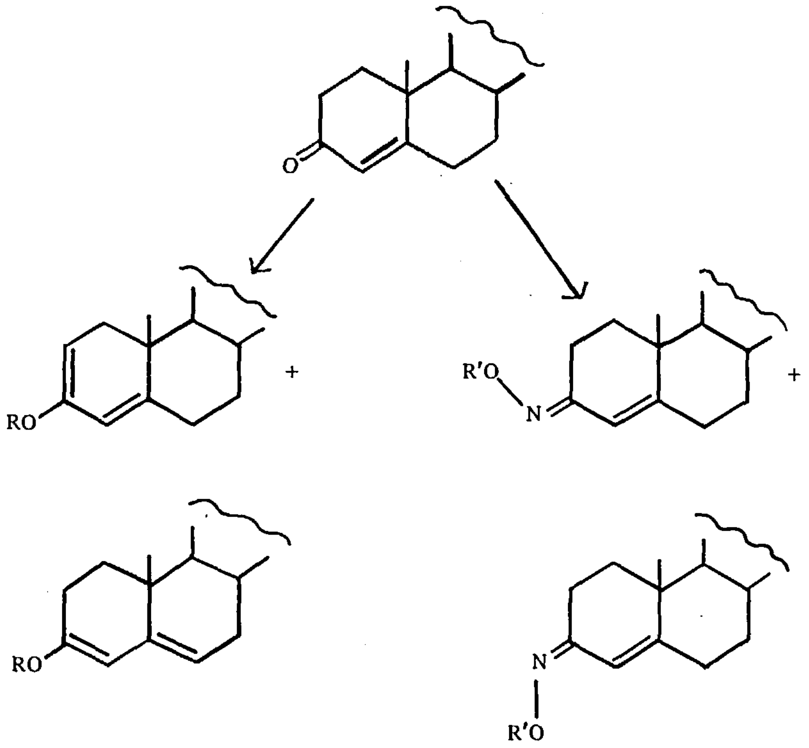
Several other derivatives have also been described for the derivatization of steroidal hydroxyl groups. It is almost impossible to judge which of them is superior. There are several factors which have to be taken into account, and it can be stated that no single derivative exists meeting all requirements. The most suitable derivatization reaction to solve a given problem has to be found separately for each case. One has to be very careful with new derivatization methods. For example, allyldimethylsilylation, which seemed to be a good reaction for GC-MS analysis,[165] has been found to be accompanied by an interfering side reaction leading to allylsiloxane derivatives. Hence, the advantage of these derivatives to the *tert*-butyl-dimethylsilyl derivatives — namely, that they are more readily formed even in the case of sterically hindered derivatives — is difficult to exploit.[166] Heptafluorobutanoyl-propenyl ether derivatives prepared by reacting axial hydroxyls with acetone followed by heptafluorobutyric anhydride excel because of their very high electron-capturing properties, but side reactions also decrease the applicability of this method.[167]

This may be the reason for the fact that new derivatives are nowadays seldom introduced. Moreover, of the several reagents described earlier, only a few seem to be in use. In the overwhelming majority of cases, trimethylsilylation or heptafluorobutyration is used.

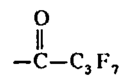
d. Keto Groups

With the exception of the 20-keto group of corticosteroids (which will be discussed separately in the subsequent section) the keto groups of steroids are not as responsible for the decomposition and irreversible adsorption as the hydroxyl groups. For this reason, the derivatization of the keto groups is not as important as that of the hydroxyl groups. Also, less versatile methods are available for the keto groups. No routine derivatization of keto groups is used in pharmaceutical analysis. However, in biological-clinical analysis where hydroxyl groups are often derivatized using the most reactive acylating or silylating reagents, keto groups are usually derivatized prior to the derivatization of the hydroxyl groups, mainly to avoid the partial silylation or acylation of the keto groups as the enol-silyl or enol-acyl derivatives.

The derivatization of the hydroxyl and ketone groups can be performed in one single step if the enolsilylation or enol-acylation reactions are made quantitative. For example, the trimethylsilylation and enol-trimethylsilylation can be carried out using *N*-methyl-trimethylsilyl-trifluoro-acetamide (MSTFA) together with potassium acetate as the silylating reagent,[168] and this reagent was successfully applied for the persilylation of urinary steroids to make them suitable for the capillary gas chromatographic profile analysis.[169] Similar enol-acylation reaction occurs when Δ^4-3-ketones are allowed to react with the same reagents as used for the most active acylating of hydroxyl groups.[170] As mentioned above, using heptafluorobutyration increases the sensitivity of response of the derivatives to electron-capture detectors, e.g., progesterone.[171] A disadvantage of the derivatization reactions based on the enolization of the keto groups is that in the case of Δ^4-3-ketones, two isomeric derivatives are formed, the ratio of which depends on the experimental conditions,[170] as is shown in the following structure.



R = Si(CH$_3$)$_3$


$$-\overset{\overset{\large O}{\|}}{C}-C_3F_7$$

R' = CH$_3$, C$_4$H$_3$, CH$_2$—C$_6$H$_5$
CH$_2$—C$_6$F$_5$, Si(CH$_3$)$_3$,
CH$_2$—CH$_2$—Cl

Methods much more frequently used are those where keto groups are transformed to oxime ether derivatives using O-substituted hydroxylamine derivatives as the reagents prior to derivatization of the hydroxyl groups. Although several other derivatives such as benzyl,[172] pentafluorobenzyl,[173] and 2-chloroethyl[174] have also been described (the latter two with electron-capturing properties), the originally described methoxime derivatives are still most frequently used. The methoxime — trimethylsilyl (MO-TMSi)[175-177] and methoxime — heptafluorobutyril (MO-HFB)[178] derivatives are prepared routinely in the determination of steroid profiles either by packed column or by capillary gas chromatography. However, in the case of Δ^4-3-ketones, two derivative (syn- and antioximes) are also formed with this reagent, as the preceding structure illustrates.

e. C-17 Side Chain of Corticosteroids

Attempts have been made to analyze corticosteroids from the very beginnings of gas chromatographic analysis of steroids. The number of publications dealing with the methodical problems of the GLC analysis of corticosteroids is undoubtedly the highest among the various groups of steroids, indicating that none of the numerous derivatization methods can be generally used. This is reflected also by the fact that no GLC methods seem to be in use for the analysis of bulk corticosteroids and pharmaceutical preparations made thereof. It is interesting to note that since the introduction of high-performance liquid chromatography for steroid analysis, the yearly output of papers dealing with the GLC analysis of corticosteroids has decreased considerably while at the same time, the relative amount of HPLC publications is higher in the corticosteroid field than in any of the other steroid groups (perhaps with the exception of cardiac glycosides). In fact, the HPLC method has several advantages compared to the GLC method for corticosteroid analysis, and use of the latter can be regarded as being expedient only in those instances where the greater sensitivity of the detection and the higher separation power of (mainly capillary column) gas chromatography are necessary to solve a particular problem.

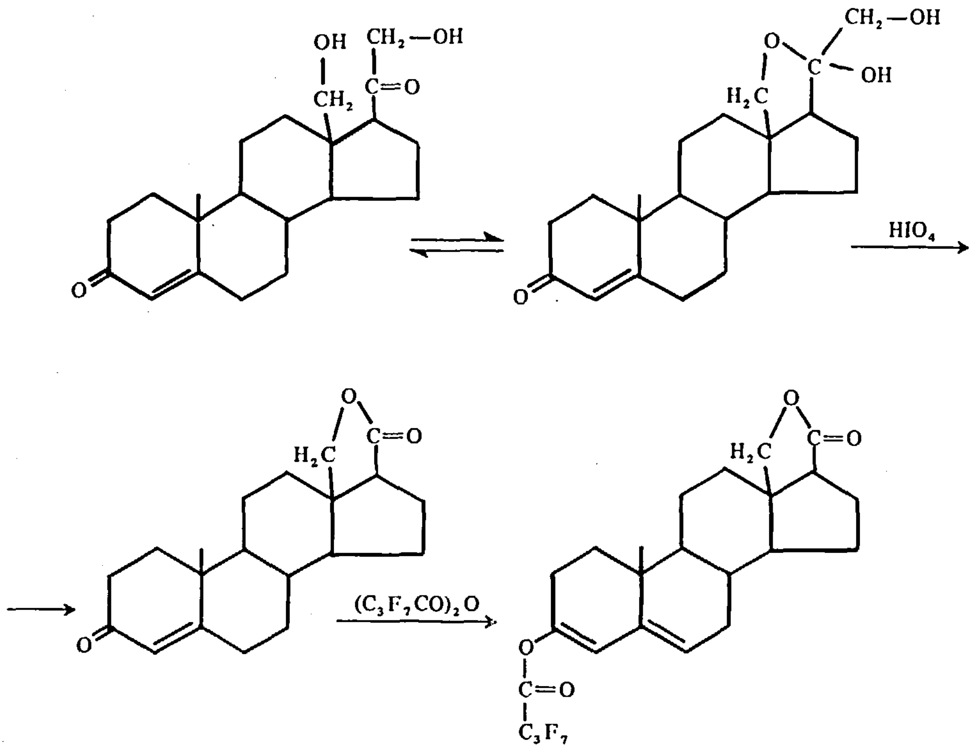
The standard monographs mentioned at the beginning of this chapter cover the fundamentals of the derivatization of corticosteroids. These fundamentals include on-column thermal degradation or oxidative splitting of the side chain and its transformation to esters, trimethylsilyl ether (TMSi) derivatives, 17,20,21-enol-persilyl derivatives, 20-methoxime-17,21-bis-TMSi derivatives, cyclic dimethylsiloxane or alkylboronate, and bis-methylenedioxy, etc. derivatives.

Of these methods, the formation of the MO-TMSi derivatives mainly by capillary column gas chromatography seems to be in general use even now for the determination of steroid profiles containing, among others, several corticosteroids. Another method is a modernized version of the classical procedure based on the oxidative splitting of the C-17 side chain. As, obviously, some selectivity is lost as a result of the oxidation step which reduces the number of the components, preliminary thin-layer or paper chromatographic separation of the individual components is usually necessary. The fractions eluted are subjected to oxidation and gas chromatography rather than the sample itself. Aldosterone and 18-hydroxy deoxycortone were oxidized with periodic acid,[179,180] while hydrocortisone and its 11-deoxy derivative were oxidized with chromium trioxide.[179] Enol-heptafluorobutyration of the oxidation products enables the electron capture detector to be used, decreasing the limits of the determination to the picogram range.

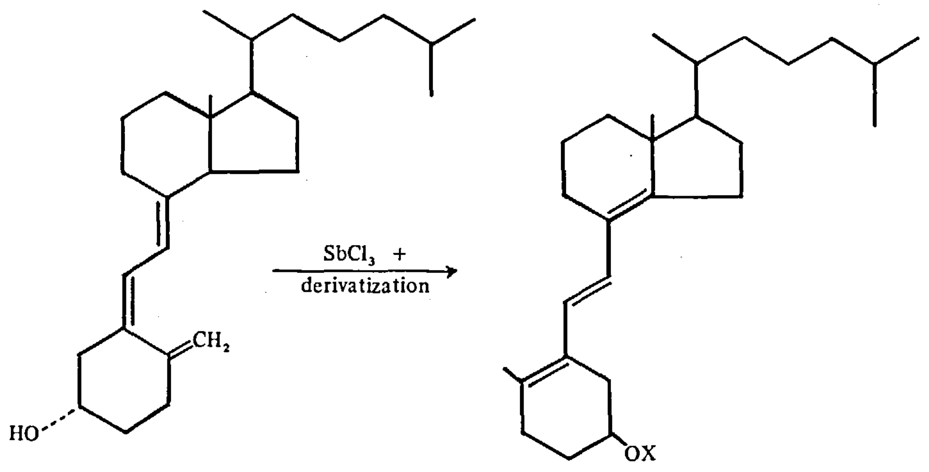
These and several other methods of this type are much more laborious than the profile methods using capillary columns. However, this seems to be unavoidable in the case of minor components of plasma, etc.

f. Miscellaneous Derivatization Techniques

The thermally unstable D vitamins can be transformed by various catalysts (anti-



mony trichloride, acetyl chloride) to the stable isotachysterols. The hydroxyl group is then derivatized in one of the usual ways (methylation,[181] heptafluorobutyration,[182] etc.). The advantage of this method compared to the on-column cyclization methods discussed above is that a single derivative is formed. The derivatives are well separated from the accompanying materials, and for this reason, this principle has found wide applications both in biological and pharmaceutical analysis. It is interesting to note that the reaction products of vitamins D_2 and D_3 are so well separated that they are often mutually used as internal standards for the others' determination.



vitamin D_3

isotachysterol$_3$

X = H, COC$_3$F$_7$, Si(CH$_3$)$_3$

The easiest way to determine the thermally unstable 4-ene-3-hydroxy or 3-acetoxy derivatives by gas chromatography is their degradation by hydrochloric acid catalysis to the stable 3,5-diene derivative. A great advantage of this principle is that this elimination reaction does not take place with saturated derivatives. Thus, they can easily be separated from the Δ^4 derivatives.[183]

2. *Applications of Capillary Columns*

Although the first papers dealing with capillary column gas chromatography of steroids were published about 10 years ago, various technical problems associated with the preparation of suitable columns has precluded widespread use of this technique until only recently. The spread of the use of the capillary column method is undoubtedly the most important develoment in the GLC analysis of steroids in the last 10 years.

The greatest advantage of capillary columns compared to packed columns is that the number of theoretical plates is about 10 to 50 times greater. For this reason, 20 to 30 steroids can be separated in a single chromatogram from each other and from a number of unidentified minor peaks. This makes this technique extremely useful, mainly in the determination of steroid hormone profiles of urine, plasma, etc.[175] The application to sterols,[184] D vitamins, and related materials,[185] as well as to bile acids,[186] have also been well documented. Figure 5 is taken from the paper of Pfaffenberger and Horning[175] and shows a typical capillary column gas chromatogram.

Up until the last few years, glass capillaries and injectors suitable for sample introduction were usually prepared by the chromatographers themselves. This caused considerable difficulties in comparison with packed column gas chromatography and the use of this technique was reasonable only in those cases where its extremely high resolution power was actually prerequisite for obtaining reliable results. This is certainly the reason for the fact that the capillary technique does not seem to have been used in pharmaceutical steroid analysis. As a fair variety of capillary columns and injectors are now commercially available, greater use of the technique is to be expected in the future.

3. *Practical Applications*

Gas chromatography has found wide application in pharmaceutical steroid analysis for the purity determination of bulk steroids and for the assay of formulations. This is reflected by the fact that several gas chromatographic methods have been included even in pharmacopoeias.[10-12] Gas chromatography is especially useful in the analysis of steroid combinations where without it, the often very different chemical and pharmacological components would have to be analyzed individually using separate analytical techniques for each component. To illustrate this, the chromatogram of a contraceptive pill containing gestogenic and estrogenic components taken from the paper of Moretti et al.[187] is shown in Figure 6. The application of gas chromatography to single component formulations can be justified by the high sensitivity and selectivity of the method enabling stability and single-tablet assays to be carried out in most instances.[14]

The main field of application of gas chormatography in steroid analysis is the determination of hormones, cholesterol, bile acids, and D vitamins in urine, blood, and other biological samples. For the assay of urine, where steroids are usually present at higher concentrations, gas chromatographic methods in general can be applied, while the determination of very low concentrations of steroids in blood is a very delicate task which can be solved only with the aid of the ultrasensitive ECD or mass spectrometric detectors. It requires special derivatization, as described in Section H.1.b above. For the solution of problems of the former type, several other methods can also be used such as colorimetry or fluorimetry (especially combined with chromatographic

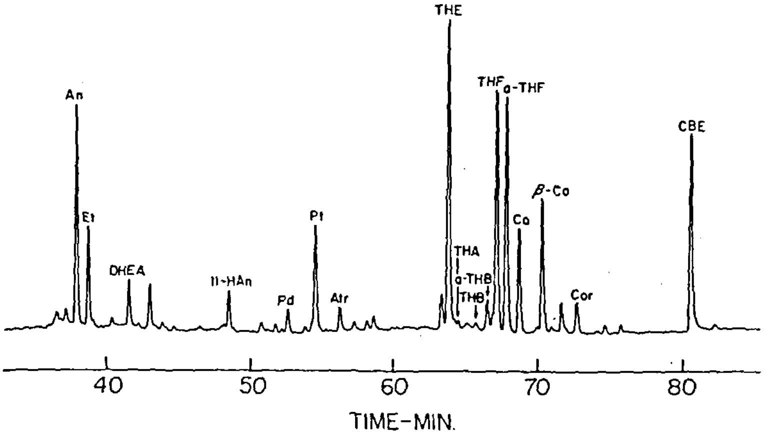


FIGURE 5. Urinary steroid metabolic profile of a premenopausal female determined by capillary gas chromatography using 60 m SE-30 glass capillar, temperature programmed at 1° C/min from 200°C. Steroids as the MO-TMSi derivatives. An, androsterone; Et, ethiocholanolone; DHEA, dehydroepiandrosterone; Pd, pregnanediol; Pt, pregnanetriol; Atr, 5-androsten-3β, 16α, 17β-triol; THE, tetrahydrocortisone; THA, tetrahydro-11-dehydrocorticosterone; THB, tetrahydrocorticosterone; aTHB, allo-THB; THF, tetrahydrocortisol; a-THF, allo-THF; Co, cortolone; β-Co, β-cortolone; Cor, Cortol; and CBE, cholesteryl butyl ether (internal reference compound). (From Pfaffenberger, C. D. and Horning, E. C., *Anal. Biochem.* 80, 329(1977). With permission.)

separation including HPLC, etc.). The only real competitors to the latter cases are nuclear methods — competitive protein binding and radioimmunoassay. Several papers deal with the comparison of gas chromatography and mass fragmentography with these methods for the solution of a given problem.[188] The conclusion is usually that both methods can successfully be applied.

In most of the cases of using gas chromatography for the determination of steroids in biological samples, the gas chromatographic determination is preceded by a long procedure. Very often, the gas chromatographic step itself is the least time and labor consuming among the steps of this complex procedure, which usually include enzymatic and/or chemical hydrolysis of the conjugates, one or more extractions and column chromatographic steps (usually on Sephadex® LH-20 or its derivatives), and derivatization (occasionally TLC purification before and/or after the derivatization step). In general, blood samples require longer pretreatment than urine samples, and the preparation of the sample for gas chromatographic assay can greatly be reduced by using capillary columns. Accurate analyses of biological samples can only be performed if the recovery of the separation is checked by using radioactively labeled steroids as indicators.

I. High Performance Liquid Chromatography (HPLC)

1. The Relations Between HPLC and Gas Chromatography

Almost exactly 10 years after the beginning of the new period created by the introduction of gas chromatography into the steroid analysis, the first papers on the use of HPLC in this field were also published. Since then, HPLC has become a very important standard method in steroid analysis. If gas chromatography had not been introduced earlier, the importance of HPLC would be even greater in the present situation. As it is, the two methods complement each other well in the steroid field. When a comparison of the possibilities of the two methods is made, steroids can be classified into four groups.

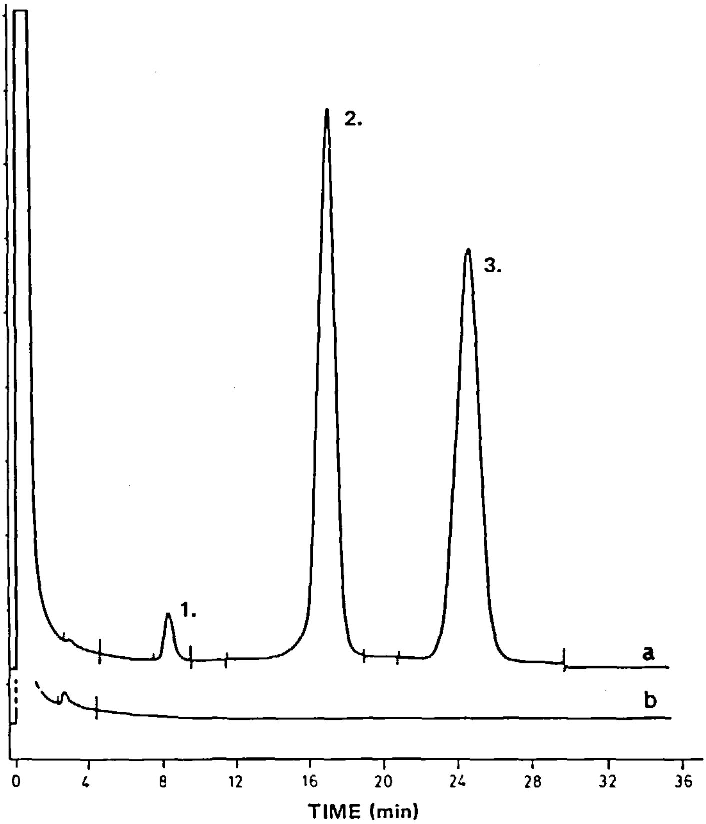


FIGURE 6. Gas chromatographic assay of a contraceptive pill. (100-cm × 2-mm column packed with 4% XE-60 on Chromosorb® W, AW, DMCS, column temperature 220°C.) Curve a, chromatogram of the tablet extract: (1), mestranol; (2), norethisterone; and (3), testosterone propionate (internal standard). Curve b, chromatogram of the extract of a placebo tablet. (Courtesy of Moretti, G., Cavina, G., Chiapetta, G., Fattori, I., and Pompi, V., *Boll. Chim. Farm*, 116, 463 (1977). With permission.)

1. In the case of those steroids whose low volatility and/or instability precludes the use of gas chromatography, HPLC offers a unique opportunity for the separation and quantitation of these derivatives which include most of the conjugates of steroid hormones, conjugates of bile acids, and some of the heat-sensitive drugs.
2. With some of the important groups of steroids such as cardiac glycosides and, to some extent, corticosteroids and D vitamins, gas chromatographic analysis can be carried out only with difficulty. One encounters such problems as tedious derivatization reactions, incomplete recovery, and barely reproducible methods which in most cases can easily be eliminated by the use of HPLC. Hence, the use of this method with these groups is certainly advantageous.
3. For most of the steroid hormones, gas chromatography and HPLC can be used with equal ease for the solution of most analysis problems. The greater resolution power and higher sensitivity of gas chromatography makes this method more suitable, for instance, where these particular features of the analytical method are of great importance such as ultramicro analysis of complex steroid mixtures. In most other cases, however, the use of both methods has been well documented,

and it is left to the analyst to decide which seems more suitable for the solution of a given problem.

4. Although in principle HPLC can be used for the separation and quantitation of all classes of steroids, in some cases the above-discussed advantages of gas chromatography seem to be so important that HPLC does not seem to have been generally accepted. These include free and esterified sterols, free or total bile acids, diosgenin and related sapogenins, etc. although on the basis of recent trends, the importance of HPLC is expected to increase in the near future in these fields, too.

No specific book has yet been devoted to the HPLC of steroids. Comprehensive chapters in general monographs, however, present sufficient material mainly for the first period of this very young, but very important field[2,5,7,117]

2. *HPLC Without Derivatization*

High performance liquid chromatograms of steroids are almost exclusively monitored by an UV detector. This is possible as the overwhelming majority of steroids possess sufficiently strong UV-absorbing groups such as the Δ^4 or $\Delta^{1,4}$-3-keto groups in androgens, gestogens, and corticosteroids; the α,β-unsaturated lactone group in digitalis glycosides; the 3,5- and 5,7-diene groups in various derivatives and D vitamins, respectively; and the aromatic ring A in estrogens, etc. Derivatives with a single isolated double bond only (sterols, sapogenins, etc.) can be detected with sufficient sensitivity at about 210 nm. Derivatives lacking even one double bond can be determined by means of the refractive index detector or by using derivatization reactions.

For chromatographic systems in use, both adsorption chromatography and reverse phase partition chromatography using almost exclusively chemically bonded stationary phases have been well documented, and any of these systems can be used for the solution of most of the problems.[189,190] For the separation of acidic derivatives and conjugates, ion-exchange columns have also been used.[191]

Of the several applications of HPLC to the analysis of sex hormones, two recent papers both solve the delicate problem of the separation of estrogen conjugates. As seen in Figure 7, van der Wal and Huber[192] separated eight conjugates within 15 min using a 100 × 4 mm Lichrosorb® RP 8 column and 40% methanol + 60% 0.1 *M* $NaClO_4$ + 0.05 *M* phosphate buffer, pH 8 at 70°C. A separation of similar quality was achieved by Musey at al.[191] using a strong anion exchanger (Partisil 10 SAX) and aqueous buffers in the pH range of 4 to 5. The delicate problem of single-tablet assay of low-dosage tablets containing ethinyl/estradiol and gestogens (ethisterone or norgestrel) was solved by Bagon and Hammond[193] using reverse-phase separation and monitoring at 212 nm.

Of the numerous applications to corticosteroids, three illustrate the use of HPLC. Loo et al.[194] separated the main corticosteroid drugs using adsorption chromatography on silica gel and applied this method for the separation and quantitation of less than 1 μg/m*l* of prednisolone in plasma. For the determination of triamcinolone acetonide in topical formulation, a reverse-phase system was used by Gordon and Wood[195] while Wikby et al.[196] compared straight- and reverse-phase partition systems for the separation of the C-22 epimers of the 16 α-hydroxyprednisolone 16,17-cyclic propylacetal. The reverse-phase system using μBondapak C_{18} and the straight-phase system using μBondapak -NH_2 were found to provide the best separation.

Chromatograms of the butenolide ring of cardiac glycosides have to be monitored at 220 nm, and the sensitivity of the measurement is not very high. Thus, most of the papers dealing with the application of HPLC to this group of steroids with UV detec-

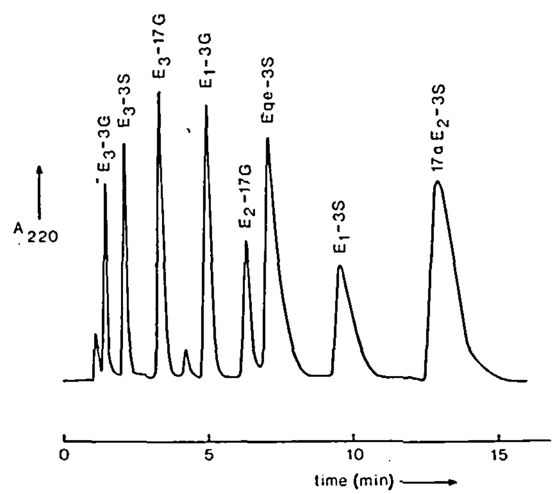


FIGURE 7. HPLC separation of estrogen conjugates. (Column: 10 cm × 4 mm packed with 5 μ Lichrosorb® RP-8. Eluent: 40 % methanol + 60% water containing 0.1 *M/ℓ* $NaCl_4$ and 0.05 *M* phosphate buffer, pH 8, 70°C.) E_3G, estriol-3β-D-glucuronide; E_3-3S, estriol-3-sulfate; E_3-17G, estriol-17β-D-glucuronide; E_1-3G, estrone-3β-D-glucuronide; E_2-17G, estradiol-17β-D-glucuronide; Eqe-3S, equilenin-3-sulfate; E_1-3S, estrone-3-sulfate; and 17β-E_2-3S, 17α-estradiol-3-sulfate. (Courtesy of van der Wal, S. and Huber, J. F. K., *J. Chromatogr.* 149, 431 (1978). With permission.)

tion have detection limits of 10 to 100 ng of cardiac glycosides. Of the numerous papers on the subject, that of Erni and Frei[197] most nicely illustrate the comparison of adsorption and reversed-phase partition chromatography. Both systems can be successfully used, as seen, for example, in Figure 8. The separation of aglycones is excellent using reversed-phase chromatography. However, for the separation of the members of, for example, the lanatoside C series, the use of adsorption chromatography is more advantageous.

In the vitamin D field, direct HPLC analysis is suitable to solve the two main problems, namely the determination of D vitamins in complex pharmaceutical mixtures and their hydroxylated metabolites in biological fluids. The absorption band of the conjugated double bond around 265 nm enables sufficiently sensitive detection. These methods can by all means be regarded as superior to gas chromatographic procedures based on double derivatization or on column transformation (see the preceding section). To illustrate the usefulness of HPLC in the pharmaceutical field, the paper of Hofsass et al.[198] is mentioned. It describes the HPLC determination of D vitamins and related substances in resins, oils, and dry concentrates in the presence of vitamin A using a silica gel column and 1.6% ethanol in isooctane as the eluent. The determination of vitamin D in milk was also carried out by using adsorption HPLC (eluent 0.6% isopropanol in hexane) and preliminary fractionation on a hydroxyalkoxypropyl Sephadex® column.[199] HPLC, as described by Stryd and Gilbertson[200] is suitable also for the determination of a few ng of 25-hydroxy-D vitamins in 1 mℓ of serum. Figure 9 shows the chromatogram of a serum extract after prefractionation on a silica gel golumn. It is rather common in the HPLC analysis of D vitamins that vitamins D_2 and D_3 are mutually used as internal standards for the determination of the other.

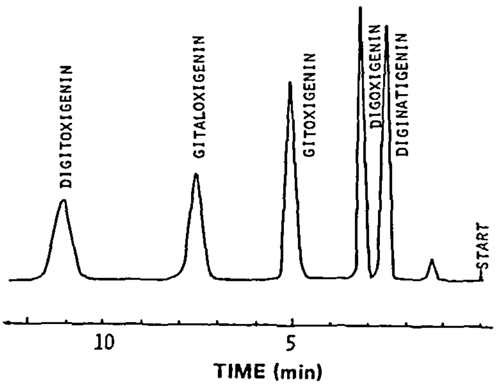


FIGURE 8. Reversed-phase HPLC separation of cardiac aglycones (Column: 30 cm × 3.5 mm packed with Nucleosil® C_{18} 10 μ. Eluent: 37% acetonitrile in water; λ = 220 nm.) (1) Diginatigenin; (2) digoxigenin; (3) gitoxigenin; (4) gitaloxigenin; and (5) digitoxigenin. (From Erni, F. and Frei, R. W., *J. Chromatogr.* 130, 169 (1977). With permission.)

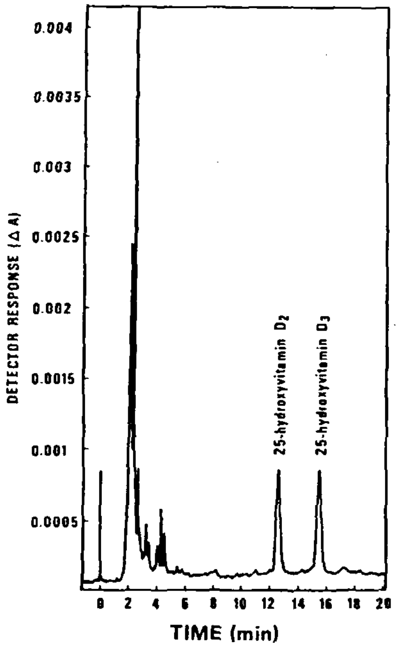


FIGURE 9. Separation of serum 25-hydroxyvitamins D_2 and D_3 (25 ng each) by HPLC after purification of the sample by silica gel column chromatography. (HPLC column: 25cm × 4.6 mm packed with Partisil® PXS silica 5μ. Eluent: hexane-ethanol 97.5:2:5; 254 nm at 0.005 aufs.) (1) 25-OH-vitamin D_2; (2) 25-OH-vitamin D_3. (From Stryd, R. P. and Gilbertson, T. J., *Clin. Chem.*, 24, 279 (1978). With permission. Copyright 1978 *Clinical Chemistry*.)

Bile acids can be detected below 210 nm only[210] and for this reason, the refractive index detector is more generally used in their HPLC analysis.[202,203] As gas chromatography has much greater resolution power in this field and its sensitivity is also much higher, the use of HPLC is reasonable in those cases only when the task is the separation and quantitation of the taurine and glycine conjugates of the individual bile acids which cannot be analyzed by chromatography. For this purpose, reversed-phase chromatography using a C_{18} silica column and methanol-aqueous acetate buffer (pH 4.7)[203] or isopropanol-ethyl acetate-aqueous ammonium hydroxide[202] as the eluents is recommended.

HPLC is even less important in the analysis of sterols. Although the use of a refractive index detector or the derivatization of their 3-hydroxy group in order that the UV detector can be used have provided sufficient means for their determination,[204] their gas ghromatographic determination is better.

Unsaturated sterols can be detected at 210 nm, too. This has been exploited by Gorog and Herenyi[205] for the determination of the epimeric 3α-acetoxy impurity in the gestogenic drug ethynodioldiacetate on the basis of their Δ^4 double bonds.

3. HPLC with Derivatization

The importance of derivatization in HPLC (which is precolumn derivatization in almost all cases) is much smaller than in the case of gas chromatographic analysis of steroids. While in the latter field there are various purposes of using derivatization (improvement of the volatility and thermal stability, increasing the selectivity and sensitivity of the detection, and improvement of the separation) in HPLC analysis of steroids, the only major purpose of derivatization is to introduce chromophores into the steroid molecules in order to enhance the applicability of the UV detector. As a result of the derivatization reaction, UV-inactive steroids (saturated sterols, etc.) can also be detected, and the spectrum of those which could be detected at 210 nm is shifted towards longer wavelengths where the detection is more convenient (i.e., a greater variety of solvents and the single wavelength detector working at 254 nm can be used). Using suitable derivatization reactions, the molar absorptivity of weak UV-absorbing steroids can be increased by 1 to 2 orders of magnitude, thus increasing the sensitivity of the determination. The effect of the derivatization on the resolution of peaks is usually not a question of primary importance and the authors of most of the papers on this subject content themselves with stating that the separation is not affected by the derivatization (in some cases improvement or deterioration have also been described).

An interesting exception to this general statement is described in the paper of Ikekawa and Koizumi[206] dealing with the HPLC separation of hydroxylated matabolites of vitamin D_3. While the epimeric pair 24R,24-$(OH)_2$-D_3 and 24S,25-$(OH)_2$-D_3 could not be separated using a silica gel column and 2% methanol in dichloromethane as the eluent, the same chromatographic system is suitable for their separation if the hydroxyl groups are transformed to their trimethylsilyl ethers.

Of the functional groups of steroids, hydroxyl groups are most frequently derivatized. Fitzpatrick and Siggia[207] introduced the benzoxylation of hydroxy steroids by benzoyl chloride in a pyridine medium leading to benzoates with absorption maximum at 230 nm (ε of 12,000 to 13,000). This method followed by reversed-phase separation was applied to sterols by Rees[204] and to sapogenins by Higgins.[208] Using *p*-nitrobenzoylation results in a further bathochromic and hyperchromic shift (λ_{max}260 nm, ε 14,800). This possibility was exploited by Nachtmann et al.[209-211] by applying the reaction to the determination of cardiac glycosides. As under the reaction conditions adopted all hydroxyl groups of the steroid nucleus and the sugar moiety (with the exception of the sterically hindered 14-hydroxyl group) react with *p*-nitrobenzoyl chloride, exceptionally high sensitivity can be attained (The molar absorptivity of a derivative containing seven reactive hydroxyl groups is approximately 10^5.) The separation of these derivatives is also extremely good: using a silica gel column and a 30:10:9 mixture of *n*-hexane, chloroform, and acetonitrile as the eluent, 10 to 15 components can be separated within 10 to 15 min (see Figure 10).

The reaction of phenolic hydroxyl group of estrogens with dansyl chloride (5-dimethylamino-1-naphtalene-sulfonyl chloride) enables these compounds to be detected at as low a concentration as 50 pg per injection. Using an adsorption column, the separation of the dansyl derivatives is also excellent (estradiol valerate, estrone, ethinyl estradiol, β- and α-estradiol could be separated within 15 min by Roos[212]).

The other functional group in steroids which is easy to derivatize to form highly absorbing products is the ketone group. Although the derivatization of ketosteroids as the 2,4-dinitrophenyl-hydrazones was described at the beginnings of steroid HPLC,[213] this reaction has been seldom used (separation of the epimers of androsterone and dehydroandrosterone[214], and determination of conjugated and esterified estrogens in dosage forms[215]).

All the above-discussed methods have been precolumn derivatization procedures. In the following, two interesting postcolumn derivatization methods are shown. On the basis of experiences with other compounds and the growing interest in this field, the importance of this technique can be expected to increase in the near future.

Gfeller et al.[216] separated cardiac glycosides using reversed-phase chromatography and passed the effluent to an AutoAnalyzer where after mixing with concentrated hydrochloric acid and dehydroascorbic acid, the detection and quantitation was performed fluorimetrically (excitation 350 nm, emission 485 nm), enabling the detection limit to be reduced to 0.5 ng. van der Wal[217] used a modified Kober reaction involving the mixing of 75% sulfuric acid with the column effluent containing estrogen conjugates of human pregnancy urine. The reaction product was monitored fluorimetrically (excitation 535 nm, emission 561 nm).

4. *Practical Applications*

As the level of the lowest steroid quantity detectable by HPLC is under 1 μg in all cases, and in advantageous cases can easily be decreased to the subnanogram range, and the resolution power of HPLC is equal or almost equal to other chormatographic techniques, HPLC can be successfully used for the solution of the majority of the analytical problems encountered in the analysis of steroids.

Within a short time HPLC has become a standard method of pharmaceutical steroid analysis. The main types of problems associated with this technique are purity control of bulk steroids with detection and quantitation of impurities down to the 0.05% level[218], determination of steroids in oil-injectables,[219,220,223] corticosteroid phosphates in aqueous solution,[221] creams and other topical formulations,[195,222,223] and assay of various tablet dosage forms, [218,223-225] among them low-dosage contraceptive pills.[193] The extremely rapid spread of HPLC in the analysis of steroid drugs can be illustrated by the fact that not more than 5 to 6 years after the appearance of the first publications, this method became official in USP XIX[10] for the assay of triamcinolone acetonide cream and triamcinolone hexacetonide suspension. As a consequence of the high resolution power of the method, it is extremely suitable for the separation of the decomposition products of the active components and is therefore an ideal method for the stability assay of the above-listed formulations. The usefulness of HPLC in the separation and quantitation of isomeric pairs of steroids should also be emphasized.[224,196,205]

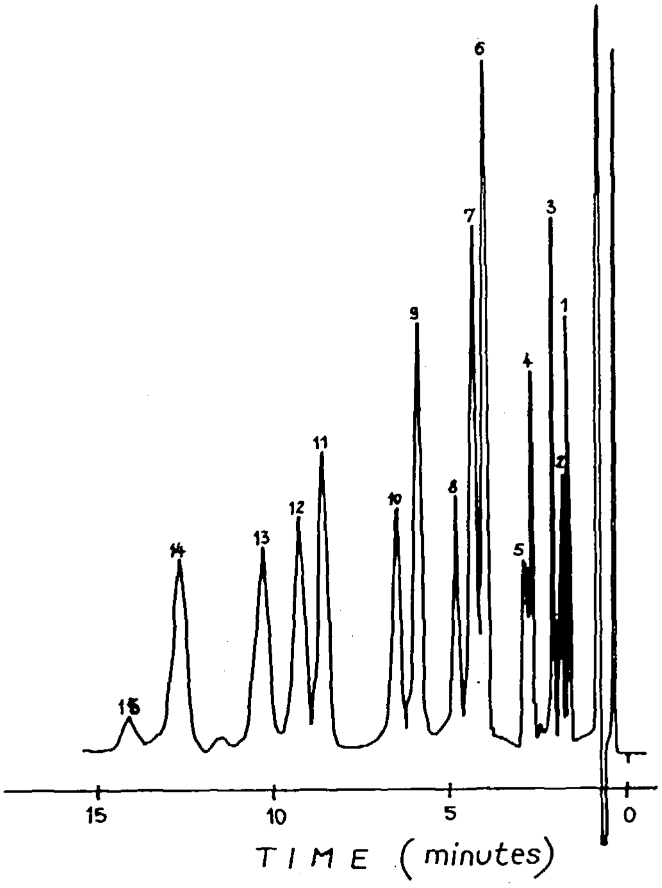


FIGURE 10. HPLC separation of the 4-nitrobenzoyl derivatives of digitalis glycosides and aglycones. (Column: 15 cm × 3 mm SI 60 5μ silica gel column. Eluent: hexane-chloroform-acetonitrile 30:10:9; 254 nm.) (1) Gitoxigenin; (2) digitoxigenin; (3) diginatigenin; (4) digoxigenin; (5) gitaloxigenin; (6) gitoxin; (7) digitoxin; (8) diginatin; (9) digoxin; (10) gitaloxin, (11) lanatoside B; (12) lanatoside C; and (15) lanatoside E. (From Nachtmann, F., Spitzy, H., and Frei, R. W., *J. Chromatogr.*, 122, 293 (1978). With permission.)

Another field where HPLC has been successfully used and where its importance is growing rapidly is the determination of steroids in biological samples. This does not cause any major problem in those cases where the concentration of the steroid is relatively high such as the determination of estrogens in pregnancy urine,[226] progesterone in rat ovarian homogenate,[227] etc. Especially useful is the application of HPLC for conjugates, e.g., the determination of estrogen conjugates in plasma[191] and bile acid conjugates in bile,[201-303] which could not be analyzed by gas chromatography. Rather delicate problems have to be solved when hydroxylated metabolites of D vitamins are determined in blood.[200]

Perhaps the most important field of application of HPLC is the determination of corticosteroids in biological fluids, as gas chromatography is suitable for their determination only after rather problematic derivatization procedures. The statement of de Vries et al.[228] is characteristic of the situation. These workers had several years experience in the routine clinical assay of urinary aldosterone by gas chromatography. After having tried HPLC they stated: " . . . HPLC in aldosterone assay in urine appeared to be more precise and more sensitive than GLC. In addition, the conversion of aldosterone into a stable derivative, required for GLC, is avoided."[228]

As the detection of unsaturated ketosteroids by a UV detector is rather sensitive, corticosteroids down to the concentration of 1 μg/100 mℓ of biological fluid can be assayed without derivatization or any serious preconcentration. This was used for, among others, the determination of cortisol in plasma[229] and urine,[230] prednisolone and other synthetic corticosteroids in plasma,[194,231] etc. Further important applications of HPLC are the determination of D vitamins[199,232] and hormones[233] in food, cardiac glycosides in plants,[234] etc.

J. Radioisotope Methods

1. Nonsaturation Methods

As a consequence of the high selectivity and sensitivity of analytical methods involving the use of radioactively labeled steroid derivatives, have long been fundamental methods of clinical and biochemical steroid analysis. Since in the past decade the overwhelming majority of interest has turned towards protein-binding methods (mainly radioimmunoassay), only a short mention is made of other methods in this section.

Before discussing the specific radioisotope methods for the assay of steroids, it has to be mentioned that in those cases when a single steroid has to be determined in a complex biological mixture and the assay procedure involves the use of several isolation and clean-up steps (extraction, column, thin-layer, or paper chromatography) reliable results can only be obtained if the losses during these steps can be determined and the final result corrected accordingly. The most convenient way to determine this correction factor is to add a radioactively labeled derivative of the steroid to be determined to the sample and measure the decrease of the radioactivity during the clean-up procedure.

The use of this technique to monitor the column chromatographic separation of steroid mixtures has been especially frequent. One of the many examples is the HPLC determination of the 26-hydroxylated metabolite of vitamin D in serum described by Gilbertson and Stryd.[235] In this method the HPLC assay is preceded by an extraction and silicic acid column chromatographic step. Tritiated 25-hydroxyvitamin D is added to the sample before the extraction step and the recoveries of the two clean-up steps are estimated by measuring the fraction of the column effluent to be analyzed by HPLC using liquid scintillation counting.

In addition to these applications, where radioactivity measurements are used only as supplementary methods in association with other quantitative procedures, various nonsaturation-type quantitative radioactive derivative methods have also been widely used in biological-clinical steroid analysis. As the importance of these methods has been greatly reduced by the rapid spreading of the saturation type methods to be discussed in the subsequent two sections, only the "double isotope method" will be described here briefly.

In this method as many radioactively labeled steroids are added to the sample as the number of the steroids to be determined, and occasionally (after suitable TLC or column chromatographic fractionation), the fractions to be analyzed are reacted with a radioactively labeled reagent which adds another kind of radioactive label to the molecule. After thorough chromatographic separation of the derivatized steroids to be determined, the ratio of the two kinds of radioactivity is determined. With the aid of the ratios measured on sample and standard, the concentrations of each steroids can then be calculated. Of the voluminous literature in this method, two reviews are mentioned dealing with the determination of corticosteroids[3a] and estrogens,[2a] respectively.

In the case of the former, the indicator steroids were the p-^{125}I-benzenesulfonates of the materials to be determined, while the derivatization was carried out by p-iodobenzenesulfonic anhydride-^{35}S, and the ratio of $^{125}I/^{35}S$ was measured. The latter paper reports on methods where the indicator compounds are tritiated estrogens, while the derivatization reaction is acetylation of the hydroxyls with ^{14}C-labeled acetic anhydride.

The advantage of this type of method is high specificity. The sensitivity, however, is much lower than that of the other radioisotope methods to be discussed in the subsequent two sections. In addition, these methods are very time and labor consuming and difficult to automate. For this reason, new double-isotope methods have been rather seldomly published in the recent years. One of the few examples is a paper reporting on the determination of corticosteroids in the placenta.[236] Here, 0.004 to 2.4 μg/g of deoxycorticosterone, 11-dehydrocorticosterone, corticosterone, cortisone, cortisol, aldosterone, and progesterone were determined from 250 to 280 g of placenta tissue using tritiated corticoids as the indicator compounds. The condensation was with ^{35}S-labeled thiosemicarbazide as the derivatization reaction, followed by several extraction and chromatographic separation steps and enzymatic hydrolysis to differentiate between free and conjugated corticosteroids.

2. *Competitive Protein Binding Methods*

Competitive protein binding methods based on the more or less specific binding of the individual steroids to the active sites of suitable proteins can be used for their ultramicro determination if their radioactively labeled analogues are added to the sample to be analyzed. The assay is based on the equilibrium between unlabeled and labeled steroids on the active sites of the protein, the concentration of the radioactive derivative in the supernatant liquid being directly proportional to the concentration of the steroid to be determined in the sample.

The sensitivity of this method is very high, enabling a decrease in the quantity of the steroids to be determined down to the picogram level. The specificity of the method is determined by the specificity of the protein. This is usually not good enough to enable a steroid to be determined in the presence of structurally closely related derivatives. For this reason, in the majority of cases a preliminary chromatographic separation step is used to increase the selectivity. For the solution of less delicate problems, however, several nonchromatographic methods have also been published.

Although competitive protein binding methods have been worked out for the determination of various types of steroids, mainly hormones, their importance has decreased in the past few years in these fields because of the spread of the even more specific radioimmunoassay methods. In those fields, however, where no radioimmunoassay methods are available, the competitive protein binding methods are the most sensitive and, if combined with preliminary chromatographic separation, they are the most specific methods suitable for both biochemical research and routine clinical analyses. A characteristic field is the determination of D vitamins and their metabolites in blood.

Of the great number of papers dealing with this subject, that of Mason and Posen[237] describes the determination of 25-OH-D_3 in human serum. The assay is carried out using 1 mℓ of serum (containing about 30 ng of 25-OH-D_3). It involves extraction with ether, column chromatography on silica gel (checking the efficiency of these steps by using tritiated 25-OH-D_3), incubation with rat kidney cytosol (binding protein) and tritiated 25-OH-D_3 in phosphate buffer, centrifugation of the precipitate using dextran-coated charcoal suspension as the separation aid, and counting the supernatant liquid using a liquid scintillation counter. A similar procedure was used by Kremer and Guillemant[238] for the determination of the 24,25-$(OH)_2$ metabolite. As is seen in Table 3, rat kidney cytosol used as the binding protein in this study, too, is sufficiently selective towards nonmetabolized D vitamins and sterols. On the other hand, however, the binding of the 25-OH and the 24,25-$(OH)_2$ metabolites is the same. Preliminary separation of the two metabolites (using Sephadex® LH-20) is necessary if the aim of the assay is to obtain values for the concentration of the individual metabolites.

3. Radioimmunoassay

As the last section of this review, radioimmunoassay (RIA), the method that has revolutionized the clinical analysis of steroids, is briefly described. Although the methodology of this technique is much more complicated than that of the competitive protein binding method, it is more selective. As a result of this and of the most recent developments, no chromatographic step is necessary in the majority of the recently published methods. From the analyst's point of view, the method has been greatly simplified. Complete kits have become commercially available in the last few years containing ready-made reagents which a few years ago had to be prepared by the analysts themselves. Within an extremely short period of time, these facts have made RIA the most frequently used routine method in biological clinical steroid analysis. It is widely used in all steroid fields where satisfactory antisera are available (e.g., various hormones, cardiac glycosides, and most recently, bile acids).

The great importance of the RIA methods in steroid analysis is reflected by the extremely great number of publications dealing with this subject. Books,[239,240] numerous reviews, and chapters in general books have been devoted to this field. The most up to date review, that published by Pratt[241] on steroid immunoassay in clinical chemistry, contains as many as 266 references. Only 10 years have passed since the development of the theoretical and practical basis of this method, and there has been an extremely rapid development during those years. Thus one has to be very careful regarding the application of "old" methods. A colorimetric, fluorimetric, gas chromatographic, etc. method for the determination of steroids published, for example, in 1973, can be regarded with high probability as being up to date even now. In many instances this is also the case with HPLC. RIA methods published at that time, however, are more or less obsolete, and the most recently published papers have to be consulted to get a true picture of the present state of the art in this field.

RIA methods are closely related to competitive protein binding methods as the basis of both methods is the competition between the steroid to be determined and its radioactively labeled derivative for the active sites of a binding protein. The main difference and at the same time the key to the higher selectivity of the RIA method, is that in this case a much more specific interaction takes place between the steroid and the protein than simple adsorption: that is, formation of an antigen — antibody complex, the steroid being the antigen.

The following is a brief outline of the main steps of the RIA procedure.

1. First is the preparation of the antiserum containing the specific antibody. This is today usually done by the manufacturers of the various kits used by most of the analysts. The preparation involves formation of chemically coupled steroid — protein (usually bovine serum albumine) complexes using bifunctional molecules such as dicarboxylic acids for hydroxy steroids and carboxymethyl hydroxylamine for ketosteroids as the bridge molecules. This immunogenic complex is then injected into experimental animals to produce the specific antiserum (haptens steroids alone are not immunogenic and can produce the antiserum bound to the protein only). The specificity of the antiserum is highly dependent on the structure of the steroid — protein complex.[242] This is a field where further devel-

Table 3
SPECIFICITY OF RAT KIDNEY CYTOSOL TOWARDS VARIOUS STEROIDS

Tested steroid	Cross reaction (%)
25-(OH)-D_3	100
24,25-$(OH)_2$-D_3	100
Vitamin D_2	1.5
Vitamin D_3	3.0
7-Dehydrocholesterol	0.0
Cortisol	0.0

From Kremer, R. and Guillemant, S., *Clin. Chim. Acta*, 86, 187 (1978). With permission.

opments are to be expected in terms of specificity of the methods (decreasing the number and extent of cross-reacting steroids).

2. The second main step is the preparation of radiolabeled steroids. These are also furnished by the manufacturers in the above-mentioned kits. Usually tritiated derivatives are used, but the use of ^{125}I labeling using, for example, iodinated tyrosine linked to the steroid through the carboxymethyl oxime moiety has also been well documented up to the present.
3. Preparation of the sample for the assay is the third main step. This usually involves an extraction step and, if necessary, TLC or column chromatographic separation to increase the selectivity of the assay. (The recoveries of these steps are usually checked using the radioactively labeled derivative of the steroid to be determined, preferably the tritiated derivative used in the following equilibration step.) The recent trends in this field, supported by the increasing specificity of the antisera, show the decrease of the importance of the sample purification, thus greatly decreasing the time and labor demand of these methods. It must be noted, however, that in many cases preliminary chromatographic separation of the steroid to be determined is used even now. This is the case in those instances when the high accuracy of the method is more important than its demand on time and when no sufficiently specific antiserum is available to solve given problems.
4. The fourth main step is incubation of the sample to be analyzed with the antiserum and the radioactive steroid in a buffered medium. The time and temperature of the equilibration is varied within wide limits in the various procedures. The most typical conditions allow the mixture to stand at 4°C overnight.
5. Separation of the steroid — antibody complex from the free steroid, and radioactivity counting represent the final major step. The separation is usually done by adding dextran-coated charcoal to the equilibrium mixture. This selectively binds the free steroid which can thus be removed by centrifugation, and the assay is based on counting the radioactivity by a liquid scintillation counter in the supernatant liquid. The standard curve which is the basis of the calculations is similarly constructed using pure steroids. A typical standard curve for the determination of plasma aldosterone taken from the paper of Tan et al.[243] is shown in Figure 11.

It must be noted that other methods also exist for the separation of free and bound steroids. Of these, first of all, the immobilization of the antibody on cellulose or on

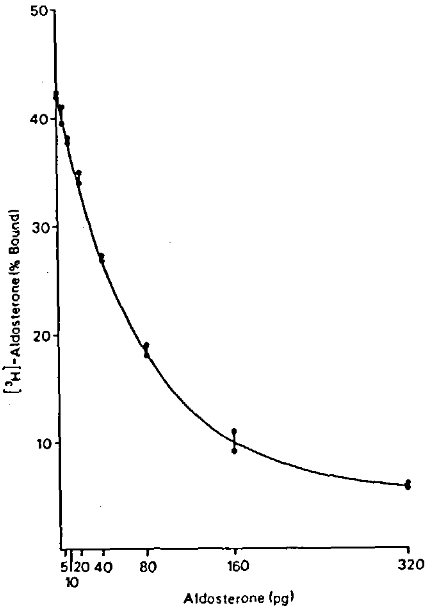


FIGURE 11. Standard aldosterone RIA curve. Antibody was obtained from rabbits immunized against the 3-carboxy-methoxy-oxime derivative of aldosterone conjugated to thyroglobulin. Incubation was 2 hr at 0 to 4° C in 0.8 m*l* of borate buffer (pH8). (From Tan, S. Y., Noth, R., and Mulrow, P. J., *Clin. Chem.*, 24, 1531 (1978). With permission. Copyright 1978 *Clinical Chemistry*.)

the wall of the test tubes supplied as parts of the commercially available kits merits mention, as this seems to be the way expected to further simplify the analysts' work in this field.

Of the hundreds of papers dealing with the application of the RIA method for the determination of steroids in biological samples, only a few characteristic examples are given here. Lijnen and Amery[244] described the determination of aldosterone in plasma. The steroids extracted from 2 m*l* of plasma sample by dichloromethane were directly subjected to RIA analysis after evaporation to dryness using 1,2-^{3}H-aldosterone and antibody raised in rabbits using aldosterone-18,21-diacetate-3-carboxymethoxime coupled to bovine serum albumin as the immunogenic hapten-protein complex. After incubation for 24 hr at 4°C, the separation was performed by the dextran-coated charcoal method. Neither in this method nor in the above-mentioned plasma aldosterone assay[243] was preliminary chromatographic separation used, as the accompanying steroids showed no cross reactivity against the antisera used. None of these workers tested tetrahydroaldosterone, while Herkner et al.[245] did and found a considerable cross reactivity (7% for both the 5β and 5α isomer). For this reason, a preliminary TLC separation was necessary when aldosterone was determined by RIA, e.g., in urine where these metabolites are present at considerable concentration.

Another field where RIA has had a great importance from the beginnings of this method is the assay of digoxin in serum and other body fluids.[246] To produce the antiserum, digoxin is bound to bovine serum albumine either at its steroid nucleus or the carbohydrate moiety. As for the labeling of digoxin, both tritiated and ^{125}I-labeled derivatives are in use, but the latter seems to be much more generally accepted.[247,248] For the separation of free and bound radioactivity, all methods mentioned earlier in this paper are in use. Most of the commercially available kits contain the antibody bound to the surface of a test tube, thus eliminating the necessity of filtration or centrifugation. The high specificity and sensitivity (down to 1 ng/mℓ or less) make the RIA method a unique tool for the routine determination of digoxin and related drugs in body fluids. It is interesting to note that the RIA method has been successfully used for the determination of digoxin in plant extracts, too. In addition to its high specificity, this method is 10^4 times more sensitive than the fluorimetric assay. Using a semiautomated version, 2000 assays could be carried out in a week.[249]

As a consequence of difficulties in obtaining suitable antisera, RIA has been introduced to bile acid analysis later than to the above-discussed classes of steroids. At present, however, suitable antisera are available and a fair number of papers have been published for the determination of cholic acid,[250] lithocolic acid,[251] etc. The RIA technique seems to become an important method for clinical analysis, although data obtained without preliminary chromatographic separation must be handled with caution because of the cross-reactivities of other bile acids and conjugates.

III. CONCLUSIONS

About 30 years ago almost exactly the same quantitative analytical methods were used by steroid analysts working in the fields of biological-clinical or industrial-pharmaceutical analysis, where the principal methods were colorimetry and fluorimetry. Since then, entirely different trends in the development of the two fields have resulted in different analytical methods. Today the methodologies of the two fields are so different that it is rather difficult to find any method used in both and even if there are such methods, they are applied from different aspects using different techniques inside a given general method.

In the field of industrial-pharmaceutical steroid analysis (analysis of bulk steroids, intermediates, raw materials, and steroid formulations), the sensitivity of the methods is not as important as in other fields; rather, it rarely occurs that steroids present at the nanogram level have to be determined. It is very important, however, that the methods adopted be selective and reliable and (mainly in the field of the assay of tablets) be suitable for automation.

Classical and polarographic methods occupy only a very narrow territory. Of the colorimetric and fluorimetric procedures, ketosteroids are usually determined by the INH method and corticosteroids by one of the tetrazolium methods. Antimony trichloride is most often used for the analysis of D vitamins, while mineral acids diluted with various organic solvents are used for almost all groups of steroids. The selectivity and reliability of these methods is often increased by thin-layer and column chromatographic separation, where the most frequently used column packings are silica gel and reversed-phase systems.

In gas chromatographic analysis, no specially selective and sensitive detectors are usually necessary. The analyses are almost exclusively done by separation on packed columns using the flame ionization detector. HPLC and TLC densitometry are two important methods which are used in almost the same form in both the industrial-pharmaceutical and biological-clinical fields.

The main differences in the demands on the analytical methods in the biological-clinical field are that

1. Much higher sensitivity is often necessary (down to the picogram level).

2. The analyst has to be able to separate and quantitate as many as 20 to 30 steroids, which are often closely related in structure, in a single sample.
3. The importance of automation is much higher.

Of the classical colorimetric and fluorimetric procedures, only a few automated versions are still used. More important are the extremely selective enzymatic methods with colorimetric and fluorimetric (and, sometimes, also with electroanalytical) ends.

Chromatographic techniques are indispensable in this field, too. Separation or purification of the samples by TLC or column chromatography (using mainly various Sephadex® preparations as the column packings) is used in most of the complex analytical procedures. If high sensitivity is required, gas chromatography with electron-capture detection is used. In the case of the estimation of steroid profiles, capillary column gas chromatography is applied. Quantitative GC-MS techniques are used in research and as reference methods. The importance of HPLC is rapidly increasing in this field, too.

The use of radioactively labeled steroids to check the recoveries of various separation steps is quite general in biological-clinical steroid analysis. Of the direct quantitative radioanalytical techniques, the competitive protein binding method and, mainly, radioimmunoassay are of prominent importance. The use of the nonradioactive variant of the latter (enzymmunoassay) is spreading rapidly.

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