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Gas Phase Chromatography of Steroids

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Gas Phase Chromatography of Steroids

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Preface

Few fields have advanced faster over the past quinquennium than separation and estimation of steroids by the technique of gas phase chromatography. A detailed and complete review of this topic would therefore be beyond the scope of the authors contributing to this monograph. It was, however, felt that a discussion of some of the highlights of this rapid advance might be of help for laboratories estimating steroids in biological samples. One of the difficulties in producing a monograph of this kind is that before it can appear in print it is likely that some of the methods it discusses will have been overtaken by better methods, so swiftly is progress now made.

No editorial power has been exercised in trying to make a uniform account of technology in this field, and the idiosyncrasies of each individual author have been left intact. Through this approach we hope that what has been lost in scholarly appearance is regained in general appeal.

Salt Lake City and Houston, May 1968

KRISTEN B. EIK-NES
EVAN C. HORNING

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Abbreviations

ACTH:	adrenocorticotrophic hormone
BSA:	*bis*trimethylsilyl acetamide
CC:	column chromatography
CDMS:	cyclohexanedimethanol succinate
DC 200:	methylsiloxane polymer
DDT:	2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane
Embaph:	Embaphase, a non-polar silicone oil
Epon 1001:	epoxy resin
F-60:	a methyl-p-chlorophenylsiloxane polymer
GLC:	gas liquid or gas phase chromatography
HCG:	human chorionic gonadotrophin
Hi Eff 8B:	cyclohexanedimethanol succinate
HMDS:	hexamethyldisilazane
id:	inside diameter
JXR:	a methylsiloxane polymer
17-KS:	17-keto steroids
MO:	methoxime
MU:	methylene unit
NGA:	neopentyl glycol adipate
NGS:	neopentyl glycol succinate
NG Seb:	neopentyl glycol sebaccate
od:	outside diameter
17-OHCS:	17-hydroxycorticosteroids
OV-1:	a dimethylsiloxane polymer
OV-17:	a methylphenylsiloxane polymer
PC:	paper chromatography
psi:	pounds per square inch
QF-1 (FS-1265):	a trifluoropropylmethylsiloxane polymer
RRT:	relative retention time
SD:	standard deviation

SE:	standard error
SE-30:	a methylsiloxane polymer
SE-52:	a methylphenylsiloxane polymer
SN:	steroid number
STAP:	a polyester (modified carbowax type)
TLC:	thin layer chromatography
TMSDMA:	trimethylsilyldimethylamine
TMSi:	trimethylsilyl ether(s)
vol:	volume
XE-60 (CNSi):	a cyanoethylmethylsiloxane polymer
Z:	a co-polymer of ethylene glycol, succinic acid and a methylphenylsiloxane monomer

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Chapter 1

Gas Phase Analytical Methods for the Study of Steroid Hormones and their Metabolites

E. C. HORNING

I. Historical

The origin of gas-liquid partition chromatography is generally taken to be a 1941 comment by A. J. P. MARTIN indicating that it should be possible to use a gas and a liquid in a partition chromatographic separation (MARTIN and SYNGE, 1941). In 1948—1949 experimental work was undertaken, and the results of the first successful experiments were published in 1952 (JAMES and MARTIN, 1952). The separation of fatty acids was studied, and the theoretical basis of GLC separations was discussed. The separation of organic amines was investigated in later work. The need for gas phase detection systems became evident as soon as compounds other than acids and bases were studied. The separation of hydrocarbons, for example, was (and is) an application of great importance to the petroleum industry, but this separation problem could not be studied effectively without suitable detectors. During the next few years many types of detection systems were developed. The "gas density balance" of MARTIN and JAMES (1956) had moderately high sensitivity. The response was on a molar basis, and this detector was used extensively at Mill Hill (National Institute for Medical Research, London). Thermal conductivity detection systems (CLAESSON, 1946; RAY, 1954) came into wide use in studies where the sample was relatively large, while the argon ionization detector with a radium foil source was used as a sensitive detection system with microgram samples (LOVELOCK, 1958). The hydrogen flame ionization detection system was developed through work in several laboratories around 1957—1958, and this detector is now used universally in quantitative work with samples down to about 0.01 μg.

During 1959—1960 the possibility of achieving a separation of steroids by GLC procedures was under study in several laboratories. A report from the University of Glasgow (EGLINTON et al., 1959) indicated that several steroids could be chromatographed with an Apiezon phase under the usual conditions of that period but with the expected result: a retention time of hours, even at the highest temperatures in use at the time. A study carried out at Bethesda (National Heart Institute) showed that steroids could be eluted with short retention times when a polar (polyester) phase was employed at high temperatures; however, decomposition of some compounds occurred under these conditions (SWEELEY and HORNING, 1960). In Holland, a group at the Unilever Laboratories showed that with a thermally-stripped siloxane polymer phase it was possible to separate several steroids (BEERTHUIS and RECOURT, 1960). The problem was solved by the demonstration of VANDENHEUVEL, SWEELEY and HORNING (1960) that thin-film columns (1—3% of phase) prepared with a thermally stable liquid phase (SE-30, a methylsiloxane polymer) could be used for the separation of a number of steroids without structural alteration. The temperature range was moderate (210—230°) and the retention times were about 15 minutes to 1 hour.

From this work it became clear that thin-film columns, prepared with deactivated supports, were entirely suitable for GLC separations of steroids and many other compounds. The supposed requirement of 5% or more of liquid phase was not theoretically sound; this view was based on inadequate recognition of the practical problem of deactivation of the support. The fact that "non-volatile" steroids could be separated indicated that the range of organic compounds that could be separated by GLC methods was far wider than had been imagined. It now seems strange that practical problems were mistaken for theoretical limitations. Separations are based upon differences in the free energy of solution of the solute in the liquid phase under the conditions of the separation, and not upon boiling point relationships. The theoretical limit for the transfer of an organic compound to the vapor phase is about C_{70} for a hydrocarbon, and this limit has not been reached. The limitation in GLC work with respect to polarity is due to hydrogen bonding (excluding ionic substances), and derivative formation may be used to reduce or eliminate this effect. Almost the entire range of organic compounds of biological interest, with the exception of high polymers, are amenable to study by GLC methods.

During the decade 1950—1960, significant changes occurred in the field of mass spectrometry which are important to the further development of gas phase analytical work. The development of high resolution mass spectrometry resulted in analytical methods of very high precision and accuracy for the study of the composition and structure of organic compounds. These methods now require computer techniques for the analysis of the experimental data (BIEMANN et al., 1964). This work at first was of interest to organic, analytical and physical chemists, but not to biochemists. However, BERGSTRÖM. RYHAGE, STENHAGEN and their associates recognized the significance for biochemistry of combining gas chromatography with mass spectrometry. Valuable features of the combined gas chromatograph-mass spectrometer developed by RYHAGE (1964) at Stockholm included a high scan speed and a "molecule separator" that permitted the use of packed GLC colums. The most recent developments include mass marking and peak matching.

It is now clear that gas phase analytical work is emerging as a new field of chemistry. All of the steps of separation, identification, quantification and structural study are carried out in the gas phase. The methods, although not familiar to chemists trained in classical techniques, are fully as rigorous and as conclusive as past methods. They are well suited for the study of complex mixtures of organic substances on a microgram or submicrogram scale, and they are of particular importance for work in the fields of biology and medicine. The use of gas phase analytical procedures is not limited to steroids, but this is a highly important area, and it is the area of work from which many current methods are derived.

The key to new discoveries lies in new methodology. It is to be hoped that the field of endocrinology will benefit from these advances which are based on the physical sciences but which find their most important use in biological problems.

II. Analytical Methods

The traditions of analytical chemistry are derived largely from analytical problems pertinent to inorganic chemistry, and in early work the steps of separation and estimation were always carried out as sequential but separate operations. There is, however, no theoretical

reason why a multicomponent analysis in which the separation and estimation steps are combined, or even in which the separation step is omitted, should not be satisfactory provided that an adequate discrimination procedure is employed at some point. In fact, methods of these kinds often offer the great practical advantages of time-saving and increased accuracy over sequential methods. GLC methods are an example of a way in which separation and estimation steps can be combined into a single operation.

Although distinct advantages are often attached to multicomponent analyses, there are many instances in biological work where single component analyses are required and where multicomponent analyses are unnecessary. Group analyses, where members of chemically or biologically related groups of compounds are determined, are by implication confined to a small number of compounds. The following brief discussion is aimed at contrasting the GLC requirements for these analytical methods.

1. Single Component Analyses

a) By High-sensitivity Non-selective Detection

The hydrogen flame ionization detector has a sensitivity limit of about 0.01 μg for most work. By using special conditions it is possible to extend this range by a factor of about 10. The detector, however, is relatively non-selective and it will respond to most organic compounds. It is therefore necessary to use samples of relatively high purity for high-sensitivity work. The major steps of purification are usually those of column chromatography (CC), thin-layer chromatography (TLC) or paper chromatography (PC). In effect, the separation conditions are designed to yield a fraction that contains only one steroid, and the function of the GLC column is to present a single component to the detector for quantification. There is little emphasis on the column as a means of separation; the requirements are nevertheless high in the sense that stable, low-bleed columns with low adsorption characteristics should be used. Isothermal conditions are employed in order to minimize base-line changes. The electrical components should be chosen for low-noise characteristics, and the area measurements should be made, if possible, by electronic integration

rather than by recorder chart measurements. A "solid" injection technique should be used to avoid solvent fronts and solvent effects on the base line and on the detector. This technique also permits an entire sample to be used; when samples are injected in solution it is not uncommon to use 1—2 μl of a 100 μl sample (discarding 98—99% of the sample). Perhaps the best current example of this type of analytical method is the blood progesterone determination developed by SOMMERVILLE and his colleagues. References to his procedure are in a later chapter.

Procedures for the estimation of urinary testosterone also fall into this class, although high-sensitivity detection is not always required. Provision for the separation of epitestosterone must be made at some point, and the bulk of the urinary steroids must be removed by one or more high capacity, low resolution procedures. These, of course, might well be GLC procedures but at present they are more likely to involve CC and TLC methods. The sample injection may be carried out by liquid (solvent) or "solid" techniques, depending upon the size of the sample.

b) By High-sensitivity Selective Detection

Several selective detection systems are now available. Of these, the most useful for steroid work is the "electron capture" detector. This detector was developed as an off-shoot of early work on the argon detector, and it is particularly useful in pesticide work. The signal is noted as a lowering of the standing current of the cell. A few steroids are known to have "electron capture" properties (LOVELOCK et al., 1963), but it is customary to prepare derivatives for this purpose. Chloracetates have electron capture properties (LANDOWNE and LIPSKY, 1963) but derivatives containing perfluoroalkyl and perfluoroaryl groups with 5 or more fluorine atoms also show this property. In earlier work applications were limited by the fact that a foil-supported tritium source was used, with a temperature limit of about 220°. The temperature limitation has been removed by the development of the nickel-63 cell, although comparatively little information has been published about steroid analytical methods using this detector.

Extensive purification of the sample and all reagents and solvents involved in sample handling are required. The GLC column usually

contributes very little to the separation and purification process; these steps are normally carried out by CC or TLC methods. Stable, low-bleed columns are used under isothermal conditions. The detection system usually does not give a response that is linearly related to the mass of the sample at all concentrations, although over a limited mass range linearity is observed (see later chapters). Measurements are usually made by comparisons of response for reference samples of the compound under study, and internal standards are recommended. Solvent injection techniques are usually employed.

The best current examples of these methods are the procedures developed by Eik-Nes and his colleagues for the estimation of testosterone (as the chloracetate) and estrone (as the pentafluorophenylhydrazone). These methods are described in later chapters.

Chloracetates may be estimated in the range of 1—10 nanograms and the sensitivity of detection of some perfluoro derivatives lies well beyond this range. It is likely that considerable emphasis will be placed on selective detection methods in the next few years. There is a possibility that mass spectrometric methods may be developed for high-sensitivity work.

2. Group Analyses

Comparatively few GLC group analytical procedures have been developed. An example is the method of Brooks et al. (1967) for the estimation of pregnanediol and estriol in pregnancy urine.

The usual detection system is the hydrogen flame ionization detector. Separations may be isothermal or temperature programmed. The sample injection may be by "solid" or solvent techniques. One or more CC or TLC separation steps are used to achieve a group separation, and the GLC column is used to separate individual members of the group.

The sensitivity is usually that of ordinary GLC work and there are no unusual requirements from the point of view of GLC techniques.

3. Multicomponent Analyses

The usual detection system for a multicomponent analysis procedure is the hydrogen flame ionization detector. Isothermal or temper-