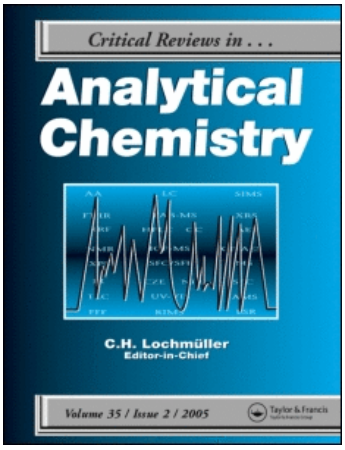

Critical Reviews in Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400837

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To cite this Article Petrak, K. and Brooks, R. V.(1973) 'Methods for the Quantitative Estimation of Nanogram and Subnanogram Amounts of Steroids in Blood and Urine', Critical Reviews in Analytical Chemistry, 3: 4, 421 — 453
To link to this Article: DOI: 10.1080/10408347308542667
URL: http://dx.doi.org/10.1080/10408347308542667

METHODS FOR THE QUANTITATIVE ESTIMATION OF NANOGRAM AND SUBNANOGRAM AMOUNTS OF STEROIDS IN BLOOD AND URINE

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I. INTRODUCTION

The determination of metabolites in human body fluids requires the development of analytical methods capable of providing both quantitative and qualitative information for a relatively large number of compounds, notably steroids, in complex mixtures. Prior to 1955, work on the isolation and identification of steroids had employed solvent partition, adsorption chromatography, digitonin precipitation, the use of Girard reagents, and a number of other techniques more common in organic chemistry. Despite the obvious progress that these techniques had made possible in the steroid field, it was apparent that other concepts would be needed in order to develop specific and quantitative methods for the determination of steroids in biological samples and especially for the determination of the relatively low concentrations of steroid hormones in blood and urine.

Much of the work on the estimation of steroids in biological extracts before 1955 was performed by bioassay techniques. Such methods, though sensitive, are relatively imprecise. Nevertheless, the technique is still used to a certain degree. The limitations of bioassay techniques are well known. No matter how strictly the conditions are maintained, it is always possible that some other substance is contributing to the test. This is also true of chemical and other assays, but compounds likely to interfere can be detected more readily.

Since 1955 a number of new methods have been developed for the determination of steroids in body fluids, and these will be reviewed here.

Spectrophotometric and fluorimetric methods have made important contributions to steroid analysis, and these methods are widely used despite the fact that their limitations for quantitative purposes are apparent. It is difficult to envisage any separation of complex biological mixtures without use of some form of chromatography — adsorption, partition, gel, etc. In its various technical forms, including paper chromatography, thin-layer chromatography, column chromatography, as well as more advanced forms such as gas-liquid or liquid-liquid chromatography, and especially in the combination of gas-liquid chromatography with mass spectroscopy, chromatography afforded a new and very powerful method for the separation and determination of steroids. The application of partition chromatography coincided in time with the availability of isotopically labeled steroids. The appearance of these steroids on the market facilitated the development of new microanalytical methods showing sensitivities of an order comparable with the concentrations at which many compounds exert their effects in biological systems. These techniques are ideally capable of exploiting the extreme delicacy of radioactive measurements combined with the chemical specificity so characteristic of many biochemical reactions. Chromatographic techniques and measurements of radioactivity have complemented each other and have yielded analytical methods of a new order of sensitivity and specificity, thus enabling a new outlook to be formed of the action of many hormones and other trace compounds.

The object of this review is to illustrate the use of various techniques for the quantitative determination of steroids at the nanogram and subnanogram levels; it does not intend to discuss all of the limitations and applications of techniques for determining steroids or to cover the extensive literature on steroid metabolism. Discussions of these subjects can be readily found elsewhere.[1-9]

II. METHODS FOR QUANTITATIVE DETERMINATION OF STEROIDS

In general, all analytical methods for determina-

tion of steroids in biological fluids include some or all of the following steps:

A. Hydrolysis of steroid conjugates
B. Extraction of steroids
C. Purification of extracts
D. Steroid derivatives formation
E. Quantitative detection of steroids in biological extracts

Let us deal with each of these steps separately.

A. Hydrolysis of Steroid Conjugates

In mammals, active steroid hormones after their production by adrenals, gonads, placenta, or fetal tissues undergo metabolic changes into inactive derivatives, in the first step as a result of a chemical reaction (reduction, hydrogenation, oxidation, etc.) and then through conjugation with glucuronic, sulfuric, or phosphoric acids or with *N*-acetylglucosamine.

Usually, before a quantitative determination of steroids at the nanogram level can be confidently attempted, the steroid conjugates are hydrolyzed into the corresponding free steroids. The original methods employed the hydrolysis of conjugated steroids with strong mineral acids (HCl, H_2SO_4) at concentrations of 10 to 15% and heating for 10 to 30 min.[10-11] The main disadvantage of these methods arises from partial decomposition of the steroids and formation of by-products (e.g., dehydroepiandrosterone is transformed into its 6β-hydroxy-3, 5-cyclo-androstan-17-one derivative).[12] Similarly, acid hydrolysis can lead to the side-chain cleavage of corticosteroids. In hydrolysis with HCl, a formation of chloride derivatives in C3 position of some steroids has been observed.[12] The hydrolyses of steroid conjugates using strong mineral acids at relatively high temperatures have now been largely given up and have been replaced by methods which use solutions of mineral acids (mainly HCl) of lower concentrations at lower temperatures; alkaline hydrolysis is less efficient.[13,14] Very often, specific enzymes are employed to hydrolyze steroid conjugates.

Apparently, there is no generally accepted method for the hydrolysis of steroid conjugates. Touchstone et al.[15] compared the enzymatic and acid hydrolyses of estriol-^{3}H-glucuronide. In this case, the enzyme hydrolysis was more reproducible and was found to result in lower amounts of by-products. Higher recoveries were found for the enzyme-hydrolyzed samples than for acid-hydrolyzed ones. For enzyme hydrolyses, 50 ml of maleate buffer solution of pH 6.8 containing 200 units of bacterial β-glucuronidase/ml was used; for acid hydrolysis, urine samples were made to 15% HCl and refluxed for 30 min. Average recovery after enzyme hydrolyses was 86 ± 6%, average recovery after acid hydrolyses was 65 ± 23% (mean ± standard deviation).

There have been several indications that other substances may interfere with the hydrolysis of steroid conjugates. A significant decrease in the yield of extractable steroid following hydrolysis may result if certain contaminating substances are present in the sample.[16-19] A. Schindler et al. performed 120 determinations of urinary estriol following acid hydrolysis in the presence of various compounds. Through the use of a radioactive standard, they showed that with an increase in density of urine samples (d = 1.001 to 1.025), a steady linear decline of estriol recovery occurred (93% to 56%).[20] In the presence of 1 to 5 g/100 ml of glucose, the recovery of estriol gradually diminished from 78.2 ± 9% to 24.9 ± 11%. Similar effects were caused by galactose and lactose. The presence of inulin, fructose, and sucrose caused even larger drops in extractable estriol (more than 95% at 5 g/100 ml), and the influences of mandelamine, methenamine, and formaldehyde on the recovery of estriol were still larger. On the other hand, dimethylamine and albumin, for example, did not influence the recovery of the steroid to any significant extent. It is quite conceivable that other steroids would behave in a similar fashion.

The fact that hot acid hydrolysis of steroids is probably the most widely used method for the hydrolytic cleavage of steroid conjugates renders these data of considerable significance. Painstaking control of steroid recovery throughout any method for the measurement of steroids is of great importance. This can be achieved by using a radioactive internal standard. The results obtained are fully valid, however, if there exists only a single conjugate of the metabolite being measured. It would not, for example, be quite adequate to correct for losses during hydrolysis of estriol even by the addition of /4-^{14}C/ estriol-3-sulphate and /^{3}H/ estriol-16-glucuronide. The detailed chemistry of the interference of various compounds present in urine with the steroid recovery is not known. Even less is known about the interferences

of various drugs and drug metabolites, and certainly caution concerning any drug excreted is justified. Slightly less important, but quite interesting and by no means negligible, is the effect of solute concentration on steroid recovery. The use of relatively highly concentrated urine samples for hydrolysis may lead to a number of erroneous conclusions when not taken into consideration, and either dilution of the sample (1:10 for urine[17]) or the use of a radioactive internal standard should be used.

Acid hydrolysis remains the least understood and the least examined part in steroid assays. While conditions are easily found which result in complete hydrolysis of the conjugated steroids, side reactions always occur. Also, under these conditions pigments are formed which are difficult to remove and which may interfere during the final quantitative determination step. There is very little to choose between using HCl or H_2SO_4. More important is to control the time of exposure of sample to the hot acid. The urine may be acidified cold and then heated for a definite time, or it may be heated to boiling before acidification. It is believed that the latter approach provides a more easily controlled time of exposure to the hot acid. The time of cooling, however, always will be dependent to a large degree on the volume of the sample. The same volume of urine should, therefore, be used for all analyses. The difficulty may also be avoided by adopting one of the procedures in which simultaneous hydrolysis and extraction are carried out. In the one-step procedure described by van Kampen and Anker[21] steroids liberated during hydrolysis are immediately taken up in the extraction fluid, and as a result virtually no degradation of the majority of naturally occurring steroids can be expected. In their procedure, two points must be observed: (1) The extraction solvent should have a density higher than 1.2, and (2) The free steroids must be easily taken up by the extraction liquid. 1,2-Dichloroethane was found to be an excellent extraction fluid for this purpose.

A comparison of two methods for the hydrolysis of steroid conjugates immediately shows the importance of choosing the right conditions for the type of assay one is attempting. Hydrolysis of urine according to van Kampen and Hoek[22] (reflux for 10 min with 10% HCl) leads to degradation of these most important steroids: >50% degradation: tetrahydrocortisol, tetrahydrocortexolone, and dehydroisoandrosterone; >25% degradation: pregnanolone (25%), pregnanediol (40%), pregnanediolone (50%), and tetrahydrocortisone (50%); slight degradation: androsterone (15%) and epiandrosterone (4%); no degradation: eticholanolone, androstenedione, 11-ketoetiocholanolone, testosterone, estrone, estradiol, estriol, cholesterol, dihydrocholesterol, and cholestanone.

On the other hand, using the continuous extraction method of van Kampen and Anker[21] (4 ml of concentrated HCl + 12 ml of 1,2-dichloroethane + 12 ml of urine sample refluxed for 10 min at about 80°C), the degradation due to acid hydrolysis is considerably diminished. In a treated mixture of eticholanolone, 11-ketoetiocholanolone, testosterone, pregnanediol, and cholestanone, the degradation of pregnanediol was brought down from 40% (the previous method) to about 1 to 2%. Similarly, the cleavage of androsterone under these new conditions had almost completely vanished. Dehydroisoandrosterone, where degradation was about 90% by the former method, showed only about 10% breakdown when continous extraction was used. The same applies for pregnanolone. Various other solvents have been used in place of dichloroethane, such as benzene[23-25] carbon tetrachloride,[26-28] and diethyl ether.[29]

The acid concentration which has been used by workers in this field varies considerably, from 0.4 to 8.0 *N*. The higher concentrations are used when the hydrolysis temperature is below the boiling point of the sample. Usually, the acid concentration lies between 1.0 and 2.0 *N*. At those concentrations, satisfactory hydrolysis seems to be achieved, providing that enough time is allowed for the reaction. Depending on the technique used (acid concentration, solvent), the time can be varied from minutes to as long as 18 hr.[29] Again, when a lower temperature of hydrolysis is used, the time is, in general, extended.

Thus, the main points to be observed when acid hydrolysis is used are

1. A constant volume sample should be used.
2. The urine should be acidified to a value which is well below the pK-values of the buffer system present in urine.
3. The sample should then be heated to a definite temperature and not simply to a boiling

point. Boiling point is likely to vary due to differing solute concentrations.

4. Mineral acid should be added to the heated sample to give the desired final acid concentration.

5. Heating should be maintained for a definite length of time.

6. The cooling time should also be controlled.

Cohen and Marrian[30,31] observed that the action of bacteria on pregnancy urine leads to the complete hydrolysis of estrogen conjugates and recognized that enzymes might be used for hydrolysis of urine steroid conjugates. Patterson[32] used preparations from *Escherichia coli* to hydrolyze conjugated estrogens. Extracts of various organs (e.g., ox spleen, calf spleen, and rat liver) have also been employed for hydrolyzing steroid conjugates.[33-36] In addition to mammalian enzymes, enzymes of bacterial origin have been used for the same purpose.[37,38]

For comparison with the conditions used when mineral acids are employed for hydrolysis, let us look at the conditions, for example, of deconjugation of urinary 17-ketosteroid glucosiduronates with β-glucuronidase. Using calf spleen extract, the hydrolysis in an acetate buffer of pH 5 takes 3 to 5 days at 37°C.[36] *E. coli* preparations hydrolyzed the above derivatives at pH 6.5 and 38°C within 24 hr.[37] The time needed for hydrolysis can, of course, be influenced by the amount of enzyme used.

Although enzymes are effective for cleaving steroid conjugates, their relatively high cost and rather slow action make their use at present inconvenient for routine assay purposes. On the other hand, their specific action is often used for research purposes and in highly specific analytical assays.[39-42] Inhibition of enzymatic hydrolyses by compounds present in biological samples is one of the major difficulties. Kushinsky et al., for example, reported[58] that saccharolactone acted as an inhibitor of both the rate and extent of hydrolysis of estrogen glucuronosides by β-glucuronidase.

As might be expected, the rates of hydrolysis of various types of conjugates differ considerably under the same conditions. Both sulfates and glucosiduronates are readily split under the conditions of acid hydrolysis described above. However, the cleavage of sulfates is much faster, as was shown by Lieberman et al.[43] and later confirmed by Buehler et al.[29] The differences in reactivities are even more pronounced when enzymic hydrolysis is used. For example, aryl sulfatases, which will hydrolyze estrone, estradiol, and estriol 3-sulphates,[44] will not catalyze the hydrolysis of the sulphates of nonaromatic steroids. A further example of this is the *alkyl* sulfatase in β-glucuronidase preparations from *Patella vulgata* and *Helix pomatia* which hydrolyse 3β-yl-Δ^5-sulfates well, 3α-yl-5α-sulfates less well, and 3α-yl-5β-sulfates hardly at all.

Two basic problems associated with hydrolysis of steroid conjugates thus are (1)that most procedures give only 40 to 80% of measurable steroid out of all steroid originally present in the sample and (2) that substances are present in samples of biological fluids, such as urine, which interfere greatly with both mineral acid and enzymic hydrolyses.[45-47] The obvious alternative is the direct assay of the steroid conjugates themselves, thus avoiding the need for hydrolysis.

B. Extraction of Steroids

It follows logically from the previous subsection that two main types of steroid extraction exist: (1) isolation of steroid conjugates and (2) isolation of free steroids liberated by hydrolysis.

1. Isolation of Steroid Conjugates

In the body, conjugated steroids are present mainly as their sodium or potassium derivatives, whose ionic character makes steroids fairly soluble in aqueous solutions. However, the polarity and solubility of the conjugate depends on the form and the position of conjugation and on the type of steroid. Many procedures have been tried for the extraction of conjugated steroid hormone metabolites from biological fluids, namely, urine and bile.[48-55]

Kornel[56] used high-voltage paper electrophoresis for separation of conjugated steroids. In this way, dehydroepiandrosterone glucuronide was separated from the corresponding sulfate in a pyridine-formic acid-acetic acid buffer (pH 2.2) at 2.000 V and 50 mA, and tetrahydrocortisone-3-glucuronide was separated from tetrahydrocortisol-3,21-disulfate. The monosulfates moved faster than glucuronides, disulfates faster than monosulfates, and trisulfates still faster. All steroids, except for the glucuronides at pH < 2.1, moved towards the anode. Recoveries of steroids after

elution from the paper ranged from 85 to 98%. Underwood and Tait[57] extracted and purified acid-labile conjugates of aldosterone from urine using partition chromatography on two columns followed by paper chromatography using two solvent systems. Kushinsky and Otterness[58] separated conjugated urinary estrogens into six major fractions by gel-filtration on Sephadex®. The flow rate, method of packing the column, size of the charge, and height of the column were all critical factors in effecting the separation. A similar technique was also employed by Beling.[51,59] A method for the isolation of steroid conjugates which are relatively unstable was described by Tamm et al.[50] The sample was made 3 *M* with NaCl and extracted twice with equal volumes of a mixture of ethyl acetate and *n*-butyl alcohol (3:1), and the extracts were evaporated to dryness. The compounds were purified further by paper chromatography. Urinary estrogen conjugates have been extracted from urine samples by a number of workers. Rourke et al.[60] extracted acidified salinized urine with ethyl acetate. Cohen[46] and Strickler et al.[61] isolated estrogen conjugates by precipitating them with $(NH_4)_2SO_4$. A detailed evaluation of various extraction methods for urinary conjugated steroids, namely corticosteroids, was presented by Kornel.[53] In all of the procedures examined in his work, the conjugates were forced into a relatively nonpolar solvent by acidification and saturation of the aqueous phase with inorganic salts. These forced extractions were found to be more selective than those using more polar solvents.

One or more of the following disadvantages limit the above methods:

1. Large volumes of solvent are used.
2. Low pH and the possibility of a reaction on steroids.
3. Unwieldy manipulative procedures.
4. Low steroid recovery.

Some of these drawbacks can be eliminated when ion-exchange chromatography is employed. Liquid-liquid extraction of conjugated steroids from biological samples by means of a liquid anion exchanger was first applied by Kushinsky and Tang[52] for the extraction of free and conjugated estrogens. Amberlite® XAD-2, a neutral cross-linked polystyrene, recently was found to adsorb steroid conjugates quantitatively from urine. After adsorption and a workup, the conjugates are readily eluted from the resin with methanol, ethanol, or tetrahydrofuran. Bradlow[62] presented a detailed study of extraction of steroid conjugates with this resin. Recovery was virtually quantitative (over 90%) for the metabolites derived from cortisol, testosterone, progesterone, estradiol, and aldosterone. Small organic molecules, such as urea and glucose, are not retained by this resin and are quantitatively recovered in the aqueous effluent. It was found that cortisol metabolites are not changed by adsorption on the resin. The capacity of Amberlite XAD-2 resin was also investigated, and it was found to be lowest for cortisol phosphate, whose recovery was only satisfactory at ratios below 25 mg/100 g of resin. However, the resin also adsorbs quantitatively unconjugated or free steroids in addition to conjugated metabolites. Other workers have also reported the use of Amberlite XAD-2 resin for the extraction of urinary steroid conjugates with equally satisfactory results. Mattox and Vrieze[63] described the use of the resin for the isolation of synthetic steroid glucuronides. Recently, Osawa and Slaunwhite[64] modified the XAD-2 polystyrene resin procedure of Bradlow[62] for a rapid assay of urinary estrogen conjugates so that 10 samples can be processed in 30 min. The resin they used adsorbed estrogen conjugates quantitatively from as much as 40 times its volume of urine. No specificity of adsorption, however, was noticed, and the extract probably contained not only all conjugates of estrone, estradiol, estriol, and other phenolic steroids but also numerous other urinary compounds.[65]

The pH of urine influences adsorption to some extent. Thus, acidic material is adsorbed more strongly from acidic than from alkaline media.[66] The pH range of 6 to 10, however, gives good quantitative results. Variations in the rate of flow of urine through the column did not affect the assay, though the flow rate during the elution step may affect the result slightly. The amount of resin used is not critical. In general, a wide range of concentration of steroid conjugates can be extracted with this resin with assurance.

2. Isolation of Free Steroids Liberated by Hydrolysis

Many of the steroid assays in use at present are based on the hydrolysis of conjugated steroids and the isolation of free steroids before the final quantitative determination is attempted. A

TABLE 1

Partition of Some Steroids Between Water and Some Organic Solvents

	$CHCl_3$	Water + EtAc	Benzene	CCl_4
Cortisone	0.027	0.057	0.9	30.0
Hydrocortisone	0.14	0.078	2.7	120.0
Testosterone	0.01	0.01	0.01	0.03
Progesterone	0.01	0.01	0.01	0.02
Androstenedione	0.01	0.01	0.01	0.056

From Silber, R. H. and Porter, C. C., in *Methods of Biochemical Analysis*, Vol. 4, Glick, D., Ed., Interscience, New York, 1954. With permission.

number of organic solvents have been used for extraction of the free steroids from the hydrolyzed samples. Some advantages as well as disadvantages are associated with using each solvent. The most generally used solvent appears to be diethyl ether despite its flammability. Its high solvent power for steroids obviously favors its use. Other widely employed solvents are benzene, carbon tetrachloride, isopentane, hexane, and 1,2-dichloroethane, and there are many others. The choice of solvent for this purpose is basically guided by the polarities of the steroids in question. For example, a nonpolar solvent such as hexane can be used to wash aqueous samples containing cortical steroids. Such solvents extract less polar compounds, leaving the compounds with a dihydroxyacetone chain in the aqueous solution. Similarly, carbon tetrachloride can be used for extracting corticosterone, estrogens, and related steroids from aqueous solutions, but 17,21-dihydroxy-20-ketosteroids are extracted only partially using this solvent.

Table 1 presents values of the partition coefficients for several steroids and water-solvent systems. Despite the importance of such data for analysis of steroids, solubility studies are scanty and uncoordinated. A systematic study of the solubilities of estrogens was presented by Ruchelman and Haines.[68] Some of their results are quoted in Tables 2 and 3. Data such as these are especially valuable for use in extraction procedures since the distribution ratio of a solute between two solvents is equal to the ratio of the solubilities of the solute in the two solvents.[69] Similar but more extensive studies would be of great value. In practice, experience and intuition provide the main guideline in choosing a particular solvent system. The general rule is to choose a solvent of the minimum polarity necessary to extract the steroids which are being measured. In this way a relatively "clean" extract may be obtained.

TABLE 2

Solubility of Estradiol in Organic Solvents at Various Temperatures (Values in γ/ml)

Solvent	15°C	25°C	30°C
THF	280,064	292,224	337,888
p-Dioxane	70,752	120,856	198,684
Acetone	42,912	70,688	89,148
Abs. MeOH	18,113	25,488	35,256
Abs. EtOH	23,872	31,344	37,274
95% EtOH	16,069	29,088	41,863
Et_2O	7,008	7,545	8,367
$CHCl_3$	2,512	4,114	6,427
CH_2Cl_2	1,084	1,926	2,671
Benzene	217.0	467.0	801.0
Toluene	148.0	331.0	515.0
Cyclohexane	5.0	13.0	22.0
Hexane	a	a	9.0

[a]Not determined.

From Ruchelman, M. W. and Haines, P., in *Advances in Gas Chromatography*, Zlatkis, A., Ed., Preston Technical Abstracts Co., Evanston, Illinois, 1967. With permission.

C. Purification of Extracts

The crude extracts contain, depending on the origin of the sample, a mixture of ketosteroids with nonketonic, acidic, phenolic, and neutral substances as well as pigments. Two main tech-

TABLE 3

Ratios of the Solubilities of Estradiol (E_2) and Estrone (E_1) in Various Solvents at 30°C

Solvent	Ratio E_2/E_1
THF	6.8
95% EtOH	6.3
Abs. MeOH	6.1
p-Dioxane	5.4
Acetone	5.2
Abs. EtOH	5.2
Et_2O	3.9
$CHCl_3$	0.4
CH_2Cl_2	0.4
Benzene	0.6
Toluene	0.5
Cyclohexane	0.7*
Hexane	2.2*

*Subject to error due to exceedingly low values of both E_2 and E_1.

From Ruchelman, M. W. and Haines, P., in *Advances in Gas Chromatography*, Zlatkis, A., Ed., Preston Technical Abstracts Co., Evanston, Illinois, 1967. With permission.

niques are usually employed for purification of these extracts.

1. Purification by Simple Partition

In general, the acidic material can be separated from the mixture by washing with saturated sodium bicarbonate solution, the phenols by washing with sodium hydroxide solution, and the amount of pigments can be decreased by washing the extract with alkaline sodium dithionite solution. After all these constituents have been removed, a solution containing a neutral fraction is left behind. The procedure described by Cohen and Marrian[30] can serve as an example. Their method for the extraction and purification of estriol and estrone from acid-hydrolyzed human pregnancy urine involved washing the urine with 10% Na_2CO_3, treatment with 0.1 *N* NaOH to separate the "estriol" fraction, and extraction of the "estrone" fraction into 1 *N* NaOH.

It has been shown[70] that the acidic fraction contains, among other things, lactic, oxalic, succinic, citric, and hippuric acids, as well as traces of hydroxyarylcarboxylic acids and substituted hydantoins believed to be formed during acid hydrolysis by condensation of urea with α-keto acids.[71] The remaining neutral fractions include mostly nonphenolic steroids, indigoids,[72] and cholesterol.[73] This particular step in estrogen analysis was later perfected by Brown.[74]

The simple partition purification can be considerably improved by making a chemical change in the steroids to be analyzed. There are now several methods of this type, most of them designed to separate complex mixtures of steroids and impurities into more or less well-defined groups. All are based on the formation of derivatives with partition coefficients differing from those of the parent steroids. Urinary estrogens, for example, after they have been separated from contaminants of considerably different polarity by the simple partitions described above, can be modified (e.g., by methoxylation) so that further simple partitions can separate contaminants similar in polarity to the unmodified estrogens. The method of Girard,[75] originally devised for the separation of ketonic from nonketonic steroids, can serve as a typical example. The method depends on the formation of water-soluble betaine hydrazones of the ketosteroids from which the nonketonic material is readily extractable. The ketosteroids may then be hydrolyzed to give the parent steroids and may be again recovered by extraction with diethyl ether. This procedure was first applied to the assay of urinary extracts by Talbot et al.,[76] closely followed by Pincus et al.,[77] Dobriner et al.,[78] and many others. Hydroxysteroids, such as estriol and estradiol, can be reacted with succinic or phthalic anhydride to form the corresponding hemiesters and separated.[77] Digitonin separation, which is based on the reaction of digitonin to give insoluble complexes with steroids possessing a hydroxyl group in the β-configuration at carbon 3, is occasionally exploited for separation of ketosteroids.[79-81] The principle of purification by derivative formation was applied very efficiently by Brown,[82] who prepared the methyl ethers of the phenolic group in estrogens by reacting them with dimethyl sulfate in alkaline solution. He then extracted these derivatives from aqueous solution with nonpolar solvents and thus achieved separation of estrogens from residual polar contaminants.

2. Purification by Chromatography

Further purification of still crude extracts can be achieved by utilizing chromatography. Meaningful quantitative assay of steroid hormones in biological fluids depends fully on the highest

possible purification of extracts before the final quantitative determination is carried out. Very often, specificity of an assay is brought about by a well-designed purification procedure. This step of the analysis is thus of a vital importance.

a. Adsorption Chromatography

In general, a fraction to be analyzed is placed at the head of the column of adsorbent and is developed by pouring through, in succession, a series of solvent mixtures or individual solvents of gradually increasing polarity. The least polar compounds appear first in the effluent and are followed by more polar compounds.

This technique was first applied to urinary steroids by Callow.[83] The studies of Lieberman et al.[84,85] extended this method to the systematic separation and isolation of urinary steroid ketones by fractional chromatography on magnesium silicate-Celite mixtures and on alumina. Heard et al.[86,87] and Stimmel[88] used aluminum oxide for the purification of estrogen fractions of urinary extracts and the method yielded much valuable information. Other workers experienced difficulties in obtaining satisfactory separations, mainly as a result of variations in the properties of aluminum oxide. Engel[89] showed that there were both technical and theoretical defects in the procedure, including variations in elution with different sets of alumina and tailing and displacement effects caused by impurities. Brown,[82] using deactivated alumina, previously shown by Stewart[90] to give a linear isotherm and therefore be free from tailing and displacement effects, standardized the properties of each batch before use and obtained very satisfactory and reproducible results.

The efficiency of adsorption of a particular compound is influenced by a considerable number of factors. The nature of the surface of the adsorbent is important and so is its surface availability. Ideally, the surface area should be completely uniform and available to molecules of any size. In practice, the surface area is far from ideal, and the available area diminishes as the molecular size of the adsorbed solute increases. Very often, although the total area available to large molecules decreases, the actual affinity for large solute molecules may increase. The surface areas of most useful adsorbents range from 100 m^2/g upwards (e.g., alumina 100 to 600 m^2/g; silica gel 300 to 800 m^2/g; Florisil®300 m^2/g). The actual chemical groups present on the surface of the adsorbent participate in the adsorption process. Surface types have been classified generally as polar and nonpolar, the guiding factor being the ability of the surface to form hydrogen bonds with the solute molecules. For example, the energies of adsorption onto alumina for aliphatic compounds containing various functional groups are known to increase in the following order: –S–; –O–; –C=N–; –COO–; –CO–; –OH; –N=; $-NH_2-$; –SO; $-CONH_2$.[91] Surface treatment and the incorporation of other materials in the adsorbent can change the specificity or even the whole character of the separation. Many modifications have been described for specific cases. In the field of steroid analysis, modification of the surface with water is the most common example. It has been shown recently that alumina deactivated with water can revert to an activated alumina grade[92] if anhydrous solvents are used. A certain amount of water must be added to solvents to maintain a particular adsorbent type during a chromatographic run. The effects of molecular size and shape of the substrate on the separation have been studied in general,[93–95] but knowledge of this influence does not necessarily help in solving a specific separation problem. The effect of solvents on the separation process is generally guided by the affinity of the surface for the solvent. For group separation, the adsorption step is carried out using a solvent more polar than the solute and the elution step with a solvent less polar than the solute for nonpolar adsorbents. The reverse procedure is used for polar adsorbents. Therefore, for the elution step the solvent is usually decreased in polarity for nonpolar adsorbents and increased for polar adsorbents. A number of so-called eluotropic series have been proposed by various authors and are often used in practice; (one of them is the series water, methanol, ethanol, propanol, diethyl ether, butanol, ethyl acetate, *n*-hexane, benzene, pyridine). A rational procedure for choosing a series of solvents for liquid-solid chromatography has recently been described by Scott and Kucera.[96]

Very careful standardization of conditions during adsorption chromatography is of paramount importance. In particular, small changes in the moisture content of the adsorbent may lead to marked alteration in its properties with resulting change of position of compounds in the elution pattern. Some independent parameter must be

used for the identification of each compound present.

b. Partition Chromatography

In adsorption chromatography, the separating power of an adsorption column depends on differences in the strengths with which the solutes are held on a solid surface. In partition chromatography, the solid serves as a support for a stationary liquid phase over which a second, immiscible, liquid phase is passed. In this case the separation of the solutes on the column is governed by the partition coefficients for these solutes between the two phases. In dilute solution, the partition coefficient of a compound does not depend on its concentration and is not affected with the presence of other solutes. The development of a suitable solvent system for a particular separation of steroid hormones may, however, be a difficult problem. The position of a compound in the effluent from the partition chromatography column is less likely to be affected by the presence of other compounds in the mixture. In case of a standardized procedure, it may be possible to make tentative identifications of compounds from their positions in the chromatogram. To achieve this, several variable factors must be controlled:

1. The packing of the columns must be uniform.

2. A constant temperature must be maintained since partition coefficients and the mutual solubilities of stationary and mobile phases vary with temperature. Therefore, the temperature during equilibration of the two phases and during the chromatography should remain within ± 0.5°C.

3. Deviations from ideal behavior must be taken into account. For most systems, a solute to be separated must have a partition ratio that is independent of the concentration of solute in the system used. If the partition ratio is not independent of concentration, tailing of the solute band occurs. This is observed in the separation of estrone and estradiol using the benzene-aqueous alkali system.[97-98]

Partition chromatography has been successfully applied to the separation of steroid hormones by a number of investigators.[99-102]

c. Paper Chromatography

The type of partition chromatography in which a sheet of paper serves as a support for the stationary phase was first described by Martin et al.[103] The use of paper as the solid support in partition chromatography eliminates the difficulties connected with packing of uniform columns. Unfortunately, in paper chromatography the fraction of the cross-sectional area of paper that is occupied by the mobile phase varies throughout the length of the paper strip.[103] It is therefore vital again to standardize the conditions under which paper chromatography is carried out and to use internal standards as often as possible. Paper chromatography can also be used for quantitative work, but in this case the technique often involves extraction of separate sections of the paper after chromatography. Impurities present in paper are often eluted with the steroids and can make the final quantitative determination inaccurate.[104] It is not always necessary to elute steroids from the paper prior to final measurement. Bush[341] eliminated this step by using his machine CASSANDRA which automatically dips the paper in reagent, dries and quantitates the spots obtained (17-oxosteroids and corticosteroids). Also, the process based on the use of *in situ* soda fluorescence of Δ^4-3-oxosteroids such as cortisol and aldosterone compares favorably with elution methods and preserves the simplicity of the paper chromatographic techniques. The precision of this process has been reported to be ± 3%.[342] Partition chromatography on paper does not seem to offer any advantage over column partition chromatography for the purification of extracts of body fluids. For practical examples, References 105 to 109 can be consulted.

d. Gel Chromatography

The gel, usually cross-linked dextran or polystyrene polymer, can be used for chromatographic separation of organic materials by molecular sieving, reversible solute-gel interaction, or partition between solvent mixtures. These characteristics have been employed for the separation of steroid hormones.[110-113] The polymer most often used for this purpose, Sephadex LH-20, is a hydroxypropylated, cross-linked dextran, which swells in polar organic solvents. Columns prepared using this material have been found to be reproducible and to contribute negligible amounts of interfering impurities. Column gel chromatography

has been used, for example, in the radioimmunoassay of plasma estrogens[114] and competitive protein binding assays of steroids.[115] Mikhail et al.[116] tested various solvent systems for the separation of neutral and phenolic steroids and recommended benzene and methanol in different proportions, with or without methylene chloride or chloroform, for the separation of the classic estrogens and heptane, isooctane, hexane, and cyclohexane (with the addition of small amounts of benzene, ethyl acetate, or chloroform together with methanol) for the chromatography of various neutral steroids.

The useful feature of the Sephadex LH-20 columns is their ability to give reproducible results for a long period of time.

D. Steroid Derivatives Formation

It is not the aim of this subsection to give a full picture of the formation of derivatives of steroid hormones. Those interested in the subject can look for a comprehensive treatment in the works of Pierce,[117] Gardi et al.,[339] and others.

The main reason for derivatization of steroids (or any other compounds) in quantitative analyses is to alter their physical properties. The resulting differences can then be utilized to effect better separation of the compounds or more sensitive quantitative estimation of the steroids. The formation, use, and the importance of each relevant derivative has been or will be mentioned in appropriate sections of this review (cf. gas-liquid chromatography, fluorimetry, radioactive steroids, etc.). In the author's opinion, the use of derivatives for simple group separation is of some importance and therefore deserves mention here.

The coordinated use of high-capacity low-resolution separations and low-capacity high-resolution separations is very effective for analytical purposes. Separations of steroids are usually based on polarity differences. Steroids of biological origin can be separated into more or less well-defined groups in their free form, but derivatization can add efficiency and specificity to the separation procedure. Acyl and methoxy derivatives have often been used in chromatographic separations; both reduce the polarity of the corresponding free steroids and thus facilitate group separations. Rosenfeld in 1964[118] and other workers later[119-121] used trimethylsilylether derivatives for preliminary fractionations. Depending on the conditions of the silylating reaction, polyhydroxy steroids and ketosteroids are completely or partially converted into silyl ethers. As a result, steroids of widely different polarity are changed into derivatives of relatively low polarity, but differences among these derivatives are still preserved by the presence of unreacted functional groups. Occasionally, the trimethylsilyl derivatives may be used, after separations by thin-layer chromatography and column chromatography, directly in quantitative studies using GLC or GLC-MS combination.[122] There are two main disadvantages connected with the use of trimethylsilyl derivatives for preliminary purifications. On the one hand, the trimethylsilylating agents tend to convert all but highly hindered hydroxyl groups into the corresponding ethers and at the same time often react with the enolic forms of steroidal carbonyl groups; on the other hand, the stabilities towards hydrolytic cleavage of some trimethylsilyl ethers, namely those derived from estrogens, are inadequate. All these factors, if not duly corrected, will definitely lead to errors in the final quantitative determination.

In an attempt to eliminate these side reactions, the use of bulky trialkylsilyl derivatives of steroids (i.e., Me_2Pr^iSi-; $MePr^i_2Si-$; Pr^i_3Si-; has been suggested by Petrak.[123] The corresponding aminosilanes have been used for the silylation reaction. The differences in reactivity towards various hydroxyl groups in steroids of the reagents used are more pronounced than in the case of trimethylsilyl reagents. For example, the silylation of estrogens using triisopropylaminosilane under certain conditions was found to be specific and quantitative for hydroxyl groups at carbon-3 of estrogens. The carbon-17 hydroxyl group was not silylated under the reaction conditions.

When trimethylsilylether derivatives of steroids are used for thin-layer chromatographic separations, a second silylation step is often necessary to get around the problem of partial hydrolysis which occurs during elution of steroids from the plate, as reported by Adlercreutz and Luukkainen.[124] The use of sterically hindered trialkylsilylether derivatives mentioned above eliminates the hydrolysis during thin-layer chromatography under the usual laboratory conditions.[123] Some relevant data are given in Table 4.

TABLE 4

Cleavage of Estrone Trialkylsilylether Derivatives in Aqueous Methanolic $HClO_4$ at 50°C

Compound	Concentration of $HClO_4$, *M*	10^3k (min^{-1})	K_{rel}
Me_3SiOE_1	0.0	200.0–300.0	50.0–80.0
$Me_2Pr^iSiOE_1$	1.18×10^{-2}	176.0	47.0
	1.13×10^{-1}	1,140.0	305.0
$MePr^i_2SiOE_1$	1.13×10^{-1}	77.6	21.0
	2.50×10^{-1}	184.0	50.0
	5.58×10^{-1}	323.0	86.0
$ClCH_2Pr^i_2SiOE_1$	5.58×10^{-1}	67.9	18.0
$Pr^i_3SiOE_1$	5.58×10^{-1}	3.74	1.0
	4.65	21.1	5.7
	9.30	31.0	8.3

E_1 – estrone; K_{rel} = k for the corresponding compound/k for $Pr^i_3SiOE_1$ in 5.58×10^{-1} $HClO_4$.

E. Quantitative Determination of Steroids in Biological Extracts

1. Colorimetry and Fluorimetry

As early as the 1930's, it was known that some steroids (e.g., estrogens and corticoids) fluoresce when heated with a strong acid such as sulfuric or phosphoric acid.[125,126] The utilization of this behavior in the quantitative determination of steroids was delayed, however, until more sensitive fluorimeters were available.

Meanwhile, the observation made by Kober in 1931[127] that the green-fluorescing, orange-colored solution obtained by heating estrone with concentrated sulfuric acid and phenol turned pink on dilution with water set the trend for some time to come. This reaction was investigated intensively, as were many other reactions of other steroids with various reagents that resulted in color changes. As a result, a definite advance in the colorimetric determination of steroids was made. Thus, for the first time, a convenient, reliable, accurate method employing a more or less specific color reaction became available for the quantitative estimation of steroid hormones at concentrations at which these compounds are found in body fluids. Later studies by Brown[82] and Bauld[97] resulted in a marked increase in the reliability of the procedure. For example, the procedure based on Kober reaction for the quantitative determination of estrogens was eventually improved by Ittrich[128] and Brown et al.[129] to the stage where as little as 0.05 to 0.1 ng of the steroid could be determined with an overall precision better than ± 4% over the range from 1 to 100 ng.

The main source of error in the quantitative determination of steroids based on a color reaction is the limited specificity of the reaction. In the case of urine extracts, various compounds are present which can produce, on treatment with a strong acid, a brown color in the spectral range of the adsorption maximum of the Kober color.

For some time the chief methods for the analysis of 17-ketosteroids were based on the reaction, first described by von Bitto,[130] adapted for steroid ketones by Zimmermann,[131,132] and modified by Callow[133] and others, of the steroid ketone with *m*-dinitrobenzene and alkali in ethanol. Under these conditions, ketones containing a methylene group adjacent to the carbonyl group react with a development of a purple color. Steroids containing ketonic groups in other positions give colors of lesser intensity.[133–135] The concentrations of alkali and of alcohol, as well as the length of time allowed for color development, influence the intensity of the color, the relative amounts of color developed by the individual compounds, and the applicability of correction factors.[136,137] The rates of color development and the final extinction coefficients under various conditions of time and concentrations of ethanol and alkali vary even within a group of ketosteroids (e.g., 17-ketosteroids).

The strictest possible definition, therefore, of experimental conditions for obtaining maximum

color yields from biological extracts is very important. Again, crude extracts, namely urinary extracts, contain materials likely to give a significant absorption in the spectral region of ketosteroids. Values of the absorbance at two different wavelengths may be used to make a correction for the increase of absorbance due to the presence of impurities.[138-140]

When fluorimeters of the necessary sensitivity became available, methods began to appear for the quantitative estimation of steroids by fluorescence reactions in aqueous sulfuric[141-145] and in phosphoric[146,147] acids. Fluorescence reactions make it possible to estimate steroid concentrations with sensitivities at least 100 times as great as can be obtained in procedures that are based on color reactions. However, the specificities of the fluorescence reactions are very often lower. For example, the accuracy of the determination of corticosteroids in plasma can be influenced by the contributions that other steroids (such as estradiol, estrone, 11β, 17α, 20β, 21-tetrahydroxy-4-pregnene-3-one and 11β, 17α-dihydroxyprogesterone) could make to the fluorescence that develops on treatment with sulfuric acid reagent.[148-151] Even after meticulous purification, urine extracts often contain substances other than those to be measured which fluoresce in aqueous solutions of strong acids[152] and it is not always easy to make a correction for nonspecific fluorescence.

It is important to know and observe the facts that are likely to change the course of the fluorescence reaction. Sulfuric acid seems to be the most satisfactory strong acid to use. The reaction carried out in phosphoric acid may be more specific[147] but is often less sensitive to steroids[153] and is easily disturbed by outside factors, including ultraviolet radiation and traces of moisture.[147] Weaker acids form steroid derivatives whose fluorescence intensities are too low, as illustrated by the reaction of formic acid with estrogens.[154]

The concentration of acid definitely influences the course of the fluorescence reaction. A generally accepted optimal amount of water is not known, partly due to differences among various laboratories arising from different ways of expressing the acid concentration and partly due to the fact that the optimal concentration of acid depends to some extent on the optical parameters of the fluorimeter.[155]

The purity of the solvent used is very important since impurities are likely to react with the strong acid and thus increase the blank.[156] For the same reason, the solvent should be reasonably stable towards aqueous sulfuric acid, for otherwise the intensity of the fluorescence varies with dilution, and the measurement is bound to be inaccurate.[157] Reagents of the highest possible purity must always be used. Trace impurities in sulfuric acid of analytical grade,[158] traces of oxidizing agents[157] and of impurities in solvents,[159] dust,[156] and, most significantly, contaminants in purified extracts of urine[160] and plasma[161] have all been shown to influence the fluorescence reaction; the combined effects of all these impurities are difficult to predict but are likely to be considerable.

The intensity of fluorescence is both time- and temperature-dependent. Studies of these dependences have been presented by Brown,[74] Diczfalusy,[159] and DeMoor.[150] Quenching and self-absorption are two other phenomena likely to decrease the intensity of fluorescence. Braunsberg and Osborn[162] showed that quenching should be caused by an increase of temperature; a decrease of viscosity, or an increase in the amount of impurities. Yet another reason for controlling the temperature of the solution during the measurement was demonstrated by Diczfalusy,[159] who examined the effect of temperature on quenching in the case of fluorescent estrogen complexes.

Although new, more specific, and more sensitive methods for quantitative determination of steroids are now available, fluorimetry remains one of the most widely used techniques for this purpose, and new procedures based on novel chemical reactions do appear from time to time.[163,164] For example, a very sensitive and more specific fluorescence reaction for testosterone, epitestosterone, and some other steroids has recently been discovered and forms the basis of the method described by Eechaute et al.[165] As little as 100 ng of testosterone per 100 ml of plasma and 2 μg of testosterone in a 24-hr urine specimen have been estimated accurately by this method.

2. Gas-liquid Chromatography

Gas-liquid chromatography has become an indispensable tool in the study of specialized problems of steroid metabolism. The main contributions of GLC to steroid analysis include the possibility of specific isolation and characteriza-

tion with quantitative estimation, sensitivity of detection, and possibilities for automation.

Gas-liquid chromatography is a kind of partition chromatography in which the moving phase is a gas; the stationary phase is a relatively nonvolatile liquid coated on a solid support. The rate of separation of the solute molecules on the column is largely governed by the relative affinity of the solute and the stationary phase. The ratio that describes the distribution of the solute between the stationary and moving phases is expressed as a partition coefficient K:

$$K = \frac{\text{concentration of solute in stationary phase}}{\text{concentration of solute in moving phase}}$$

The individual compounds are detected as they emerge from the column. The signal from the detector (flame ionization detector, electron-capture detector, argon ionization detector, mass spectrometer) is amplified, and the signal is then recorded (e.g., using a moving chart recorder).

Reportedly, the first practical demonstration of the separation of steroids by GLC was described by Vanden Heuvel et al. in 1960.[166] Since then a range of conditions have been tried for specific steroid separation tasks. Columns with numbers of theoretical plates between 2,000 and 100,000 are commonly employed. With the improvement of the thermal stability of the stationary phases, the temperature range for general analytical use was extended from 100° to 300-350°C. Retention times are usually between 5 and 30 min, but for a complete separation of some very complex crude biological extracts, 1 to 2 hr may be needed. There is a vast literature that deals with both the theoretical and the technical side of gas-liquid chromatography.[167-170]

During the last decade, GLC has been shown to be of great value in the separation of pure steroids.[171-173] The successful application of this technique to the estimation of steroids depends again primarily on the purity of the extract applied to the column.

GLC methods for estimating steroids in body fluids usually consist of a combination of the following steps:[174-176]

1. Addition of an internal standard to the sample.
2. Isolation of steroids from the sample.
3. Hydrolysis of steroid conjugates.
4. Purification of the crude extract, using conventional techniques (e.g., column chromatography, thin-layer chromatography, etc.) with or without the formation of a derivative.
5. Detection and quantitative measurement.

The direct injection of blood or urine into a gas chromatograph has yet to be accomplished as a valid determinative process for steroids. So far, extraction of steroids from body fluids has always been needed. Samples of biological origin analyzed by GLC vary from crude extracts that contain numerous components to highly purified extracts that contain only a single steroid. In the former case, distortion and overlap of peaks in the resulting chromatographic tracing make an accurate quantitative evaluation impracticable if not impossible. When a highly purified extract containing a single steroid is subjected to GLC analysis, the sensitivity of the detection system can be employed to obtain quantitative determination at the nanogram or subnanogram level,[177] and the gas chromatographic properties of the steroid are used only as an additional proof of the specificity of determination. The sensitivity of the GLC technique to organic compounds allows detection of steroids in reasonable volumes of body fluids (e.g., 50 to 200 ml of urine or as little as 0.1 to 10 ml of plasma) and compares favorably with all other methods used for this purpose. The specificity of GLC detection for compounds in complex mixtures cannot usually be matched by other techniques. The detection systems that are normally used in combination with GLC, however, are not specific for any single steroid, and detection specificity must be obtained indirectly. Ionization detectors are widely used; these detectors measure changes in conductivity of the flowing gas stream with changes in the gas composition.[178,179] The flame ionization detector still remains the detector of choice for the estimation of all but the lowest concentrations. This detector measures the electric conductivity of gases in a hydrogen flame. A certain specificity can be added to this detector by modifying it with alkali-metal salts.[180] Detectors modified in this way have been used for the selective detection of compounds containing phosphorus or halogens.[181,182] A similar specificity with even higher sensitivity is achieved when the so-called electron-capture detector is used.[183-187] A radio-frequency discharge detector, developed by Karmen and Bowman,[188] gives relatively large signals from low

TABLE 5

Mean Values (μg/l) of Estrogens in Maternal and Cord Plasma and Amniotic Fluid in Normal Pregnancy at Term

Estrogen	Maternal plasma		Cord plasma		Amniotic fluid	
	Free	Conjugate	Free	Conjugate	Free	Conjugate
Estriol	6.5	124.0	137.0	1,260.0	56.2	932.0
Estrone	11.1	79.2	24.3	26.2	5.6	8.0
Estradiol-17β	19.0	4.4	7.4	3.4	+	1.4
Estradiol-17β + unknown	–	0.9	–	+	–	–
11-Dehydro-17α-estradiol	–	1.4	–	2.2	–	2.0
2-Methoxyestrone	2.2	2.1	1.4	2.8	+	+
16-Epiestriol	+	6.3	1.7	5.4	–	5.7
17-Epiestriol	–	–	+	1.0	+	1.9
16α-Hydroxy-estrone	0.8	27.8	2.6	46.6	2.7	24.7
16β-Hydroxy-estrone	–	4.1	1.4	9.3	–	17.8
16-Oxo-estradiol	0.9	15.5	3.4	60.7	3.6	26.5
15α-Hydroxy-estrone	+	3.4	2.7	5.5	1.0	5.5

From Aldercreutz, H. and Luukkainen, T., *Ann. Clin. Res.*, 2, 365 (1970). With permission.

concentrations of organic substances, and its use in combination with GLC has recently been revived.[189]

The usefulness of the mass spectrometer as a detector for the identification and estimation of compounds in biological samples has been recognized without any doubt. Even a nanogram or subnanogram quantity of a compound injected into the GLC instrument will give a valid spectrum. Still higher sensitivity can be achieved by continuous monitoring of a single ion that is characteristic of the compound under observation. Luukkainen[190] has described the determination of 4 pg of pregnanediol by measurement of the m/e 117 ion. Estrogens have been estimated by this technique in pregnancy urine, maternal and cord plasma, and amniotic fluid.[191] The results obtained (Table 5) show the great potentialities of gas chromatography. Ninety-one fractions from these fluids were investigated by GLC-MS combination, and in 61 of them the identity of the estrogens could be definitely established.

Interpretation of the mass spectra of the trimethylsilyl derivatives of steroids has been facilitated by the introduction of perdeuterotrimethylsilylating reagents.[192] A method for selective silylation using these reagents has been described recently[193] which is based on the differences among the silylation rates for various hydroxyl groups.

Obviously, the strength of the GLC methods lies in the ability of this technique to provide information on unidentified compounds of clinical importance in a mixture. To increase the practicability of GLC methods for the use in routine clinical chemistry, it would be particularly useful if simpler "clean-up" procedures were available since at present there is no saving of time or labor in using such methods as compared to others such as colorimetry. Also, many GLC methods designed to give high sensitivity, as for example the method of Wotiz et al.[194] for the determination of very low concentrations of steroids in peripheral blood samples using ECD, require a considerable degree of expertise on the part of the analyst and do not seem at present to be capable of much simplification. They also tend to operate at the limit of their sensitivity.

The full power of GLC as a tool in biological research and diagnostic investigation thus lies in its ability to reveal rapidly a wide spectrum of results. The pattern of these results is often more useful

diagnostically than a result of one individual determination.[195] So-called "steroid profiles," described by Gardiner et al.[196] some years ago and by van Kampen et al.,[197-201] are now attracting considerable interest, and the availability of suitable capillary columns has made it possible to extend the method.[202-204] "Steroid-profile analysis" is a term that denotes the simultaneous determination of all the steroid metabolites present in a biological sample. It is not likely to replace group determinations, but it could be used in cases where an occurrence of "abnormal" steroids is likely.[205] A metabolic system is likely to change under various circumstances, and very often a change in the concentration of one compound in the body induces changes in the concentrations of other related compounds. There are many applications of this use of the GLC technique, e.g., for studies of individual steroids in the metabolic network, for comparative and developmental biochemical studies, etc. An interpretation of steroid profiles, however, is not simple. Considerable variations in the quantitative relationships of individual components in the spectra are very often observed, though the origin of the changes may be very difficult to trace. Another practical limitation of the steroid profile approach is in the fact that some groups of steroids are secreted in much larger amounts than others: for example, glucocorticoids and androgens occur in tens of mg, while estrogens and aldosterone occur in tens of μg, so a scheme suited for the fractionation of one such group will not suit the other.

a. Steroid Derivatives for Gas-liquid Chromatography

The classical derivatives of steroids used in organic chemistry are rarely of any use in gas chromatographic work since these derivatives are usually prepared for reasons (solubility, melting point, reactivity, etc.) that are irrelevant to gas chromatography. The steroid derivatives for use in GLC should have an appropriate degree of volatility, should be thermally stable, and should preferably be less polar than the parent compounds. Although a specific GLC method for the direct determination of free steroids has been described,[206] it is generally unwise, especially in case of polyfunctional steroids, to attempt GLC separation without derivative formation, for irreversible absorption and/or thermal decomposition on the column can occur. The use of derivatives of steroid hormones in GLC can definitely bring about some improvements in the method and lead to superior vapor-phase behavior by reducing adsorption and increasing the separation factors. For the tentative identification of an unknown steroid, the conversion of portions of the sample into different derivatives can give a clue to the identity of the steroid through the comparison of retention times. Moreover, the formation of steroid derivatives allows the sensitivity of GLC technique towards the modified steroid (e.g., chlorine-containing derivatives for use with ECD) to be increased, which can under certain conditions of reaction lead to higher specificity of the method. Derivatization of some temperature-sensitive steroids, such as corticosteroids, makes it easier to find optimal conditions for the GLC analysis under which the steroid in question is stable. Two functional groups commonly present in steroids, hydroxyl and carbonyl, need to be converted into derivatives.

i. Derivatives for Hydroxyl Group

Acetates – Wotiz and Martin[207] were the first to report that estrone, estradiol, and estriol could be separated on GLC columns after conversion to acetates. Rather high temperatures, however, are required for GLC analyses of steroid acetates, even when relatively short columns are used. The acetates have been used for estrogen estimation[208,209] and have also been applied to the determination of other steroids.[210] It has been shown[211] that an 18-acetate group is eliminated from 18,21-diacetates during GLC analyses. The stability of acetates in solution is high. The steroidal hydroxyl group can also be protected by other acyl groups, such as propionyl or butyryl, if an increase in retention time is desired.

Haloacyl derivatives – Because the conversion of steroids to formates, acetates, and ethyl carbonates increases the retention volumes beyond the limit of practicability, the vapor-phase properties of other derivatives have been studied. The problem was initially attacked by converting the steroids into trifluoroacetates.[212] Since these esters were among the first derivatives used for steroids in GLC, their usefulness is not widely known, but in identification work they are superior to acetates because of their better behaviors under GLC conditions. After the introduction of the ECD into gas chromatography,[213] the

haloacyl derivatives of steroids again became important. The extreme sensitivity of the ECD towards these derivatives can be exploited for the analysis of biological samples which have been extensively purified. Another haloacyl derivative, heptafluorobutyrate, seems also to be relatively volatile and to show strong electron-capture properties.[214-216]

Trimethylsilylether derivatives – It has been found that trimethylsilylether (TMS) derivatives are almost ideal for the GLC analysis. The hydroxyl-substituted steroids are converted into TMS ethers when treated with a silanizing agent. This leads to "stabilization" of the hydroxyl group, decreases adsorption on the column, and changes the volatility of the steroid relative to the stationary phase. Therefore, better resolution and determination of steroids can be achieved. Steroidal TMS derivatives are easy to prepare, and their gas chromatographic properties are good since peaks of virtually theoretical shape may be obtained. Stereochemical differences of epimers are also accentuated.[217] The formations of TMS derivatives are generally quantitative, and they emerge unchanged from GLC columns. They also behave excellently under the conditions employed for mass spectroscopic analysis, giving clear molecular ion peaks and characteristic fragmentation patterns.[218,219] If necessary, TMS ethers can be readily and quantitatively hydrolyzed, and new derivatives can be synthesized. Their usefulness in analytical work has been confirmed by a great number of investigations.[217,220] The advantages of silylation of highly polar polyhydroxy steroids are pronounced. The severe tailing of free estriol, for instance, is almost absent from the tris-TMS-derivative; retention times on selective phases are shorter, and resolution is improved. "Double" derivatives obtained by selective methylation of the phenolic C-3 hydroxyl group followed by silylation of any other hydroxyls have also been studied.[221]

The ketone group may be left in underivatized form for some steroids, but partial formation of enol ethers often occurs under silylating conditions. Chambaz et al.[222] found that the degree of enol formation for the steroid ketones was dependent on the nature of the silylating conditions and on the degree of steric hindrance of the ketone group. The order of reactivity for ordinary ketones was found to be 4-ene-3-one = 3-one > 17-one > 20-one; no reaction was observed for the 11-one group. The most powerful silylating agents for enol ether formation were mixtures of trimethylsilylimidazole with bis-trimethylsilylacetamide and trimethylchlorosilane and of bis-trimethylsilylacetamide with trimethylchlorosilane, which corresponds with observations made for silylation of hydroxyl groups. The formation of only a single derivative is always desirable for quantitative GLC analysis, and that is why another double derivative, an oxime-TMS-ether, is used for ketonic steroids (cf. later).

Van Kampen and Anker[200] critically evaluated the silanization of urinary steroids. They concluded that silanization is certainly not a necessity when using an apolar stationary phase. The last disturbing polar activities of such a phase can easily be overcome by silanization of the column instead of the steroid. With a typical polar stationary phase, however, silylation of steroids is almost indispensable. When using steroid derivatives, the advantages and disadvantages of the alternative procedure should be carefully considered.

TMS ethers undergo both acid- and base-catalyzed hydrolysis. The ease of this reaction for various TMS ethers is roughly as follows: TMS esters > TMS (oxime) ethers > TMS (phenol) ethers > TMS (alcohol) ethers.[223]

Successful determinations of the glucuronides of androsterone and etiocholanolone by GLC have been reported recently.[224,225] The derivative used was the methyl ester trimethylsilyl ether or the trimethylsilyl ester trimethylsilyl ether; the latter formed directly by reaction with bis-trimethylsilylacetamide.

The rate of silylation of a hydroxyl group in a steroid depends on whether the hydroxyl group is primary, secondary, or tertiary.[226,227] Several comparative studies aimed at establishing quantitative differences among the silylating rates have been made. It is often difficult to generalize the results since the experiments are usually oriented toward one specific analytical problem.[228,229]

The formation of TMS derivatives of steroids was first described by Luukkainen et al.[217] in 1961. Many methods for formation and separation of these derivatives have since been published.[117] Recently, Chambaz and Horning[230] investigated the conversion of human steroids to their TMS derivatives for GLC studies in more detail and pointed out that it is not easy to recommend an optimum set of conditions for all analytical

purposes. The following examples indicate the range of silylation rates one has to deal with.

The 3β-ol(eq) and 3α-ol(eq) groups in 5α-cholestan-3β-ol and 5β-cholestan-3α-ol, respectively, are converted completely to ethers in about 4 hr during a noncatalyzed silylation (with, for example, a 1:1 mixture of hexamethyldisilazane and pyridine) at room temperature. Hindered hydroxyl groups (such as the 11β-ol group in 5β-androstan-3α,11β-diol-17-one) show no reaction whatsoever under these conditions. When bis-trimethylsilylacetamide is used to silylate steroids in the absence of solvent at room temperature, groups which react quantitatively include those at the 3, 16, *sec*-17, 20 and 21 positions.

The addition of a catalyst usually results in a major change in the rate of reaction. Thus, at room temperature a 10:10:1 mixture of hexamethyldisilazane, pyridine, and trimethylchlorosilane silylates the 3-ol group in 11β-hydroxyetiocholanolone within a few minutes and converts the 11β-ol group to the TMS ether over about 7 to 8 hr. Very highly hindered hydroxyl groups, such as the *tert*-17α-ol groups in steroids that also contain a 20α- or 20β-ol group, are not converted to the corresponding TMS derivatives under these conditions. However, when a silylating mixture containing equal parts of trimethylsilylimidazole, bis-trimethylsilylacetamide, and trimethylchlorosilane was used[230] at 60° for silylating 5β-pregnan-3α-, 17α,20α-triol, complete conversion of all hydroxyl groups to TMS ethers was observed.

Positions for substitution which are encountered for mammalian hydroxysteroids include 1, 2, 3, 6, 7, 11, 12, 15, 17, 18, 19, 20, 22, 25, and 26, while carbonyl groups are located at positions 1, 3, 6, 7, 11, 12, 16, 17, 18, or 20. The number of functional positions to be silylated varies from one to five or six, and higher levels of substitution may occur. It is important to establish appropriate conditions for derivative formation prior to GLC separation. The results in Reference 230 indicate that any of the following conditions may be used for most analytical studies:

1. A noncatalyzed reaction with bis-trimethylsilylacetamide at room temperature.
2. A catalyzed reaction with a mixture of bis-trimethylsilylacetamide and trimethylchlorosilane or a mixture of bis-trimethylsilyltrifluoroacetamide and trimethylchlorosilane at room temperature or at 60°.
3. A catalyzed reaction with a mixture of trimethylsilylimidazole, bis-trimethylsilylacetamide, and trimethylchlorosilane at 60 to 80°.

Halogen-containing silyl ethers – Thomas, Eaborn, and Walton some years ago[231] described the synthesis of steroidal chloromethyldimethylsilyl ethers and reported the GLC properties of the products. Bromomethyldimethylsilyl and iodomethyldimethylsilyl ethers for GLC of steroids were introduced later.[232] Kinetic results[123] indicate that these halogen-containing silyl ethers of steroids are more stable to acid-catalyzed hydrolysis than the corresponding TMS ethers. They are less volatile than TMS derivatives but allow separation of epimers. These derivatives, and especially the iodomethyldimethylsilyl ethers, are very sensitive to ECD by the ^{63}Ni detector.[233] Studies made by Thomas[234] compare favorably the sensitivity of iodomethyldimethylsilyl method with competitive binding assays. The minimum detectable quantity of halogensilylethers has been reported to be in the range 10^{-10} to 10^{-12} g, which indicates that the GLC-ECD method is sensitive enough to justify the use of these derivatives in ultramicroanalysis.

Other derivatives for hydroxyl groups – The uses of some other steroid derivatives have been reported. Besides those already mentioned, hexadecafluorononoates and eicosafluoroundecanoates[235] have been used in conjunction with ECD detectors. The following derivatives of estrogens were prepared by Exley et al.[236] and used for GLC-ECD:perfluorooctanoic esters; hexafluoroglutaryl esters; 2:2, 3:3, 4:4-hexafluoro-5-oxo-6-thianonates; 17-pentafluorophenylhydrazones; iodobenzenesulphonyl and iodobenzoyl esters; and iodinated estrogens.

ii. Derivatives for Carbonyl Groups

Dimethyl hydrazones – The first derivatives of steroids for use in GLC analysis were dimethyl hydrazones.[237] These derivatives have good GLC behavior and can also be used for determination of the number and nature of the reactive keto groups present in a steroid molecule.

Methyloximes – The use of these derivatives was first reported by Sheps et al.[238] for the isolation of estrone sulfate, and their successful application for GLC, MS, and NMR studies was made by Fales and Luukkainen.[239] Methyloximes can also be used for characterization of steroidal

ketones. The thermal stabilities of some methoximes, however, are too low[239] to permit their use in GLC.

O-Methyloxime-trimethylsilylether derivatives (MO-TMS) – These derivatives were first used for GLC of corticosteroids by Gardiner and Horning[196] and were found to stabilize the side chain of corticosteroids during GLC. MO-TMS derivatives are easy to prepare, and their GLC behavior is good. It has been demonstrated[196] that 11β-hydroxy, 17α-hydroxy, and 11-keto groups do not participate in this derivative formation.

Benzyloximes (BO) and benzyloxime-trimethylsilyl derivatives (BO-TMS) – The complete conversion of all hydroxyl groups in a mixture of, for instance, human urinary steroids may lead to difficulties during the separation using ordinary packed GLC columns, and the use of a highly efficient column may be necessary. An alternative approach is to use benzyloxime and BO-TMS derivatives. These derivatives are thermally stable,[196] have good chromatographic properties, show characteristic retention behaviors, and have easily identifiable mass spectra. BO-TMS derivatives have been used, mainly by Horning et al., for studies of urinary steroids.[240]

Trimethylsilylether-enol-trimethylsilylether derivatives – This type of derivative was first suggested for the gas phase studies by Chambaz and Madani.[241,242] Trimethylsilylether-enol-trimethylsilylether derivatives are formed as a result of a base-catalyzed silylation procedure using, for example, bis-trimethylsilylacetamide, hexamethyldisilazane, or any other common silylating agent together with a nucleophilic agent such as potassium or sodium acetate. Acid catalysis could also be used[243] but under these conditions, the formation of enol-TMS from the keto group is not strictly quantitative, and a formation of additional reaction products as a result of an oxisilylation process is possible; the exact conditions for a base-catalyzed formation of the title derivatives have been described by Chambaz et al.[244]

Molecular modification – The chromatographic behavior of a steroid molecule can also be modified by bringing about a more substantial change than takes place in a simple derivatization reaction. The best-known application is the estimation of corticosteroids by oxidation to the corresponding 17-ketosteroids before GLC.[245] Rapp and Eik-Nes used successfully the γ-lactone of aldosterone for GLC with electron-capture detection.[246] The γ-lactone of tetrahydroaldosterone behaves in a similar fashion.[247]

In routine clinical work, the selection of methods for estimation of steroids in body fluids is usually guided by criteria of reliability, practicability, and by convention. Although GLC techniques can be used to obtain reliable estimates of many steroids in biological samples,[248] other techniques (utilizing spectrophotometry, saturation analysis techniques, etc.) which often offer equally reliable results are considered more practical methods for the purpose. Full interpretation of complex chromatograms with the help of data-retrieval systems[249] should show in the near future that GLC analysis of crude biological samples is also possible for hormone assays in routine clinical chemistry.

3. Liquid-liquid Chromatography

The potential of liquid-liquid chromatography as a technique for the separation of complex biological mixtures has been recognized for some time now and is becoming more and more popular in research laboratories. High-pressure liquid-liquid chromatography has been developed during the last 5 years and has rendered liquid chromatography comparable with gas-liquid chromatography in speed, resolution power, sensitivity, and simplicity of operation.

The most important characteristics of an up-to-date liquid-liquid chromatography instrument are

1. A pressure system able to supply degassed dust-free solvent against pressures up to 21 MN/m^2 and at flow rates up to 10 ml/min.
2. An injection system for introducing samples onto the column head.
3. A column, 20 to 100 cm long and 2 to 5 mm in diameter, packed with appropriate solid material (20 to 50 μm in diameter) and preferably equipped with a temperature-control system.
4. A detector, not larger than 10 μl in volume at the column outlet. The detection is usually based on: a change in an overall property of the eluent, such as its index of refraction or electrical properties or a change of a simple property of the eluent (usually a spectroscopic property, such as ultraviolet or infrared absorption, colorimetry etc.).
5. Separation of solute from solvent fol-

TABLE 6

Retention Data for Some Adrenal Corticosteroids

Steroid	t_R, min	Relative $t_{R'}$	k
6β-Hydroxy-cortisone	3.87	0.123	0.046
Aldosterone	4.26	0.406	0.151
4-Pregnene-17α, 20β, 21-triol-3, 11-dione	4.75	0.761	0.284
Cortisone	5.08	1.000	0.373
4-Pregnene-11β, 17α, 20β, 21-tetrol-3-one	5.27	1.14	0.424
Cortisol	5.71	1.46	0.543
11-Dehydrocortico-sterone	6.05	1.70	0.638
Corticosterone	8.10	3.19	1.19
4-Pregnene-11β, 17α-diol-3, 20-dione	9.24	4.01	1.50
4-Pregnene-17α-ol-3, 11, 20-trione	11.29	5.50	2.05
11-Deoxycortisol	11.86	5.91	2.21
Cortisone 21-acetate	14.31	7.69	2.87
4-Pregnene-20β, 21-diol-3-one	36.6	23.8	8.89
Deoxycortico-sterone	47.6	31.8	11.86

Column: 485 mm x 2 mm i.d.; eluant: water; Flow: 0.25 ml/min; packing: 24% LA-1 on CTFE.

From Siggia, S. and Dishman, R. A., *Anal. Chem.*, 42, 1223 (1970). With permission.

TABLE 7

Retention Data for Some Estrogens

Steroid	t_R, min	Relative $t_{R'}$	k
Estradiol-17α-glucosiduronic acid	3.60	0.008	0.003
Estriol	3.98	0.305	0.109
17-Epiestriol	4.02	0.336	0.120
16-Ketoestrone	4.20	0.477	0.170
16-Ketoestradiol	4.87	1.000	0.357
16-Epiestriol	7.30	2.90	1.03
Equilinen	25.8	17.3	6.18
Estradiol	39.8	28.3	10.1
2-Methoxyestrone	40.4	28.8	10.3
Equilin	43.0	30.8	11.0
Estrone	56.4	41.3	14.7
17-Epiestriol triacetate	–	a	
17β-Estradiol-17-acetate	–	a	

[a]No elution observed under these conditions after 2 hr.

Column: 485 mm x 2 mm i.d.; eluant: water, pH 11.5 (NaOH); Flow: 0.25 ml/min; packing: 28% LA-1 on CTFE.

From Siggia, S. and Dishman, R. A., *Anal. Chem.*, 42, 1223 (1970). With permission.

lowed by determination of the solute (e.g., by GLC with wire detector).

The detectors that respond to changes in spectroscopic properties are at present the most sensitive ones, being able to detect solutes at concentrations as low as 10^{-9} g/l.

The separating power of liquid-liquid chromatography has been demonstrated many times over.[250-252] The use of liquid chromatography in the steroid field can be illustrated by the results obtained by Siggia and Dishman,[253] who separated structurally similar androgens, estrogens, progestins, and adrenal corticosteroids. Some of their retention data are given in Tables 6 and 7.

The practical results published so far, however, do not prove that the technique has advanced enough to be successfully used for quantitative determinations of hormones in low-titer body fluids.[254]

4. Radioactive Compounds in Quantitative Determination

a. Labeled Steroids Internal Standards

Any procedure for the accurate and specific determination of steroids in biological samples at the nanogram level requires a number of purification steps. As a result, losses of the compounds to be estimated are inevitable, and the importance of using labeled internal standards as a way of correcting for these losses is paramount.

Labeled internal standards have been used in many spectrophotometric procedures, for example, for the colorimetric[255,256] and fluorimetric[257] estimations of urinary testosterone and plasma testosterone,[262] the fluorimetric determination of urinary aldosterone,[258] the fluorimetric determinations of progesterone[259] and estrogens[260,261] in plasma, etc.

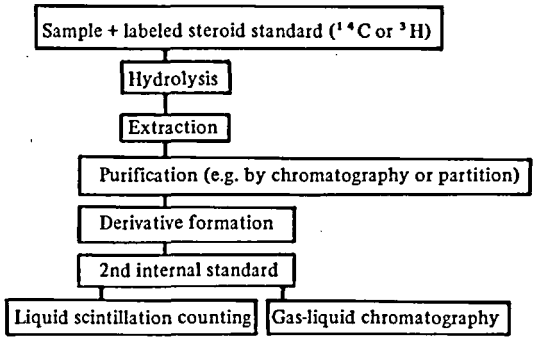

FIGURE 1. Radioactive steroids used as internal standards in steroid analysis of body fluids.

The usage of labeled steroids as internal standards has become a basic procedure in connection with GLC quantitative assays. The technique usually involves a combination of individual steps as illustrated by Figure 1.

This technique has been used for various classes of steroids, including progesterone,[263] testosterone, androstenedione and dehydroepiandrosterone,[264] estrogens[265] in plasma and corticosteroids,[266] testosterone and epitestosterone[267,268] in urine, and many others.

The radiochemical purity of the standard should be tested before it is used in a quantitative determination. Internal standards containing ^{14}C are recommended because of their purities and because their quality can easily be established by thin-layer chromatography analysis. Unfortunately, ^{14}C-labeled steroids of the required specific activity (about 50 to 60 mCi/mM) are not always available. The choice of labeled steroid depends mainly on the expected range of concentration of the steroid to be estimated in biological samples. If this concentration is in the nanogram region, ^{3}H-labeled steroids should be used if the corresponding ^{14}C steroid of adequate specific activity is not available.[269] A disadvantage of using ^{3}H-labeled steroids as tracers, however, arises from the existence of isotope effects.[343]

b. Double Isotope Technique

In this technique, isotopic derivatives are formed of the compound to be estimated, and the radioactivity of the isolated and extensively purified individual steroid hormone or a class of steroids is measured. Early attempts applied the isotope derivative technique to the determination of amino acids.[270] The reagents in this case were ^{131}I and ^{35}S-pipsyl chlorides used in combination with ^{3}H and ^{14}C steroids as internal standards. Later, ^{3}H-acetic anhydride, which was first used as the labeled reagent for aldosterone[271] and for the determination of cortisol,[272] and ^{35}S-thiosemicarbazide, which was first used for the determination of testosterone,[273] found wide application. Other reagents found occasional use, including ^{35}S-p-toluene-sulfonic anhydride or ^{35}S-pipsyl anhydride for the estimation of corticosteroids;[274] ^{3}H-borohydride for progesterone;[275] ^{82}Br,[276] ^{131}I, and ^{125}I-pipsyl chlorides[277] and ^{35}S-p-iodobenzene sulfonyl chloride[278,279] or ^{35}S-thiosemicarbazide[280] for the determination of testosterone.

The sensitivity and specificity of most double isotope derivative formation techniques is high, but the procedure involves elaborate purification. It is therefore time-consuming and consequently not always convenient for routine clinical investigations. The technique is used only when no better procedure for the determination of low concentrations of steroids is available or for the evaluation of the sensitivity and specificity of a new technique.

c. Saturation Analysis Techniques

Techniques of this category are based on the phenomenon of specific protein binding and vary according to the type of protein employed and the subsequent specific reactions involved:

1. *Competitive protein binding assay* – based on the specialized binding properties of a protein.
2. *Radio-ligand binding assay* – involving a protein or a nonprotein chelating agent.
3. *Radioimmunoassay* – the protein used is an antibody.
4. *Radioenzymatic assay* – the protein used is an enzyme.

The first experiments on the interaction of biologically active compounds, including steroid hormones, with proteins were described at the beginning of this century by Heidenhain.[281] Since then the interest in steroid interactions with proteins spread through various interrelated fields, such as biochemistry, endocrinology, and medicine, and eventually into analytical chemistry.

Immunochemical principles have been employed for the determination of steroid hormones

at extremely low concentrations by Beiser and Erlanger,[282] who measured the effects of testosterone, estradiol, and progesterone in inhibiting agglutination of steroid hormone-HSA conjugates erythrocytes. The maximal measurable amounts were found to be 0.05 μg/ml of testosterone and 0.06 μg/ml of estradiol. The sensitivity of immunochemical steroid determination is increased by several orders of magnitude by utilizing the principle of competitive binding – the technique used originally for the radioimmunoassays of insulin, growth hormone, thyroid stimulating hormones, gonadotropins, etc.[283]

The basic principle of radioimmunoassay is the competition between labeled and unlabeled steroids for the specific binding sites of an antibody. The labeled steroid is noncovalently bound to the antibody; the unlabeled steroid of the same structure displaces the radioactive one in proportion to its concentration, and the unbound and bound labeled steroid fractions are separated. A series of experiments using known amounts of nonradioactive steroid gives a calibration curve. This principle of radioimmunoassay has been applied to the determination of steroid hormones with a steroid-protein complex as antigen. Immunoglobulins, for example, are proteins which can interact with high specificity with the very compounds that participate in their formation. These compounds are known as antigens. The steroids as such are not immunogenic but can be made antigenic when covalently bound to proteins. Used in this way, a steroid can initiate the production of an antibody which is unique in its structure, reflecting the antigenic specificity of the steroid that was directly responsible for its formation.[284] In an ideal case, the result is an in vivo formation of specific binding sites for any steroid that can be covalently bound as a hapten to a protein. The preparations of steroid-binding immunoproteins along these lines were studied by a number of researchers.[285-288]

Competitive protein binding also involves competition between labeled and unlabeled hormone for the binding sites of a protein, but instead of an antibody, a naturally occurring protein with a high affinity for the particular steroid being measured is used. Such a protein may be found in the plasma (for some steroids) or perhaps in a target tissue. The flow chart in Figure 2 illustrates the individual steps in this type of assay.

Let us briefly consider each step in turn.

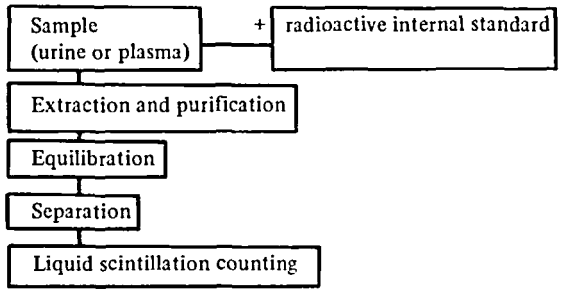

FIGURE 2. Saturation analysis of steroids in body fluids.

i. Extraction and Purification

Most of what has been said about extraction and purification above is also valid here. These processes must be effective enough to overcome interferences coming from any or all of the following sources:

1. The compound to be determined is bound to a substance high molecular weight material.
2. Compounds are present in the sample that could cross-react with the binding protein and thus affect the final estimation.
3. Compounds are present that could influence the equilibrium constant of the reaction between the compounds to be measured and the binding protein.
4. Some material may be present which could interfere in the separation of the bound and free steroid fractions.
5. The concentrations of the compound to be estimated may be below the limit of detection of the method.

A fuller discussion of these points can be found in Reference 324.

ii. Equilibration

The binding protein has to fulfill two basic requirements: Its reaction with the steroid under investigation must be both highly specific and quantitative. The binding proteins have a wide spectrum of binding affinities, and it is therefore rare for this reaction to be entirely specific. Zimmering et al.[289] observed that at steroid concentrations below 3×10^{-6} *M*, a marked steroid specificity to the higher-affinity sites occurs, but the sites with lower affinity values showed extensive cross-reactivity.

The properties of the binding protein do, of course, depend on the way of preparation, but its specificity can be markedly improved by suitable subsequent purification. As reported by Zimmering et al.,[289] antibodies against the testosterone-17-bovine serum albumin-conjugate purified by specific precipitation technique showed binding affinities corresponding to association constants of about $0.9 \times 10^5\ M^{-1}$, whereas the same antibody purified by salt fractionation and diethylaminoethyl-cellulose chromatography exhibited apparent affinity to the hapten of 3.5 to $3.9 \times 10^5\ M^{-1}$. These values are relatively low (as compared, for example, to association constants in the range of 10^9 to $10^{10}\ M^{-1}$ for antibodies produced against steroid conjugates with bovine or rabbit serum albumin, as observed by Midgley and Niswender[290]) but indicate well the importance of purification. Naturally, the true value of the apparent affinity constant for each binding protein can by obtained only after the complete isolation of each complex is achieved.

An important approach to the purification which has not been mentioned so far in this review, but which is particularly relevant to the specific purification of antibodies, is the method of affinity chromatography. In affinity chromatography, the hapten-specific immunoglobulins are adsorbed onto an immunoadsorbent made of an insoluble matrix (such as bromoacetylcellulose) onto which the steroid or other substance to be determined is covalently attached and then eluted.[291] Inman et al.[292] used the acyl-azide derivative of cross-linked polyacrylamide to bind amino steroids quantitatively. At present, elution from the specific adsorbents presents some difficulties.[293] Also, the choice of specific adsorbents is somewhat limited. The preparation and purification of antibodies to steroids have recently been discussed by Thorneycroft et al.[294]

iii. Separation of Unreacted and Reacted Fractions

In this step an ideal separation should be achieved; that is, the free and bound fractions should be totally separated in a very short time. A large number of techniques have been suggested, and, indeed, are in common use, for this separation. Few, if any, separation methods, however, meet the above requirements, and therefore there is usually a significant loss of sensitivity and accuracy. Also, during the separation, association and dissociation reactions between reactants should cease at the same time, for if they do not, there may be a further loss of specificity. The methods for separation of free and reacted steroids so far described can be summarized as follows:

1. **Techniques based on inequality of migration of bound and free steroid:** chromatoelectrophoresis,[295] electrophoresis,[296] and gel filtration.[297,298]

2. **Techniques which depend on the isolation of free steroid:** ion exchange,[299] adsorption onto dextran-coated charcoal,[300] adsorption onto silica or talc,[301] and adsorption onto cellulose powder.[302]

3. **Techniques based on isolation of bound steroid:** double antibody precipitation with filtration[303] or centrifugation,[304] salt precipitation,[305] solvent fractionation,[306] dioxane precipitation,[307] enzyme proteolysis,[308] immunoradiometric method,[309] and solid-phase antibody technique.[310]

All these methods have contributed to some extent to the development of saturation analysis techniques. Chromatoelectrophoresis was of great importance at first but as saturation analysis progressed, simpler separation procedures were needed. The technique may, however, serve as a reference in comparing most other techniques despite being time-consuming and requiring the use of radioactive hormone of higher specific activity than is needed in other separation techniques. It is not a general method for all steroid hormones.

The classical charcoal method is useful because of its simplicity, efficiency with small antigen molecules, high precision, and relatively low cost.

The chemical precipitation techniques, though generally simple from the practical point of view, have the disadvantage that the precipitation is hardly specific and other proteins are likely to separate from the solution with the hormones.

The fairly simple double antibody techniques are applicable to all saturation-analysis systems, including haptenic substances of low molecular weight like steroid hormones. The double antibody methods known so far depend on:

1. The action of immunoprecipitating serum to precipitate soluble antigen-antibody complexes.[311]

2. Employing the first antiserum in an insol-

ubilized form after earlier precipitation with the immunoprecipitating antiserum.[303]

3. The use of immunoprecipitating antiserum conjugated to a solid support.[312]

The disadvantages of the double antibody techniques are the considerable difficulty in assessing the quality variations from serum to serum, the variations in the behavior of the second antibody, and the fact that the specificity of the assay can vary with the amount of carrier protein used.

Solid phase antibodies were first used for the isolation of the corresponding antigens by Sehon et al.[313] in 1959. The method has since become an established procedure.[314] Several distinct methods have appeared so far, each employing an original way of coupling of antibody to a solid matrix:

1. Precipitation by second antibody.[303]
2. Covalent bonding to an insoluble polymer.[315]
3. Antibody adsorbed to the inner surface of assay tube.[316]
4. Antibody adsorbed onto bentonite particles.[317]
5. Antibody entrapped within acrylamide gel.[318]
6. Use of polymerized antiserum.[319]

By the use of antibodies coupled to a water-insoluble polymer the separation procedure can be simplified to the stage where a simple centrifugation is sufficient to separate free and bound fractions. Thus, in *an indirect assay* technique described by Wide,[320] the sample to be assayed is mixed with the required amount of radioactive tracer antigen, and a measured amount of an immunosorbent consisting of insoluble polymer-coupled antibodies directed against the particular antigen is added. After overnight incubation the solids are centrifuged and washed, and the radioactivity of the solid phase is measured. The increase of the polymer radioactivity is inversely proportional to the quantity of antigen present in the original incubation mixture. In *a direct assay* using immunosorbents, as described for example by Wide et al.[321] and by Miles et al.,[322] the labeled antibodies are added to the sample to be tested; after incubation, an immunosorbent made up of the antigen coupled to a solid phase is added, which then binds the free antibodies. In this type of assay, the amount of the particular antigen present in the sample can be directly related to the radioactivity in the liquid phase.

Solids used as insoluble carriers for antigens or antibodies are of two basic types: (1) those that involve physical adsorption (charcoal, glass, some polymers) and (2) those that employ functional groups on the surface of the solid which can couple covalently with the substances to be bound (e.g., functional polymers).

The immunosorbents used should be of high overall stability, should exhibit high capacity to bind the antigen, should have little tendency to nonspecific adsorption of proteins and polypeptides, and should be readily dispersed to allow taking aliquots with a measured amount of material.

iv. Liquid Scintillation Counting

The distribution of estimated steroid between free and bound fractions can be determined by counting either fraction alone, or both, using various methods. The statistical implications of alternative procedures can differ. The correct interpretation of isotopic distribution is very important if information about the true compositions of analyzed mixtures is to be obtained. For example, increasing the time of counting of samples or increasing the specific activity of the tracer theoretically improves the precision of the measurement, but at a certain point such arrangements can become uneconomic. Full discussions of this subject can be found in a number of publications.[323,324]

Tritiated steroids are almost universally used in existing saturation assays for steroids. ^{14}C-labeled steroids cannot usually be used because of their low specific activities. The use of some other isotopes including ^{125}I and ^{131}I[325] has been suggested. The choice of radioactive isotopes, however, may be somewhat limited by the fact that the derivatization may sometimes result in a decrease in immunoreactivity of the labeled antigen.

Saturation-analysis techniques for steroids are improving rapidly. At present, however, there are many possible inefficiencies in these assays, including nonequilibrium conditions, instability of the bound complex, inefficiency of adsorption of free steroid, adsorption to the reaction vessel, etc. Hopefully, it should not be difficult to eliminate these drawbacks once they have been recognized.

For a demonstration of this, one can consult the studies of Wilson et al.[326] and others.[327,328]

The nature of the data obtained as a result of these techniques requires that statistical analysis be carried out. The customary methods for data processing, as used for bioassay data, are not directly applicable here. Some time ago, Rodbard et al.[329] described the statistical problems associated with analyses of such data. Since then computer programs have been developed which make full automation of data processing for these data possible.[330]

5. Other Methods

a. Polarography

Polarography, an analytical technique based on the behavior of a polarized electrode in an electrolyte solution, has been used for the quantitative determination of steroids in biological extracts. It is only rarely used in clinical practice at present but is very sensitive and can be reasonably specific and therefore deserves mention in this review.

In brief, polarographic methods of estimation of steroids in body fluids (in this case mainly in blood) depend on the fact that some compounds are reduced at a definite potential at the dropping mercury electrode and give a characteristic polarographic wave, the height of which is a linear function of concentration. The majority of natural steroids are not reduced at the dropping mercury electrode during polarography and derivatives of them, usually their condensation products with Girard reagent T (betaine hydrazide),[75] are used instead. The half-wave potential of the cathodic wave obtained is characteristic of the type of steroid, (being about -1.4 V vs. S.C.E. for 17-ketosteroids, -1.2 V for Δ^4-3-ketosteroids, and -1.5 V for 20-ketosteroids). Similarly, the product of the condensation of estrone with Girard reagent T gives characteristic polarographic waves.[331] A method applicable to all phenolic steroids was proposed by Heusghem.[332]

The polarographic method offers certain advantages over, for instance, the colorimetric methods since it gives in one measurement the indication of the nature of the compounds as well as the quantitative results. The sensitivity of polarography to electroactive substances has been reported to be as high as 5×10^{-8} *M*.[333]

b. U.V. Reflectance Spectroscopy

Recently, Fredholm[334] suggested the application of ultraviolet reflectance spectra for the determination of estrone. The sensitivity, specificity, and precision of the method were reported to be high. Ten separate determinations of the amount of estrone in each of five samples, containing from 1.5 to 7.0 μg of estrone, gave a maximum relative deviation of 10%. The theoretical sensitivity limit of the method is about 5 ng of estrone per mm^2 of reflecting area.

III. SUMMARY

A vast number of methods exist for quantitative determination of steroids in biological samples based on the above-mentioned techniques. It is difficult to recommend a single method which should be used for a particular study.

Spectrophotometric techniques, although relatively simple, may occasionally lack detection sensitivity. The basically nonspecific gas-liquid chromatographic techniques, though very sensitive, may require excessive time for analysis and relatively expensive equipment and are thus often felt to be impracticable. The latest advances in the combined gas liquid chromatographic-mass spectrometric technique make the sensitivity of the technique comparable to radioimmunoassays while retaining the unique specificity of mass spectrometric detection. At present, however, this technique is not yet sufficiently developed for use in routine work, and the equipment needed is very expensive. Double-isotope derivative assays are very laborious, frequently involving up to five or six chromatographic purification steps. Methods based on protein binding are relatively simple, can be made highly specific, and are highly sensitive. It seems that competitive protein binding and radioimmunoassays will find more and more use for steroid hormone assays in clinical practice.

The limit of detection is of vital importance in evaluating the suitability of a technique for quantitative determinations at the nanogram level, and is given in Table 8 for each of a number of techniques applicable to determinations of steroids.

The specificity of a quantitative method is no less important and must always be established in the context of its usage. Very often the specificity and reliability reported by one laboratory cannot be reproduced when applied under slightly differ-

TABLE 8

Sensitivity of Detection Methods for Steroids

Method	Sensitivity of detection (ng)
U.V. absorption or spectrophotometry	100–2,000
Fluorescence	5–50
Isotope labeling	
^{14}C or ^{3}H acetate	5–50
^{3}H	2–5
^{35}S-thiosemicarbazide	1–2
^{82}Br	0.2–1
Competitive protein binding	1–10
GLC detection	
Argon ionization	20–50
FID	5–20
ECD	0.05–2
MS	As low as 5×10^{-3}
Radioimmunoassay	As low as 5×10^{-3}

ent circumstances by other workers. Thus, specificity is always relative.[335]

All techniques for quantitative determination of steroid hormones in body fluids mentioned in this review are designed to evaluate the function of steroid-producing glands by the determination of the steroid metabolites excreted with the corresponding biological fluid. It is a well-known fact, however, that the excretion does not always represent the real production of biologically active steroids.[336,337] Indirect analysis, which measures the production rate, was recently reviewed by Thijssen.[338]

The following is a list of abbreviations other than units and symbols and of trivial and systematic names used in this review.

BO	benzyloxime
GLC	gas-liquid chromatography
ECD	electron-capture detector
MO	methyloxime
MS	mass spectra
TMS	trimethylsilyl
Aldosterone	4-pregnene-11β,21-diol-3,20-dione-18-al
Androstenedione	4-androstene-3,17-dione
Androsterone	3α-hydroxy-5α-androstan-17-one
Cholesterol	5-cholesten-3β-ol
Cholestanone	5α-cholestan-3-one
Corticosterone	4-pregnene-11β,21-diol-3,20-dione
Cortisone	4-pregnene-17α,21-diol-3,11,20-trione
Cortisol	4-pregnene-11β,17α,21-triol-3,20-dione
Dihydrocholesterol	5α-cholestan-3β-ol
11-Dehydrocorticosterone	4-pregnene-21-ol-3,11,20-trione
Dehydroisoandrosterone	3β-hydroxy-5-androsten-17-one
Deoxycorticosterone	4-pregnene-21-ol-3,20-dione
11-Deoxycortisol	4-pregnene-17α,21-diol-3,20-dione
Epiandrosterone	3β-hydroxy-5α-androstan-17-one
16-Epiestriol	1,3,5(10)-estratrien-3,16β,17β-triol
17-Epiestriol	1,3,5(10)-estratrien-3,16α,17α-triol
Equilin	1,3,5(10),7-estratetraen-3-ol-17-one
Equilinen	1,3,5(10),6,8-estrapentaen-3-ol-17-one
Estradiol	1,3,5(10)-estratriene-3,17β-diol
Estriol	1,3,5(10)-estratriene-3,16α,17β-triol
Estrone	3-hydroxy-1,3,5(10)estratriene-17-one
Etiocholanolone	3α-hydroxy-5β-androstan-17-one
Hydrocortisone	Δ^4-pregnene-11β,17α,21-triol-3,20-dione
6β-Hydroxycortisone	4-pregnene-6β,17α,21-triol-3,11,20-trione

16-Ketoestradiol	1,3,5(10)-estratrien-3,17β-diol-16-one
16-Ketoestrone	1,3,5(10)-estratrien-3-ol-16,17-dione
11-Ketoetiocholanolone	3α-hydroxy-5β-androstane-11,17-dione
2-Methoxyestrone	1,3,5(10)-estratrien-2-methoxy-3-ol-17-one
Pregnanediol	5α-pregnane-3α,20α-diol
Pregnanediolone	3α,17α-dihydroxy-5β-pregnan-20-one
Pregnanolone	3α-hydroxy-5β-pregnan-20-one
Pregnenolone	3β-hydroxy-5-pregnen-20-one
Progesterone	Pregn-4-ene-3,20-dione
Testosterone	17β-hydroxy-4-androsten-3-one
Tetrahydrocortexolone	3α,17α,21-trihydroxy-5β-pregnan-20-one
Tetrahydrocortisol	3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one
Tetrahydrocortisone	3α,17α,21-trihydroxy-5α-pregnane-11,20-dione

ACKNOWLEDGMENTS

I would like to thank all who contributed to this review by their work. In particular, I am grateful to the publishers and authors who have given permission to reproduce some of the data used here: Interscience Publishers, Inc. (Silber, R. H. and Porter, C. C.), Table 1; Preston Technical Abstracts Co. (Ruchelman, M. W. and Haines, P.), Tables 2 and 3; Finnish Medical Society Duodecim (*Ann. Clin. Res.*, Adlercreutz, H. and Luukkainen, T.), Table 5; and the American Chemical Society (*Anal. Chem.*, Siggia, S. and Dishman, R. A.) Tables 6 and 7.

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