HIGH RESOLUTION GAS CHROMATOGRAPHY
IN STEROID ANALYSIS

AN INTRODUCTION TO THE USE FOR CLINICAL PURPOSES

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INTRODUCTION

1.1 INTRODUCTION

This thesis deals with the gas chromatographic analysis
of steroids. Hormones, steroids and steroid hormones are
names to designate a class of substances of great bio-
chemical importance. If there is no ambiguity the name
steroids will be used. They have in common that they con-
tain a system of four hydrocarbon rings, one five ring
and three six rings. The saturated parent molecule is
called sterane (fig. 1.1): it is not known to occur in
nature.
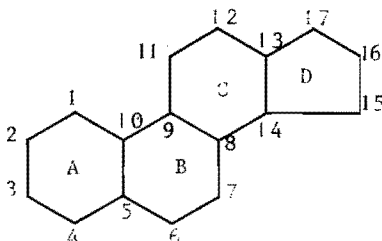
Fig. 1.1 Sterane.

Steroids can be considered as derived from sterane by
substitutions and dehydrogenation; the only hetero atom
is oxygen. Long side chains do not occur, nevertheless
the number of conceivable steroids is considerable. If
however only steroids as occurring in nature are consid-
ered, it appears that certain structural rules are obey-
10 ed from which the so called isoprenoid rule is important.

These rules will reduce the number of natural steroids
with respect to the conceivable steroids appreciably;
the number of natural steroids may be well less than,
say, 10^5. According to recent estimates roughly 7000
steroids have been isolated or synthesised.

Steroids as they occur in natural samples usually con-
stitute mixtures of fair complexity. With the progress-
ion of the analytical methods the complexity is more
fully recognized. The type of steroids as well as their
spectrum of concentrations have appeared to be charact-
eristic of the sample in question. It thus has appeared
that the family of steroids occurring in plant material
is different of that of for instance body fluids. Also
steroids from one plant will be different from those of
another plant: steroids occurring in blood do not all
occur in urine and the other way round. Usually steroids
occurring in one natural sample show a considerable var-
iety. About 10 years ago the number of different ster-
oids isolated from body fluids amounted to 80. Today,
mainly as a result from the powerful methods of analys-
is, this number has increased to a few hundred, which
is still small as compared to the number of known ster-
oids, and too large to be amenable for complete analys-
is.

Recognizing that in some cases a complete as possible
analysis may be desirable, one of the main problems in
steroid analysis is what to analyze for. For some time
it has been common use to focus the attention mainly to
steroids of more or less biological activity. Today it
is recognized that steroids of little or no biological
activity at all cannot be ignored, among other things,
because they carry important information. There are two
extreme cases: one is the complete as possible analysis
of all steroids detectable and (or) separable, leading
to a two dimensional pattern of the steroid types and

their concentrations. The other case is the analysis for
one or a small number of steroids as for instance will
be the case in future pregnancy control. In this thesis
these two objectives have been kept in mind. In an at-
tempt to develop a gas chromatographic analysis of ster-
oids in urine, the practical aspect of applying this
method routinely in hospital laboratories has continuous-
ly been kept in mind. By doing this, techniques appli-
able to steroid analysis in a much broader sense have
been obtained.

1.2 A FEW PRELIMINARY OBSERVATIONS ABOUT STEROIDS

Because of the great complexity of the chemistry of ster-
oids various classifications have been made. One method
is to divide the steroids into two major groups: one group
containing the compounds with more than 21 carbon atoms,
among which the sterines, the vitamines D, the bile acids,
the cardiac glycosides, the sapogenins and the steroid
alkaloids; the second group contains steroids of 18, 19
or 21 carbon atoms, respectively for instance oestrogens
(18 C atoms) androgens (19 C atoms) and the adrenocortic-
al hormones (21 C atoms). The word hormone is used to in-
dicate the hormonal activity; at the same time, however,
this word is used only for compounds with 18, 19 or 21
carbon atoms. Among the latter group there are many ster-
oids with neither hormonal nor biological activity; the
name steroid hormones, therefore, is confusing. On the
other hand however, this designation could be useful to
distinguish between steroidal hormones and proteinic hor-
mones. This thesis will confine itself mainly to some as-
pects of the analysis of the C_{18}, C_{19}, C_{21} only; they are
found in the human body and they are referred to either
12 as steroid hormones or steroids.

1.3 STEROIDS (HORMONAL) IN BODY FLUIDS

The oestrogens (derived from oestrane, fig. 1.2) are
characterized by a phenolic A ring, the OH group in 3
position, and a CH_3 group at the 13th carbon atom. Spec-
ific oestrogens with their own characteristic properties
are obtained from this parent molecule by substituting
oxygen functions at various places. These oxygen funct-
ions are either $=0$ or $-OH$
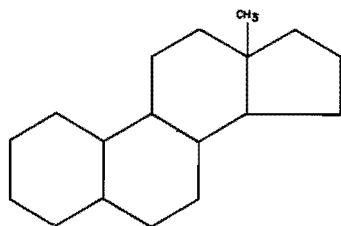
Fig. 1.2   Oestrane.

The androgens (C_{19}) may be conceived as derivatives of
the following parent molecule, androstane (fig. 1.3). In-
dividual androgens are formed by the substitution of ox-
ygen functions and (or) partly dehydrogenation in the
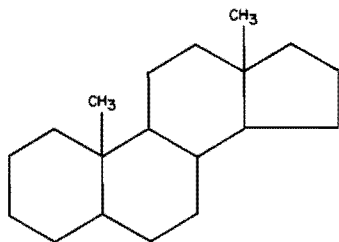
Fig. 1.3   Androstane (etiocholane).

ring system. They have in common a keto group at the
17th C atom. The oestrogens and androgens are collect-
ively called sex hormones, many of them maintain the   **13**

primary and secondary sex qualities, moreover they can be conceived too as catalysts for certain biological processes in the human body.

The C_{21} steroids possess at the 10th and 13th carbon atom a CH_3 group, just as the androgens and they contain further a C_2H_5 group at C atom 17. They can be conceived to have been derived from the parent molecule pregnane (fig. 1.4). Again by subsitutions and ring dehydrogenations the individual and specific members of this group are formed. In the C_{21} group distinction is made between "gestational" compounds (e.g. progesterone) which are sex hormones and the "corticosteroids" which are indispensable for the maintenance of life.
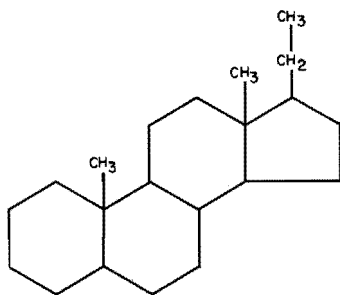
Fig. 1.4   Pregnane (allo pregnane).

1.4   ABOUT CLINICAL SIGNIFICANCE

The steroid hormones are secreted by the gonads and the adrenal gland. The secretion is stimulated by proteinic hormones of the anterior lobe of the pituitary gland, carried to these organs by the blood. By administration of radio active labelled steroids to the human body much has been elucidated about the place and nature of action as well as of the metabolism of primary products. Many functions in the human body appear to correlate

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with the rates of secretions of the primary biosynthetic products and the rates of excretion of the metabolic products in e.g. urine. From this short survey the clinical significance of these substances will be obvious. Many a disorder in the organism can be related to deviations of nature and concentration as appears from the analysis of steroids in body fluids.

1.5 CONCENTRATIONS AND QUANTITIES OF STEROIDS

The analysis of steroids in natural samples is difficult not only because of the complexity of the mixture but to a great extent also because in many instances steroids occur in extremely low concentrations. To illustrate this it is mentioned, that steroids as are known to be present in body fluids are produced in quantities of 10^{-10} gram for the lowest concentration to 10^{-3} gram per 24 hours (roughly 2 liters of urine or per 100 ml blood). The steroids do not occur as such but as conjugates of glucuronic acid and of sulfuric acid. The extraction of steroids from body fluids is therefore always preceded by some hydrolysis pretreatment. Many of the free steroids are converted or degraded by mineral acid hydrolysis methods. For this reason enzymatic hydrolysis must be preferred, although this is much more time consuming. The enzymatic hydrolysis time may be reduced by taking an excess of the enzymatic reagent and by carrying out the hydrolysis at somewhat elevated temperature (reference in chapter 3). Since the enzymes are proteins the temperature limit is about $60^{o}C$. Since, however, the enzymatic reagent which is prepared from the juice of the stomach of snails, is very expensive a great excess is justified only if small samples can be used. Classical steroid analysis based on colorimetric measurements require one twentieth of a 24 hour collect- **15**

ion and more of urine. In this thesis it will be shown,
that the quantity of urine used in the gas chromatograph-
ic analysis can be reduced to ml and even sub ml quantit-
ies. For such small samples an excess reagent is econom-
ically fully justified. It is obvious that one of the
main advantages of the enzymatic hydrolysis, as compared
to mineral acid hydrolysis, is that little or no inform-
ation is lost.

1.6 A FEW WORDS ABOUT CLASSICAL METHODS OF STEROID ANALYSIS

Although steroids constitute a class of substances that
are much alike, group separations have appeared to be
possible. For instance the phenolic steroids can be sel-
ectively extracted because of their phenolic ring. Clas-
sical steroid analyses as are used today in the hospit-
al laboratories are based on group separation (eventual-
ly further fractionation) and a colorimetric or fluori-
metric measurement for the quantitative determination.
These methods are going to be used for some time to come.
It is not likely that in the near future gas chromato-
graphic analyses are going to replace them except in
those laboratories where chemical instrumentation and
automation of analyses have been adopted. A more wide
spread use can only be expected if fool proof routine
analysis methods become available. The present thesis
is intended to be a contribution to this philosophy.

1.7 SUMMARY OF THE POSITION

Gas liquid chromatography involves partition between a
moving gas phase and a stationary liquid phase, and the
substances separated must therefore, be volatile. This

requirement is much less restrictive than it might appear at first sight. Several reagents are now available which allow quantitative conversion of polar low volatile compounds to non-polar more volatile derivatives, the most popular derivatives probably being trimethylsilylether (TMS$_i$) derivatives. Using TMS$_i$-ethers excellent gas liquid separation can be obtained with most steroids. The surprising high stability of compounds which have to be believed to be fairly labile is probably due to the complete absence from the destructive agents water, oxygen and light ensured by the nature of the separation process. Steroids obtained from natural samples are either contaminated with other material from the natural sample or the sample has undergone extensive pretreatment in order to isolate the required steroids.

In all practical cases the amounts of steroids will be small to very small. Extensive pretreatment has the advantage that colorimetric methods may be useful, although our analytical techniques today are not well enough developed to make sure that during pretreatment the required steroids are either isolated quantitatively or sufficiently reproducible. The lowest possible pretreatment has the advantage that no material is lost. Since the nature of the contaminants are not known and certainly vary from sample to sample, colorimetric methods are of little value. Even gas chromatography using short packed columns (as believed to be the only possibility for analysis) will not separate contaminants from steroid peaks with reliability to make quantitative analysis possible. During the development of analytical methods for steroids by means of GLC as described in the following pages, the analytical problems have been kept relatively simple, the pretreatment of the sample have been carried out with care in order that all possible information be preserved. It has appeared that this philosophy leads to what in information theory is called pattern

recognition. The next step is to develop a method of improved resolution, peaks that do overlap on short packed columns can be completely resolved if capillary or open hole columns are used. Admittedly the interpretation of the "pattern", the high resolution chromatogram, is still one of the main problems. It would be an attestation of short-sightedness, however, to mask peaks in order to simplify a chromatogram. It is believed that close cooperation with clinicians and methodic study of the patient soon will learn to which of the many unknown peaks the attention will have to be focussed.

This thesis deals with an investigation to improve the resolution of the separation and to simplify at the same time the analysis procedures, keeping in mind the goal, the use for clinical routine purposes. On the one hand this philosophy has lead to the development of a capillary column technique with regard to high resolving power; on the other hand simplification of the methods is emphasised with regard to routine purposes.

The advent of gas chromatography in steroid analysis in 1959 has lead to a better understanding of the complexity of the matter and the recent progress in high resolution separation will enhance probably the knowledge about the complexity of the field in question.

THE METHODS OF ANALYSIS

2.1 INTRODUCTION

In general it can be said, that the GC method is very welcome in the field of steroid analysis, however the pitfalls encountered in GC techniques are numerous. In spite of this fact it is encouraging, that many people appear not to be shyed off by these difficulties. The reasons for this are obvious. The very sensitive detection systems in GC are preeminently suitable for steroid analysis. The high resolution of the technique and hence better separations, enlarges the amount of information. Time consuming methods can be replaced by the faster GC techniques, whereas at the same time the accuracy is improved.

On the other hand the conventional methods, predominantly based on chemical determinations of steroids, are still of great use. For this reason and because of the fact, that many pretreatments of the biological samples in GC techniques apply to conventional techniques as well as to GC methods, there will be given here a short survey of a number of standard procedures.

2.2 CONVENTIONAL METHODS OF ANALYSIS

It is almost impossible to give a general survey of the methods in question within the framework of this thesis. Therefore some general lines will be considered on which these methods are based.   **19**

The steroids which are found in body fluids are mainly
present as conjugates of sulfuric acid and glucuronic
acid. It is very often necessary to submit the natural
specimen to hydrolysis to obtain the free steroids. The
major group of conjugates can be hydrolysed by the act-
ion of mineral acids (2.1). The sample is than exposed
during 10 to 30 minutes to e.g. hydrochloric acid (acid
concentration between 5 and 15%) at a temperature of 80
to 100°C.

It is known however, that several labile conjugates are
degraded to artifacts (2.2). This can be avoided by
enzymatic hydrolysis. One uses for this purpose β-gluc-
uronidase from bacterial origin (2.3) or from animal
sources (2.4, 2.5, 2.6). For the steroid sulfates are
used different types of sulfatases either from liver or
from snakes (2.7, 2.8, 2.9, 2.10, 2.11, 2.12). There are
also commercially available preparations which contain
β-glucuronidase as well as sulfatase which bring the
hydrolysis to completion (2.13, 2.14). The hydrolysis
methods are rather time consuming and different authors
claim, that times between 24 and 98 hours are required.
There are however faster methods which are used for ex-
ample in the hydrolysis of conjugated estrogens by the
enzymes of Helix Pomatia digestive juice (2.15). With
this method the time can be reduced to as little as 30
minutes.

The hydrolysis is followed by an extraction with some
organic solvent. In this procedure the choice of the
solvent and the number of extractions are important,
which is dependent on the distribution coefficients of
the steroids between the two phases (2.16, 2.17, 2.18,
2.19). Diethylether or dichloroethylene are good solv-
ents for the extractions of 17-ketosteroids and estrog-
ens. The more polar steroids must be extracted by more
20 polar solvents such as chloroform and ethyl acetate.

The formation of emulsions in the extracts of ether or
of benzene is very cumbersome and can be avoided by the
use of cold solvents and (or) by the addition of sodium-
sulfate or "Bradosol" (CIBA S.A. Basel). The acids and
the phenolic substances are removed by washing of the
organic extracts with 0.1-2.5 N sodiumhydroxide. The
phenolic steroids (e.g. estrogens) are isolated accord-
ing to the well known procedures (2.20, 2.21).

After these steps the sample is ready for separation. It
would carry too far to give a detailed description of all
the methods used. Chromatographic methods such as paper
chromatography, column chromatography (Al_2O_3) and thin
layer chromatography are of great use. All these methods
are described in literature of which a detailed survey is
given by Oertel (2.22).

For the determination of the fractions, obtained by chrom-
atography, use is made of specific detection methods such
as fluorescence, colorimetry, spectrometry etc.. To that
purpose the steroids are transformed into other compounds
which have specific properties for the detection.

Among the above indicated methods there are relatively
fast methods, but the most of them are very time consum-
ing which is a general disadvantage of the conventional
methods.

2.3 GAS CHROMATOGRAPHY

After the first publication about GC in 1952 (2.23) a
serial number of publications and books appeared, which
are dealing with the theory about GC in detail. Among
these are mentioned several handbooks to which is referr-
ed (2.24, 2.25, 2.26, 2.27, 2.28). This section is only
dealing with short notes on the theory.   **21**

2.3.2 THE COLUMN

The most important part of the gaschromatograph is the column. This is mostly a glass or metal tube (about 0.5-6 meters length, 1-6 millimeters inside diameter) which is packed with an inert granular material. An inert carrier gas (mobile phase) is passing continuously through the packing, the surface of which is coated with a thin film of a non volatile liquid (stationary phase). The inert packing material serves as a support for the stationary phase. For open hole tubular columns the wall of the column serves as a support.

If the partition of the compounds between the two phases, given by the distribution coefficient k (eqn. 2.1), is different, than in principle the compounds can be separated.

$$k = c_L/c_G \qquad \text{(eqn. 2.1)}$$

c_L and c_G are respectively the concentrations of the compound in the liquid phase and the gas phase.

2.3.3 RETENTION TIME

The capacity ratio, indicated by k', represents the ratio of the amounts of compound, distributed between the two phases.

$$k' = k \cdot V_L/V_G \qquad \text{(eqn. 2.2)}$$

V_L and V_G are the volumes of respectively the liquid phase and the gas phase. The time t_R required for the elution of a compound is given by the following equation:

$$t_R = t_0 \ (1+k') \qquad \text{(eqn. 2.3)}$$

where t_o is the time which elapses between the injection and the elution of a non retarted component. The retention time is characteristic of a component and hence the tool for identification. When the concentration of a component is low enough, k is constant(linear chromatography)for a defined system at a defined temperature. The quantity t_o is dependent on the carrier gas velocity. Variations in temperature, in carrier gas velocity, in the ratio V_L/V_G and in the dimensions of the columns make it impossible to compare the retention times from one to another instrument. For this reason the retention data are given for example as retention times relative to one component out of the mixture. There are several systems among which the Kovats retention index system
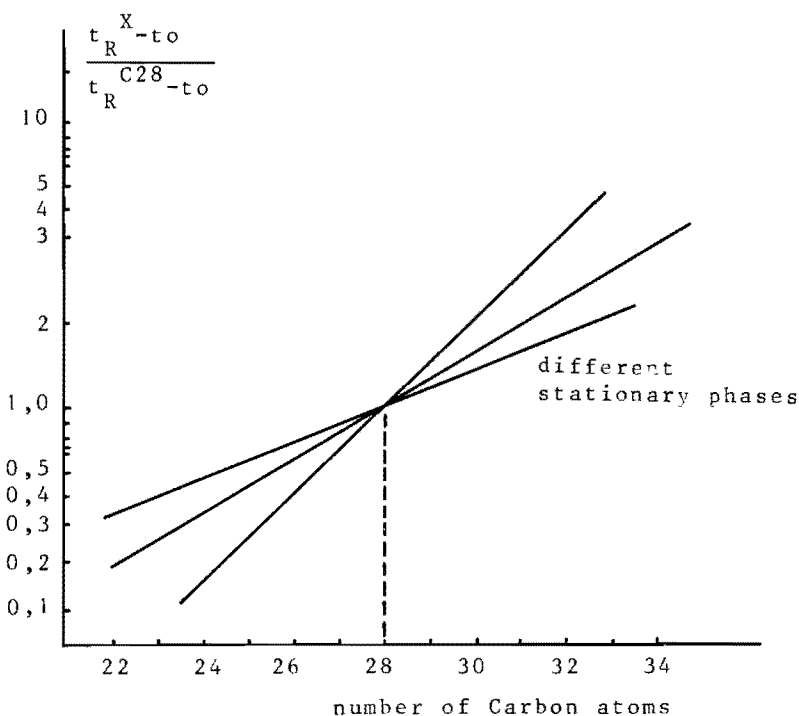
Fig. 2.1   Plot from which the steroid index can be calculated.

(2.29, 2.30) has appeared to be attractive. For steroids
several systems are in use. These systems are compiled
in the book of Wotiz (2.31). Among these systems there
are methods in which the relationship of chromatographic
behaviour and steroid structure are considered such as
e.g. steroid numbers (SN).

In this thesis use will be made of a retention index in
which the logarithm of the retention time of the comp-
ound X (t_R^X), relative to the retention time of C-28 n-
paraffin (t_R^{C-28}), is given as a linear function of the
number of carbon atoms of the series of n-paraffins.
This is illustrated in fig. 2.1, in which different
plots are obtained for different stationary phases.

2.3.4 PLATE THEORY

It is convenient in GC to use the concept of the theoret-
ical plate. The column may be conceived as divided into
a number of equal parts. The height equivalent to a
theoretical plate (HETP) is the length of such a part
of the column in which the partition process can be cons-
idered as to have come to equilibrium. It is obvious that
H(ETP) is among many things also dependent on the nature
of the compound in study. The number of plates (n) is
represented in equation 2.4, in which L is the length
of the column.

$$n = L/H$$ (eqn. 2.4)

2.3.5 RESOLUTION

When a compound is injected the input curve is about a
δ-function. Because of the fact that not all molecules

of this compound have the same residence time, the out-
put curve will be approximately of the gaussian type
(fig.2.2.a).This curve represents the residence time
distribution of the molecules, in which σ, the standard
deviation, is a measure of the spreading.

The degree of separation of two compounds 1 and 2 is call-
ed the resolution and is expressed as (fig. 2.2.b)

$$R_{21} = \frac{t_{R_2} - t_{R_1}}{\sigma_1} \qquad \text{(eqn. 2.5)}$$
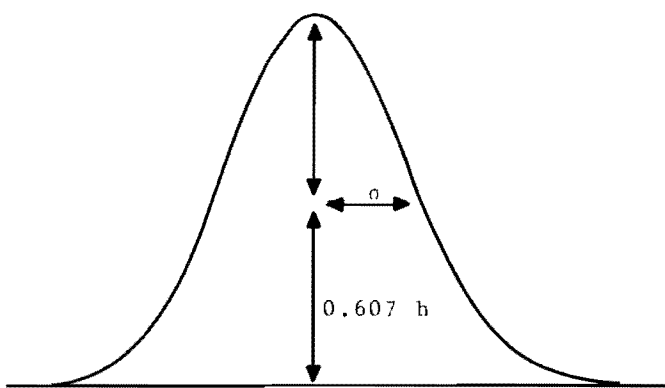
Fig. 2.2a Gaussian curve.
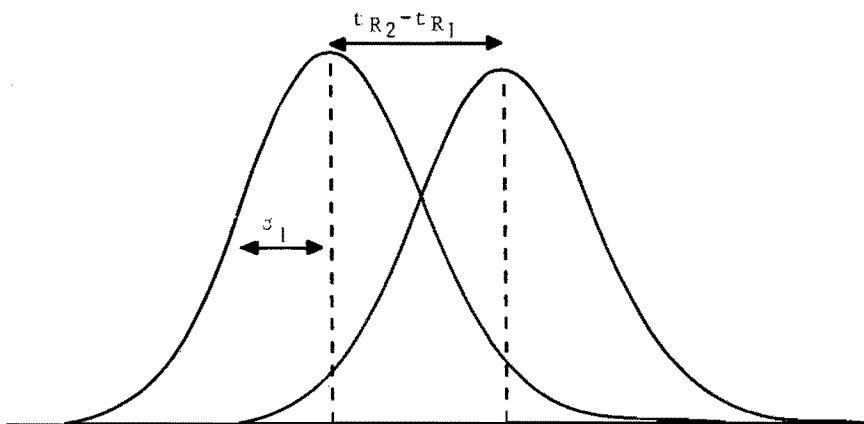
Fig. 2.2b Definition of Resolution.

Equation 2.4 can also be written as:

$$n = L/H = t_R^2 / \sigma^2 \qquad \text{(eqn. 2.6)}$$

The resolution R_{21} can be rewritten, substistuting eqns. 2.3 and 2.6, resulting in the following form which is most frequently used:

$$R_{21} = \frac{(\alpha-1)}{1+1/k_1'} \quad \sqrt{L/H} \qquad \text{(eqn. 2.7)}$$

The quantity $\alpha=k_2'/k_1'$ is equivalent to the relative volatility of the two components in that particular system. When $\alpha=1$, no separation takes place. When R equals 4 the separation is complete. The parameters in this formula can be varied and the result on the separation can be predicted.

2.3.6   COLUMN PERFORMANCE

It is supposed in the plate theory, that in each "discrete" plate the equilibrium is attained, although GC is a continuous process, in which in both gas and liquid phases the sample is capable of going in all directions. The partition process will never come to equilibrium in GC. Both diffusion and non-equilibrium have to be taken into account in the description of the influence of the experimental variables upon column performance. By van Deemter (2.32) is given a general approach to this problem, resulting in the following formula in which u is the average linear velocity of the carrier gas.

$$H = A + B/u + Cu \qquad \text{(eqn. 2.8)}$$

A, B and C are terms representing the spreading of the solute band. The coefficient A accounts for the fact, that the molecules run along paths of unequal lengths,

caused by the column packing. The coefficient B repres-
ents the spreading, caused by the longitudinal diffus-
ion in the gas phase. The resistance to mass transfer,
due to the non-equilibrium in the partition process, is
expressed by the term C. Fig. 2.3 shows the relationship
between plate height and gas velocity. The optimum cond-
itions for a given column can be found from the curve
which is experimentally determined.

u, avarage linear gas velocity
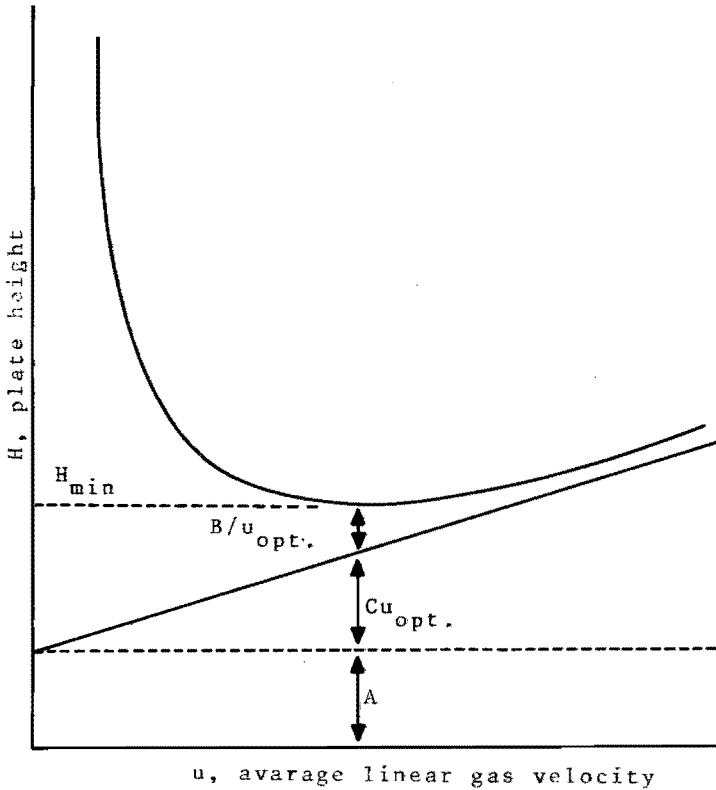
Fig. 2.3   Relation between plate height and linear gas
           velocity.

It is therefore of practical importance to test a new
column in order to find the optimum conditions. Freq-

uently it is more practical to run the chromatographic process in the curve right of the optimum.

As a rule of thumb a value for k' of about 3 appears to be a good compromise between analysis time (eqn. 2.3) and resolution (eqn. 2.7), however in the field of steroid analysis values up to 10 do occur.

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PRETREATMENT OF THE SAMPLE

3.1   INTRODUCTION

As mentioned in chapter 2 the hydrolysis of steroid con-
jugates in body fluids can be carried out in different
ways. The hydrolysis by means of mineral acids (3.1)
still widely used with regard to its speed, has been
avoided in this work because degradation and formation
of artifacts are far from negligible.

Steroids in body fluids e.g. urine are mainly present
as conjugates of glucuronic acid and of sulfuric acid.
These conjugates as such can hardly be chromatographed,
because of the fact, that decomposition of these com-
pounds will occur at the high temperatures which are
necessary in order to obtain a reasonable vapor press-
ure, required for gas chromatography.

It is common use to start the hydrolysis with an amount
of urine of one-twentieth of a 24 hr collection which
appears to be 50 to 100 ml. In the method developed
here use can be made of only 100 to 1000 microliters
of urine. In those cases where the concentrations of
the steroids in urine are very low, use is made of
amounts up to 10 ml. This scaling down results in a
simplification as required for routine analyses. Also
a reduction in sample size is saving on time as well
as on cost of chemicals. The concentration of enzyme
in the method as described below, is so large, that not
30 only the speed of hydrolysis is increased by a factor

of ten or more but also the influence of inhibitors
(which are always present in urine) on the reaction
is reduced, whereas yet the absolute amount of enzyme
is small as compared with the amounts commonly used in
conventional techniques. Neither the precision of the
analysis nor its quantitative aspect is affected by
this method.

3.2 ENZYMATIC HYDROLYSIS

Enzymatic hydrolysis as proposed by many authors requir-
es incubation times ranging from 24 hours up to about
100 hours, except e.g. the hydrolysis at elevated temp-
erature as shown by Scholler et al. (3.2). For the ap-
plication of the analysis of oestriol and 5β-pregnane-
3α, 20α-diol a fairly fast hydrolysis method should be
used. The analysis of these compounds is indicative for
disorders in the foeto-placental unit in case of rapid
decreasing excretions over a 24 hour period. It is
therefore of importance that the analysis is relatively
fast, whereas the reproducibility is emphasised more
than the achievement of an absolute result.

There are many parameters which are affecting the hy-
drolysis with regard to speed and to yield of free ster-
oids. These parameters are: temperature, enzyme concen-
tration (nature and activity of the preparation involv-
ed), incubation time and pH which must be kept constant
by adding a buffer solution. It is not within the scope
of this thesis to investigate the enzymatic hydrolysis.
It is however necessary to choose the experimental cond-
itions such that a reasonable compromise is made between
time of hydrolysis and yield of free steroids. For the
analysis of oestriol and pregnanediol in particular in
pregnancy a standard method will be proposed.   **31**

Although a detailed hydrolysis study has not been att-
empted, the many parameters involved require a few hun-
dreds of analyses to be done to get some idea of the
process. Two identical series of experiments have been
set up at: 37°C

          pH 5.2
          incubation time 30, 60, 90, 120, 150 and 180
          minutes
          urine/buffer ratio 5 to 1
          enzyme concentration 0.05, 0.10, 0.15, 0.20
          and 0.25 ml helicase/ml urine.

The enzyme being the digestive juice from Helix Pomatia,
containing 100.000 units "Fishman" of β-glucuronidase
and 800.000 units "Roy" of sulfatase is used. For this
purpose use is made of a urine of a pregnant woman. The
recoveries of oestriol (O) and 5β-pregnane-3α, 20α-diol
(P) are studied by means of GC. The reproducibility of
the enzymatic hydrolysis was poor. The standard deviat-
ion amounted to 6.6% of the avarage. In this figure are
also involved other contributions which will be discuss-
ed below. The urine, mentioned above, was also hydrol-
ysed during 24 hours with an enzyme concentration of
0.1 ml/ml urine. The avarage yield of O and P at all
enzyme concentrations and at 180 minutes agreed very
well on the result of the 24 hours hydrolysis as shown
in table 3.1.

This means that the hydrolysis is brought to completion
within 3 hours at the used enzyme concentrations of
which the 0.05, 0.10 and 0.15 ml enzyme have appeared
to give the best results. The standard deviation over
all measurements, described above, being 6.6% of the
avarage is to a certain extent a reflection of the con-
tributions of the extraction, of the evaporation of the
extraction solvent, of the conversion into steroid der-
32 ivatives and of the GC process, whereas a considerable

Table 3.1   Agreement between the 3 hours and the 24 hours
hydrolysis.

enzyme concentration in ml/ml urine	Avarage yield in µg/ml urine of P after 180 min. of 4 experiments each	Avarage yield in µg/ml urine of O after 180 min. of 4 experiments each	Avarage yield in µg/ml urine of P after 24 hours of 2 experiments	Avarage yield in µg/ml urine of O after 24 hours of 2 experiments
0.05	47.8	37.4		
0.10	49.7	40.0	46.9	38.1
0.15	49.2	41.8		
0.20	43.2	35.4		
0.25	43.9	35.0		
	av. 46.8	37.9		

contribution is coming from the hydrolysis of which the
reproducibility is poor. One of the major reasons for
this has appeared to be the change in pH for which en-
zyme reactions are sensitive. This is further investig-
ated.

In order to start from a urine of pH 5.2, the urine is
titrated to this pH either by 1N HCl or by 1N NaOH. This
is carried out using an automatic titrator which is add-
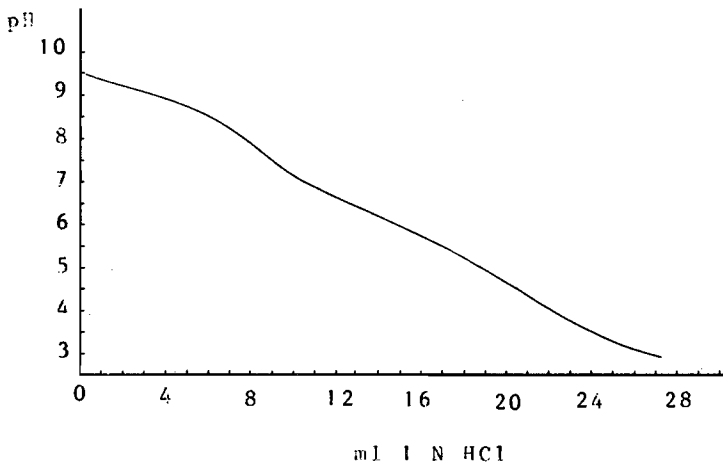
Fig. 3.1   Titration curve of urine.

ing the acid to 10 ml urine with increments of 0.1 ml.
The attainment of the right pH by adding increments of
0.1 ml 1N HCl is dependent now on the buffer capacity
of the urine itself. This is shown in fig. 3.1 which is
a titration curve of the urine in question, brought at
pH 9.52 before the start of the experiment.

From fig. 3.1 can be seen that the change of the pH is
0.025 pH units per added increment around pH 5.2. The
buffer capacity at higher pH is slightly better. This
method of pH adjustment is acceptable since the change
in pH is 0.025 units per increment.

Another point is to keep the urine at pH 5.2 during the
hydrolysis and more important, as has appeared, after
the addition of enzyme. Table 3.2 shows the pH change
after addition of enzyme for two different amounts of
acetate buffer (0.2 molar).

*Table 3.2 Influence of the enzyme quantity on the Ph of
the urine, differently buffered.*

enzyme in ml/ml urine	pH urine/buffer 5 / 1	pH urine/buffer 1 / 1
0	5.23	5.22
0.05	5.30	5.28
0.10	5.34	5.30
0.15	5.38	5.30
0.20	5.40	5.30
0.25	5.41	5.31

From this table it is concluded that the urine/buffer ratio 1 to 1 is preferred to the ratio 5 to 1 at which the pH change of 0.18 is not acceptable with regard to the sensitivity of the enzyme reaction.

Another series of experiments have been done in order to study the recoveries of free O and P at conditions derived from the experiments described above. The hydrolysis of the urine of the same patient, collected at an other day, is carried out at $37^{O}C$, at pH 5.2 with a ratio urine/0.2 molar acetate buffer of 1 to 1. As variables are taken the hydrolysis time from 30 to 180 minutes and the enzyme concentration from 0.05 to 0.25 ml/ml urine. It is started from one portion of urine which is adjusted to pH 5.2 and which is buffered 1 to 1. This portion is divided into 10 equal portions and transferred to glass vessels. To every 2 vessels (for a duplicate hydrolysis) respectively 0.05 - 0.10 - 0.15 - 0.20 - 0.25 ml enzyme/ml urine is added. After every 30 minutes up to 180 a sample is taken out of each vessel and saturated with solid sodiumhydrocarbonate to stop the hydrolysis and to make the sample suitable for extraction. The recoveries of O and P are studied by means of GC. The results of these experiments are illustrated in table 3.3.

In the standard deviation of these results one should take into account, apart from the contribution of the hydrolysis procedure, the contributions of the extraction, of the evaporation, of the conversion into steroid derivatives and of the GC process. The standard deviation is amounting to 4% of the avarage (extreme values neglected) which is considerably better than in the first experiments due to the better pH control. The relation between the yield of O and P and the incubation time is plotted in fig. 3.2 for different amounts of enzyme. The plotted points being the avarage of two separate hydrolyses are connected with each other by straight lines. **35**

*Table 3.3   The influence of incubation time and enzyme
concentration on the yield of O and P.*

Time in min.	Number of exp.	P µg/ml urine	O µg/ml urine	Enzyme ml/ml urine
	1	15.7	6.8	0.05
	2	15.3	7.8	
	3	19.1	11.7	0.10
	4	19.4	11.9	
30	5	22.3	12.7	0.15
	6	23.9	14.5	
	7	28.2	16.1	0.20
	8	27.4	15.0	
	9	33.2	14.8	0.25
	10	34.5	13.8	
	11	19.0	13.2	0.05
	12	18.9	13.7	
	13	21.6	14.8	0.10
	14	20.6	14.3	
60	15	25.0	17.7	0.15
	16	23.2	17.2	
	17	27.0	19.1	0.20
	18	23.0	15.2	
	19	27.6	15.9	0.25
	20	31.0	17.4	
	21	20.3	17.3	0.05
	22	19.0	15.5	
	23	21.6	17.7	0.10
	24	25.1	21.2	
90	25	23.2	19.4	0.15
	26	21.4	17.6	
	27	23.4	17.8	0.20
	28	21.8	14.9	
	29	24.1	15.8	0.25
	30	25.0	16.4	

Time in min.	Number of exp.	P µg/ml urine	O µg/ml urine	Enzyme ml/ml urine
120	31	14.8	14.1	0.05
	32	14.8	14.1	
	33	19.4	17.8	0.10
	34	14.4	13.9	
	35	27.5	12.2	0.15
	36	20.0	16.0	
	37	19.1	15.8	0.20
	38	18.8	12.6	
	39	19.7	11.4	0.25
	40	18.2	11.9	
150	41	25.6	17.6	0.05
	42	18.6	13.7	
	43	19.9	16.0	0.10
	44	20.4	16.1	
	45	22.8	18.7	0.15
	46	18.5	14.0	
	47	20.3	15.0	0.20
	48	17.4	12.2	
	49	19.6	14.7	0.25
	50	19.0		
180	51	20.8	13.4	0.05
	52	19.9	12.7	
	53	19.6	14.6	0.10
	54	19.7	15.3	
	55	20.3	15.5	0.15
	56	19.4	13.9	
	57	20.7	15.7	0.20
	58	19.6	14.0	
	59	18.9	13.5	0.25
	60	20.0	16.8	
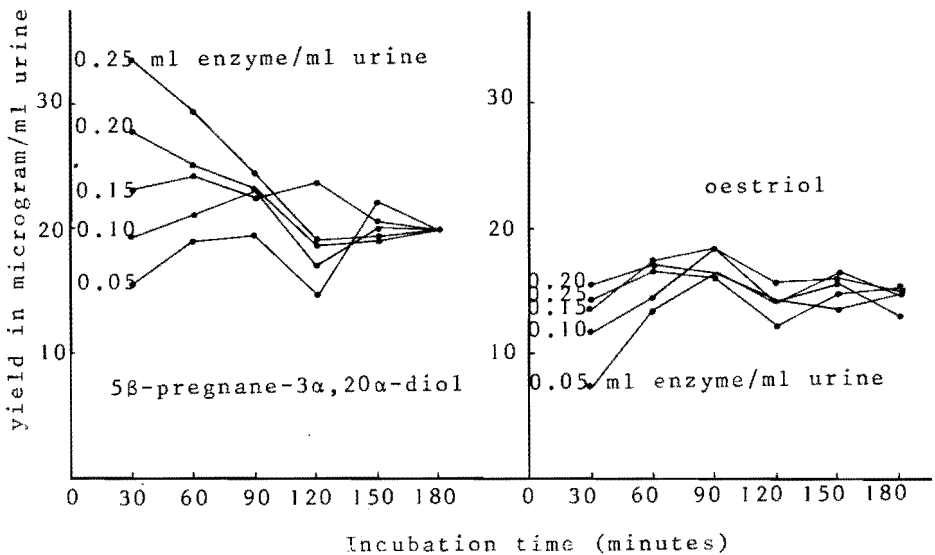
Fig. 3.2   Relation between hydrolysis time and yield of
free oestriol and 5β-pregnane diol for differ-
ent enzyme concentrations (ml enzyme/ml urine).

For O an almost steady increase of the yield up till 90
minutes and simultaneously an increase of the yield with
increasing enzyme concentrations are observed. From
there on up to 180 minutes the lines are slightly dropp-
ing and are converging to an avarage yield at 180 min.
which is equal to the recovery obtained at these condit-
ions after 24 hours with 0.1 ml enzyme/ml urine. For P
an exceptional relation is found in an almost dropping
yield as function of time for almost all enzyme concentr-
ations. The yield at 30 minutes for instance is increas-
ing with increasing enzyme concentrations which is log-
ical. Again here the lines are converging to an avarage
value which has also been found for the 24 hour hydro-
lysis at 0.1 ml enzyme/ml urine. Such relations could
not be found in the past, because the hydrolysis proced-
ures required 24 hours and more with in general less en-
38 zyme. This should be investigated further by others.

Until this matter has been studied more thouroughly it
is proposed to choose the experimental variables as
follows. In order to obtain more reproducible results
one should take an incubation time of 180 minutes and
an enzyme concentration of 0.1 ml/ml urine, supported
by the knowledge that at 0.1 ml enzyme/ml urine the
yield, obtained from a 24 hour hydrolysis, is the same.
Hence the hydrolysis can be considerably speeded up as
compared to conventional enzymatic hydrolysis. The pH
control should be emphasised and starting from 0.5 ml
urine an equal amount of acetate buffer (0.2 molar)
should be used. It has appeared to be very convenient
to carry out the entire process in a teflon stoppered
test tube.

3.3 EXTRACTION

To the hydrolysate is added 5 ml of diethylether and the
appropriate amount of C24-n-paraffin as an internal
standard for quantitative analysis. The test tube, after
having been closed, is thouroughly shaken. The water
layer in the test tube is then gradually saturated with
anhydrous sodium sulphate. The gradual addition converts
the aquous layer into a saturated solution and by the
salting out effect the steroids, still for a small am-
ount present in the water layer, are driven into the
ether layer quantitatively. Further addition of sodium
sulphate takes up all the water and after centrifuging,
the ether layer can be decanted easily. The amount of
ether is then divided into 3 to 5 portions and more if
desired, dependent on the amounts of steroids present
in the extract and dependent on the quantity, required
for a GC analysis. Each portion is transferred into a
small glass cup (volume 1.5 ml) from where the evapor-
ation of the solvent can be carried out.   **39**

3.4   THE EVAPORATION OF THE EXTRACTION SOLVENT

Much attention is payed to the evaporation technique,
which is carried out by means of a completely uncommon
method. The amount of steroids, present in the extract,
is very small and amounts in this method to about 10
micrograms as a maximum with no limit to lower quantit-
ies. This minute amount must be collected in a small
space of a few microliters in order to be capable of
both derivatizing the sample with a small amount of
reagent and carrying almost the whole mixture over to
the top of a GC column.

For this purpose use is made of a pyrex glass capillary
of a design as is illustrated in fig. 3.3. The thick
end of the capillary is connected with a vacuum pump,
whereas the thin end is immersed in the ether extract.
Depending on the length of the thin part of the capill-
ary a relatively low absolute pressure (< 25 mm Hg) is
applied by means of which the ether is evaporated. On
the other hand the steroids are collected quantitative-
ly in the conical part of the capillary where in fact
the evaporation of the solvent takes place. The effic-
acy of the system is based on the high speed of evapor-
ation of the ether.

3.4.1   DRAWING APPARATUS FOR GLASS CAPILLARIES

The evaporation in a capillary as described above is de-
pendent on several parameters of which the diameter of
the thin part and the thick part as well as the shape
of the conical piece of the capillary have appeared to
be most important. The speed of evaporation for a given
capillary at a definite temperature depends in the first
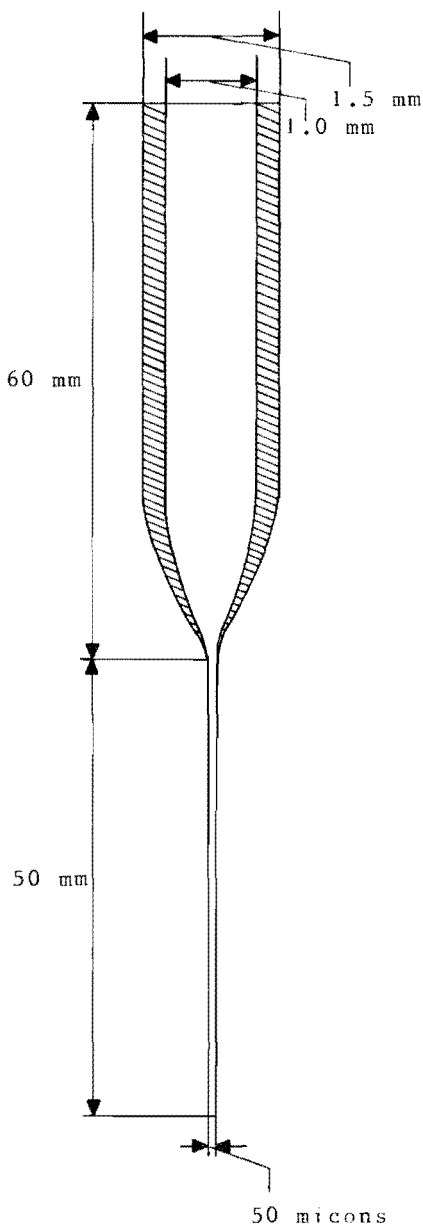**40**   instance on the absolute pressure applied and secondly

Fig. 3.3   Glass capillary showing form and dimensions.

on the viscosity of the extract. During the evaporation
the conical part of the capillary is covered by ice on
the outside due to the withdrawal of heat from the sur-

rounding humid air. This ice formation decreases the
the speed of evaporation which can, when desired, be
avoided by applying a stream of hot air on that partic-
ular part of the capillary. The parameters which have
influence on the quality and the speed of evaporation
must be eliminated as much as possible. For the exper-
iments at the start of this work the capillaries have
been made manually. This involves practical difficulties,
whereas the evaporation could not be carried out repro-
ducibly. To eliminate most of the variable quantities
the capillaries must be standardized with respect to
the dimensions with great precision. This has been
reached by constructing a drawing machine by which the
capillary is obtained in an automatic manner.

The machine in question is presented in fig. 3.4 togeth-
er with the cross section X - X' of fig. 3.5. The glass
capillary (b fig. 3.4), dimensions 100 x 1.0 x 1.5 mm
either commercially available (or drawn e.g. on a Desty-
Goldup machine (3.3)) is put in position by inserting it
in a silicone rubber O-ring (fig. 3.5). The capillary
when heated by the heating coil will come down by grav-
ity, the speed of drawing being accelerated by fixing a
brass weight of cylindrical shape (C) at the bottom end
of the capillary (also by means of an O-ring). The free
fall is damped by the glass tube (d) that fits snuggly
around the brass weight. By moving down the lever (a)
the contact (f) is actuated and the heating coil (cross
section 3.5) is rapidly heaten to dark red. It has app-
eared that with an outside diameter of the brass weight
(10 mm) and inside diameter of the glass tube (10.4 mm)
a constant downward velocity is reached almost instant-
aneously and a very uniform conical profile results. The
machine-made capillaries have precisely equal dimensions
by which means the evaporations are carried out with

42 great reproducibility. Fig. 3.6 shows a photograph of

a:lever
b:glass capillary
c:weight (6.8g)
  O.D. 10 mm
d:centre tube (glass)
  length 30 cm
  (is variable)
  I.D. 10.4 mm
e:Power supply
  (10V)
f:switch
g:heating coil
  holder with
  heating coil
  frame, see
  cross section
  XX' in fig.3.5
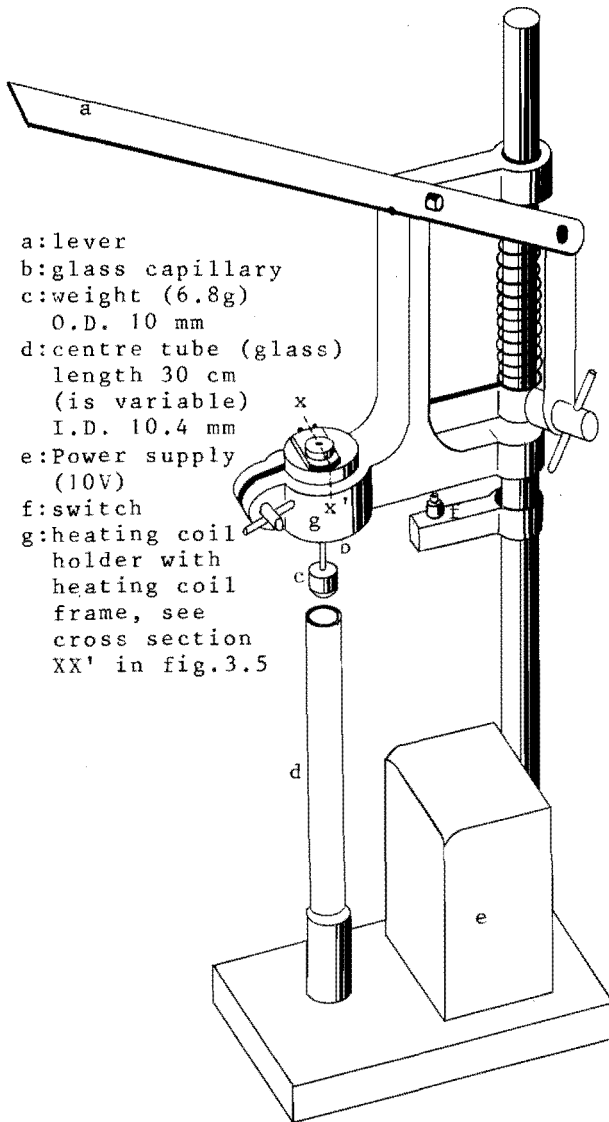
Fig. 3.4 Drawing apparatus for glass capillaries.

a: lever   e: power supply,

b: glass capillary   10V

c: weight, 6.8 g, O.D. 10 mm   f: switch

d: centre tube, length 30 cm   g: heating coil

*   I.D. 10.4 mm   frame, see fig.3.5*

For further explanation, see text. **43**

X ———————————————— silicone rubber

————heating coil frame
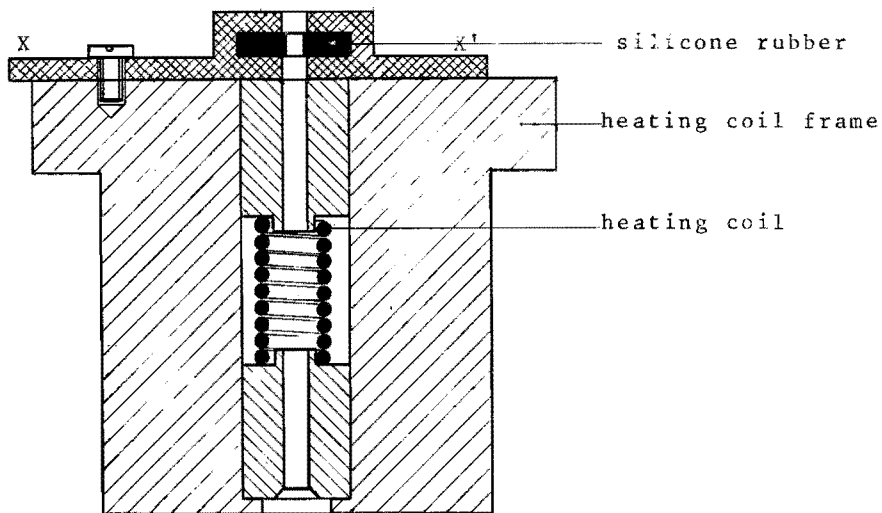
————heating coil

*Fig. 3.5 Cross section X-X' of fig. 3.4. Frame with
         heating coil.
         Coil: length 7 mm, I.D. 2.5 mm. Coil material
         NiCr (40/60), wire diameter 0.5 mm. VA = 60.
         The glass capillary is at the top end fixed
         in a silicone rubber ring.*

the capillaries. In this picture they are covered with
a waterfilm by immersion in order to make the outside
wall visible, whereas they are partly filled with merc-
ury for the same reason.

The outside diameter of the thin part of the capillary
is easy to measure with a micrometer and amounts to 50
microns. The inside diameter is estimated at 40 microns.

3.4.2   THE PERFORMANCE OF THE EVAPORATION

As is shown in the diagram (fig. 3.7) the evaporation
can be carried out simultaneously for a number of ex-
44 tracts. The connections of the capillaries with the
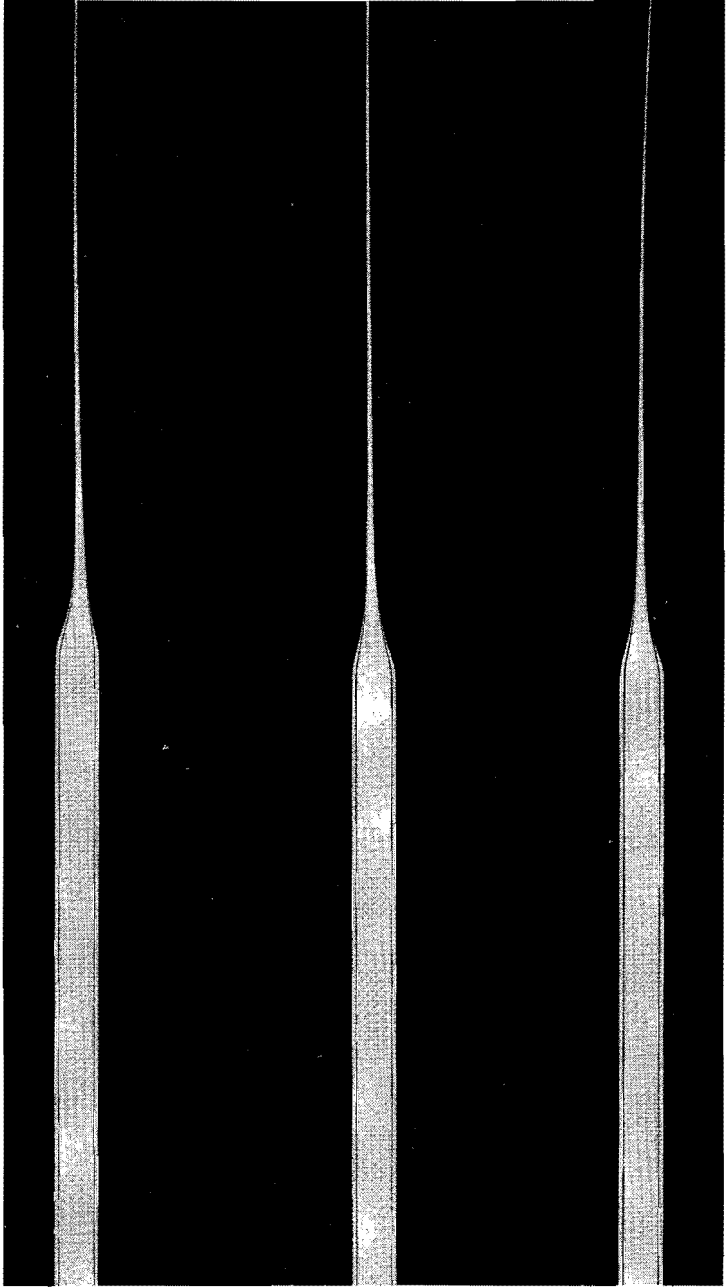
Fig. 3.6   A picture showing machine made capillaries and
their reproducible forms.
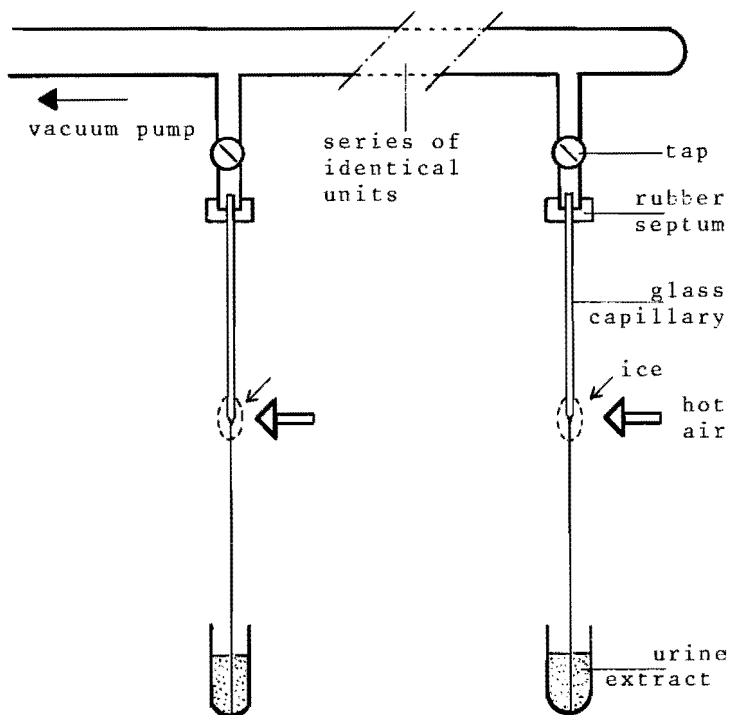
*Fig. 3.7   Diagram showing the procedure of evaporation.
It can be performed simultaneously for a ser-
ies of extracts as is shown.*

vacuum pump are made by plastic tubing. The glass cups
containing the extracts are placed in position, where-
as hot air, when desired, is supplied on the evaporat-
ion zone. The evaporation is started by opening the
taps. At an absolute pressure of roughly 2 mm Hg, 1 ml
of ether extract is evaporated in about 15 minutes. Be-
cause of the uniformity of the capillaries the speed of
evaporation is constant.

The shape of the conical part has appeared to be very
important. A too smooth transition from the thin part
to the thick part of the capillary results in a contin-
uously sucking up of the solvent which then disappears

into the vacuum line as a liquid. A very sharp transition however results in an irregular spattering of droplets which disappear also into the vacuum line. The shape of the transition has been found experimentally. The angle of the cone has to amount to between 30 and 45°.

The rate of feeding and the rate of evaporation must be equal which is attained for these capillaries by choosing a low absolute pressure and by adapting the length of the capillary in such a way, that the input does not exceed the output. At high absolute pressures (> 25 mm Hg) the speed of evaporation is too small and the solvent is creeping up to the wall of the capillary.

It must be emphasized, that all the experimental variables such as absolute pressure, temperature of the supplied hot air etc. are dependent on the dimensions of the capillary only.

3.5   STEROID DERIVATIVES

In order to impart greater stability and greater volatility to the steroid molecule and in order to prevent absorption of the polar steroid molecule on the solid support during GC, it has appeared to be of great importance to derivatise steroids before the GC analysis.

There are many reagents available among which hexamethyl-disilasane (HMDS) and bis-trimethyl-silylacetamide (BSA) are the most important by means of which the hydroxy groups are derivatised into trimethylsilyl-ethers. For oestrogens use is sometimes made of the acetate derivatives. When an electrone capture detector is used the derivatives of choice are with bromine, chlorine or fluorine.

The most common derivatives for the purpose of GC are
trimethylsilylethers of which the preparation is based
on the procedure of Sweeley et al. (3.4). In almost all
these procedures trimethylchlorosilane (TMCS) acts as a
catalyst. In order to keep the steroids solved in the
reagent pyridine is often added. Using BSA it is not
necessary to add pyridine because BSA acts preeminent-
ly as a solvent as well. The reaction proceeds as foll-
ows:

$$2ROH + CH_3 - CO - N \left[S_i(CH_3)_3 \right]_2 (BSA) \rightarrow$$

$$2ROS_i(CH_3)_3 + CH_3 - CO - NH_2$$
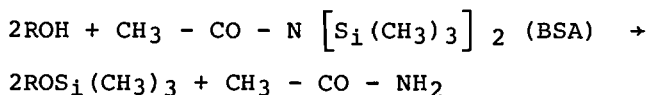
In general the reaction takes a few minutes time and
the conversion is almost quantitative.

In this work only BSA is used as reagent, containing
10% of TMCS as a catalyst. It has appeared, that this
mixture converts the hydroxy groups which are not ster-
ically hindered, into the ethers within 5 minutes at
room temperature. This reaction time is tested by the
following experiment.

Five microgram of each compound, oestrone, oestradiol-
17β, oestriol, 5β-pregnane-3α, 20α-diol and n-tetra-
cosane as an internal standard are exposed to an excess
of the reagent during different times at room temperat-
ure. The recoveries of the trimethylsilyl ethers are
studied relative to n-tetracosane (chosen as unity) by
a GC analysis as is shown by table 3.4.

It is well known, that the 11-hydroxy group in steroids
requires more time to derivatize in the TMS_i-ether caus-
ed by steric hindrance. The reaction time can be short-
ened for this case by adding more catalyst (1 to 5) and
48 by applying higher temperatures (3.5).

Table 3.4 The minimum required reaction time for the conversion of not sterically hindered ster-oids into TMS$_i$-derivatives using BSA-TMCS (10:1).

Recoveries relative to n-tetracosane					
Reaction time in minutes	n-tetra cosane	oestrone	oestra-diol-17β	5β-preg-nane-3α, 20α-diol	oestriol
5	1.00	1.07	1.32	1.29	1.33
15	1.00	1.09	1.29	1.28	1.36
30	1.00	1.07	1.28	1.24	1.36
60	1.00	1.11	1.30	1.28	1.36

When the sample is collected in the glass capillary aft-er evaporation of the solvent, the reagent amounting to roughly 2 microliters and more if desired, when more mat-erial is available, is sucked up into the capillary by means of flexible plastic tubing. The capillary is then sealed off at both ends to protect the sample against moisture. The TMS$_i$-derivatives are fairly stable but in humid atmosphere they may conceivably hydrolyse which should be avoided. The sample is now ready for injection on to the top of a GC column.

3.6 PURITY OF SOLVENTS AND REAGENTS

The detection systems in gas chromatography are very sensitive so that impurities present in solvents which are used in the pretreatment of the sample, may disturb the GC analysis. The ether extract, containing the ster- **49**

oids, is evaporated in the glass capillary, as describ-
ed above. Non volatile impurities in the ether mainly
peroxides are also collected in the glass capillary.
When the amount of steroids compared with the amount of
impurities collected in the capillary is of the same
order the GC analysis is completely upset. This is ex-
emplified on the analysis of the residue which is obt-
ained after evaporation of 1.5 ml of "chemical pure"
ether only, curve 1 in the chromatogram shown in fig.
3.8. Curve 2 represents the same analysis, but now the

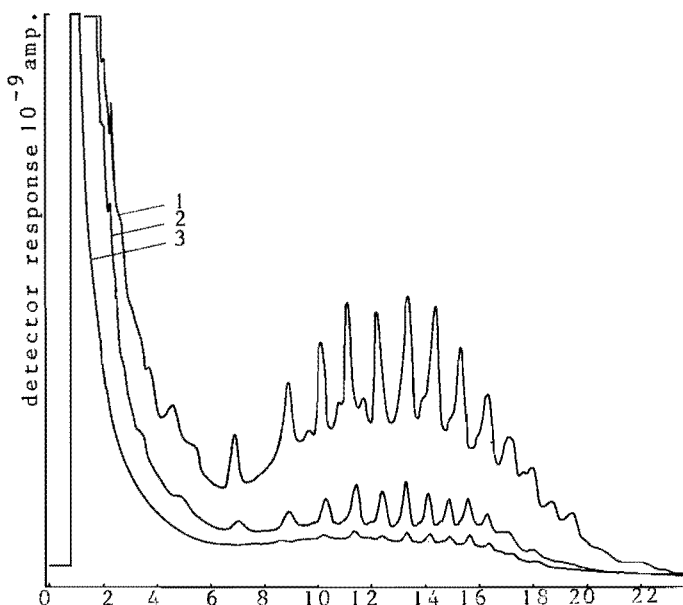*Fig. 3.8 A temperature programmed gas chromatographic*
analysis of the non volatile peroxides collect-
ed from 1.5 ml ether at three grades of purity
(see text). Column 160 cm x 4 mm, 3.8% SE-30
on chromosorb Q 80-100 mesh. Carrier gas flow
46 ml N_2 per min. Initial temperature $140^{o}C$,
TP rate $5^{o}C/min.$, final temperature $250^{o}C$.
Temperature of injection system and FID both
$250^{o}C$.

ether is prepurified by means of ferrosulphate in order
to remove peroxides. This ether is stored in a dark
bottle over anhydrous calcium chloride and it is furth-
er used for one week. When the prepurified ether is el-
uted over Al_2O_3 (Woelm basic, activity grade 1) the re-
maining peroxides are removed to that extent at which
gas chromatography at the used condition of sensitivity
is not disturbed. This is illustrated in curve 3 of fig.
3.8. The third purification step is carried out with
the aid of a column of 20 cm length and of 10 mm inside
diameter, filled with Al_2O_3. The first 20 ml of eluted
ether are wasted. In the next 100 ml the amount of per-
oxides are acceptably low for the purpose of GC. It
should be observed that this elution should be done
just before use because there are possibly peroxides
present. It should be considered too, that the format-
ion of peroxides is catalysed by peroxides, so that
purified ether keeps longer as the ether is cleaner.

Another experience about the purity of reagents should
be pointed out here. The commercial available reagents,
such as BSA, TMCS, HMDS etc. are mostly delivered in
glass bottles sealed by rubber serum caps, which is con-
venient for use. The amount of constituents, however,
coming from the rubber is sometimes considerable and
these substances are very often interfering with the
GC analysis. This can be avoided by using only fresh
distilled reagents which should be stored in glass
stoppered vessels.

One of the reasons for which the glass capillary has
been developed, is to have a perfectly clean injection
syringe. Syringes are very difficult to clean and cause
very often troubles in GC. When the glass capillary is
used as syringe another capillary can be used for each
injection. The glass capillary is scrupulously clean,   **51**

because of the high temperature at which they are manu-
factured. It has appeared that these capillaries are
suitable for the direct sampling on to capillary columns
which will be described in chapter 6.

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STEROID ANALYSIS WITH PACKED COLUMNS

4.1 INTRODUCTION

The hart of the gas chromatograph is the column. There-
fore much attention must be payed to the preparation of
analytical columns. A simple theory, as is given in
chapter 2, will serve as a guide to the achievement of
column efficiency. During the last few years much effort
is devoted to the development of efficient columns for
steroid analysis and there are certainly good columns
commercially available. Nevertheless there must be made
here some remarks with regard to steroid analysis.

4.2 COLUMN

In steroid analysis it is very important to set high de-
mands on quality of the column. The support has to be
inert to a great extent. Therefore acid washed and sil-
anised diatomaceous earth is commonly used for the pur-
pose (4.1). The use of glass columns which is commonly
preferred to the use of stainless steel columns, is un-
necessary for most purposes. The steroid derivatives
such as TMS_i-ethers and acetates are stable enough and
the reason for which glass columns should be used is
mostly to avoid decomposition of bromine, chlorine and
fluorine derivatives when an electrone capture detector
is used. Mostly the injection ports, provided with flash
heaters, give rise to decomposition of thermally labile
substances. Also for this reason it is better to bring **53**

the sample directly on to the column itself. It has appeared, that reagents, such as BSA-TMCS, used in excess and introduced on the top of the column together with the sample, are affecting the stationary phase which is probably decomposed. As a consequence the solid support becomes more active and this in turn will give rise to a partial decomposition of the steroid derivatives. This could be concluded from an apparent decreasing of the response factors of the sample components of a standard mixture. By replacing the packing of the top of the column with fresh material the effect disappears. The stationary phase must be thermally stable at temperatures which are applied in steroid analysis and which are varying from 200 to 260°C. The bleeding of the stationary phase causes a lot of troubles. There is a way to minimise the bleeding of the stationary liquid. This procedure is used in this work for all SE-30 (silicone gum) columns. The column, filled with the coated support, is placed in an oven at 350°C for one hour. The thermally labile constituents of the polymer are degraded to more volatile compounds which are eluted from the column at 300°C with a gentle stream of nitrogen (5 ml/min) within an hour. The column is ready for use after a conditioning at a temperature 25°C above the operation temperature of the intended GC analysis and at a normal flow of carrier gas for 24 hours. This method has appeared to be satisfactory in obtaining a stable polymer for this purpose. An extra advantage of the method is, that the SE-30 polymer gets the opportunity at the high temperature to spread out homogeneously over the granular material.

4.3 THE PERFORMANCE OF THE GC ANALYSIS OF THE MAIN OESTROGENS AND 5β-PREGNANE-3α, 20α-DIOL

The above mentioned steroids are mainly found in the urine of pregnant and non pregnant females. These hormones are of clinical importance which will be discussed below. The GC analyses of these compounds in pregnancy is described in section 4.4.

A standard mixture of the compounds, oestrone, 17β-

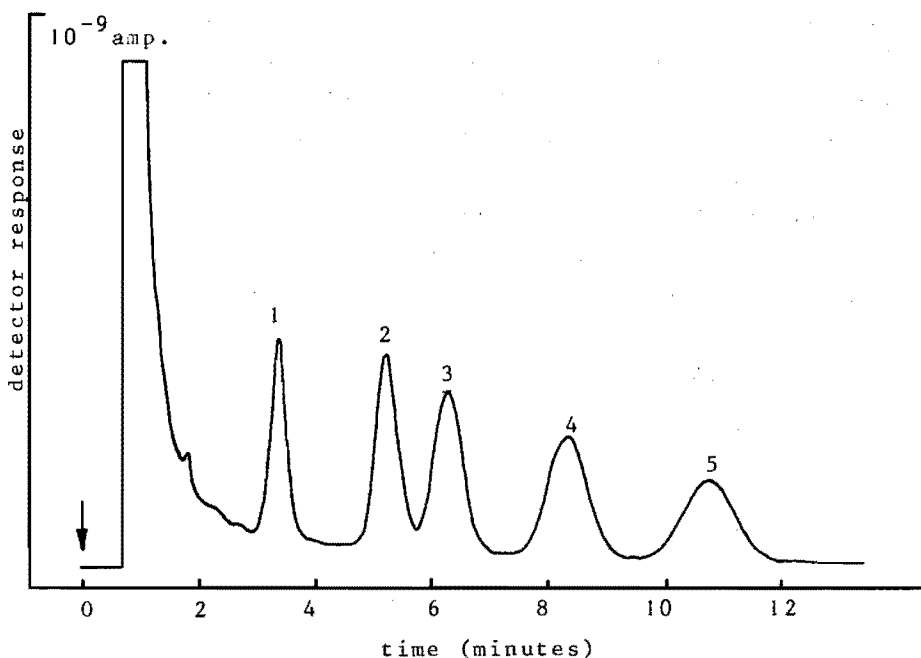Fig. 4.1 Analysis of a standard mixture of the TMS_i-derivatives of oestrone (2), 17β-oestradiol (3), 5β-pregnane-3α, 20α-diol (4), oestriol (5) and n-tetracosane (1) as internal standard. Column 160 cm x 4 mm, 3.8% SE-30 on chromosorb Q 80-100 mesh. Carrier gas flow 45 ml N_2/min., temperature of injection port, column and FID $250^{o}C$. Injected amount 0.1 μl, containing about 1 μg of each steroid.

oestradiol, 5β-pregnane-3α, 20α-diol, oestriol and
n-tetracosane as internal standard, is prepared in
ethyl alcohol of which a small portion is taken up in
ether. This solution is treated as is described in
chapter 3. In the final step the sample, collected in
the glass capillary, is exposed to an excess of two
microliters of the reagent BSA-TMCS (10:1) for 5 minut-
es. From this mixture 0.1 microliter, containing about
1 microgram of each steroid derivative, is injected, on
to the top of the column. The result is shown in fig.
4.1, in which the experimental conditions are mentioned.
The resolution which may appear not too bad, still
leaves to be desired. On the one hand the separation
between steroids must be improved, on the other hand,
the contamination of steroid peaks with the tail of the
solvent will impair the analysis. In standard analyses
the tailing of the solvent peak is still relatively
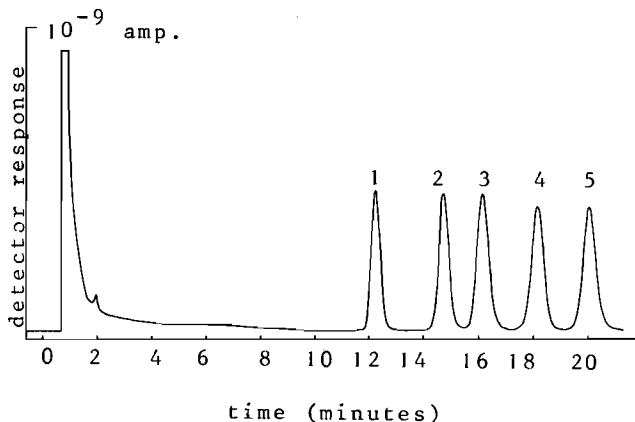small, as compared with the tailing obtained in the

time (minutes)

*Fig. 4.2   Temperature programmed analysis of the same
standard mixture as described in Fig. 4.1 with
the same column. Initial temperature 140°C,
final temperature 250°C, TP rate 5°C/min. Tem-
perature of injection port and detector (FID)
both 250°C.*

analysis of urine extracts of which 1 μl of solvent has
to be injected. This is ten times more than the amount
used in the standard (fig. 4.1). In order to avoid the
overlap of the solvent peak with other peaks temperat-
ure programming (TP) is used which has simultaneously
the advantage of improving the separation of the ster-
oid derivatives. This TP analysis is shown in fig. 4.2
in which the same standard mixture is used. The experi-
mental conditions are mentioned in the figure.

4.4 THE REMOVAL OF THE SOLVENT DURING THE GC PROCESS
AS A FURTHER IMPROVEMENT

It has appeared in the temperature programmed process,
that at an initial temperature of 140°C the steroid
derivatives are remaining on the top of the column, in
fact they are travelling very slowly at this temperat-
ure. For instance the velocity of oestrone-TMS$_i$ at 140°
C is 0.2 cm/min which is 160 times slower than at 250°C
at which the velocity amounts to 32 cm/min. Use is made
of the principle of collecting non volatile material on
a relatively cold place, whereas the volatiles,such as
the solvent in steroid analyses, are removed. The solv-
ent is the source of many a trouble e.g. the presence
of the solvent during the GC analysis is affecting the
quality of the separation and the reproducibility of the
analysis, specially when the solvent is tailing consider-
ably. In steroid analysis the reagent, commonly also
used as a solvent, is injected together with the steroid
derivatives by means of which the detector is getting
dirty and hence noisy. In order to avoid all these
troubles it was decided to find means of removing the
solvent at the top of the column. The system developed
for this purpose which is referred to the "trapping
proces_" is outlined in fig. 4.3.   **57**

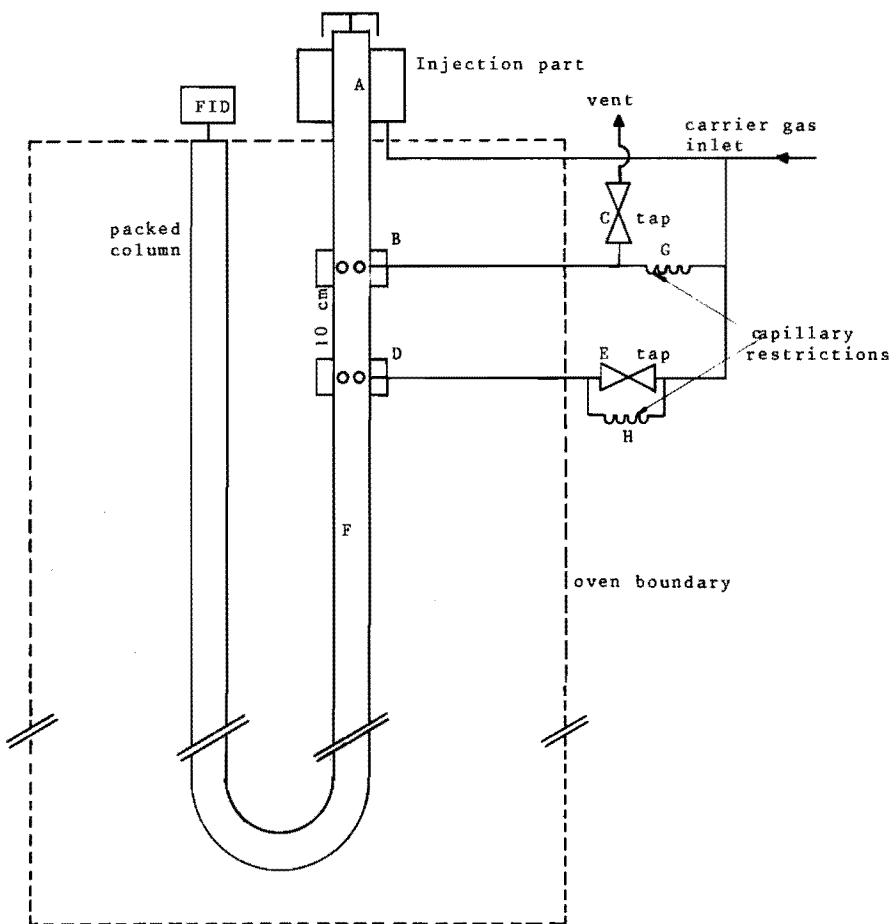Fig. 4.3 Flow pattern diagram of the "trapping" system
in order to remove the solvent at the top of
the column. See text for explanation.

A length of 10 cm of column is surrounded by the inject-
ion port (due to the design of the used apparatus) and
it has a temperature of 250°C. The rest of the column is
at 140°C at the start of the TP analysis. Before the in-
jection both taps (C and E) are open. A stream of gas

now is flowing to vent along ABC. Simultaneously a
"backflush" stream of gas coming through tap E leaves
the system to vent along DBC. The inlet system is flow
controlled. That part of the gas which is not leaving
to vent is flowing through the column from D into dir-
ection F. There is in this situation almost no flow
through the capillaries (G and H) of which the resist-
ance against flow is big as compared to the resistance
of the packed column itself. In this situation the solv-
ent, containing the sample, is injected. This solvent
is carried to vent within two minutes according to the
flow diagram described above. The steroid derivatives
remain somewhere between A and B. Two minutes after in-
jection both taps are closed simultaneously and at the
same time temperature programming is started. In this
situation the main stream of gas is coming from the top
A of the column. The column is at B and at D perforated.
Through the small holes at these places the sample is
capable of going into BC and into DE. When there is how-
ever a gas stream at B and at D into the column, then
this effect is negligible. Therefore the inlet of the
carrier gas is connected via capillary restrictions with
B and D, so that a small part of the total gas flow is
supplied at these points. In order to supply a few per-
cent of the total carrier gas flow at B and at D the
lengths of the capillaries are chosen respectively for
G and H 65 cm and 35 cm (I.D. 0.25 mm).

The same analysis, as is carried out in fig. 4.2, is
shown in fig. 4.4, using this system. The quality of
the separation is not affected by the use of this proc-
edure. The negative base line at the start is caused by
the very small flow of carrier gas trough the detector
during the "trapping process". It is of course possible
to evaporate the solvent before the GC analysis. One
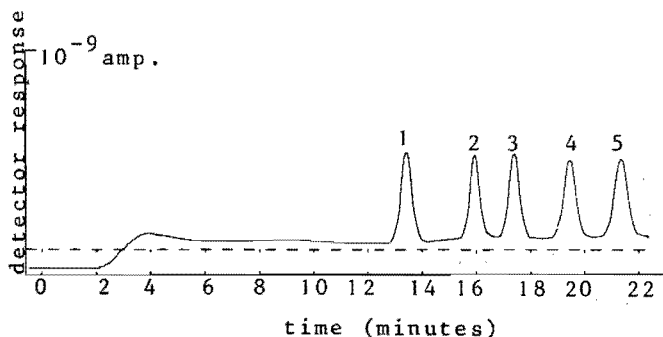then is faced, however, with the problems of injecting   **59**

Fig. 4.4   Temperature programmed analysis of the same
           standard mixture as in Fig. 4.1 with the same
           column, using the trapping system. The exper-
           imental conditions, temperatures, carrier gas
           etc. are the same as in Fig. 4.2.

solids and the inherent difficulties are enhanced be-
cause of the smallness of the sample. This means for
routine purposes that another step has to be done which
is time consuming and which is introducing errors (loss-
es of material etc.)

4.5   THE ANALYSIS OF OESTRIOL AND 5β-PREGNANE-3α, 20α-DIOL IN PREGNANCY

An aliquote of a 24 hr urine specimen is treated as is
described in chapter 3. The TMS_i-derivates are injected
on to the top of the column. Both the experimental con-
ditions and the trapping procedure are the same as des-
cribed in section 4.3. The result of the analysis is
shown in fig. 4.5. The oestriol peak and the 5β-pregnane-
3α, 20α-diol peak are predominating the other peaks in
the chromatogram which accounts for the fact, that high
excretion rates of these compounds occur during late
pregnancy of which this analysis is an example (37 weeks
60 gestation). The amounts of oestriol and 5β-pregnane-3α,

20α-diol are only determined (respectively 36 mg/24 hr
and 34.5 mg/24 hr) because of the importance of the rat-
es of excretion in pregnancy (section 4.6). The quantit-
ation is performed by the aid of n-tetracosane as intern-
al standard, which is chosen, because no other substance
is interfering with n-C_{24} at that place in the chromato-
gram.
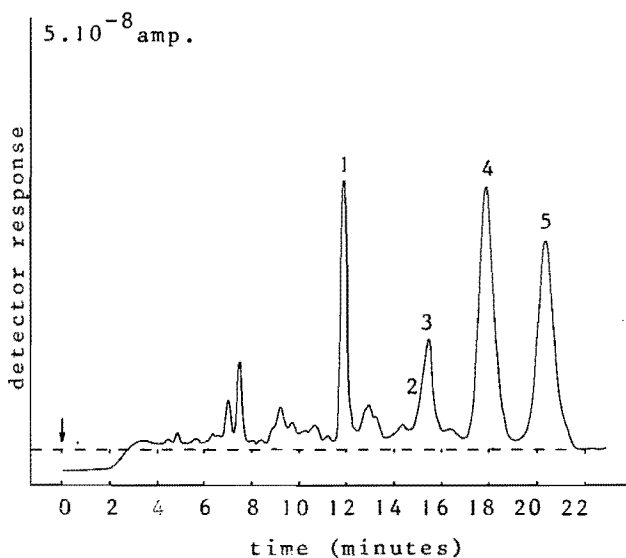
*Fig. 4.5   A TP analysis of a urine extract of a pregnant
woman (37 weeks gestation), using the trapping
system. Column 160 cm x 4 mm, 3.8% SE-30 on
chromosorb Q 80-100 mesh. Carrier gas flow 45
ml N_2/min. Initial temp. 140^oC, final temp.
250^oC, TP rate 5^oC/min. Temp. injection port
and FID both 250^oC. Sample components in the
order of microgram n-tetracosane (1), 17β-
oestradiol (2), pregnanolone (3), 5β-pregnane-
3α, 20α-diol (4) and oestriol (5). Other peaks
are not identified. The injected amount of liq-
uid is 1 μl (BSA-TMCS).*

The analysis of oestrogens in urine during the menstrual cycle constitutes a much more involved problem. The reason for this is that the excreted amounts are approximately 1000 times smaller than in late pregnancy, whereas contaminants such as other steroids and impurities predominate entirely in the mixture and disturb the analysis. How this problem should be solved is proposed in the next section.

4.6   THE CHOICE OF THE METHOD OF ANALYSIS AND THE CLINICAL SIGNIFICANCE OF SOME HORMONES

4.6.1   INTRODUCTION

A conservative estimate of the number of steroids as isolated or synthesised is about 7000. This is of course considerably lower than the number of conceivable steroids as derived from the skeleton and combination of possible substituents. Steroid hormones as occurring in natural samples constitute mixtures of high complexity, although it appears, that nature makes a careful selection of theoretically conceivable isomers. Nevertheless steroids occurring in plants are of different nature as those occurring e.g. in body fluids. In urine a few hundred steroids are known to occur and the number of steroids occurring in blood exceeds that of those occurring in urine. The concentrations of the individual steroids in body fluids is low; some steroids occur in extremely low concentration corresponding to an excretion of 10^{-10}g per 24 hours or even less, other may under circumstances occur in concentrations corresponding to excretion in the order milligram per day. The combination of the large variety together with the wide range of concentration demonstrates the complexity of the problem of steroid analysis. From the above it will

be obvious that the methods of analysis to be adopted
will depend upon the nature and the required accuracy
of the desired information. As will be mentioned later
the sample pretreatment has to be considered carfully
since in this part of the analysis often much informat-
ion is lost.

The general procedure in steroid analysis up till today
is considered first in order to see how the problems of
analysis have been approached during the last few dec-
ades. From bioassays the functions of definite steroid
hormones have been determined. These compounds are anal-
ysed by isolating them one after another from the biol-
ogical specimen, using a very specific method. Because
of the complexity of the mixtures, described above, ex-
tensive prepurifications and preseparations have to be
done for this isolation. The amount of information which
is possibly present in these complex mixtures and which
is probably of great importance, is lost by these proc-
edures of pretreatment. During the past many examples of
these procedures are described in literature among which
is mentioned the determination of oestrogens according
to Ittrich (4.2), the determination of 17-ketosteroids
according to Zimmermann (4.3.) and the determination of
corticosteroids according to Norymberski (4.4). In these
procedures the desired group is removed from the mixt-
ure by a separation method, specific for a functional
group in the molecule. A further separation of the ind-
ividual components in such groups is exemplified by e.g.
the method of Brown J.B. (4.5) for oestrogens which is
too extensive to be discussed here. In general all these
methods try to isolate one compound, which is very time
consuming.

With the advent of GC in steroid analysis in 1959 not
much has been changed compared with the methods of anal-
ysis used in the time before. The crude extract of body **63**

fluids is in the same way extensively pretreated, so
that only a few compounds remain in the last step which
is the detection of the quantaties by means of one of
the sensitive GC detectors. The sensitivity of these de-
tectors is undoubtedly suitable, considering the amount
of available material in the natural sample. The GC
method, however, is in the first instance a separation
technique which requires a highly sensitive detector in-
herent to the method itself. Therefore extensive use
should be made of the resolving power of this technique.
It is therefore emphasised to make use of prepurificat-
ions and preseparations only when it is strictly neces-
sary and to be not afraid of feeding the GC column with
a complex mixture in order to be capable of evaluating
the information in the final result by means of system-
atic assays. An ideal solution would be to inject simply
the urine extract or the blood extract on to a GC col-
umn. This is not yet possible because of a lack of re-
solving power, but the encouraging results obtained by
capillary columns of which a start is made in this thes-
is and the knowledge that improvement of resolving power
is possible, will lead to follow another line of thinking
in which the separation is much more emphasised.

4.6.2 5β-PREGNANE-3α, 20α-DIOL AND THE MAIN OESTROGENS

The analysis of oestriol and 5β-pregnane-3α, 20α-diol in
pregnancy, described in section 4.5 is an example in
which the crude extract, liberated from acids, is used
for a GC analysis after derivatising to trimethylsilyl-
ethers. The excreted amounts in late pregnancy are so
large as compared with contaminants that the GC analysis
is not impaired. The excreted amounts of the main oestro-
gens and 5β-pregnane-3α, 20α-diol in urine during preg-
nancy are illustrated in fig. 4.6 and fig. 4.7 for heal-

μg/24 hr.
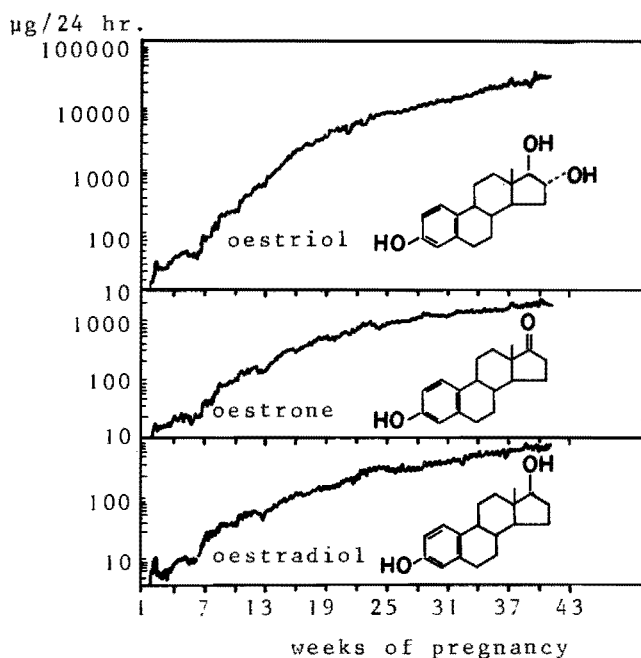
Fig. 4.6 Avarage urinary excretion of the classical oestrogens during normal pregnancy. Results are on logarithmic scale.

thy women as an avarage. For a detailed interpretation of fig. 4.6 and fig. 4.7 is respectively referred to the literature (4.5, 4.6). The reason for which this analysis is carried out is to have an indication for the function of the placenta in the pregnanediol output and for the foetal function in the oestriol output. When for example the amount of excreted oestriol in late pregnancy is reduced very sharp over a 24 hour period, than this is indicative for an imminent abortion. This calls for a fast analysis. This is of course possible using a relatively fast enzyme hydrolysis and afterwards a GC analysis or an analysis according to Ittrich (4.2) only for oestriol. High accuracy is not demanded.

mg/24 hr

5β-pregnane-3α,20α-diol

70
65
60
55
50
45
40
35
30
25
20
15
10
5

weeks of pregnancy

8   12   16   20   24   28   32   36   40
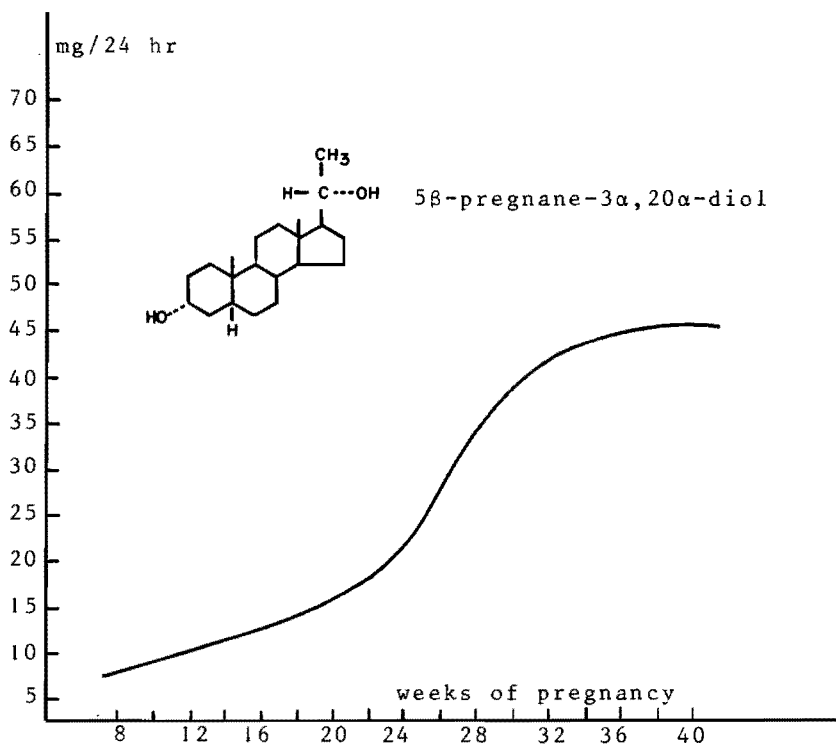
Fig. 4.7   Urinary excretion   of 5β-pregnane-3α, 20α-diol
in normal pregnancy, showing the avarage value.

The excretion pattern of 5β-pregnane-3α, 20α-diol during
the menstrual cycle is shown in fig. 4.8. The excreted
amounts are of the order of milligrams per 24 hours. Ex-
tensive prepurifications are not necessary in order to
analyse this compound by means of GC. During the menstr-
ual cycle the function of progesterone (see fig. 4.9)
can be followed by studying the excreted amounts of 5β-
pregnane-3α, 20α-diol as one of the major metabolites
in urine, since progesterone is secreted by the corpus
luteum and since it is responsible for preparing the
endometrium for pregnancy. For a detailed description
of the metabolism of progesterone reference is made to
the literature (4.6).
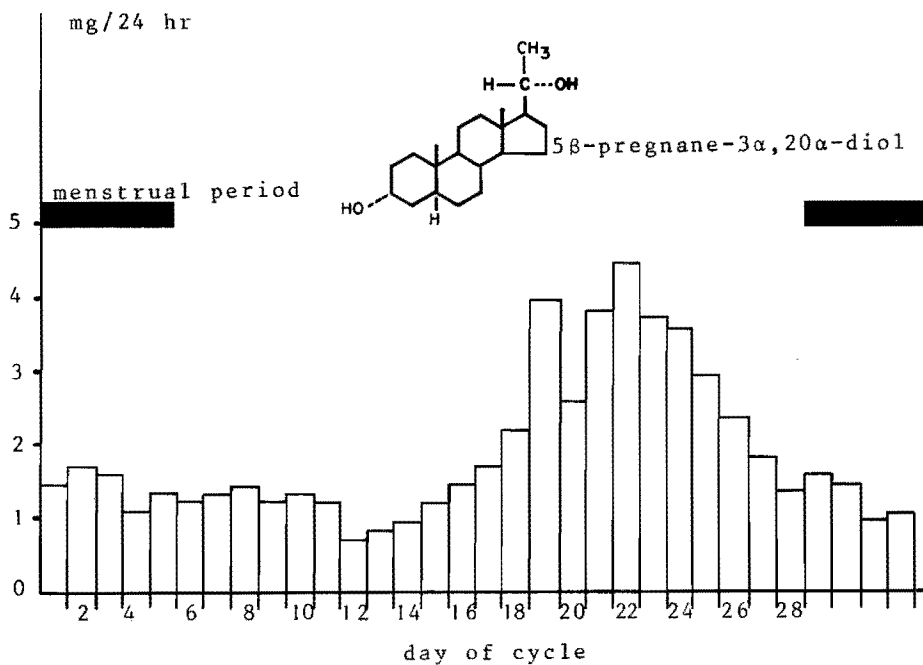
Fig. 4.8 Urinary excretion of 5β-pregnane-3α, 20α-diol
in a normal menstrual cycle.
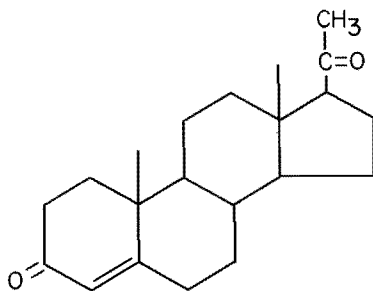
Fig. 4.9 Progesterone.

The analysis of the main oestrogens during the menstru-
al cycle is much more complicated because of the very
small amounts excreted in urine during a 24 hour period.
The excreted amounts as an avarage of healthy women are
illustrated in fig. 4.10. For an explanation of the pat-
terns reference is made to the literature (4.6). Using
the method of Brown J.B. (4.5) extensive prepurificat-
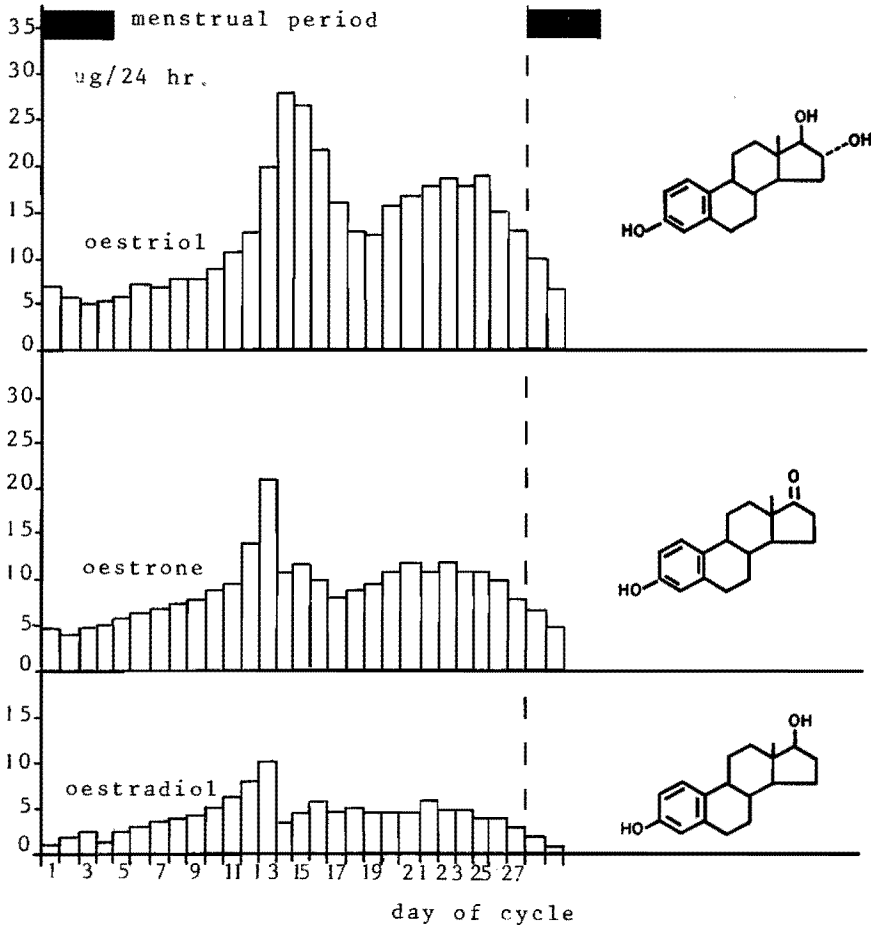
Fig. 4.10 Avarage urinary excretion pattern of the three
classical oestrogens during the menstrual
cycle of healthy individuals.

ions and preseparations are applied which is due to the
subsequent method of chemical determination by the use
of the Kober colour reaction for the separated oestro-
gens. The oestrogens can be analysed by means of gas
chromatography after less prepurification and less pre-
separation, which is only dependent on the quality of
the separation technique and the sensitivity of the
method. The degree of pretreatment is determined by the
quality of the separation. The classical oestrogens can
be analysed during the menstrual cycle with regard to
sensitivity and resolution by the use of capillary col-
umns with a normal FID, which is shown in the next
chapter.

For blood extracts the same problems occur. The concen-
trations of steroids are very low and are varying from
the nanogram level to the microgram level per 100 ml of
plasma. For children these levels are lower. Hence not
only high demands must be made on the quality of the GC
separation, but also to the sensitivity of the detection
with regard to the small available amounts. A flame ion-
isation detector for instance is measuring mass per unit
time. Capillary columns have the property of handling
very small samples. At the same time peaks are very nar-
row. This means that a certain mass is eluted in a short
time and hence the detectability (mass per unit time) is
favourable.

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STEROID ANALYSIS WITH OPEN HOLE TUBULAR COLUMNS

5.1 INTRODUCTION

Steroids as obtained from natural samples constitute a
mixture of fair complexity. It is therefore surprising,
that so little has been published on the use of open
hole or capillary columns for this purpose.

In 1960 Chen and Lantz (5.1) reported the GC separation
of a few steroids using capillary columns, which appears
today to be the only reference. The succesful attempts,
made by Groenendijk (5.2) et al. and illustrated in this
chapter, have lead to develop the capillary column tech-
nique further.

There are of course several reasons for which this tech-
nique is avoided for steroid analysis. The use of stream
splitters, required for the sampling on to capillary col-
umns and the very low concentrations of steroids, as av-
ailable in the natural sample, have shyed away people
from trying seriously. As has been shown clearly in
Cramers' thesis (5.3) using stream splitters the width
of the input peak into the column is also dependent on
the boiling point of the component in question. This
means for steroid analysis that the mixing tube of the
sample device should have the temperature of the highest
boiling component in the mixture in order to obtain a
reasonably sharp input band. The temperature which
should then be applied, is much too high so that decom-
position will occur. These temperatures may rang up to

400°C and even higher as compared to a series of n-paraffins which have vapor pressures of the same order as the steroids in question. Another disadvantage of the splitting device has appeared to be the quantitative interpretation of the analysis (5.3).

An adequate solution of this problem is to develop a methode of direct injection on to capillary columns by means of which the high resolution technique comes available in particular for the very complex mixtures of natural steroid samples.

The sampling technique will be discussed in detail in chapter 6 with regard to the overloading caused by the solvent which has to be injected together with the sample. In order to show the analyses on capillary columns use is made in this chapter of a direct injection system which is in principle derived from systems out of the packed column technique and which is modified to minimise dead volumes.

5.2   SAMPLING

The steroids dissolved in the BSA-TMCS reagent are injected with a syringe into a glass insert in which the capillary column is placed. This is shown in fig. 5.1. In order to avoid overlap of the solvent peak with sample components a very small amount of liquid less than 0.05 µl is injected. This can be done fairly reproducibly by expelling the liquid out of the syringe and by injecting the liquid which is remaining in the needle of the syringe. The top of the sample device is cooled by tap water which is necessary to protect the silicone rubber septums. The volume of the glass insert ($\pm$ 25 µl) is kept as small as possible and it is almost a part of the column itself. A disadvantage of this system is that
the injected amounts of liquid must be very small which
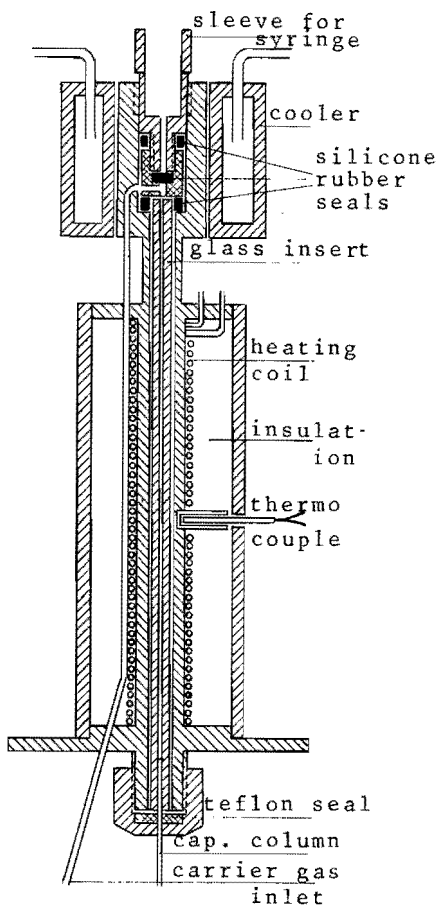
Fig. 5.1   A sampling device for direct sampling on to
          capillary columns. For further explanation
          see text.

is feasible for standard samples of pure compounds, but
not always for natural samples. In the latter the con-
centrations of steroids in the solvent are small in gen-
eral, so that more liquid has to be injected. This has
a number of adverse effects, among which overlapping of
the solvent peak with sample components and reducing of
the quality of the separation. In chapter 6 a method
will be discussed in which it is tried to give a reason-
able solution for this problem.   **73**

5.3 PACKED COLUMNS VERSUS CAPILLARY COLUMNS

The resolving power of capillary columns is illustrated best by comparing the results obtained for capillary columns with those of packed columns.

Fig. 5.2   The analysis of a standard mixture of the TMS$_i$-derivatives of oestrone (1), 17β-oestradiol (2), 5β-pregnane-3α, 20α-diol (3) and of oestriol (4) on a 20 m capillary column at 240°C, using the injection system of section 5.1. Experimental: column 20 m x 0.25 mm stainless steel, stationary phase SE-30, linear carrier gas (N$_2$) velocity 9.4 cm/sec. Temp. column and FID both 240°C, injection port 260°C. Injected amount less than 10 nanograms each.
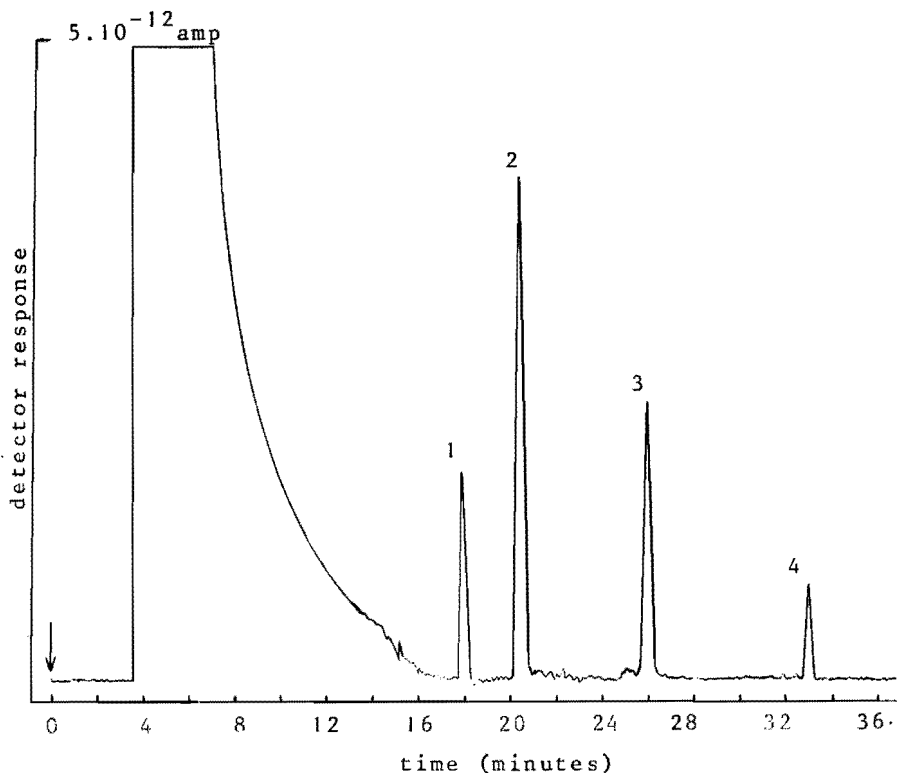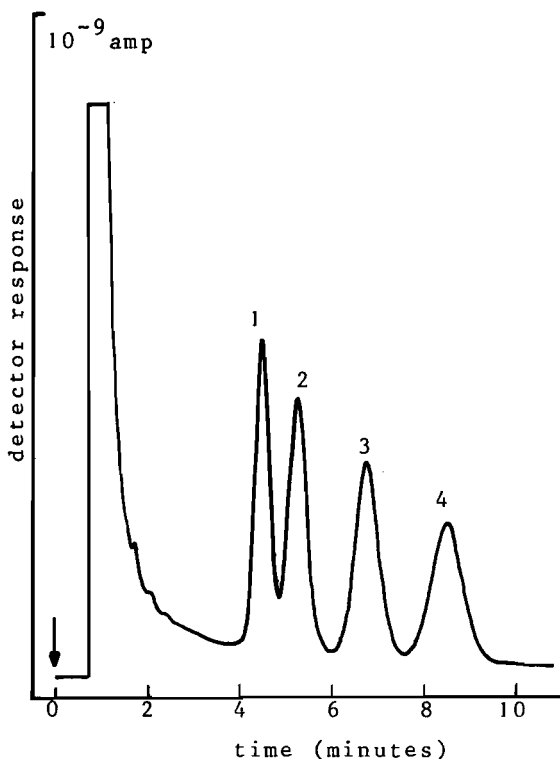
Fig. 5.3 *The analysis of a standard mixture of TMS_i-derivatives of oestrone (1), 17β-oestradiol (2), 5β-pregnane-3α, 20α-diol (3) and of oestriol (4) on a packed column at $240^{\circ}C$. Experimental: Column 160 cm x 4 mm stainless steel, stat. phase 3.8% SE-30 on chromosorb Q 80-100 mesh. carrier gas flow 45 ml N_2/min. Temp. column $240^{\circ}C$. Temp. injection port and FID both $250^{\circ}C$. Injected amount about 1 µg each.*

For this purpose use is made of a capillary column and a packed column both on which a qualitative analysis is carried out at comparable conditions. This is exemplified on an analysis of the three main oestrogens and 5β-pregnane-3α, 20α-diol as their TMS_i-ethers on both columns. The analyses on both columns are carried out at

approximately optimum conditions. The experimental quant-
ities are presented below the figures. The results   of
the analyses on both capillary and packed column are
shown respectively in fig. 5.2 and fig. 5.3. From these
figures the resolving power of capillary columns for
this purpose is illustrated evidently. The resolution
R (chapter 2) between oestrone and 17β-oestradiol for
the capillary column amounts to 20, whereas for the
packed column R equals 4. This means, that for the
packed column no other component can be placed between
oestrone and 17β-oestradiol without overlapping, where-
as for the capillary column 4 peaks, sufficiently sep-
arated, can be placed between these two compounds, since
a sufficient separation is obtained for R=4 in that way
as is mentioned in the literature (5.4).

The amount of injected material for the packed column
amounts to approximately 1 microgram of each component,
whereas the amount used for the capillary column is less
than 10 nanograms. The latter is an advantage of the use
of capillary columns in particular with regard to the
small amounts in most cases available in natural samples.
The sharper the peak the less material has to be used
for the GC analysis, because a FID is measuring mass per
second .

For a more complex mixture the advantage of the use of
a capillary column is shown in fig. 5.4. In this analys-
is the experimental conditions are adapted to the anal-
ysis problem and are slightly changed as compared with
the one in fig. 5.2. The TMS_i-ethers of epiandrosterone,
11-ketoandrosterone and 11-ketoetiocholanolone seem to
be eluted as one peak. From the literature (5.5) it is
known, that the components in this system have slightly
different distribution coëfficients and separation should
be possible . The column, however, which is used, is rel-
76 atively short and it is certainly possible to improve
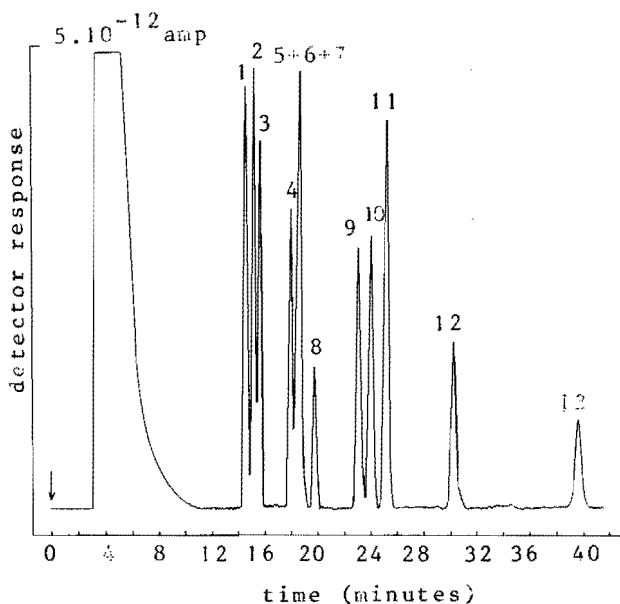
Fig. 5.4 The analysis of a more complex standard mixt-
ure of the TMS_i-derivatives of dehydroandros-
terone (1), androsterone (2), etiocholanolone
(3), dehydroepiandrosterone (4), epiandroste-
rone (5), 11-ketoandrosterone (6), 11-keto-
etiocholanolone (7), oestrone (8), 17β-oestra-
diol (9), 11β-hydroxyandrosterone (10), 11β-
hydroxyetiocholanolone (11), 5β-pregnane-3α,
20α-diol (12) and oestriol (13), at $230^{o}C$ on
a SE-30 capillary column (20 m x 0.25 mm).
Column temp. $230^{o}C$, FID $230^{o}C$, injection port
$260^{o}C$. Linear gas velocity 10.7 cm/sec.

the separation of these components by enlarging the
length of the column and by changing the experimental
conditions. As a comparison the same mixture of the TMS_i-
ethers is analysed on a packed column at the same condit-
ions as used in fig. 5.3 in order to show the attainable **77**

resolution. This analysis is presented in fig. 5.5. The
overlap of several peaks is hardly to be avoided in this
isothermal analysis and it is evidently shown, that such
complex mixtures are not separable in this way. The us-
ed column could be improved slightly, but it will not
yet give the required resolution.
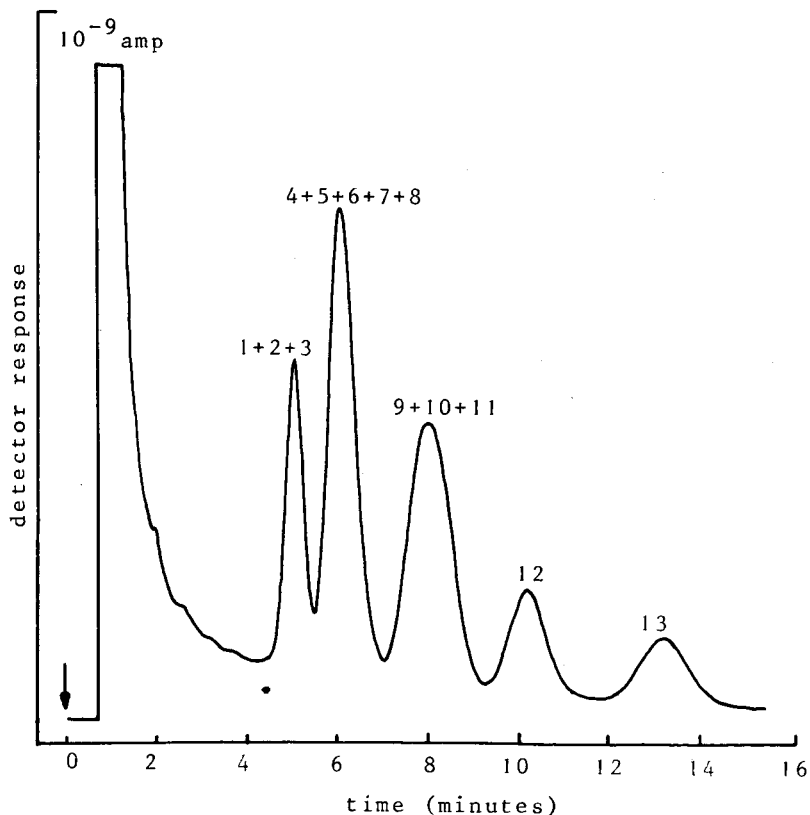
*Fig. 5.5   The analysis of the same standard mixture as
in Fig. 5.4 on a 160 cm x 4 mm packed column,
3.8% SE-30 on chromosorb Q, at 230°C. The same
compound numbers are used. Further the same
conditions as in Fig. 5.3, except column tem-
perature.*

To analyse still such mixtures with packed columns use
must be made of preseparations in groups and of select-
ive stationary phases for an analysis of each group.

On the other hand temperature programming improves some-
times the resolution as is shown in fig. 5.6, in which
a TP analysis is carried out with the same mixture as
in fig. 5.5. The experimental conditions are presented
below the figure.
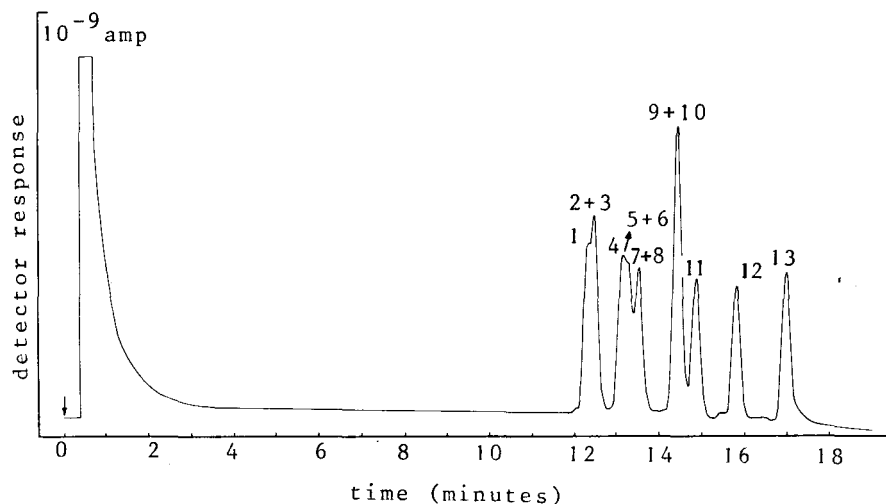
*Fig. 5.6   Temperature programmed analysis of the same
          mixture as in Fig. 5.4. The same compound num-
          bers are used. Used column as in Fig. 5.5. In-
          itial temperature 140°C, prog. rate 5°C/min.,
          final temperature 250°C.*

The analyses described above on a capillary column are
performed with the use of pure compounds. These standard
mixtures can be prepared using a small amount of solvent
so that the concentration of steroid derivatives is
large. This means that these analyses could be performed
using the injection system of section 5.2. Only for the
natural samples, where the amount of available material   **79**

is sufficient, an analysis can be done by means of this method of injection. This is shown in fig. 5.7 in which the urine extract of a woman, after a habitual abortion, is analysed as TMS_i-derivatives. This urine has been pre-treated as described in chapter 3. In this figure not all peaks could be identified by the lack of reference compounds and by the fact, that also impurities are present in the sample.
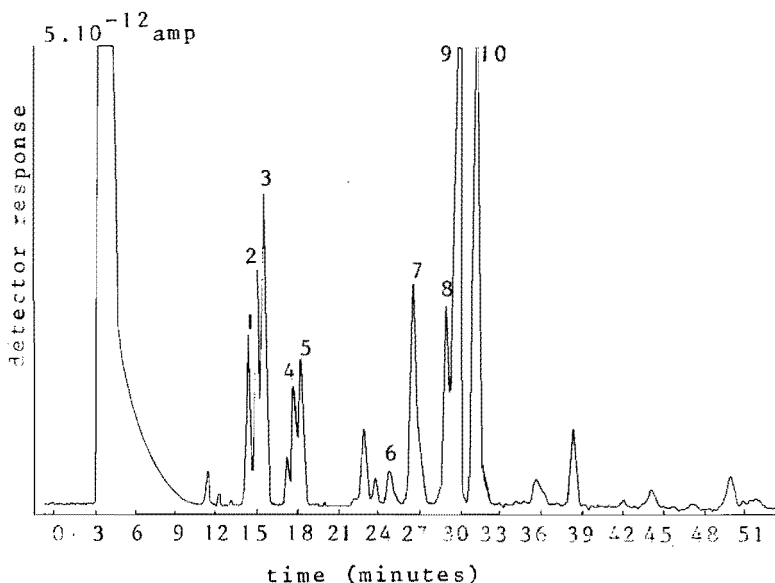
Fig. 5.7 *The analysis of a urine extract of a woman af-*
ter a habitual abortion on a 20 m x 0.25 mm
SE-30 capillary column at 230°C. The same con-
ditions as in Fig. 5.4 are used. Identified
compounds as TMS_i-derivatives. Dehydroandros-
terone (1), androsterone (2), etiocholanolone
(3), dehydroepiandrosterone (4), epiandroste-
rone + 11 ketoandrosterone + 11 ketoetiocho-
lanolone (5), 11ß-hydroxyandrosterone (6),
11ß-hydroxyetiocholanolone (7), 5ß-pregnane-
3α, 20ß-diol (8), 5ß-pregnane-3α, 20α-diol (9)
and pregnenediol or/and i-androstanolone (?)(10).

The analysis of oestrogens with capillary columns, as
shown in this chapter, needs less than 10 nanograms of
material. This means that this technique is capable of
analysing the classical oestrogens excreted during the
menstrual cycle, starting from an amount of urine of the
order of 10 ml. The selective removal of the oestrogens
from the extract is an easy procedure because of the
phenolic A-ring present in all oestrogens. This proced-
ure is strongly simplifying the analysis with regard to
the danger of other compounds and impurities interfering

5.4 COATING OF CAPILLARY COLUMNS

It is commonly known, that coating of a thin film of
stationary liquid on the wall of the column is a diffi-
cult procedure in particular with silicone gums on stain-
less steel. The most common method is to press a plug of
a solution of the stationary phase (2-10%) through the
column by which means the wall is wetted. The solvent is
then evaporated by an adequate stream of gas, whereas a
film of stationary liquid remains on the wall of the col-
umn. The affinity of the stationary liquid for the nat-
ure of the column material is of great importance. It
has appeared that the affinity of SE-30 stationary liq-
uid for stainless steel is not too bad, however it is
rather difficult to bring on the wall a thin homogeneous
film the quality of which is very dependent on the used
coating procedure. The above indicated procedure has ap-
peared to be insufficient so that another method is
followed in this work.

The capillary column (after the usual cleaning process)
is filled with a 5% solution of SE-30 in benzene. The
column closed at one end, is placed in an oven at 90°C.
In the first instance the main portion of the solution   **81**

is expelled out of the column at the open end at this temperature . When no solvent occurs any more, a gentle stream of nitrogen (0.2 ml/min) is supplied on the column for one hour in order to remove the remaining solvent. The column is then heated to 300°C without any gas flow for two hours. During this procedure the silicone gum is assumed to be spread out homogeneously over the wall of the column, whereas thermally instable material in the gum is degraded. The column is then cooled to 260°C (about 20°C above operation temperature). A normal carrier gas flow is then supplied on the column in order to clean and in order to condition the column before use. This process is continued for 24 hours.

5.5 IDENTIFICATION

As is illustrated in chapter 4 the resolving power of packed columns is fairly insufficient for the analysis of complex mixtures. This means that from the qualitative point of view the identification by means of retention indices is not very attractive. For capillary columns the identification by means of retention indices becomes more interesting in particular in the analysis of more complex mixtures. The retention times using capillary columns are more reproducible, because the eluted peak maxima are sharp and hence better defined and because the experimental conditions, such as carrier gas velocity, are more constant as compared with packed columns. In order to investigate how accurate a retention index can be measured, the indices of most of the above mentioned steroids as their TMS$_i$-derivatives are determined on a ov-17 stationary phase which is chosen for the sake of convenience. The index mentioned in chapter 2 is given for a number of steroids together with the

standard deviation as obtained from 5 to 10 measurements

for each steroid. In this procedure the logarithm of the relative retention times (relative to n-octacosane) is calculated and this quantity is plotted against the number of carbon atoms of a series of n-alkanes. From the function which is then obtained, the indices are calculated. The results are presented in table 5.1.

Table 5.1   Retention indices of some TMS_i-derivatives of steroids on OV-17 stat. phase at $256^{O}C$; the standard deviation is given in index units.

Compounds as TMS$_i$-derivatives	Retention time relative to n-C$_{28}$	Retention index	Standard deviation
n-tetracosane	0.338	24.00	(reference)
Androsterone-TMS$_i$	0.980	27.89	0.01
Dehydroandrosterone-TMS$_i$	0.980	27.89	0.01
n-octacosane	1.000	28.00	(reference)
Etiocholanolone-TMS$_i$	1.030	28.12	0.01
5β-pregnane-3α,20αdiol-TMS$_i$	1.300	28.95	0.02
Epiandrosterone-TMS$_i$	1.320	29.03	0.01
Dehydroepiandrosterone-TMS$_i$	1.320	29.03	0.01
17β-oestradiol-di-TMS$_i$	1.350	29.09	0.01
11β-hydroxyandrosterone-di-TMS$_i$	1.370	29.17	0.02
11β-hydroxyetiocholanolone-di-TMS$_i$	1.450	29.36	0.03
11-ketoandrosterone-TMS$_i$	1.545	29.61	0.01
11-ketoetiocholanolone-TMS$_i$	1.545	29.61	0.01
oestrone-TMS$_i$	1.920	30.40	0.02
oestriol-tri-TMS$_i$	2.090	30.71	0.04

From table 5.1 it can in general be concluded, that such an identification tool is fairly reliable. At higher index numbers the standard deviation however is increasing due to the increasing width of the eluted peaks of which the maximum is becoming more and more unsharp and hence less reproducibly to measure.

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SAMPLING OF HIGH BOILING COMPOUNDS ON TO OPEN HOLE TUBULAR COLUMNS

6.1 INTRODUCTION

There are many demands set on sampling on to capillary columns with regard to overloading of the column and to band broadening. Sample sizes in the microgram and sub microgram range must be fed to the column in a very narrow band. Stream splitters are applied in order to sample these minute amounts out of a stream containing sample in the order of milligrams. For a detailed discussion on the performance and the working of stream splitters and of a few other direct sample devices reference is made to Cramers' thesis (6.1). In the following a completely different approach is made in the development of a direct sample device suitable for high boiling compounds.

The result of the GC analysis is a peak of the gaussian type. The standard deviation of this peak σ is a measure of the band broadening caused by the GC process as well as the sampling. This phenomenon is expressed in mathematical form in equation 6.1, in which σ_i is the standard deviation of the input band, whereas σ_t is the standard deviation of the output curve, caused by the GC process only.

$$\sigma^2 = \sigma_i^2 + \sigma_t^2 \qquad\qquad \text{(eqn. 6.1)}$$

The relationship between the decrease of the resolving power of the column and the contribution of sampling to band broadening can be calculated from the formula which **85**

can be derived by substituting eqn. 6.1 in eqn. 2.5
(chapter 2).

$$R_{12} = \frac{tR_2 - tR_1}{\sqrt{\sigma_i^2 + \sigma_t^2}} \qquad \text{(eqn. 6.2)}$$

When σ_i tends to zero, R_{12} approaches to a maximum,
whereas $\sigma \simeq \sigma_t$. Hence:

$$R_{12} = \frac{(tR_2 - tR_1)/\sigma_t}{\sqrt{1 + \sigma_i^2/\sigma_t^2}} = \frac{R_{12}max}{\sqrt{1 + \sigma_i^2/\sigma_t^2}} \quad \text{(eqn. 6.3)}$$

Equation 6.3 is showing the relation between the resol-
ution and the influence of sampling. The decrease of
resolving power of the column as a percentage of $R_{12}max$
for increasing values of σ_i is represented in table 6.1.

A decrease of 10% in resolution being acceptable for a
well working injection system means that σ_i is required
to be smaller than 0.5 σ_t. This limit is used in the
following to qualify the injection system. The quality
of the injection system is tested by measuring σ_i and
by calculating the decrease in resolution caused by σ_i.

Table 6.1   The influence of sampling on the resolution.

Decrease of resolution in % of $R_{12}max$	σ_i expressed in σ_t
1	σ_i = 0.1 σ_t
3	" = 0.25 σ_t
10	" = 0.5 σ_t
30	" = σ_t
55	" = 2 σ_t

6.2   THE PRINCIPLE AND THE PERFORMANCE OF THE INJECTION SYSTEM

The principle on which the following injection system is based, is the same as is described in chapter 4 in the "trapping" system. The sample is directly transferred into the capillary column. This sample which is consisting of a small amount (microgram, sub microgram range) of high boiling material (such as steroids) and of an excess of solvent, passes after injection a cold zone of 5 mm length. In this zone the high boiling material is collected, whereas the solvent is running through this zone into the column. When the solvent has left the column, the cold zone is heated quickly and the sample is allowed to move through the column.

The performance of a system which is capable of doing so, has introduced many practical problems for which the following system provides a good solution, see fig. 6.1. The injection is made by the aid of the glass capillary (chapter 3) in which the sample, dissolved in an excess of solvent, is present. The method of transferring the sample into the glass capillary is somewhat different for synthetic mixtures as for, for instance, steroids obtained by extraction and evaporation of the extraction solvent. In the case of for instance a synthetic mixture the steroids usually are dissolved in a solvent and filling of the glass capillary is done by putting in the solution the tip of the capillary which fills itself by capillary action. For steroids extracted from a natural specimen, the sample is already in the capillary in the solid state and a small amount of solvent is added by suction. The glass capillary is placed in a teflon seal which is put in position as is shown in fig. 6.1. The thin end of the capillary has an outside diameter of 50 microns, whereas the column inner diameter amounts to **87**
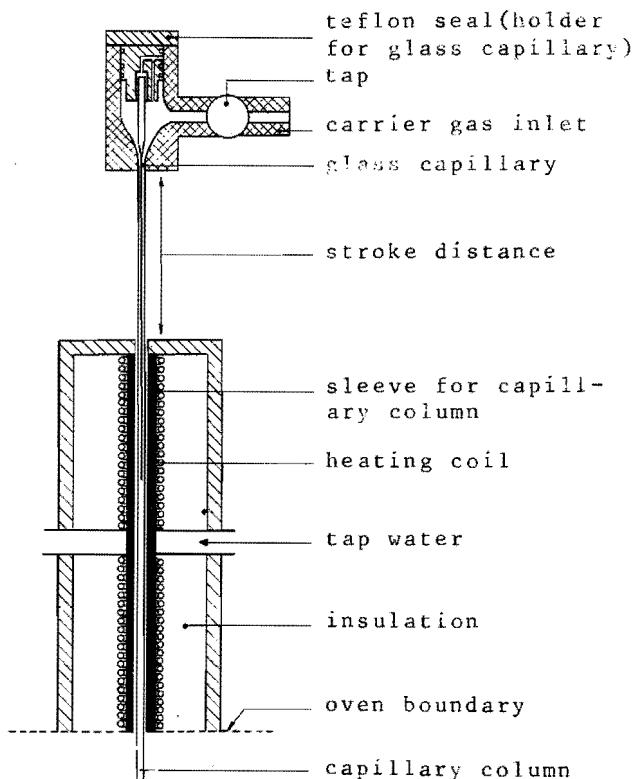
*Fig. 6.1 Outline of the direct sample device. For ex-
planation see text.*

250 microns, so that this manipulation is very easy. The
stainless steel capillary column (O.D. 0.55 mm, I.D.
0.25 mm) is surrounded over a distance of 65 mm by a
stainless steel tubular sleeve (O.D. 1.0 mm, I.D. 0.6
mm) which in turn is enclosed by a heating coil as shown.
The middle of the sleeve protrudes into a stainless steel
tube (O.D. 5 mm, I.D. 4 mm) trough which a cool medium
is flowing. The temperature of the system above and be-
low the cool zone is about 250°C. By opening the tap the
carrier gas is transferring the sample into the column.
The solvent is passing the cool zone, whereas the high
boiling compounds are collected between the bottom end

of the glass capillary and the cool zone. When the solvent has left the column, the cool zone is heated quickly by moving the column itself down through the sleeve over a stroke of 35 mm.

From the experience with trapping systems, used in GC, it has appeared, that fog formation occurs easily due to the sharp transition between the hot and the cool section. The temperature profile over the tubular sleeve in this system must be known and it is therefore measured with a thermocouple at different conditions. The temperature of the middle of the hot zones is kept at 240°C. The temperature of the middle of the cool section is adjusted by choosing the temperature of the cool medium for which tap water, silicone oil or air can be used. The resulting temperature profiles are illustrated in fig. 6.2. Under the conditions as illustrated in fig. 6.2 no fog formation could be detected.

For the sake of convenience air is used as cool medium. The cool zone reaches then a temperature of 45°C at a consumption of about 10 liters air/min of room temperature. The system is working very well under these circumstances.

The reason for which the temperature profiles also must be known is to determine the length of the stroke which the capillary column should make in order to bring the cool section of the column completely into the hot region for injection. At this point it must be observed that this stroke is also dependent on the place where the glass capillary is ending above the cool section.It has appeared from experiments that the end of the glass capillary should lie between 125 and 150°C which means very near to the cool section (see fig. 6.2).   **89**

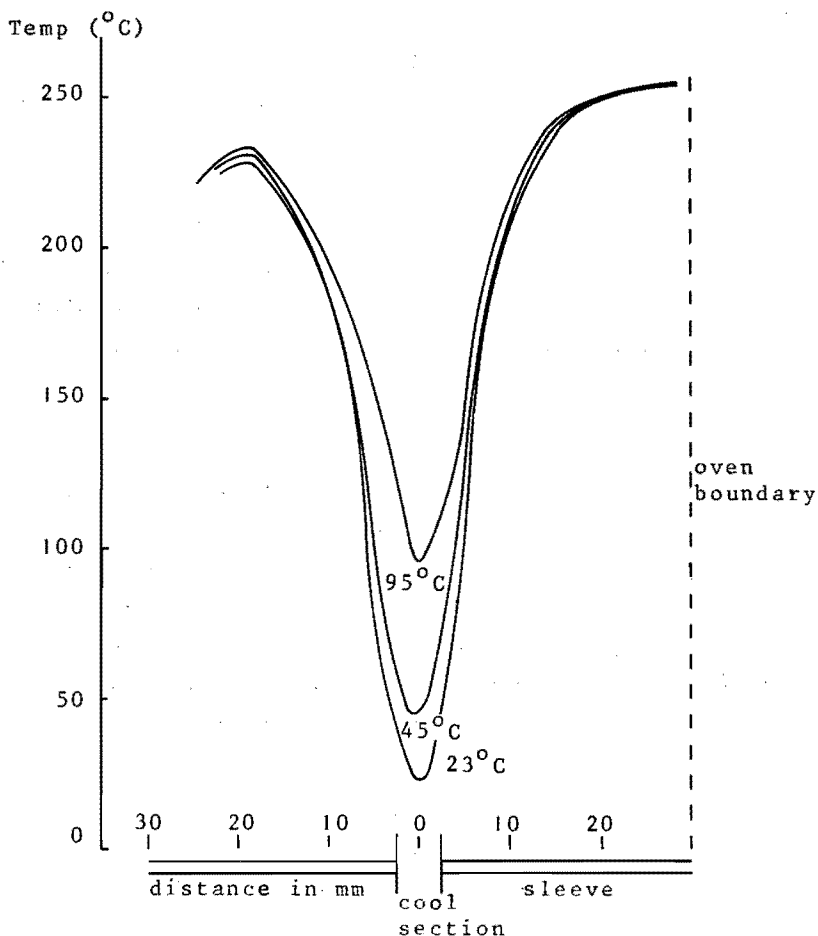Fig. 6.2 *Temperature profiles of the injection system at different temperatures of the cool section.*

The carrier gas is supplied on the glass capillary (to expell the sample) as well as around the capillary in order to prevent, that material would creep up between the glass capillary and the wall of the column. It has appeared that this effect introduces a dead volume, when **90** no gas is flowing around the capillary.

6.3   TESTING OF THE SAMPLE DEVICE

Testing a sample device is always based on measuring the
contribution to band broadening caused by injection. For
this purpose the injection port is directly connected
with the detector in order to eliminate band broadening
by the GC process. A standard compound is then injected,
the standard deviation σ_i of the obtained peak is meas-
ured and it is compared with the standard deviation σ_t
caused by the GC process at usual conditions.

However for the system in question a very short but coat-
ed (in this case OV-17 stat. phase) capillary column (40
cm length, 0.25 mm I.D.) must be used as a connection be-
tween injection system and detector, because the stat-
ionary phase is essential to take up the sample in the
cool section. The testing experiment is carried out at
250°C for injection system, column and detector, whereas
the cool section is 45°C, cooled by air of room temper-
ature. The injected standard substance is n-C_{28}-paraffin.
Doing so, the result being a peak of an almost gaussian
type, has a standard deviation which is composed by three
effects leading to peak broadening.

First the width of the input plug which is in this sys-
tem smaller than the distance between the bottom end of
the glass capillary and the middle of the cool zone, a-
mounts to about 5 mm, as described in the foregoing sect-
ion. This means that at a usual linear carrier gas veloc-
ity of 10 cm/sec this plug will pass the detector in 0.05
seconds. Considering the standard deviation of a peak in
a capillary chromatogram, being in the order of seconds
for columns of 30 m length and 0.25 mm internal diameter,
this first effect can completely be neglected as shown
by the figures of table 6.1 since, when σ_t is 5 sec.,
being an acceptable measure, $\sigma_{i_1} = 0.01\ \sigma_t$.   **91**

Using the experimental conditions, as described above,
the second and third effect leading to band broadening
are caused by heating the sample after it has been coll-
ected in the cool section and by the GC process, however

Table 6.2   Standard deviation for $n-C_{28}$ at conditions
described for different residence times in
the cool section.

Residence time in the cool section in min	σ_t sec.
1	0.30
1	0.27
2	0.28
2	0.26
5	0.33
5	0.26
10	0.29
10	0.29
15	0.30
20	0.24
20	0.24
30	0.25
30	0.25
45	0.29
45	0.25
68	0.30
	$\sigma_{t_{av.}}$ 0.27

of short duration. The avarage standard deviation of the
peak obtained after injection of the standard n-octaco-
sane amounts to 0.27 at the above mentioned conditions
of temperature and at a linear carrier gas velocity of
25 cm/sec. This experiment is done at different resid-
ence times of the collected $n-C_{28}$ in the cool section
which is illustrated in table 6.2. The registration of
the peaks has been made by means of a UV (ultra violet)
recorder.

Table 6.2 shows obviously that residence times up to an
hour do not affect the result which is important for dev-
eloping an automatic injection system as will be con-
sidered below. Because the second and third effect could
not be measured independently, the raio of their contri-
butions to σ must be estimated. The main contribution is
coming from the heating process, because the GC process
on a short column, as described above, requires little
time. As long as both effects, combined, are small as
compared to the peak width obtained from a usual capill-
ary chromatogram, it is justified to devote no effort to
investigate the individual contributions of both effects.
This is illustrated in fig. 6.3 in which a standard chro-
matogram on a 15 m OV-17 column is shown, using the in-
jection system in question. For instance the standard
deviation (σ) of $n-C_{28}$ amounts to 7.5 seconds. The stan-
dard deviation, σ_i, caused by injection is 0.27 sec.
Hence $\sigma_i = 0.035 \ \sigma_t$ ($\sigma \ \underset{\sim}{\sim} \ \sigma_t$) which means that the de-
crease in resolution (R) is less than 1% of R_{max} (see
table 6.1), whereas a 10% decrease is supposed to be ac-
ceptable.

The chromatogram in fig. 6.3 shows at the start the sol-
vent peak (0.1 µl of benzene) which has been eluted first
before injecting the substances, which are collected in
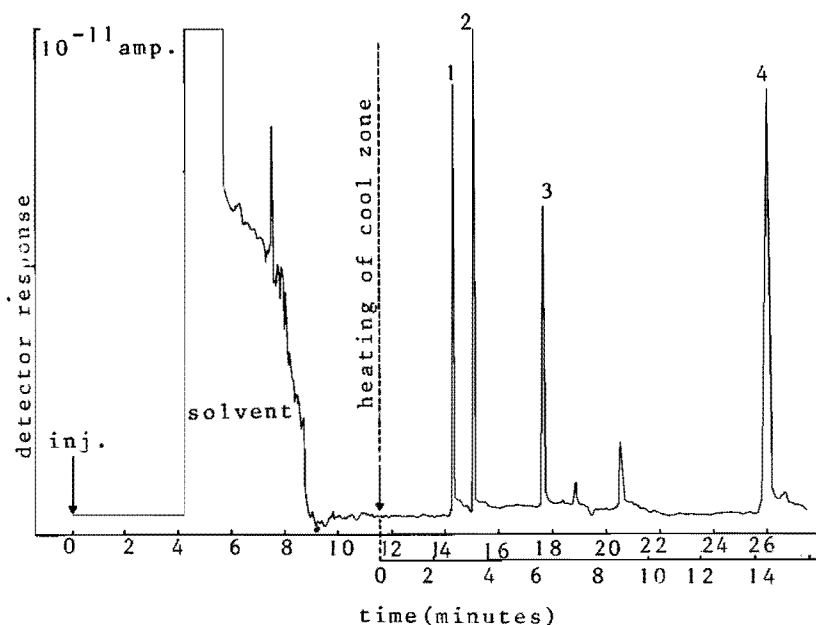the cool zone. This is, apart from the quality of the

Fig. 6.3 *Analysis of a mixture of C_{20}, C_{22}, C_{24} and C_{28}-n-paraffins (resp. 1, 2, 3 and 4) using the direct sample device of section 6.2. Temperature injection system $250^{\circ}C$ (cool zone $45^{\circ}C$), column and FID $250^{\circ}C$. Column 15 m x 0.25 mm, stat. phase OV-17. Linear carrier gas velocity 10 cm/sec.*

injection, described above, one of the main advantages of the system which allows to feed the top part of the column with large amounts of solvent without any danger for decrease in resolving power.

The same result has been obtained using the injection system of chapter 5 section 5.1 and using the principle of the cool zone. An experiment has been done by simply cooling the capillary column below the injection system by tap water. For this purpose a cooling tube has been coiled around the column. After the sample has been in-

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troduced, there is waited as long as necessary for elut-
ing the solvent peak. Then the water in the cool tube is
quickly and completely removed by air, so that the cool
zone is allowed to be heated by the oven to column temp-
erature. The result of a steroid analysis on a 20 m SE-
30 column (I.D. 0.25 mm) is shown in fig. 6.4 according
to this procedure. Comparing this result with those ob-
tained in chapter 5, where amounts smaller than 0.05 µl
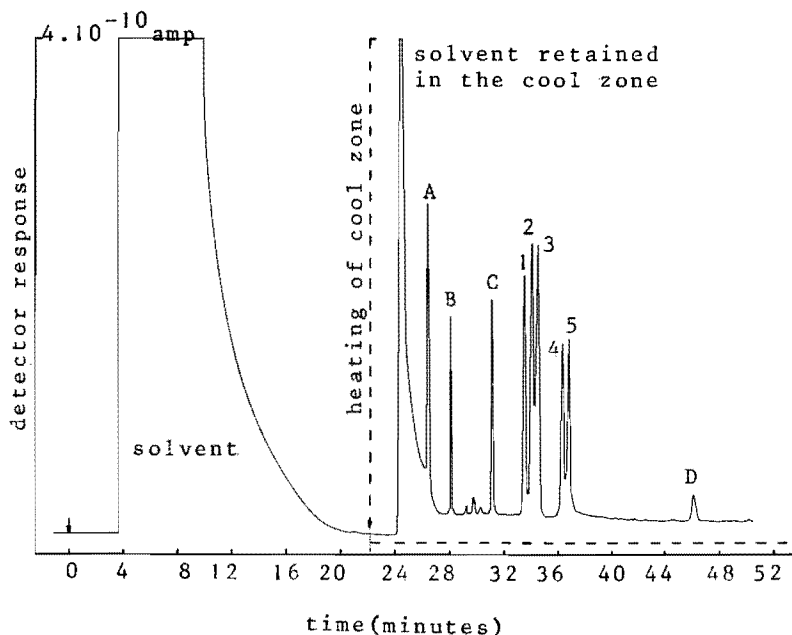of solvent are injected, it is obvious that in this way,

time(minutes)

Fig. 6.4   *Analysis of a standard mixture of dehydroan-*
drosterone (1), androsterone (2), etiocholano-
lone (3), dehydroepiandrosterone (4) and epi-
androsterone (5), using the cooling principle
as described. Temperature injection port 250°.
Cool zone 20°C. Temperature column and detec-
tor 225°C. Column 20 m x 0.25 mm, stat. phase
SE-30, linear gas velocity 13 cm/sec. A, B, C
and D are respectively C_{20}, C_{22}, C_{24} and C_{28}-
n-paraffins.

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although 0.1 μl is used, overlap of the solvent peak with steroid peaks can be avoided.

As an improvement the injection system for e.g. steroids must be developed further with respect to a method by which means the solvent can be removed. The same way could probably be followed as is shown in chapter 4 for packed columns. The dimensions being in the order of a millimeter and less are becoming more and more important and will introduce practical construction problems.

6.4 A FUTURE APPLICATION OF THE COOLING PRINCIPLE IN AN AUTOMATED INJECTION DEVICE

As has been shown in the foregoing section the sample is retained for hours in the cool zone. This means that a fairly simple system of automated injection could probably be made. For this purpose the column should be provided at the top by a series of successive hot and cool zones, equidistant, so that it becomes possible to move the sample by simultaneously heating the cool zones and cooling the hot zones very quickly from one zone to the next one in the direction of the carrier gas flow. This is outlined in the diagram of fig. 6.5.

When the first sample (1), collected in the top cool zone, is heated, it will go to the next cool zone, which has been hot just before. The first sample now is pushed up from position I to position II. Now the second sample (2) is introduced, whereas the hot zones have been interchanged for the cool ones, so that position III is obtained. Position IV and V are obtained in the same way. When the injection system has been loaded with as many samples as there are cool zones, a programme adapted to the duration of the GC process must be started in order

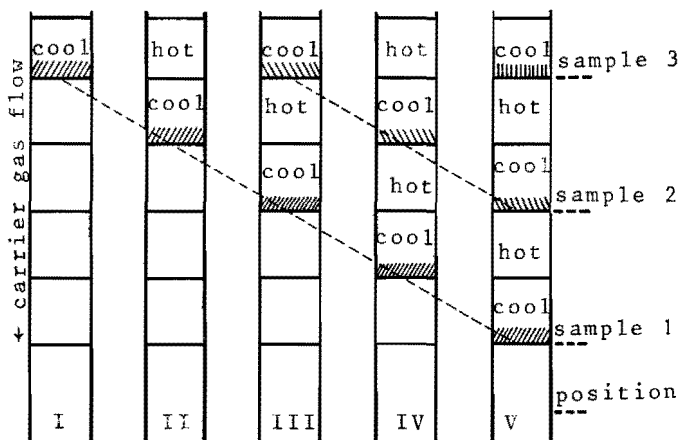**96** to inject the samples automatically one after each other.

Fig. 6.5   Diagram of the loading of the automatic sample system showing the positions of the samples.

The latter could probably be done by the use of the mechanical system described in section 6.2

REFERENCE

6.1   Cramers, C.A., Thesis, Technological University Eindhoven, The Netherlands (1967).

SUMMARY

Substances derived from sterane are called steroids. Many
steroids occur in nature, quite a few also have been pre-
pared by synthesis. Out of an odd 10.000 known steroids
only a few hundred are known to occur in the human body
either as primary products from biosynthesis or as their
metabolites. Their importance became known from methodic-
al investigations during the last few decades. As the an-
alytical methods to assay steroids improved, the knowled-
ge increased. Particularly through the improvement of the
analytical methods we now believe to know the complexity
of the problem in analytical respect as well as with res-
pect to their function in the human body.

For some time it has been believed that the attention
had to be focussed on steroids of biological activity.
Today we know that essential information is obtained
from practically all steroids. Since the quantities pro-
duced in the body may vary from 10^{-10} gram to as much as
10^{-2} gram per 24 hours only the most sophisticated meth-
ods of analysis can be considered. Up till now mainly
methods using group separations and colour reactions
have been used in the practice of the clinical laborat-
ory. The surprising development in gas chromatographic
separations of steroids, however, appear to have opened
a new and promising approach. The obvious gain as compar-
ed to the "classical" methods is gain in time and a tre-
mendous gain in information, although people familiar
with the classical methods appear not to be too much in-
terested in the latter aspect.

The purpose of this thesis is manyfold. On the one hand
it will be aimed to develop a routine method of analysis
taking a few hours, sample pretreatment included. On the
other hand the sample pretreatment with all it's practic-
al aspects has been developed not only as a previous
step before the fairly easy gas chromatographic separat-
ion of those steroids present in high concentrations in
body fluids, but it has been developed specially as a
previous step before the rather difficult separation of
steroids present in low concentrations in body fluids
using capillary or open hole tubular columns. It will be
shown that this sample pretreatment involves the use of
small samples of body fluids mainly urine (0.1 - 1 ml)
and blood (in most cases up to 10 ml) which is practic-
ally always a small fraction of what has been used in
the existing methods. The reduction in sample size is
essential in order to improve on analysis time, in ord-
er to reduce the cost·and last not least to make adapt-
ion to automatic analysis possible.

It hardly needs to be said that the utmost will be re-
quired from the detectors from which the flame ionisat-
ion detector is the one of choice. The reduction in an-
alysis time is for a main portion due to some simplific-
ation in sample pretreatment. It has appeared that the
simplification leaves a sample of much greater complex-
ity. Rather than reducing the complexity we should aim
in developing methods to obtain the maximum information
possible. Although for many a practical application
packed columns of good resolution are satisfactory (lar-
ge concentrations e.g.), it is to be expected that fut-
ure development will require very high resolution as is
obtainable by using capillary columns which will be
shown. The next step undoubtedly will be the correlat-
ion of this information with functions in the human body
by means of methodical examinations. Simultaneously **99**

methods of codification and digitalization of the chrom-
atogram have to be developed.

In this thesis a successful attempt to solve a number
of instrumental aspects, such as the practice of sample
pretreatment, improvement of packed column techniques,
introduction of capillary column techniques and methods
of direct sampling on to capillary columns, is describ-
ed. It is believed that this thesis is the first step
which may lead to the introduction of the high resolut-
ion capillary gas chromatography in steroid analysis.

SAMENVATTING

Stoffen afgeleid van steraan worden steroiden genoemd.
Vele van deze stoffen komen in de natuur voor, vele
echter zijn alleen bekend door synthese.Van een, naar
ruwe schatting 10.000 bekende steroiden, komen er, voor
zover bekend, slechts een paar honderd voor in het men-
selijk lichaam of als primaire biosynthetische produk-
ten of als de metabolieten daarvan. Uit methodisch kli-
nisch onderzoek is komen vast te staan, dat deze stoffen
belangrijk zijn, en dat er verband bestaat tussen de hoe-
veelheden der af- of uitgescheiden stoffen en diverse
functies in het menselijk lichaam.

De analyse van steroiden heeft velen, gedurende de
laatste vier decennia, bezig gehouden. De enorme inspan-
ningen, die men zich getroost heeft om een bepaald ste-
roid uit een complex mengsel te isoleren en nadien te
bepalen met behulp van een kleurreactie, heeft een stem-
pel gedrukt op de thans beschikbare technieken voor de
analyse van steroiden. Bij de opkomst van de gaschroma-
tografie toegepast op steroiden voor het eerst in 1959
zijn de conventionele analyse methoden in principe ge-
handhaafd gebleven en wordt de gaschromatografie in de
meeste gevallen slechts gebruikt als hulpmiddel bij- en
als aanvulling van de bestaande technieken.

In dit proefschrift wordt aangetoond, dat de gaschroma-
tografie bij uitstek een analytische scheidingsmethode
is voor de analyse van steroiden in tegenstelling tot
de gangbare mening, dat deze methode voornamelijk ge-
schikt is voor de detectie van zeer kleine hoeveelheden.

Aan de steroid analyse gaat steeds een voorbewerking
vooraf, zoals hydrolyse, extractie, zuivering, indampen
van het extract en de chemische omzetting in stoffen,
die voldoende vluchtig en stabiel zijn voor de gaschro-
matografische analyse. Bij deze voorbewerking is er in
dit proefschrift naar gestreefd om zo weinig mogelijk
informatie te verliezen ten gevolge van deze voorbehan-
deling. Verder is de praktische uitvoering door middel
van miniaturisering vereenvoudigd.

Het probleem bij de steroid analyse is tweeledig. Ener-
zijds bezit het mengsel, dat in lichaamsvloeistoffen
voorkomt veel componenten, anderzijds komen deze stof-
fen voor in een concentratie spectrum, dat varieert van
10^{-10} gram tot 10^{-2} gram stof, geproduceerd per dag. Men
heeft voor de analytische oplossing van dit probleem een
methode nodig met zowel een groot scheidend vermogen als
een hoge gevoeligheid. De gaschromatografische techniek
beantwoordt aan beide eisen beter dan iedere andere me-
thode.

Voor de eenvoudigere problemen (hoge concentraties van
enkele componenten, die men wil analyseren) wordt het
gebruik van gepakte kolommen besproken met verbeteringen
in de uitvoering, zoals het injectiesysteem, waarbij het
oplosmiddel, waarin het monster is opgelost, selectief
tijdens injectie verwijderd wordt. Voor de meer gecom-
pliceerde analyse problemen (groot aantal componenten,
lage concentraties) is het gebruik van capillaire kolom-
men de aangewezen weg. Men heeft tot op heden echter
vrijwel geen gebruik gemaakt van capillaire kolommen
voor de steroid analyse, omdat er geen systemen waren
om de voor deze kolommen vereiste, microgram en submi-
crogram hoeveelheden stof splitloos te injecteren. In
dit proefschrift wordt aangetoond, dat er diverse moge-
lijkheden zijn om deze kleine hoeveelheden rechtstreeks
102 op de kolom te brengen. De aanzienlijke winst in oplos-

send vermogen van de capillaire kolom wordt aangetoond door de resultaten ervan te vergelijken met die van gepakte kolommen.

Daar in veel gevallen slechts een fractie van het natuurlijke monster, dat men na de voorbehandeling overhoudt, uit steroiden bestaat is het noodzakelijk voor injectie dit residu op te nemen in een oplosmiddel, waarvan de hoeveelheid enkele duizenden malen groter is dan de hoeveelheid aanwezige steroiden. Bij injectie zal het oplosmiddel de capillaire kolom geheel overladen. Voor de oplossing van dit probleem is een systeem voor directe injectie van dergelijke monsters ontwikkeld.

Door de grote winst aan informatie bij het gebruik van capillaire kolommen zal de toepassing van de steroid analyse ongetwijfeld op een ander en hoger niveau komen. Dit proefschrift is een eerste stap in deze richting.

DANKBETUIGING

LEVENSBESCHRIJVING

Op verzoek van de Senaat volgen hier enkele persoonlijke
gegevens over de schrijver van dit proefschrift.

Hij werd geboren te Weert op 26 juni 1937. Na het door-
lopen van de gymnasiale opleiding alfa, werd de studie
vervolgd op het Bisschoppelijk College te Weert, waar
hij in 1958 het diploma H.B.S.-B behaalde. Na een onder-
breking van 2 jaar ter vervulling van de militaire dienst-
plicht, werd de studie in september 1960 voortgezet aan
de Technische Hogeschool te Eindhoven, waar hij in janu-
ari 1965 het ingenieursexamen aflegde in de afdeling
Scheikundige Technologie. Op 1 februari 1965 trad hij
als wetenschappelijk medewerker in dienst bij de Tech-
nische Hogeschool te Eindhoven op de Afdeling Instrumen-
tele Analyse. Hier werd in samenwerking met het klinisch
chemisch laboratorium van het St. Elisabeth ziekenhuis
te Tilburg een aanvang gemaakt met het onderzoek, dat
leidde tot dit proefschrift.

STELLINGEN

1. Gezien de tijdafhankelijke parameters in kolomproces-
   sen verdient het aanbeveling het begrip retentievolu-
   me te vervangen door het begrip retentietijd.

2. De kwalitatieve en kwantitatieve interpretatie van
   de gaschromatografisch verkregen steroid spectra van
   urine-extracten is voor nawerking door andere labora-
   toria niet bruikbaar, omdat de streng gestandaardi-
   seerde condities, waarbij de experimenten zijn uit-
   gevoerd, niet uitvoerig worden beschreven.

   Van Kampen, E.J. and Hoek, W. Clin. Chem. Acta, **16**,
   442-444 (1967).
   Hoek, W. and van Kampen, E.J. Clin. Chem. Acta, **19**,
   371-381 (1968).

3. De beoogde nauwkeurigheid bij de ijking van standaard-
   stroommeters, gebaseerd op de nieuwe definitie van de
   ampère, is niet dan met zeer grote moeite haalbaar.

   Schweers, J. en van Vianen, P. Natuurkunde op corpus-
   culaire grondslag, deel
   IV, Malmberg, Den Bosch
   1964.

4. De Curie-temperatuur van ferromagnetische conductoren,
   welke gebruikt worden bij de pyrolyse van organische
   stoffen, is minder goed gedefinieerd dan de tempera-
   tuur, die verkregen wordt bij de ontlading van een
   condensator over een nauwkeurige weerstand.

Giacobbo, H., Simon, W. Pharm. Acta Helv. $\underline{39}$, 162
(1964).

Giacobbo, H., Diplomarbeit, ETH Zürich (1964).

5. Door het gebruik van grote hoeveelheden citraatbloed
bij hartoperaties met behulp van een hart-longmachine
treedt vaak metabole alkalose op. De door Maas ge-
geven verklaring voor dit verschijnsel is niet correct.

   Maas, A.H.J. Proefschrift, pag. 163, Rijksuniversi-
             teit, Utrecht (1967).

6. De verhouding van het aantal college-uren en het aan-
tal praktikum-middagen bij de moderne academische op-
leiding, is bij hogescholen groter dan bij universi-
teiten en neemt bij de eerstgenoemde steeds meer toe.
Dit komt niet overeen met de praktische aard van de
beroepen, die door ingenieurs worden uitgeoefend.

   Studiegidsen van Universiteiten en Hogescholen,
   1966/1967.

7. Grote ziekenhuizen zouden naast een medisch en econo-
misch directeur een technologisch "manager" als mede-
directeur moeten hebben.

8. Bij het meten van effectieve diffusie-coëfficiënten
bij moleculair zeven heeft de vorm en de grootte van
de korrel geen invloed.

   Zikanova, thesis, Tschechoslowakische Akademie der
             Wissenschaften, 1965.
   Gaschromatographie, p. 517, Deutsche Akademie der
                   Wissenschaften, Berlin 1968.

Eindhoven, 18 juni 1968   P.S.H. Kuppens