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CONTENTS

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CONVECTION IN CONTINUOUS-FLOW ELECTROPHORESIS

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(Received April 4th, 1977)

SUMMARY

The various types of convection possible in an electrophoresis device are indicated and criteria are presented from which estimates can be made of the importance of convection in the separation. These criteria also indicate design options possible to suppress or reduce convection effects.

Specific consideration is then given to the convection induced by Joule heating in a representative continuous-flow electrophoresis configuration. Detailed solutions for the associated velocity and temperature distributions are presented and it is shown how they are influenced by the buffer through-put, wall cooling, and electric field intensity.

Significant distinction is pointed out between counter- and co-flow operation. With the latter mode it would appear that larger gap devices could be successfully run in a normal gravitational environment.

INTRODUCTION

The occurrence of natural convection in electrophoresis is thought to be detrimental because of the resulting mixing of the fluid. It is, therefore, important to establish criteria that will indicate under what conditions the convective flow will occur and what the nature of the flow is. Such information will indicate the design options available to eliminate or minimize such flows and how to scale meaningful models.

The basic configuration for continuous electrophoresis (see Fig. 1) is essentially a rectangular parallelopiped with the height, h, large relative to the width, w, and the depth, d. The ratio d/w is small also. The electrode length is denoted by L. In Fig. 1 the electrodes are shown located on the end walls; in some configurations they are placed on the side walls.

There are basically two modes of natural convection, *viz.*, conventional convection and unstable convection. These can occur separately or together in a given configuration. Conventional convection is generated immediately by a density gradient that is normal to the gravitational vector. Unstable convection can occur when the density gradient is parallel to but opposed to the gravity vector. The onset of this motion is not immediate but depends strongly on the geometry; once this motion
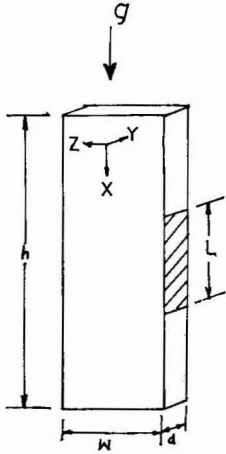
Fig. 1. Cell configuration.

starts, however, it causes much more mixing, in general, than conventional convection. Conventional convection, thus, results when the fluid and wall temperatures are different as with wall cooling and Joule heating. Furthermore, when the buffer flow is downward the fluid is heated as it proceeds along the cell. A vertical temperature (density) gradient, therefore, is imposed on the fluid with the higher temperature oc-
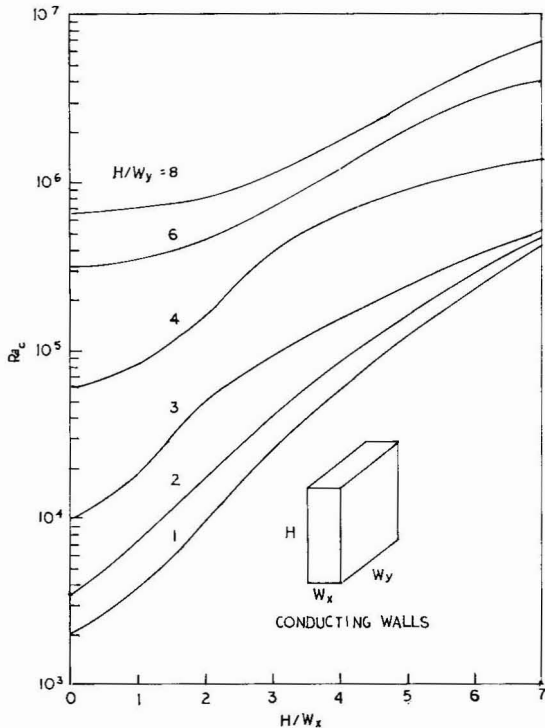
Fig. 2. Critical Rayleigh number as a function of aspect ratios (Catton[1]).

curring at the lower end of the cell, so that the heavier fluid is above the lighter. In such an unstable configuration no fluid motions are induced until a critical tempera-ture (density) gradient, or non-dimensionally, a Rayleigh number is exceeded; this critical value depends on the fluid properties but, more importantly, on the geometric configuration. The relation of the critical Rayleigh number with the two aspect ratios of significance for the configuration of interest herein is presented in Fig. 2 to (ref. 1).

From the above it should be evident that both types of convection are possible in a continuous-flow electrophoresis cell. One may dominate the other depending on the design and operating conditions. Thus, it is necessary to make estimates for each type of convection.

CONVECTION CRITERIA

In order to obtain some quantitative results let us assume that a representative configuration has $h = 30.5$ cm, $w = 5.08$ cm, $d = 0.508$ cm, and $L = 10.16$ cm. Let us also assume that the buffer properties are similar to water so that the kinematic viscosity, $v = 1.4 \times 10^{-2}$ cm^2/sec, the volumetric expansion coefficient $\beta = 0.18 \times 10^{-3}$ °K^{-1}, the thermal conductivity, $k = 0.56$ W/m °K, and the Prandtl number $Pr = 10$.

Convectional convection

The dimensionless parameter that indicates the ratio of buoyancy to viscous forces[2] is the Grashof number, $Gr = \beta g \Delta T l^3 / v^2$ where g is the gravitational force, ΔT denotes a characteristic temperature difference, and l a characteristic length. For the specific example being considered the difference in temperature between the fluid and the wall is taken to be 5 °K and the characteristic length $l = d/2 = 0.254$ cm so that $Gr = 73.8$. An estimate of the velocities induced under these conditions can be made from

$$u = \sqrt{Gr} \, (2v/d) = \sqrt{\beta g \Delta T (d/2)} = 0.472 \text{ cm/sec.}$$

From this expression it is clear that the most convenient design option to reduce convection is to reduce the characteristic length.

Unstable convection

The parameter that determines the onset of unstable convection[2] is the Rayleigh number.

$$Ra_c = Pr \, Gr_c = Pr \, \frac{g \Delta \varrho_c \, L^3}{\varrho v^2} = \frac{\beta g \Delta T_c \, L^3}{v^2}$$

where $\Delta \varrho$ is a characteristic density difference and the subscript c denotes the critical condition. For the cell in the vertical orientation $h/d = H/W_x = 60$, $h/w = H/W_y = 6$. For these conditions it can be estimated from Fig. 2 that $Ra_c = 2 \times 10^6$. With $L = 10.16$ cm the critical temperature difference can be determined from

$$\Delta T_c = 2 \times 10^6 \, \frac{v^2}{Pr \beta L^3 g} = 0.214 \text{ °K}$$

A difference in temperature (from bottom to top) greater than this value will lead to unstable convection.

If the cell is placed horizontally on the large side walls the configuration simulates an unbounded horizontal fluid layer half of which is heated from below. The critical Rayleigh number for such a situation[3] is 1101. Thus the characteristic length is $d/2 = 0.254$ cm and the critical temperature difference is $\varDelta T_c = 7.5\ °$K. The considerable stabilization obtained from a simple change in cell orientation is obvious. However, in this configuration conventional convection would result because the horizontal temperature difference is normal to the vertical gravity vector.

It must be noted that the values of the critical Rayleigh numbers have been determined for situations where all fluid motions are due solely to gravitational effects. It has been shown that, for proper conditions, superposed co-flows do not alter the critical Rayleigh numbers. However, for counter-flows such as would result with downward buffer flow there is no information.

Combined force and natural convection

In continuous-flow electrophoresis the fluid motions generated by buoyancy occur simultaneously with the forced flow of the buffer. The importance of the buoyancy-induced motions relative to the forced ones can be estimated[2] from the ratio of the Grashof and square of the Reynolds numbers:

$$\frac{Gr}{Re^2} = \frac{\text{buoyancy force}}{\text{inertia force}} = \frac{g\varDelta\varrho l^3}{\varrho v^2}\left(\frac{v^2}{U^2 l^2}\right) = \frac{g\varDelta\varrho l}{\varrho U^2}$$

where U is the buffer velocity. Convection will be negligible if

$$\frac{g\varDelta\varrho l}{\varrho U^2} \ll 1$$

This inequality indicates the design options possible to minimize or eliminate the effects of natural convection is a combined flow field.

It must be emphasized that the criteria given above are useful to obtain qualitative and order-of-magnitude estimates. Some of the variables used above could be written in terms of others, *e.g.*, $\varDelta T$ could be expressed in terms of the electrical power used. Also, in applying them all coupling mechanisms must be kept in mind. For example, from the last inequality presented above one might think that all convection problems could be avoided by increasing the buffer velocity. However, the sample residence time would be reduced accordingly and that trade-off would have to be considered.

CONVECTION INDUCED BY JOULE HEATING

Order-of-magnitude estimates are useful but it would be of interest to examine some details now. Let us, therefore, find the velocity and temperature distributions in the buffer generated by Joule heating. To this end consider the fully developed flow of a quasi-incompressible viscous fluid in a channel like that shown in Fig. 1. The wall temperatures are taken to be constant and equal because of wall cooling and an

electric current through the fluid causes Joule heating. The analysis of ref. 4 can easily be modified to the present case to yield

$$\theta = \theta_w + \frac{\sigma E^2 l^2}{2k} [1 - (Y/l)^2] \tag{1}$$

$$u = \frac{l^2 [1 - (Y/l)^2]}{2\mu} \left\{ - \frac{dP}{dX} - \varrho\beta g\theta_w - \frac{5}{12} \frac{\varrho\beta g\sigma E^2 l^2}{k} \left[1 - \frac{(Y/l)^2}{5} \right] \right\} \tag{2}$$

where $\theta = T - T_s$ is the temperature difference, T_s is a reference temperature (here, the buffer temperature before it enters the electric field), θ_w is the wall temperature difference, σ is the electric conductivity, E is the electric field intensity, l half the gap distance $(d/2)$, μ the absolute viscosity and P the pressure. Note these solutions (with a coordinate stretching) are applicable to fluids with variable viscosities and thermal conductivities.

From eqns. 1 and 2 it can be seen how each of the design and operating conditions influence the velocity and temperature profiles. For example, the factor of the last term of eqn. 1 equals the difference between the maximum fluid temperature and the wall temperature due to Joule heating. In eqn. 2 the first term on the right can be directly related to the buffer through-put, the second term is due to wall cooling, and the last to Joule heating. Note that the signs in the above equations correspond to the coordinate system indicated on Fig. 1; in particular X increases downward.

It is essential to understand that the buffer and sample flows can be directed in the direction of the gravity vector (downward) or opposed to it (upward). The former is the one usually considered although a few devices actually operate in the latter fashion. If the buffer flow is downward the first and third terms in eqn. 2 are of opposite signs $(dP/dX$ is negative) which indicates that the convective flow induced by Joule heating opposes the downward buffer flow. The sign of the second term is determined by $T_w - T_s$ which for cooled walls is negative so that the second term is positive and, thus, enhances the first term. If, however, the buffer flow is directed upwards the first and third terms are both negative so that Joule heating enhances the buffer flow. Thus, the direction of the buffer flow (or orientation of the device) profoundly influences the resultant flow. For downward buffer flows the convection opposes it and can ultimately destroy the parabolic profile. The convection will reinforce upward buffer flows so that the velocity profile will always be parabolic with different scales, $i.e.$, the flow is cocurrent.

The buffer volume flow-rate, Q^*, can be related to the pressure gradient as[5]

$$- \frac{1}{\mu} \frac{dP}{dX} = \frac{2}{3} \frac{Q^*}{wl^3}$$

so that eqn. 2 can be written as

$$u = \frac{l^2 [1 - (Y/l)^2]}{2\mu} \left\{ \frac{2}{3} \frac{Q^*}{wl^3} - \varrho\beta g\theta_w - \frac{5}{12} \frac{\varrho\beta g\sigma E^2 l^2}{k} \left[1 - \frac{(Y/l)^2}{5} \right] \right\} \tag{2a}$$

From this equation it is obvious that a reduced-gravity environment would reduce the Joule heating effect. However, it will be useful to investigate the conditions under

which the Joule heating will be detrimental in general for downward buffer flows ($Q^* > 0$).

The velocity distribution can now be seen to take on different shapes depending on the relative magnitudes of the design variables. Thus, if

$$\frac{2}{3} \frac{Q^*}{wl^3} \geqslant \frac{\varrho\beta g}{\mu} \left[\theta_w + \frac{\sigma E^2 \, l^2}{2k} \right] \tag{3}$$

the profile is a parabola as in Fig. 3a. If, however,

$$\frac{\varrho\beta g}{\mu} \left(\theta_w + \frac{5}{6} \frac{\sigma E^2 \, l^2}{2k} \right) < \frac{2}{3} \frac{Q^*}{wl^3} < \frac{\varrho\beta g}{\mu} \left(\theta_w + \frac{\sigma E^2 \, l^2}{2k} \right) \tag{4}$$

the flow in the vicinity of the axis (along which the sample flows) is retarded as in Fig. 3b. Finally, if

$$\frac{2}{3} \frac{Q^*}{wl^3} < \frac{\varrho\beta g}{\mu} \left(\theta_w + \frac{5}{6} \frac{\sigma E^2 \, l^2}{2k} \right)$$

the velocity near the axis will actually be reversed as shown in Fig. 3c.
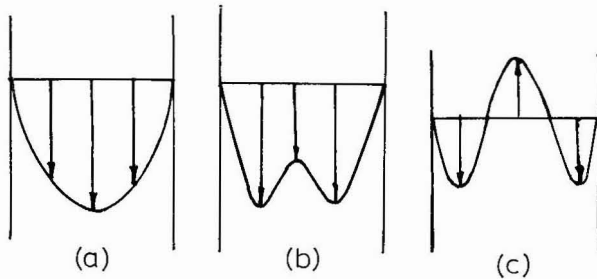
Fig. 3. Velocity profiles. a, Parabolic profile; b, retarded profile; c, reversed profile.

An estimate of the relative magnitudes of velocities induced by buoyancy (due either to wall cooling or Joule heating) can be made by comparing the corresponding terms of eqn. 2 evaluated at the channel axis ($Y = 0$). Thus, for example, the velocities induced by Joule heating compared to the buffer average velocity, U is

$$\frac{u_J}{U} = \frac{5}{24} \frac{\varrho\beta g\sigma E^2 \, l^4}{\mu k U} = \frac{5}{24} \frac{Gr_J}{Re} \tag{5}$$

where $Gr_J = \dfrac{\beta g\sigma E^2 \, l^2}{v^2}$ and $Re = U \cdot l/v$.

Again design options to reduce the convective effects are indicated in eqn. 5.

A separate analysis was made to determine the factors that influence the separation of a particle in a flow field with an electric field. To that end the trajectory was determined of a particle with a diameter d_i having a charge q_i subject to a Stokes drag

force and an electric field. Diffusion effects were neglected. It was found that the ratio of the maximum distance normal to the flow direction to the length of flow D is

$$\frac{X_{max.}}{D} = \frac{q_i E}{3\pi\mu d_i v_f} \tag{6}$$

where v_f is the fluid velocity due both to the forced and natural-convection flows. Eqn. 6 is, in effect, a measure of the resolution and the factors that influence it are indicated therein. This equation is strictly valid only for thick double layers. For thin double layers which are more likely in the electrophoresis of biological materials the right side of eqn. 6 contains a small factor (inversely proportional to the Debye length) due to electrokinetic streaming[6]. It can be observed that it would be beneficial to reduce the fluid velocity to improve resolution. That could, of course, be done by reducing the buffer flow.

SPECIFIC EXAMPLES

The general considerations treated above will now be applied to two specific continuous-flow electrophoresis devices: one representative of those currently used for analysis and one proposed to operate with considerably greater through-put in space. They will be referred to herein as the ground-based apparatus and the space flight apparatus. Representative values for the buffer properties are: $\sigma = 7.3 \times 10^{-4}$ mho/cm, $\beta = 0.18 \times 10^{-3}\ ^{\circ}K^{-1}$, $k = 0.5$ W/m $^{\circ}K$, and $v = 1.4 \times 10^{-2}$ cm²/sec.

Ground-based apparatus
For this device the following representative values will be used: $g = 980$ cm/sec², $E = 86$ V/cm, $l = 0.075$ cm, $w = 7$ cm, and $L = 45$ cm. To determine the maximum temperature rise in the buffer due to Joule heating eqn. 1 is evaluated at the axis $(Y = 0)$ to yield:

$$\theta_{max.} - \theta_w = T_{max.} - T_w = \frac{\sigma E^2 l^2}{2k}$$

so that for the values given above $T_{max.} - T_w = 2.76\ ^{\circ}C$. The Grashof number, which represents the ratio of buoyancy to viscous forces, is

$$Gr = \frac{\beta g (T_{max.} - T_w) l^3}{v^2} = 1.03$$

This indicates that the flow will be relatively slow and laminar. Evaluation of all the terms in eqn. 2a with $Q^* = 15$ cm³/min $= 0.25$ cm³/sec indicates that even with buffer downflow the velocity profile will be parabolic, *i.e.*, the first term is dominant. Thus, the maximum velocity (at the axis, $Y = 0$) is

$$u_{max.} = \frac{l^2}{2} \left[\frac{2Q^*}{3wl^3} - \frac{\beta g \theta w}{v} - \frac{5}{12} \frac{\beta g \sigma E^2 l^2}{kv} \right] = 0.15 \text{ cm/sec}$$

with $\theta_w = -2$ °C. Note that estimating the velocity from the expression $u = \sqrt{Gr}\,(\nu/l)$ presented in the section Convection criteria yields a value of 0.189 cm/sec. For the given electrode length, $L = 45$ cm, the residence time, τ, is

$$\tau = L/u_{\text{max.}} = 300 \text{ sec}$$

If, on the other hand, the buffer flow were upward, $u_{\text{max.}} = 0.168$ cm/sec and $\tau = 268$. In view of the previous discussion concerning the differences with downward and upward buffer flows it can be seen that in the former case the Joule heating results in a lower maximum velocity and, therefore, leads to a greater residence time whereas the reverse is true for upward buffer flow which is increased by the Joule heating.

Increases in the electric field intensity will ultimately modify the parabolic velocity profile with downward buffer flow. However, the field intensity can be increased with upward buffer flow. The residence time may be reduced by such increases but the electrode length can be increased to compensate for this. With upward buffer flow the field intensity increases is limited only by the maximum fluid temperature difference permissible for the particular biological samples. For the specific conditions treated herein and for $(T_{\text{max.}} - T_w) = 5$ °C, the maximum field intensity could be 117 V/cm. Even with this field intensity the residence time would still be approximately 200 sec.

Space-flight apparatus

Since greater through-put is desired for this device the gap width is increased to 0.5 cm so that $l = 0.25$ cm. Also $w = 5$ cm, $L = 10$ cm, and $E = 69$ V/cm. All other values are the same as previously used. Thus, it is found that $(T_{\text{max.}} - T_w) = 19.4$ °C and the Grashof number is 273 based on the earth's gravity ($g = 980$ cm/sec^2). Such a low value for Gr implies that the convection will be laminar and relatively slow and would not ordinarily be detrimental. However, the difficulty arises from the counterflow. The velocity profile determined from the specified values for this case and eqn. 2a for downward buffer flow is a reversed-flow one like that in Fig. 3c and this would, therefore, be unacceptable. If the buffer flow were upward a parabolic profile would be obtained. However, the velocities would be so large that the residence time would be too short. To compensate for this the electrode length would have to be increased. Clearly, these difficulties could be overcome for both orientations in a space vehicle where the gravitational force is reduced by five or six orders of magnitude. (Note that g is in the numerator of the Joule heating term in eqn. 2.) However, the analysis has indicated a number of design options that could permit the apparatus to be designed with the large gap and to operate on earth. This would require a decrease in field intensity and an increase in the electrode length with possibly a reduction in residence time. For example, if a residence time of 100 sec were acceptable an increase in the electrode length to 100 cm and a decrease in field intensity to 27 V/cm would then permit operation of the wider-gap device on earth. Other trade-offs are, of course, possible. From the relations presented herein design or operating charts can be developed that relate the electric field intensity, maximum temperature difference, gap width, residence time, electrode length, and buffer flow-rate in order to indicate the trade-offs explicitly. These would also indicate the bounds within which large-gap devices could operate in a normal gravitational environment.

SUMMARY

A number of criteria are presented that permit estimates to be made of various convective effects on continuous-flow electrophoresis. Design options possible to eliminate or reduce these effects are indicated therefrom. The detailed velocity and temperature distributions are then presented as functions of the buffer flow-rate, wall cooling, and Joule heating. Several different flow regimes are delineated in this way and the significant differences between counterflow and co-flow operation are indicated. In particular, it is shown that it appears possible to operate a large-gap device in a normal gravitational environment if the buffer flow is upward (co-flow). It should also be mentioned that with upward buffer flow the possibility of a thermal instability due to heating from below need not occur (with proper design) because the heated (electrode) region overlays the region of incoming cool buffer flow. Thus, the upward buffer flow configuration seems to be free of at least two possible causes of remixing. Such a configuration has been utilized to separate simple dyes[7,8].

The emphasis throughout the present paper is to gain some understanding of the relevant phenomena in order to make clear the design options. Other hydrodynamic aspects (such as electro-osmosis, stream stability, Taylor dispersion, and sample concentration) of continuous flow electrophoresis need to be investigated in greater detail so that proper design can be made of such devices for preparative purposes.

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OPTIMIZATION OF OPERATING PARAMETERS FOR GLASS CAPILLARY COLUMN GAS CHROMATOGRAPHY

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SUMMARY

The relationship between analysis time and separation with glass capillary columns was studied. Optimal operating parameters for achieving the shortest analysis times consistent with degree of separation were determined experimentally. It was found that the optimum column temperature is governed by retention characteristics of the solutes and the optimum column length by the required magnitude of separation. The optimum carrier gas flow-rate was found to be a function of the column length. Emphasis is placed on using capillary column length as an operating parameter.

INTRODUCTION

The obvious advantage of open tubular columns in comparison with packed columns is their extremely high resolution. Analyses involving compounds of similar retention times require high resolution. This in turn requires a long analysis time. Another important but less apparent advantage is that open tubular columns require shorter analysis times than packed columns to achieve given separations. Faster analysis is by no means less important than greater resolving power. In order to achieve the fastest analysis, all the operating parameters must be optimized with respect to the time of analysis. It is a formidable task to establish the optimum conditions when all operating parameters are considered. This is not necessary, as we are concerned here with column performance under nearly optimum conditions. This would still result in considerable time saving relative to other arbitrarily chosen conditions. The purpose of this study has been to improve our understanding of the relationship of the numerous operating parameters with respect to the analysis time.

The effect of chromatographic conditions upon retention time and resolution has been studied by a number of workers. The Van Deemter equation in which HETP is expressed as a function of the carrier gas velocity is widely accepted and serves as a basis for further development of chromatographic theory. Most of the parameters have been, therefore, studied in light of the theoretical plate model[1].

Purnell and Quinn[2] explored the best means for achieving fast analysis. Gid-

dings[1] examined the validity of the theory and derived an approach to rapid analysis. Open tubular columns were also studied; the absence of "multi-path effect" of the solid support (or eddy diffusion) made the use of these columns ideal for such study[3].

From Golay's equation for open tubular columns (see Fig. 1), it can be seen that the highest number of theoretical plates obtained at the optimum average linear velocity (where the height equivalent to a theoretical plate (HETP) is minimum) is not the maximum attainable when the analysis time is concerned. For the same given period, a column with increased length at proportionally increased gas velocity would produce more theoretical plates. Based on the assumption that Golay's equation is dependent on column length, increases of the two would result in ever increasing theoretical plates. However, the increase must be finite in reality (as discussed later). Thus by incorporating both column length and average linear velocity simultaneously into the operating parameters, the column efficiency can be maximized. This gas velocity is greater than the optimum average linear velocity and is usually referred to as "optimum practical gas velocity" (OPGV). This concept is very valuable for the pursuit of fast analysis. Scott and Hazeldean[4] originally defined the OPGV as "the linear velocity at which the HETP curve tends to become linear" (see Fig. 1).

Van Deemter Plot for Open Tubular Column
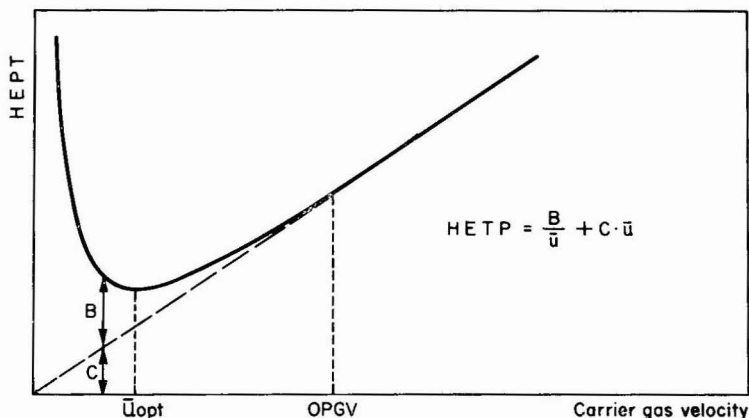
Fig. 1. Plot of HETP vs. carrier gas velocity for open tubular columns based on the given equation. Optimum average linear velocity ($\bar{u}_{opt}$) and optimum practical gas velocity (OPGV) are indicated.

Desty et al.[5] attempted to express the column performance as the ratio of the number of effective theoretical plates to the analysis time (N/t_R). Since most chromatographic analyses deal with resolution of more than one component, the effective theoretical plates are more expressive of the degree of separation than the values related to theoretical plates. They[5] defined the OPGV more explicitly as the velocity for the maximum of N/t_R. Ettre and March[6] suggested a plot of resolution vs. analysis time for comparison of column performance. It is also our belief that the actual measurement of the resolution of a pair of selected solutes will permit direct comparison of various columns and valid evaluation of parameters within a column, if experimental conditions are carefully controlled.

The usefulness of relatively short capillary columns for rapid analysis was advocated by Gouw et al.[7]. Previously developed theories are helpful in studying this fast analysis problem. However, one of the most critical restrictions on the use of HETP and its related variables (e.g., Golay equation, etc.) for this problem is that the relationship of HETP vs. average linear velocity is considerably affected by the pressure gradient (i.e., by column length) (discussed later in detail). Indeed, Desty et al.[5] warned of the error in assuming that the Van Deemter plot is little affected by column length.

While most of the parameters concerning both the column design and its operation influence performance, three of the most conveniently variable parameters were chosen for study. Unlike packed columns, the glass capillary or wall-coated open tubular columns (WCOT) can be trimmed to any desired length with little effort. The column length was, therefore, considered as an operational parameter rather than a column design parameter even though this is not yet commonly practiced. Moreover, this is imperative for approaching the optimum conditions necessary for rapid analysis.

The other two parameters chosen, carrier gas velocity and column temperature, are the commonly changed operational parameters. Previous work on this subject did not treat the effect of temperature as an interrelating variable with respect to other parameters. The column temperature does affect not only the retention times but also the relative retention which makes the mathematical treatment cumbersome, but it cannot be ignored or set constant to evaluate the true column performance; it must be treated as one dimension of the interrelating parameter matrix.

The rest of the parameters, such as column diameter and the stationary phase film thickness, were considered as part of the column design, hence not constituting additional dimensions in this study. From the practical point of view, the emphasis should be placed on the above three parameters.

EXPERIMENTAL

Column coating

Coiled glass capillary tubing (0.50 mm O.D., 0.25 mm I.D. × over 80 m) was drawn from flint glass by a modified Shimadzu glass drawing machine Model GDM-1. The capillary tubing was coated with the liquid phase by thermostatic method. The tubing was filled with SE-30 solution in pentane (4 mg/ml) and one end flame-sealed. The glass capillary was then driven into a specially designed heated oven (200°) at constant speed (2 cm/sec) starting from the open end. At the entrance of the oven, an additional stainless-steel tube was installed and heated to 250° for instant heat transfer to the capillary. Evaporated solvent vapor was forced to escape through the open end of the capillary tubing into the oven. After completion of the drying step, the solvent vapor remaining in the column was removed by vacuum. The detailed procedure is reported by Jennings et al.[8]. This coating method is quite reproducible in achieving uniform liquid phase film thickness and satisfactory separation efficiency. The column was cut to desired length and both ends straightened by microflame.

Instrumentation

A Hewlett-Packard Model 5731A gas chromatograph with dual-flame ionization detector was used. Variable-ratio inlet splitters were installed and the detector

TABLE I

GAS CHROMATOGRAPHIC CONDITIONS

Parameter	Value
Inlet split ratio	80–100:1
Carrier gas flow	0.1–2.0 ml/min
Detector make-up gas flow	30 ml/min
Hydrogen flow	30 ml/min
Air flow	240 ml/min
Inlet temperature	250°
Column temperature	115–160°
Detector temperature	250°

slightly modified so that the column effluent entered the flame tip directly. Conditions were adjusted as shown in Table I. All gases were supplied through pressure regulators.

Parameters examined

Column length. Four column lengths were chosen for study. To minimize variation of column efficiency resulting from variations in the coating procedure, 7.5-m and 40-m columns were made from one long column. The other two columns (15 m and 25 m) were made from the 40-m column after experiments using that length column were completed. The efficiency and liquid phase loading did not change during this study.

Temperature. Chromatographic data were collected under isothermal conditions at four different temperatures (115°, 130°, 145° and 160°) with all four columns.

Carrier gas flow. Carrier gas flow was varied by regulating the inlet pressure. Flow condition was expressed in terms of average linear velocity measured by the retention time of methane. For each set of condition (column length and temperature) the average gas velocity was varied from *ca.* 20 cm/sec to 100 cm/sec for at least six data points.

Retention time range of solutes. The test mixture for the measurement of column efficiency was composed of a series of straight-chain hydrocarbons (C_{11}–C_{16}) dissolved in heptane. This mixture allowed examination of theoretical plates and resolution of hydrocarbon pairs at wide ranges of the partition ratio. In order to express the physical properties of the solutes for further generalization, their retention behaviour was expressed in terms of Kováts' retention indices, I[9]. The retention property range examined in this experiment was, therefore, from $I = 1100$ to $I = 1600$. Each injection included a small amount of methane.

Measurement

Retention times and widths at half peak height of all peaks were measured. Column performance at each set of operating condition was evaluated by resolution of neighboring hydrocarbon peaks, theoretical plates and effective theoretical plates. The equations used are given below:

$$R = \frac{1.18 \, (t_{R2} - t_{R1})}{(W_{h2} + W_{h1})}$$

$$n = 5.54 \times \left(\frac{t_R}{W_h} \right)^2$$

$$N = 5.54 \times \left(\frac{t'_R}{W_h} \right)^2$$

where R is resolution*, n number of theoretical plates, N number of effective theoretical plates, t_R retention time, t'_R adjusted retention time, W_h peak widths at half height.

H.E.T.P. vs Average Linear Velocity
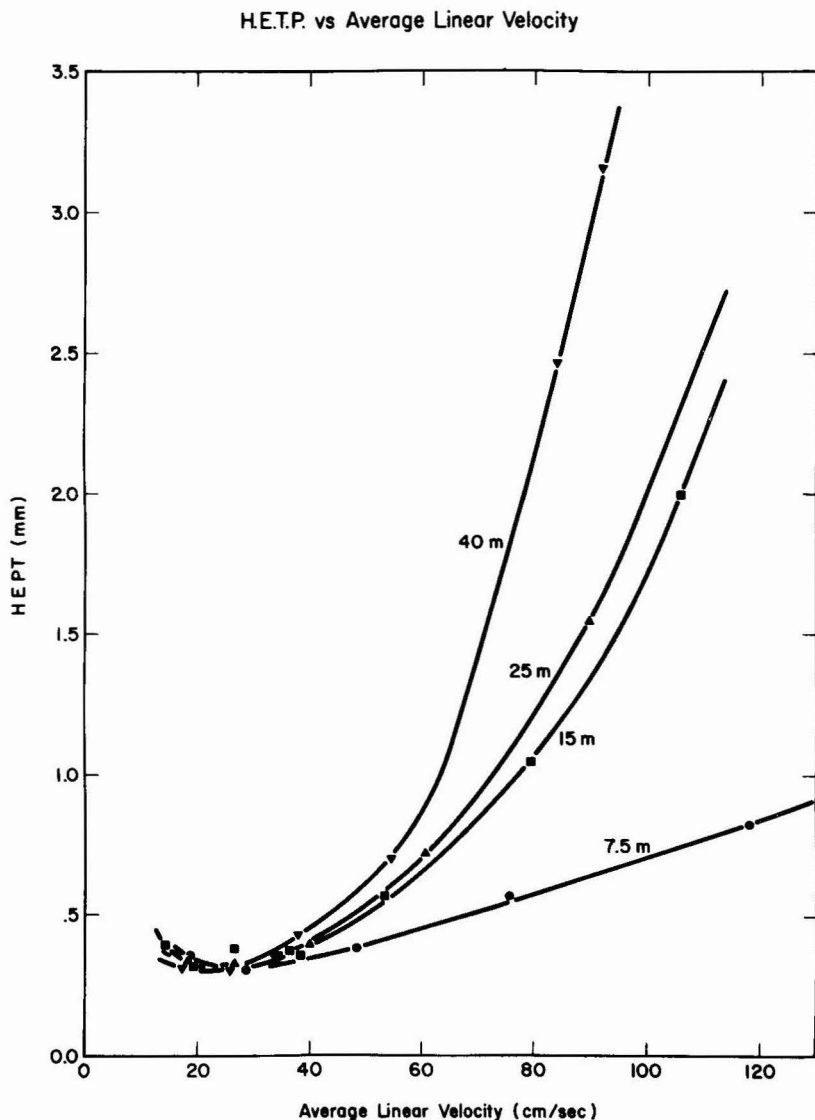
Fig. 2. Plot of HETP vs. average linear velocity measured by tetradecane peak at 130° on 0.25 mm I.D. SE-30 columns.

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* The equation is equivalent to the commonly used expression, $R = 2(t_{R2} - t_{R1})/(W_2 + W_1)$. The factor is adjusted for the peak width measured at half peak height of a gaussian curve.

RESULTS AND DISCUSSION

Because of the compressibility of carrier gas, a gas chromatographic (GC) column always exhibits a pressure gradient. Hence, a uniform linear velocity can never exist. Moreover, when column length is changed, the linear velocity profile throughout the column will be affected. Since the band broadening process is a function of carrier gas linear velocity at a particular position in the column, most conventionally used terms such as the number of theoretical plates or HETP value are of limited validity. Fig. 2 shows the relationship of average linear velocity to HETP.

Variation in column length has little effect upon HETP when the average linear velocity is near the optimum. However, at increased flow the longer columns have much greater HETP value than the shorter ones. The 40-m column exhibits little greater resolution than the shorter columns at an average linear velocity of 80 cm/sec or above. This is perhaps because with a longer column, there is a greater spread of linear velocities than with a shorter column.

It follows that carrier gas flows expressed as average linear velocity are of limited validity when variations in column lengths are concerned, as are equations using these values. For the same reason, the curve shown in Fig. 1 has large deviation from actually measured values (Fig. 2). Since the Van Deemter curve shifts with increase in column length, the value of OPGV cannot be a constant value.

Choice of carrier gas

Helium is a commonly used carrier gas primarily because of its high diffusivity, allowing faster analysis with only slightly greater HETP compared to nitrogen. Fig. 3 shows that analysis using helium as carrier allows a significant reduction of the analy-

H.E.T.P. vs Average Linear Velocity
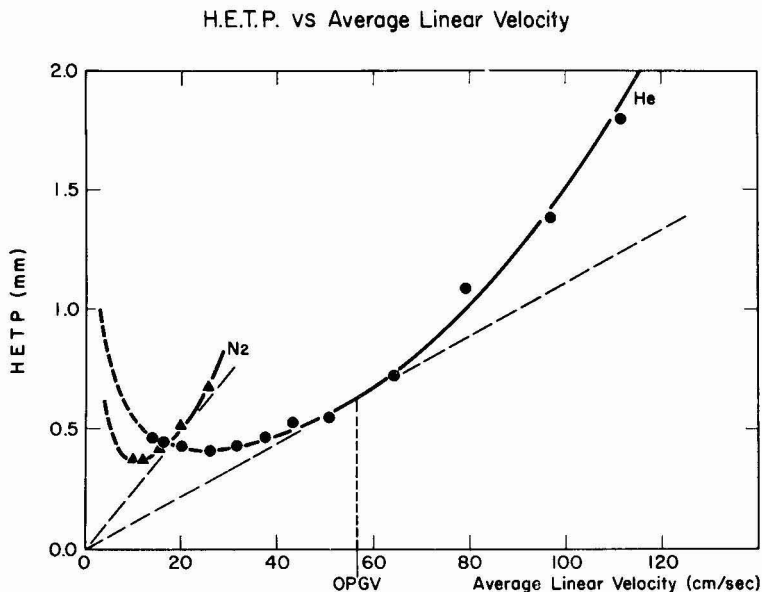
Fig. 3. Effect of carrier gas on HETP at various velocities. Measured by tricosane peak at 230° on 20 m × 0.28 mm I.D. SE-30 column.

sis time at increased flow compared to use of nitrogen. Hydrogen has been demonstrated to be an even better carrier gas for fast analyses; however, it was not examined in this study.

Velocity and length

Fig. 4 shows the effect of the carrier gas flow on the analysis time and resolution for four columns at 130°. The solid curves were obtained when the column length was fixed to the four values. The tangential broken line (maximum resolution curve) was drawn to facilitate estimating the resolution attainable for continually changed column length.

Resolution vs Analysis Time

Effect of Column Length and Flow
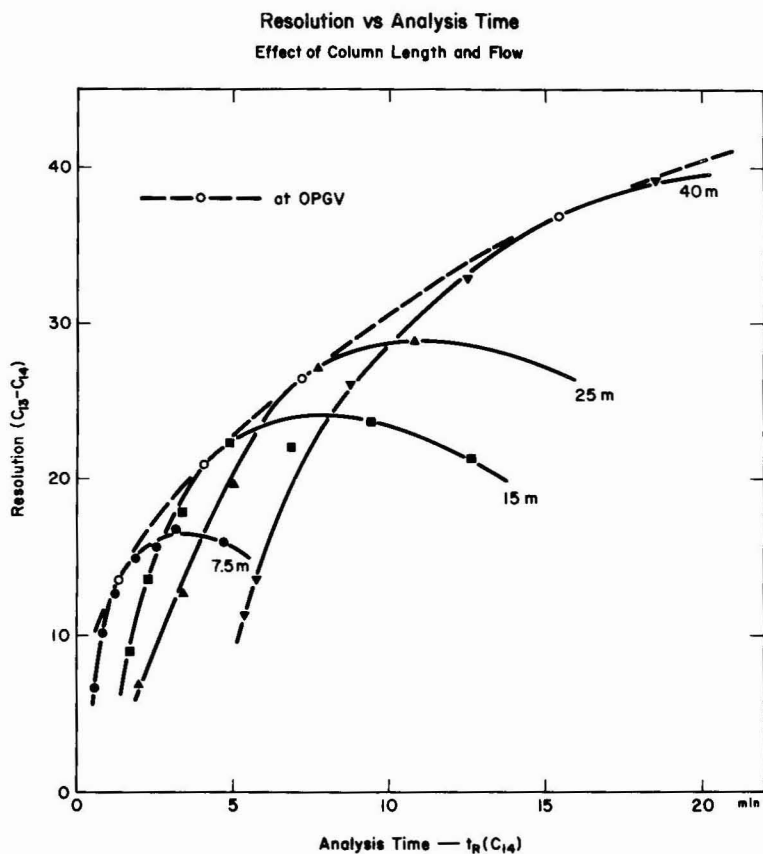
Fig. 4. Effect of column length and carrier gas flow on resolution and analysis time. Measured by tridecane and tetradecane peaks at 130° on 0.25 mm I.D. SE-30 columns. The broken curve (maximum resolution curve) indicates the highest resolution attainable from the capillary column of 0.25 mm I.D.

The maximum resolution curve can be drawn for each set of temperature and the retention property (retention index of the peak). It is apparent that the highest resolution from a given column is not the optimum with respect to analysis time. Also, the condition which results in the maximum value of R/t_R for the given column can be

outperformed by shorter columns. It can be noted from the graph that each column length has only one average linear velocity at which the column is used optimally when the column length is allowed to be an operating parameter. At the temperatures other than 130°, the same effects were observed. Measurement of the other pair of hydrocarbon peaks also showed a similar trend.

Temperature and retention character

The maximum resolution attainable for each pair of peaks for various temperatures were examined with the aid of graphs similar to Fig. 4. Among the four temperatures examined, the temperature of 130° showed more resolution than others for the C_{13} and C_{14} hydrocarbons. Similarly, for the C_{15}/C_{16} pair, a temperature of 160° exhibited higher resolution for analysis time than the other three temperatures. As already reported in 1960[4], there is an optimum temperature for a pair of solutes at which the highest resolution can be obtained for a given analysis period. Fig. 5 was plotted in order to generalize this temperature–retention relationship. The partition ratio was chosen for the horizontal axis. As the partition ratio of a solute is independent of either column length or flow condition (as the phase ratio was kept constant in this study) the plot is valid to examine the maximum resolution curve where both length and the flow were variables.

Minimum Analysis Time vs Partition Ratio For Fixed Resolution
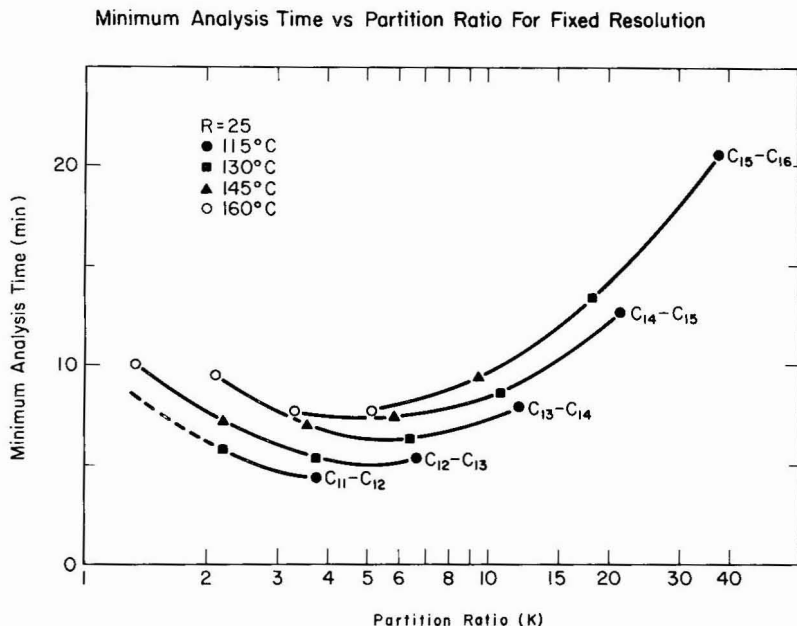
Fig. 5. Minimum analysis time required to produce a fixed resolution ($R = 25$) of the hydrocarbon pairs *vs.* partition ratio (k) measured at the second peak of each pair. Minimum analysis time was obtained from maximum resolution curve at $R = 25$ in each condition.

In Fig. 5, the minimum analysis time was determined as the analysis time required to achieve the resolution of 25 (an arbitrarily chosen value) along the maximum resolution curve (Fig. 4). In other words, each data point in Fig. 5 represents the

shortest analysis time for the pair of hydrocarbons by allowing the length and flow to be optimal. It is noted from the graph that the partition ratio of about 6 results in the shortest analysis time for the desired resolution regardless of the retention behavior. Since in the range from 3.5 to 8 the curves are flat, the temperature is not critical; *i.e.*, as long as the temperature was set to give the partition ratio of the solutes from 3.5 to 8 (in terms of temperature, *ca.* $\pm$ 8° from the optimum), the maximum resolution curve will be similar and all very close to the optimum. However, greater departure from that optimum range (either very large or very small partition ratio) causes the required analysis time to be much longer.

It is interesting to note that a hydrocarbon pair of longer chain length (*e.g.*, C_{15}/C_{16}) requires a longer analysis time to achieve the same degree of resolution in comparison with a solute pair of smaller retention indices (*e.g.*, C_{14}/C_{15}). By generalizing the statement, for the same difference in retention indices for two solutes, the pair with larger retention indices requires longer analysis time than a pair of smaller retention. As an example, the pair of methyl decanoate ($I = 1368$) and methyl 1-decenoate ($I = 1338$) will require a longer retention time for complete separation than the pair of methyl hexanoate ($I = 869$) and methyl 1-hexenoate ($I = 839$). This is so even though in both cases the structural difference within the pair is saturated/mono-unsaturated and ΔI for both pairs is 30. This can be accounted for by the relative retention decrease with increased temperature. For the pair with larger retention indices, temperature must be elevated to put the partition ratio in the suitable range; therefore, the relative retention becomes closer to unity which in turn results in complete separation becoming more difficult.

Overall relationship

Up to this point, the interrelating parameters were discussed while others were set constant. We can summarize as follows:

(1) There is an optimum temperature at which a given pair of solutes can be best separated in the shortest time. The optimum temperature is governed by the retention properties of the solutes regardless of desired resolution. That is, column temperature should be adjusted to obtain a partition ratio of 6 for the later peak.

(2) Once the desired degree of separation is defined, there is only one optimum column length and its corresponding optimum value of average linear velocity. For a given column (fixed diameter, film thickness and coating efficiency), the maximum resolution curve will facilitate estimation of the optimum column length.

Using this approach, one can select the three operating parameters with a minimum of effort at or close to the optimal conditions.

In practice, column length is usually kept constant, and the only operating parameters varied are temperature and carrier gas flow. Let us examine the merit of employing the additional parameter, *i.e.*, varied column length. Fig. 6 shows the degree of time saving by this method. The point where all three curves join indicates the best choice of all the parameters (temperature, length, flow).

The "curve for varied flow" was plotted at various gas velocities while the temperature was set constant to give the partition ratio of 6. The "curve for varied temperature" was obtained by increasing and decreasing temperature for the optimum value while the average linear velocity was kept constant. In both cases the same column was used. In both directions from the original condition point, manipulation by
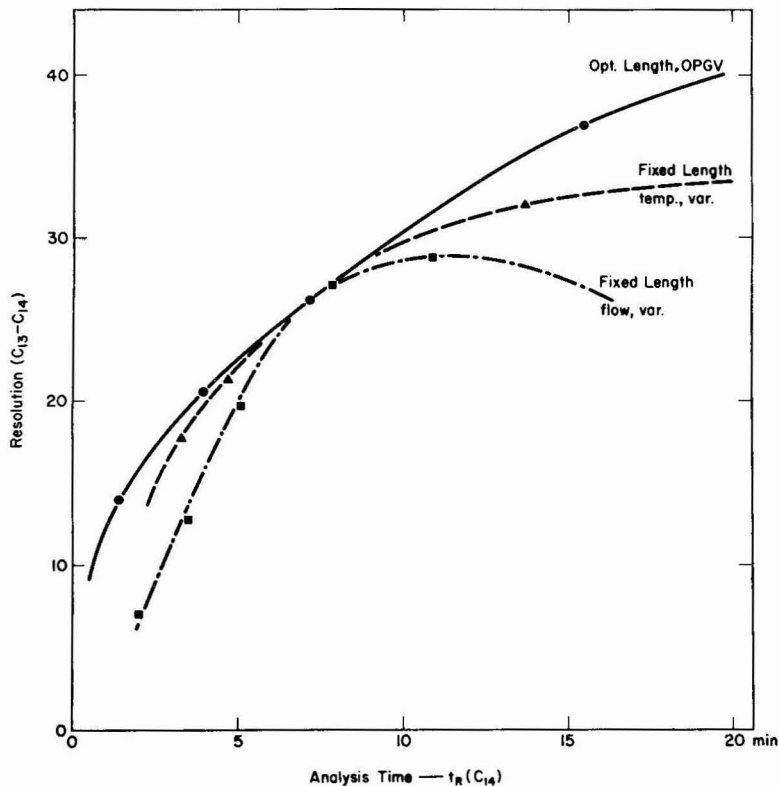
Fig. 6. Effect of varied parameters on resolution and analysis time. Top curve (maximum resolution curve) was obtained at 130° (near optimum) by adjusting the column length and flow. Second curve was obtained by changing only the temperature. The bottom curve was obtained by changing only flow at 130°.

temperature gives higher resolution than by flow. The temperature curve is not significantly remote from the "curve for optimum length and flow" (maximum resolution curve). Therefore, one can simply raise temperature to take advantage of time saving with slight departure from the "maximum resolution curve". On the other hand, in order to achieve greater resolution than the original point, time saving by optimum column length with OPGV (increase in length with concomitant flow adjustment) can be considerable. This is because the slope of the maximum resolution attainable constantly decreases. Therefore, when the relative retention of the solute pair is very close to unity (*i.e.*, difficult to separate), increase of column length and re-adjusting the average linear velocity result in considerably greater time saving than simply lowering temperature with the same column.

ACKNOWLEDGEMENT

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SIMPLIFIED METHOD FOR THE DETERMINATION OF RESIDUES OF CARBOFURAN AND ITS METABOLITES IN CROPS USING GAS–LIQUID CHROMATOGRAPHY–MASS FRAGMENTOGRAPHY

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SUMMARY

The gas–liquid chromatographic behaviour of 2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate (carbofuran), its 3-keto-, and 3-hydroxy-derivatives, their respective phenolic hydrolysis products and the heptafluorobutyryl (HFB) derivatives of the carbamates and phenols were studied by examining the column effluent using chemical ionization mass spectrometry. In contrast to the behaviour of the carbamates, their HFB derivatives consistently produced ions having intensities proportional to the quantities injected. The common base-peak ion at 228 a.m.u. was used to quantitate these materials at the 0.02–1 ppm level in field-treated carrots, celery, tomatoes and corn with minimal sample preparation.

INTRODUCTION

The determination of traces of insecticidal carbamates is difficult and development of precise methods for the measurement of residue levels has not kept pace with those techniques available for organochlorine and organophosphorus pesticides. Recent reviews[1,2] of carbamate analysis have surveyed the use of gas–liquid chromatography (GLC) for the analysis of carbofuran (CF) and its metabolites 3-hydroxy-carbofuran (HO-CF) and 3-ketocarbofuran (CO-CF) (Fig. 1). The suitability of GLC
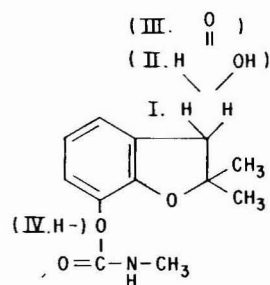
Fig. 1. Chemical structures of carbofuran (I) and its metabolites:3-Hydroxycarbofuran (II), 3-ketocarbofuran (III) and associated phenols (IV).

for the analyses of monomethyl carbamates is a matter of some controversy[3,4]. In general it appears that thermal decomposition is not a major obstacle when the amounts of CF are greater than 1 ng. At this level responses for equivalent amounts of CO-CF and HO-CF are often much smaller. To reduce thermal decomposition and/or to improve sensitivity, derivatives suitable for GLC separation and detectable with electron-capture detectors (ECD) have been prepared. The degree of analytical success attained with these derivatives has been somewhat dependent on the substrate being analyzed. For example, the analysis of the 2,4-dinitrophenyl ethers of the phenols derived from arylcarbamates appears an acceptable method for the analysis of the carbamates in soil. The carbamates are extractable from the substrate under conditions which do not extract the corresponding phenol residues which otherwise would interfere[5–7]. In the case of plant and animal material and water, the analyst is not so fortunate. More elaborate procedures have been devised[8] based on the separation of phenols utilizing their weak acidity compared to the neutral carbamate.

Unfortunately CO-CF and HO-CF are much more susceptible to hydrolysis by the alkaline reagents required to effect the separation than CF is. As a result, accurate analyses of these oxidation products are difficult or impossible to obtain using such methods. A more acceptable alternative, free of this problem, is the derivatization of the intact carbamates. Acylation[9–11] and alkylation[12,13] of the N-methylcarbamates have been reported and acylations have been more fully investigated, particularly with fluorinated carboxylic acid anhydrides which produce acylated carbamates and phenols having strong electron-capturing characteristics. We have successfully combined such procedures with GLC–ECD for the rapid analyses of HFB derivatives of CF and carbofuran phenol (CF-P) in phosphate buffers and biological media. However, interfering responses have largely precluded the use of the ECD for the analyses of these derivatives in plant and animal material except for the work reported by Wong and Fisher[14]. The polar nature of the carbamates hinders their separation from potentially interfering materials prior to derivatization, and separation subsequent to derivatization is rendered impossible by the hydrolytic instability of these derivatives. The use of an electrolytic conductivity detector in the halogen mode for the analysis of the heptafluorobutyryl (HFB) derivatives of a number of methylcarbamates has been reported recently[15]. It lacks the sensitivity attainable with the ECD and remains relatively non-specific since materials which will interfere with the analysis of many of the carbamates are also detected in the crop extracts.

The mass spectrometer appeared a potentially useful alternative to the ECD for this type of analysis. Using specific ion monitoring, sensitivities in the picogram range are attainable for many materials. The carbofuran compounds, and their HFB derivatives, contain only one nitrogen atom and possess odd-number molecular weights; in a chemical ionization mass spectrum their large, protonated molecular ions (and protonated fragments containing the carbamyl nitrogen) will appear at even mass-to-charge (m/e) values. Their phenols, having lost the nitrogen atom, will appear at odd m/e values together with most of the potentially interfering materials of vegetable origin which, fortuitously, present a very low background at even m/e values above 200 a.m.u. This provides the basis for a more specific detection system, with very high sensitivity, for derivatives of intact carbamates.

We wish to report the development of a simple method for the analysis of

carbofuran and its two common oxidation products based on the determination of HFB derivatives by chemical ionization (CI) mass fragmentography in crude crop extracts and to record our observations on the GLC and mass spectrometric (MS) properties of the materials pertinent to this analysis.

MATERIALS AND METHODS

Instrumentation

A Micro-Tek 220 gas chromatograph fitted with a 120 cm × 4 mm I.D. glass column packed with 5 % OV-1 on 100–200 Varaport-30 and equipped with a flame ionization detector (FID) and a ^{63}Ni electron-capture detector was used for preliminary studies on the quantitative preparation, the separation and the stability of the HFB derivatives. For experiments involving MS and subsequent residue analysis, a Finnigan 9500 gas chromatograph was fitted with a similar column and coupled directly to a Finnigan 3200 quadrupole mass spectrometer equipped with a CI source and a three-channel Promim* specific ion monitor. The most satisfactory mode of operation for our purpose was to use methane at 5–10 ml/min as the combined carrier–CI reagent gas. This flow-rate provided the optimum CI source pressure (1 Torr) and the column was operated isothermally (145°) to give efficient GLC resolution of the carbamate derivatives of interest. The major portion of the solvent was diverted from the mass spectrometer for up to 3 min following injection. Conventional CI mass spectra of the GLC eluates were recorded with a light-beam oscillograph. Mass chromatograms and fragmentograms were obtained by recording the respective ion integrator or specific ion monitor outputs with a multi-channel paper-chart recorder and peak heights were measured for analysis. All instrument operation and data extraction were performed manually.

Chemicals and crops

Analytical grade CF, CO-CF, HO-CF, CF-P were supplied by Niagara FMC, (Burlington, Ontario Canada). The 3-hydroxycarbofuran phenol (HO-CF-P) and 3-ketocarbofuran phenol (CO-CF-P) were prepared by suitable hydrolysis of the parent carbamate. Standard solutions were prepared at 100 μg/ml in benzene and diluted as required. Reagent-grade chloroform and benzene were purified and glass-distilled in our laboratory and were free of interfering responses. The heptafluorbutyric anhydride (HFBA) (Pierce, Rockford, Ill., U.S.A.) and pyridine (Fisher, Pittsburgh, Pa., U.S.A.) were used as received.

Samples of carrots, tomatoes, celery and corn were from crops grown and treated with CF at conventional levels for insect control at our field station.

Extraction

Crops were extracted by the acid digestion procedure of Cook et al.[16] within a day of harvest and the chloroform extracts of the hydrolysates were stored over anhydrous sodium sulfate in a freezer until analyzed. Appropriate aliquots of the chloroform extracts were solvent-exchanged to benzene in preparation for analysis.

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* Promim is a registered trademark of The Finnigan Corporation, Sunnyvale, Calif., U.S.A.

Derivatization

Heptafluorobutyrylation was carried out by treating the carbamate or phenol (up to 100 μg), or the crop extract (equivalent to 10 g of crop) in 5–10 ml of benzene with 4 drops of pyridine and 0.1 ml of HFBA at room temperature for 15–16 h (overnight). If the reaction mixture was to be analyzed by GLC–MS without water-washing, it was convenient to dilute 1 ml of the standard at 10 × the desired final concentration, or the extract equivalent to 10 g of crop, to about 8 ml in a 10-ml volumetric. The pyridine and HFBA were then added and the reaction allowed to proceed. Before analysis the samples were made up to volume with benzene. When the reaction mixture was to be washed free of excess reagent, as was required for analysis by FID or ECD in some preliminary experiments, or to reduce background noise on a second or third specific ion-channel on the mass spectrometer, the reaction was carried out with 5 ml of an appropriate concentration of standard or crop extract in a test tube fitted with a PTFE-lined screw cap. After reaction the benzene was shaken with 5 ml of water three times. The water was removed each time after centrifugation using a disposable pipette. The washed benzene was dried with sodium sulfate before analysis. Washed samples were analyzed within 24 h to minimize changes due to hydrolysis of the derivative; unwashed samples were normally used within 5 days of preparation.

Mass spectra, chromatographic behaviour, ion selection and calibration

Mass spectra were determined between 125–650 a.m.u. on the GLC eluate from injection of 50–100 ng of CF, CO-CF and HO-CF and amounts of the CF-HFB, CO-CF-HFB and HO-CF-DIHFB equivalent to these amounts of carbamate. The intensities of the major ions were measured and normalized and pertinent data for the HFB derivatives are given in Table I. Mass fragmentograms were recorded using the equivalent of 1 ng of each of the carbamate HFB derivatives and three channels of Promim, one set at *m/e* 228 and the others at appropriate values. A composite fragmentogram is shown in Fig. 2. The intensities of the ions at 228 a.m.u. were measured for 4–7 injections of various amounts of CF-HFB and HO-CF-DiHFB

TABLE I

METHANE-CHEMICAL IONIZATION MASS SPECTRA OF THE HFB DERIVATIVES OF CARBOFURAN, 3-KETOCARBOFURAN AND 3-HYDROXYCARBOFURAN

Compound injected	Relevant major ions (m/e)	Relative abundance (%)	Structural assignment, protonated molecular ion of
CF-HFB (MW = 417)	418	20	CF-HFB
	361	9	CF-P-HFB
	228	100	Methylamine-HFB
	165	30	CF-P
CO-CF-HFB (MW = 431)	432	27	CO-CF-HFB
	375	14	CO-CF-P-HFB
	228	100	Methylamine-HFB
	179	43	CO-CF-P
HO-CF-DiHFB (MW = 629)	416	37	HO-CF-DiHFB – HFB Acid
	359	16	HO-CF-P-DiHFB – HFB Acid
	228	100	Methylamine-HFB
	163	42	HO-CF-P – H_2O
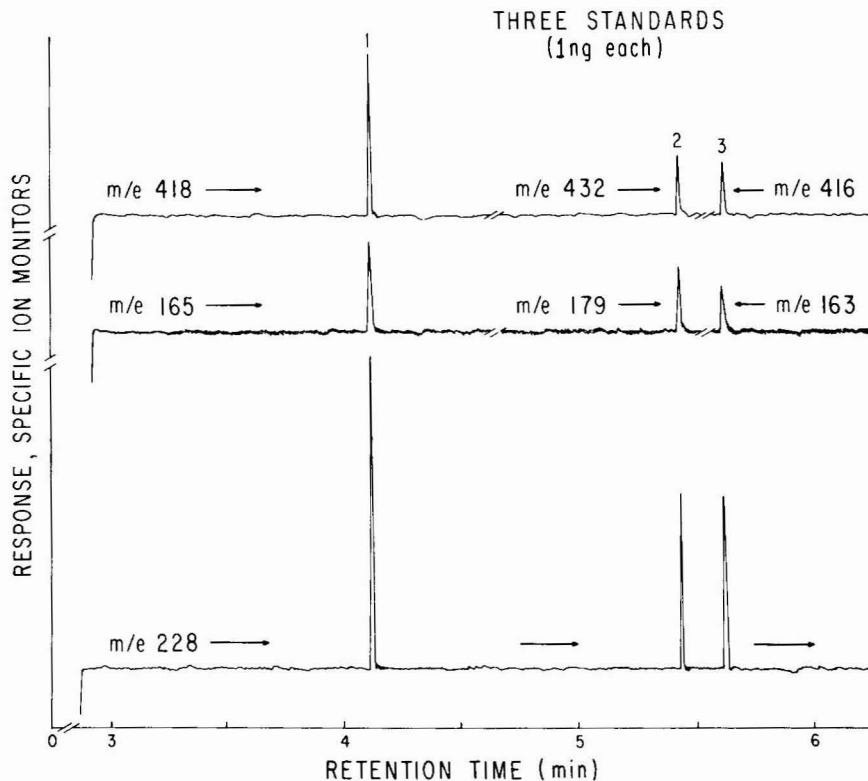
Fig. 2. Reconstituted mass fragmentogram of the HFB derivatives of carbofuran and metabolites. 1 — CF-HFB, 2 — CO-CF-HFB, 3 — HO-CF-DiHFB.

equivalent to between 10 and 1000 pg of carbamate and the results are summarized in Fig. 3. The respective coefficients of variation (CV % = 100 × standard deviation/mean), for the responses for the HFB derivatives equivalent to 10, 25, 50, 100, 250, 500 and 1000 pg of CF and HO-CF were 16.7, 8.6; 8.7, 9.7; 6.1, 5.9; 3.3, 1.6; 1.5, 0.9; 3.0, 2.8 and 0.6, 1.5. The 228-a.m.u. ion from CO-CF-HFB behaved similarly but was not as rigorously determined because preliminary analysis of the crops indicated significant amounts of this metabolite were not present. Constant (100 pg) amounts of un-derivatized CF, CO-CF and HO-CF were also injected and, at this level, only phenolic fragment ions at 165, 179 and 163 a.m.u. were observed at the carbamate retention times. The much shorter retention times for similar amounts of directly injected CF-P, CO-CF-P, HO-CF-P, and their HFB derivatives were also observed by not activating the solvent-divert valve.

Residue analysis

        Samples of derivatized crop extracts (usually 1 μl and unwashed) at a concentration equivalent to 1 g/ml were injected into the chromatograph and the intensities of the ions at 228 a.m.u. coincident with the retention times of CF-HFB, CO-CF-HFB and HO-CF-DIHFB were measured. From this preliminary determination an estimate of the concentration of these components, if present, was made by comparison with the calibration responses for derivatized standards. Samples of the
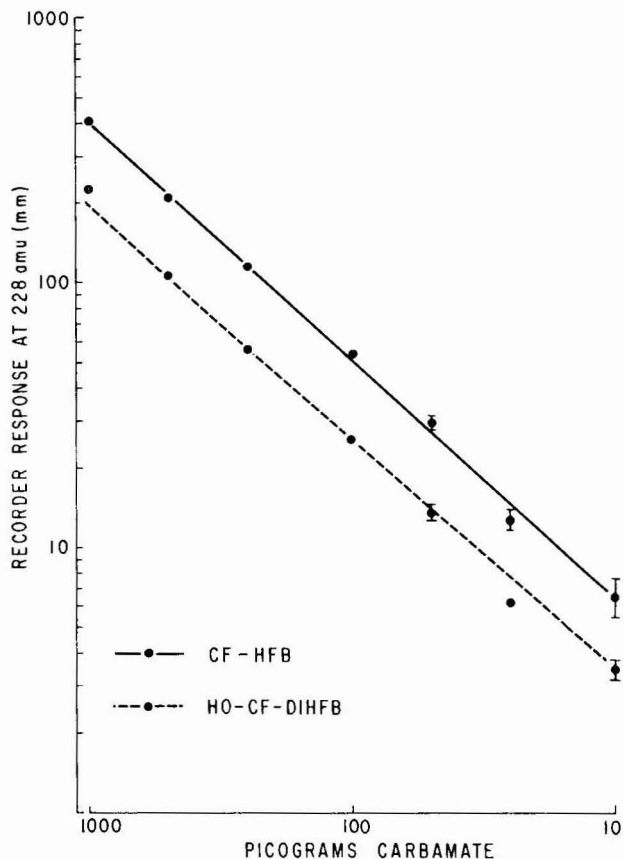
Fig. 3. Response characteristics of the *m/e* 228 ion produced by methane-CI of various quantities of CF-HFB and HO-CF-DiHFB eluted from GLC.

control extract were then fortified with the component(s) of interest at slightly above and below the estimated concentration(s) and derivatized as usual. The unknown was then re-analysed by comparison of the 228-a.m.u. intensity with that of the component(s) in the fortified control.

RESULTS AND DISCUSSION

Extraction

The acid digestion procedure of Cook *et al.*[16] was chosen as it appears to be the best available for CF and its degradation products in crops[17]. It is generally known that considerable quantities of HO-CF can be conjugated in plants[18-20] and although the toxicological significance of HO-CF in this form is not known it seems best to determine the total carbamate present rather than only the portion soluble in organic solvents. In future it may prove worthwhile to distinguish between the two forms. Although the acid digestion procedure is commonly accepted, it should be noted that in the original report the fortification recoveries of CF and HO-CF varied

from 57 to 84% and 51 to 101%, respectively. It is not clear whether the variation arises in the extraction or in the subsequent analysis. We plan to examine this aspect more carefully in future work.

Derivatization

The development of procedures for the perfluoroacylation of N-methylcarbamates has been thoroughly reviewed by Dorough and Thorstenson[2]. In our hands the procedure of Seiber[11] failed to convert CO-CF and HO-CF quantitatively to their respective mono- and di-HFB derivatives. Increasing the reaction time or the amount of acid anhydride did not improve the conversion markedly. For the procedure adopted, based on the work of Shafik *et al.*[21], the minimum time for completion of the reaction was not examined as the time allowed fitted conveniently into our work schedule and provided quantitative conversion. The amounts of reagent used were shown to be sufficient to derivatize completely at least 100 μg of each carbamate in the presence of the extractives from 10 g of each crop studied. The derivatives are relatively stable in the reaction mixture and some samples have been stored in capped hypo vials in the dark at room temperature for 2–3 weeks without showing significant changes in the concentrations of the carbamate derivatives. Changes in the concentration of the derivatives in water-washed samples were usually detectable after 2–3 days under the same conditions.

Mass spectra, chromatographic behaviour, ion selection and calibration

The mass spectra of a variety of N-methylcarbamates produced by electron impact (EI) and CI of samples introduced via the probe inlet have been reported[22,23]. Under these conditions the corresponding phenol, or protonated phenol in the case of methane-CI, is observed as the base peak. Samples of CF, CO-CF and HO-CF introduced at sub-nanogram levels into our methane-CI system *via* the GLC behaved similarly; the respective protonated molecular ions at 222, 236 and 238 a.m.u. were not seen but ions were observed at 165, 179 and 163 a.m.u. corresponding respectively to $[CF-PH]^+$, $[CO-CF-PH]^+$ and $[HO-CF-PH-18]^+$. Free phenols, injected for comparison, had much shorter retention times than the carbamates so there is no question that the signals observed were due to the fragmentation of a portion of the intact carbamate that had survived passage through the GLC column. The intensities of these ions varied erratically when constant amounts were injected and this, coupled with the fact that they are at odd *m/e* in CI–MS and would undoubtedly suffer from interference from crop extractives led us to abandon further thought of utilizing a combination of GLC of underivatized carbamates and methane CI–MS for analysis. Attempts to produce protonated molecular ions having even *m/e* values by use of a "softer" CI reagent were fully successful with isobutane but this led to a more rapid fouling of the ion source and necessitated too frequent cleaning for routine use. The stability and linearity of the intensities of these $[MH]^+$ ions from the carbamates were not examined.

Mumma and Khalifa[24] have reported that EI fragmentation of the trifluoroacetyl (TFA) derivatives of carbaryl and its metabolites produced the TFA derivative of the corresponding phenols as the major fragment and the TFA derivative of methylamine as a minor fragment. In our system, the methane-CI fragmentation of CF-HFB, CO-CF-HFB and HO-CF-DiHFB fortuitously produced the even *m/e* ion

at 228 a.m.u. corresponding to the protonated HFB derivative of methylamine (*i.e.,* protonated N-methyl heptafluorobutyramide) as the base peak for all three compounds, as well as providing additional ions (see Table I and Fig. 2) which can be used for further identification when sample size and level of interference permit. The composite mass fragmentogram shown in Fig. 2 illustrates the use of these ions for the identification of the compounds eluted. The *m/e* 228 ion response shown was recorded at the same sensitivity range for the equivalent of 1 ng of each carbamate while the other responses shown were recorded at various ranges. The intensity of the 228-a.m.u. ion was directly proportional to the amounts of CF-HFB and HO-CF-DiHFB injected over the range equivalent to 10–1000 pg of carbamate demonstrating its suitability as a basis of analysis (see Fig. 3). Predictably the larger coefficients of variation occurred at the lower levels. The data indicated that the standard error for estimating CF and HO-CF is about ±10% at the 25-pg level. Although the methane-CI fragmentation produces the same base-peak ion for each HFB derivative, the compounds are all sufficiently well separated by GLC (see Figs. 2 and 4), to permit accurate analysis based on the 228-a.m.u. ion signal. The method should be applicable to any N-methylcarbamate-HFB derivative. For our purposes HFBA was preferred over trifluoroacetic anhydride and pentafluoropropionic anhydride as the
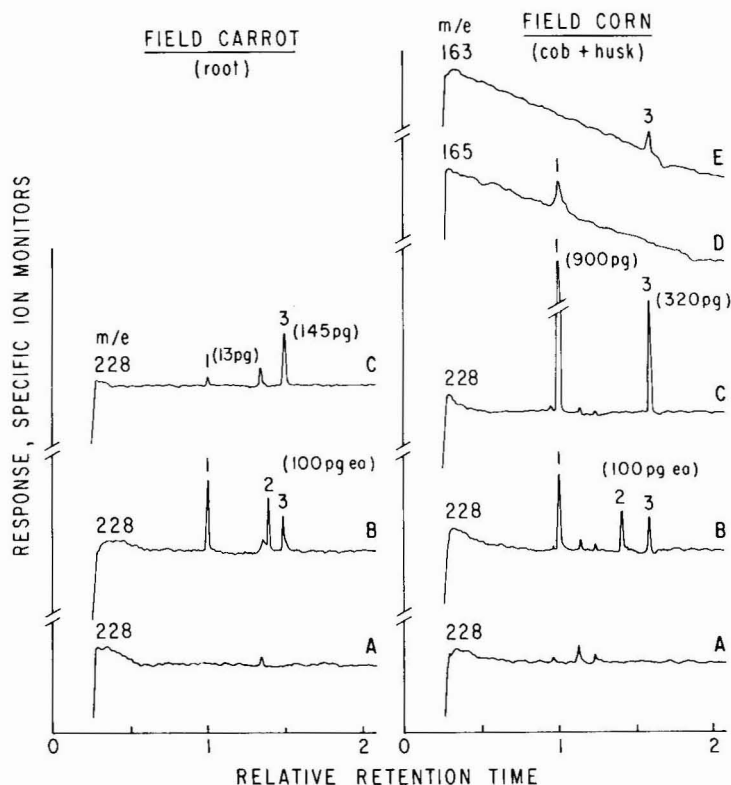
Fig. 4. Reconstituted fragmentograms of HFBA-derivatized carrot and corn extracts used for analysis. A, Extract from control samples; B, extract from crop controls fortified with 0.1 ppm (1) CF, (2) CO-CF and (3) HO-CF; C, D and E, extract from field-treated crop.

derivatizing agent because the increase in molecular weight shifts the selected ion to high a.m.u. values where potential interference is lessened.

Residue analysis

Using the 228-a.m.u. ion for analysis, no interference was observed at the retention times of CF-HFB and HO-CF-DiHFB in samples of carrot, celery, corn (kernels, leaves and stalks, cobs and husks) and tomato extracts that had been prepared for analysis as described, *i.e.* evaporation to a level equivalent to 1 g/ml and reaction with HFBA. Fig. 4 shows typical results on carrot (root) and corn (cob and husk). Each fragmentogram was produced from the equivalent of 1 mg of crop. The residue levels of CF and HO-CF in the corn were sufficiently high to permit confirmation of the components with a second channel of Promim but for the low levels in the carrot the signals on these channels could not be seen above background noise unless the derivatized extract was water-washed to reduce interference. In some cases a small interference was observed for CO-CF-HFB which would limit the minimum level of detectability for this component below 0.05 p.p.m.; no levels above this were observed in any of the extracts. Derivatized carrot, celery and corn extracts contained material(s) which eluted between 25 and 30 min and produced a 228-a.m.u. ion. This of course, does not interfere with the analysis but does lengthen the analysis time considerably. Samples were analyzed by comparison with fortified controls rather than pure standards to eliminate any possibility of the extractives interfering with the formation of the derivative(s) and invalidating the results.

The injection of the equivalent of 1–2 mg of crude crop may offend the classical residue analyst. In our work, we have not observed a deterioration in column performance due to repeated analyses of crop samples. The time saved and the accuracy of the results obtained using this method will rapidly make up for the slight inconvenience and cost of replacing the GLC column when it is required. In using samples which have not been washed free of the large excess of derivatizing reagent a column length of 90–120 cm was found optimum. Shorter columns began to suffer from lack of resolution of CO-CF-HFB and HO-CF-DiHFB while longer columns required considerable time to elute residual HFBA which appeared to alter the response characteristics of the system even when no interference was observed at m/e 228. The ion source of a mass spectrometer is subject to fouling with organic materials. The continual presence of relatively large amounts of methane in a CI source accentuates this problem and cleaning was required at 4–5 week intervals to maintain the signal-to-noise levels required.

CONCLUSION

A simple yet sensitive method has been developed for the analysis of carbofuran, 3-ketocarbofuran and 3-hydroxycarbofuran in carrots, celery, corn and tomatoes based on the GLC separation of the heptafluorobutyryl derivatives of the insecticides prepared in crude extracts followed by their chemical ionization to a common ion and the measurement of its intensity with a quadrupole mass spectrometer. Sensitivity is at least 0.05 ppm for all three materials.

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PREFERENTIAL SOLVATION OF POLY(DIMETHYLSILOXANE) AND POLY(METHYL METHACRYLATE) IN BENZENE–METHANOL MIXTURES BY GEL PERMEATION CHROMATOGRAPHY

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SUMMARY

Preferential solvation λ parameters for the ternary systems benzene–methanol–poly(dimethylsiloxane) and benzene–methanol–poly(methyl methacrylate) have been determined by gel permeation chromatography. When benzene is preferentially adsorbed by the polymer, good agreement is found between λ values determined by this method and by light scattering and dialysis equilibrium. However, when methanol is preferentially adsorbed by the polymer, discrepancies arise. The differences are discussed in terms of interactions between the solvent and the chromatographic support.

INTRODUCTION

The preferential solvation of polymers in binary solvent mixtures has been studied by different physicochemical techniques, such as dialysis equilibrium[1], light scattering[2], ultracentrifugation[3] and densitometry[4], and spectroscopic techniques such as nuclear magnetic resonance[5], fluorescence inhibition[6] and infrared spectroscopy[7,8]. On the other hand, it is a known experimental fact that the frequent presence of vacant peaks in gel permeation chromatograms[9], when working with mixed solvents. In this case, as Berek *et al.*[10] have recently shown, if the magnitude of the vacant peak is proportional to the amount of polymer injected and its elution volume corresponds to that of one of the components of the mixture, the quantitative evaluation of the preferential solvation parameter becomes possible.

In this paper we report results on the determination of the preferential solvation parameter by gel permeation chromatography (GPC). Two ternary systems, the preferential adsorption of which has been established by classical techniques, have been chosen to test the reliability of the GPC method. The systems are poly(dimethylsiloxane)–benzene–methanol[4] and poly(methyl methacrylate)–benzene–methanol[11]. The second of these systems seemed to us of particular interest due to the appearance of an inversion in its preferential solvation.

EXPERIMENTAL

All of the measurements were carried out on a commercial Waters Assoc., Model ALC/GPC 202 liquid chromatograph, equipped with a 6000-p.s.i. pump, a differential refractometer unit R401 and a U6K universal injector, admitting sample sizes from 1 μl up to 2 ml. The differential refractometer cell was thermostatted at 22.0° and the elution volumes were determined from the weight of eluent. The solvent reservoir and the waste container were connected in order to avoid changes in mixture composition. The constancy in mixture composition was tested by refractometry. A Pharmacia SR 25 column (45 × 0.25 cm I.D.) packed with Spherosil XOA 200 of high granularity (100–200 μm) was used. The flow-rate was 1.0 ml/min, and the volume of the injected polymer solution was always 2 ml corresponding to the total loop volume.

The benzene and methanol solvents were purified and dried in the usual way. The polymer solutions were prepared immediately before injection using solvent mixtures from the solvent reservoir. The poly(dimethylsiloxane) test polymer was a commercial sample from Rhone Poulenc (Paris, France). From light scattering, $\bar{M}_w = 62,000$ and from independent GPC measurements, the polydispersity ratio, $(\bar{M}_w/\bar{M}_n) = 1.3$. The two atactic poly(methyl methacrylate) samples had molecular weights $(\bar{M}_w)$ of 210,000 and 550,000 from light scattering.

RESULTS AND DISCUSSION

The system benzene (1)–methanol (2)–poly(dimethylsiloxane) (3)

In Fig. 1 are shown the chromatograms of poly(dimethylsiloxane), PDMS, at different polymer concentrations in benzene–methanol (90:10). Two negative peaks corresponding to solvated polymer ($V_e = V_0 = 72.0$ ml) and to non-adsorbed
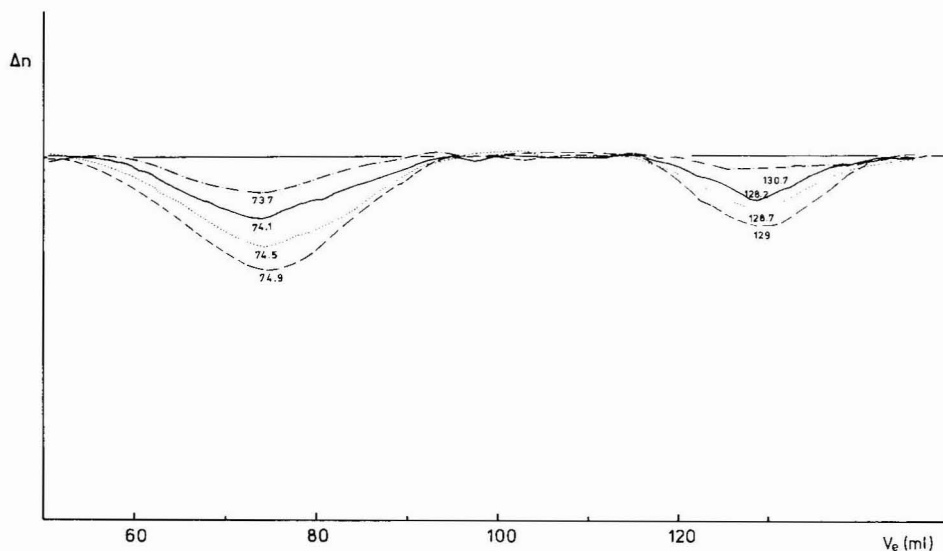
Fig. 1. Chromatograms of PDMS ($\bar{M}_w = 62,000$) in a benzene–methanol mixture (90:10), at different polymer concentrations: $-\cdot-$, 2.01; ———, 4.36; $\cdot\cdot\cdot$, 5.03 and $-\ -$, 6.25 mg/ml.
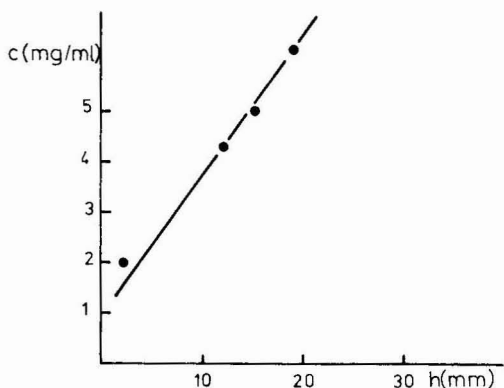
Fig. 2. Dependence of the height of the vacant peak on PDMS concentration in benzene–methanol (90:10).

methanol ($128.2 < V_e < 130.7$ ml) are seen. As shown in Fig. 2, the heights of the vacant peaks are proportional to the amount of injected polymer. This indicates that the polymer is preferentially solvated by benzene[10].

PDMS chromatograms obtained at several eluent compositions are shown in Fig. 3. Even taking into account the differences in polymer concentration of the injected solutions (see Table I), it can be seen that the size of the polymer peak decreases with increasing amount of methanol in the mixture. This behaviour could be expected since the refractive index of the mixture approaches that of the polymer on increasing the methanol concentration. The vacant peak, however, follows the opposite trend, i.e., it increases with increasing methanol content. This means that
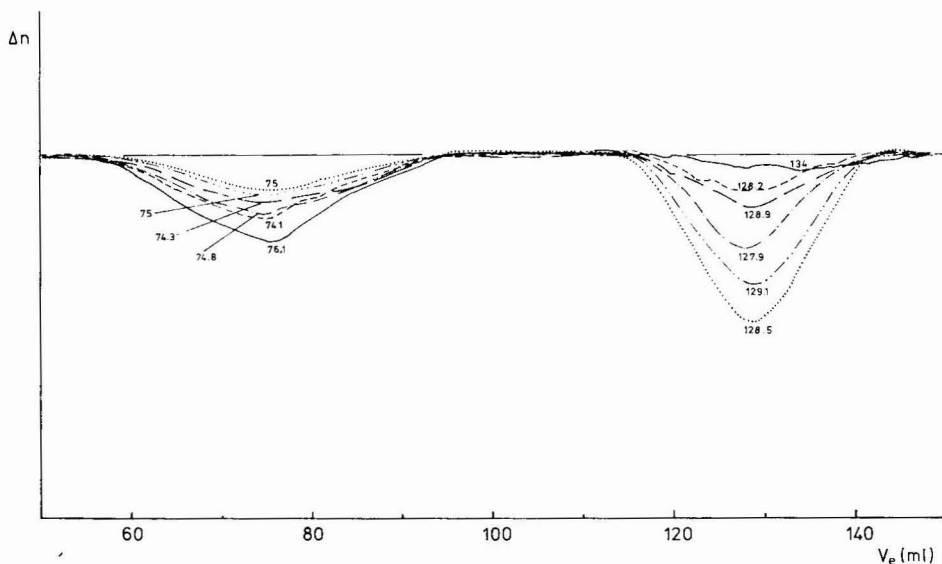
Fig. 3. Chromatograms of PDMS in different benzene–methanol mixtures. v_2: ———, 0.05; – – –, 0.10; – – – –, 0.13; – · – ·, 0.16; – · · – · ·, 0.20; · · ·, 0.25.

the preferential solvation of the polymer by benzene increases with increasing amount of the methanol in the mixture.

From the above considerations, the way in which preferential solvation takes place, and its dependence on mixture composition, can be easily visualized. The next step is to relate, in a quantitative form, the size of the vacant peak to the parameter λ, which is usually employed to evaluate the prefential solvation phenomenon. When a solvent (1) is preferentially adsorbed by a polymer in a ternary system solvent (1)–solvent (2)–polymer (3), λ can be defined as the change in volume fraction of component 1 with respect to the polymer concentration in the dialyzed solvent at infinite dilution. The expression for λ would be:

$$\lambda = \frac{dv_1}{dc_3} = -\frac{dv_2}{dc_3} \tag{1}$$

Keeping in mind the above formula, the following experimental procedure was followed for the determination of λ.

Before injecting any polymer, a methanol solution of known concentration in a given solvent mixture was injected. The difference in the volume fraction of benzene between the above solution and the mixture, Δv_1^0, was related to the height of the methanol peak, h_1. Subsequent injection of polymer solution will cause a vacant methanol peak, height h_2, which can be related to the change in the volume fraction of benzene, Δv_1, due to its preferential adsorption:

$$\Delta v_1 = \Delta v_1^0 \cdot \frac{h_2}{h_1} \tag{2}$$

Replacing the differentials in eqn. 1 by increments and substituting into eqn. 1 the Δv_1 value given by eqn. 2, λ may be expressed as:

$$\lambda = \frac{\Delta v_1^0}{c_3} \cdot \frac{h_2}{h_1} \tag{3}$$

The results of the different chromatograms performed for the system benzene (1)–methanol (2)–PDMS (3) are in Table 1: v_1 is the mole fraction of benzene; c_2 is the concentration of methanol injected resulting in a peak of height h_1, corresponding to a change Δv_1^0 in the volume fraction of benzene; c_3 is the polymer concentration in g/ml resulting in a vacant methanol peak of height h_2 and elution volume V_e. The λ values were calculated from eqn. 3. The elution volumes corresponding to the vacant peaks in the different mixtures ranged from 128.0 to 129.0 ml, except for the mixture having the lowest methanol content for which the elution volume of the vacant peak appeared at 134.0 ml. As Fig. 3 shows, this effect may be due to retention of methanol by the chromatographic support. Static differential refractometry measurements performed independently support this hypothesis. Effectively, after a short equilibration time of *ca.* 2 h, methanol is preferentially adsorbed by the Spherosil. Although the amount of methanol adsorbed remains constant independent of the methanol content in the mixture, mixtures containing small amounts of methanol will exhibit higher relative retentions those containing larger amounts.

TABLE I

DETERMINATION OF THE PREFERENTIAL SOLVATION PARAMETER λ FOR THE SYSTEM BENZENE (1)–METHANOL (2) –PDMS (3) ($\bar{M}_w = 62000$) FROM GEL PERMEATION CHROMATOGRAMS AT DIFFERENT MIXTURE COMPOSITIONS

v_1	c_2 (ml/100 ml)	$\Delta v_1^0 \cdot 10^3$	c_3 (mg/ml)	h_1 (mm)	h_2 (mm)	V_e (ml)	λ (ml/g)
0.95	0.231	2.19	5.07	54 ± 1	4 ± 2	134.0	0.03 ± 0.02
0.90	0.242	2.18	4.36	50 ± 1	10 ± 1	128.2	0.10 ± 0.01
0.87	0.245	2.13	3.93	49 ± 1	14 ± 2	128.9	0.16 ± 0.03
0.84	0.240	2.02	5.29	47 ± 1	26 ± 1	127.9	0.21 ± 0.01
0.80	0.251	2.01	6.24	42 ± 1	36 ± 1	129.1	0.28 ± 0.01
0.75	0.248	1.86	6.80	37 ± 1	46 ± 2	128.5	0.34 ± 0.02

This peak broadening effect at low methanol compositions hampers the estimation of the heights of the vacant peaks with the result that the calculated λ values may suffer higher relative errors.

Fig. 4 shows a plot of the calculated values of λ *versus* v_2, together with the results of Hert and Strazielle[4] from light scattering and differential refractometry after dialysis equilibrium. The small difference in molecular weight between the earlier sample and ours (58,000 and 62,000, respectively) will hardly be reflected in the λ values. The agreement between the two sets of λ values is remarkable. Moreover, the GPC technique is not limited by the composition of the mixture as occurs with light scattering. In the ternary system studied, it is not possible to determine λ by light scattering, for mixtures of composition $v_2 > 0.13$, as pointed out by Hert and Strazielle[4].
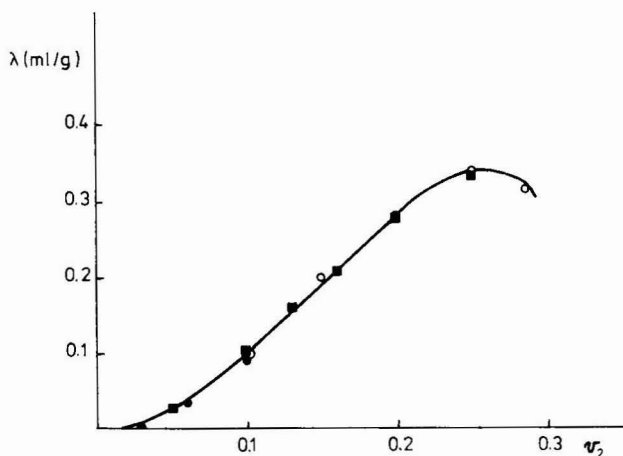
Fig. 4. Dependence of the parameter λ on mixture composition for the system benzene–methanol–PDMS. (■) GPC values; (○) values from refractometry after dialysis[4]; (●) values from light scattering[4].

The system benzene(1)–methanol(2)–poly(methyl methacrylate) (3)

In Fig. 5a are shown the chromatograms of poly(methyl methacrylate), PMM ($\bar{M}_w = 550,000$), at three different polymer concentrations in benzene–methanol

(70:30). Fig. 5b provides evidence for the preferential solvation phenomenon. Chromatograms of the two PMM polymers with $\bar{M}_w = 210,000$ and 550,000, respectively, are shown at several mixture compositions in Figs. 6 and 7. It can be seen that the elution volumes for both polymers are similar. This is due to the fact that both polymers fall outside the effective separation range of the Spherosil column used. Since we are interested only in the vacant peaks the above fact is unimportant.
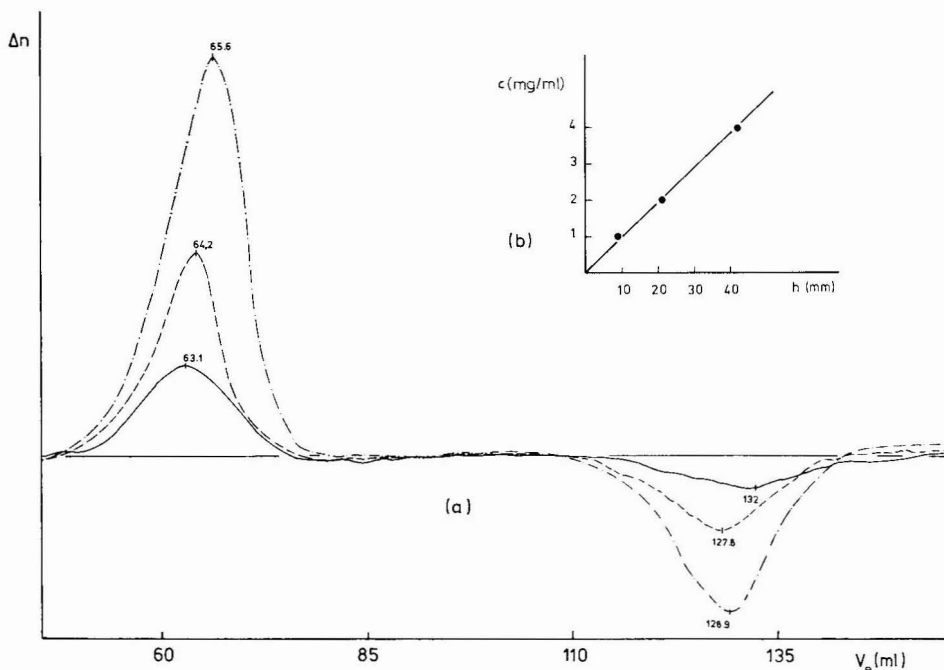
Fig. 5. (a) Chromatograms of PMM ($\bar{M}_w = 550,000$) in a benzene–methanol mixture (70:30), at different concentrations: ———, 1.03; – – –, 0.202; – · –, 0.402 mg/ml. (b) Dependence of the size of the vacant peak on PMM concentration.

All the chromatograms in Figs. 6 and 7 show the corresponding vacant peaks. Positive peaks due to benzene appear in mixtures of low methanol content. In these cases, methanol is preferentially adsorbent by the polymer. On the other hand, negative peaks appear in methanol-rich mixtures and in these cases benzene is preferentially adsorbed. The analysis of this inversion phenomenon indicates that it takes place at $0.16 < v_2 < 0.17$, in accordance with published results determined by classical techniques[11]. The elution volumes of the vacant benzene peaks are coincident with the elution volume of the peak obtained by benzene injection. This is not the case for the vacant methanol peaks. Their elution volumes and sizes follow the same trends as in the benzene–methanol–PMDS system discussed above.

Table II collects the chromatographic parameters and the λ values calculated from eqn. 3. The λ values are plotted *versus* v_2 in Fig. 8. The solid line in Fig. 8 represents λ data determined from dialysis equilibrium by Pouchly *et al.*[11]. Unfortunately, since these workers did not indicate the molecular weight of the polymer studied, it is not possible to make any comparisons. Measurements of λ by dialysis equilibrium
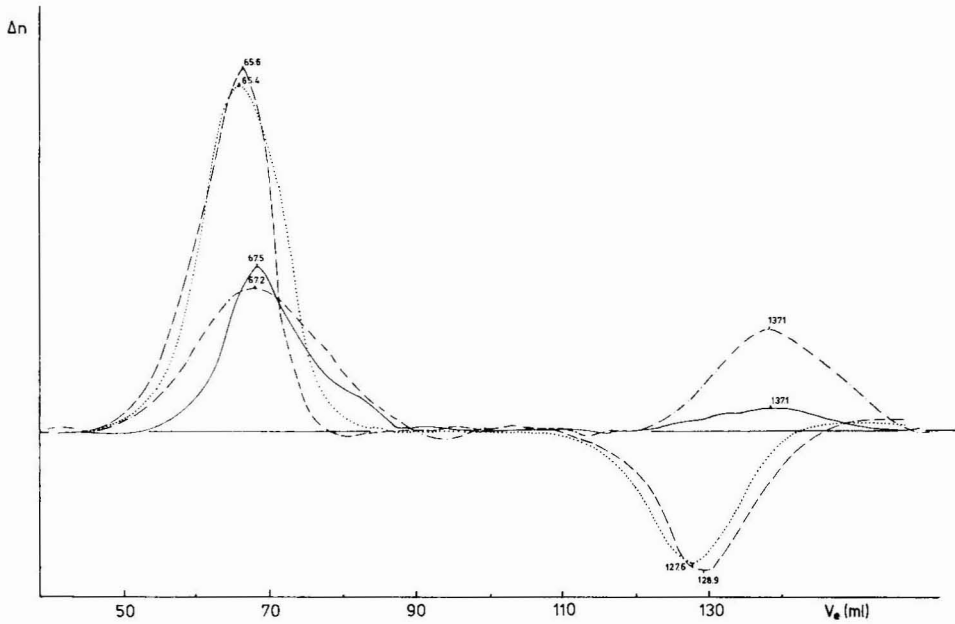
Fig. 6. Chromatograms of PMM ($\bar{M}_w = 210{,}000$) in different benzene–methanol mixtures. v_2:
$- \cdot - \cdot$, 0.08; ————, 0.17; – – –, 0.30; $\cdots$, 0.40.
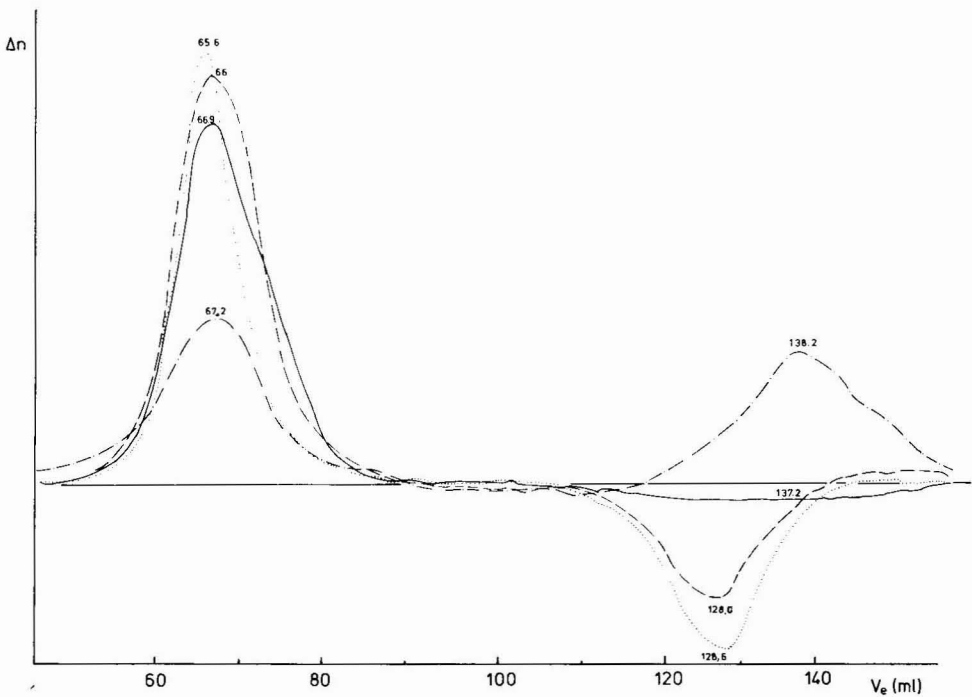
Fig. 7. Chromatograms of PMM ($\bar{M}_w = 550{,}000$) in different benzene–methanol mixtures. v_2: $- \cdot - \cdot$,
0.08; ————, 0.16; – – –, 0.30; $\cdots$, 0.40.

TABLE II

DETERMINATION OF THE PREFERENTIAL SOLVATION PARAMETER λ FOR THE SYSTEMS BENZENE (1) – METHANOL (2) – PMM (3) ($\bar{M}_w = 210000$ AND 550000) FROM GEL PERMEATION CHROMATOGRAMS AT DIFFERENT MIXTURE COMPOSITIONS

Sample	v_1	c_2 (ml/100 ml)	$\Delta v_1^0 \cdot 10^3$	c_3 (mg/ml)	h_1 (mm)	h_2 (mm)	V_e (ml)	λ (ml/g)
PMM 210,000	0.92	1.901*	1.52	14.99	11 ± 1	36 ± 2	138.2	−0.33 ± 0.06
PMM 550,000	0.92	1.901*	1.52	15.02	11 ± 1	30 ± 2	137.1	−0.28 ± 0.06
PMM 210,000**	0.83	0.261	2.17	6.01	46 ± 1	5 ± 1	137.2	0.009 ± 0.002
PMM 550,000	0.84	1.571*	2.51	6.01	22 ± 1	6 ± 1	137.1	−0.11 ± 0.01
PMM 210,000	0.70	0.275	1.93	4.00	64 ± 1	32 ± 3	128.0	0.24 ± 0.03
PMM 550,000	0.70	0.275	1.93	4.02	64 ± 1	42 ± 4	128.9	0.31 ± 0.04
PMM 210,000	0.60	0.283	1.70	3.98	52 ± 1	45 ± 1	128.6	0.37 ± 0.02
PMM 550,000	0.60	0.283	1.70	4.00	52 ± 1	40 ± 1	127.6	0.33 ± 0.02

* The pure component injected was benzene, so the numbers are really c_1 values.
** The h_1 and h_2 values were determined at half and double the sensitivity with respect to the rest of the chromatograms.

were therefore made for these systems ($\bar{M}_w = 220,000$ and 550,000) in this laboratory. The results are also shown in Fig. 8.

It can be seen that the results of the GPC and dialysis equilibrium measurements differ for negative values of λ. A plausible explanation for this may be as follows. When the polar methanol is preferentially adsorbed by PMM, notable adsorption and retention phenomena may take place between the polymer solvated by methanol and the Spherosil support. As a result, the amount of methanol surrounding the polymer may increase with respect to that of the thermodynamic equilibrium and
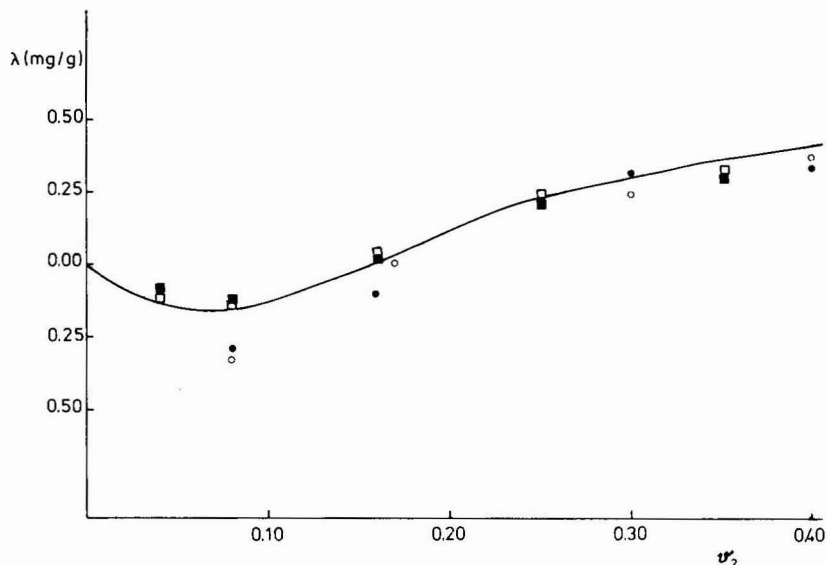
Fig. 8. Dependence of the parameter λ on mixture composition for the system benzene–methanol–PMM. (○) and (●) GPC values; (□) and (■) values from refractometry after dialysis. Open symbols for $\bar{M}_w = 210,000$, filled symbols for $\bar{M}_w = 550,000$. Solid line, dialysis equilibrium values[11].

the size of the benzene peak, and hence the absolute values of λ, will increase. A possible way of correcting the low negative λ values obtained by GPC may be by the use of a chromatographic support having larger diameter pores. Exploratory differential refractometry experiments have shown that the adsorption of polar solvents by Spherosil decreases with increasing pore size.

ACKNOWLEDGEMENTS

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ANALYSIS OF $C_{19}O_3$ STEROIDS BY THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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SUMMARY

The separation of twenty-six saturated and two unsaturated $C_{19}O_3$ steroids has been studied by thin-layer chromatography on silica gel F_{254}, with seven mobile phases, and by gas–liquid chromatography on packed columns with four stationary phases; combination of both techniques permitted separation of all the test compounds. The mass spectra of the steroids were obtained by gas chromatography–mass spectrometry and are presented. Fragmentation processes have been studied, and characteristic ions that may be used for multiple ion detection or lead to identification of biologically produced $C_{19}O_3$ steroids are discussed.

INTRODUCTION

Enzymic hydroxylation leading to 5α-reduced $C_{19}O_3$ steroids has been studied in numerous human, murine and canine tissues, including liver[1,2] and prostate[3,4] and perianal glands[5]. Hydroxylation has been reported to occur at the 2ξ-position[1–3], the 6ξ-position[3,4,6], the 7ξ-position[2,4,5], the 15α-position[2] and the 16α-position[1,6], the naturally occurring $C_{19}O_2$ substrates being mainly 3-oxo-, 17β-hydroxy- or $3\xi,17\beta$-dihydroxy-compounds[1,6]. This study of $C_{19}O_3$ steroids has therefore been limited to those having oxygen functions at the positions cited above. Most of these compounds are not commercially available, and their reported identification is based on comparison of their chromatographic mobilities[1–3] and mass spectra[1,2,4–6] with those of custom-made reference compounds. Few studies of their behaviour in thin-layer chromatography (TLC) and gas–liquid chromatography (GLC), or of their fragmentation during mass spectrometry (MS), have been systematically reported[1,7]. Accordingly, we have obtained reference compounds, established conditions for the resolution of mixtures and sought criteria for identification. In this paper we report the mobilities of some $C_{19}O_3$ steroids in TLC and their retention indices in GLC, and present their mass spectra.

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EXPERIMENTAL

Unsaturated steroids

Testosterone and androst-5-ene-3β,16α,17β-triol were purchased from Sigma (St. Louis, Mo., U.S.A.). Androst-5-ene-3β,16β,17β-triol and 5α-androst-2-en-17-one were obtained from Steraloids (Pawling, N.Y., U.S.A.). The androst-5-ene-3β,17β-diol was obtained from Merck (Darmstadt, G.F.R.).

Saturated C$_{19}$O$_3$ steroids

3β,11β-Dihydroxy-5α-androstan-17-one was a commercial sample from Ikapharm (Ramat Gan, Israel), and 17β-hydroxy-5α-androstane-3,6-dione and 3β-hydroxy-5α-androstane-6,17-dione were obtained from Dr. P. Ofner (Boston, Mass., U.S.A.). 3β-Hydroxy-5α-androstane-7,17-dione was a gift from Sir Ewart R. H. Jones (Oxford University, Oxford, Great Britain) and was also obtained by incubating iso-androsterone (Merck) with *Rhizopus nigricans*[8]. 5α-Androstane-3,6,17-trione and 5α-androstane-3,7,17-trione were obtained by oxidation of 17β-hydroxy-5α-androstane-3,6-dione and 3β-hydroxy-5α-androstane-7,17-dione, respectively, with chromium trioxide. 6α,17β-Dihydroxy-5α-androstan-3-one and 3α,17β-dihydroxy-5α-androstan-6-one were from Sir Ewart R. H. Jones, who also kindly provided some 3β,17β-dihydroxy-5α-androstan-6-one. This last-named steroid was also obtained by specific reduction of the 3-oxo-group in 17β-hydroxy-5α-androstane-3,6-dione with lithium tri-*tert.*-butoxyaluminate (Merck) for 15 min at 0°. Further reduction by lithium tetrahydroaluminate of the three dihydroxy-steroids mentioned above yielded 5α-androstane-3β,6α,17β-triol, 5α-androstane-3α,6β,17β-triol and its 3β-epimer, respectively. 3β,17β-Dihydroxy-5α-androstan-7-one was prepared by a modification of the method of Valcavi *et al.*[9]. The diacetoxy-derivative of androst-5-ene-3β,17β-diol was prepared and specifically oxidized at the 7-position with sodium chromate (Merck). Hydrogenation on platinum dioxide of the resulting 3β,17β-diacetoxy-androst-5-en-7-one yielded a mixture of 3β,17β-diacetoxy-5α-androstan-7α-ol and the epimeric 7β-ol. Before separation of these two epimers, 3β,17β-diacetoxy-5α-androstan-7-one was prepared by oxidation of a portion of the mixture with chromium trioxide. Hydrolysis carried out with methanolic sodium hydroxide produced the expected 3β,17β-dihydroxy-5α-androstan-7-one. The remaining mixture of 3β,17β-diacetoxy-5α-androstan-7α-ol and the epimeric 7β-ol was resolved by column chromatography on alumina, and each of the isolated and crystallized epimers was hydrolyzed with methanolic sodium hydroxide. Thus, 5α-androstane-3β,7α,17β-triol and its 7β-epimer were obtained; the identity of these steroids was checked by GLC and GLC–MS in comparison with authentic samples provided by Dr. J. C. Orr (St Johns, Newfoundland, Canada).

3β,7β-Dihydroxy-5α-androstan-17-one, its 7α-epimer and 3β,6α-dihydroxy-5α-androstan-17-one were obtained from incubations of isoandrosterone with *Rhizopus nigricans*[8]. Reduction of these compounds with potassium tetrahydroborate yielded major quantities of 5α-androstane-3β,7β,17β-triol, its 7α-epimer and 5α-androstane-3β,6α,17β-triol, respectively.

5α-Androstane-2β,3β,17β-triol and 5α-androstane-2α,3α,17β-triol were kindly provided by Dr. Y. Collet (Collège de France, Paris). The synthesis of 5α-androstane-2β,3α,17β-triol was carried out as follows: 5α-androst-2-en-17-one was reduced with

potassium tetrahydroborate; 2α,3α-epoxy-5α-androstan-17β-ol was then obtained by the action of *m*-chloroperbenzoic acid (overnight in benzene medium), and opening of the epoxy-ring with 2 *N* sulphuric acid (Shopee *et al.*[10]) yielded 5α-androstane-2β,3α,17β-triol.

5α-Androstane-3β,11β,17β-triol and its 3α-epimer were obtained by reduction with potassium tetrahydroborate of 3β,11β-dihydroxy-5α-androstan-17-one (Ikapharm), and its 3α-epimer (Sigma), respectively.

5α-Androstane-3β,15α,17β-triol was obtained from Dr. E. M. Chambaz (Grenoble, France), and 5α-androstane-3β,16α,17β-triol and its 3α-epimer were purchased from Steraloids.

Thin-layer chromatography

Thin-layer plates pre-coated with silica gel 60 F$_{254}$ (Merck) were used in this study. Steroids were applied in 20–30 μg amounts and the plates were developed in unsaturated tanks with different solvent systems (see Table I). Visualization of the steroids on the chromatograms was achieved by spraying with sulphuric acid–methanol (7:3, v/v) and heating at 120° for 15 min.

Derivative formation

All derivatives were prepared from 50 μg of free steroid. The trimethylsilyl (TMS) derivatives were prepared (in 30 min at 60° or overnight at room temperature) after addition to the dry steroid of 100 μl of NO-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 10 μl of pyridine. In some instances, 10 μl of trimethylchlorosilane (TMCS) were added to the reaction mixture; TMCS behaves as a catalyst[11] in the formation of TMS derivatives, and thus residual free hydroxyl-groups in the steroid molecules were eliminated.

Gas–liquid chromatography

Carlo Erba (Milan, Italy) GI-452 and Pye Unicam (Cambridge, Great Britain) 104-84 chromatographs, each equipped with flame ionization detectors, were used, with nitrogen as carrier gas (40 ml/min). The operating conditions and stationary phases are given in Table II.

Gas–liquid chromatography–mass spectrometry

A double-beam MS-30 mass spectrometer (AEI, Manchester, Great Britain) coupled by a silicone-membrane separator to a Pye Unicam 104 gas chromatograph was used. As previously described[12], the chromatograph was fitted with a glass capillary column (60 m × 0.3 mm) coated with OV-101 and operated at 265°. Helium was used as carrier gas and added at the column exit before the molecular separator at a make-up flow of 20–25 ml/min[12]. The steroid derivatives were injected through an all-glass solid injector[13]; the temperatures of the molecular separator, introduction line and ion source were 215°, 250° and 200°, respectively. In the mass spectrometer, the energy of the bombarding electrons was 24 eV and the ionizing current was 100 μA; mass spectra were taken at a scan speed of 3 sec per decade at a resolution of 1000.

RESULTS

Thin-layer chromatography

All TLC was carried out in the presence of testosterone, and the R_F values of the steroids (relative to that of testosterone) are presented in Table I. From visualization of the steroids on the chromatograms, we estimated that sufficient separation between steroid pairs was achieved when these values differed by at least 0.05. In all the systems tested, resolution between $3\alpha,17\beta$-dihydroxy-5α-androstan-6-one and $3\beta,17\beta$-dihydroxy-5α-androstan-7-one was unsatisfactory; the same was true for 5α-androstane-$3\alpha,16\alpha,17\beta$-triol and its 3β-epimer and for 5α-androstane-$3\beta,6\alpha,17\beta$-triol and the analogous $3\beta,7\alpha,17\beta$- and $3\beta,15\alpha,17\beta$-triols.

TABLE I

TLC OF TESTOSTERONE AND $C_{19}O_3$ STEROIDS

The mobile phases used were: A, chloroform–ethyl acetate (4:1, v/v) once; B, chloroform–ethyl acetate (4:1, v/v) twice; C, benzene–ethyl acetate (3:1, v/v) twice; D, cyclohexane–ethyl acetate (3:2, v/v) twice; E, cyclohexane–ethyl acetate–ethanol (45:45:10, v/v/v) once; F, chloroform–ethanol (9:1, v/v) once; G, benzene–ethanol (9:1, v/v) three times.

Steroid	Mobility (relative to testosterone) in mobile phase						
	A	B	C	D	E	F	G
Testosterone	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Androst-5-ene-$3\beta,16\alpha,17\beta$-triol	0.03	0.07	0.06	0.25	0.56	0.68	0.35
Androst-5-ene-$3\beta,16\beta,17\beta$-triol	0.35	0.40	0.42	0.77	0.80	0.80	0.51
5α-Androstane-3,6,17-trione	1.06	1.00	1.10	0.90	0.91	1.08	1.32
5α-Androstane-3,7,17-trione	1.28	1.11	1.08	0.90	0.90	1.12	1.31
3β-Hydroxy-5α-androstane-6,17-dione	0.31	0.30	0.23	0.34	0.69	0.75	0.68
3β-Hydroxy-5α-androstane-7,17-dione	0.48	0.50	0.34	0.51	0.71	0.80	0.71
17β-Hydroxy-5α-androstane-3, 6-dione	0.60	0.60	0.51	0.64	0.79	0.83	0.80
$3\alpha,17\beta$-Dihydroxy-5α-androstan- 6-one	0.17	0.19	0.16	0.37	0.72	0.74	0.56
$3\beta, 6\alpha$-Dihydroxy-5α-androstan-17-one	0.04	0.06	0.02	0.10	0.45	0.60	0.48
$3\beta, 7\alpha$-Dihydroxy-5α-androstan-17-one	0.10	0.12	0.05	0.21	0.61	0.64	0.49
$3\beta, 7\beta$-Dihydroxy-5α-androstan-17-one	0.11	0.13	0.07	0.19	0.54	0.61	0.46
$3\beta,11\beta$-Dihydroxy-5α-androstan-17-one	0.32	0.34	0.43	0.80	0.93	0.74	0.64
$3\beta,17\beta$-Dihydroxy-5α-androstan- 6-one	0.17	0.19	0.13	0.30	0.65	0.65	0.54
$3\beta,17\beta$-Dihydroxy-5α-androstan- 7-one	0.21	0.23	0.20	0.40	0.68	0.69	0.58
$6\alpha,17\beta$-Dihydroxy-5α-androstan- 3-one	0.18	0.21	0.17	0.43	0.73	0.63	0.55
5α-Androstane-$2\alpha, 3\alpha,17\beta$-triol	0.10	0.12	0.11	0.38	0.65	0.55	0.53
5α-Androstane-$2\beta, 3\alpha,17\beta$-triol	0.06	0.08	0.09	0.33	0.67	0.45	0.48
5α-Androstane-$2\beta, 3\beta,17\beta$-triol	0.17	0.20	0.19	0.57	0.83	0.63	0.56
5α-Androstane-$3\alpha, 6\beta,17\beta$-triol	0.14	0.13	0.12	0.35	0.69	0.63	0.51
5α-Androstane-$3\alpha,11\beta,17\beta$-triol	0.06	0.08	0.09	0.36	0.76	0.56	0.50
5α-Androstane-$3\alpha,16\alpha,17\beta$-triol	0.02	0.03	0.03	0.14	0.50	0.41	0.39
5α-Androstane-$3\beta, 6\alpha,17\beta$-triol	0.04	0.06	0.02	0.13	0.46	0.43	0.42
5α-Androstane-$3\beta, 6\beta,17\beta$-triol	0.05	0.07	0.04	0.25	0.60	0.50	0.47
5α-Androstane-$3\beta, 7\alpha,17\beta$-triol	0.06	0.05	0.03	0.17	0.48	0.40	0.38
5α-Androstane-$3\beta, 7\beta,17\beta$-triol	0.06	0.06	0.04	0.21	0.50	0.46	0.42
5α-Androstane-$3\beta,11\beta,17\beta$-triol	0.05	0.06	0.07	0.35	0.70	0.47	0.53
5α-Androstane-$3\beta,15\alpha,17\beta$-triol	0.09	0.06	0.04	0.15	0.46	0.43	0.39
5α-Androstane-$3\beta,16\alpha,17\beta$-triol	0.05	0.07	0.07	0.21	0.57	0.54	0.42

Gas–liquid chromatography

Retention indices on four stationary phases were calculated and expressed according to Kováts[14] for each steroid derivative and the free triketones. The operating conditions and data are shown in Table II.

TABLE II

RETENTION INDICES OF TMS DERIVATIVES FROM TWENTY-SIX $C_{19}O_3$ STEROIDS AND TWO TRIONES IN GLC

The GLC systems used were as follows: I, 2.1-m × 4-mm column with 3.08 % of OV-101 at 263°; II, 2.1-m × 4-mm column with 1.82 % of DEXSIL at 263°; III, 3.1-m × 3-mm column with 1.57 % of OV-7 at 270°; IV, 2.1-m × 4-mm column with 3.08 % of SP-2250 at 280°. In each system the inert support was Gas-Chrom Q (100–120 mesh).

Steroid	Retention index in system			
	I	*II*	*III*	*IV*
Androst-5-ene-3β,16α,17β-triol	2889	2886	2899	2941
Androst-5-ene-3β,16β,17β-triol	2914	2923	2922	2972
5α-Androstane-3,6,17-trione	2789	3127	3080	3346
5α-Androstane-3,7,17-trione	2584	2740	2808	2797
3β-Hydroxy-5α-androstane-6,17-dione	2826	3063	3022	3230
3β-Hydroxy-5α-androstane-7,17-dione	2790	2987	2962	3137
17β-Hydroxy-5α-androstane-3, 6-dione	2920	3093	3026	3210
3α,17β-Dihydroxy-5α-androstan- 6-one	2793	2893	2872	2970
3β, 6α-Dihydroxy-5α-androstan-17-one	2755	2861	2861	2972
3β, 7α-Dihydroxy-5α-androstan-17-one	2685	2777	2767	2862
3β, 7β-Dihydroxy-5α-androstan-17-one	2798	2910	2883	2998
3β,11β-Dihydroxy-5α-androstan-17-one	2849	2968	2932	3043
3β,17β-Dihydroxy-5α-androstan- 6-one	2899	3024	2984	3100
3β,17β-Dihydroxy-5α-androstan- 7-one	2860	2956	2948	--
6α,17β-Dihydroxy-5α-androstan- 3-one	2856	2990	2938	3037
5α-Androstane-2α, 3α,17β-triol	2755	2749	2741	2760
5α-Androstane-2β, 3α,17β-triol	2737	2714	2713	2730
5α-Androstane-2β, 3β,17β-triol	2869	2881	2854	2882
5α-Androstane-3α, 6β,17β-triol	2697	2675	2672	2701
5α-Androstane-3α,11β,17β-triol	2743	2740	2739	2771
5α-Androstane-3α,16α,17β-triol	2802	2802	2773	2807
5α-Androstane-3β, 6α,17β-triol	2854	2817	2823	2856
5α-Androstane-3β, 6β,17β-triol	2775	2748	2775	2810
5α-Androstane-3β, 7α,17β-triol	2692	2654	2688	2716
5α-Androstane-3β, 7β,17β-triol	2836	2844	2833	2868
5α-Androstane-3β,11β,17β-triol	2854	2864	2851	2887
5α-Androstane-3β,15α,17β-triol	2846	2811	2833	2849
5α-Androstane-3β,16α,17β-triol	2898	2896	2907	2945

Problems in the formation of tri-TMS derivatives were encountered with steroids bearing a 6β-hydroxyl-group (5α-androstane-3α,6β,17β-triol and its 3β-epimer). Thus, di-TMS derivatives with a free 6β-hydroxyl-group, as confirmed by MS, were obtained when TMCS was not added to the BSTFA–pyridine reaction mixture. Retention indices on OV-101 were 2784 and 2876 for 3α,17β-di-TMS-5α-androstan-6β-ol and the 3β,17β-di-TMS-5α-androstan-6β-ol, respectively. Derivatization of all hydroxyl-groups was obtained when TMCS was present in the reaction

mixture. No enol-trimethylsilyl ethers were formed when 3-oxo- or 17-oxo-steroids were subjected to silylation with BSTFA–TMCS (10:1) at room temperature.

In contrast to TLC, the separation of $3\alpha,17\beta$-dihydroxy-5α-androstan-6-one from $3\beta,17\beta$-dihydroxy-5α-androstan-7-one, and of 5α-androstane-$3\beta,7\alpha,17\beta$-triol from 5α-androstane-$3\beta,6\alpha,17\beta$-triol and 5α-androstane-$3\beta,15\alpha,17\beta$-triol, (as TMS derivatives), was achieved on all the stationary phases tested; nevertheless, separation of androst-5-ene-$3\beta,6\alpha,17\beta$-triol from 5α-androstane-$3\beta,15\alpha,17\beta$-triol was incomplete with each phase.

In all systems, steroids oxygenated in the axial configuration (2β-, 3α-, 6β- and 7 α-positions) yielded lower retention times than the respective 2α-, 3β-, 6α- and 7β-epimers with an equatorial configuration. The greater flatness of the latter molecules may favour interactions with the stationary phase and so explain the difference in mobilities. In addition, 16α-oxygenated steroids had lower retention times than their 16β-epimers. Such a phenomenon may be explained by co-participation of the 16β- and 17β-radicals in interaction with the stationary phase.

Resolution of 3β-hydroxy-5-ene from 3β-hydroxy-5α-reduced steroids was difficult on packed columns of limited efficiency. In contrast, molecules differing by only 9 index units were completely resolved on glass capillary columns. In the course of this work, capillary columns coated with OV-101 were used for different separations and identifications in synthesis-reaction mixtures.

Mass spectra of $C_{19}O_3$ steroids

All mass spectra of hydroxylated steroids were those of TMS derivatives. Only fragments with a relative intensity of 5% or more are shown in the figures.

5α-Androstane-3,6,17-trione and 5α-androstane-3,7,17-trione (Fig. 1). As expected, both molecules show a molecular ion at $m/e = 302$, but the fragmentation patterns are quite different. Thus, fragments at $m/e = 255$ and 137 are characteristic of 5α-androstane-3,7,17-trione, and those at $m/e = 273$ and 123 only occur with 5α-androstane-3,6,17-trione.

The mass spectra from these two triones were discussed by Obermann *et al.*[15] and by Hammerschmidt *et al.*[16]. Our results agree with the characteristic frag-
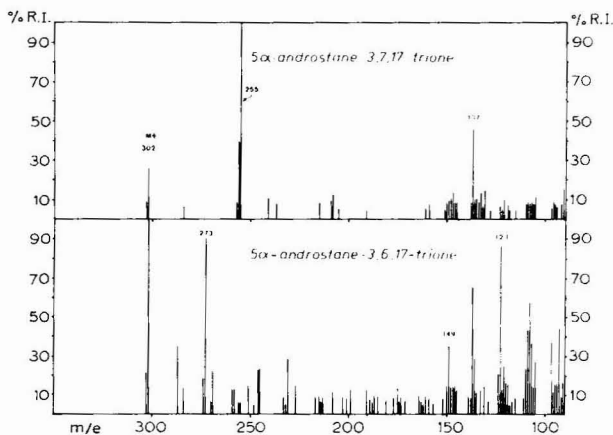
Fig. 1. Mass spectra of 5α-androstane-3,7,17-trione and 5α-androstane-3,6,17-trione.

mentation patterns of the two molecules, but there are differences as regards the relative intensities of low-mass ions. The cited authors explained formation of the ion at $m/e = 273$ by loss of H$_2$O and CHO[15]. Our finding of another ion at $m/e = 274$ with a relative intensity higher than expected for an isotopic ion suggests the loss of C$_2$H$_5$ (M −29) from carbons 1 and 2 of 5α-androstane-3,6,17-trione and loss of CO (M −28) from the 6-oxo-function[16]. Such fragmentation would be characteristic of a trione incorporating a 6-oxo-group. On the other hand, the fragmentation mechanism described by Djerassi *et al.*[17] for 6-oxosteroids may be involved in the formation of fragments at $m/e = 137$ and 109.

3β-Hydroxy-5α-androstane-7,17-dione, 3β-hydroxy-5α-androstane-6,17-dione and 17β-hydroxy-5α-androstane-3,6-dione (Fig. 2). The molecular ion for the TMS derivatives of these three molecules is at $m/e = 376$, but the fragmentation patterns and relative intensities of the fragments are different. The base peaks are at $m/e = 329$ (M −47), 361 (M −15) and 129 for the respective compounds.
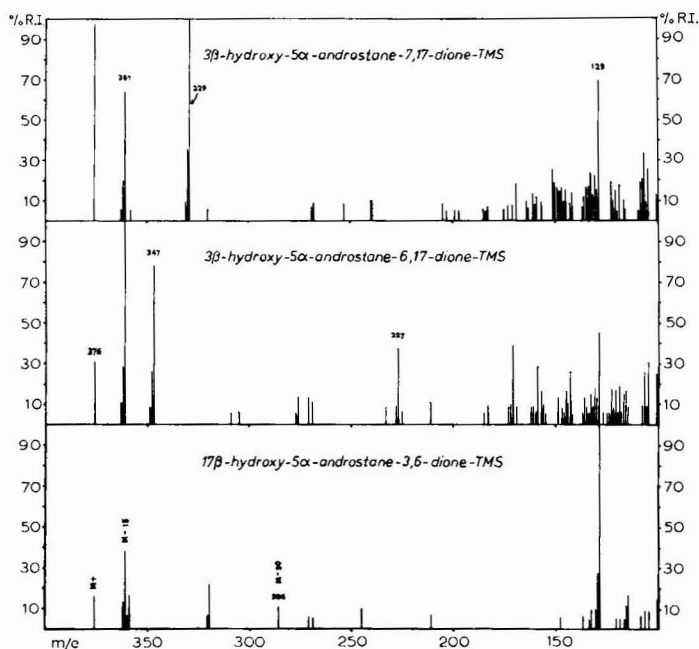
Fig. 2. Mass spectra of TMS derivatives of 3β-hydroxy-5α-androstane-7,17-dione, 3β-hydroxy-5α-androstane-6,17-dione and 17β-hydroxy-5α-androstane-3,6-dione.

Comparison between the spectra of 3β-hydroxy-5α-androstane-7,17-dione and that of 5α-androstane-3,7,17-trione shows that the same fragment (at 47 a.m.u.) is lost by both molecules; this implies that such loss is characteristic of the 7,17-dione function. A one-step mechanism for the loss of H$_2$O and CHO· is suggested by the metastable ion found at 287.8 a.m.u., which corresponds to the transition $m/e = 376$ to $m/e = 329$ for the dioxosteroid. Formation of an ion at $m/e = 129$ in the spectrum of 3β-hydroxy-5α-androstane-7,17-dione may arise through 7-enol formation and elimination of water[18] as follows:
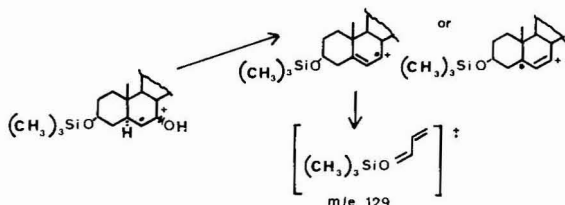
Such structures agree with elimination of an ion of 129 a.m.u. according to the classical mechanism[19]. Further, it was noted that loss of silanol, which corresponds to the M−90 ion (*m/e* = 286) is very low when compared with that of the TMS derivatives of 5α-androstanediols.

The spectrum of 3β-hydroxy-5α-androstane-6,17-dione differs from that of the analogous 7,17-dione through elimination of an ion of 29 a.m.u. (*m/e* = 347) and an ion at *m/e* = 227. It was also noted that the ion at *m/e* = 139 described by Hammerschmidt and Spiteller[16] as characteristic of 3β-hydroxy-5α-androstane-6,17-dione was not present. The base peak at *m/e* = 129 in the spectrum of 17β-hydroxy-5α-androstane-3,6-dione may result from ring-D fragmentation when an -O-TMS group is in the 17β-position[19]; other fragments can be related with loss of that -O-TMS. Some previously described fragments[19] were not observed.

3β,6α-Dihydroxy-5α-androstan-17-one, 3α,17β-dihydroxy-5α-androstan-6-one and its 3β-epimer and 6α,17β-dihydroxy-5α-androstan-3-one. These four molecules bear oxygen functions in the 3-, 6- and 17-positions of the androstane skeleton and differ only by the positions of the two hydroxyl-groups and that of the ketone (Fig. 3). When analyzed as TMS derivatives, they all exhibit a molecular ion at *m/e* = 450 and have similar fragmentation patterns, but different relative intensities. Thus, the relative intensities of the ions at *m/e* = 345 [M−(90+15)] and *m/e* = 270 [M−(2×90)] differ significantly for 3α,17β-dihydroxy-5α-androstan-6-one, its 3β-epimer and 6α,17β-dihydroxy-5α-androstan-3-one, even though these three molecules have the same reference fragment at *m/e* = 129.

In contrast, 3β,6α-dihydroxy-5α-androstan-17-one has its base peak at *m/e* = 271. Fragmentation in this steroid is induced by both the 17-oxo- and the 3β- and 6α-O-TMS groups. The base peak at *m/e* = 271 [M−(90+89)] is unusual for a di-TMS derivative of the androstane series. In this instance, elimination of silanol seems to follow a 1–3 mechanism[20]: elimination from the 6α-position involves the H atom on carbon 8, which is a β-position; the distance is then too large for silanol liberation and results in elimination of -O-TMS as a free radical (89 a.m.u.). Such a mechanism creates an unsaturated site in ring B and favours the formation of the ion at *m/e* = 129, positive charges being located either on the M−129 fragment (231 a.m.u.) or on the "lost" ion at *m/e* = 129.

The 17-ketone is characterized by some fragments involving loss of water, *viz.*, 271 → 253, and 231 → 213 (metastable at *m/e* = 196.9), or ethylene elimination, *viz.*, 271 → 243 (ref. 21).

The spectra of the three other steroids are similar. As expected, the base peak is at *m/e* = 129 (see ref. 19). We cannot explain the M−56 ion that also appears in spectra of TMS derivatives of 5α-androstanediols.

3β,7α-Dihydroxy-5α-androstan-17-one, its 7β-epimer and 3β,17β-dihydroxy-5α-

Fig. 3. Mass spectra of TMS derivatives of 3β,6α-dihydroxy-5α-androstan-17-one, 3α,17β-dihydroxy-5α-androstan-6-one, its 3β-epimer and 6α,17β-dihydroxy-5α-androstan-3-one.

androstan-7-one. These three molecules bear oxygen functions in positions 3, 7 and 17 of the androstane skeleton and differ only in the positions of the two hydroxyl-groups and that of the ketone (Fig. 4). When analyzed as TMS derivatives, each exhibits a molecular ion at $m/e = 450$ and similar fragmentation patterns, but the relative intensities of the fragments differ. Thus, the molecular ion at $m/e = 450$, the fragment at $m/e = 435$ (M−15) and that at $m/e = 129$ are the most abundant for 3β,17β-dihydroxy-5α-androstan-7-one, 3β,7β-dihydroxy-5α-androstan-17-one and its 7α-epimer, respectively. With these last two steroids, the ion at $m/e = 129$ can be explained by loss of silanol from position 7, involving the H atom on carbon 5. The resulting unsaturation on carbon 5 favours elimination of $m/e = 231$ and $m/e = 129$ fragments from the ion at $m/e = 360$ (M−90). Loss of water (231 → 213) and of ethylene (360 → 332) involves the 17-oxo-group and agrees with the similar losses described by Djerassi for the TMS derivative of 3β-hydroxyandrost-5-en-17-one[19]. No satisfactory explanation can be given for the ion at $m/e = 243$.

Loss of silanol is more extensive for the 7α-epimer and may be explained by the distance between the -O-TMS and the 5α- and/or the 9α-H being shorter than for the 7β-epimer.
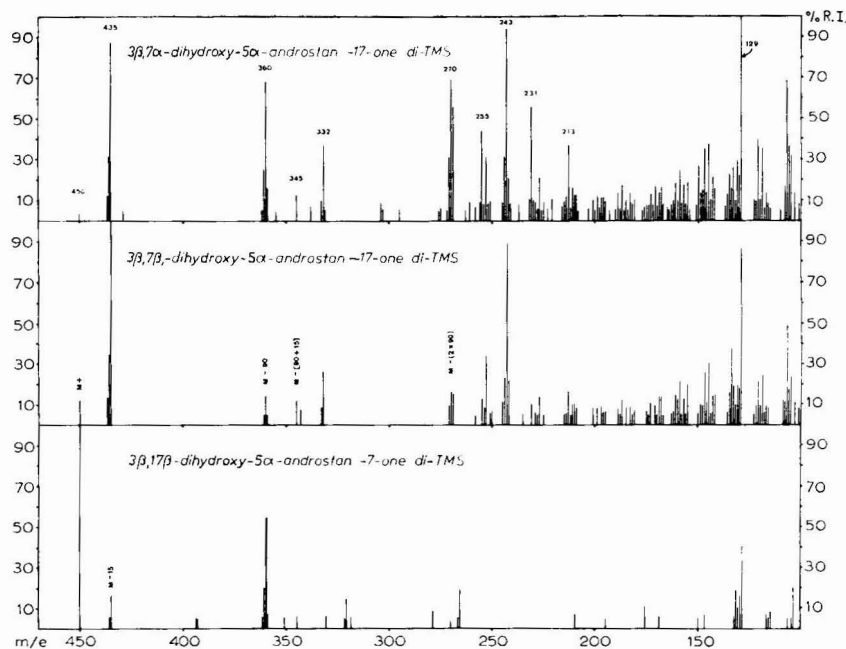
Fig. 4. Mass spectra of TMS derivatives of $3\beta,7\alpha$-dihydroxy-5α-androstan-17-one, its 7β-epimer and $3\beta,17\beta$-dihydroxy-5α-androstan-7-one.

The spectrum of the di-TMS derivative of $3\beta,17\beta$-dihydroxy-5α-androstan-7-one shows the base peak to be the molecular ion. Comparison of this spectrum with that of the analogous derivative of 5α-androstane-$3\xi,17\xi$-diol[22] suggests that a 7-oxo-function results in stabilization of the molecule.

$3\beta,11\beta$-Dihydroxy-5α-androstan-17-one. This compound, when analyzed as a TMS derivative (Fig. 5), differs from those giving the spectra shown in Figs. 3 and 4 by the 11β-hydroxyl-group. Fragmentation agrees with previously published data[23] and resembles that of the steroids mentioned above, but the most abundant fragment is at $m/e = 156$ and cannot be satisfactorily explained. Most of the fragmentation results from loss of silanol (90 a.m.u.) or ring D (56 a.m.u.) according to Zaretskii[21] and loss of a methyl group (15 a.m.u.) or from a combination of these losses.

Androst-5-ene-$3\beta,16\alpha,17\beta$-triol and its 16β-epimer. When these compounds are analyzed as TMS derivatives, they both yield base peaks at $m/e = 129$ and have
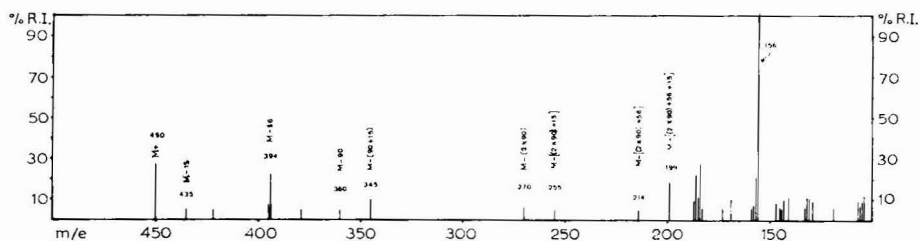
Fig. 5. Mass spectrum of TMS derivative of $3\beta,11\beta$-dihydroxy-5α-androstan-17-one.
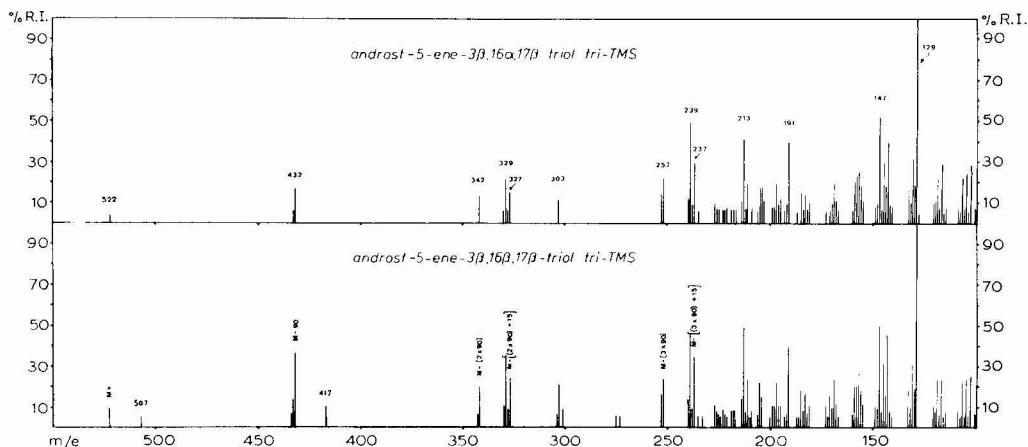
Fig. 6. Mass spectra of TMS derivatives of androst-5-ene-3β,16α,17β-triol and its 16β-epimer.

identical fragmentation patterns; the relative intensities of the fragments are not significantly different (see Fig. 6). The ion at $m/e = 147$ may originate from ring D according to Sloan *et al.*[24], and the other fragments are those expected from TMS derivatives of 5(6)-unsaturated androstenediols.

5α-*Androstane-2α,3α,17β-triol, its 2β-epimer and 5α-androstane-2β,3β,17β-triol.* The mass spectra of TMS derivatives for these steroids are identical with those presented by Lisboa[7] and show a molecular ion at $m/e = 524$ (Fig. 7); the fragmen-
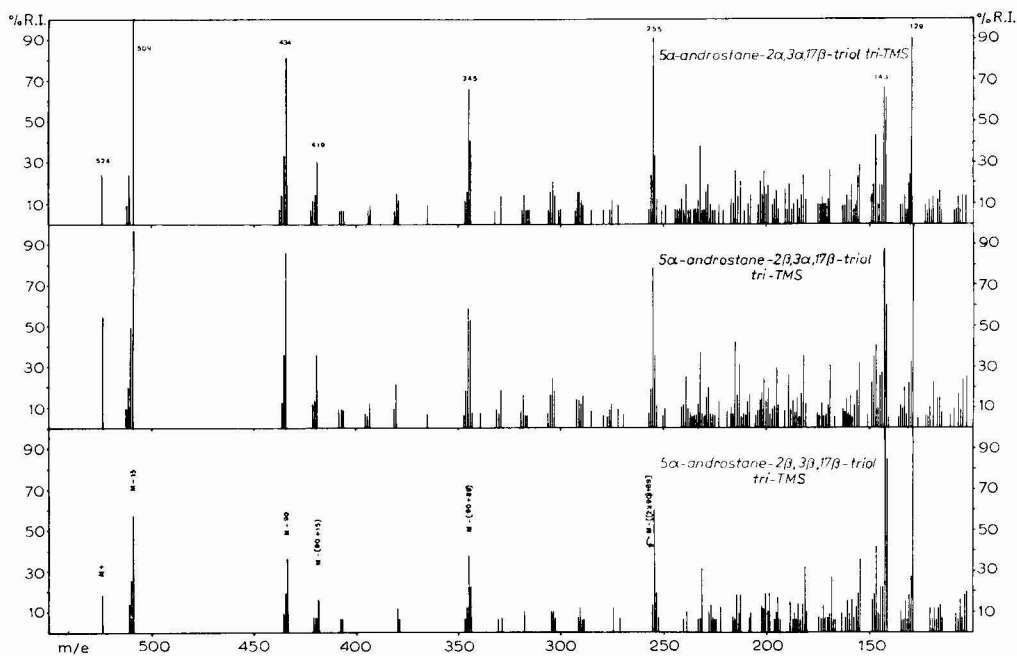
Fig. 7. Mass spectra of TMS derivatives of 5α-androstane-2α,3α,17β-triol, its 2β-epimer and 5α-androstane-2β,3β,17β-triol.

tation patterns are identical, but have different relative intensities. Thus, the base peak is at $m/e = 509$ (M−15) for 5α-androstane-2α,3α,17β-triol whereas the most abundant fragment of the other two steroids is at $m/e = 129$. The patterns show successive losses of fragments of either $m/e = 90$ or $m/e = 89$. This difference may be explained by the proximity of the hydroxyl-groups on carbons 2 and 3 of the androstane skeleton. Such loss of fragments of $m/e = 89$ is characteristic of TMS derivatives of 2ξ,3ξ-dihydroxysteroids. No explanation is as yet available for the ion at 143 a.m.u.

   5α-Androstane-3α,6β-17β-triol, its 3β-epimer and 5α-androstane-3β,6α,17β-triol. The mass spectra of the TMS derivatives of these three epimers are closely related. They show the same molecular ion at $m/e = 524$ and a base peak at $m/e = 129$ (see Fig. 8). The fragmentation patterns are identical, with little differences in relative intensities, and are governed by the -O-TMS functions.
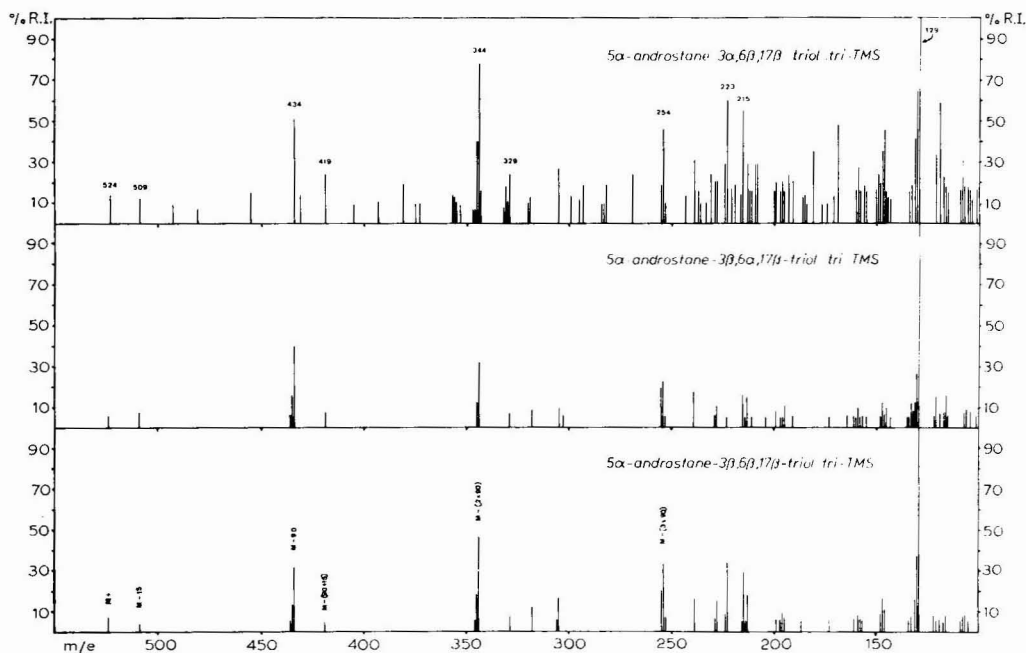
Fig. 8. Mass spectra of TMS derivatives of 5α-androstane-3α,6β,17β-triol, its 6α-epimer and 5α-androstane-3β,6β,17β-triol.

   5α-Androstane-3β,7α,17β-triol and its 7β-epimer. The mass spectra of the TMS derivatives of these epimers show the same molecular ion at $m/e = 524$ and identical fragmentation patterns (see Fig. 9). The base peaks are at $m/e = 434$ (M−90) for 5α-androstane-3β,7β,17β-triol and $m/e = 393$ (M−131) for the 7α-epimer, thus permitting differentiation between the compounds. The fragment at 393 a.m.u. corresponds with the loss of ring D and determines the expected diminished intensity of the ion at $m/e = 129$. It is also noteworthy that peaks attributable to fragmentation of rings A, B and C in both steroids (masses lower than 217 a.m.u.) have an unusually low intensity.
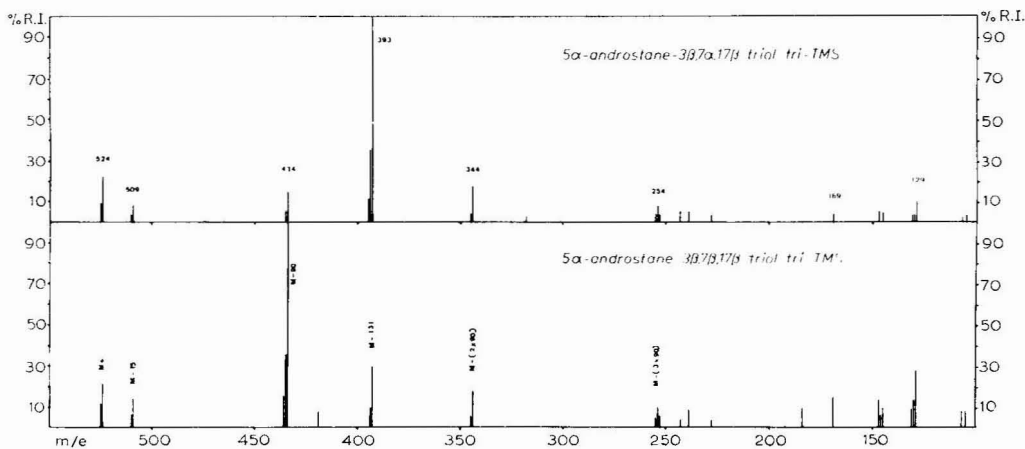
Fig. 9. Mass spectra of TMS derivatives of 5α-androstane-3β,7α,17β-triol and its 7β-epimer.

5α-Androstane-3α,11β,17β-triol and its 3β-epimer. The mass spectra of the TMS derivatives of these epimers show the same molecular ion at $m/e = 524$, the same base peak at $m/e = 169$ and identical fragmentation patterns (see Fig. 10). This last ion may be explained by loss of ring D and carbons 13, 14 and 19. Further loss of a chain containing carbons 19, 13, 12, 11 and the 11-O-TMS may correspond with the ion at $m/e = 143$ (see ref. 25).

5α-Androstane-3β,15α,17β-triol. The TMS derivative of this triol gives a spectrum (see Fig. 11) with a molecular ion at $m/e = 524$ and a base peak at $m/e = 217$ as previously reported[2]. This last ion was attributed by Gustafsson *et al.*[26] to the breaking of C_{13}–C_{17} and C_{14}–C_{15} bonds; the ion at $m/e = 191$ results from complete transfer of the 17-O-TMS group to position 15 (see ref. 26).

5α-Androstane-3α,16α,17β-triol and its 3β-epimer. In agreement with Lisboa[7], the mass spectra of the TMS derivatives of these epimers show identical fragmentation patterns with close relative intensities (see Fig. 12). The molecular ion is at
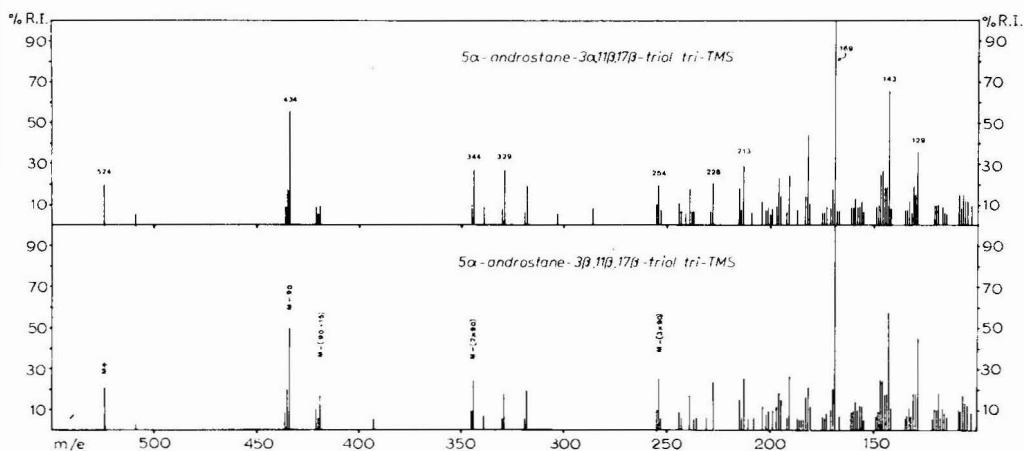
Fig. 10. Mass spectra of TMS derivatives of 5α-androstane-3α,11β,17β-triol and its 3β-epimer.
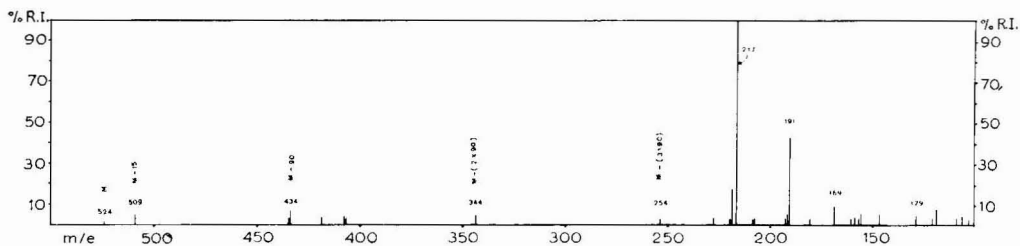
Fig. 11. Mass spectrum of TMS derivative of 5α-androstane-3β,15α,17β-triol.

$m/e = 524$ and the base peak at $m/e = 191$. This last ion may be explained by a complex transfer of 17-O-TMS to position 16. The fragmentation is also consistent with that observed for the TMS derivatives of androst-5-ene-3β,16ξ,17ξ-triol epimers (see Fig. 6).
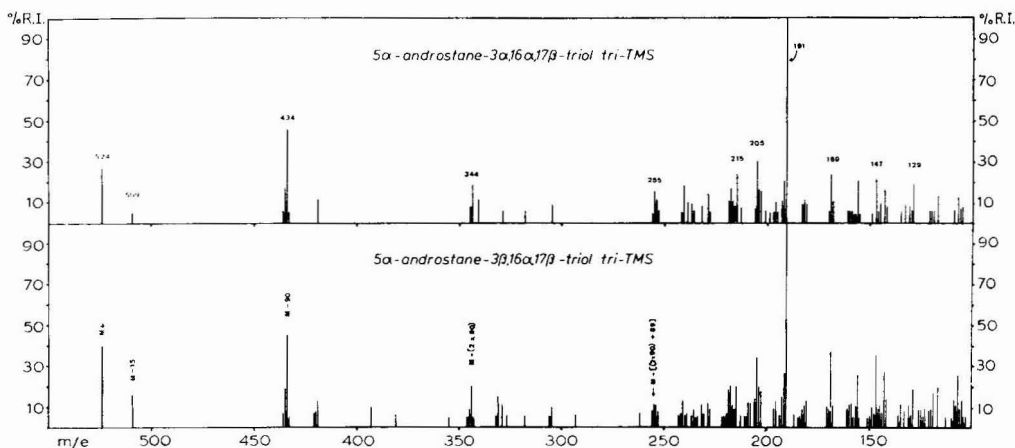
Fig. 12. Mass spectra of TMS derivatives of 5α-androstane-3α,16α,17β-triol and its 3β-epimer.

Characteristic fragmentation of TMS derivatives of dihydroxy-5α-androstanones

   The m/e values for characteristic fragments obtained from these derivatives are shown in Table III; fragmentation differences between steroids may be used for multiple ion detection or for confirmation of identity.

Characteristic fragmentation of TMS derivatives of androstanetriols

   The m/e values of characteristic fragments obtained from these derivatives are shown in Table IV; the differences may prove useful for confirming the identity of a steroid or in multiple ion detection.

DISCUSSION

   The chromatographic data and mass spectra presented in this paper are limited to twenty-eight $C_{19}O_3$ steroids. Many more such steroids may be chemically available, but their number was limited both by their availability and by our choice of molecules that could be naturally occurring[1-6]. TLC, GLC and GLC–MS are the most common

TABLE III

VALUES OF m/e FOR FRAGMENTS OF TMS DERIVATIVES OF DIHYDROXYANDROST-ANONES

Positions of hydroxyl-groups	Position of oxo-group	Characteristic m/e values
3β, 6α	17	271, 253, 231, 203, 137
3α,17β	6	129
3β,17β	6	129
6α,17β	3	129
3β, 7α	17	435, 360, 332, 270, 255, 243, 231, 213, 129
3β, 7β	17	435, 253, 243, 129
3β,17β	7	450, 360
3β,11β	17	394, 214, 199, 156

TABLE IV

VALUES OF m/e FOR FRAGMENTS OF TMS DERIVATIVES OF ANDROSTANETRIOLS

Positions of hydroxyl-groups	Characteristic m/e values
3β,16α,17β(5-ene)	329, 327, 239, 237, 191, 147, 129
3β,16β,17β(5-ene)	329, 327, 239, 237, 191, 147, 129
3α,16α,17β	524, 434, 205, 191, 169, 147
3β,16α,17β	524, 434, 205, 191, 169, 147
2α, 3α,17β	509, 345, 255, 143
2β, 3α,17β	509, 345, 255, 143
2β, 3β,17β	509, 345, 255, 143
3α, 6β,17β	434, 344, 233, 215, 129
3β, 6α,17β	434, 344, 129
3β, 6β,17β	434, 344, 129
3β, 7α,17β	393
3β, 7β,17β	434
3α,11β,17β	228, 213, 169, 143, 129
3β,11β,17β	228, 213, 169, 143, 129

techniques currently used in steroid-metabolism research for the separation and identification of transformation products; in such instances, the structure of the initial compound being known, only a limited number of new hydroxylated steroids may be formed[1,4].

We tested our C$_{19}$O$_3$ steroids by TLC in seven different solvent mixtures, all but one of which differed from those used by Gustafsson et al.[1]. Our purpose was to extend their study and to avoid both multiple development and the use of acetic acid in the mobile phases.

In GLC, the TMS derivatives of hydroxylated steroids give clear symmetrical peaks. Calculated retention indices generally differ between molecules and between stationary phases; nevertheless, effective resolution of two steroids also depends on the efficiency of the column. Thus, a column with 2000 theoretical plates will separate (with a resolution factor greater than 1) two steroids differing by only 20 index units.

Use of capillary columns with 100,000 theoretical plates[27] may permit the separation of molecules differing by 5 index units with a resolution factor of 1.40. The separation of molecules by GLC must be achieved before the GLC can be coupled with MS.

Few papers report mass spectra of $C_{19}O_3$ steroids as their TMS derivatives. Those reported by Gustafsson *et al.*[1] and by Lisboa[7] differ only slightly from those discussed here. Thus, a base peak at $m/e = 344$ was reported for 5β-androstane-3β,6β,17β-triol instead of at $m/e = 129$, but our fragment at $m/e = 344$ had an intensity 80% of that of the peak at m/e 129. Such a difference may be explained by the fact that the published spectrum[1] was given by a micro-amount of steroid obtained after double enzymic transformation.

Our investigation of the fragmentation of the studied molecules during MS may be used as a criterion for multiple ion detection or to ascertain the identity of $C_{19}O_3$ steroids of biological origin; identification may not be achieved, but useful leads for confirmatory experiments may be obtained.

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QUANTITATIVE GAS CHROMATOGRAPHIC ANALYSIS ON SUPPORT-COATED OPEN TUBULAR CAPILLARY COLUMNS

I. ANALYSIS OF ISOMERIC ETHYLPHENOLS

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SUMMARY

Gas chromatographic methodology has been developed for quantitative analysis of samples of *o*-, *m*-, and *p*-ethylphenols. Samples are dissolved in a dichloromethane solution of the internal standard, anisole, and are then derivatized with N-trimethylsilylimidazole to form the silyl ethers. The samples are chromatographed with similarly prepared standards on a stainless-steel OV-17 support-coated open tubular (SCOT) column.

Specificity, linearity, precision and stability of the samples are discussed, and chromatograms obtained on the SCOT column are compared to those obtained on a packed analytical column.

This report describes one of the first quantitative gas chromatographic analytical methods to use a SCOT (capillary) column.

INTRODUCTION

The great value of gas chromatography (GC) for highly reliable quantitative analyses of a wide variety of compounds is immediately apparent to all who familiarize themselves with its literature. GC is one of the most practical and widely used techniques for determinations at almost any concentration, including trace levels. However, the majority of applications involve conventional packed columns which have a limited separating efficiency.

Although successful high-efficiency capillary column separations were achieved more than a decade ago, the versalility and application (especially for quantitative analyses) of such columns is subject to argument. Highly satisfactory chromatograms are normally obtainable in roughly the same time on packed or capillary columns, but quantitative capillary column applications are often dismissed due to reluctance to work with somewhat more sophisticated (and more expensive) instrumentation. The latter usually includes an inlet splitter which may itself be a source of error in quantitative work[1].

Because of insufficient accuracy and precision often related to unreliable

sampling methods, and due to instrument installation problems, relatively few quantitative analyses have been reported using capillary GC techniques. Most work is qualitative, dealing with petrochemical[2,3], biomedical[4-6] and environmental[7-9] applications. Mattsson and Nygren[10] have reported on the determination of a variety of polychlorinated biphenyls and chlorinated pesticides in sewage sludge by use of a capillary column, but results were reported with relatively wide variation. McCallum and Cairns reported conditions for quantitative analysis of cannabinoids using a support-coated open tubular (SCOT) column without stream splitting[11] but reported no numerical results. Only very recently have the quantitative determination of chlorophenols[12] and the determination of the anti-depressant psychotropic drug Nomifensine in human plasma[13] been reported.

Handled properly, capillary columns can be a powerful tool for quantitative analyses of substances (especially isomers) not satisfactorily handled on packed analytical columns usually due to incomplete resolution. The ethylphenols are used as starting materials and intermediates in a wide variety of organic syntheses but suffer from incomplete resolution of the *meta* and *para* isomers on typical packed columns[14]. Precise and accurate quantitation is difficult if not impossible under such conditions. This paper reports the quantitative analysis of the ethylphenols on an OV-17 SCOT column.

EXPERIMENTAL

Reagents

The "distilled in glass" dichloromethane was obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Anisole (used as internal standard) was "certified" grade from Fisher Scientific (Fair Lawn, N.J., U.S.A.). N-Trimethylsilyl-imidazole (TMSI) was purchased from Pierce (Rockford, Ill., U.S.A.). Regisil was from Regis (Morton Grove, Ill., U.S.A.). Commerical samples of *m*- and *p*-ethyl-phenols were purified by distillation to 98 % or more. *o*-Ethylphenol was used "as is" from Aldrich (Milwaukee, Wis., U.S.A.).

Ethylphenol samples for analysis were prepared as follows. 50 mg of ethyl-phenol (for linearity, 0–100 mg) were weighed into a test tube (Cat. No. 99447; Corning, Corning, N.Y., U.S.A.) to which 10.0 ml of 2.5 mg/ml anisole in dichloro-methane were added. The tube was closed with a screw cap and a PTFE-lined septum (Microsep F-138, Canton Bio-Medical Products, Boulder, Colo., U.S.A.) and 0.5 ml of TMSI (or Regisil for packed-column experiments) was added. After heating the tubes for 30 min at 80°, they were cooled and the contents were diluted to approx. 50 ml with dichloromethane. Solutions were mixed and a portion of each was trans-ferred to auto-injector vials (Hewlett-Packard No. 5080-8712) for GC.

Equipment

The gas chromatograph was a Hewlett-Packard Model 5711A dual-flame instrument equipped according to Hewlett-Packard instructions with a Model 18704 inlet splitter and the appropriate injection port and detector connections. A Brooks dual GC mass-flow controller Model 5840 calibrated for helium was used to control the column carrier flow (set at 10.0 ml/min) and the flame make-up gas flow (30 ml/min) (Brooks Instrument Div., Emerson Electric, Hatfield, Pa., U.S.A.). A split ratio of

about 2 was used. The capillary column was a standard SCOT column (No. 008-0218), coated with OV-17, from Perkin-Elmer (Norwalk, Conn., U.S.A.). Its dimensions were 50 ft. × 0.02 in. I.D. and it was of stainless steel with 1/16-in. female fittings. The oven was operated at 115°, the injection port at 200° and the detector at 250°. A Hewlett-Packard Model 7671A automatic injector was used to inject 0.5-μl samples.

For packed-column chromatograms, a Hewlett-Packard Model 402 high-efficiency dual-flame gas chromatograph was used with an oven temperature of 70°, an injection port temperature of 70°, a detector temperature of 125°, and a helium flow of 60 ml/min. The column was a 4 ft. × 1/8 in. I.D. glass U-tube packed with 3.8% UC-W-98 on 80–100 mesh Gas-Chrom Z prepared by the funnel coating method[15].

Peak areas were calculated using an on-line calculation program from the expanded RTE 2100 computer system (Hewlett-Packard, Avondale, Pa., U.S.A.). Statistical evaluation of the results was accomplished by use of a program[16] available through a time-shared DEC-10 computer system (Digital Equipment, Marlborough, Me., U.S.A.).

RESULTS AND DISCUSSION

Ethylphenol isomers are very useful starting materials and intermediates in a variety of organic reactions. For a highly selective, reproducible assay, complete resolution of the isomers on the GC column is necessary. When a routine screening program[17] failed to yield conditions which would completely resolve the *ortho*, *meta*,
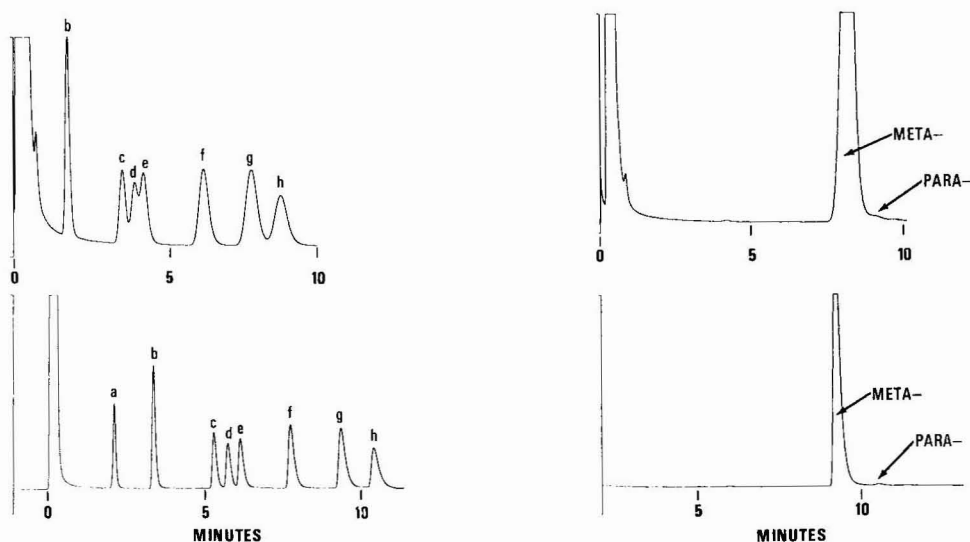
Fig. 1. Gas chromatograms of phenol mixture. Top, packed column; bottom, SCOT capillary column Peaks: a = anisole (in solvent front in upper trace), b = phenol, c = o-cresol, d = m-cresol, e = p-cresol, f = o-ethylphenol, g = m-ethylphenol, h = p-ethylphenol. For chromatographic details see text.
Fig. 2. Gas chromatograms for approx. 0.5% p-ethylphenol in m-ethylphenol. Top, packed column; bottom, capillary column. For chromatographic details see text.

and *para* isomers, attention was turned to SCOT columns for a quantitative analysis.

Chromatograms in Figs. 1 and 2 illustrate results for the packed and capillary columns and demonstrate some problems that are overcome by use of the capillary column for assay. Fig. 1 shows comparative chromatograms of the ethylphenols mixed with some potential impurities: phenol and *o*-, *m*- and *p*-cresol. Note that resolution of the *m*- and *p*-ethylphenols on the packed column is incomplete but that they are totally resolved on the capillary column in about the same analysis time. This *meta–para* resolution problem is very critical when *m*-ethylphenol samples are to be assayed for low levels of the *para* isomer. Fig. 2 illustrates the problem and it is obviously much easier to get good accuracy and precision of determination in the case of the capillary column. With the bad overlap on the packed column, the analyst encounters difficulty in determining the area of the *p*-ethylphenol peak and has reproducibility problems in determining the *m*-ethylphenol peak area. Since *m*-ethylphenol is on the leading side of the *p*-ethylphenol peak, determination of small quantities of it in the presence of the latter is not a problem on either column.

For each ethylphenol isomer the linearity of response to concentration from 0 mg/ml to about 2 mg/ml was tested. In all cases the coefficient of determination was greater than 0.999 and the log–log slope was between 0.995 and 1.010. The maximum percent deviation from a least squares line was 1.6 for *o*-, 0.4 for *m*- and 1.1 for *p*-ethylphenol.

Table I shows the results of precision testing for each of the separate ethylphenol isomers. Over twenty separate replicates were prepared of each isomer to determine the number of replicates necessary to assure that the assay results are $\pm 2\%$ of the true value at a confidence limit of 95%. For an assay, equal numbers of replicates of each sample and of the appropriate standard are prepared.

TABLE I

PRECISION DATA FOR ETHYLPHENOL POSITIONAL ISOMERS

Parameter	Ortho	Meta	Para
No. of samples tested	24	23	23
Standard deviation	4.36×10^{-2}	3.78×10^{-2}	3.75×10^{-2}
Mean normalized response	3.15	3.17	4.67
RSD	1.38	1.19	0.80
Method RSD*	1.96	1.69	1.14
Replicates for 95% confidence ratio of 2%	7	5	2

* Method RSD = RSD $\times \sqrt{2}$ to compensate for variation from samples *and* standards in assay[16].

Calculations for a typical assay are done as follows. For each replicate standard or sample, the peak area of the ethylphenol is divided by the peak area of the anisole (internal standard). These are then divided by the respective weights to give a normalized response. For each sample and standard the normalized replicate responses are averaged and the final purity of the sample (using *p*-ethylphenol as an example) is calculated:

$$\% \text{purity of sample} = \frac{\text{avg.norm. response } p\text{-ethylphenol sample}}{\text{avg.norm. response } p\text{-ethylphenol standard}} \times \% \text{purity standard}$$

Since a large sample load, computer or other instrumental problems may occasionally cause delay in chromatographing the prepared solutions, the stability of *m*-ethylphenol solutions prepared as described was tested. The solutions were chromatographed, then allowed to stand in the auto-injector vials for 24 h, and then re-chromatographed. The average response for ten samples was 99.5 % of the original response. The results obtained after a delay of 24 h indicated that the solutions were acceptably stable under the described conditions. It was assumed that the stabilities of the *o*- and *p*-ethylphenol solutions would be similar.

No quantitative results were generated from the packed-column work due to inadequate resolution. Therefore, attention was focused on capillary column experiments.

Several lots of *m*- and *p*-ethylphenol were assayed and, in instances where possible, results were compared to those found using a UV method based on hydrogen-bonding differences. The results compared favorably.

CONCLUSIONS

This paper describes one of the first reported quantitative analyses done on GC SCOT capillary columns. The method is accurate, highly precise, and selective for the determination of ethylphenol positional isomers. Futher work is in progress to test applicability of capillary columns to quantitative analysis in GC.

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CHROM. 10,162

SINGLE-STEP SEPARATION OF MAJOR AND RARE RIBONUCLEOSIDES AND DEOXYRIBONUCLEOSIDES BY HIGH-PERFORMANCE LIQUID CATION-EXCHANGE CHROMATOGRAPHY FOR THE DETERMINATION OF THE PURITY OF NUCLEIC ACID PREPARATIONS

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SUMMARY

A method is described for the separation of 13 major and minor ribo- and deoxyribonucleosides in a single chromatographic run using high-performance liquid chromatography on strongly acidic cation-exchange columns. The method proved useful for the routine determination of small amounts of ribonucleic acid impurities in deoxyribonucleic acid preparations and *vice versa*. About 3% or even less of nucleic acid contamination in a given sample can be easily detected and quantitatively determined under the conditions used.

INTRODUCTION

The quantitative determination of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) impurities in DNA or RNA samples during the isolation procedure still represents a difficult task, particularly if small amounts of DNA or RNA (less than 10^{-4} g) have to be processed and if the impurity is 3% or less of the total sample.

Purification of DNA preparations from contaminating RNA is commonly achieved by CsCl centrifugation[1], and the amount of RNA which is found at the bottom of the centrifuge tube may then be determined quantitatively. The separation of contaminating DNA from RNA or of DNA and rRNA from tRNA can be achieved by selective precipitation or extraction procedures[2,3], by chromatographic methods[4,5] or by polyacrylamide gel electrophoresis[6]. The fractions containing the impurities or minor components are pooled and quantitatively determined. These methods, however, are more or less time consuming and require at least two subsequent steps of processing, one for the separation and another for the quantitative determination of the minor residues.

In the course of our recent work on the isolation and characterization of DNA[7]

and RNA[8] we developed a simple and rapid method for the determination of RNA impurities in DNA preparations and *vice versa*. The procedure depends on the separation and quantitative determination of ribo- and deoxyribonucleosides in a single chromatographic run using high-performance liquid cation-exchange chromatography. Prior to this the nucleic acid preparation is enzymatically hydrolysed by ribo- and deoxyribonucleases, by phosphodiesterase and phosphatase.

In this paper the feasibility of this method in routine analytical work and its sensitivity are demonstrated by means of an RNA preparation from *Escherichia coli* and a DNA preparation of the sponge *Geodia cydonium*.

MATERIALS AND METHODS

DNA of the sponge *Geodia cydonium* was isolated according to Hönig *et al.*[9] from sponges harvested in the Northern Adriatic near Rovinj (Yugoslavia). RNA was isolated according to Aviv and Leder[10] from *E. coli* ATCC 11303.

Ribonuclease I (RNase I, EC 3.1.4.22) was purchased from Miles Laboratories, Slough, Great Britain, ribonuclease T_1 (RNase T_1, EC 3.1.4.8), deoxyribonuclease I (DNase I, EC 3.1.4.5, 1000 U/ml), snake venom phosphodiesterase (PDase, EC 3.1.4.1, 2 mg/ml), and alkaline phosphatase (APase, EC 3.1.3.1, 1 mg/ml) were from Boehringer, Mannheim, G.F.R.

Strongly acidic cation-exchange resin (type M-71, particle diameter 10–12 μm) was purchased from Beckman, München, G.F.R. The reagents, obtained from E. Merck, Darmstadt, G.F.R., were of the highest available purity.

Adenosine (rAdo), cytidine (rCyd), guanosine (rGuo), inosine (rIno), uridine (rUrd), deoxyadenosine (dAdo), deoxycytidine (dCyd), deoxyguanosine (dGuo), deoxyinosine (dIno), and deoxythymidine (dThd) were obtained from Papierwerke Waldhof-Aschaffenburg, Mannheim, G.F.R., pseudouridine (Ψrd, natural isomer), ribothymidine (5-methyluridine, me[5]Urd), and 5-methyldeoxycytidine (me[5]dCyd) from P. L. Biochemicals, Milwaukee, Wisc., U.S.A.

For the preparation of reference solutions the (deoxyribonucleosides were dissolved in standard buffer solutions at pH 6.0 and 7.0. The solutions, which exhibited absorbances of 2 to 3 at λ_{max}, were diluted before use with 0.4 M ammonium formate, pH 4.6, in the ratio 1:10.

The isolated DNA and RNA samples were separately dissolved in 0.01 M Tris–HCl + 0.1 M NaCl, pH 7.9, at a concentration of 0.5 mg/ml. A 1.0 ml sample of each solution was digested with 10 μg each of RNase I and RNase T_1 for 4 h at room temperature. Then 200 μl of DNase I, 100 μl of PDase, and 200 μl of APase, together with 1.0 ml of Tris–HCl, pH 9.4, and 50 μl of 0.6 M MgCl$_2$ solution were added and the mixture reincubated for 4 h at 37 °.

The ribo- and deoxyribonucleosides were separated in a Varian LCS-1000 liquid chromatograph, equipped with a 254 nm UV flow-cell detector and a diaphragm-piston pump (Orlita, Giessen, G.F.R.). A 150 × 0.18 cm I.D. stainless steel tube was filled with cation-exchange resin according to Scott and Lee[11], and the column eluted with 0.4 M (with respect to the NH$_4^+$ concentration) ammonium formate, pH 4.6, at a linear flow velocity of 4.7 cm/min (flow-rate 7.2 ml/h) at 40° column oven temperature.

RESULTS AND DISCUSSION

The total separation of the major ribo- and deoxyribonucleosides from each other, from inosine and deoxyinosine and some rare nucleosides within a single column-chromatographic run has not previously been reported.

As compared to similar devices which have been reported earlier for the separation of the major deoxyribonucleosides and of deoxyinosine[12], for the major and some rare ribonucleosides[13], for a minor deoxyribonucleoside from DNA hydrolysates[14], and for 6-thiopurine ribonucleosides and bases[15], only the column length and the column temperature had to be changed to 150 cm and 40°, respectively. By these means the nucleoside pairs dIno–rGuo, rCyd–dAdo, and particularly dGuo–rAdo, were separated. The separation of Ψrd–rUrd–dThd–rIno and dCyd–me^{5}dCyd was easily achieved on even shorter columns. The me^{5}Urd fraction, however, if added to the reference sample, eluted in between rUrd and dThd without resolution from either nucleoside. An elution pattern as obtained from the separation of 13 ribo- and deoxyribonucleosides on the 150 cm column is shown in Fig. 1.

The elution pattern of a separation of the deoxyribonucleosides obtained from an enzymatic hydrolysis of DNA from *Geodia cydonium* which was slightly con-
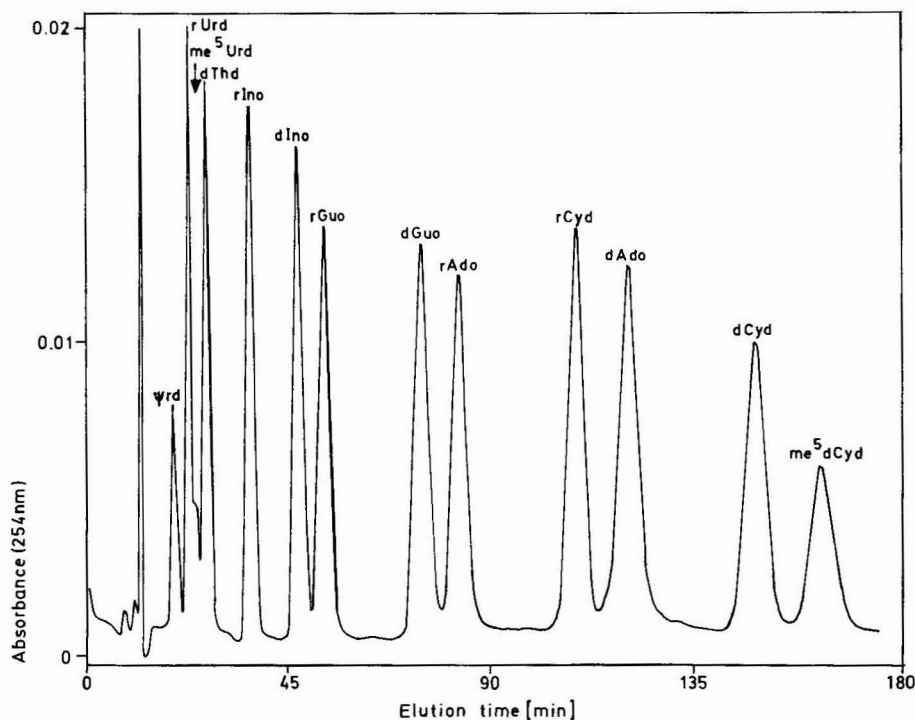
Fig. 1. Elution pattern obtained from the separation of a synthetic mixture of 13 major and rare ribo- and deoxyribonucleosides (for chromatographic conditions see Materials and Methods). Injected volume: 28 μl. Amounts of nucleoside (pmole): ψrd, 90; rUrd, 200; me^{5}Urd, 55; dThd, 240; rIno, 200; dIno, 200; rGuo, 180; dGuo, 220; rAdo, 220; rCyd, 660; dAdo, 220; dCyd, 580; me^{5}dCyd, 450.
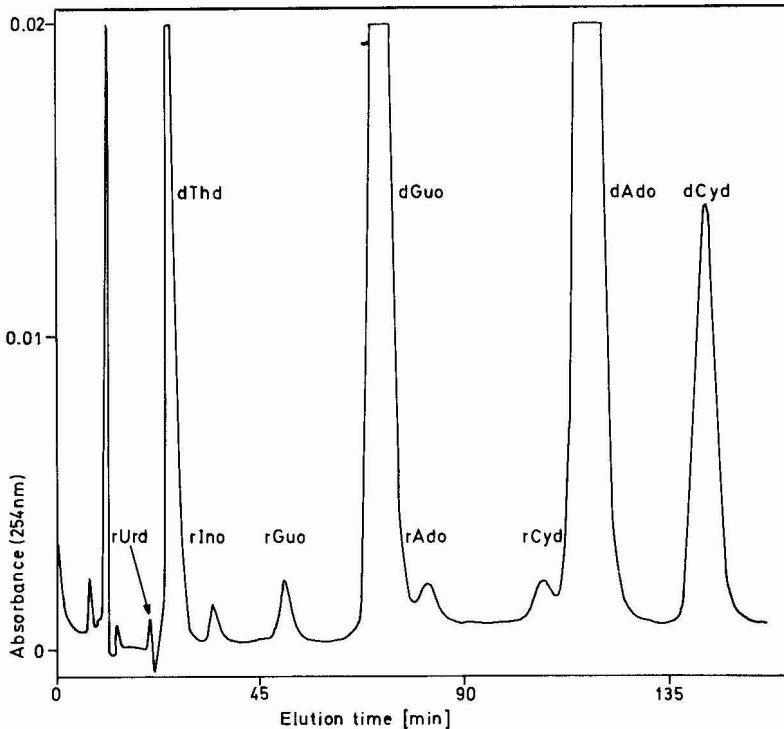
Fig. 2. Elution pattern obtained from the separation of a *Geodia cydonium* DNA hydrolysate containing *ca.* 3% of RNA impurities (for chromatographic conditions see Materials and Methods). Injected volume: 15 μl.

taminated with RNA is shown in Fig. 2. A concentration of *ca.* 3% of RNA in the DNA sample is easily detectable.

The elution pattern of a separation of the ribonucleosides obtained from an enzymatic hydrolysis of RNA from *E. coli* which contained *ca.* 3% of DNA is shown in Fig. 3. As the RNA preparation contains a relatively high amount of rare bases, additional peaks (Ψrd and the unidentified compounds A and B) are obtained in the elution diagram together with the major ribo- and deoxyribonucleoside fractions. To demonstrate the sensitivity of detection 150 pmole of dAdo was added to the sample which was injected onto the column.

As compared to a method of Schrecker *et al.*[16] who used thin-layer chromatography for the separation of deoxyribo-, ribo- and arabinonucleosides, the method described here is much more sensitive. Even unlabelled ribo- and deoxyribonucleosides can be detected in amounts less than 30 pmole.

Aoyagi *et al.*[17] recently described a method for the separation of ribo- or deoxyribonucleosides which, however, required previous group separation[18] if the ribo- and deoxyribonucleoside of a given base were to be distinguished.

A separation method for ribo- and deoxyribonucleosides described by Duch and Laskowski[19] working with an Aminex A-7 column (25 × 0.24 cm I.D.) which is eluted with ammonium formate of various molarities at pH 4.55 gave poor separation of rUrd and dThd. Furthermore, the elution diagrams showed broad peak
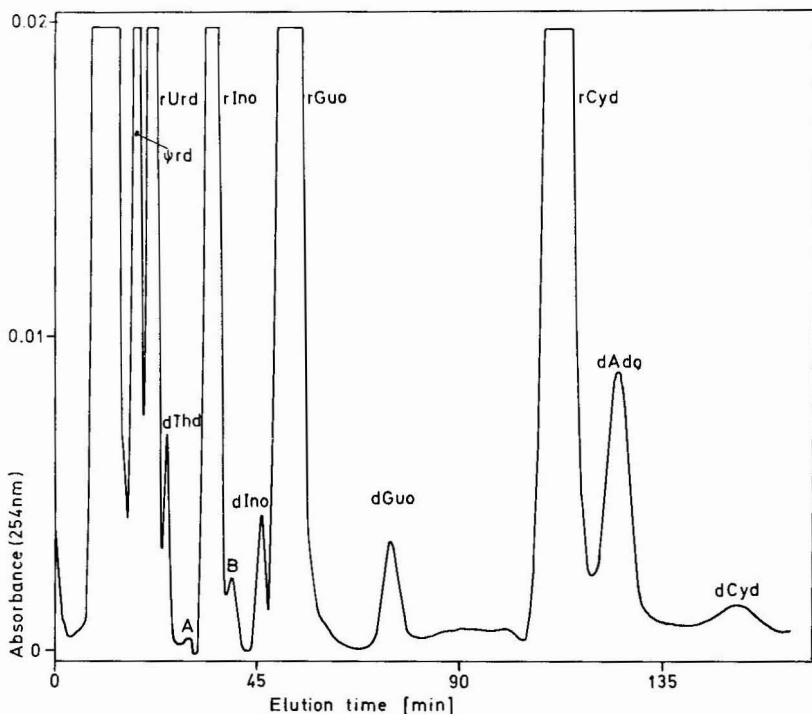
Fig. 3. Elution pattern obtained from the separation of an *E. coli* RNA hydrolysate containing *ca.* 3% of DNA impurities (for chromatographic conditions see Materials and Methods). Injected volume: 30 μl; dAdo (150 pmole) was added to the injected sample.

shapes although the separation was finished within 60 min. Owing to this disadvantage quantitative measurements had to be carried out by cutting the peaks and weighing the paper. Obviously the system was not optimized with respect to the molarity of the eluent, resulting unfavourable values for the capacity factors of strongly retained compounds[20].

The system described here clearly separates rUrd from dThd, and the major ribo- and deoxyribonucleosides from each other, as well as from Ψrd, rIno, dIno, me⁵Urd, and me⁵dCyd. Even at the end of the elution diagram, peak widths are sufficiently narrow although a total separation lasts for *ca.* 3 h. Hence, quantitative determinations of ribo- and deoxyribonucleosides are easily obtained with small standard errors using simple peak-height measurements if an integrator is not available. The sensitivity of this method for the determination of impurities of RNA in DNA samples and *vice versa* was found to be better than 0.07% of a nucleoside.

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Note

Pluronics as liquid phases for capillary gas–liquid chromatography

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A large number of liquid phases have been recommended and, in the interests of communication between laboratories and the reproduction of published work by other workers, it is desirable to limit them to a reasonable number of widely used phases. Furthermore, products with low purity and ill-defined molecular structures should be eliminated.

The introduction of new phases is justified in only two instances: either the new material, owing to its particular molecular structure, is able to fill a gap between existing phases, or it is able to replace an existing phase because of superior characteristics. The Pluronic materials (Fluka, Buchs, Switzerland) belong to the second category. They have occasionally been used as liquid phases in the early years of gas–liquid chromatography (GLC)[1,2], but did not become popular. The introduction of glass capillary columns has led to a further evaluation of known liquid phases and a search for superior products. In our work we re-examined the Pluronics and as a result they have now replaced certain well known phases that we had used for many years.

PLURONIC MATERIALS

Emkalyx-Pluronics (Pluronics) are widely used as detergents. Compared with similar chemicals they are exceptionally pure and structurally well defined. According to information from the original producer, Wyandotte Chemical Corp., Pluronics are made by condensing propylene oxide with propylene glycol. The resulting chain is then extended on both sides by the addition of controlled amounts of ethylene oxide, yielding polar endgroups on a hydrophobic central chain.

The different types available vary in two respects: the length of the polypropylene glycol chain, and the amount of polyethylene glycol added. The molecular weight varies between 1000 and 14,000.

The polarity of the materials increases with increasing percentage of polyethylene glycol. The viscosity increases with molecular weight and the percentage of polyethylene glycol (the materials are generally solids at room temperature if the polyethylene glycol content exceeds 50% of the polymer).

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Polarity range

The polarity of a Pluronic containing 90% of polypropylene glycol is similar to that of Ucon LB 550, whereas that of a Pluronic containing 80% of polyethylene glycol is similar to that of Carbowaxes.

Pluronic L 61

Pluronic L 61 (Pl. 61) contains 90% of polypropylene glycol and has an average molecular weight of 2000. It has chromatographic characteristics almost identical with those of Ucon LB but has several advantages. On a glass surface covered with barium carbonate[3] it shows less bleeding. Its upper temperature limit is about 30° higher; there were no signs of deterioration (higher bleeding, increased adsorption and reduced retention) after heating at 200° for several days. Hence barium carbonate pre-treated columns with Pl. 61 can be used up to a maximum temperature of 220° in programmed runs.

In addition, Pl. 61 also possesses the outstanding low-temperature characteristics of Ucon LB. Its low viscosity at 25° (Brookfield viscosity 285 cP) and its freezing point of −29° lead to a high column efficiency at sub-ambient temperatures with a relatively high retention for a given film thickness.

Pluronic L 64

Pl. 64, a liquid containing 40% of polyethylene glycol and with a molecular weight of *ca.* 2900 has similar chromatographic properties to Ucon HB 5100 and Emulphor ON 870. The reasons for replacing these two phases with Pl. 64 are the same as those mentioned above. The bleeding is very low (again on catalytically inactive surfaces such as barium carbonate), probably owing to its high purity. The upper temperature limit is increased: on neutral or basic supports such as barium carbonate the columns withstand temperatures of 240–250° for several days (depending on the film thickness). On very acidic supports (columns that give no peak for 2,6-dimethylaniline) this limit is decreased (as for all polyglycols) to about 220°, but for short time periods the temperature limit is again about 240°.

The maximum film thickness that is stable on barium carbonate columns is about 0.8 μm; thicker films occasionally form droplets.

Pluronic F 68

Pl. 68 is a solid containing 80% of polyethylene glycol and a molecular weight of 8350. The melting point is 52°. The polarity of Pl. 68 lies between that of Ucon HB or Emulphor and that of Carbowax 20,000, and is closest to that of Ucon H 90,000.

The lower temperature limit is near the melting point of the phase; Pl. 68 behaves very reasonably above 60°. The upper temperature limit was found to be between 250° and 260° for long-term use (barium carbonate surface). This limit is probably due to degradation of the phase. Therefore, it is of no use coating columns with materials of higher molecular weight, with higher low-temperature limits, higher viscosity and no increased thermal stability.

COATING PROCEDURE

As described elsewhere[3] we use columns (Pyrex or soft glass) pre-treated with

concentrated barium hydroxide solution. Deactivation, although not essential, gives some improvement regarding adsorption of polar materials, mainly for Pl. 61 and less for Pl. 64 and 68. We apply a 0.1% solution of Carbowax 1.000 and heat the column after drying it at 280° for 10 min.

The Pluronics can be coated dynamically using methylene chloride solutions of the phases of concentration between 10 and 50% (depending on the film thickness desired), followed by a plug of mercury. For unknown reasons, the mercury plug behind Pl. 64 solutions on neutral or acidic surfaces may cause problems as it acquires a black tail, which is deposited in the column after some time. In this instance we prefer dynamic coating without mercury, using about 50% less concentrated solutions.

A film of 0.1 μm of Pl. 64 is obtained by using about a 15% solution with a mercury plug; 40% solutions without mercury yield films of Pl. 64 of thickness about 0.65 μm. Dynamic coating using Pl. 64 without solvent with mercury at 120–160° yields films more than 1 μm thick, which are too thick to be stable. Columns with the coatings described should show practically no tailing for 1-octanol (depending on the film thickness and deactivation used). On all glycol phases, especially with the polarity of Pl. 61, aldehyde peaks are very sensitive to acid–base effects; on neutral or basic columns they tail or disappear completely. The separation efficiencies are the same as for all columns of similar type. Columns of length 20 m and I.D. 0.3 mm should attain a separation number (TZ, according to Kaiser) of 30–35 when measured with the C_{13} and C_{14} n-alkanes.

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Note

High-performance liquid chromatography of peptides

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Usually, mixtures of peptides are separated by thin-layer chromatography[1] or ion-exchange column chromatography[2], although high-performance liquid chromatography (HPLC) has been used for the isolation of single peptides[3,4,5]. In this communication, a reversed-phase method is described for the separation of small peptides of different polarities and chain lengths. The advantages of octadecylsilane (ODS, reversed-phase) columns in comparison with ion-exchange columns are: (1) they are pressure-stable; (2) re-equilibration of the columns to their initial conditions after use of a gradient is more rapid; (3) bleeding seldom occurs; (4) ODS is resistant to most organic solutes at neutral and acid pH; (5) retention on ODS is less dependent on temperature; and (6) ODS can be re-used many times without deterioration of its resolving properties.

MATERIALS AND METHODS

Peptides (Serva, Heidelberg, G.F.R.), phosphoric acid and potassium phosphate (p.a. quality; Merck, Darmstadt, G.F.R.) were used. Methanol (p.a.; Merck) and water were distilled at least three times before use; the latter with addition of alkaline potassium permanganate during the second distillation to oxidise trace impurities that might give spurious peaks on the chromatogram. The chromatograph assembly (Waters Assoc., Königstein, G.F.R.) consisted of two pumps (model 6000), a programmer (model 660), an injector (model U6K) and a detector (Perkin-Elmer, model LC 55). The column (200 × 4 mm I.D.) was packed by the slurry method at 6000 p.s.i. with 5-μm ODS particles (Nuleosil 5 C-18; Macherey and Nagel, Düren, G.F.R.) and its temperature was maintained at 31° during chromatography. A linear gradient was applied, the initial eluent being 0.05 M potassium dihydrogen phosphate (adjusted to pH 2 with phosphoric acid) and the final eluent being methanol; the gradient was terminated after 60 min. The sample was 5 μl of peptide solution (1 mg/ml), the flow-rate was 3 ml/min, and the detection wavelength was 230 nm.

RESULTS AND DISCUSSION

Preliminary experiments showed that acid pH and the addition of potassium phosphate resulted in narrower peaks and better resolution; probably this is because
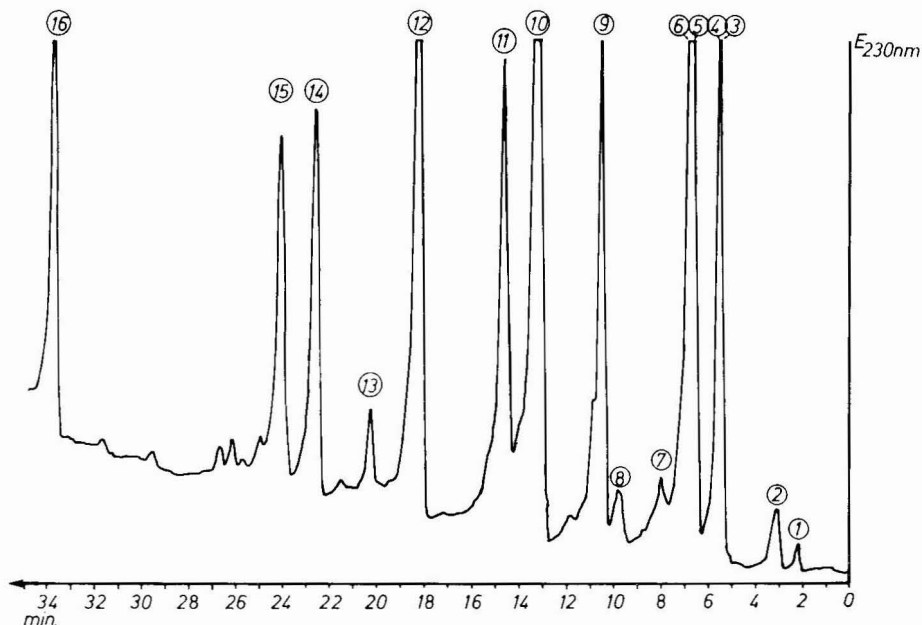
Fig. 1. Chromatographic profile showing separation of a peptide mixture. For conditions of separation see "Materials and Methods", and for identities of numbered peaks see Table I.

TABLE I

SEPARATION OF PEPTIDES BY HPLC

For conditions of separation see "Materials and Methods".

Peak No.	Peptide	Retention time, min
1	Val-Gly	2.1
2	Gly-Val	3.0
3	Tyr-Gly	5.5
4	Leu-Gly	5.5
5	Gly-Tyr	6.8
6	Gly-Leu	6.8
7	Phe-Gly	8.0
8	Gly-Phe	9.8
9	Ala-Ala-Tyr-Ala-Ala	10.5
10	Gly-Trp	13.2
11	Phe-Tyr	14.6
12	Leu-Trp-Met-Arg	18.3
13	Unknown*	20.2
14	Met-Glu-His-Phe-Arg-Trp-Gly	22.5
15	Leu-Leu-Val-Tyr	24.0
16	Renin inhibitor (an octapeptide)	33.6

* Probably an impurity.

peptides are more strongly bound to ODS at acid pH, and thus retention times are increased.

The results are shown in Fig. 1 and Table I, from which it can be seen that only two pairs of the peptides tested were not separated. From the values presented, it can be deduced that the retention time of a peptide in this system is determined by its polarity. The C-terminal amino acid of a dipeptide seems to be of particular importance in this respect, *e.g.*, the retention times of Gly-Tyr and Tyr-Gly are different, the retention time being longer when the less polar Tyr is the C-terminal amino acid; the same is true for other dipeptides. The polarities of some of the amino acids used, as indicated by their solubility in water, is shown in Table II (see also ref. 6). With oligopeptides, the pentapeptide Ala-Ala-Tyr-Ala-Ala, in which the polar Ala predominates, is retained to a much lesser extent than the tetrapeptide Leu-Leu-Val-Tyr, which is composed of non-polar amino acids.

TABLE II

SOLUBILITIES OF SOME AMINO ACIDS IN WATER AT 25°

Amino acid	Solubility, g/100 g of water*
Glycine	24.99
L-Alanine	16.51
L-Valine	8.85
L-Phenylalanine	2.96
L-Leucine	2.19
L-Tryptophan	1.13
L-Tyrosine	0.45

* Values taken from ref. 6.

The chain length of a peptide also influences its retention on the column, oligopeptides usually having longer retention times than dipeptides. However, non-polar dipeptides (*e.g.*, Gly-Phe) are eluted at about the same time as oligopeptides predominantly composed of polar amino acids (such as Ala-Ala-Tyr-Ala-Ala).

The elution profiles so obtained suggest that mixtures of small peptides can be separated by using reversed-phase HPLC as described here.

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Note

Modification of quantitative thin-layer chromatography by elution

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Thin-layer chromatography (TLC) is of interest as a method for quantitative micro-analysis, and many procedures[1] (planimetry[2,3], photodensitometry[4–6], and photometry[7,8]) have been developed. Although the spectrophotometric determination of eluted fractions is generally applicable, appreciable error ($\pm 4\%$) can arise in scraping off and eluting the spots, and some difficulties are encountered with polar samples of low solubility. Here we describe a technique for circumventing these difficulties, and also improving the accuracy of quantitative analysis of substances separated by TLC.

EXPERIMENTAL

Chromatograms were developed on Merck PLC silica gel F_{254} pre-coated plates (5×1.2 cm; layer thickness 2 mm; Catalogue No. 5717/0012). The test mixture was brought into solution and applied to the start as a band from a 10-μl hypodermic syringe; a gentle stream of air was used during sample application to keep the spots less than 2 mm in diameter. Generally, the concentration of the solution was 0.1 to 0.5% (w/v) and 4 to $12\,\mu$l were applied. The chromatogram was developed by the ascending technique in a cylindrical tank lined with filter-paper to saturate the atmosphere with the developing solvent and so prevent the so-called edge-effects[9]. After development, the chromatogram was removed, and dried under reduced pressure for 15 min. The bands of substances being assayed were located in 254-nm radiation and outlined with use of a narrow metal spatula or a blunt pencil.

After the spots had been marked, the plate with the layer on it was divided into small pieces by using a glass-cutter. Each row of spots, and a magnetic-stirrer bar, were placed in a centrifuge tube, and the weight (W_1) of this tube was determined. A 1-ml portion of eluent was added to each tube, and the gel was scraped off the glass with a spatula. Approximately 9 ml of eluent were then added, and the mixtures were stirred for at least 1 h (even for extremely polar substances, stirring for 4 h was sufficient) by means of a rotating electromagnet.

After elution, fine adsorbent particles that could give rise to light scattering were separated by centrifuging (2800–3500 g; 20 min), and the centrifuge tube was re-weighed (W_2). The absorbance of the clear eluate was measured at the appropriate characteristic wavelength. A blank zone of adsorbent was taken from a clean TLC

plate at the same distance from the start as the sample spot had migrated, and this material was used to provide a blank absorbance. The apparent weight of substance in the spot was calculated from the molar absorption coefficient (ε) as follows.

$$W = \frac{A \cdot M \cdot W_s}{d \cdot \varepsilon} \tag{1}$$

where:

    W = the apparent weight (mg) of substance in the spot,

    A = the absorbance,

    $W_s = W_2 - W_1$, the weight (g) of solvent used,

    M = the molecular weight of the substance, and

    d = the specific gravity of the solvent.

From eqn. 1, the extent of extraction (%) is given by $100 W/W_0$ where W_0 represents the true weight of substance applied to the layer. Thus, the weight of substance in a mixture can be obtained from the extent of extraction and W.

RESULTS

Our method has the following features. We used a certain amount of water as co-solvent in the eluent, which led to standard deviations of less than 1 %, even with such polar substances as 4-nitroaniline and an acylurea derivative (I), as shown in Table I. It appears that large deviations (*ca.* $+4\%$) and extraction percentages higher than 100 in non-aqueous tetrahydrofuran or ethanol solution are attributable to the scattering of incident light by dispersed particles of silica gel. The co-existent water

I
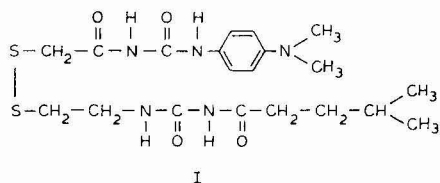
TABLE I

PERCENTAGES OF EXTRACTION[*]

Compound	Eluent[**]	Extraction (%)[***]	Standard deviation (%)
I	100% Tetrahydrofuran	119.9	± 4.1
I	90% Tetrahydrofuran	104.8	± 2.0
I	80% Tetrahydrofuran	101.0	± 1.0
I	70% Tetrahydrofuran	86.0	± 2.0
4-Nitroaniline	100% Ethanol	107.3	± 4.3
4-Nitroaniline	90% Ethanol	105.3	± 0.9
4-Nitroaniline	80% Ethanol	100.5	± 0.8
4-Nitroaniline	50% Ethanol	98.4	± 1.2

  [*] Calculated (see text).

  [**] Percentage of organic solvent in mixture with water when appropriate.

  [***] Average of at least 5 values.

may assist in solubilizing these particles or, conversely, in preventing their dispersion in the solvent. Moreover, we found that the standard deviations increased slightly when the amount of water added as co-solvent exceeded a certain level.

We used 5 × 1.2 cm plates instead of the conventional 20 × 20 cm plates for reasons of economy, and preferred a layer thickness of 2.0 mm to the generally used 0.25 to 1.0 mm in order to prevent "creeping". The results in Table I show that our method is applicable to a wide variety of substances, including highly polar ones difficult to determine accurately by ordinary methods.

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Note

Gas chromatographic determination of methylthiouracil residues in meat and organs of slaughtered animals

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Although most of the European countries prohibit the use of thyroid-inhibiting substances in cattle breeding, this practice is still frequent. The most important thyreostatic used is a cyclic thioureide: 2,3-dihydro-2-thioxo-4-1*H*-pyrimidinone, the 6-methyl derivative of which is usually called methylthiouracil (MTU) and the 6-propyl derivative, propylthiouracil (PTU).

R = CH₃ : MTU
R = C₃H₇ : PTU
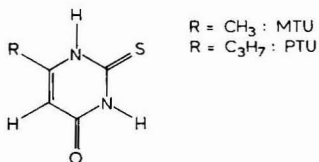
Several methods have been proposed to control the treatment of carcasses before their commercial distribution. One type of method permits the detection of organ modifications in the carcass of an animal fed with a thyreostatic drug, for example histological examination of the thyroid gland[1]. There are also procedures for the detection of residues in tissues of treated animals: colorimetry[2,3], UV detection after one-dimensional thin-layer chromatography (TLC)[4] or after high-pressure liquid chromatography[5] and fluorescence detection after two dimensional TLC[6]. The latter type of method is very sensitive but it does not allow an easy determination of the residues. Therefore we have developed a gas chromatographic (GC) analysis for the determination of MTU residues in animal tissues which makes use of the highly specific flame photometric detector.

EXPERIMENTAL

Apparatus

A Tracor Model 560 gas–liquid chromatograph was used with a flame photometric detector (394-nm filter and response linearizer) and a glass column (1.8 m × 4 in. I.D.) packed with 3% OV-1 on Chromosorb W HP (80–100 mesh). Operating conditions: carrier gas (nitrogen) flow-rate, 35 ml/min; hydrogen flow-rate, 40 ml/min;

air flow-rate, 170 ml/min; inlet temperature, 200°; oven temperature, 180°; detector temperature, 175°.

Solvents and reagents

Analytical-grade solvents and methyl iodide were purchased from Merck (Darmstadt, G.F.R.) and were redistilled in an all-glass apparatus. Reference compounds were obtained from Fluka (Buchs, Switzerland) for MTU and from Aldrich-Europe (Beerse, Belgium) for PTU. Sephadex "lipophilic" LH 20-100 was purchased from Sigma (St. Louis, Mo., U.S.A.). The Sephadex columns were glass tubes (5 mm I.D.) filled with a suspension of Sephadex in benzene–methanol (85:15) to a height of 10 cm and stored in this solvent until use.

Extraction and clean-up

Thyroid gland tissues. A 0.5-g amount of tissue was sampled and placed in an extraction tube; 20 μg of PTU were then added as internal standard (0.1 ml of an ethanolic solution containing 200 μg/ml). After homogenization in 1 ml of distilled water, the mixture was defatted by washing three times with 3 ml of light petroleum (b.p. 40–60°). MTU was then extracted by shaking the mixture three times with 3 ml of ethyl acetate. The combined ethyl acetate fractions were dried under a nitrogen stream on a water bath at 50°. The extract was then dissolved in 0.5 ml of benzene–methanol (85:15), transferred to the top of a Sephadex column and eluted with the same solvent. The first 3 ml which were eluted were discarded and the following 4 ml were collected in a reaction tube.

Other tissues (liver, kidney, muscle or fat). Due to the lower residue level present in these tissues, a 2-g amount was sampled and homogenized in 3 ml of distilled water after addition of 2 μg of PTU as internal standard (0.1 ml of an ethanolic solution containing 20 μg PTU per ml). The following extraction steps were the same as described above for thyroid glands.

Reaction

After evaporation of the 4-ml eluate under a nitrogen stream, 1 ml of a 0.1 M solution of potassium acetate in ethanol and 50 μl of methyl iodide were added to the residue. The reaction tube was stoppered and placed in a water bath at 55° for 70 min. Ethanol was then evaporated and the solid was redissolved in 1 ml of distilled water. The methylation derivatives were extracted three times with 1 ml of benzene. The combined benzene extracts were dried and the residue was finally dissolved in 1 ml of benzene. An amount of 2–5 μl of this solution was injected into the chromatograph. The mean retention times of methylation derivatives of MTU and PTU were 124 and 190 sec, respectively.

Determination

For calibration, the methylation reaction was performed, as described above for tissue extracts, on known quantities of standard (for thyroid gland tissue, 10,20, 40, 60 and 80 μg of MTU and 20 μg of PTU as internal standard; for other samples 0.5, 1, 2, 4 and 8 μg of MTU and 2 μg of PTU). Aliquots of 2 μl of the standard solutions were injected into the chromatograph. The calibration graph was obtained by plotting the ratio of the peak area of MTU to the peak area of PTU as a function of concentration.

RESULTS AND DISCUSSION

Methylation reaction

This reaction was performed in order to transform MTU in a volatile compound. As shown in Table I and II, under the conditions described, mass spectrometry, elementary analysis and proton magnetic resonance spectroscopy were in agreement wih the introduction of two methyl groups into the molecule of MTU. The IR spectrum showed that the carbonyl group remained unchanged. All these data are in agreement with the formulation of the derivative as 3,6-dimethyl-2-(methylthio)-uracil. The same reaction was observed when thiouracil was treated with dimethylformamide dimethylacetal in acetonitrile[7]. We found that the above derivative was also the main product formed on treatment of MTU with dimethyl sulphate in 0.1 *N* aqueous sodium hydroxide, with dimethylformamide dimethylacetal in pyridine (Methyl 8; Pierce, Rockford, Ill., U.S.A.) or with methyl iodide in 1 *N* aqueous sodium hydroxide. However, under all these conditions, side reactions were always observed and other derivatives formed in variable amounts obscured the final chromatogram.

Fig. 1 represents the course of the methylation reaction of 20 μg of MTU and 20 μg of PTU under our conditions. The two compounds were found to react at practically the same rate.

Clean-up

The use of gel chromatography in an organic medium was found to be very useful as a final clean-up step. This procedure allowed us to reduce the number of purification steps. Extracts purified in this way were very clean and interferences were not detected, whatever the tissue analysed. Fig. 2 shows the elution profile of MTU and PTU from a Sephadex column. This column may be reused after careful washing with the elution solvent.

TABLE I

ELEMENTARY ANALYSIS (%) OF THE METHYLATION DERIVATIVE OF MTU

	Calculated	*Found*
C	49.41	49.4
H	5.88	6.0
N	16.47	16.5

TABLE II

PROTON MAGNETIC RESONANCE SPECTRUM IN CDCl$_3$–HEXAMETHYLDISILOXANE
Molecular mass, 170; IR (KBr disc), ν_{CO} at 1667 cm^{-1}.

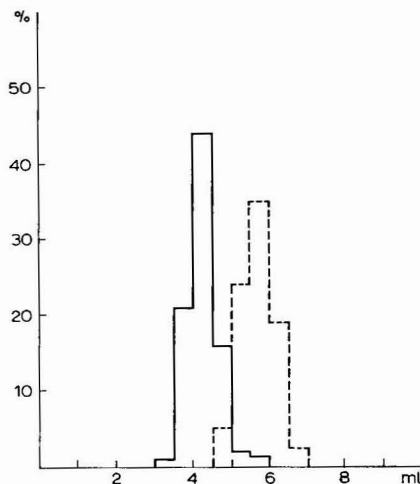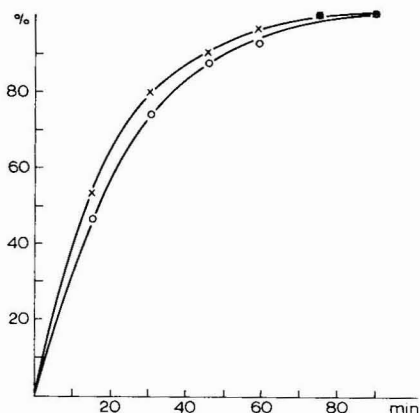| *Chemical shift (ppm)*	*Signal*	*Coupling constant (Hz)*
2.15	3H (*d*) 6-CH$_3$	0.6
2.50	3H (*s*) 2-SCH$_3$	—
3.41	3H (*s*) 3-NCH$_3$	—
5.96	1H (*q*) 5-H	0.6

Fig. 1. Course of reaction of MTU (○) and PTU (×) with methyl iodide.
Fig. 2. Elution profile of PTU (full line) and of MTU (broken line) from a Sephadex column.

Determination

Determinations performed on samples of different tissues to which MTU and PTU had been added showed that the recovery of these two compounds is of the order of 40–60 % depending on the nature of the tissue. The determination of MTU in 10 muscle samples to which 2 μg of MTU had been added gave a mean value of 2.05 μg (S.D. 0.21). The detection limit for MTU was found to be 0.5 ng. For samples containing very low levels of residue, the extract of 2 g of tissue may be reduced to a final volume of 0.2 ml and 5 μl of this solution may be injected into the chromatograph without notable interferences. With a mean recovery value of 50 %, the detection limit is thus of the order of 10 ppb.

CONCLUSION

Our method presents a very simplified clean-up procedure and gives quantitative results very easily. It is also of the same order of sensitivity as the official method used in our country for the detection of thyreostatic residues in meat and organs of cattle[6].

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Note

Gas–liquid chromatographic determination of dextromethorphan in serum and brain

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Dextromethorphan is widely accepted as a safe and effective antitussive agent, for its activity seems generally to be the same as that of codeine both in terms of potency and in mechanism of action. Unlike codeine and related drugs, dextromethorphan and its active metabolite dextrorphan are in a sense unique in that, although chemically related to opioids, they seem to be non-addictive[1–8]. Nevertheless, some information from toxicologists and neuropsychiatrists regarding rapid deterioration owing to habituation to dextromethorphan[9] has stimulated experimental research showing that this agent can prevent morphine abstinence and induce addiction in dog[10], and in the mouse and rat[11].

On the other hand the W.H.O. Expert Committee on Drug Dependence, examining the available reports, considered that the frequency of abuse was so low that it does not constitute a serious social problem. However, the Committee did recommend strict monitoring of dextromethorphan consumption to detect any sign of addiction[12]. Subsequently, a W.H.O. Scientific Group on Opiate Analgesics and Antitussive Agents remarked that, in high doses, dextromethorphan can cause mental changes in some people, although the drug seems to be "little or not addicting" (ref. 13).

Thus we have attempted to achieve a reliable procedure for the qualitative and quantitative evaluation of dextromethorphan and dextrorphan in serum and brain in order to assess whether the addiction induced in animals might be related to the persistence of these agents, or to some conformation changes in the opiate receptors independently of stereospecific binding affinities[14].

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EXPERIMENTAL

Reagents

Dextromethorphan and dextrorphan were obtained from Roche (Basel, Switzerland), imipramine from Geigy (Basel, Switzerland) and chloroform and diethyl ether from Merck (Darmstadt, G.F.R.).

Apparatus

A Carlo Erba Fractovap GV gas chromatograph equipped with a dual flame ionization detector was used. The glass columns (4 mm I.D.) were packed with 3 % OV-17 on Gas-Chrom Q (100–200 mesh) (Carlo Erba, Milan, Italy). Operating conditions: Injection temperature, 280°; column temperature, 250°; detector temperature, 270°; nitrogen flow-rate, 40 ml/min; hydrogen flow-rate, 35 ml/min; air flow-rate, 400 ml/min.

Extraction procedure (Fig. 1)

Rat serum. 20 μl of methanol containing 1000 μg/ml of imipramine (internal standard), 0.2 ml of 1 N NaOH and 8 ml of chloroform were added to 1 ml of serum in a glass centrifuge tube, where they were mixed for 30 sec on a rotatory mixer and then centrifuged for 10 min at 2000 g. The aqueous phase was discarded, and the chloroform layer was transferred to a clean tube and evaporated to dryness under a stream of nitrogen in a water bath at 50°. After evaporation, the residue was redissolved in 50 μl of methanol and 1 μl was injected into the gas chromatograph.
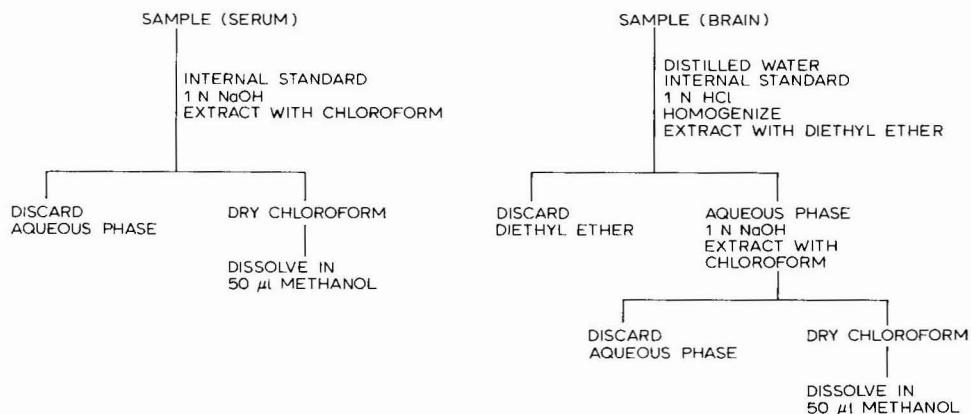

Fig. 1. Outline of the extraction procedure.

Rat brain. 300 mg wet weight of rat brain were homogenized in a glass potter in the presence of 8 volumes of distilled water, 20 μl of methanol containing 1000 μg/ml of imipramine (internal standard) and 0.2 ml of 1 N HCl. The homogenate was transferred to a glass centrifuge tube, and 10 volumes of diethyl ether were added (with respect to the initial fresh tissue). The reagents were mixed for 30 sec on a rotatory mixer and then centrifuged for 20 min at 2000 g. The organic phase was discarded and the aqueous phase was transferred to another centrifuge tube, alcalinized with 0.2 ml of 1 N NaOH and extracted with 6 ml of chloroform by mixing for 30 sec on a rotatory

mixer and centrifuged for 10 min at 2000 g. The aqueous phase was discarded, and the chloroform layer was transferred to a clean tube and finally evaporated to dryness as for the serum extract. After evaporation, the residue was treated in the same way as the serum extract and 1 μl was injected into the gas chromatograph.

Calculations

Known amounts of dextromethorphan (0.5-30 μg) and 20 μg of imipramine (internal standard) were added to serum or rat brain and extracted as previously described. The extracts were chromatographed and calibrations curves were constructed by measuring the peak-area ratio of dextromethorphan to the internal standard. These standard curves (Fig. 2) were used to calculate the unknown concentrations of samples.
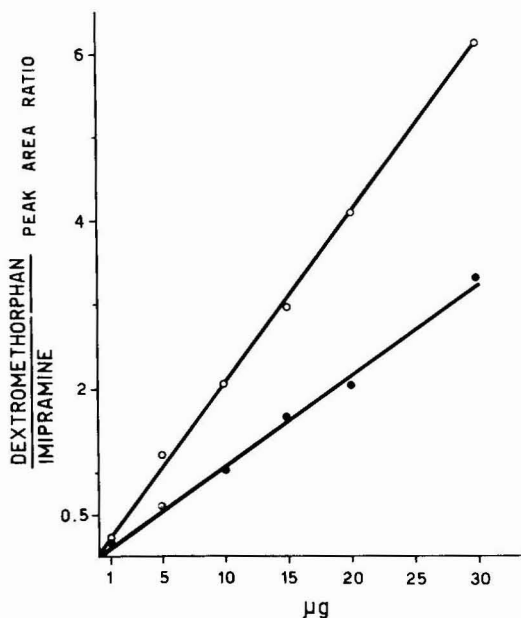

Fig. 2. Standard calibration curves for concentrations of dextromethorphan extracted from serum (○—○) and from brain (●—●).

RESULTS AND DISCUSSION

Under the gas chromatographic (GC) conditions described above, dextromethorphan and imipramine (internal standard) appeared within 6 min in the order shown in Fig. 3. Dextromethorphan exhibited a retention time of *ca.* 4 min, and the internal standard of *ca.* 5 min. The peak area ratio for various concentrations of drug and internal standard were plotted against drug concentration in μg per ml of serum or μg per 300 mg of rat brain and gave good linearity up to 30 μg of drug added. The back-extraction of brain samples, by acidification in diethyl ether followed by alkalinization and re-extraction in chloroform, removed interfering peaks and thus allowed a better measurement of the peak.
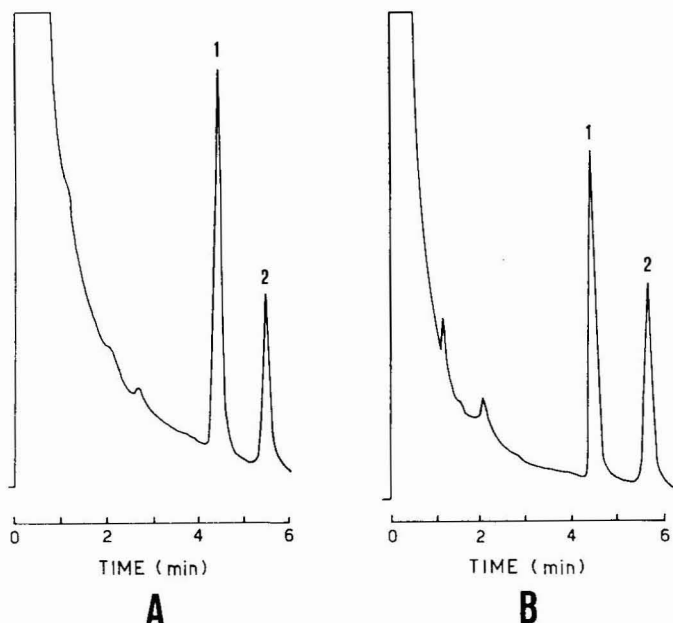
Fig. 3. Gas chromatograms of dextromethorphan (1) and imipramine (2) (internal standard) obtained from serum (A) and brain extracts (B).

The recovery of dextromethorphan from samples and the reproducibility of the method are illustrated in Table I. The reproducibility of the method was tested by analyzing five times a mixture of the two drugs extracted from specimens. The results demonstrate that there is a good recovery and an excellent reproducibility. It was demonstrated that the extracts (stored at room temperature) were stable for almost a week.

TABLE I

RECOVERY OF DEXTROMETHORPHAN FROM RAT SERUM AND BRAIN, AND THE REPRODUCIBILITY OF THE METHOD FROM FIVE ANALYSES

	Serum		Brain	
	10 µg/ml	20 µg/ml	5 µg/300 mg	10 µg/300 mg
Recovery (%) (mean ± S.D.)	85 ± 5.5	97 ± 4.7	87 ± 4.7	93 ± 3.7
Reproducibility	10 ± 0.16	20 ± 1.0	5 ± 0.1	10 ± 1.0

By use of the same procedure, we achieved good results for the determination of the metabolite dextrorphan through a further step in the extraction: the alkaline aqueous phase, after extraction of dextromethorphan, was acidified, then made alkaline (pH 8.5) with solid sodium bicarbonate and finally extracted with ethyl acetate. Dextrorphan exhibited a retention time of ca. 6 min under the above GC conditions. Therefore the peak of the internal standard lies between those of dextro-methorphan and its metabolite.

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Note

Gas–liquid chromatographic determination of pseudoephedrine and norpseudoephedrine in human plasma and urine

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Methods have been described for the identification and determination of pseudoephedrine (PS) in biological materials. Cummins and Fourier[1] made heptafluorobutyryl (HFB) derivatives of ephedrines and by using gas–liquid chromatography (GLC) with an electron capture detector (ECD) were able to analyze blood levels in man. A different approach was that of Kuntzman *et al.*[2] who made an acetyl derivative of PS with tritiated acetic anhydride and, after thin-layer chromatography, quantified the drug by scintillation counting. Bye *et al.*[3] determined the plasma concentration of PS by GLC using a nitrogen-sensitive detector. We modified the previously published GLC–ECD assay[1] of the HFB derivatives of the ephedrines to allow determination of PS and its observed metabolite, norpseudoephedrine (NPS) in plasma and urine of children with renal tubular acidosis.

EXPERIMENTAL

Reagents

Benzene AR grade, 0.01 N hydrochloric acid, 4 N sodium hydroxide, pyridine sequential grade (Pierce, Rockford, Ill., U.S.A.) and heptafluorobutyric anhydride (HFBA) (Pierce) were used.

Apparatus

A Varian Aerograph Model 1400 GLC instrument equipped with a scandium tritide ECD and Varian A-25 recorder was used. The chromatographic column was 1/8 in. O.D. × 6 ft. glass, packed with 3% OV-17 on 100–120 mesh Gas-Chrom Q.

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The column was conditioned for 24 h at 275° before using. Nitrogen was used as the carrier gas at a flow-rate of 6 ml/min. The injector and detector temperatures were 210° and 200°, respectively. The column temperature was 150°.

Procedure

Biologic samples, 0.2 ml of serum or 0.1 ml of urine, were placed into 20-ml test tubes to which 0.2 ml of a stock solution of 1-(1)-methylbenzylamine (internal standard) and 0.1 ml of 4 N NaOH were added. This mixture was extracted with 3 ml of benzene for 30 min using a rotatory extractor. Following centrifugation at 1000 g for 5 min after extraction, 2 ml of the benzene solution was removed and placed in another tube with a PTFE-lined cap. To this tube 0.1 ml of 10% pyridine (diluted with benzene) and 0.02 ml of HFBA were added; the samples were mixed on a Vortex mixer for 20 sec and the tubes were capped. The mixture was allowed to stand at room temperature for 4 h, and then washed three times with 3 ml of cold 0.01 N HCl.

Standard curves were derived from assays of duplicate samples of plasma and urine in the concentration ranges 0.14 to 0.84 μg per 0.2 ml for PS, 0.10 to 1.30 μg per 0.2 ml for NPS and 0.05 to 0.5 μg per 0.2 ml for ephedrine (E). The concentration of the ephedrines was obtained by calculating the ratio of peak heights of the ephedrines to that of the internal standard and relating this to a constructed calibration curve of ephedrines in plasma or urine. Plasma and urine samples were frozen for varying periods of time and ephedrines content determined periodically.

RESULTS AND DISCUSSION

Well-resolved symmetrical peaks were obtained for the ephedrines (Fig. 1). PS, E and NPS were differentiated and identified by their retention times. However, the method did not separate NPS from norephedrine (NE). The retention time for the internal standard was 2.8 min, 3.8 min for NE and NPS, 5 min for E, and 7.5 min for PS. This method has the sensitivity required to determine the serum and urine levels of PS in man at the doses used therapeutically (5 mg/kg). As little as 0.7 ng of PS could be easily detected, smaller levels could be measured if necessary by utilizing larger samples.

Because only one extraction was required before derivatization, the method lent itself to multiple sample analysis with minimal effort and equipment. The ECD response was linear between 0 and 1000 ng for the HFB derivatives of PS and NPS. Plots of peak height ratio to concentration of PS, NPS, and E (μg per 0.2 ml plasma) are shown in Fig. 2. The linear equations for each plot (concentration in μg per 0.2 ml) and the regression coefficients are:

Peak height ratio of PS = 0.010 + 1.770 conc. ($r^2 = 0.9967$)

Peak height ratio of NPS = 0.039 + 0.707 conc. ($r^2 = 0.9953$)

Peak height ratio of E = $-0.001 + 0.676$ conc. ($r^2 = 0.9966$)

No significant difference was noted between standard curves prepared from urine or plasma samples as indicated in Table I for PS. Twelve repeat analyses of a plasma sample containing 0.84 μg per 0.2 ml of PS gave a mean concentration of 0.849 and a standard deviation of 0.018.

The benzene extracts contain less water and other undesirable products, so the derivatization of ephedrines was accomplished without the need for several acid–
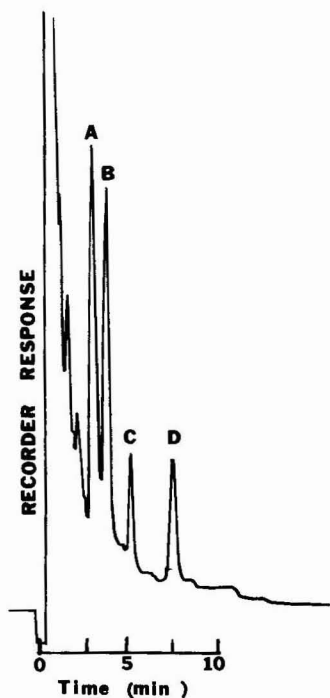
Fig. 1. Chromatogram of the HFB derivatives of L-(−)-d-methylbenzylamine (internal standard) (A); DL-norpseudoephedrine (B); ephedrine (C); pseudoephedrine (D).
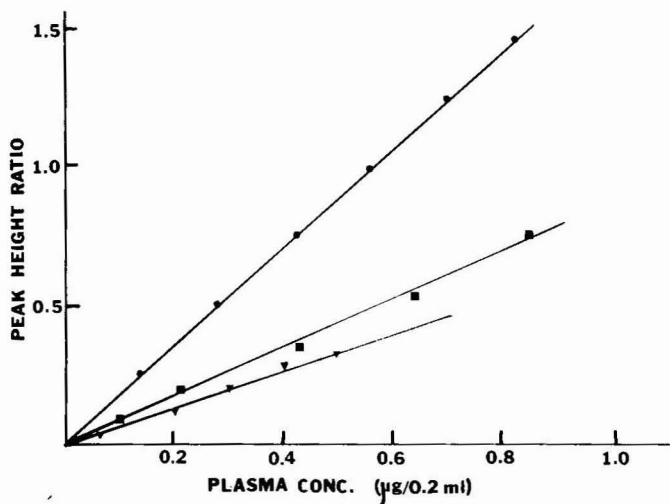

Fig. 2. Calibration curves relating the peak ratios to the concentration of PS (●), NPS (■), and E (▼) present in 0.2-ml plasma samples.

TABLE I

PEAK HEIGHT RATIO *VS.* CONCENTRATION FOR PSEUDOEPHEDRINE IN DUPLICATE PLASMA AND URINE SAMPLES

Concentration (μg per 0.2 ml)	Peak height ratio	
	Plasma	Urine
0.14	0.26	0.26
	0.26	0.24
0.28	0.48	0.52
	0.50	0.50
0.42	0.75	0.76
	0.74	0.70
0.56	1.00	0.97
	1.07	0.99
0.70	1.27	1.25
	1.25	1.25
0.84	1.47	1.47
	1.48	1.48
Slope	1.770	1.754
Intercept	0.010	0.006
r^2	0.9967	0.9982

base purification steps and drying of the solvent extract. The acid extraction after derivation removes both the pyridine and excess HFBA; thus no interfering substances were found in the many samples analyzed and no deterioration of the ephedrines occurred with storage or freezing (see Table II). The acid washes carried out under cold conditions prevent any hydrolysis that might occur. The HFB derivatives of the ephedrines themselves are stable for weeks in benzene at room temperature.

The method described for analysis of ephedrines with a very sensitive Sc^3H ECD allowed detection of nanogram amounts of ephedrines in plasma and urine. Only small amounts of samples are required. This results in a simplified extraction

TABLE II

STABILITY STUDY OF PSEUDOEPHEDRINE IN FROZEN PLASMA AND URINE SAMPLES

Day	Measured concentration	
	Plasma (μg per 0.2 ml)	Urine (μg per 0.1 ml)
0	0.22	0.36
2		0.36, 0.39
3	0.22	
4		0.35, 0.35
6	0.23	
7		0.35, 0.36, 0.38
10		0.36, 0.35
20	0.22	0.35, 0.35
33	0.20	
39	0.22	
60	0.24	

procedure and reduces the amount of interfering substances in the samples. A typical plasma concentration–time curve of PS following oral administration of 5 mg/kg dose to a normal volunteer is depicted in Fig. 3. Our laboratory is currently utilizing this GLC assay to characterize the pharmacokinetics and the determinants of renal excretion of PS and NPS. We are studying the possibility of both urine pH and flow dependent excretion kinetics.
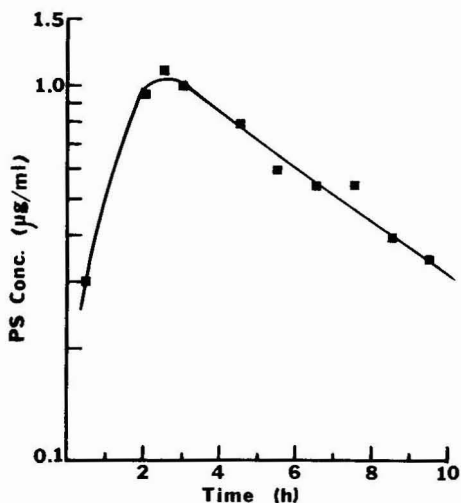

Fig. 3. Typical serum concentration–time curve for pseudoephedrine after oral administration of 5 mg/kg to a normal volunteer.

ACKNOWLEDGEMENT

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Note

Gas chromatographic method for the determination of dextropropoxyphene and nordextropropoxyphene in human plasma, serum and urine

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Cases of acute poisoning by overdosing of dextropropoxyphene, which is a widely used analgesic, have appeared with growing frequency during the past few years. A method for the relatively rapid determination of dextropropoxyphene and its major metabolite nordextropropoxyphene is thus required. Previously described gas chromatographic (GC) methods[1–3] are based on determining dextropropoxyphene and nordextropropoxyphene (Fig. 1) without derivatization, and great problems with on-column decomposition have been described[4–6].
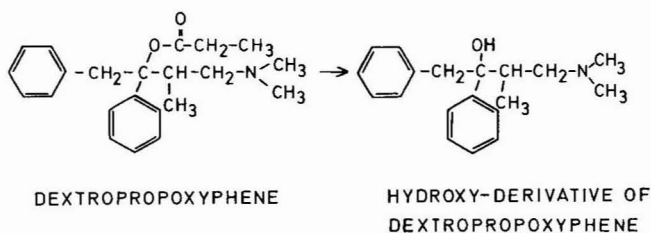

Fig. 1. Structural formulae of dextropropoxyphene and its hydroxy derivative.

This paper describes a method which solves these problems by reduction of the drugs before gas chromatography, using the easily handled and non-explosive sodium bis-(2-methoxyethoxy)aluminium dihydride[7]. The method is specific for the determination of dextropropoxyphene and nordextropropoxyphene, and has a reasonable reproducibility and sensitivity.

MATERIALS AND METHODS

Materials

Dextropropoxyphene hydrochloride was a gift from A/S Alfred Benzon Copenhagen, Denmark), nordextropropoxyphene hemicitrate and α-*d*-pyrroliphene hydrochloride (internal standard) were gifts from the Lilly Research Laboratories (Indianapolis, Ind., U.S.A.). The reagents were of analytical quality except for the sodium bis-(2-methoxyethoxy)aluminium dihydride, which was Merck zur Synthese (Merck, Darmstadt, G.F.R.). This reagent was diluted 1:10 with dry toluene just before use. The toluene was dried over sodium-lead alloy (Merck) for at least 12 h before use.

Extraction and reduction procedure

To 2.5 ml of plasma was added 1.0 ml of 1 *M* carbonate buffer, pH 10, 200 μl of pyrroliphene solution (0.13 mmole/l) and 5.0 ml of *n*-butyl chloride. The mixture was shaken for 5 min, and after centrifugation for 5 min at 1300 *g* and cooling in a dry ice–acetone bath for 1 min, the *n*-butyl chloride phase was easily decanted into a fresh glass vessel with 2.0 ml of 0.6 *N* HCl. After shaking for 5 min followed by centrifugation the *n*-butylchloride phase was aspirated off and discarded. The acid phase was made alkaline by the addition of 600 μl of 20 % NaOH. After mixing, 5.0 ml of chloroform was added. The chloroform–NaOH mixture was shaken for 5 min, and after centrifugation for 5 min the aqueous phase was aspirated off and discarded. The chloroform phase was filtered through phase-separating paper and evaporated to dryness at room temperature under nitrogen. The residue was dissolved in 3 ml of dry toluene. Under a weak stream of nitrogen it was reduced by 2 ml of 6 % (0.3 mole/l) sodium bis-(2-methoxyethoxy)aluminium dihydride. This reagent was added dropwise, and the mixture was heated to dryness at 100° (*ca.* 15 min) while being frequently shaken. After cooling, 3 ml of water was added slowly, and subsequently 5 ml of methylenechloride, and the mixture was shaken for 5 min. After centrifugation the water phase was aspirated off and discarded. The methylene chloride phase was filtered through phase-separating paper and evaporated to dryness at room temperature under nitrogen. The residue was dissolved in 25 μl of ethylacetate (containing 1 % of triethylamine). Samples of 0.7 μl were analysed by GC. Quantitation was by peak height ratio.

Gas chromatography

A Model 900 gas chromatograph equipped with a nitrogen–phosphorus detector (Perkin-Elmer, Norwalk, Conn., U.S.A.) with the following operating conditions: a 1.8 m × 2 mm I.D. glass column was packed with 2.8 % OV-210 and 3.2 % OV-1 on 80–100 mesh Chromosorb W HP; carrier gas (helium) flow-rate, 40 ml/min; air flow-rate, 135 ml/min; hydrogen flow-rate, 2.7 ml/min; injector and detector temperatures, 300°; the oven was programmed from 210° to 230° at 10°/min. The rubidium bead heating was set at 6.5–7.5.

RESULTS

Evaluation of the analytical procedure

Fig. 2 shows chromatograms obtained from plasma analysis. Exactly the same chromatograms are seen when serum or urine is analysed and in no cases are there interfering peaks from the blank (Fig. 2A).

Plots of the standard curves of the two drugs over the range 1–30 μmole/l were linear and passed through the origin. The same curves are obtained when urine or serum is analysed.

The reproducibility of replicate analyses of plasma samples containing different concentrations is recorded in Table I. Table II compares these results with those from a mass fragmentographic method[8].

Specificity

The following drugs were investigated for possible interfering peaks in the
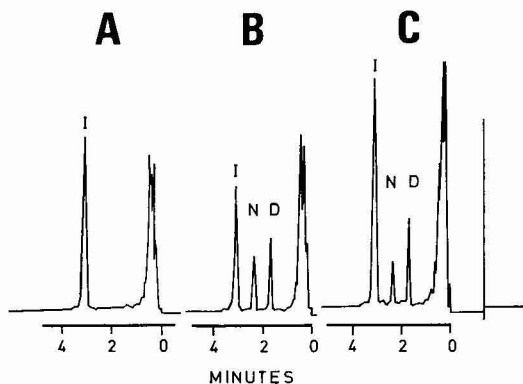
Fig. 2. Chromatograms of human plasma extracts, analysed as described in the text. A, Blank plasma; B, plasma to which dextropropoxyphene and nordextropropoxyphene were added (5.3 and 3.9 μmole/l, respectively); C, plasma from a patient overdosed with dextropropoxyphene. D = Dextropropoxyphene; N = nordextropropoxyphene; I = internal standard.

TABLE I

REPRODUCIBILITY OF REPLICATE SIMULTANEOUS ANALYSIS (MEANS FROM FIVE SAMPLES OF EACH CONCENTRATION) OF DEXTROPROPOXYPHENE HYDROCHLORIDE AND NORDEXTROPROPOXYPHENE HEMICITRATE ADDED TO HUMAN PLASMA

Compound	Plasma concentration (μmole/l)	Coefficient of variation (%)
Dextropropoxyphene	1.0	8.5
	4.9	2.2
	16.0	4.0
Nordextropropoxyphene	1.0	4.6
	4.9	4.9
	11.7	8.3

TABLE II

DETERMINATIONS OF STANDARDS PREPARED BY ADDING DEXTROPROPOXYPHENE AND NORDEXTROPROPOXYPHENE TO HUMAN PLASMA

A, Expected value; B, our results; C, results from a mass-fragmentographic method[8].

Sample No.	Dextropropoxyphene (μmole/l)			Nordextropropoxyphene (μmole/l)		
	A	B	C	A	B	C
1	1.2	1.3	1.4	0.83	0.8	0.88
2	0	0	0	0.89	1.0	0.86
3	6.1	6.2	6.6	4.1	4.4	4.2
4	0	0	0	4.4	4.7	4.2
5	12	11.8	13	8.3	8.9	8.5
6	0	0	0	8.9	9.6	9.1

same region as dextropropoxyphene, nordextropropoxyphene and pyrroliphene: benzodiazepines, barbiturates, common phenothiazines, common thioxanthenes, common tricyclic antidepressants and the most common drugs of abuse (codeine, methadone, morphine, ketobemidone, dextromoramide, pethidine and cocaine).

Only one of these drugs, chlordiazepoxide, showed peaks which might interfere with the determination of dextropropoxyphene. However, by changing the temperature programming, it was possible to separate the peaks.

DISCUSSION

The great advantage of this method is that it overcomes all problems of on-column decomposition. It has been performed without trouble by different technicians during the past 18 months. The time taken to process 18 samples (extraction and reduction procedure) is *ca.* 6 h, and a little less for fewer samples.

The lower limit of detection in our earlier published method [6] was *ca.* 5 μmole/l, because of interfering peaks. Use of a nitrogen–phosphorus detector led to the disappearance of these peaks and the limit was lowered to 0.25 μmole/l. Changing the column from 3% OV-17 to a mixture of 2.8% OV-210 and 3.2% OV-1 improved the linearity from 0.25–15 μmole/l to 0.25–30 μmole/l. This range of detection is sufficient for toxicological plasma (serum) determinations. Urine samples, however, frequently require dilution before the determination.

In 25 patients who had taken overdoses of dextropropoxyphene, the plasma levels of dextropropoxyphene and nordextropropoxyphene were determined to 0.4–9.2 μmole/l and 0.7–8.6 μmole/l, respectively.

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Note

Photolysis of volatile nitrosamines* at the picogram level as an aid to confirmation

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Many nitrosamines (NAs) are potent animal carcinogens[1]. They also have been suggested as a potential source of environmental carcinogens for humans. Considerable worldwide research effort is underway to isolate, quantitate, and identify these substances in foodstuffs. The usual method of separating volatile NAs from other food components involves extraction, distillation, solvent partitioning, and column and gas–liquid chromatography (GLC)[2]. Nitrogen-specific detectors, such as the Coulson or Hall and the alkali flame ionization detectors, have been used for NA determination, but they lack specificity. Fine *et al.*[3] developed a thermal energy analyzer (TEA) which can be used as a detector for GLC[4] and high-pressure liquid chromatography[5]. The TEA is claimed to be specific for NAs since the N–NO group is cleaved by pyrolysis, then the liberated NO radical is reacted with ozone to yield a chemiluminescent response[6]. Despite this, a number of non-nitrosamines, particularly nitro compounds, give a weak TEA response[7]. In addition, because of the great sensitivity of the instrument, picogram quantities of NAs can be detected at concentrations too low for mass spectral (MS) confirmation when present in samples derived from natural products. As an aid in determining whether these small GLC–TEA peaks are NAs, we developed a procedure based on the rapid and complete photolytic decomposition of NAs by ultraviolet (UV) light[8,9]. The method and results obtained in the analytical nitrosamine system used in our laboratory are reported herein.

EXPERIMENTAL

Reagents

All solvents and chemicals were reagent grade or better and were used without further purification. They were checked by GLC to ensure the absence of impurities.

Photolysis

Standard thin-walled melting point capillary tubes (1.6–1.8 × 100 mm) (Kimax-51*** or equivalent) were thoroughly washed with acetone, dried, and sealed at

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* *Note*: Nitrosamines are potentially carcinogenic and should be handled with care.
** Agricultural Research Service, U.S. Department of Agriculture.
*** Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

one end prior to use. Twenty microliters of solutions, containing either the following seven NAs: dimethyl-, methylethyl-, diethyl-, methylbutylnitrosamine, nitroso-piperidine, -pyrrolidine, and -morpholine, or sample extract, were introduced into the tubes using a 25-μl syringe. This volume was selected for convenience although 10–60 μl could be used. After sample introduction, the tubes were immersed in liquid nitrogen and sealed with a small flame. Incomplete closure could result in solvent evaporation during photolysis and give poor results. The tubes were then placed in a Chromato-Vue cabinet on a platform *ca.* 5 cm from a 366-nm UV lamp. Sample controls consisted of tubes covered with aluminium foil or other opaque material placed adjacent to those being photolyzed. After the appropriate time interval, the tubes were opened and the solutions were removed with a 10-μl syringe.

Apparatus

Approximately 6 μl of the test material were injected into a Varian-Aerograph Model 2740 gas chromatograph interfaced with a TEA. The 275 cm × 3 mm stainless-steel column was packed with 16 % Carbowax 20M-TPA on 60–80 mesh Gas-Chrom P. The column was programmed from 130 to 190° at 4°/min with an argon carrier flow of 85 ml/min. The injector port temperature was 190°. The TEA operating conditions were: catalytic pyrolyzer at 450°, cold trap at −150° and a vacuum of 2 mm Hg.

Nitrosamine analysis

The procedure for the determination of volatile NAs is a modification of the multidetection method described by Fazio *et al.*[10], with only minor changes in the quantity of some of the chemicals used. Fried bacon or other product (25 g) was used with 0.5 μg of methylethylnitrosamine (MENA) added as an internal standard prior to digestion with methanolic KOH or direct extraction with methylene chloride. A 1.0 ml methylene chloride concentrate was obtained for GLC separation and quantitation. For these experiments 40 μl of concentrate were used for the photolyzed and unphotolyzed (control) tubes and the rest was reduced to *ca.* 0.1 ml for confirmation by GLC–high-resolution MS under conditions previously reported[11].

RESULTS AND DISCUSSION

An average of 50 % of the seven NAs disappeared within 10–15 min when we subjected a methylene chloride solution containing 0.5 ng/μl of each NA to the experimental conditions for photolysis. After 60 min, none of the NAs could be detected.

To determine the effect of solvent, we photolyzed 1 ng/μl of the same NAs in hexane, methanol, or water. The NAs in hexane disappeared completely within 60 min. After 90 min, NA decomposition ranged from 98 to 100 % in methanol and from 82 to 92 % in water. For photolysis in the latter two solvents under these experimental conditions, a minimum of 2-h exposure to UV light is recommended. The photolytic stability of NAs, particularly in aqueous solution, has been reported[12,13]. While the addition of acid may facilitate NA photolytic degradation[14,15], we found it was not necessary for our purposes.

We photolyzed decreasing concentrations of the seven NAs in methylene

chloride to determine a minimum level at which this technique could still be utilized. Decomposition was complete at all concentrations. We measured a minimum of 12 pg/μl of each NA, or a total of 250 pg, under our usual GLC and detection conditions. An increase in injection size and lower TEA attenuation would significantly increase the sensitivity of this method.

We then applied this photolysis procedure to samples derived from fried bacon. The chromatograms of the same sample unphotolyzed and following UV irradiation (Fig. 1) show three peaks of interest. The peak at 4.5 min is the MENA internal standard and is equivalent to 60% recovery of this NA. Methylethylnitrosamine is not normally found in fried bacon. The other peaks correspond to the retention times of dimethylnitrosamine (DMNA) and nitrosopyrrolidine (NPy). The concentration of the two NAs is equivalent to 2 μg/kg DMNA and 6 μg/kg NPy with respect to the quantity of fried bacon used. After photolysis, the three peaks disappeared thus providing additional presumptive evidence of the presence of these NAs. Under our conditions, 3 μg/kg or greater of NA can be confirmed by MS with a high degree of reliability, except when the sample contains a large amount of interfering material that prevents the determination of the exact mass of the parent ion. In this particular sample, NPy could easily be confirmed by MS but the small amount of DMNA would make MS confirmation extremely difficult without additional sample scale-up and clean-up. This method has been successfully applied to the analysis of volatile NAs in other food products and types of materials including fried bacon drippings, cured sausage, fish and cheese products, tobacco smoke condensate, deionized water, gastric contents, and saliva.
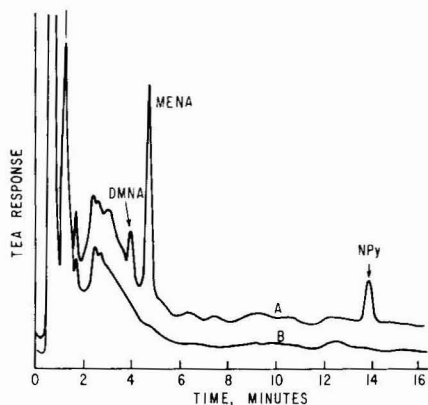

Fig. 1. GLC–TEA chromatograms of 5.8 μl methylene chloride concentrate from fried bacon containing 50 pg/μl DMNA, 300 pg/μl MENA and 144 pg/μl NPy. A, unphotolyzed; B, after photolysis (vertical scale offset for A and B).

The sample solutions must be transparant to UV light. The efficiency of denitrosation is adversely affected by the presence of highly colored material and suspended matter. Fortunately, the use of an added NA to obtain recovery data for the separation and isolation procedure is a good indicator to determine the completeness of the denitrosative degradation. We occasionally observed skewed shaped peaks, having a retention time greater than NPy in photolyzed but not in unphotolyzed fish samples.

These peaks, probably secondary reaction products, do not significantly affect the utility of the photolysis procedure. We also found several samples that gave a TEA response in the GLC region where volatile NAs would be expected to elute. These peaks did not disappear upon photolysis, thus indicating that they were probably not due to NAs. The identification of these compounds is currently under investigation.

For very low levels of NAs that cannot be confirmed by MS or for researchers without mass spectrometer facilities, photolysis at 366 nm of a small portion of the sample concentrate offers a simple, rapid, and sensitive method for the presumptive evidence of NAs. It is desirable to obtain MS confirmation of NAs where possible, because this is still the best method for determining the identity of these compounds at present. However, photolysis affords an alternate means of validating the positive response of the TEA, providing information concerning the possible presence of heterofore unknown NAs, and identifying the response due to non-NAs.

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Note

Rapid and sensitive method for the determination of antipyrine in biological fluids by high-pressure liquid chromatography

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Although no longer used as an antipyretic and analgesic drug, antipyrine has been widely employed recently as a drug for various pharmacokinetic studies. It has been used to study the influence of diseases[1–3], environmental factors[4], diet[5], drugs[6] and genetic factors[7,8] on drug metabolism in man. Antipyrine is well suited for this purpose as it is almost completely absorbed after oral administration, extensively metabolized, mainly by hydroxylation, and all of the metabolites formed are excreted in the urine[9,10]. Its rate of decline in plasma, which is taken as a measure of the drug-metabolizing capacity of the individual tested, correlates closely with the rate of formation of one of its main metabolites, 4-hydroxyantipyrine[11]. At doses between 1.8 and 20 mg per kg body weight its plasma half-life is not dose-dependent[12].

In most studies, the method of Brodie *et al.*[13] for the determination of antipyrine has been used. This method is rather time consuming, requires 2 ml of plasma and is of low sensitivity which means that, in spite of high doses of 20 mg/kg, levels can be measured for not longer than 18–24 h. Under certain conditions, for example uremia, some metabolites seem to interfere with the measurement of the parent compound, mimicking a prolongation of half-life where, in fact, when measured by a more specific gas–liquid chromatographic (GLC) assay, the half-life was appreciably decreased[3]. Several GLC assays have been described, requiring between 0.5 and 3 ml of plasma or saliva and an evaporation step, and the lower limit of detection is *ca.* 0.5–2.0 μg/ml (refs. 14–18).

Recently, a radioimmunoassay for the determination of antipyrine was reported which requires small plasma samples and is of high sensitivity[12]. Since the antibody is not commercially available, this method requires the preparation of the antibody which may cause some problems. Welch *et al.*[19] recently described a sensitive method using thin-layer chromatography and spectrodensitometry.

We report a method which we think is superior to the methods published. This method uses high-pressure liquid chromatography and is sensitive, specific, accurate and rapid. With this method, *ca.* 1500 samples have been analyzed to our satisfaction during the last 1.5 years.

MATERIALS AND METHODS

Chemicals

All of the chemicals and solvents used were of analytical grade and were purchased from Merck (Darmstadt, G.F.R.).

Apparatus

The liquid chromatographic system consisted of a Spectra Physics 3500 B liquid chromatograph. A Zeiss PM 2 LC spectraphotometer (cell volume, $10 \mu l$; path length, 10 mm) was used as the UV detector and operated at 254 nm. A stainless-steel column (250 mm × 6 mm O.D. × 3 mm I.D.) packed with $5\text{-}\mu m$ spherical silica gel (Spherisorb 5μ; Spectra Physics, Darmstadt, G.F.R.) was used. The mobile phase was dichloromethane–methanol–ammonia (25% in water) (98:1.8:0.2) with a flow-rate of 1.6 ml/min (pressure, 80 bar). The mobile phase was degassed by ultrasonification and the system was operated at room temperature.

Procedure

Plasma or saliva (0.5 ml) was pipetted into a tapered test-tube and 2.5 μg of the internal standard, 4-aminoantipyrine (dissolved in 20 μl of distilled water) and 0.1 ml of 0.1 N NaOH were added. The sample was mixed for a few seconds on a Vortex-type mixer and 1 ml of dichloromethane was added. The contents of the tube were again mixed for 2 min (*e.g.*, on a Heto Rotamix test-tube rotator). After centrifuga-tion (1500 g), the aqueous phase was aspirated and an aliquot portion of the organic phase (*ca.* 100 μl) was injected on to the column.

RESULTS AND DISCUSSION

Under the chromatographic conditions described, the following chromato-graphic parameters were observed at a linear velocity of 0.40 cm/sec.

	antipyrine	*4-aminoantipyrine*
Retention time	4.6 min	6.4 min
Capacity factor, k'	3.5	5.0
Number of plates, N	2500	2400
HETP	0.1 mm	0.1 mm

The known metabolites of antipyrine, norantipyrine, 4-hydroxyantipyrine, 3-hydroxymethylantipyrine and 3-carboxyantipyrine, were all separated from anti-pyrine and 4-aminoantipyrine and did not interfere with the determination of anti-pyrine. Interfering peaks could not be detected in analyses of plasma samples of non-medicated members of the staff or of patients receiving a variety of drugs (phenytoin, primidone, phenobarbital, carbamazepine, phenacetin, aspirin, digoxin, vitamins or penicillin).

Quantitation was done by use of a Hewlett-Packard Model 3850 A integrator, from the peak-area ratios of antipyrine to the internal standard, 4-aminoantipyrine. A linear relation was obtained for the range tested (0.1–30 μg/ml). Almost the same accuracy was obtained by peak-height ratio calculation.

Fig. 1 shows the chromatograms obtained from plasma and saliva before and after the oral administration of an aqueous solution of 10 mg per kg antipyrine to a volunteer.

Precision

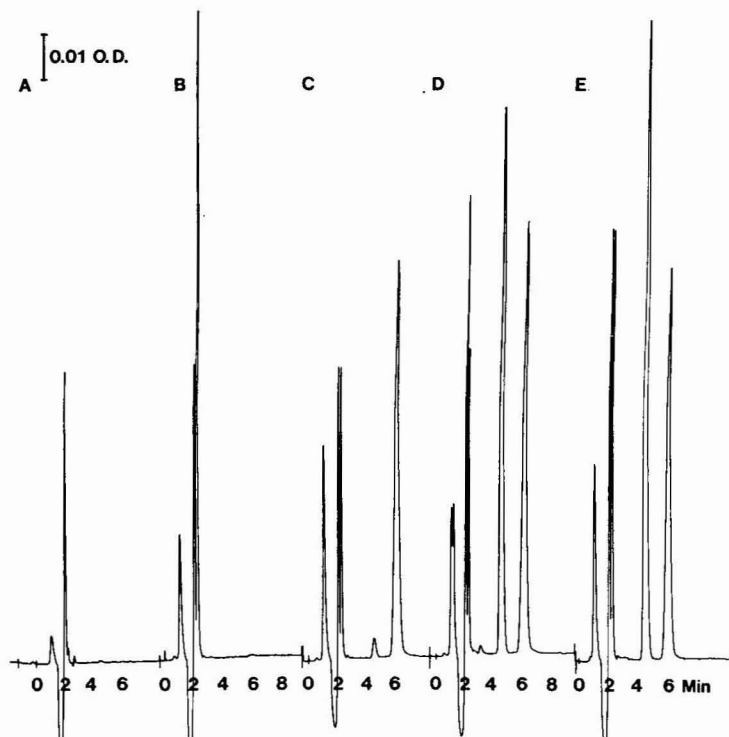The precision of the method for the determination of antipyrine was obtained

Fig. 1. Chromatograms of plasma and saliva extracts. Aliquots of 50 μl of plasma or saliva extract were injected. Serum (A) and saliva (B) blanks were recorded before the administration of antipyrine. The retention times were 4.6 min for antipyrine and 6.4 min for 4-aminoantipyrine. Extracts from plasma containing 0.12 μg/ml (C) and 2.5 μg/ml (D) of antipyrine, and from saliva containing 3.3 μg/ml of antipyrine (E) were then chromatographed.

by analyzing pooled plasma and saliva containing 5 and 0.5 μg/ml. The coefficient of variation (10 samples of each concentration were analyzed) was ± 3.5% for 5 μg/ml and ± 4.8% for 0.5 μg/ml antipyrine, respectively. Day-to-day precision obtained by analyzing pooled plasma and saliva containing 5 μg/ml antipyrine at different days over a 6-month period was ± 3.9% (n = 30).

Accuracy

The accuracy of the method was checked by adding known amounts of antipyrine to drug-free plasma and analyzing the samples as described. Table I shows the results of this experiment. The coefficient of correlation was 0.99, thus demonstrating the good accuracy of the method.

Sensitivity

With a sample volume of 0.5 ml of either plasma or saliva, antipyrine concentrations as low as 0.1 μg/ml can be detected.

TABLE I

ACCURACY OF THE DETERMINATION OF ANTIPYRINE

Sample	Amount of antipyrine (μg/ml)	
	added	found
A	0.30	0.28
B	5.00	4.90
C	2.50	2.35
D	0.60	0.57
E	10.00	9.90
F	0.50	0.53
G	15.00	14.90
H	0.40	0.40

Specificity

The specificity of our method was assessed by collecting the antipyrine peaks eluted from the column from different subjects and recording the mass spectra of these peaks (direct-inlet system). These mass spectra were identical with those obtained from reference compounds. Since only a single extraction step and no evaporation of organic solvent is required, in contrast to the published gas chromatographic (GC) methods, and retention times are short, 50–60 samples can be analyzed by one technician within a normal working day.

The results indicate that the method described is sensitive, specific, rapid, precise and accurate. The half-lives of antipyrine measured with this method are in close agreement with the published half-lives measured by different methods[10]. The high sensitivity of the method allows the exact measurement of antipyrine for several half-lives, or at lower doses of antipyrine than normally used. The main advantage of the method is its speed. It allows at least twice as many analyses to be carried out as compared with the method of Brodie *et al.* and the GC methods. The column has now been in use for more than 1.5 years. About 1500 samples have been analyzed without loss of column efficiency, which is also an advantage compared with GC methods.

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Note

Reversed-phase high-performance liquid chromatography of doxycycline

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Of the tetracyclines, doxycycline is one of the most widely used in broad spectrum antibiotic therapy. It is particularly characterized by its greater chemical stability and chemotherapeutic activity compared with the parent molecule tetracycline. The structural formulae of some tetracyclines are presented in Table I.

TABLE I

STRUCTURES OF TETRACYCLINES
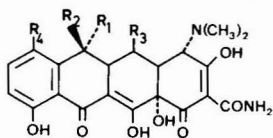
R_1	R_2	R_3	R_4	*Common name*
CH_3	OH	H	H	Tetracycline
CH_3	OH	OH	H	Oxytetracycline
H	OH	H	Cl	Demethylchlortetracycline
CH_3	H	OH	H	Doxycycline
$=CH_2$		OH	H	Methacycline

Microbiological[1,2] and fluorimetric techniques[3-6] are used to determine the levels of doxycycline in biological samples. However, both methods suffer from a lack of selectivity. To overcome this problem, a chromatographic system capable of separating the drug from interfering materials and analogues is required.

Up to now, only one paper has reported the use of gas–liquid chromatography (GLC) for separating tetracyclines[7]. However, owing to their considerable molecular weight and high degree of polarity, the GLC of these compounds suffers from several problems. Moreover, because these structures are chemically unstable in basic media, derivatization is extremely difficult. In contrast to GLC, high-performance liquid chromatography (HPLC) combined with UV detection seems to be more suitable. Several ion-exchange systems, using pellicular packing materials[8-12], have been described, but none of them was sufficiently sensitive for our purposes. Better results have been reported with various reversed-phase systems[13-19], although often

drastic pH conditions[15,16] or gradient elution[14,17] were required in order to provide acceptable resolution. Most of these methods have been applied only to relatively simple systems, *e.g.*, the determination of impurities in tetracycline formulations[13-17]. Only one paper has dealt with the separation of doxycycline from three other commonly used tetracyclines on a pellicular RP 18 column[19]. However, in common with other packing materials of this type a low efficiency was obtained.

Current interest in our laboratory in the determination of doxycycline in biological materials has led to the development of a new HPLC system, which permits the separation of the compound from four other tetracyclines.

EXPERIMENTAL

Apparatus

A Varian Model LC 8500 liquid chromatograph with gradient capability was used. Injections were made with the aid of a Valco sampling valve (Model CV-6-UHPa-C20), equipped with a 10-μl loop. A Vari-Chrom variable-wavelength detector was operated at 350 nm.

Chromatographic conditions

The following reversed-phase columns were studied.

(a) *Bonded phase: octadecyl chain.* (1) 50 cm $\times$ 2.1 mm I.D. (stainless steel), containing 35–50-μm Vydac RP 18 (Varian, Brussels, Belgium). (2) 25 cm $\times$ 4.6 mm I.D. (stainless steel), containing 5-μm RP 18 (R.S.L., St. Martens-Latem, Belgium).

(b) *Bonded phase: octyl chain.* (3) 25 cm $\times$ 4.6 mm I.D. (stainless steel), filled with 5-μm LiChrosorb RP 8 (Merck, Darmstadt, G.F.R.). (4) 25 cm $\times$ 3 mm I.D. (stainless steel), filled with 7-μm LiChrosorb RP 8 (Merck).

Eluent systems. The eluent systems consisted of mixtures of acidic buffer solutions (pump A) and acetonitrile or methanol (pump B), except for the Vydac column (No. 1), where pump A contained 0.15 M ammonium carbonate solution (adjusted to pH 8.4 with 13 M ammonia solution).

Chemicals and reagents

All tetracyclines were kindly provided by Pfizer (Brussels, Belgium), except demethylchlortetracycline, which we obtained from Lederle (Brussels, Belgium). Samples for injection were made by dissolving the tetracyclines (hydrochloride salts) in methanol to give concentrations of 1 mg/ml. The organic solvents acetonitrile and methanol, both from Merck, were of analytical grade. Freshly doubly distilled water was used to prepare buffer solutions, as follows. (1) A citrate–phosphate buffer of pH 2.2, according to McIlvaine[20], was prepared by mixing 980 ml of 0.1 M citric acid with 20 ml of 0.2 M disodium hydrogen orthophosphate solution. (2) Glycine buffers of pH 3.1 and 2.1 were prepared by adding 15 and 45 ml, respectively, of 0.2 M hydrochloric acid to 85 and 55 ml, respectively, of a solution containing 1.5 g of glycine and 1.17 g of sodium chloride per 100 ml.

RESULTS AND DISCUSSION

The high polarity of tetracyclines makes them unsuitable for adsorption chro-

matography on silica gel and, as confirmed in many studies reversed-phase chromatography appears to be more promising. Using simple solvent combinations such as acetonitrile–water or methanol–water, either no symmetrical doxycycline peak or a very poor separation from other tetracyclines was obtained. To enhance the selectivity of the chromatographic system, the incorporation of a "modifier" in the mobile phase seemed necessary.

A previously described mobile phase[19], containing 8 % (v/v) of methanol in 0.05 M ammonium carbonate solution, proved to be unsuccessful on the Vydac RP 18 column (No. 1), the doxycycline appearing as a broad, strongly tailing peak. However, we considerably improved the peak shape by adding 13 M ammonia solution to a final pH of 8.4 as a tailing suppressor. In addition, we obtained an increased efficiency with more concentrated solutions, $e.g.$, 0.15 M ammonium carbonate, and replacement of methanol with acetonitrile also gave a similar effect. The separation of oxytetracycline, doxycycline, demethylchlortetracycline and tetracycline was achieved with as little as 4 % (v/v) of acetonitrile in the mobile phase, as shown in Fig. 1.

TIME (MINUTES)
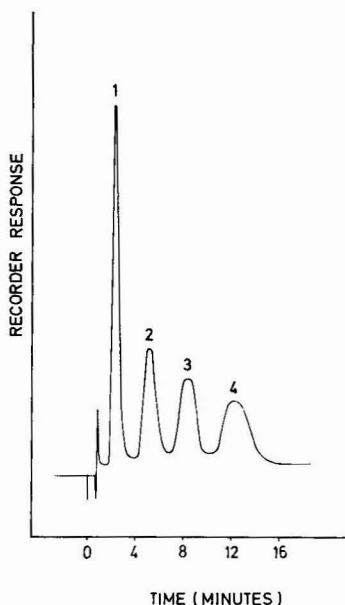
Fig. 1. Separation of four tetracyclines. Column: 50.0 cm × 2.1 mm I.D., Vydac RP 18. Eluent: 0.15 M ammonium carbonate–acetonitrile (96:4) + 13 M ammonia solution to pH 8.4. Flow-rate: 60 ml/h. Temperature, 20°; pressure, 500 p.s.i. Peaks: 1 = oxytetracycline; 2 = doxycycline; 3 = demethylchlortetracycline; 4 = tetracycline.

Unfortunately, the low efficiency of the pellicular packing material limits the sensitivity of detection. We therefore applied the modified solvent system to a totally porous RP 18 column (No. 2). Again, the peaks tailed badly and very high concentrations of organic solvents (up to 95 % of methanol) were necessary in order to elute the required compounds. This effect was thought to be due to secondary adsorption on the porous silica[15]. Some workers have assumed that the latter phenomenon is due to

presence of divalent cations, such as calcium[18], and a small amount of a chelating agent, *e.g.*, EDTA is therefore often added to the mobile phase[7–9,11–13,17–19]. We also performed chromatography with 0.005 M disodium EDTA incorporated in´an acetonitrile–water eluent. Although we obtained no better results with the RP 18 column, a shorter bonded alkyl chain, *i.e.*, an octyl chain (column No. 3) proved to be more suitable. In fact, on the latter column system doxycycline chromatographed as a symmetrical peak, but could not be separated from four other tetracyclines. This effect was probably due to non-optimization of the pH.

Knox and co-workers[15,16] studied the addition of inorganic acids (*e.g.*, perchloric acid) to the eluent, giving pH values of 1.0–1.5. We examined various buffers, containing organic acids such as citric and tartaric acid, which also serve as chelating agents. Initially, maximum retention of tetracyclines on an apolar stationary phase would be expected near the isoelectric point (pH 5), where they exist in their most lipophilic form. However, using a citrate buffer of this pH, a particularly poor resolution between five tetracyclines was obtained on the RP 8 column. The retention times increased considerably when the pH was decreased. Maximum resolution was achieved with a citrate–phosphate buffer of pH 2.20, which is the lower limit of this buffer system (Fig. 2). Doxycycline and methacycline, whose structures are closely related, are still incompletely resolved. The two small peaks (2 and 4) are probably caused by some degradation products, perhaps epimers at the C_4 position. Analogous results were obtained with the latter eluent on a second RP 8 column (No. 4). No significant improvement in resolution was obtained when the pH was decreased
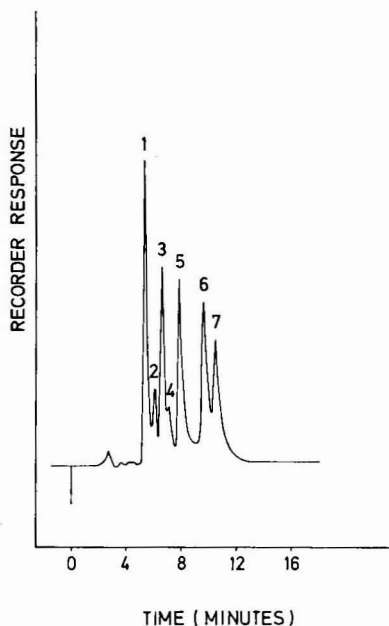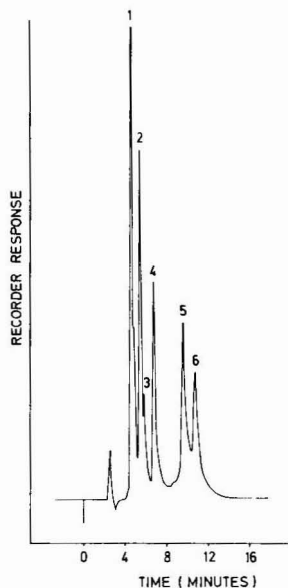

Fig. 2. Separation of five tetracyclines. Column: 25.0 cm × 4.6 mm I.D., LiChrosorb RP 8 (5 μm). Eluent: citrate–phosphate buffer (pH 2.20)–acetonitrile (65:35). Flow-rate: 80 ml/h. Temperature, 20°; pressure, 3900 p.s.i. Peaks: 1 = oxytetracycline; 3 = tetracycline; 5 = demethylchlortetracycline; 6 = methacycline; 7 = doxycycline; 2 and 4 = unidentified.

further by using pure citric or tartaric acid solutions as the eluent. As shown in Table II, the capacity ratios increased until a maximum value was reached, whereas doubling the citric acid concentration resulted in less retention. Changing the nature of the organic acid had little or no effect, as shown by the replacement of citric acid with tartaric acid.

TABLE II

CAPACITY RATIOS (k') OF DOXYCYCLINE AT DIFFERENT pH VALUES

Column: 25 cm $\times$ 3 mm I.D., LiChrosorb RP 8 (7 μm). Flow-rate: 80 ml/h. Acetonitrile: 29 % (v/v) (pump B).

Aqueous eluent (pump A)	pH	k'
Citrate–phosphate buffer	2.20	4.6
Citric acid (0.1 M)	2.15	6.9
Tartaric acid (0.1 M)	2.15	6.9
Citric acid (0.2 M)	1.98	5.9

To elucidate the factors that influence retention and resolution, another acidic buffer system combined with a chelating agent, *viz.*, a glycine buffer containing 0.005 M penicillamine hydrochloride (pH 3.1), was examined. This eluent gave a very similar chromatographic pattern on the first RP 8 column (No. 3) in comparison with a citrate–phosphate buffer of identical pH. Furthermore, to establish whether the chelating agent is obligatory, penicillamine was omitted, and no significant differences in peak shape, resolution or retention time were observed. The separation could also be optimized by decreasing the pH to 2.1, as shown in Fig. 3.

Fig. 3. Separation of five tetracyclines. Column: 25.0 cm $\times$ 4.6 mm I.D., LiChrosorb RP 8 (5 μm). Eluent: glycine buffer (pH 2.1)–acetonitrile (72:28). Flow-rate: 80 ml/h. Temperature, 20°; pressure, 3700 p.s.i. Peaks: 1 = oxytetracycline; 2 = tetracycline; 4 = demethylchlortetracycline; 5 = methacycline; 6 = doxycycline; 3 = unidentified.

CONCLUSION

The elucidation of the mechanism of the retention of tetracyclines in 'a reversed-phase chromatographic system involves several problems. Complex formation with metal ions should not be considered crucial, as eluents without any chelating agent (*i.e.*, glycine buffers) also yield acceptable resolution. However, satisfactory results with the latter solvents were obtained only on "old" columns that had previously been used with EDTA and citrate–phosphate buffers. Apparently, some "modification" of the packing material takes place. Re-filling the top of a column with fresh packing material resulted in a total loss of resolution. Therefore, pre-equilibration with a chelating agent is probably required.

The pH of the mobile phase was found to be most important. Under the experimental conditions, tetracyclines exist mainly in their cationic form. The ion-pair approach of Knox and co-workers[15,16] provides an acceptable explanation of why positively charged molecules are retained by hydrocarbon stationary phases. According to this theory, the nature of the counter ion should be the key to the chromatographic behaviour. In contrast to the results of Knox and co-workers[15,16], the retention times and resolution were considerably affected by pH variations, rather than by the nature of the anions (citrate, phosphate, tartrate or glycinate). It is not clear why the retention decreases when the acid concentration (or the pH) reaches a certain limit.

The method described permits the rapid and simple separation of five tetracyclines. This system seems to be sufficiently selective and provides a basis for the analysis of doxycycline in biological materials.

NOTE ADDED IN PROOF

After submission of this paper, another article on the same subject appeared in this journal[21].

A reversed-phase liquid chromatographic system using a RP 18 column to separate oxytetracycline, tetracycline and chlortetracycline was described. No chelating agent was incorporated in the mobile phase consisting of 40 % of acetonitrile in 0.01 M phosphate buffer of pH 2.4.

As we have discussed above, the latter is in contradiction with several former experiments[18,19], as well as with some of our own observations. This confirms once more the existing controversy about this problem.

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Note

An ultramicro high-performance liquid chromatographic method for assaying ion-pair species of benactyzine

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Current methodology for measuring the concentration of benactyzine in various types of therapeutic drugs is based primarily on colorimetric and spectrophotometric methods[1–4]. In most cases, pre-treatment of the sample is required prior to analysis. Hence, many of the procedures used in these methods are long and tedious. The sensitivity is limited. Benactyzine levels of less than 5 μg are difficult to measure.

We have recently developed a highly sensitive ion-pair high-performance liquid chromatographic (HPLC) method for determining benactyzine in various therapeutic drugs using reversed-phase chromatography. The method is capable of quantifying levels of the compound as low as 200 ng on column. Analysis time is 6 min per sample. No pre-treatment is required. As a rather simple and specific method, a high degree of precision and accuracy is possible when utilizing this new procedure.

EXPERIMENTAL*

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) Model ALC/GPC 204 liquid chromatograph, equipped with two Model 6000A high-pressure pumps, a 660 solvent programmer, a U6K loop injector, a 254-nm UV detector, a Houston Instrument series A 5000 Omni-Scribe dual-pen recorder and a Columbia Scientific Supergrator-3 integrator were used to complete this study.

Reagents

All solvents used for these chromatographic separations were of spectro quality or analytical grade. Acetonitrile was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). PIC B-7 reagent (1-heptane sulfonic acid) was purchased from Waters Assoc. Stock standard solutions of benactyzine hydrochloride, benzilic acid and *p*-sulfanilic acid (Aldrich, Milwaukee, Wis., U.S.A.) were used to prepare all working standards. Quality control was maintained by sulfanilic acid internal standards.

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* The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.

Procedure

A pre-packed 30 cm × 3.9 mm I.D. μBondapak C_{18} column (Waters Assoc.) was used to separate benactyzine and benzilic acid. The mobile phase consisted of 0.01 M 1-heptanesulfonic acid mixed with acetonitrile and was prepared by dissolving 20 ml of the pre-packaged reagent PIC B-7 in 480 ml of glass-distilled water. The pH of the solution was 3.40.

Utilizing both pumps of the HPLC system along with the 660 solvent programmer, a 35–65% mixture of acetonitrile to PIC reagent was isocratically pumped through the column. The flow-rate was 1.5 ml/min. Operational pressures ranged between 1200 and 1500 P.s.i. All separations were performed at ambient temperatures. Two microlitres of sample were introduced onto the column through a continuous-flow loop injector. The detection limit of the method was 100 ng on-column at the lower absorbance range of 0.005 A. Peak areas were measured by an on-line computing integrator.

RESULTS AND DISCUSSION

The recent introduction of ion-pair HPLC as a new and innovating technique has created the impetus for developing simpler[1], speedier and more sensitive analytical methods. As such, we have developed an ultramicro HPLC method to measure benactyzine in various types of therapeutic drugs. Benactyzine hydrochloride, which is a mild anti-depressant and anti-cholinergic agent is often administered to patients for reducing autonomic responses due to emotional provoking stresses.

Our primary interest for developing an improved method for quantifying benactyzine was due to the instability of this compound under certain conditions. We wanted to observe its fate when subjected to various pH and temperature gradients. Benzilic acid, the oxidative by-product, which is formed during hydrolysis, is ineffective as a therapeutic modality. This method is also capable of separating and quantifying benzilic acid.

A series of standards and experimental sample were analyzed. Fig. 1 represents the calibration curve of benactyzine standards containing concentrations ranging from 200 through 1000 ng. Each point plotted on the graph is an average of five separations for the denoted quantity. A linear relationship was observed for all absorbance
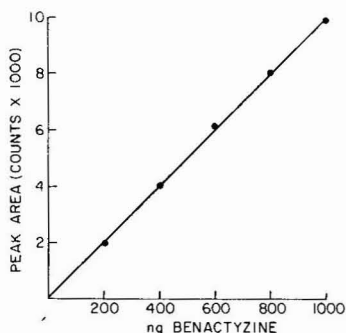
Fig. 1. Calibration curve of benactyzine detected at 254 nm, 0.005 A. Column: 30 cm × 3.9 mm I.D., μBondapak C_{18}.

ranges studied (0.005–0.02 A). The precision of the method was excellent. The co-efficient of variation for these multiple analyses ranged from 0.5 to 1.2%.

In three chromatograms, which depict the separation of benactyzine and bén-zilic acid, the sensitivity and resolution of the method are demonstrated. Fig. 2 shows the separation of a 2-μl aqueous standard solution containing 400 ng/μl of benacty-zine and 5 ng/μl of sulfanilic acid. This standard solution was stable for three weeks when refrigerated at 4°.

On the contrary, benactyzine hydrochloride prepared in 0.01 N NaOH was highly unstable. The chromatogram shown in Fig. 3 is a partially hydrolyzed sample of benactyzine, which was heated in boiling water at 90° for 30 sec. During the period
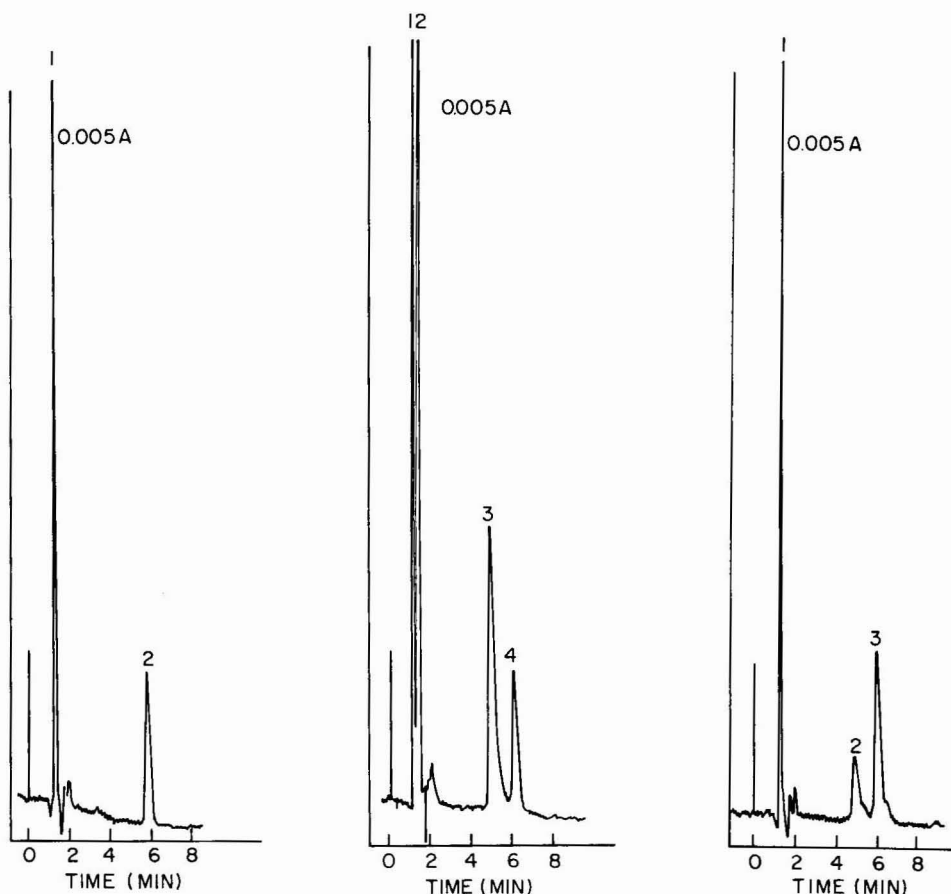
Fig. 2. Separation of a standard solution of (1) 10 ng sulfanilic acid (internal standard) and (2) 800 ng benactyzine. Mobile phase: 35% acetonitrile and 65% PIC B-7 reagent. Flow-rate: 1.5 ml/min. Column temp.: 20°. Chart speed: 0.5 cm/min.

Fig. 3. Chromatogram of a 2.0-μg sample of benactyzine, heated in 0.01 M NaOH at 90° for 30 sec. Peaks: (1) unknown; (2) sulfanilic acid; (3) benzilic acid; (4) benactyzine.

Fig. 4. Chromatogram showing the formation of benzilic acid in 0.01 M NaOH at room tempera-ture. Separation includes (1) sulfanilic acid, (2) benzilic acid and (3) benactyzine.

of hydrolysis, 60% of the benactyzine was converted to benzilic acid. The total conversion occurred after 60 sec.

When an identical sample of benactyzine was prepared and allowed to stand at room temperature, the results were similar. Benactyzine oxidized to benzilic acid but at a much slower rate. In the chromatogram of Fig. 4, 3% of benactyzine was oxidized to benzilic acid in 10 min. Upon leaving the remaining portion of the sample overnight at room temperature, more than 90% of benactyzine had oxidized.

Conversely, benactyzine hydrochloride was relatively stable in 0.01 N HCl at room temperatures, 37° and 90° under similar experimental conditions as were used in the 0.01 N NaOH study.

From this study, we were able to demonstrate the applicability of this new analytical technique in measuring the concentration of benactyzine. Obviously, an analytical system offering the advantages of improved sensitivity, specificity and simplicity should have an immediate application in minimizing many of the disadvantages that beset conventional analytical methodologies.

The advantages of ion-pair HPLC are self-evident as was demonstrated by this study.

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CHROM. 10,154

Note

Separation of some polyhydric alcohols by high-performance liquid chromatography

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The separation of polyhydric alcohols by various types of chromatography has been reported by several authors. Thin-layer chromatography on various adsorbents, with use of such complexing agents as boric acid[1], ammonium borate[2] and lead(II) in alkaline medium[3], has been applied to separations of mannitol and sorbitol in the presence of other polyhydric alcohols and sugars[4–8]. Ion-exchange resins can be used for the partition chromatography of polyhydric alcohols, retention of a polar non-electrolytic compound occurring by partition between adsorbed water at the surface of the resin and the mobile phase; separation have been described by Samuelson[9] and others[10–13].

Recently, I have shown the feasibility of separating some polyhydric alcohols on silica gel to which aminoalkyl groups had been chemically bonded, by using water–acetonitrile as mobile phase[14]. This normal-phase partition system gave good separations of many polyhydric alcohols, but could not be used to separate xylitol from arabitol (which is necessary in the analysis of cellulose hydrolysates).

The detection of small amounts of compounds with a differential refractometer poses serious problems. By forming the nitrobenzoate derivative, as described by Nachtmann *et al.*[15,16], I was able to improve the sensitivity by a factor of several thousand.

EXPERIMENTAL

The apparatus and column systems were as described previously[14,17,18]; separation parameters are given in the legends to the chromatograms.

Materials

The columns were packed with Aminex Q-15-S (Bio-Rad Labs., Richmond, Calif., U.S.A.), LiChrosorb SI-60, 5 μm (Merck, Darmstadt, G.F.R.) or an aminopropylsilica gel[14].

Reagents

4-Nitrobenzoyl chloride puriss.p.a. (Fluka, Buchs, Switzerland) was twice recrystallized from *n*-pentane puriss.p.a. (Merck); pyridine puriss.p.a. (Fluka) was refluxed for 2 h over potassium hydroxide puriss.p.a. (Fluka), distilled at 115° and

stored over potassium hydroxide tablets. It is essential for trouble-free derivatization that these reagents be as pure as possible. 4-Dimethylaminopyridine purum (Fluka), sodium hydrogen carborate Ph.H.VI (Siegfried, Zofingen, Switzerland), sodium carbonate puriss.p.a. (Fluka), chloroform for UV spectroscopy (Fluka), n-hexane purum (Merck), dichloromethane puriss.p.a. (Merck) and Uvasol acetonitrile (Merck) were used without further purification.

Standards

The polyhydric alcohol standards were obtained in highly pure grades from Fluka and from Sigma (St. Louis, Mo., U.S.A.).

Derivatization

The polyhydric alcohols were dissolved in pyridine (the concentration of this sample solution should not exceed 5 mg/ml), 50 μl of this solution were placed in a 10-ml round-bottomed flask, and 150 μl of a fresh solution prepared by dissolving 100 mg of 4-nitrobenzoyl chloride in 1 ml of pyridine were added. The mixture was well shaken and allowed to react for 10 min at room temperature, then pyridine was removed by heating at 80° *in vacuo* (water pump), and the residue was dried under high vacuum; special attention must be paid to this step, as any trace of pyridine will give rise to a group of peaks that can interfere with the separation of the derivatives. The pyridine-free residue was dissolved in 2 ml of a solution of 250 mg of 4-dimethyl-aminopyridine in 100 ml of 5% aqueous sodium carbonate in order to hydrolyze the excess of reagent. The solution, which was usually turbid, was well shaken and kept at room temperature for 10 min, then extracted with 2 ml of chloroform, and the extract was washed once with 2 ml of 5% aqueous sodium hydrogen carbonate and twice with 3 ml of 0.05 N hydrochloric acid containing 5% of sodium chloride (*cf.* Nachtmann *et al.*[16]). A portion of the chloroform extract was directly injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Separation systems

The separation of a few glycols and sugar alcohols on an aminoalkylsilica gel column is shown in Fig. 1; this system provides rapid separation at room temperature, and both aqueous and alcoholic samples can be analyzed directly. However, sorbitol and mannitol are eluted at the same k' value, and xylitol and arabitol are not completely separated from each other. By using a column of Aminex Q-15-S (Fig. 2), sorbitol and mannitol can easily be separated, as can xylitol and arabitol, but sorbitol and xylitol are eluted together. Disaccharide alcohols, *e.g.*, maltitol, are eluted at lower k' values than monosaccharide alcohols in this system.

Complete separation of all the above-mentioned polyhydric alcohols was possible by adsorption chromatography on silica gel after derivatization (Fig. 3). The main advantage of derivatization, however, is the gain in sensitivity; a 10,000-fold improvement for monosaccharide alcohols is typical. The derivatization step was found to be linear in the range 2 to 750 μg for monosaccharide alcohols, and the NMR
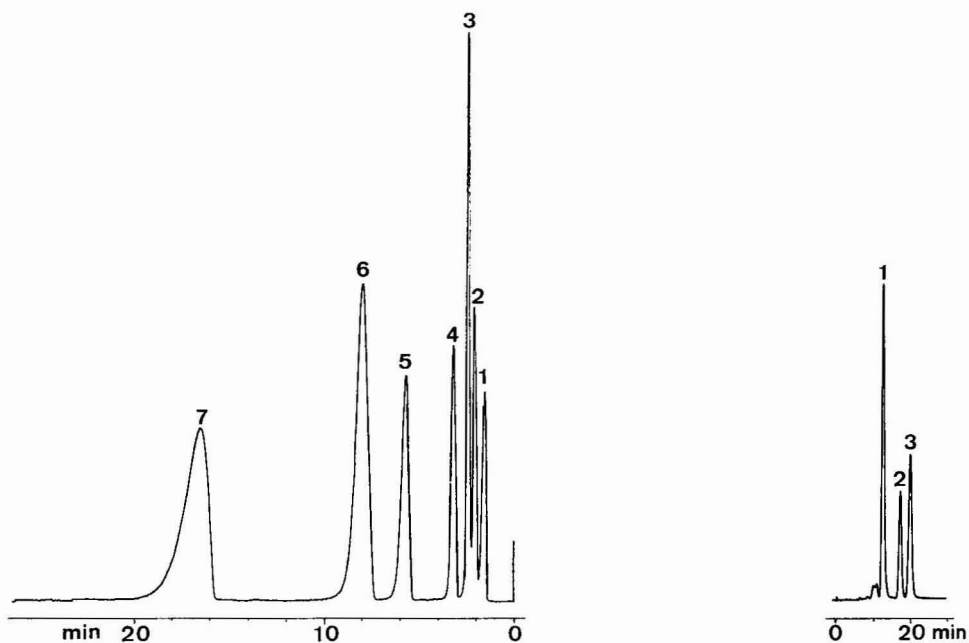
Fig. 1. Chromatogram of polyhydric compounds on a column (250 × 3 mm) of aminoalkylsilica gel (5 μm) at room temperature with a mobile phase of water–acetonitrile (1:3) at 1.0 ml/min. Peaks: 1 = water; 2 = propylene glycol; 3 = ethylene glycol; 4 = glycerol; 5 = xylitol; 6 = sorbitol; 7 = maltitol.

Fig. 2. Chromatogram of polyhydric compounds on a column (1 m × 4 mm of Aminex Q-15-S (22 μm) at 85° with a mobile phase of water at 0.6 ml/min. Peaks: 1 = maltitol; 2 = xylitol; 3 = arabitol.
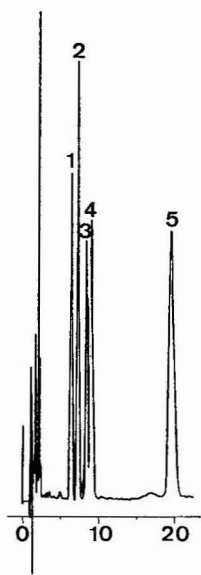
Fig. 3. Chromatogram of polyhydric alcohol derivatives on a column (250 × 3 mm) of LiChrosorb SI-60 (5 μm) at room temperature with a mobile phase of hexane–chloroform–acetonitrile (5:2:1) at 0.8 ml/min. Peaks: 1 = arabitol; 2 = xylitol; 3 = mannitol; 4 = sorbitol; 5 = maltitol.
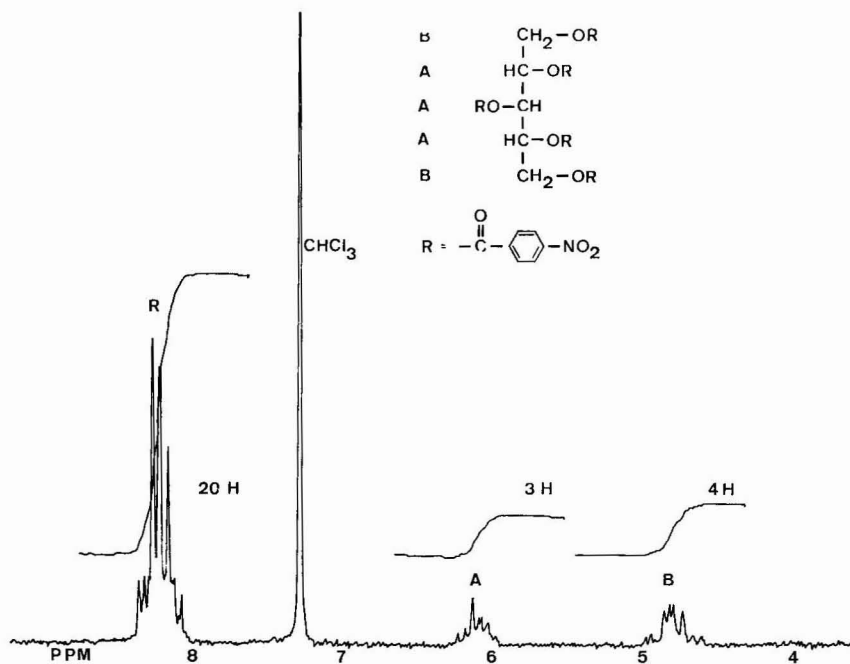
Fig. 4. Section of the 100-MHz nuclear magnetic resonance spectrum of the 4-nitrobenzoyl derivative of xylitol.
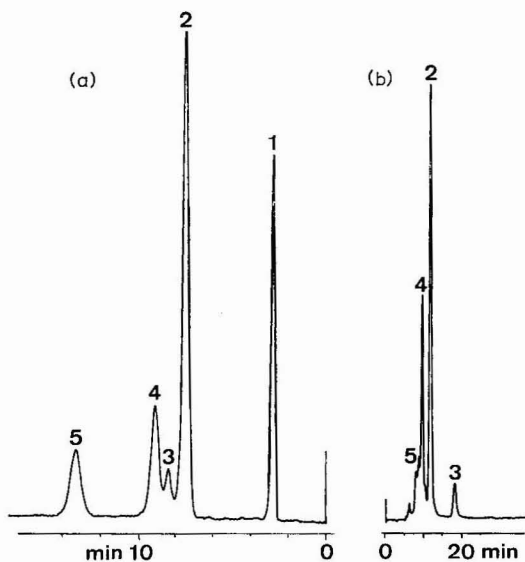
Fig. 5. Chromatograms of apple juice. Conditions: (a) as in Fig. 1, but with water–acetonitrile (1:4) as mobile phase; (b) as in Fig. 2. Peaks: 1 = water; 2 = fructose; 3 = sorbitol; 4 = glucose; 5 = saccharose.

spectra (Fig. 4) of the derivatives showed that all hydroxyl groups had been converted[19].

Applications

Determination of sorbitol in apple juice. The apple juice can be injected directly. Fig. 5a shows the separation on an aminoalkylsilica gel column, and Fig. 5b is the chromatogram of the same sample on Aminex Q-15-S. The Aminex system has the advantage of a baseline-separated sorbitol peak, which facilitates determination of this alcohol. The retention time is short, and, since no sample preparation or clean-up is necessary, the entire analysis is simple.

Determination of humectants in tobacco. The humectants most commonly in tobacco (propylene glycol, ethylene glycol, glycerol and sorbitol) can be identified and determined in a single operation. The powdered tobacco is extracted with water, the extract is cleaned-up on a short column (a Pasteur pipette) filled with an aminoalkyl-silica gel (200 mesh) prepared in the same manner as the packing material for the aminoalkylsilica gel column[14], and then injected. The chromatogram of such an extract is shown in Fig. 6.
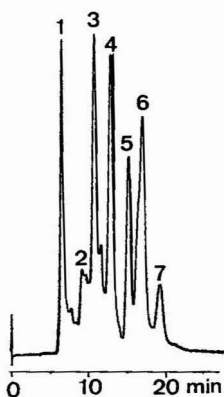
Fig. 6. Chromatogram of tobacco extract (conditions as in Fig. 2). Peaks: 1 = higher polysaccharides; 2 = disaccharides; 3 = glucose; 4 = fructose; 5 = glycerol; 6 = ethylene glycol and propylene glycol; 7 = sorbitol.

Determination of xylitol in tooth-paste. Since tests at the University of Turku, Finland[20], have shown that xylitol has an anti-caries effect, this sweetener has become increasingly used in, *e.g.*, dietary food products, soft drinks, chewing-gum and tooth-paste. Its determination by liquid chromatography is simple, as the product itself, a solution or an extract of it can usually be directly analyzed. Tooth-paste was mixed with water to form a thin slurry, which was then centrifuged, the supernatant liquid was filtered over Celite, and the filtrate was injected into the liquid chromatograph. The chromatogram (Fig. 7) of this filtrate shows all the polyhydric alcohols present in the paste.

The high-performance liquid chromatographic systems discussed here have the following advantages over other techniques: analyses are rapid; the compounds can often be assayed directly, without prior separation from common monosaccharides;

Fig. 7. Chromatogram of tooth-paste extract (conditions as in Fig. 1). Peaks: 1 = water; 2 = ethylene glycol; 3 = glycerol; 4 = xylitol.

and with very small amounts of sample, the sensitivity can be improved by derivatization with 4-nitrobenzoyl chloride.

ACKNOWLEDGEMENT

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Note

Determination of (d, l)-6-chloro-α-methylcarbazole-2-acetic acid in plasma by high-performance liquid chromatography

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The drug (d,l)-6-chloro-α-methylcarbazole-2-acetic acid (Ro 20-5720) is used as an antiinflammatory agent. For determination of the substance in the normal dose range, an analytical method with high specificity and a sensitivity in the nanomole range is necessary. Previously described methods for the analysis of the drug lack both specificity and sensitivity[1].

The high-performance liquid chromatographic (HPLC) method described here was based on reversed-phase ion-pair partition. This technique gives separation systems of high efficiency. The retention is determined by the properties of the mobile phase[2–4]. By the use of fluorimetric detection, high sensitivity can be achieved in the analysis of fluorescent compounds. The fluorescence of carbazole compounds was described by Bender et al.[5].

EXPERIMENTAL

Reagents

An acetate buffer solution (0.1 M, pH 5.0) was prepared. Diethyl ether (Uvasol), methanol (Uvasol), acetic acid (Merck, Darmstadt, G.F.R., p.a. quality) and tetrabutylammonium hydroxide (TBA-OH) (Eastman, Kodak, Rochester, N.Y., U.S.A. 25.9% in methanol) were used.

Standard solutions

Compound 1 (Fig. 1), molecular weight 273.72, melting point 206.0–208.0° (with decomposition), of pharmaceutical grade supplied by Hoffmann-La Roche (Basle, Switzerland) was used as an analytical standard. A stock solution was prepared by dissolving 100 mg of the compound in 100 ml of methanol (A) and diluting 1:9 to yield a solution of 0.1 mg/ml (B). From solutions A and B working solutions in plasma were made, containing 20 and 1 μg/ml. The working solutions were diluted with plasma to give 10 batches covering the range from 0.07 to 14.6 μmoles/l (20 ng/ml to 4 μg/ml).

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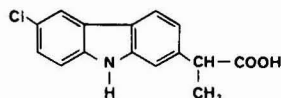
Compound 1   pKa = 4.73
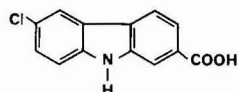
Compound 2
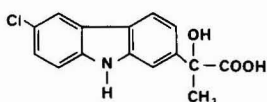
Compound 3   pKa = 3.70

Fig. 1. Structures of compounds. 1 = (*d,l*)-6-Chloro-α-methylcarbazole-2-acetic acid; 2 = 6-chloro-2-carbazolecarboxylic acid (internal standard); 3 = 6-chloro-α-hydroxy-α-methylcarbazole-2-acetic acid (metabolite).

Internal standard solution

Compound 2 (Fig. 1), 6-chloro-2-carbazolecarboxylic acid, was supplied by Hoffmann-La Roche. A stock solution was prepared by dissolving 10.0 mg of the compound in 100 ml of methanol; 1.0 ml of the stock solution was added to 1000 ml of the buffer solution (250 μl to 1000 ml of buffer for the lower standard curve, 0.07–0.7 μmole/l).

Chromatographic apparatus and preparation of the mobile phase

The liquid chromatography system consisted of a high-pressure pump (Waters Assoc., M-6000), a loop injector (Waters Assoc., U6K) and a fluorescence detector (Schoeffel, FS 970). The excitation wavelength was 300 nm and a 370-nm cut-off filter was used. A μBondapak C_{18} separation column ,30 cm $\times$ 4 mm, was used.

The mobile phase, containing the counter ion, was prepared from de-gassed, triply distilled water and methanol (30:70). A 10.0-ml volume of TBA-OH solution was added to 1000 ml of the water–methanol mixture and the pH was adjusted to 7.3 with acetic acid. In order to prevent contamination of the mobile phase with air, the solution was stored under nitrogen and the air inlet of the bottle was fitted with a charcoal filter. The flow-rate of the mobile phase was 1.5 ml/min.

Analysis of plasma

In the analytical procedure, an autopipette (Autochem Instruments, Lidingö, Sweden) was used to increase the reproducibility of the pipettings.

Into a 15-ml tube, 100 μl of plasma (1.0 ml for concentrations below 1.46 μmole/l) and 1.0 ml of buffer solution containing the internal standard (4.0 ml for concentrations below 1.46 μmole/l) were introduced. The mixture was extracted with 5 ml of diethyl ether on a reciprocating shaker for 10 min. After centrifugation at 1000 g for 10 min, the supernatant ether phase was transferred into another 15-ml tube with a conical bottom. The diethyl ether was evaporated off under a stream of nitrogen at 30°, the residue was dissolved in 100 μl of methanol and 10 μl of the solution were injected for HPLC–fluorescence analysis.

In vivo study

Six healthy volunteers (four males and two females) were used. One 75-mg capsule or one 75-mg tablet was ingested on an empty stomach (overnight fasting) together with 0.1 l of water. Venous blood samples were drawn 0.5, 1, 2, 3, 4, 6, 12, 24 and 48 h following administration, no food intake being allowed until after the 4-h samples had been taken. The trial was performed with the cross-over method. The drug was administered in a random manner in the order of capsule–tablet or tablet–capsule. The period between ingestion of the two formulations was 2 weeks.

RESULT AND DISCUSSION

Analytical procedure

The chromatographic system used (reversed-phase ion-pair partition), is not temperature sensitive and room temperature can be used throughout, but the column
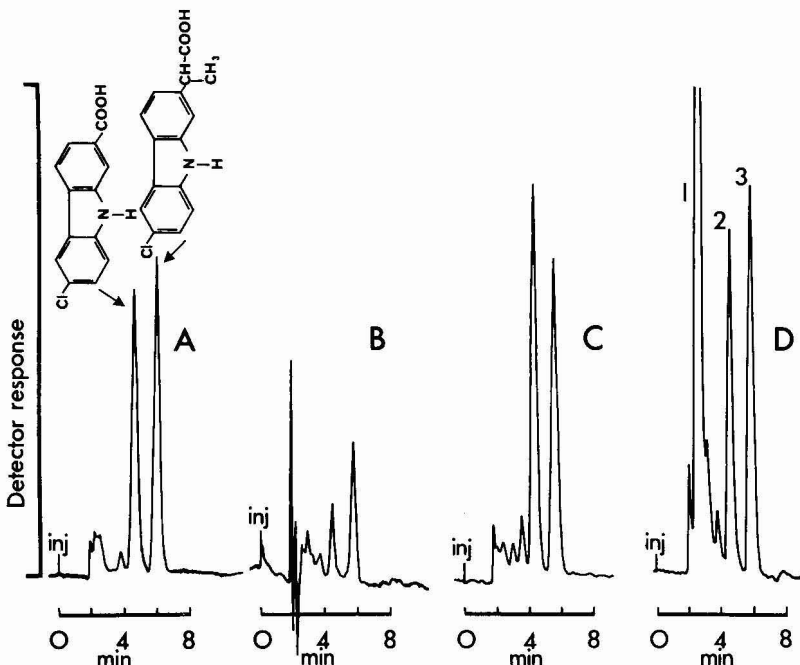
Fig. 2. Chromatograms from analysis of plasma samples. A, standard sample (307 ng/ml of compound 1); B and C, UV and fluorescence detector responses, respectively, to the 30-min sample from a normal subject after administration of 100 mg; D, 60-min sample from a plasma containing salicylate. Peaks: 1, salicylate; 2, compound 2; 3, compound 1.

must be equilibrated with the mobile phase at a flow-rate of 1.5 ml/min for 45 min before use. With a counter ion concentration of 10 mM, the water content of the mobile phase must be 25–30%, depending on the number of theoretical plates in the column, in order to achieve a good resolution and a retention time of about 6 min.

Fig. 2A shows a typical chromatogram from a standard sample of compound 1, Fig. 2B shows the chromatogram obtained with plasma from a patient when a UV detector (LDC Spectromonitor) was used at the absorption maximum of 238 nm, and Fig. 2C shows the chromatogram obtained with fluorimetric detection with excitation at 300 nm for the same plasma sample. The results show a higher sensitivity accompanied by increased specificity for the fluorimeter.

The absorption and luminescence properties of compound 1 are well documented[1,5] and the fluorescence spectrum for compound 1 in the mobile phase is in accordance with the spectrum obtained by De Silva *et al.* in ethanol and 1% glacial acetic acid[1]. The fluorescence was linear over a wide range of concentrations (Fig. 3).

The simple sample pre-separation makes it possible to analyze at least 150 samples in 3 days. The recovery of the drug from plasma varied from 84 to 94% (Table I). The error of the method was calculated for two plasma levels (Table II).
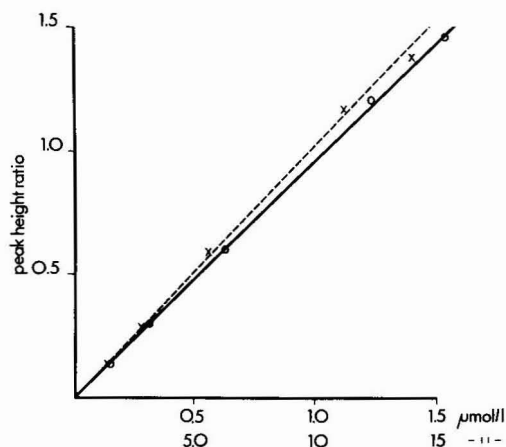
Fig. 3. Standard curves: peak-height ratio of compound 1 to compound 2 *versus* concentration of compound 1. Amounts extracted: $\times$, 0.14–1.4 μmol/l from 1 ml of plasma; $\bigcirc$, 1.54–15.4 μmol/l from 100 μl of plasma.

TABLE I

EXTRACTION RECOVERIES

Results obtained by adding the internal standard after the extraction procedure and comparison with a non-extracted methanol standard series. Each figure represents the mean of 4 observations.

Amount of compound 1 (μmole/l)		Recovery (%)
Added	Found	
1.54	1.37	89.5
3.07	2.59	84.3
6.17	5.50	89.1
12.3	11.2	91.0
15.4	14.4	93.5

TABLE II

EXPERIMENTAL ERRORS FOR THE DETERMINATION OF COMPOUND 1 IN PLASMA

Determined by duplicate analysis. $S = \sqrt{\dfrac{\Sigma d^2}{2n}}$, where d is the difference between the duplicate analysis.

Range ($\mu mole/l$)	n	Mean ($\mu mole/l$)	Error(s) of the method ($\mu mole/l$)	% of mean
0.14– 1.4	12	0.30	0.034	11.4
1.54–15.4	95	10.9	0.460	4.21

Specificity of the method

For routine clinical use, the specificity of the method was checked for some substances with antiinflammatory activity. Phenylbutazone, indometacin, phenacetin and naproxene did not interfere. Salicylates, on the other hand, are detectable under the chromatographic conditions used but appear just after the front in the chromatogram (Fig. 2D). The metabolite 6-chloro-α-hydroxy-α-methylcarbazole-2-acetic acid (compound 3, Fig. 1) does not interfere under the conditions used. It gives intense fluorescence on excitation at 300 nm and, after several injections of a large amount of this metabolite, the baseline will rise slightly. The columns have been used for more than 300 injections with only a small decrease in the theoretical plate number.
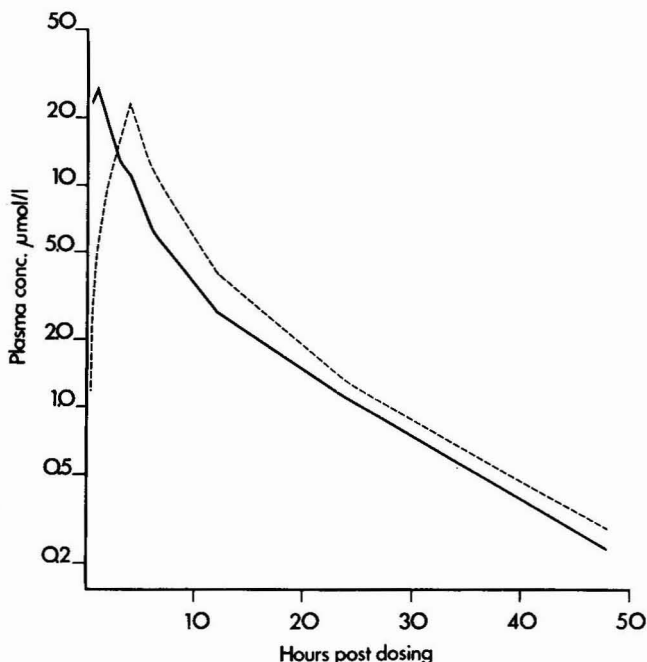
Fig. 4. Plasma level fall-off curve of a subject after administration of 75 mg of compound 1 as tablet (solid line) and as capsule (broken line).

In vivo study

The plasma level fall-off curves ranged from a maximum of 15.6–43.1 μmoles/l after 1–4 h to 0.073–0.555 μmole/l after 48 h for capsules, and from a maximum of 14.8–30.0 μmoles/l after 0.5–4 h to 0.036–0.475 μmole/l after 48 h for tablets. A plasma level fall-off curve is shown in Fig. 4. There was no consistent difference in plasma level attributable to the form of galenic preparation.

The variation of the peak level is probably due to the fact that the substance is poorly dissolved in the acidic medium and is therefore dependent on the acidity in the stomach and the stomach emptying time. Probably the irregularities in the first part of the plasma concentration curve can be explained by the same mechanism. The intra- and inter-individual variations of the "peak time", if due to this mechanism, will make any conclusion on the relationship between the galenical preparation and resorption time irrelevant.

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Note

Separation of thiamine and its derivatives on a Sephadex column

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The separation of thiamine on a Decalso column[1] is currently employed for the isolation of this vitamin from biological substrates. A Dowex ion-exchange material may also be employed[2]. The oxidation products of thiamine and its derivatives may be separated on a Sephadex gel column[3].

During a study of the interaction of the fungicide folpet with thiamine[4], a useful separation of the interaction products was obtained on a Sephadex G15 column. Thus, the possibilities of the separation of thiamine and its derivatives on a column of this type have been studied in this paper.

EXPERIMENTAL

0.5–1.0 ml of a 10^{-3} M solution of thiamine or one of its derivatives (thiamine monophosphate, thiamine diphosphate, thiamine disulphide and thiochrome, respectively) were placed on a Sephadex G10 column; a mixture of all of these compounds (each 10^{-3} M) was placed on the column in the same way. The samples were eluted with water and/or 0.01 M hydrochloric acid. (The column was equilibrated with 500 ml of HCl prior to separation.) Further experiments were carried out by use of a stepwise elution (100 ml of water, then 200 ml of 0.01 M HCl) and gradient elution (a linearly increasing amount of 0.01 M HCl in water).

Thiamine and its derivatives were identified in the separated fractions after gel chromatography by using thin-layer chromatography (TLC)[5,6]. The separated thiamine was determined after oxidation with potassium ferricyanide by the thiochrome method[1]; thiamine diphosphate was determined in the same way after enzyme hydrolysis.

RESULTS AND DISCUSSION

The elution of thiamine and its derivatives by water and 0.01 M HCl, respectively, is shown in Fig. 1. Thiamine diphosphate is the only derivative separated by water elution; thiamine and the other derivatives (thiamine monophosphate, thiamine disulphide and thiochrome) are retained on the column and must be eluted with 0.01 M HCl.

The stepwise elution with water and 0.01 M HCl resulted in the separation of
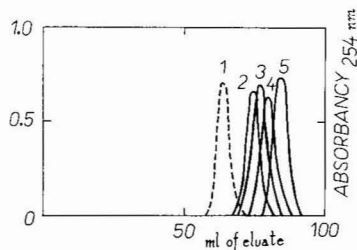
Fig. 1. Separation of thiamine and its derivatives on the Sephadex G10 column. For procedure see text. $- - -$, Elution with water; ———, elution with 0.01 M HCl. Peaks: 1 = thiamine diphosphate; 2 = thiamine monophosphate; 3 = thiamine disulphide; 4 = thiamine; 5 = thiochrome. Column, 400×26 mm ($V_0 = 63$ ml, $V_t = 105$ ml).
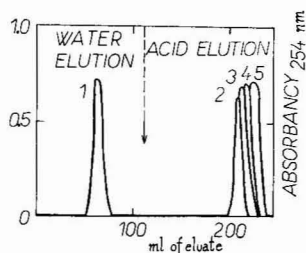
Fig. 2. Stepwise elution of thiamine and its derivatives from the Sephadex G10 column. For procedure see text. Other details as in Fig. 1.

thiamine diphosphate from the mixture of the other substances (Fig. 2). Gradient elution by water containing an increasing amount of 0.01 M HCl brings about the separation of thiamine diphosphate from thiamine monophosphate and the mixture of thiamine, thiamine disulphide and thiochrome. The last three compounds are incompletely separated (Fig. 3).
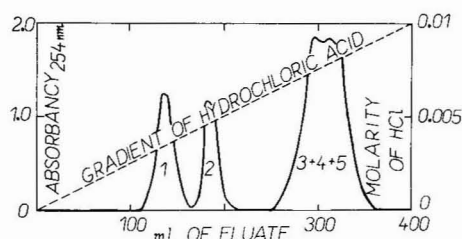
Fig. 3. Linear gradient elution of thiamine and its derivatives from the Sephadex G10 column. For procedure see text. Column, 700×26 mm ($V_0 = 121$ ml, $V_t = 253$ ml). Other details as in Fig. 1.

The determination of thiamine and thiamine diphosphate in the eluate after gel chromatography demonstrates the suitability of this separation technique. Thus, even if the separation on the Sephadex column requires more time in comparison to an ion-exchange column, there is no loss whatsoever during the whole procedure.

The elution pattern of thiamine and its derivatives does not change when the sample contains 0.08 M sodium sulphate. (Sodium sulphate would be formed in the neutralization of an acid extract of a biological sample prior to column separation.)

The method is suitable for the separation of thiamine diphosphate from thiamine monophosphate and from the mixture of thiamine, thiamine disulphide and thiochrome, and the subsequent determination by the thiochrome method. Even if the separation of thiamine, thiamine disulphide and thiochrome is incomplete, these cómpounds can be determined in the following way. One portion of the separated fraction is used to determine thiochrome fluorimetrically; a second portion is used to estimate the sum of thiamine and thiochrome after oxidation with potassium ferri-

cyanide and a third portion is used to determine all three components after primary reduction of thiamine disulphide by sodium thiosulphate[4,7] and subsequent application of the thiochrome method.

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Note

Surface-layer sorbents for group analysis of aromatic hydrocarbons in petroleum distillates

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For the determination of aromatic content of petroleum distillates, having boiling points up to 220°, various schemes of analysis of gasoline group composition were used.

The separation of aromatic hydrocarbons is usually effected on columns with cyanoethylated stationary liquids[1,2] followed by chromatographic analysis of the individual separated aromatics[3,4]. The following methods have also been used to determine the total amount of aromatic hydrocarbons: liquid adsorption chromatography on silica gel, a variant of fluorescence indicator analysis[5]; adsorption on calcium zeolite 10X at 300–350° followed by removal of the aromatic hydrocarbons by programming the temperature to 400° (ref. 6–8) or by using different chemical methods[9,10].

In the works cited[1–8] the research workers were faced with complicated chromatographic analyses of the group compositions of petroleum distillates containing aromatics, and in many cases only their total content is determined.

The present paper describes a method of gas adsorption analysis of aromatics based on their preliminary isolation by adsorption on silica gel and subsequent analysis on a surface-layer sorbent[11,12]. This method has been applied to the analysis of aromatic hydrocarbons having boiling points within the range 80–235°, and a separation of the aromatics into groups is made according to the number of carbon atoms in molecules.

Silica gel dust was used as a fine-grained adsorbent. It was impregnated with a 0.25 % solution of sodium hydroxide in order to shorten the analysis time, decrease the analysis temperature and to make possibile the group analysis of the aromatics.

EXPERIMENTAL AND RESULTS

In a surface-layer sorbent the active agent (sorbent) is uniformly distributed over the exterior surface of a grain, and the sorptive processes do not take place throughout the grain but only in a shallow surface layer equal to the depth of the layer of the active adsorbent. This explains the high efficiency of surface-layer sorbents which is attained by decreasing the resistance to mass transfer and shortening the diffusion paths of compounds to be chromatographed (see, for instance, ref. 13).

In the present work, the adsorbent was prepared by coating Celite-545 (80–100 mesh) with silica gel dust by mechanical shaking for 10 h and subsequent removal of the excess of dust. The fine-grained particles of silica gel powder enter the macropóres of the large porous carrier with a small specific surface area and are held in it by adhesive forces. As an active adsorbent we used silica gel developed by the All-Union research institute of petroleum refining especially for fluorescence indicator analysis. Characteristics of the silica gel used were as follows:

Bulk density, 0.64 g/ml.

Geometric structure: surface, 550–650 g/ml; pore volume, 0.5–0.7 ml/g; medium radius of pores, 14–23 Å.

In order to avoid the possible overlapping of paraffinic and naphthenic hydrocarbons, the straight gasoline cut (b.p. 45–220°) under investigation was subjected to separation on a liquid column with a fluorescent indicator to isolate the aromatics. The indicator used in this analysis permits a clear determination of the zones of not only the paraffinic naphthenic aromatics, but also of the olefinic hydrocarbons in the case of catalytic gasoline analysis in daylight (without a UV light source).
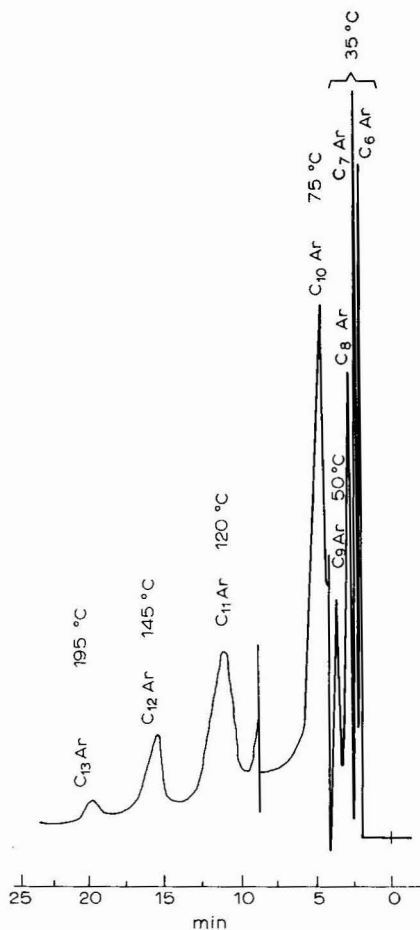
Fig. 1. Chromatogram of a concentrate of C_6–C_{13} aromatic hydrocarbons.

The analysis of the C_6–C_{13} aromatic hydrocarbon concentrate showed that, owing to a substantial decrease in the catalytic activity of the silica gel as a result of alkaline treatment the temperature of separation of the aromatics of a given mass decreased below their boiling points by an average of 50°. Thus benzene, toluene and C_8 aromatics were eluted at 35°; increasing the separation temperature to 50° led to the separation of C_9 hydrocarbons, and C_{10}, C_{11}, C_{12} and C_{13} aromatics were separated when the temperature was increased further to 195° at the rate of 8°/min. The separation temperature of C_{13} aromatics having boiling points of *ca.* 235° was 195° (Fig. 1).

The analysis was carried out on a serial TSVET-100 chromatograph equipped with a flame ionization detector. The column was a stainless steel tube (1.00 m × 3 mm) filled with silica gel dust impregnated with a 0.25 % solution of NaOH on Celite-545 (80–100 mesh) (20 % of carrier weight). The carrier gas (nitrogen) flow-rate was 25 ml/min, and the total elution time was 20–25 min. Quantitation of a chromatogram as shown in Fig. 1 was carried out by using the coefficients obtained in the analysis of artificial mixtures of aromatics under similar conditions.

The method described has permitted the determination of the group distribution of aromatics in a straight run petroleum distillate boiling within the range 45–220°.

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CHROM. 10,186

Note

Die Auftrennung und Remissionsmessung *in situ* von Flavonoiden auf Hochleistungs-Dünnschichtchromatographie-Fertigplatten Kieselgel 60

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Die meisten der bisher in der Literatur beschriebenen quantitativen Bestimmungen von Flavonoiden stellen Gesamtbestimmungsverfahren dar. Im besonderen Masse werden dabei die Chelatbildung mit mehrwertigen Metallionen[1] und die Umsetzung mit arylsubstituierten Borsäuren[2] zur photometrischen Bestimmung herangezogen. Mit der Strukturaufklärung zahlreicher, natürlich vorkommender Flavonoide wurde die Möglichkeit zur selektiven Erfassung dieser Naturstoffe geschaffen. Für die Auftrennung der Flavonoidgemische bietet sich vor allem die Dünnschichtchromatographie (DC) an. Über eine anschliessende, quantitative Erfassung durch Direktauswertung der UV-Absorption und Fluoreszenz *in situ* wird im Zusammenhang mit den Silymarinen von Halbach und Görler[3] und Wagner und Mitarbeitern[4] berichtet. Spiegl und Mitarbeiter[5] untersuchten die Möglichkeit der qualitativen Analyse von chromatographisch aufgetrennten Flavonoiden durch Remissionsmessung. Durch Detektion mit Na-azetat, $AlCl_3$ und HCl können an Hand der Verschiebung der Absorptionsbanden gegenüber den Normalspektren strukturelle Merkmale wahrgenommen werden. Nach Angaben der Autoren eignen sich als Sorbentien für die Remissionsmessung besonders Cellulose und Polyamid. Die Absorptionskurven der Remissionsspektren der auf Kieselgel aufgetrennten Flavonoide sind hingegen nach Spiegl und Mitarbeiter[5] sehr abgeflacht und zeigen keine ausgeprägten Maxima.

Für chemotaxonomische Untersuchungen an flavonoidführenden Pflanzen und für die Beobachtung der Flavonoidbildung in Gewebekulturen ist es notwendig, über eine einfache und empfindliche Methode zur qualitativen und quantitativen Bestimmung dieser Naturstoffe zu verfügen. In der vorliegenden Arbeit wurde nun die Möglichkeit einer DC-Auftrennung von Flavonoiden mittels der Hochleistungs-Dünnschichtchromatographie (HPTLC, Nano-DC) und einer anschliessenden, quantitativen Auswertung durch Remissionsmessung *in situ* untersucht.

EXPERIMENTELLER TEIL

Qualitative Untersuchung

Sorptionsschicht, HPTLC-Fertigplatten Kieselgel 60 F_{254} (10 × 10 cm; Merck, Darmstadt, B.R.D.). Das Auftragen der Flavonoidlösungen erfolgt mit einer Mikrokapillare 0.75 nl der Fa. Merck (1 cm vom unteren Plattenrand mit einem seitlichen

Abstand von 5 mm). Laufstrecke, 8 cm. FG 1 (für Aglykone): Benzol–Äthylazetat–Ameisensäure (40:10:5), Entwicklungszeit *ca.* 25 Min; FG 2 (für Glykoside): Aceton–Äthylmethylketon–Ameisensäure (50:35:5), Entwicklungszeit *ca.* 25 Min.

Nach Vertreibung des Fliessmittels im warmen Luftstrom werden die Flavonoide im UV_{254} betrachtet und anschliessend mit Naturstoffreagenz (1 % in Methanol) und mit Polyäthylenglykol (PEG) 4000 (5 % in Äthanol) detektiert (Betrachten im UV_{366}).

Remissionsmessung

Die Absorptionskurven wurden ohne Detektion durch Direktauswertung mit

TABELLE I

R_F-WERTE UND ANFÄRBUNG EINIGER FLAVONOIDE NACH DETEKTION MIT NATURSTOFFREAGENZ UND PEG AUF HPTLC-KIESELGELSCHICHTEN

Aglykone wurden mit FG 1, Glykoside mit FG 2 entwickelt.

Flavonoid	Farbe	R_F Wert
Aglykone		
3-OH-Flavon	hellblau	0.83
Galangin	blassgelb	0.76
Kämpferid	hellblau	0.71
Chrysin	violett	0.68
Pectolinarigenin	blaubraun	0.60
Acacetin	gelbbraun	0.59
Digicitrin	blaubraun	0.57
Naringenin	ocker	0.56
Kämpferol	hellgelb	0.55
Rhamnetin	orange	0.53
Jaceosidin	blaubraun	0.51
Apigenin	gelb	0.47
Chryseriol	gelborange	0.44
Diosmetin	gelborange	0.42
Quercetin	rotorange	0.40
Morin	hellblau	0.38
Nepetin	orange	0.37
Luteolin	orange	0.35
Fisetin	rotorange	0.30
Dihydroquercetin	rotbraun	0.27
Myricetin	rot	0.23
Dihydrofisetin	rotbraun	0.18
Scuttelarein	braunblau	0.14
Glykoside		
Quercitrin	rotorange	0.82
Myricitrin	rot	0.77
Vitexin	gelb	0.75
Apigenin-7-glucosid	gelb	0.71
Luteolin-7-glucosid	orange	0.66
Hyperosid	orange	0.51
Naringin	blaugrau	0.42
Vitexinrhamnosid	gelb	0.37
Lúteolin-5-glucosid	hellblau	0.35
Rutin	orange	0.21
Robinin	gelbgrau	0.12

einem Spektralphotometer (Zeiss PMQ 3) im Bereich von 500 bis 240 nm aufgezeichnet (Parameter: siehe unten). Einwandfreie Spektren werden bei Aglykonen im Mengen-bereich von 20 bis 30 ng, bei Glykosiden von 30 bis 40 ng erhalten.

Quantitative Untersuchung

Zur quantitativen Analyse werden die Flavonoidlösungen mit einer Platin–Iridium Festvolumenauftragekapillare (0.1 nl; Fa. Antech) aufgetragen (1 cm seit-licher Abstand der Flecken voneinander). Nach dem Entwickeln und dem Vertreiben des Fliessmittelgemisches wird durch Remissionsmessung in situ bei einer Wellen-länge von 360 nm die Absorption gemessen (Spektralphotometer Zeiss PMQ 3; Spaltbreite 6 mm, Monochromatorspalt 0.5 mm, Dämpfung 3, Servogor S-Schreiber, Spreizung 20 mV quantitativ, 50 mV qualitativ). Der Durchschnitt der mittleren Standardabweichungen beträgt $\pm$ 2.1 %.

ERGEBNISSE

Als Sorptionsschicht wurde HPTLC-Kieselgel 60 (10 × 10 cm) verwendet. Die Auftrennung der uns zur Verfügung stehenden Flavonoid-Aglykone und -Glykoside (Tabelle I) erfolgte mit den Fliessmittelgemischen FG 1 und 2.

Die Flavonoidgemische wurden mit einer Mikrokapillare 0.75 nl aufgebracht. Das Sichtbarmachen der aufgetrennten Verbindungen erfolgte —neben der Be-trachtung im UV_{254}— durch Detektion mit Naturstoffreagenz nach Neu[2] und PEG[5]. Die Nachweisgrenze liegt bei der Detektion mit Naturstoffreagenz bei etwa 5–8 ng.

Neben den R_F-Werten und der spezifischen Anfärbung der betreffenden Fla-vonoide können auch die Absorptionskurven der Remissionsmessung zur Identifizie-rung herangezogen werden. Zur Bestimmung der UV-Absorption *in situ* wird das
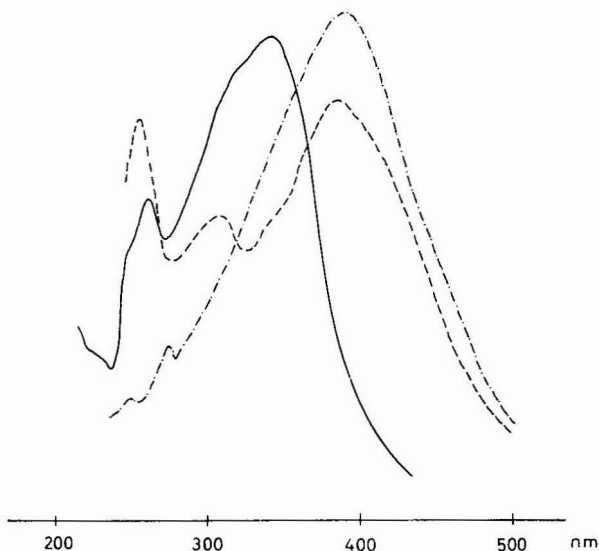
Fig. 1. Remissionsspektren einiger Flavonoid-Aglykone. ———, Acacetin; – – –, Quercetin; – · – · – Fisetin.
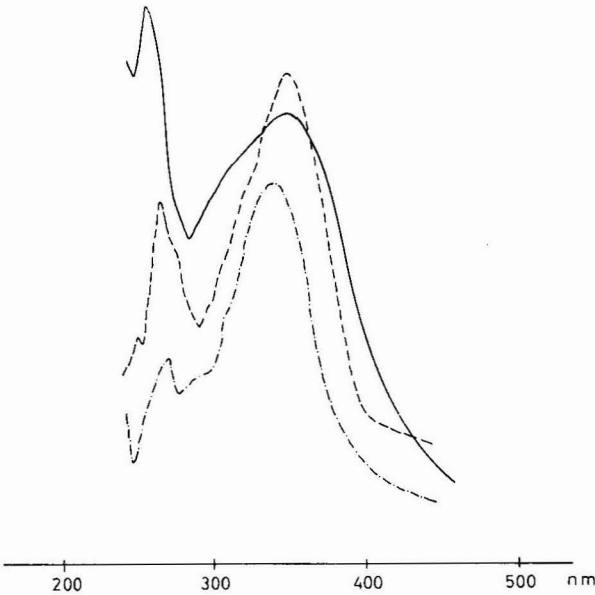
Fig. 2. Remissionsspektren unterschiedlich glykosidierter Flavonoide. ———, Rutin; – – –, Robinin; – · – · –, Apigenin-7-glucosid.

Fliessmittelgemisch im warmen Luftstrom von der Platte vertrieben und die Remissionsspektren im Bereich von 500 bis 240 nm aufgenommen (Fig. 1 und 2 zeigen die Spektren einiger repräsentativer Flavonoid-Aglykone und -Glykoside). Alle von uns untersuchten Flavonoide weisen zwischen 330 und 390 nm ein deutlich ausgeprägtes
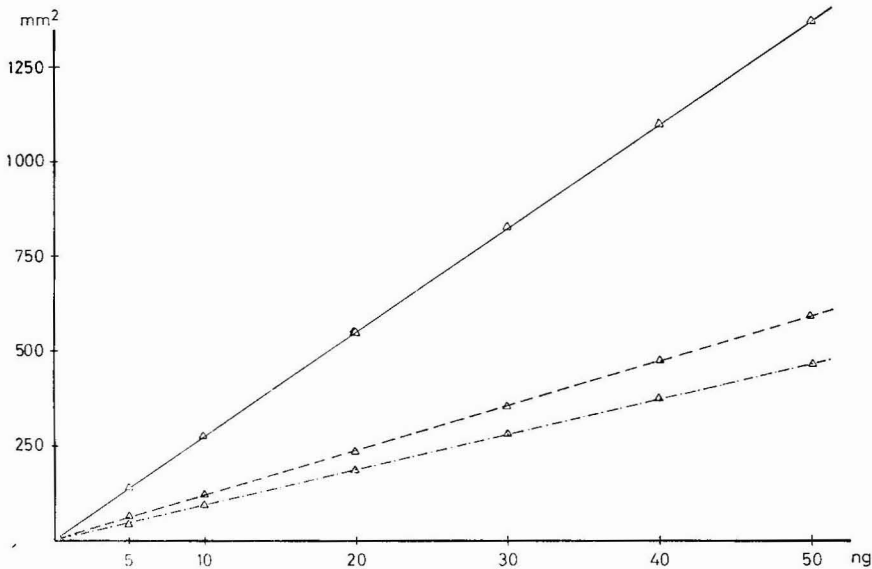
Fig. 3. Durch Remissionsmessung erstellte Eichkurven einiger Flavonoid-Aglykone. ———, Acecatin; – – –, Quercetin; – · – · –, Fisetin.

Maximum auf. Damit ist auch die Voraussetzung für eine quantitative Auswertung gegeben.

Inwieweit man durch die so erhaltenen Absorptionsspektren vor und nach Umsetzung mit Na-azetat, AlCl$_3$ und HCl auf die Struktur der Flavonoide schliessen kann, ist Gegenstand unserer derzeitigen Untersuchungen. Zur quantitativen Erfassung der Flavonoide durch Remissionsmessung auf Kieselgel-HPTLC-Schichten wurden einige Modellflavonoide mit einer Platin–Iridiumkapillare aufgebracht und die Eichkurven erstellt (siehe Fig. 3 und 4). Die Nachweisgrenze liegt für Aglykone bei 5 ng und für Glykoside bei 8 ng.
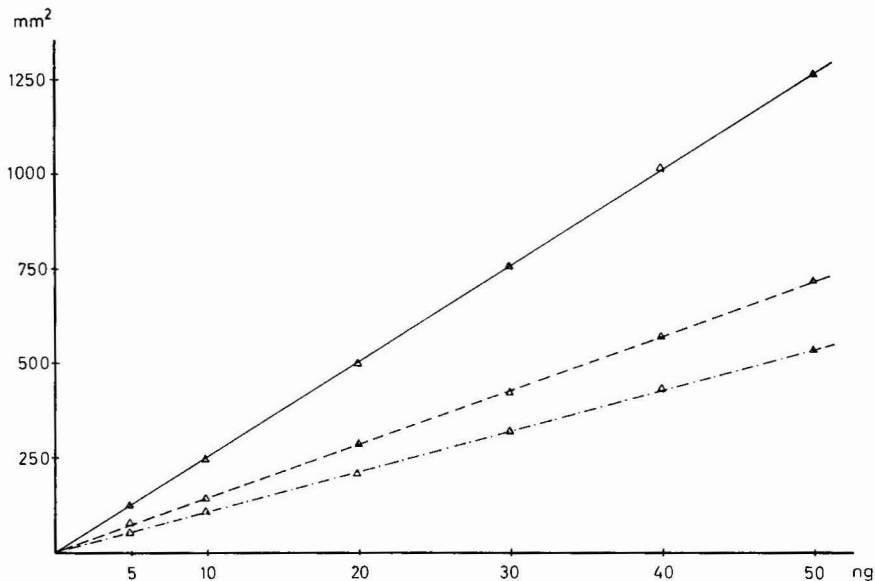
Fig. 4. Durch Remissionsmessung erstellte Eichkurven unterschiedlich glykosidierter Flavonoide. ———, Apigenin-7-glucosid; – – –, Robinin; ———, Rutin.

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Errata

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Page 183, Table I, heading of column 5 should read "*THP*", and heading of column
6 should read "*THF*".

Page 184, Table III, last data of columns 2, 3, 4 and 5 (3   8   19.66   17.86)
should be deleted.

J. Chromatogr., 134 (1977) 524–528

Page 525, section *Gas Chromatography*, 1st line, "Hewlett-Packard Model 419 gas
chromatograph" should read "Packard Model 419 gas chromatograph".

J. Chromatogr., 138 (1977) 97–110

Page 106, Table V, last line, first column: "(i)" should read "(ii)".

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APPARATUS

N-1065

SWEEP CO-DISTILLER

The new Kontes sweep co-distiller is for the clean-up of samples prior to analysis. Extraction is eliminated with this technique. The unit can be used for organophosphates, polychlorobiphenyls and chlorinated pesticides, as well as organic fumigants, herbicides and fungicides. A bibliography is available on request.
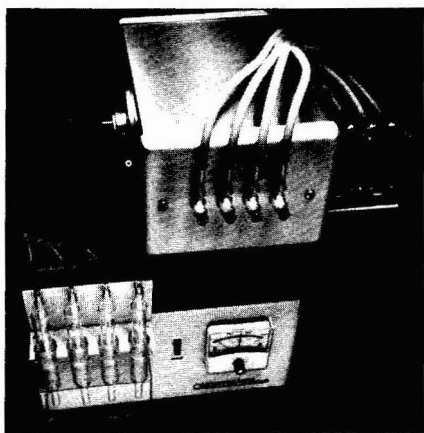
For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.
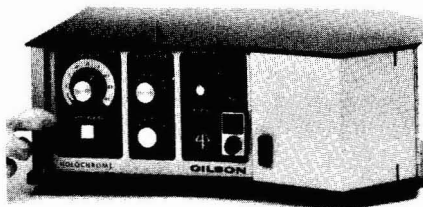
N-1064

UV–VISIBLE ABSORPTION MONITOR

The new Gilson "holochrome" absorption monitor for LC features double-beam optics. Holographic monochromator optics provide continuous calibrated wavelength adjustment from 190 to 600 nm. Interchangeable flow cells have volumes of 8 and 70 μl (10 mm light path). The pressure limit is 37 bar. There is a built-in heater for cold-room operation and an optional disc gel scanning attachment.

N-1053

MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPH

The Familic-100 from Japan Spectroscopic (JASCO) is a modular high-performance liquid chromatograph equipped with a micro column 150 mm × 0.5 mm I.D. of Teflon which is operated at flow-rates of 2–16 μl/min. The sample (as small as 0.1 μl) is first collected in a short pre-column before delivery to the main column. The Familic is designed to operate with a UV detector.

NEW NITROGEN–PHOSPHORUS GC DETECTOR

Tracor Instruments have introduced a new nitrogen–phosphorus gas chromatography detector (Model 702) which provides burn-out protection for the alkali source. An exclusive desensitizing mode allows routine use of chlorinated solvents and derivatizing reagents not possible with previous designs.

TLC SOLVENT SELECTOR SYSTEM

The SelectaSol^{TM} solvent selector system from Schleicher & Schuell allows the simultaneous comparison of up to 16 different solvents and/or samples on a single standard-size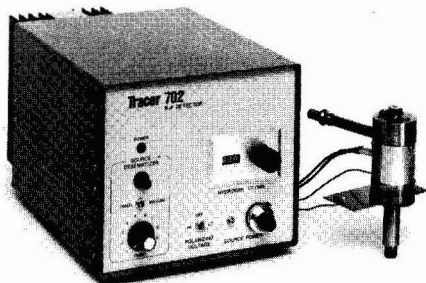 (20 cm × 20 cm) TLC plate. Each chromatogram (16 40-mm, 4 87-mm or one 170-mm diameter) is fed from its own solvent well via a wick and developed by the circular technique. Sealing by gaskets ensures atmospheric solvent saturation. An instructional bulletin is available from the manufacturer.
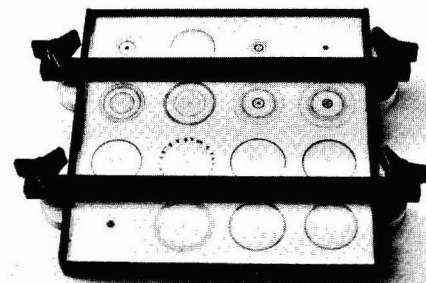
HAMILTON SYRINGES

Two new gas- and liquid-tight syringes are available from Hamilton. The 1701 has a capacity of 10 μl, the 1702 a 25-μl capacity. The Teflon-tipped plunger makes the syringe gas- and liquid-tight.

NEW BOOKS

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Modern practice of gas chromatography, edited by R.L. Grob, Wiley-Interscience, New York, London, Sydney, Toronto, 1977, xx + 654 pp., price £16.00, US$28.00, ISBN 0-471-01564-4.

An introduction to clay colloid chemistry, by H. van Olphen, Wiley, Chichester, New York, Sydney, Toronto, 2nd ed., 1977, ca. 336 pp., price ca. US$24.70, £14.40, ISBN 0471-01463-X.

Liquid chromatography in practice, by P.A. Bristow, hetp, Wilmslow, 1977, ca. 265 pp., price £10.00, US$20.00 (paperback), £14.00, US$28.00 (hardback), £5.00, US$10.00 (microfiche), ISBN 0-9504833-0-3 (paperback), 0-9504833-1-1 (hardback), 0-9504833-2-X (microfiche).

Photochemistry – 6 (6th IUPAC Symposium, Marseille, 1976; previously published in *Pure Appl. Chem.*, Vol. 49, No. 3), edited by A. Gilbert, Pergamon, Oxford, 1977, 162 pp., price £18.00, US$32.00, ISBN 0-08-021201-8.

Nonaqueous solutions – 5 (5th International Conference, Leeds, 1976; previously published in *Pure Appl. Chem.*, Vol. 49, No. 1), edited by J.B. Gill, Pergamon, Oxford, 1977, 124 pp., price £13.00, US$23.75, ISBN 0-08-021202-6.

Polymerization of heterocycles (ring-opening), (previously published in *Pure Appl. Chem.*, Vol. 48, No. 3), edited by S. Penczek, Pergamon, Oxford, 1977, ca. 126 pp., price £10.00, US$18.00, ISBN 0-08-021367-7.

How to name an inorganic substance – Guide to the use of nomenclature of inorganic chemistry (the red book), by W.C. Fernelius, Pergamon, Oxford, 1977, 35 pp., price £2.80, US$5.00, ISBN 0-08-021982-9.

Stereochemistry – Fundamentals and methods, Vol. 1, Determination of configurations by spectrometric methods, edited by H.B. Kagan, Thieme Verlag, Stuttgart, 1977, ca. 260 pp., price ca. DM88.00, ISBN 3-13-132501-1.

Stereochemistry – Fundamentals and methods, Vol. 4, Absolute configurations of 6000 selected compounds with one asymmetric carbon atom, edited by H.B. Kagan, Thieme Verlag, Stuttgart, 1977, xx + 602 pp., price ca. DM158.00, ISBN 3-13-132801-0.

Filter aids and materials – Technology and applications, by H.T. Driscoll, Noyes Data Corporation, Park Ridge, N.J., 1977, x + 307 pp., price US$39.00, ISBN 0-8155-0658-9.

Surfactants and sequestrants 1977 – Recent advances, (Chemical Technology Review No. 89), by S.J. Gutcho, Noyes Data Corporation, Park Ridge, N.J., 1977, 291 pp., price US$39.00, ISBN 0-8155-0661-9.

Liquid chromatography of polymers and related materials (Chromatographic Science Series, Vol. 8), edited by J. Cazes, Marcel Dekker, New York, Basel, 1977, x + 180 pp., price SFr.65.00, ISBN 0-8247-6592-3.

Instrumental organic elemental analysis, by R. Belcher, Academic Press, New York, London, 1977, ISBN 12-08595-0-5.

The total synthesis of natural products, Vol. 3, edited by J. ApSimon, Wiley, Chichester, New York, Sydney, Toronto, 1977, *ca.* 832 pp., price *ca.* US$ 38.10, £ 22.50, ISBN 0471-02392-2.

Analytical applications of NMR, by D.E. Leyden and R.H. Cox, Wiley, Chichester, New York, Sydney, Toronto, 1977, *ca.* 448 pp., price *ca.* US$33.00, £19.45, ISBN 0471-53403-X.

Chemistry of coal utilization, Vols. 1 and 2, edited by H.H. Lowry, Wiley, Chichester, New York, Sydney, Toronto, 1977, 2080 pp., price US$63.45, £37.50 (2-vol. set), ISBN 0471-02494-5.

Amino acids, peptides and proteins, by H.D. Jakubke and H. Jeschkeit, Macmillan, New York, London, 2nd ed., 1977, price £10.50, ISBN 333-17886-6.

Analytical and quantitative methods in microscopy (Society for Experimental Biology Seminar Series 3), Cambridge University Press, London, New York, 1977, price £9.00, paperback £4.00, ISBN 521-21404-1, paperback ISBN 521-29141-0.

Molecular spectroscopy, edited by A.R. West, Heyden & Son, London, 1977, price £30.00, ISBN 85501-303-6.

Gas chromatographs as industrial process analysers, by D.J. Huskins, Adam Hilger, Bristol, 1977, xv + 166 pp., price £10.00, ISBN 0-85274-137-5.

Chemical analysis of additives in plastics (International Series on Analytical Chemistry, Vol. 46), by T.R. Crompton, Pergamon, Oxford, 2nd ed., 1977, price £11.10, ISBN 08-020497-X.

IP standards for petroleum and its products, Part 1, Methods for analysis and testing, 2 Vols., Heyden & Son, London, 36th ed., 1977, price £36.00, ISBN 85501-309-5.

Aquametry, Part 1, A treatise on methods for the determination of water, by J. Mitchell, Jr. and D.M. Smith, Wiley, Chichester, 1977, *ca.* 646 pp., price *ca.* £22.45, US$38.00, ISBN 0471-02264-0.

Advances in infrared and Raman spectroscopy, Vol. 3, edited by R.J.H. Clark and R.E. Hester, Heyden & Son, London, 1977, 286 pp., price £17.50, US$ 35.00, DM 112.00, ISBN 0-85501-183-1.

Compendium of organic synthetic methods, Vol. 3, by L.S. Hegedus and L.G. Wade, Jr., Wiley, Chichester, 1977, *ca.* 400 pp., price *ca.* £12.00, US$ 20.30, ISBN 0-471-36752-4.

Treatise on analytical chemistry, Part 3, Vol. 4, edited by I.M. Kolthoff, P.J. Elving and F.H. Stross, Wiley, Chichester, 1977, *ca.* 688 pp., price *ca.* £30.00, US$ 46.00, ISBN 0-471-02765-0.

Scientific analysis on the pocket calculator, by J.M. Smith, Wiley, Chichester, 2nd ed., 1977, *ca.* 512 pp., price *ca.* £12.00, US$ 20.25, ISBN 0-471-03071-6.

Applications of ion selective membrane electrodes in organic analysis, by G.E. Baiulescu and V.V. Cosofret, Ellis Horwood/Wiley, Chichester, 1977, *ca.* 250 pp., price *ca.* £14.00, US$ 26.60, ISBN 0-85312-060-9.

Fine particles in gaseous media, by H.E. Hesketh, Ann Arbor Sci. Publ., Ann Arbor, Mich./Wiley, Chichester, 1977, *ca.* 200 pp., price *ca.* £18.40, US$ 30.25, ISBN 0-250-40182-7.

Carbohydrate chemistry 8, edited by K. Onodera, Pergamon, Oxford, 1977, 172 pp., price £15.00, US$ 27.00, ISBN 0-08-02201-0 (previously published in *Pure Appl. Chem.*, Vol. 49, No. 8).

Houben Weyl: Methoden der Organischen Chemie, Band V/2a, Alkine, Di- und Polyine, Allene, Kumulene, edited by E. Müller, Georg Thieme, Stuttgart, 4th ed., 1977, *ca.* 1280 pp., price DM 880.00 (subscription price DM 792.00), ISBN 3-13-202604-2.

Advances in mass spectrometry, Vol. 7 (7th Int. MS Conf., Florence, August–September, 1976), edited by N.R. Daly, Heyden & Son, London, 1977, 1600 pp., price £72.00, US$ 144.00, DM 460.00, ISBN 0-85501-305-2.

Physical organic chemistry 3, by A. Fruchier, Pergamon, Oxford, 1977, 110 pp., price £9.50, US$ 17.00, ISBN 0-08-021197-6 (previously published in *Pure Appl. Chem.,* Vol. 49, No. 7).

Colloid and surface science, edited by E. Wolfram, Pergamon, Oxford, 1977, 108 pp., price £11.00, US$ 20.00, ISBN 0-08-021570-X (previously published in *Pure Appl. Chem.,* Vol. 48, No. 4).

Critical evaluation of some equilibrium constants involving alkylammonium extractants, by A.S. Kertes, Pergamon, Oxford, 1977, 36 pp., price £3.30, US$ 6.00, ISBN 0-08-021591-2.

Photochemical processes in polymer chemistry 2 (2nd IUPAC Micro Symp.), edited by G. Smets, Pergamon, Oxford, 1977, 136 pp., price £13.60, US$ 24.50, ISBN 0-08-021205-0 (previously published in *Pure Appl. Chem.,* Vol. 49, No. 4).

Particle size analysis, edited by J.D. Stockham and E.G. Fochtman, Ann Arbor Sci. Publ., Ann Arbor, Mich./Wiley, Chichester, 1977, *ca.* 140 pp., price *ca.* £16.75, US$ 27.00, ISBN 0-250-40189-4.

Microprocessors; Fundamentals and applications, edited by W.C. Lin, IEEE Press/Wiley, Chichester, 1977, 344 pp., price £14.50, US$ 25.00 (cloth); £6.65, US$ 11.45 (paper); ISBN 0-471-03115-1 (cloth), 0-471-03114-3 (paper).

Handbook of derivatives for chromatography, edited by K. Blau and G.S. King, Heyden & Son, London, 1977, 576 pp., price £24.00, US$ 48.00, DM 154.00, ISBN 0-85501-206-4.

Handbook of analysis of synthetic polymers and plastics, by J. Urbański, W. Czerwiński, K. Janicka, F. Majewska and H. Zowall, Ellis Horwood, Chichester/Halsted (Wiley), New York, 1977, 494 pp., price £25.00, US$ 47.50, ISBN 0-85312-020-X.

MEETING

INTERNATIONAL CONFERENCE ON COMPUTERS AND OPTIMIZATION IN ANALYTICAL CHEMISTRY

The International Conference on Computers and Optimization in Analytical Chemistry will be held from April 5–7, 1978, in Amsterdam,The Netherlands.

Some years ago "Computers in Analytical Chemistry" was the theme of successful conferences in Mainz and Vienna. The continuation of this series of conferences is a logical consequence of the continuous and even increasing interest in this area. The application of formal optimization techniques in analytical chemistry now attracts wide attention and because of the strong links between computerization and optimization, the theme of the third conference will be "Computers and Optimization in Analytical Chemistry". The scientific committee expects the combination of topics to be of benefit to scientists active or interested in both areas.

The scientific programme will include invited plenary lectures, and invited and submitted research papers. Topics are: design and use of computer systems for data acquisition and data processing; computer controlled analysis; application of microprocessors; data retrieval, pattern recognition, artificial intelligence, etc.; development and application of formal techniques for design, optimization and evaluation of analytical procedures and results; application of system theory, operations research, information theory, mathematics, statistics and other chemometrical techniques in analytical chemistry. Discussions on these topics will be organised.

Papers presented at the conference will be refereed for publication in a special issue of *Analytical Chimica Acta/Computer Techniques and Optimization.*

Further details are available from the Conference Secretary, Laboratory for Analytical Chemistry, Nieuwe Achtergracht 166, Amsterdam, The Netherlands.

CALENDAR OF FORTHCOMING MEETINGS

November 7–11, 1977
Amsterdam, The Netherlands

Chromatography '77. 12th International Symposium on Advances in Chromatography

Contact:
Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A. (Further details published in Vol. 131; program published in Vol. 136, No. 2)

November 14–15, 1977
Tübingen, G.F.R.

Mass Spectrometry and Combined Techniques in Medicine, Clinical Chemistry and Clinical Biochemistry

Contact:
Dr. H.M. Liebig, Abteilung Innere Medizin IV, Medizinische Klinik der Universität Tübingen, Otfried-Müller-Strasse 10, D-7400 Tübingen, G.F.R. (Further details published in Vol. 137, No. 2)

December 9–11, 1977
San Francisco, Calif., U.S.A.

Clinical Toxicology, Pharmacology and Carcinogenic Hazards

Contact:
Medical Symposia, Division of Letters and Sciences, University of California Extension, Berkeley, Calif. 94720, U.S.A.

February 27–March 3, 1978
Cleveland, Ohio, U.S.A.

29th Annual Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy

Contact:
Robert W. Baudoux, United States Steel Corporation, Research Laboratory, MS 57, Monroeville, Pa. 15146, U.S.A.

April 5–7, 1978
Amsterdam, The Netherlands

International Conference on Computers and Optimization in Analytical Chemistry

Contact:
Conference Secretary, Laboratory for Analytical Chemistry, Nieuwe Achtergracht 166, Amsterdam, The Netherlands. (Further details published in Vol. 140, No. 3)

April 18–21, 1978
Munich, G.F.R.

Biochemische Analytik 78

Contact:
Dr. Rosmarie Vogel, P.O. Box 200324, D-8000 Munich 2, G.F.R.

April 18–22, 1978
Munich, G.F.R.

Analytica 78

Contact:
Münchener Messe- und Ausstellungsgesellschaft, Messegelände, P.O. Box 121009, D-8000 Munich 12, G.F.R.

April 27–28, 1978 Montreal, Canada	**Canadian Chromatography Conference** Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario K6H 5V7, Canada

May 21–24, 1978 Hluboká, Czechoslovakia	**6th International Symposium on Biomedical Applications of Chromatography** Contact: Assoc. Professor Dr. K. Macek, Physiological Institute of the Czechoslovak Academy of Sciences, Budejovická 1083, Prague 4, Czechoslovakia (Further details published in Vol. 143, No. 6)

June 13–16, 1978 Ghent, Belgium	**2nd International Symposium on Quantitative Mass Spectrometry in Life Sciences** Contact: Prof. Dr. A. De Leenheer, Laboratoria voor Medische Biochemie en Klinische Analyse, De Pintelaan 135, B-9000 Ghent, Belgium

June 29–30, 1978 Stockholm, Sweden	**World Chromatography Conference** Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario K6H 5V7, Canada

August 20–25, 1978 Dublin, Ireland	**Euroanalysis III** Contact: Secretariat Euroanalysis III, Institute for Industrial Research and Standards, Ballymun Road, Dublin 9, Ireland (Further details published in Vol. 137, No. 1)

September 25–29, 1978 Baden-Baden, G.F.R.	**12th International Symposium on Chromatography** Contact: Geschäftsstelle der Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, P.O. Box 900440, 6000 Frankfurt/ Main 90, G.F.R. (Further details published in Vol. 140, No. 2)

GENERAL INFORMATION

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (full-length papers), short communications and notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (e.g., Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

Title. The title of the paper should be concise and informative. Since titles are widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the authors' full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are *not* to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

Summary. Full-length papers and review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers etc. remain legible after reduction. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using arabic numerals. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the authors' expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

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1 A. T. James and A. J. P. Martin, *Biochem. J.,* 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography,* Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds,* Vol. IV, *Heterocyclic Compounds,* Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976,* Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication". The *Journal of Chromatography; Journal of Chromatography, Biomedical Applications* and *Chromatographic Reviews* should be cited as *J. Chromatogr.*

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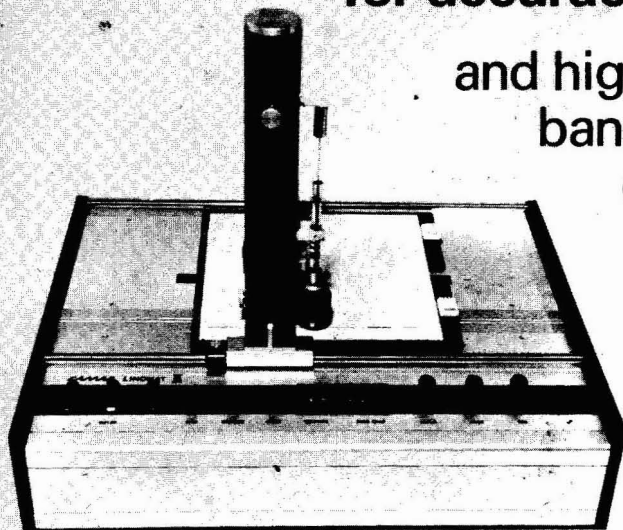
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