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Journal of Chromatography	166/1 166/2 167	168/1 168/2	169 170/1	170/2	171 172	173/1 173/2	174/1	174/2 175/1 175/2	The publication schedule for the volumes 176–180 and for further *Chromatographic Reviews* issues (Vol. 165) will be published later.				
Chromatographic Reviews				165/1			165/2						
Biomedical Applications		162/1	162/2	162/3	162/4	163/1	163/2	163/3	163/4	164/1	164/2	164/3	164/4

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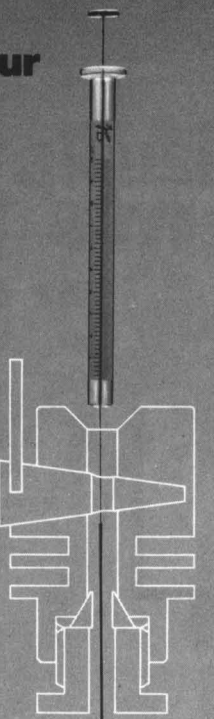

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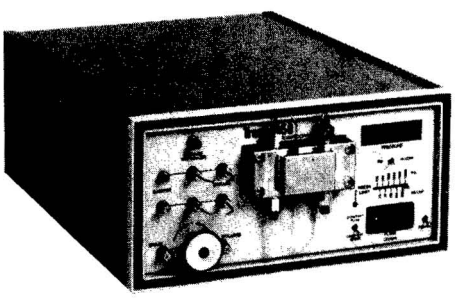

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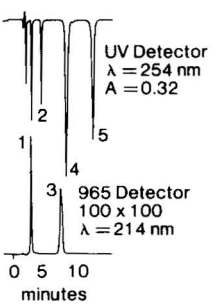

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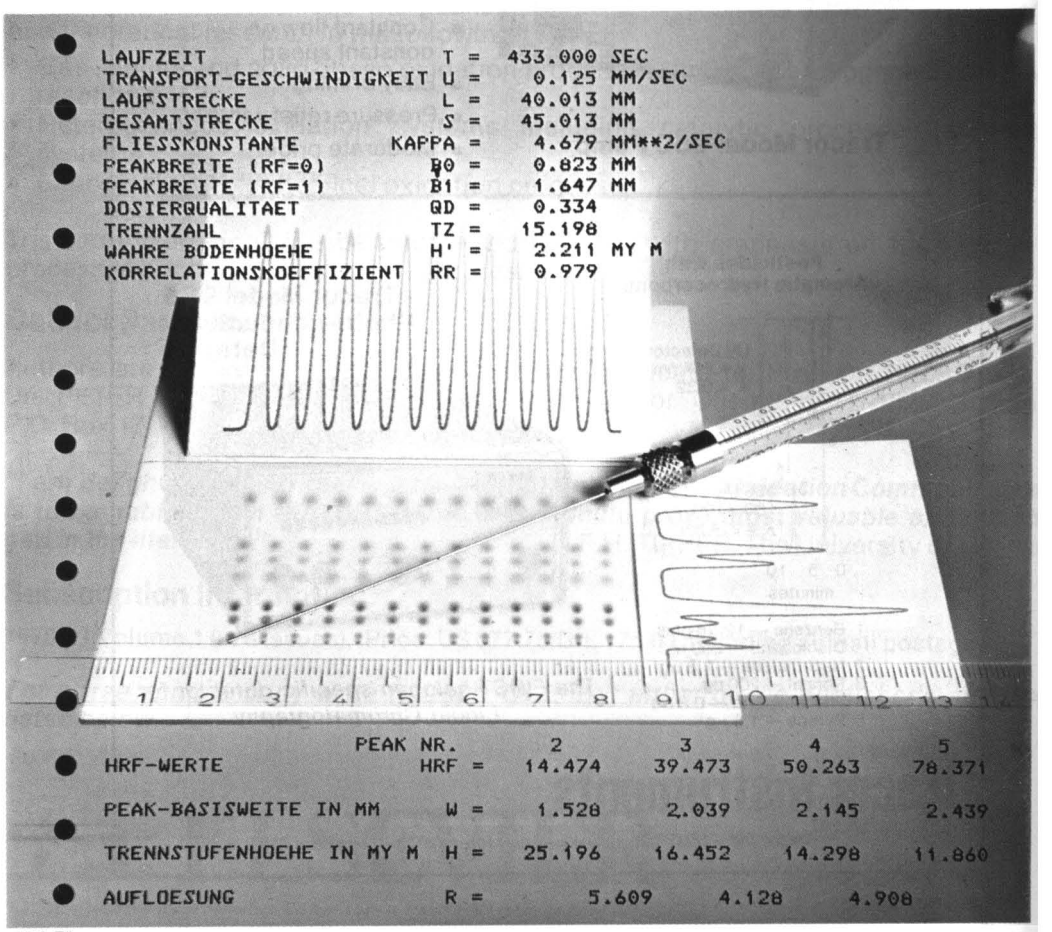

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MASS TRANSFER IN IDEAL AND GEOMETRICALLY DEFORMED OPEN TUBES

I*. IDEAL AND COILED TUBES WITH CIRCULAR CROSS-SECTION**

KURT HOFMANN and ISTVÁN HALÁSZ

Angewandte Physikalische Chemie, Universität des Saarlandes, 6600 Saarbrücken (G.F.R.)

(Received August 16th, 1978)

SUMMARY

A summary of the hydrodynamics relevant to open tubes is presented. The band broadening of unretained samples in ideal and coiled tubes with circular cross-sections (0.25–4 mm I.D.) was measured with *n*-heptane and water as eluents. The lack of any appreciable interference from instrumental factors is substantiated by the experimentally determined diffusion coefficients and critical Reynold's numbers. It is shown that carefully prepared copper tubes behave as ideally as the corresponding glass tubes. With decreasing tube diameter, wall roughness leads to greater radial mass transfer. This effect is evident in 0.25-mm I.D. copper tubes and 0.5-mm (or even 0.75-mm) I.D. stainless-steel tubes, and can be used to advantage in constructing heat exchangers, connecting tubes or reaction detectors in liquid chromatography.

It is shown for the first time that the onset of turbulence can be detected with considerably higher sensitivity by means of *h versus u* curves than by the *K versus u* curves used previously.

It is shown that at the onset of turbulence the specific permeability decreases by a factor of about 3. Band broadening in the turbulent region was found to be ten times greater than that calculated on the basis of the friction theory of Taylor, apparently because laminar conditions prevail in a substantial portion of the tube cross-section.

If an ideal tube is coiled, its specific permeability decreases with increasing velocity. Furthermore, coiling stabilizes "laminar" flow and the beginning of the turbulent region is shifted to higher Reynold's number (*i.e.*, higher velocities).

It is shown that, as the inner diameter of the open tube decreases, higher linear velocities are required in order to decrease band broadening substantially (*e.g.*, by a factor of 5) for coiled tubes than for ideal tubes. For example, a linear velocity of 250 cm/sec is necessary to achieve this factor of 5 with 0.25-mm I.D. tubing, even though the presence of secondary flow is indicated by the *K versus u* curve at $u > 10$ cm/sec. For liquid chromatographic separations tubes narrower than 0.25 mm I.D. are essential. Therefore, at acceptable linear velocities (< 50 cm/sec), the coiling of an

* Part II: I. Halász, *J. Chromatogr.*, 173 (1979) 229.

** Part of the Ph.D. Thesis of Kurt Hofmann, University of Saarbrücken, Saarbrücken, 1975.

ideal tube will not reduce band broadening appreciably in comparison with that in straight (ideal) tubes.

Secondary flow effects at high velocities, however, may decrease the h values markedly. The h value in a coiled tube of 1.2 mm I.D. was 290 times smaller at $u = 250$ cm/sec than in a corresponding ideal tube.

INTRODUCTION

In gas chromatography, the principal advantage of open tubes (capillary columns) over packed columns lies in the considerably greater number of theoretical plates (n) that can be generated per unit pressure drop[1]. In liquids the diffusion coefficients are about 10^4 times smaller than in gases, which results in greater band broadening in liquid mobile phases. Further, the *ca.* 100-fold higher viscosity of liquids compared with gases requires a greater pressure drop over the column to achieve the same linear velocities for similar tube geometries. Consequently, efforts have constantly been made to apply the advantages of open tubes to high-performance liquid chromatography (HPLC).

This paper compares the behaviour of ideal open tubes in the region of laminar and turbulent flow and discusses the radial mass transfer within them. In Part II, potential applications of ideal and coiled open tubes in liquid chromatography are discussed. Part II also describes the advantages of other types of geometrically deformed tubes[2–11], which stem primarily from the more rapid radial mass transfer within them. This is not only essential in liquid chromatography, but is also desirable for heat exchangers[8], mixing tubes (including reaction detectors), and connecting tubing between the sampling device and the column and between the column and the detector in HPLC.

In classical hydrodynamics, the various types of flow (*e.g.*, laminar and turbulent) are described in terms of the specific permeability, K, (which will be abbreviated to permeability) as a function of the cross-sectionally averaged linear velocity, u. It will be shown that the relative band broadening, h, (also called the height equivalent to a theoretical plate) is a more sensitive function of the type of flow than the permeability. Therefore, the ordinary chromatographic method may be very useful in hydrodynamics.

This paper describes only the properties of inert samples ($k' = 0$), as band spreading can only increase with increasing capacity ratios, k'.

As chromatographers are not always conversant with the basic principles of hydrodynamics, a brief description will be presented first. It will be demonstrated by measurements in ideal tubes that no additional band broadening accrues from the apparatus. Finally, the hydrodynamic properties of geometrically deformed tubes (coiled, squeezed, twisted and undulated) fabricated from various column materials (glass, copper, stainless steel, aluminium and Teflon) will be described.

FUNDAMENTAL PRINCIPLES OF HYDRODYNAMICS

In this summary, the usual detailed and thorough treatment of hydrodynamics will be dispensed with.

Liquid flow in ideal tubes

Ideal open tubes are straight tubes with a constant circular cross-section, a smooth inner wall and smooth inlet and outlet surfaces. The tube must be long enough that disturbances at the inlet and outlet are negligibly small.

The compressibility of liquids[12] will always be neglected.

For laminar flow in ideal tubes, it will be assumed that:

(a) there are co-axial layers in the flowing medium where the velocity is constant;

(b) except for diffusion flow, no radial flow takes place;

(c) the cross-sectionally averaged linear velocity can be described by means of the Hagen–Poiseuille equation[13,14]:

$$u = \frac{K \Delta P}{\eta L} = \frac{r^2 \Delta P}{8 \eta L} \tag{1}$$

(see list of symbols at the end of the paper);

(d) the maximum linear velocity at the centre of the tube is

$$u_{max} = 2\,u \tag{2}$$

(e) for "real" tubes the length of the inlet disturbance, L_e, (ref. 15) is given by

$$L_e = 100\,d = 200\,r \tag{3}$$

(f) the Reynold's number, Re, is less than 2000–2300, where

$$Re = \frac{\varrho d u}{\eta} = \frac{2 r u}{\nu} \tag{4}$$

(g) the permeability, K, is not a function of the linear velocity;

(h) the wall of the tube is inert, *i.e.*, there is no interaction between the wall and the liquid phase.

Mass transfer processes in ideal tubes

As shown by Taylor[16], dispersion in flowing liquids is not an isotropic property. Aris[17] extended the theory of Taylor to include axial diffusion for dispersion in flowing liquids and gave the dynamic diffusion coefficient as

$$D_{\mathrm{dyn}} = D + \frac{r^2 u^2}{48 D} \tag{5}$$

Based on additional conclusions of Aris, D_{dyn} may be substituted in the unidimensional Einstein diffusion equation[18]:

$$\sigma^2 = 2 D_{\mathrm{dyn}} t \tag{6}$$

and rewritten[5] in chromatographic nomenclature as

$$h \equiv \frac{\sigma^2}{L} = \frac{2D}{u} + \frac{r^2 u}{24D} \tag{7}$$

For $u > 0.01$ cm/sec, the first term on the right-hand side of eqn. 7 becomes negligible for liquids (this is identical with the approach of Taylor), and thus the Aris theory to a good approximation can be written as

$$h = \frac{r^2 u}{24D} \tag{8}$$

Golay[1] extended the Aris theory to include mass transfer at non-inert tube walls. It should be noted here that the Golay equation is not valid even for coiled tubes, let alone geometrically deformed tubes.

Turbulent flow in ideal tubes

To a first approximation, if $Re > 2300$ the flow becomes turbulent. With the onset of turbulence a substantial distortion of the flow profile[15,19] occurs, in relation to laminar flow. As a result of the radial flow that appears on reaching turbulence,

$$u_{max} = 1.22u \tag{9}$$

For turbulent flow the length of the inlet or outlet disturbance is

$$L_e = 40d \tag{10}$$

Formally, eqn. 1 can also be applied to the turbulent region by taking into account that K decreases monotonically with increasing flow-rate, as will be shown experimentally. In the transition region from laminar to turbulent flow the permeability decreases sharply at first and then more gradually. The theory of turbulent flow[11] will not be discussed here, as it is beyond the scope of this paper.

In chromatographic discussions, it has frequently been suggested that operation in the turbulent region should optimize dispersion and mass transfer both for gas[2] and liquid chromatography. However, it is sometimes overlooked that the high velocities and lower (poorer) permeabilities necessitate a higher pressure drop over the column by up to a factor of 3. This aggravates almost all instrumental problems. It is also open to question whether the rate of mass transfer in (or on) the stationary phase can keep up with this high linear velocity of the mobile phase. At such high velocities, the risk of mechanical erosion should not be underestimated.

Liquid flow in coiled tubes with a circular cross-section

In coiled tubes with a circular cross-section, a radial mixing flow occurs, which is called secondary flow[20,21]. This secondary flow is caused by the centrifugal forces, f. These forces can be related to the volume element, V, for convenience and can be described by

$$\frac{f}{V} = \frac{\varrho u^2}{R} \tag{11}$$

where ϱ is the density of the flowing medium and R the distance from the tube axis. This disrupts the flow profile to the extent that the radial mixing equalizes the velocity differences to a certain extent. This radial mass transport is desirable for chromatography and heat exchangers. Whenever secondary flow occurs, the permeability becomes a monotonically decreasing function of the flow-rate even in the "laminar" region ($Re < 2300$) and the onset of turbulence is displaced to greater Reynold's numbers.

Coiling produces the least geometric change in ideal tubes, but it is then no longer possible to give an explicit mathematical description of the flow profile or of the mass transfer phenomena. These problems become even more difficult for more complex geometric forms such as oval cross-sections (squeezed) and/or twisted shapes. In such instances only the experimental results can be presented, systematically if possible.

EXPERIMENTAL

Chromatographic equipment

The measurements were performed with a home-made apparatus[11,22,23]. A piston diaphragm pump (Orlita, Giessen, G.F.R., Type S 15) was used to provide a pulseless[23] flow of eluent. The sampling device[22] and the home-made UV detector (254 nm) were adapted to the tubes[11], so that the inlet and outlet disturbances were negligible. The dead volumes of the apparatus were minimized and the detector response times were optimized to measure real phenomena within the limits of error given.

Preparation of glass tubes

A sophisticated apparatus was developed[11] by means of which even glass tubes with an oval cross-section could be twisted reproducibly and coiled.

Treatment of metal tubes

The metal tubes were rinsed with methylene chloride before use. In order to smooth and harden the material (copper), the straight tubes, before being stretched, were carefully coiled around a *ca.* 11-cm diameter cylinder under a light tensile strain. Any residual ripples in the tubes were negligible, as evidenced by the fact that essentially literature values were measured for Re_{crit} and D. The copper capillary and stainless-steel tubing with more or less smooth inner surfaces were obtained from Schoeller (Hellenthal/Eifel, G.F.R.) and the aluminium tubing with a smooth inner surface from Cochius (Frankfurt/Main, G.F.R.).

Mobile phases and samples

The following eluent/sample systems were used: (a) *n*-heptane/1 vol.-% benzene in *n*-heptane; (b) methylene chloride/1 vol.-% benzene in methylene chloride; and (c) water/5 vol.-% acetone in water.

The amounts of sample varied between 2 and 10 μl, depending on the tube diameter. All measurements were carried out at room temperature.

For the calculation of Reynold's numbers, the following values were used:

	Density (g/cm^3)	*Dynamic viscosity* (*Poise*)	*Kinematic viscosity* (*Stokes*)
n-Heptane	0.68	$4 \cdot 10^{-3}$	$5.9 \cdot 10^{-3}$
Water	1.0	$10 \cdot 10^{-3}$	$10 \cdot 10^{-3}$

Evaluation of the chromatograms

The relative band broadening (h values) was calculated by means of the equation

$$h = \frac{Lw^2}{16t_0^2} \tag{12}$$

The h values were usually reproducible to better than $\pm 5\%$.

The permeability, K, of the tubes was calculated by means of eqn. 1. Manometers of grade 1.0 were used. Except for unusually difficult cases, the accuracy of these measurements was better than $\pm 10\%$. The determination of the centre of mass of some peaks with ideal tubes was particularly difficult.

MEASUREMENTS WITH IDEAL TUBES

n-Heptane as mobile phase

Most measurements were carried out with 10-m tubes. As will be shown, the pre-treated copper usually behaved ideally and therefore no measurements with glass tubes are described here.

In the following sections, typical experimental results will first be discussed in detail for the 1-mm I.D. tubing. All other results, for tubing between 0.25 and 4 mm I.D., will be presented subsequently in order of increasing diameters.

Fig. 1 shows the permeability, K, of an ideal copper tube (1 mm I.D. $\times$ 2 mm O.D.) as a function of the cross-sectionally averaged linear velocity, u. The turbulent region ($Re = 2040$) was attained at $u_{crit} = 120$ cm/sec. The K values in the laminar

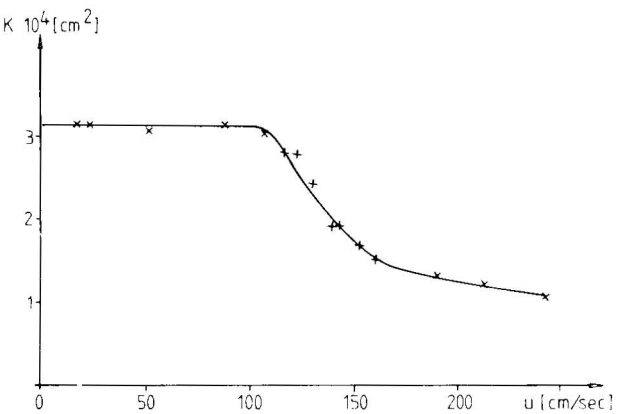

Fig. 1. K *versus* u curve for an ideal open tube (I.D. 1 mm). Copper tube (1.0 mm I.D. $\times$ 2.0 mm O.D.), $L = 10$ m. Eluent, *n*-heptane; sample, benzene; room temperature.

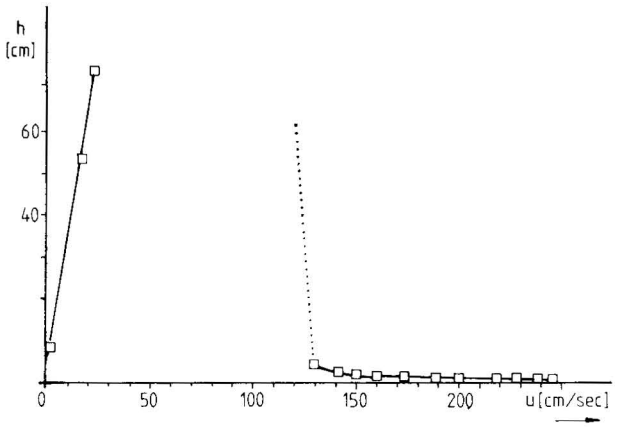

Fig. 2. *h versus u* curve for an ideal open tube. Conditions as in Fig. 1.

region correspond to those calculated via eqn. 1 ($K_{theo} = 3.1 \cdot 10^{-4}$ cm^2). When u was increased to 2 u_{crit} (240 cm/sec) the permeability deteriorated by a factor of 3. The experimental scatter shown in Fig. 1 is characteristic of the accuracy attainable with ideal tubes.

Fig. 2. depicts the *h versus u* plot for the same copper tube. In the laminar region, even careful and rapid sample application yielded asymmetric peaks if the residence time, t, in the tube was not long enough. As can be seen, the h values could be calculated if $u < 30$ cm/sec ($t > 33$ sec). In this region, the experimental h values agree with those calculated from the Taylor approximation (eqn. 8). In the laminar region with $30 < u < 120$ cm/sec the peaks were asymmetric and therefore could not be evaluated (dotted line in Fig. 2). At higher velocities the dotted line shows the calculated (eqn. 8) h values. In the turbulent region ($u > 120$ cm/sec), very symmetrical peaks were obtained. At $u = 130$ cm/sec the relative band broadening decreased abruptly ($h = 2.5$ cm) and continued to diminish slowly with increasing u. As later measurements showed, and as has already been pointed out earlier[5], an *h versus u* plot is a substantially more sensitive indicator of the type of flow of a liquid than is the hydrodynamic quantity K.

Measurements similar to those discussed above were carried out at room temperature with 10 m × 0.5–2-mm I.D. copper tubes. The results are summarized in

TABLE I

EXPERIMENTAL PARAMETERS DETERMINED IN IDEAL OPEN TUBES

Eluent, *n*-heptane; sample, benzene; room temperature.

I.D. × *O.D. of tube (mm)*	u_{sym} *(cm/sec)*	u_{turb} *(cm/sec)*	h_{turb} *(cm)*	$D_{calc} \cdot 10^5$ *(cm^2/sec)*
0.5 × 1.5	78	230	2.3	3.3
0.75 × 2.0	35	170	2.2	3.4
1.0 × 2.0	25	130	2.0	3.4
1.2 × 2.0	20	115	2.5	3.4
1.4 × 2.0	15	90	3.0	3.6
2.0 × 4.0	6.4	60	3.0	4.1

Table I. The first column lists the inner or outer diameter of the tube. The second column (u_{sym}) indicates the maximum linear velocity at which symmetrical peaks are still obtained and at which the rise of the *h versus u* curve is linear. The next two columns (u_{turb} and h_{turb}) give the velocity and the relative band broadening at incipient turbulence, respectively. It is noteworthy that the onset of turbulence was almost always manifest at lower velocities in *h versus u* curves than in *K versus u* plots, even though the velocity differences were within the probable error limits (*ca.* 5–10%). The calculated diffusion coefficients, D_{calc}, of benzene in *n*-heptane (last column) will be discussed later. In this study the onset of turbulence occurred between Reynold's numbers of 2040 and 2160.

The data in Table I substantiate that the apparatus had been carefully constructed and that the inlet and outlet disturbances, with exception of the last row of results, were negligible.

In contrast to the tubes evaluated in Table I, the roughness in 0.25 mm I.D. × 1.5 mm O.D. tubes was not negligible (Fig. 3). The *h versus u* curve is linear up to $u = 70$ cm/sec, then flattens out, passes through a maximum and declines sharply. The measurements were extended to $u = 515$ cm/sec, the onset of turbulence ($Re = 2180$). However, complete turbulence could not be achieved, as is evident from the *h versus u* curve. In "rough-walled" small-diameter tubes, the transition from laminar to turbulent flow appears to take place continuously.

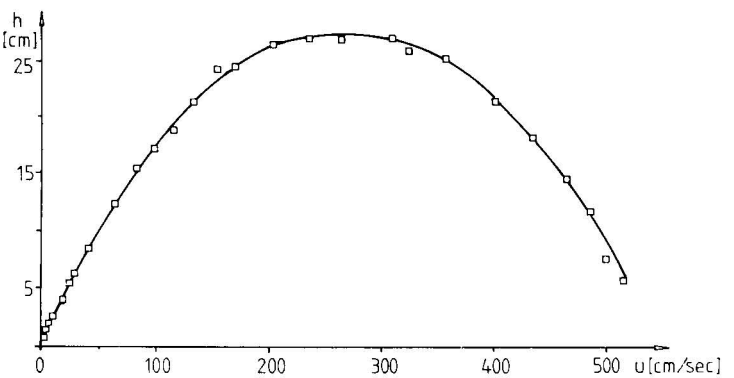

Fig. 3. *h versus u* curve for an open tube (I.D. 0.25 mm) with wall roughness. Conditions as in Fig. 1, except 0.25 mm I.D. × 1.5 mm O.D.

It should be noted that the degree of unevenness of the inner wall of the copper tubing was independent of the diameter. Hence, the smaller the inner diameter, the greater is the effect of the roughness, as can be seen from Table I and Fig. 3. This effect is considerably more pronounced with stainless-steel tubes. A small inner diameter and a rough wall destroy the classic laminar flow and enhance radial mass transfer. Such tubes are to be preferred as connecting tubing for liquid chromatographic equipment and heat exchangers. However, with decreasing diameter clogging of these tubes becomes more likely, especially if they are connected after a packed column where particles may be discharged.

Band broadening in the turbulent region of open tubes. The friction theory of turbulent flow[24] was used by Taylor to calculate dispersion in tubes in that region for

the first time. It is postulated that radial fluctuations extending to the tube wall are superimposed on the axial flow of liquid. A calculation based on this friction theory yields $h = 7.4 \cdot 10^{-2}$ cm for a 1-mm I.D. tube and an eluent velocity of $u = 176$ cm/sec ($Re = 3000$) (p. 45 in ref. 11). The experimental h value in Fig. 2, however, is at least 10 times greater. This discrepancy can be explained by the Prandtl boundary layer theory of turbulent flow [19,25,26]. Based on our experimental results, up to 20% of the cross-section of a "turbulent" flow consists of a laminar layer flow. In other words, in the turbulent region the experimentally determined dispersion is at least 10 times that calculated from the friction theory.

Determination of diffusion coefficients (D) in ideal tubes. The diffusion coefficient of a substance in a particular eluent can be determined from the ascending portion of an *h versus u* curve with the aid of eqn. 8. As can be seen from the first five values in the last column in Table I, the D_{calc} value for benzene in *n*-heptane is calculated to be $3.3 \cdot 10^{-5}$–$3.6 \cdot 10^{-5}$ cm²/sec at room temperature. These values are based on the low sample concentrations (< 100 ppm) encountered in open chromatographic columns. A value of $D = 3.7 \cdot 10^{-5}$ cm²/sec is calculated for this system from the Wilke–Chang equation[27]. Such excellent agreement must be regarded as fortuitous, but it nevertheless does indicate that mechanical mixing (due to inlet turbulence, wall roughness, etc.) was negligible in the copper tubes used.

Water as mobile phase

Water was selected as the second mobile phase because its kinematic viscosity, ν, is about double that of *n*-heptane and because it is frequently employed in chromatography. The dispersion properties of acetone (as sample) in water were determined in all of the copper tubes described in the previous section. The inner diameters of the tube calculated using eqn. 1 were independent of whether water or *n*-heptane was the eluent. The shapes of the *h versus u* curves for water were similar to those obtained for *n*-heptane (Fig. 2). The most important experimental results are summarized in Table II.

TABLE II

EXPERIMENTAL PARAMETERS DETERMINED IN IDEAL OPEN TUBES

Eluent, water; sample, acetone; room temperature.

I.D. × O.D. of tube (mm)	u_{sym} *(cm/sec)*	u_{turb} *(cm/sec)*	h_{turb} *(cm)*	$D_{calc} \cdot 10^5$ *(cm²/sec)*
0.5 × 1.5	20	330	5.3	1.1
0.75 × 2.0	16	280	2.5	1.3
1.0 × 2.0	8	222	2.0	1.4
1.2 × 2.0	8	170	4.0	1.3
1.4 × 2.0	8	167	5.0	2.6

Because of the higher kinematic viscosity of water, u_{sym} is smaller than with *n*-heptane. However, u_{sym} is not strictly proportional to D. Before complete turbulence is reached, the peaks are symmetrical and the h values decrease very steeply with increasing velocity, as a result of which the determination of u_{turb} and h_{turb} is imprecise. The same is true of the measurements of u_{sym}. The diffusion coefficient of acetone in water was calculated to be $1.1 \cdot 10^{-5}$–$1.4 \cdot 10^{-5}$ cm²/sec.

The roughness of the wall of the 0.25 mm I.D. × 1.5 mm O.D. copper tubing was also noticeable in this instance. The turbulent region with water could not be attained because the pressure drop of about 500 atm required to achieve $u = 800$ cm/sec was not compatible with the sampling device used. As shown in Fig. 4, h rises linearly to $u = 40$ cm/sec and then increases more slowly until a plateau of $h \approx 60$ cm is reached. Particularly noteworthy is the large experimental scatter when $u > 160$ cm/sec, which is greater than the experimental uncertainty. When the wall roughness is not negligible, characteristic curves are obtained such as those shown in Fig. 4.

The inlet and outlet disturbances in 1.4 mm I.D. × 2.0 mm O.D. tubes also led to excessively high calculated diffusion coefficients, as shown in Table II.

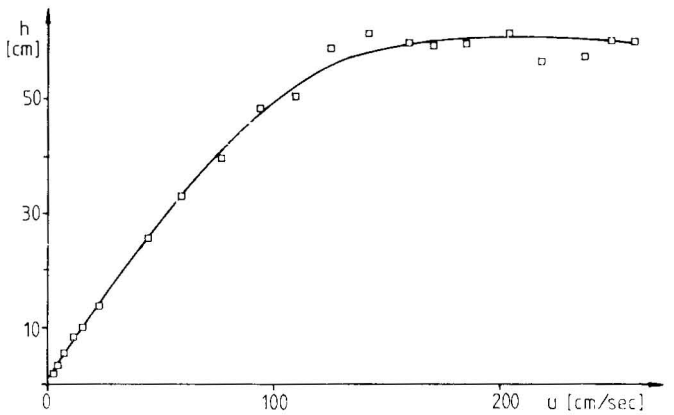

Fig. 4. *h versus u* curve for an open tube (I.D. 0.25 mm) with wall roughness. Copper tube (0.25 mm I.D. × 1.5 mm O.D.), $L = 10$ m. Eluent, water; sample, acetone; room temperature.

MEASUREMENTS ON COILED TUBES WITH A CIRCULAR CROSS-SECTION

n-Heptane as mobile phase

In order to simplify the comparison between the flow properties of ideal and coiled tubes, the lengths and inner diameters of the copper tubes were kept constant and the measurements were carried out with *n*-heptane as mobile phase and benzene as sample. Unless stated otherwise, the coil diameter was always 12 cm.

Fig. 5 depicts the permeability as a function of the velocity in a 1.0 mm I.D. × 2.0 mm O.D. coiled copper tube (solid line). For comparison, the data for the corresponding ideal tube, which have already been presented in Fig. 1, are included (broken line).

As can be seen from Fig. 5, the permeabilities of the ideal and coiled tubes coincide only at very low velocities. The permeability of the coiled tubes decreases steadily with increasing u and continues thus into the turbulent region of the ideal tube. For liquid flow in coiled tubes, the critical velocity corresponding to the onset of turbulent flow can no longer be determined accurately.

Fig. 6 shows that the dispersion in the coiled tube is greatly reduced as a result of the secondary flow. The difficulties with sample introduction in the case of

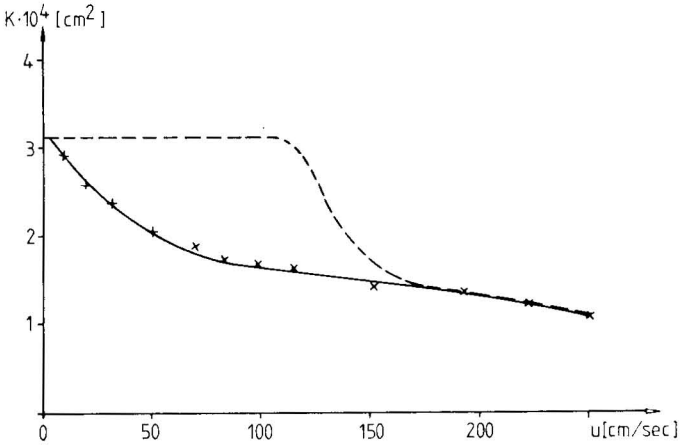

Fig. 5. *K versus u* curve for a coiled tube (I.D. 1 mm). Copper tube (1.0 mm I.D. × 2.0 mm O.D.), $L = 10$ m. Coil diameter: 12 cm. Eluent, *n*-heptane; sample, benzene; room temperature. Solid line, coiled tube; broken line, ideal tube.

the ideal tube, *i.e.*, peaks that cannot be evaluated (Fig. 2), are absent here as a result of the extensive mixing created by the secondary flow. However, the experimental scatter in the velocity range 30–120 cm/sec is greater than for the ideal tubes.

Turbulence in coiled tubes appears later ($Re = 2900$, $u \approx 170$ cm/sec) than in ideal tubes. Coiling stabilizes "laminar" flow as a result of the secondary flow.

A comparison of Figs. 5 and 6 indicates that *h versus u* plots are a more sensitive indicator than *K versus u* curves of the onset of turbulent flow.

In the laminar region the ratio of *h* values for ideal and coiled tubes increases with increasing velocity. As is evident from Fig. 6 and calculated from eqn. 8, this ratio is about 40 at $u = 120$ cm/sec ($h_{ideal} = 380$ cm and $h_{coil} = 9$ cm, $D = 3.3 \cdot 10^{-5}$ cm²/sec).

The *h versus u* and *K versus u* plots for the 0.25 mm I.D. × 1.5 mm O.D. coiled copper tubes are presented in Fig. 7. The measurements were carried out only up to $u = 250$ cm/sec ($Re = 1060$). Owing to wall roughness, the permeability was lower than the calculated theoretical value of $1.95 \cdot 10^{-5}$ cm², even at low velocity; it de-

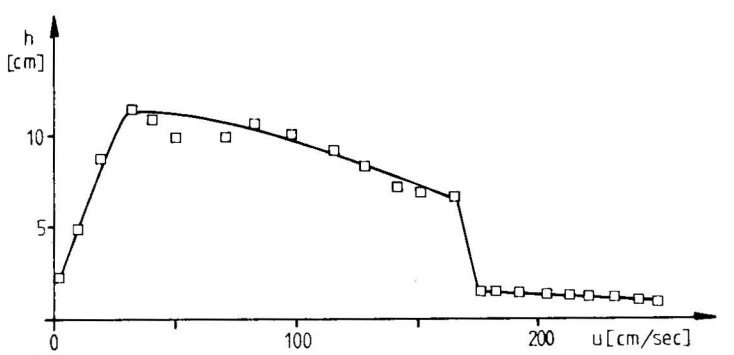

Fig. 6. *h versus u* curve for a coiled tube (I.D. 1 mm). Conditions as in Fig. 5.

creased with increasing velocity from $1.5 \cdot 10^{-5}$ to $1.0 \cdot 10^{-5}$ cm^2. It is noteworthy that owing to wall roughness the same permeability ($1.5 \cdot 10^{-5}$ cm^2) was found in a straight tube of the same diameter (Fig. 3), although in this instance K remained independent of velocity up to $u = 250$ cm/sec.

The *h versus u* curve in Fig. 7 is considerably flattened as a result of the secondary flow caused by the coiling. Even though secondary flow also occurs in the straight tube owing to wall roughness, the h value at $u = 250$ cm/sec is 5 times smaller in Fig. 7 than in Fig. 3. These results again indicate that *h versus u* curves are a considerably more sensitive indicator than *K versus u* plots of the appearance of secondary flow.

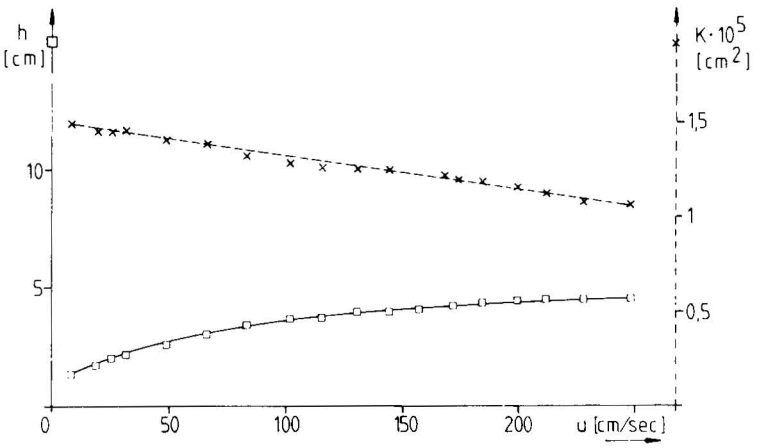

Fig. 7. *h versus u* (□) and *K versus u* (×) curves for a coiled tube (I.D. 0.25 mm). Conditions as in Fig. 5, except 0.25 mm I.D. × 1.5 mm O.D.

The shape of the curves obtained with 0.5 mm I.D. × 1.5 mm O.D. coiled copper tubes was similar to that shown in Fig. 7, although because of the larger diameter the h and K values were correspondingly higher[11].

To verify that the method of coiling employed resulted in no changes in the cross-section, measurements were performed on 0.5-mm I.D. tubes with O.D. 1.5 and 3.2 mm. The shapes of the *h versus u* and *K versus u* curves for both tubes were very similar[11].

Fig. 8 shows the data for a 0.75 mm I.D. × 2.0 mm O.D. coiled copper tube. Even though $Re = 2300$ was attained at $u = 180$ cm/sec, neither curve indicates the onset of turbulence. In this instance also, secondary flow stabilizes laminar flow and displaces the transition to higher velocities.

The permeability decreases monotonically even at low velocities and at $u = 205$ cm/sec attains about half of the $K_{theo} = d^2/32$.

Band broadening increases to its maximum value ($h = 11$ cm) at $u = 70$ cm/sec and declines slowly to 8 cm at $u = 205$ cm/sec. The relative dispersion continues to decrease with increasing tube diameter as a result of the increased rate of mass transfer brought about by the secondary flow. At $u = 70$ and 150 cm/sec the experimental values for coiled tubes (Fig. 8) are 50 and 150 times smaller, respectively, than those calculated via the Taylor equation (eqn. 8) for ideal tubes where no secondary

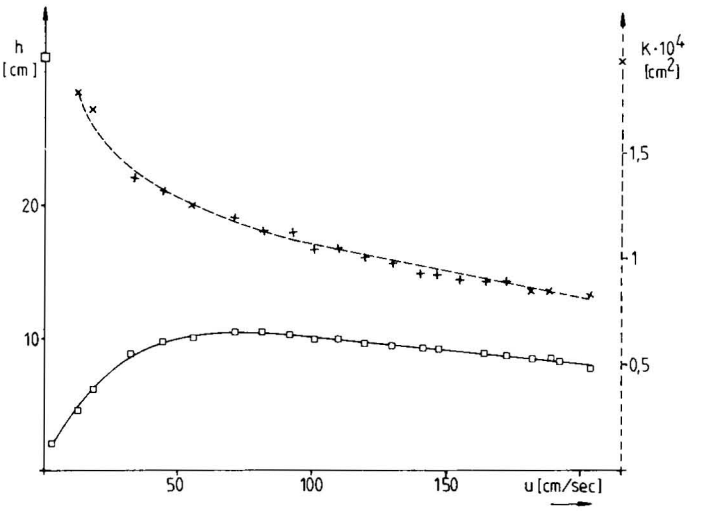

Fig. 8. *h versus u* (□) and *K versus u* (×) curves for a coiled tube (I.D. 0.75 mm). Conditions as in Fig. 7, except 0.75 mm I.D. × 2.0 mm O.D.

flow occurs. Hence, although there is no firm theoretical basis for it, experimentally this factor is found to increase with increasing inner diameter of the open tube. The same coil radius is assumed in this comparison.

The data for the 1.2 mm I.D. × 2.0 mm O.D. copper tubes (Fig. 9) indicate that the permeability is the same as for ideal tubes ($K_{ideal} = 4.5 \cdot 10^{-4}$ cm^2) at very low velocities. From $u = 10$ cm/sec K decreases monotonically with increasing u and continuously approaches the value for turbulent flow; at $u = 100$ and 140 cm/sec ($Re = 2040$ and 2860), for example, K amounts to 42 and 40 %, respectively of K_{ideal}. These curves show no obvious onset of turbulence. Within the limits of error, these tubes exhibited the same permeability at the corresponding u values as the ideal tubes in the turbulent region.

The dispersion in these tubes increases up to $u = 20$ cm/sec ($h = 11.5$ cm) and passes through a maximum, and the onset of turbulence is indicated distinctly at $u = 140$ cm/sec ($Re = 2860$) by the inconsistency of the *h versus u* curve. From $h = 4$ cm at the latter velocity, the dispersion decreases to 1.5 cm at $u = 150$ cm/sec and then gradually to 1 cm at 200 cm/sec.

Similar results were also obtained with 1.4 mm I.D. × 2.0 mm O.D. and 2.0 mm I.D. × 3.2 mm O.D. coiled copper tubes, as shown in Fig. 9.

To illustrate the experimental difficulties and to point out possible misinterpretations, data for the 4.0 mm I.D. × 6.0 mm O.D. coiled tubes are presented in Fig. 10. The peaks obtained with a coil diameter of 12 cm for both 10- and 20-m lengths could not be evaluated. Symmetrical peaks were obtained only with an 80-cm coil diameter, but even for the 20-m length (upper curve) the ascending branch of the *h versus u* curve could not be determined. Between $u = 5$ cm/sec ($h = 55$ cm) and $u = 40$ cm/sec ($h = 22$ cm) the curve decreases continuously, and turbulence sets in at $u = 45$ cm/sec ($Re = 3060$, $h = 5$ cm). This linear velocity corresponds to a flow-rate of 340 ml/min. The limitations of the pump output prevented measurements at higher

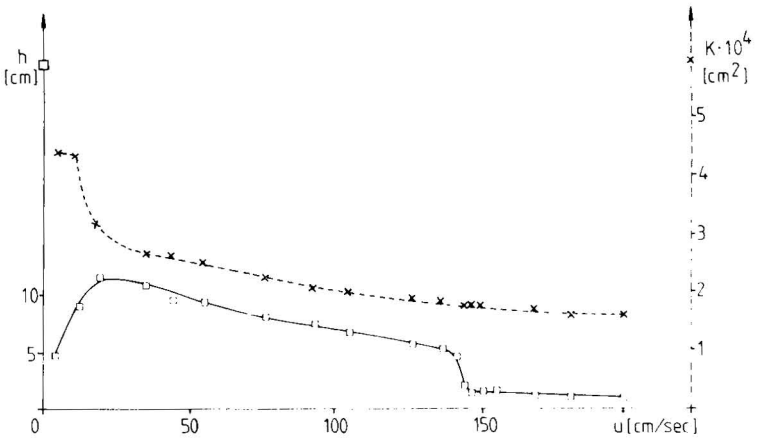

Fig. 9. *h versus u* (□) and *K versus u* (×) curves for a coiled tube (I.D. 1.2 mm). Conditions as in Fig. 7, except 1.2 mm I.D. × 2.0 mm O.D.

velocities. The pressure drop over this column was less than 0.2 bar and could not be measured accurately with the manometer used. Therefore, a *K versus u* curve was not evaluated.

The lower curve in Fig. 10 depicts the data for the 10-m tube (80-cm coil diameter). The inlet and outlet disturbances were obviously appreciable and determined the shape of the curve.

Water as mobile phase

The dispersion of acetone in water was determined in all of the coiled tubes (I.D. 0.25–4.0 mm[11]) as for *n*-heptane. The onset of turbulence in water occurs at

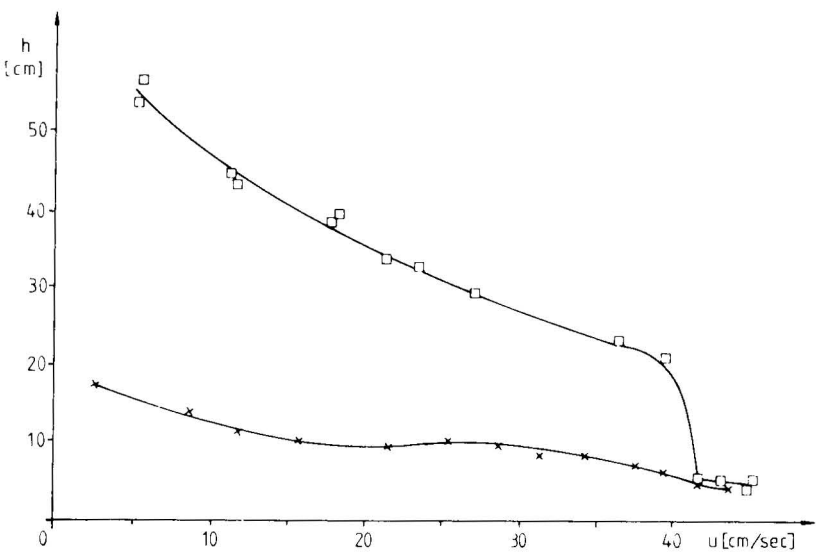

Fig. 10. *h versus u* curve for a coiled tube (I.D. 4 mm). Conditions as in Fig. 5, except coil diameter 80 cm. Column length: □, 20 m; ×, 10 m.

substantially higher velocities because its kinematic viscosity is higher than that of *n*-heptane. It is noteworthy, but still unexplainable, that considerably more asymmetric peaks were obtained that could not be evaluated in comparison with *n*-heptane. For this reason, the data for the 1.0 mm I.D. × 2.0 mm O.D. tube, which were designated as typical in this work, are not shown. To shorten the presentation, only selected results will be discussed, as similarly shaped curves were obtained for both water and *n*-heptane at the same Reynold's numbers.

The measurements for the 0.25 mm I.D. × 1.5 mm O.D. tube (Fig. 11) were carried out only up to $u = 260$ cm/sec ($Re = 650$). Wall roughness becomes noticeable as in Fig. 7. With increasing velocity the h values rise monotonically to a plateau from $u = 50$ cm/sec ($h = 9.5$ cm) to $u = 240$ cm/sec ($h = 11$ cm) and then decline slowly.

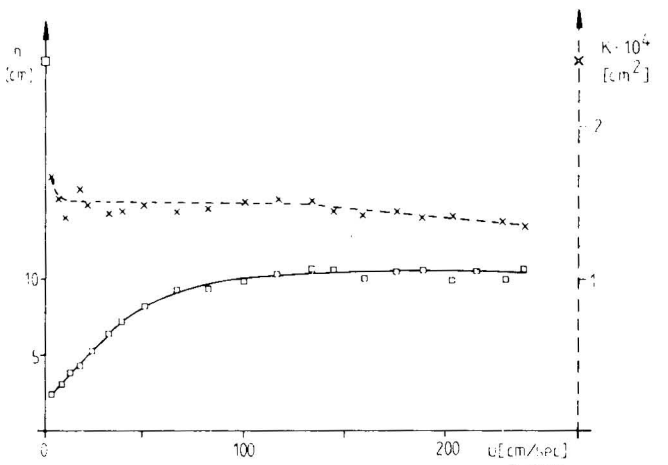

Fig. 11. *h versus u* (□) and *K versus u* (×) curves for a coiled tube (I.D. 0.25 mm). Copper tube (0.25 mm I.D. × 1.5 mm O.D.), $L = 10$ m. Coil diameter: 12 cm. Eluent, water; sample, acetone; room temperature.

Compared with that of ideal tubes, the permeability was about 10% lower at $u = 5$ cm/sec and 25% lower from $u = 10$ cm/sec upwards. From 150 cm/sec the permeability decreased gradually.

For the 0.75 mm I.D. × 2.0 mm O.D. tubes (Fig. 12), h reaches its maximum value of 26 cm at $u = 40$ cm/sec and decreases to 17.5 cm at $u = 122$ cm/sec. From this velocity to 300 cm/sec the peaks are too asymmetric for evaluation. The onset of turbulence occurs between 330 and 365 cm/sec ($Re = 2480$ and 2730) and the h values drop to 2.2 cm. Hence, laminar flow in coiled tubes is stabilized in water also and turbulence is displaced to higher Reynold's numbers. The permeability decreases continuously with increasing velocity to the values in turbulent flow.

Fig. 13 contains the results for 1.2-mm I.D. tubes. The *h versus u* curve passes through a maximum at $u = 25$ cm/sec ($h = 31.5$ cm) and then decreases monotonically to $h = 6$ cm at $u = 215$ cm/sec ($Re = 2580$). Turbulence sets in at $u = 263$ cm/sec ($Re = 3160$) and h is 1.5 cm. The permeability decreases continuously with increasing velocity and the onset of turbulence is not obvious from this curve.

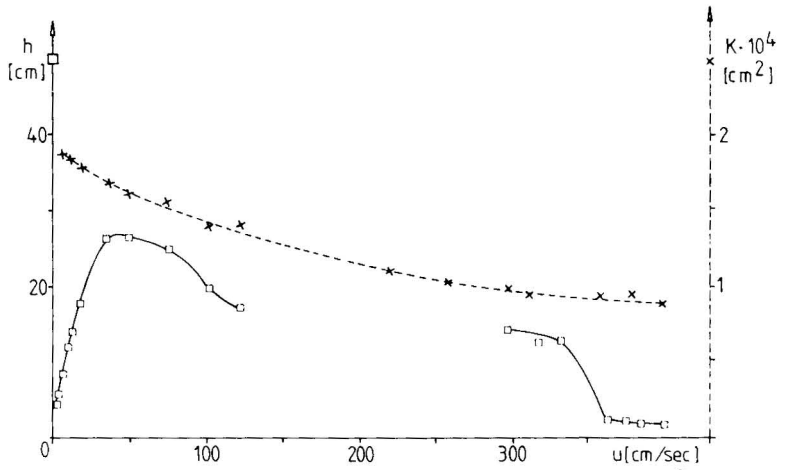

Fig. 12. *h versus u* (□) and *K versus u* (×) curves for a coiled tube (I.D. 0.75 mm). Conditions as in Fig. 11, except 0.75 mm I.D. × 2.0 mm O.D.

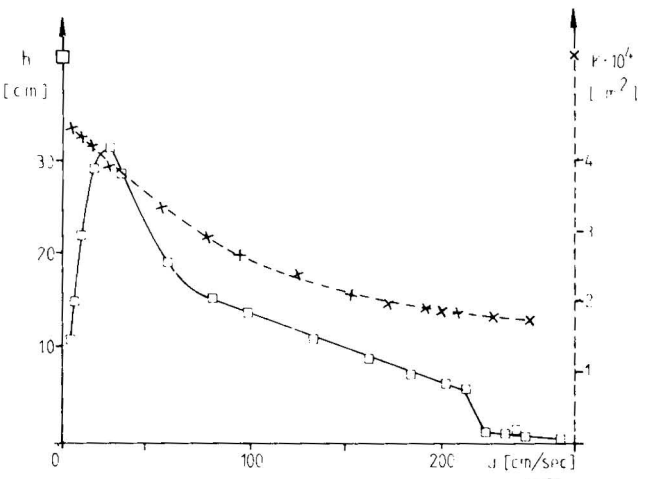

Fig. 13. *h versus u* (□) and *K versus u* (×) curves for a coiled tube (I.D. 1.2 mm). Conditions as in Fig. 11, except 1.2 mm I.D. × 2.0 mm O.D.

The shapes of the *h versus u* and *K versus u* curves for 1.4-, 2.0- and 4.0-mm I.D. tubes are similar to that shown in Fig. 13. Thick- and thin-walled tubes with the same inner diameter display no appreciable differences in their *h versus u* or *K versus u* curves.

ACKNOWLEDGEMENTS

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SYMBOLS

d (cm)	inner diameter of the open tube
D (cm^2/sec)	interdiffusion coefficient of the sample in the mobile phase
D_{calc} (cm^2/sec)	D calculated via eqn. 8
D_{dyn} (cm^2/sec)	defined in eqn. 5
F (cm^3/sec)	flow-rate of the eluent
h (cm)	height equivalent to a theoretical plate or relative band broadening
h_{turb} (cm)	h at u_{turb}
K (cm^2)	specific permeability as defined in eqn. 1
K_{theo} (cm^2)	$= d^2/32$
k'	$= (t_R - t_0)/t_0$ = capacity ratio
L (cm)	column length
L_e (cm)	length of the inlet (or outlet) turbulence as defined in eqn. 3 or 10
n	number of theoretical plates
ΔP (bar)	pressure drop over the column
r (cm)	radius of the open tube
Re	Reynold's number, as defined in eqn. 4
Re_{crit}	critical Reynold's number
t (sec)	time
t_0 (sec)	retention time of an inert sample
t_R (sec)	retention time of a retarded peak
u (cm/sec)	cross-section averaged linear velocity of the mobile phase
u_{crit} (cm/sec)	minimum velocity in the turbulent region
u_{max} (cm/sec)	maximum velocity of the mobile phase
u_{sym} (cm/sec)	maximum linear velocity in the laminar region at which symmetrical peaks are obtained
u_{turb}	u at incipient turbulence
w (sec)	peak width
η (poise)	dynamic viscosity
ϱ (g/cm^3)	density
ν (stokes)	$= \eta/\varrho$ kinematic viscosity
σ (cm)	standard deviation of a Gaussian curve

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MASS TRANSFER IN IDEAL AND GEOMETRICALLY DEFORMED OPEN TUBES

II*. POTENTIAL APPLICATION OF IDEAL AND COILED OPEN TUBES IN LIQUID CHROMATOGRAPHY

ISTVÁN HALÁSZ

Angewandte Physikalische Chemie, Universität des Saarlandes, 6600 Saarbrücken (G.F.R.)

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SUMMARY

It has been shown experimentally in Part I that the peak broadening in an open tube with a liquid mobile phase can be described solely by the $C_m u$ term of the Golay equation, if the inner diameter is small ($< 100\ \mu$m) and the linear velocity is not extremely high (< 1 m/sec). With this assumption, optimal values achievable in capillary columns are calculated for the number of theoretical and effective plates, the speed of analysis, the number of plates per unit pressure drop and the peak volume. These parameters yield results which are hardly, if at all, better than those generated in packed columns in routine liquid chromatography. Even if the instrumental problems, including the coating of the open tube, are neglected, capillary columns will be of interest in liquid chromatography only if secondary flow can be generated within the open tubes.

INTRODUCTION

In earlier publications[1,2] it was shown experimentally that in ideal tubes the band broadening for an inert peak can be described with the help of the equations of Taylor[3], Aris[4] or Golay[5–7] if (a) the linear velocity of the eluent is less than 20 cm/sec, (b) the inner diameter of the tube is less than 500 μm and (c) the unevenness of the wall is negligible. This limiting velocity increases with decreasing inner diameter of the open tube, and with decreasing kinematic viscosity of the eluent. The linear velocity limit given above is valid for the "worst" eluent used in liquid chromatography (LC), *i.e.*, for *ca.* 45% (v/v) methanol in water.

Coiling the column to a diameter of about 12 cm stabilizes the laminar flow. In the laminar region secondary flow[8,9] diminishes the dispersion. It was shown experimentally that although there is no firm theoretical basis for it, the desired influence of the secondary flow declines with decreasing inner diameter of the tube. With the boundary conditions mentioned above the effect of the secondary flow can be neglected.

* Part I: ref. 2.

The application of open tubes in LC in the turbulent region will not be discussed in this paper for the following reasons:

(a) In this region the specific permeability of an open tube is at least a factor of 3 less than that for laminar flow[1,2].

(b) The h values in the turbulent region are about ten times higher[1,2] than those calculated on the basis of the friction theory of Taylor[10], because laminar conditions prevail in an appreciable portion of the cross-section of the tube.

(c) The pressure drop over the column is high, because of the high linear velocities required and because of the great pneumatic resistance of a long column with a small inner diameter.

(d) The injection of small samples is difficult if the inlet pressure is high.

On the basis of the experimental results for the laminar region discussed above, it will be assumed that the dispersion of a sample in ideal or coiled tubes (with circular cross-sections) can be described by the Taylor[3], Aris[4] and Golay[5-7] equations, if the inner diameter of the tube is less than 500 μm and the linear velocity of the eluent is less than 20 cm/sec. The limiting velocity can be increased to 100 cm/sec if the inner diameter of the open tube is less than 100 μm. A coil diameter of about 10 cm or more is assumed.

In this paper the B/u and $C_s u$ terms of the Golay equations[5-7] will be neglected, and consequently for retarded peaks in ideal open tubes the maximum efficiency is calculated. In a following paper these maximum values will be compared with the experimental data. The relative advantages and disadvantages of capillary and packed columns will be discussed.

MAXIMUM NUMBER OF PLATES GENERATED IN AN OPEN TUBE

In an ideal open tube, the height equivalent to a theoretical plate, h, can be calculated on the basis of the Golay equation[5-7]:

$$h = \underbrace{\frac{2\gamma D}{u}} + \underbrace{\frac{1 + 6k' + 11(k')^2}{(1 + k')^2}} \cdot \frac{d^2}{96D} \cdot u + \frac{2k'}{3(1 + k')^2} \cdot u \qquad (1)$$

$$= \frac{B}{u} + \underbrace{f \cdot C_m^* u} + C_s u =$$

$$= \frac{B}{u} + C_m u + C_s u$$

where

$$f = \frac{1 + 6k' + 11(k')^2}{(1 + k')^2} \qquad (2)$$

The factor f is given in Table I as a function of k', including also the extreme values for the capacity ratios.

It is sometimes overlooked that the Golay equation is valid only for straight tubes, as is implicit in the statement of Golay[7]: "...the average speed of flow has a parabolic profile along any cross-section parallel to the direction of flow,...". This statement, however, is valid only for straight tubes, and elsewhere it is impossible to describe explicitly the flow profile. Further, the Golay equation is an "extension of

TABLE I

FACTORS f, f^*, F' AND F^* AS A FUNCTION OF THE CAPACITY RATIO, k'

k'	f	f^*	F'	F^*
0	1.000	∞	∞	∞
0.05	1.204	441.0	531.0	557.6
0.075	1.308	205.4	268.8	289.0
0.1	1.413	121	171.0	188.1
0.15	1.624	58.78	95.44	109.8
0.25	2.040	25.00	51.00	63.75
0.30	2.243	18.78	42.11	54.74
0.40	2.633	12.25	32.25	45.15
0.50	3.000	9.000	27.00	40.50
0.75	3.816	5.444	20.78	36.37
0.914	4.278	4.385	18.76	35.91
1.0	4.500	4.000	18.00	36.
1.25	5.074	3.240	16.44	36.99
1.50	5.560	2.778	15.44	38.60
1.75	5.975	2.469	14.76	40.59
2.0	6.333	2.250	14.25	42.75
2.5	6.918	1.960	13.56	47.46
3.0	7.375	1.778	13.11	52.44
5.0	8.500	1.440	12.24	73.44
10.0	9.595	1.210	11.61	127.7
20.0	10.252	1.103	11.30	272.3
∞	11.000	1.000	11.00	∞

Taylor's method"[7] and eqn. 1 is reduced to the Taylor or Aris equations for an inert peak ($k' = 0$). These equations again are valid only for straight tubes, as pointed out by Aris[4]: "In three recent papers, Sir Geoffrey Taylor has discussed the dispersion of soluble matter in a fluid flowing in a straight tube[3,10,11]".

On the other hand, in gas chromatography the Golay equation is always an excellent approximation for coiled tubes, because of the high interdiffusion coefficients of gases ($D_g \approx 10^{-1}$ cm^2/sec).

The peak broadening with a liquid mobile phase can only be calculated using the Golay equation if the inner diameter of the tube is less than 100 μm (coil diameter about 10 cm) and the linear velocity of the eluent is less than 100 cm/sec, as is usual in LC. A further assumption is that no secondary flow is caused by the unevenness of the wall.

From experience, it is a tolerable approximation that in eqn. 1 $\gamma = 1$. By differentiation of eqn. 1 the velocity at the minimum of the h *versus* u curve, u_{Min}, is

$$u_{\mathrm{Min}} = \frac{D}{d}\sqrt{\frac{192}{f}} = \frac{13.86D}{d\sqrt{f}} \tag{3}$$

In routine LC, $u \gg u_{\mathrm{Min}}$. Consequently, the B/u term in eqn. 1 will be neglected. The upper limit for this approach, however, must be stated: in a 15-μm I.D. tube with n-heptane as eluent, u_{Min} for an inert peak is 0.28 cm/sec.

Also for an inert peak the $C_s u$ term is zero. Consequently, the minimum h value, h_0, is generated for an inert peak.

The maximum number of theoretical plates, n_{max}, for a retarded peak is generated if we assume an extremely rapid mass transfer in the stationary phase, *i.e.*, we neglect also the $C_s u$ term in eqn. 1:

$$n_{max} = \frac{L}{h_{min}} = \frac{96DL}{fd^2u} = \frac{96Dt_0}{fd^2} = \frac{n_0}{f} \tag{4}$$

where n_0 is the number of theoretical plates generated for an inert peak ($k' = 0$). In eqn. 4 the B/u and the $C_s u$ terms of in eqn. 1 are neglected. It can be seen in eqn. 4 that for a retarded peak n_{max} is always less than n_0, because $f > 1$.

Using the same assumption, the minimum h value for a retarded peak (h_{min}) is

$$h_{min} = \frac{fd^2u}{96D} = fh_0u \tag{5}$$

Apart from the factor f in eqn. 4, the validity of the Taylor equation is assumed for calculating h in both inert and retarded peaks.

As is shown in eqn. 4 n_{max} is a linear function of the hold-up time, t_0, of the column only if the eluent and sample (*i.e.*, D for a given temperature) and the inner diameter of the column, d, are given. If the column is elongated by a given factor β, the linear velocity has to be increased by the same factor β to achieve the same hold-up time. Consequently, h also increases by a factor of β while n_{max} remains unchanged. Therefore, n_{max} is a function of the ratio of the column length over the linear velocity of the eluent ($t_0 = L/u$), as was shown in eqn. 4.

For difficult separations in chromatography, the relative retention α is small and it is a reasonable approximation[12,13] that

$$R \equiv \frac{\Delta t_R}{w} = \frac{\alpha - 1}{4\alpha} \cdot \frac{k'}{1 + k'} \cdot \sqrt{n_2} = \frac{\alpha - 1}{4\alpha} \cdot \sqrt{N_2} \tag{6}$$

with

$$n = \left(\frac{1 + k'}{k'}\right)^2 N = f^* N \tag{7}$$

In eqn. 6 the theoretical plate number, n_2, and the effective plate number, N_2, both refer to the later emerging peak. Thus, for difficult separations ($\alpha < 1.2$) the resolution is an extremely sensitive function of α and of $R \approx \sqrt{N}$. From the point of view of the separation, N (not n alone) is of interest, as is shown in eqn. 6.

The maximum number of effective plates, N_{max}, (the B/u and $C_s u$ terms of the Golay equation are again neglected) can be calculated if eqns. 4 and 7 are combined:

$$N_{max} = \frac{96Dt_0}{d^2ff^*} = \frac{n_0}{ff^*} = \frac{n_0}{F'} \tag{8}$$

where

$$F' = \frac{1 + 6k' + 11(k')^2}{(k')^2} \tag{9}$$

The dimensionless factors f^* and F' as functions of k' are given in Table I together with their extreme values. As shown in Fig. 1, f increases slowly, but f^* and F' decrease extremely sharply with increasing capacity when $0 \leqslant k' \leqslant 2$. It is this region which is the most important in practice.

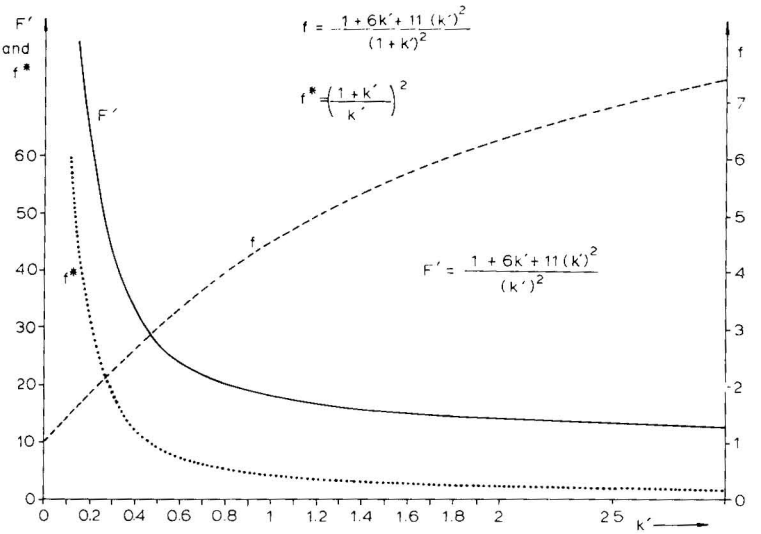


Fig. 1. The factors f, f^* and F' as functions of the capacity ratios.

IMPORTANCE OF THE REGION OF SMALL CAPACITY RATIOS ($k' < 2$)

The hold-up time, t_0, of a capillary column is usually long compared with that of a packed column of similar efficiency. Therefore, with increasing k' the analysis time increases rapidly.

The weight (or volume) of the stationary phase per unit column volume is much smaller in coated open tubes than in packed columns. Therefore, the maximum sample size per unit column volume is smaller if open tubes are used. Consequently, the concentration of the sample in the eluent at the peak maximum is usually smaller by a factor of 10 (or even more) in conventional open tubes. As the retention time of the compounds increases the peak height decreases, if the sample size remains constant. Because of the higher density of the eluent the concentrations of the sample in LC are smaller than in gas chromatography. Hence in LC, with increasing k', the possibility that the signal from the detector will become smaller than the noise level of the detector becomes much greater. *With increasing capacity ratios not only does the time of analyses increase, but the peaks can become undetectable.*

In efficient open tubes the first retarded peak with $k' = 0.05$ used to be separated from the inert peak. Let us assume that the relative retention of the first and second retarded peak is $\alpha = 1.2$. Rearranging eqn. 6 the effective number of plates necessary for a given separation, N_{ne}, is

$$N_{ne} = \left(\frac{4R\alpha}{\alpha - 1}\right)^2 \tag{10}$$

For example, if $\alpha = 1.2$ and baseline resolution ($R = 1.5$) is wanted, then 1296 effective plates are required for this separation. All of the other pairs of peaks with $\alpha = 1.2$ (or higher) will have a resolution of $R > 1.5$ because N increases with increasing capacity ratios (because of the increase in factor f') if the mass transfer in the stationary phase (*i.e.*, the $C_s u$ term in eqn. 1) is neglected. Approximately constant α values are typical if homologous series are to be separated. With these assumptions, thirteen peaks (excluding the inert peak) can be resolved if $0.05 \leqslant k' < 0.5$. Between $k' = 0.5$ and 1 only four peaks can be separated, and also between $k' = 1$ and 2.

Qualitatively, the picture remains unchanged (as shown in Table II) if the opposite assumption is made: $n_0 = 30{,}000$ and $R = 1.5$ are required for all the neighbouring peaks. Further, in a hypothetical sample the retentions of the compounds should be free to change, as demonstrated in Table II. In the third column of Table II the relative retentions, α, of the pair of compounds with $k' = 0.05$ and 0.09473 are in the lane of $k' = 0.09473$ tabulated. The same is true for the effective number of plates necessary for the seperation of these two compounds, N, in the last column. As shown in the third column, the relative retentions, α, decrease considerably with increasing k' in the range $0 < k' < 0.5$ (*i.e.*, from $\alpha = 1.90$ to 1.22). The number of effective plates for the first retarded compound is only $N = 56$, although here $n \approx 25{,}000$. The N values increase and the n values decrease with increasing k'. For infinite

TABLE II

SEPARATION OF A HYPOTHETICAL MIXTURE IN AN OPEN TUBE WITH BASELINE RESOLUTION ($R = 1.5$) IF $n_0 = 30{,}000$

$B/u = C_s u = 0$.

Number of samples	k'	α	n	N
0	0	—	30 000	0
1	0.05000	—	24 915	56
2	0.09473	1.89453	21 566	161
3	0.14493	1.53003	18 721	300
4	0.20137	1.38940	16 313	458
5	0.26488	1.31538	14 281	626
6	0.33641	1.27003	12 568	796
7	0.41702	1.23962	11 124	963
8	0.50791	1.21797	9 907	1124
9	0.61045	1.20188	8 880	1276
10	0.72616	1.18955	8 012	1418
11	0.85677	1.17986	7 276	1549
12	1.00422	1.17210	6 651	1670
13	1.17072	1.16580	6 119	1780
14	1.35874	1.16060	5 666	1880
15	1.57109	1.15629	5 277	1971
16	1.81094	1.15266	4 945	2052
17	2.08187	1.14960	4 658	2126
18	2.38791	1.14700	4 412	2192
19	2.73363	1.14478	4 199	2251
20	3.12418	1.14287	4 014	2304
21	3.56539	1.14123	3 854	2351
22	4.06385	1.13980	3 715	2393
23	4.62698	1.13857	3 595	2430
24	5.26317	1.13750	3 489	2464

k' the maximum number of theoretical plates is $n_{max} = 2727$, and of course $N_{max} = 2727$ also. If $R = 1.5$ is required the optimal resolution is $\alpha = 1.13$. This relative retention, however, is achieved in practice in the capacity ratio region around $k' = 5$, as shown in Table II.

In calculating the values in Table II, it was again assumed that $B/u = C_s u = 0$. In practice, the higher the capacity ratios the less this approach is valid. With increasing k' the $C_s u$ term usually increases, and consequently the plate numbers will become smaller than is indicated in Table II and the α values will remain more or less constant if R is constant as is claimed.

The maximum speed of separation, *i.e.*, N_{max}/t_R, occurs at $k' = 0.914$, as will be shown later. If only a few compounds have to be separated, the stationary phase and eluent have to be selected so that the compounds will be eluted around $k' = 1$. If the capacity ratios are smaller, longer columns have to be used to generate enough plates for the required resolution and the velocity of the eluent has to be increased to achieve unchanged hold-up times. For both of these reasons the inlet pressure must be increased.

To summarize, the region of small capacity ratios ($k' < 2$) is of extreme importance in LC with open tubes because of (a) the time of analysis, (b) the detectability, (c) the peak capacity, (d) the speed of analysis in this region and (e) possible pressure limitations.

CALCULATED MAXIMUM PLATE NUMBERS FOR DIFFERENT ELUENTS

It will be demonstrated with some numerical values that in capillary-column LC only the effective plate numbers are of importance. Further, the required geometry of the open tubes will be outlined.

In the following it will be assumed that the molecular weight of the compounds to be separated is smaller than 400 and that room temperature applies. The product of the diffusion coefficient, D, and the viscosity of the eluent, η, is constant. The values for *n*-heptane are

$$\begin{aligned} D\eta &= (3 \cdot 10^{-5}\ \mathrm{cm^2/sec}) \cdot (4 \cdot 10^{-3}\ \mathrm{poise}) \\ &= 1.2 \cdot 10^{-7}\ \mathrm{dyne} \\ &= 1.2 \cdot 10^{-12}\ \mathrm{newton} \end{aligned} \qquad (11)$$

As shown in eqns. 4 and 8, the plate number is linear in t_0. To generate enough plates, $t_0 = 10$ min was assumed, *i.e.*, the time of analysis for $k' = 2$ is 30 min. For routine work a longer time of analysis is not usually acceptable.

With these assumptions (*i.e.*, $B/u = C_s u = 0$, $t_0 = 10$ min and $D\eta = 1.2 \cdot 10^{-7}$ dyne) and with the help of eqns. 4 and 8 the maximum plate numbers for different eluents were calculated as functions of the inner diameter of the column and of the capacity ratios of the samples at room temperature. In Table III the eluent is *n*-heptane. The results are impressive: for an open tube of 30-μm I.D. 192,000 theoretical plates are calculated for the inert peak. As a consequence of the *f versus k'* function (Table I), n decreases with increasing capacity ratio. However, for $k' = 2$ more than 30,000 theoretical plates are calculated. On the other hand, n_0 is inversely

TABLE III

MAXIMUM NUMBER OF THEORETICAL PLATES, n, GENERATED IN OPEN TUBES OF DIFFERENT INNER DIAMETERS AND FOR VARIOUS k' VALUES

t_0 = 10 min. Eluent, n-heptane. D $3 \cdot 10^{-5}$ cm²/sec. η $4 \cdot 10^{-3}$ poise. Room temperature.

I.D. (*μm*)	*k'*								
	0	*0.1*	*0.25*	*0.5*	*0.75*	*1.0*	*1.5*	*2.0*	*2.5*
30	192,000	135,860	94,118	64,000	50,344	42,667	34,532	30,316	27,752
50	69,120	48,909	33,882	23,040	18,112	15,360	12,432	10,914	9991
65	40,889	28,941	20,049	13,633	10,717	9089	7356	6458	5912
85	23,917	16,924	11,724	7972	6267	5315	4302	3776	3457
100	17,280	12,227	8471	5760	4528	3840	3108	2728	2498

proportional to d^2. If only n_0 = 30,000 is required, the inner diameter of the tube has only to be smaller than 77 μm.

The picture becomes worse, but more realistic, if the effective plate numbers, N, are calculated for n-heptane as eluent (Table IV). The baseline separation (R = 1.5) of a pair of compounds with α = 1.3 is a simple problem in chromatography. They can be resolved with N = 675, as can be calculated with eqn. 10. This condition, however, is fulfilled only above the dashed line in Table IV. If a pair of compounds emerge around k' = 0.1, and α = 1.3 is required, only columns with an inner diameter smaller than 30 μm can be used if t_0 = 10 min.

TABLE IV

NUMBER OF EFFECTIVE PLATES, N, GENERATED IN OPEN TUBES OF DIFFERENT INNER DIAMETERS AND FOR VARIOUS k' VALUES

t_0 = 10 min. Eluent, n-heptane. D $3 \cdot 10^{-5}$ cm²/sec. η $4 \cdot 10^{-3}$ poise. Room temperature.

I.D. (*μm*)	*k'*							
	0.1	*0.25*	*0.5*	*0.75*	*1.0*	*1.5*	*2.0*	*2.5*
30	1123	3765	7111	9241	10,667	12,432	13,474	14,159
50	404	1355	2560	3327	3840	4475	4851	5097
65	239	802	1515	1968	2272	2648	2870	3016
85	140	469	886	1151	1329	1549	1678	1764
100	101	339	640	832	960	1119	1213	1274

Diffusion coefficients in water are about 2.5 times less than in n-heptane. Consequently, the corresponding plate numbers are smaller by the same factor, as shown in Tables V and VI. As demonstrated in Table V with water as eluent, the inner diameter of the open tube has to be less than 50 μm if n_0 = 30,000 is required. With a 30-μm I.D. column, baseline separation for α = 1.3 is with $k' < 0.12$ not achieved. Here, as in Table IV, baseline separations for α = 1.3 are only achieved

TABLE V

NUMBER OF THEORETICAL PLATES, n, GENERATED IN OPEN TUBES OF DIFFERENT INNER DIAMETERS AND FOR VARIOUS k' VALUES

t_0 = 10 min. Eluent, water. D = 1.2 · 10^{-5} cm²/sec. η = 1.0 · 10^{-2} poise. Room temperature.

I.D. (μm)	k' 0	0.1	0.25	0.5	0.75	1.0	1.5	2.0	2.5
30	76,800	54,344	37,647	25,600	20,124	17,067	13,813	12,126	11,101
50	27,648	19,564	13,553	9216	7245	6144	4973	4365	3996
65	16,360	11,576	8019	5453	4287	3636	2942	2583	2365
85	9567	6769	4690	3189	2507	2126	1721	1511	1453
100	6912	4891	3388	2304	1811	1536	1243	1091	999

TABLE VI

NUMBER OF EFFECTIVE PLATES, N, GENERATED IN OPEN TUBES OF DIFFERENT INNER DIAMETERS AND FOR VARIOUS k' VALUES

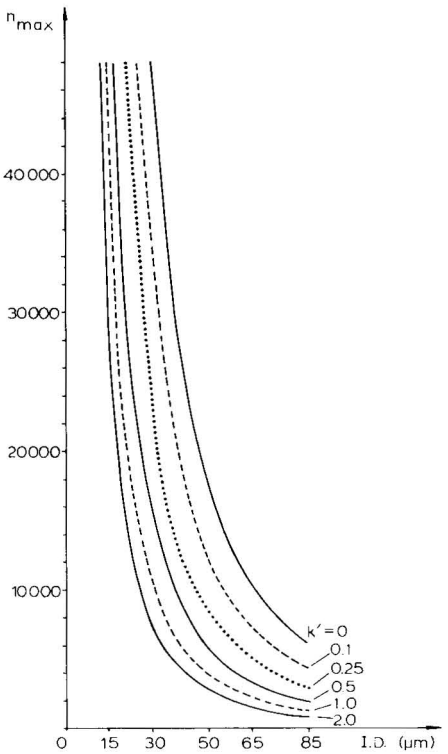
t_0 = 10 min. Eluent, water. D = 1.2 · 10^{-5} cm²/sec. η = 1.0 · 10^{-2} poise. Room temperature.

I.D. (μm)	k' 0.1	0.25	0.5	0.75	1.0	1.5	2.0	2.5
30	449	1506	2844	3696	4267	4973	5389	5664
50	162	542	1024	1331	1536	1790	1940	2039
65	96	321	606	787	909	1059	1148	1206
85	56	188	354	460	531	619	671	687
100	40	136	256	333	384	448	485	510

with the conditions above the dashed line in Table VI. With water as eluent only columns with I.D. < 50 μm are of interest.

The methanol–water mixture has its maximum viscosity (0.016 poise) if the volume ratio is around 45–55. Unfortunately, similar eluent compositions are often required if the separation is made with reversed phases. In Fig. 2 the curves of n_{max} *versus* inner diameter of the column are shown for different capacity ratios. The corresponding plate numbers here are four times less than with *n*-heptane as eluent. Therefore, 15 μm I.D. is also included in Fig. 2. The higher the viscosity of the eluent (*i.e.*, the lower the diffusion coefficients), the smaller are the inner diameters required to solve routine problems in LC. On increasing the viscosity of the eluent by a factor of four, the columns have to be elongated by the same factor in order to generate identical plate numbers. The curves in Fig. 2 demonstrate again one of the problems of comparing the efficiency of open tubes and packed columns in LC: in contrast to the well packed columns in open tubes, n is a sensitive function of the capacity ratios.

It was shown in eqn. 10 that for a baseline resolution (R = 1.5) the relative retention, α, is only a function of the effective plate number, N. In Fig. 3 both N and α are shown as a function of the inner diameter of the open tube. The eluent here is the same as in Fig. 2. For example, in Fig. 3 it can be seen directly that if two com-



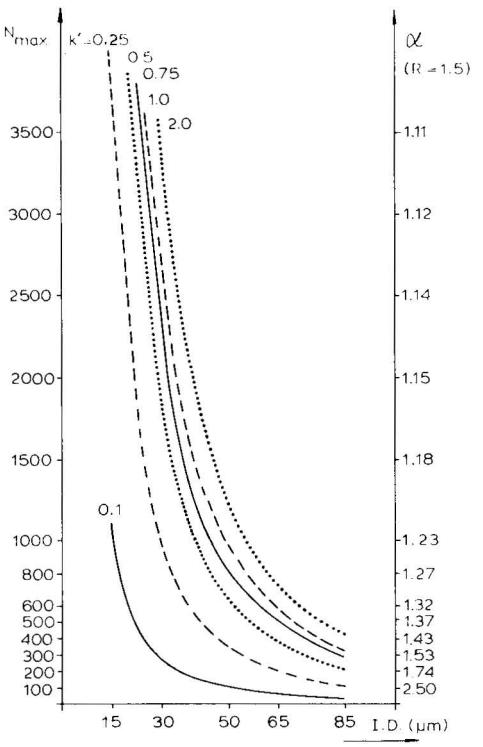


Fig. 2. Maximum number of theoretical plates as a function of the inner diameter of the capillary column for different capacity ratios. Eluent, methanol–water (45:55, v/v). Hold-up time, 10 min.

Fig. 3. Curves of N_{max} and α *versus* inner diameter for different k' values. Eluent, methanol–water (45:55). t_0, 10 min.

pounds emerge around $k' = 0.25$ and baseline resolution is required in a 30-μm I.D. column, then the relative retention of these substances has to be greater than $\alpha = 1.23$. With a 50-μm I.D. capillary column only compounds with $\alpha > 2.5$ can be resolved in the neighbourhood of $k' = 0.1$. Bearing in mind that in all of the calculations $B/u = C_s u = 0$ was assumed, it seems to be evident that for routine separations in LC, open tubes of I.D. 30 μm or less are required if the viscosity of the eluent is about $1 \cdot 10^{-2}$ poise.

Rearranging the Hagen–Poiseuille equation[14,15] for ideal tubes we obtain

$$L = \frac{d^2 \Delta P}{32 u \eta} \tag{12}$$

On combining eqns. 4 and 12:

$$n_{max} = \frac{3 D \Delta P}{f u^2 \eta} \tag{13}$$

or, at room temperature with the approximation described in eqn. 11

$$n_{max} = \frac{3.6 \cdot 10^{-7} \Delta P}{f u^2 \eta^2} \tag{14}$$

In eqn. 14 all of the parameters are given in c.g.s. units (1 bar $\approx 10^6$ dyne). The maximum outlet pressure of the micropumps is limited. If ΔP is constant, n_{max} is inversely proportional to the square of the linear velocity and to the square of the viscosity of the eluent. It can be seen in eqn. 13 that, with ΔP constant, n_{max} will be achieved at u_{Min} (*i.e.*, the minimum of the *h versus u* curve), as described in eqn. 3. Of course, in this u region the B/u term is not negligible. Comparing eqn. 13 or 14 with eqn. 12 it can be seen that if u, η and ΔP are given, the ratio d^2/L has to be constant. Otherwise, for a given d the value of L can be calculated.

If u is small (and consequently h is also small) the column length, L, has to be short in order to achieve reasonable analysis times. A short column and a small h value result in a very small peak volume, and consequently the volume of the detector has to be extremely small. Because of the volume of the detector cell, long columns (and high linear velocities) are required in LC with open tubes. Between the two contradictory statements in this paragraph, a compromise has to be found. The best choice is dependent on the maximum outlet pressure of the pump and the cell volume of the detector. It should be mentioned that the velocity at the minimum of the *h versus u* curve is inversely proportional to the inner diameter of the open tube, as shown in eqn. 3.

SPEED OF ANALYSIS IN OPEN TUBES

Rearranging eqn. 8 and bearing in mind that $t_R = t_0(1+k')$, the maximum number of plates generated per unit time is

$$\frac{n_{max}}{t_R} = \frac{96D}{fd^2(1+k')} \tag{15}$$

or

$$\frac{N_{max}}{t_R} = \frac{96D}{d^2F'(1+k')} = \frac{96D}{d^2F^*} = \frac{n_0}{t_0F^*} \tag{16}$$

where

$$F^* = F'(1+k') = \frac{1+6k'+11(k')^2}{(k')^2} \cdot (1+k') \tag{17}$$

The factor F^* as a function of k' is given in Table I and has its minimum at $k' = 0.914$. The optimal speed of analysis, defined in terms of the number of effective plates generated per unit time, is achieved in this capacity ratio region. It should be remembered that with packed columns the maximum of N/t_R is generated with $k' = 2$ if n is not a function of k', because the $(k')^2/(1+k')^3$ *versus* k' curve has its maximum at this point.

The assumption in this paper is that $u \gg u_{Min}$. Consequently, the speed of analysis is independent of the velocity of the eluent, because h is a linear function of u.

In a 30-μm I.D. tube with *n*-heptane as eluent, analyses are very rapid, as shown in Fig. 4. N_{max}/t_R is extremely low at small k' values, passes through a maximum at $k' = 0.914$ and decreases sharply with increasing capacity ratios. There is only a narrow range of capacity ratios where the optimal speed of analysis is achieved. If only a few compounds have to be separated, a system (*i.e.*, stationary phase, eluent composition and sample) has to be chosen where the compounds emerge in the neighbourhood of $k' = 1$. As was pointed out earlier and as shown in Fig. 4, capacity ratios greater than $k' = 2$ are of limited interest in routine LC if open tubes are to be used.

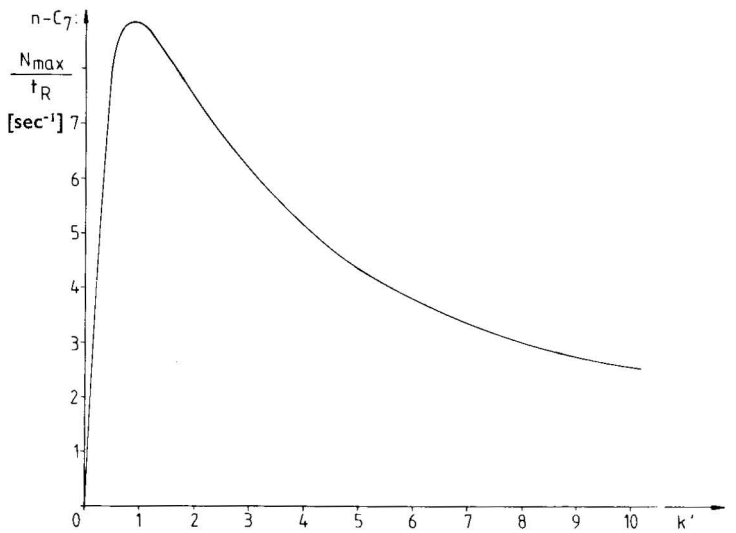


Fig. 4. Maximum speed of analysis, N_{max}/t_R, *versus* k' in a 30-μm I.D. capillary column. Eluent, *n*-heptane.

In Fig. 5 the speed of analysis is shown for water and methanol–water eluents in capillary columns with different inner diameter as a function of k'. Although optimal boundary conditions ($B/u = C_s u = 0$) are assumed, the speed of analysis in the 50-μm I.D. column is again surprisingly low compared with the N/t_R values generated in columns packed with 10- or 5-μm particles.

PEAK VOLUME AND SAMPLING SYSTEM

The minimum peak volume (*i.e.*, $B/u = C_s u = 0$), defined as 4ω, can be calculated with eqn. 5 and with $\sigma = \sqrt{hL}$:

$$\begin{aligned} 4\omega_{min} &= 4\sigma r^2 \pi = \pi d^2 \sqrt{hL} \\ &= 0.32 d^3 \sqrt{\frac{fuL}{D}} \\ &= 0.32 u d^3 \sqrt{\frac{ft_0}{D}} \end{aligned} \tag{18}$$

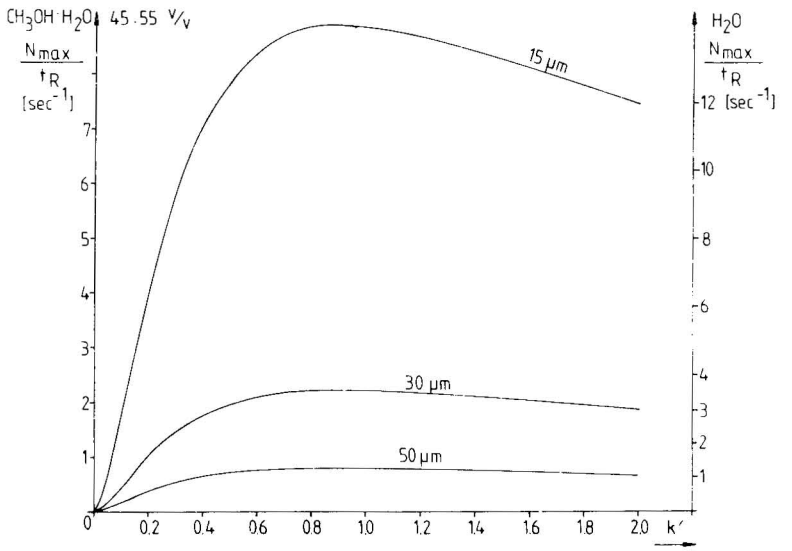


Fig. 5. N_{max}/t_R *versus* k' curves for open tubes with different inner diameters. Eluent, water (right-hand axis) and methanol–water (45:55) (left-hand axis).

N.B., the peak volume is proportional to d^3. Because of the factor f, which is a function of k', the minimum 4 ω_{min} will be calculated or measured for the inert peak.

It must be pointed out again that ω is a linear function of u, as shown in eqn. 18. If the peak volume has to be increased by a given factor β (because of the problem of the cell volume of the detector), the linear velocity (*i.e.*, the pressure drop over the column) has to be increased by the same factor β. To keep the number of plates and the time of analysis constant, the length of the column therefore has to be increased. Consequently, the pressure drop over the column must increase by a factor of β^2.

Because of the sampling system, peak broadening outside the column and the restricted speed of mass transfer in the stationary phase (*i.e.*, $C_s u > 0$), the measured peak volumes will be greater than that calculated above, and will increase with increasing k'.

NUMBER OF PLATES GENERATED PER UNIT PRESSURE

As pointed out by Golay, the primary advantage of open tubes (also called Golay or capillary columns) in gas chromatography is the substantially greater number of plates generated per unit pressure drop compared with packed columns. High efficiency can then be achieved using long columns. However, the linear velocity must be sufficiently high to obtain acceptable analysis times.

Rearranging eqn. 13:

$$\frac{n_{max}}{\Delta P} = \frac{3D}{fu^2\eta} = \frac{3Dt_0}{fu\eta L} \tag{19}$$

or with eqn. 9:

$$\frac{N_{max}}{\Delta P} = \frac{3D}{F'u^2\eta} \tag{20}$$

or at room temperature with the approximation described in eqn. 11:

$$\frac{N_{max}}{\Delta P} = \frac{3.6 \times 10^{-7}}{F'u^2\eta^2} \tag{21}$$

In eqn. 21 all of the parameters are again given in c.g.s. units. As shown in the equations above, the number of plates generated per unit time is independent of the inner diameter of the column, because both h and the permeability K are proportional to d^2.

As shown in eqn. 21, $N_{max}/\Delta P$ is inversely proportional to u^2 and η^2 and, because of the change in F', it increases with increasing capacity ratio. In Table VII some $N_{max}/\Delta P$ values are given for different eluents and different velocities when $k' = 1$. This capacity ratio (where $F = 18$) was chosen because the maximum speed of analysis is in this region. Of course, $N_{max}/\Delta P$ is smaller by a factor of about 30 for compounds with $k' = 0.05$ than for the values shown in Table VII. In the second column of Table VII, chromatographic column lengths, L, are given assuming a hold-up time of 10 min. The number of effective plates, of course, decreases with increasing velocity. In Table VII linear velocities of $u < 0.1$ cm/sec are of no interest, because $u \gg u_{Min}$. The problems of low velocities and short columns were discussed earlier.

TABLE VII

MAXIMUM NUMBER OF EFFECTIVE PLATES GENERATED PER UNIT PRESSURE (BAR) WITH DIFFERENT ELUENTS AND FOR $k' = 1$

u (cm/sec)	L* (m)	Eluent		
		n-Heptane	*Water*	*Methanol–water (45:55, v/v)*
0.1	0.6	125,000	20,000	7810
0.5	3	5000	800	313
1	6	1250	200	78.1
10	60	12.5	2.00	0.781
50	300	0.5	0.08	0.031

* For the calculation of L it was assumed that $t_0 = 10$ min.

The number of theoretical plates generated per unit time, $n_{max}/\Delta P$, is a factor 4.5 smaller for an inert peak than for a retarded peak with $k' = 1$ because of the factor f in eqn. 19. This is also of importance if the efficiencies of capillary and packed columns are compared.

PROBLEMS WITH IDEAL OPEN TUBES IN LIQUID CHROMATOGRAPHY

In Table VIII some typical optimal parameters are given for open tubes without secondary flow for $t_0 = 10$ min. The inner diameters of the tubes ranged from 30 to

100 μm. The $u_{min,0}$ values in the second column were calculated with eqn. 3 for an inert peak, because this value is smaller than that for a retarded peak. Because the B/u term is always neglected, u was chosen so as to be always seven times greater than $u_{min,0}$. When $t_0 = 600$ sec and when u is defined, then the column length L is defined also. The other parameters in this table were calculated with the assumptions and equations described earlier. The effective plate numbers were calculated for $k' = 1$ (*i.e.*, N_1), because the speed of analysis is optimal in this range.

TABLE VIII

PEAK VOLUME, PRESSURE DROP AND SPEED OF ANALYSIS WITH IDEAL OPEN TUBES AND PACKED COLUMNS

Eluent, *n*-heptane. $t_0 = 600$ sec. $D = 3 \cdot 10^{-5}$ cm^2/sec. $\eta = 4 \cdot 10^{-3}$ poise.

d (μm)	$u_{min,0}$* (cm/sec)	u (cm/sec)	L (m)	ΔP (bar)	n_0 ($k' = 0$)	N_1 ($k' = 1$)	$n_0/\Delta P$ (bar^{-1})	$N_1/\Delta P$ (bar^{-1})	n_0/t_0 (sec^{-1})	N_1/t_1 (sec^{-1})	$\overline{4\omega_0}$ (μl)
30	0.14	1.0	6	8.53	192,000	10,667	22,500	1250	320	8.9	0.0386
30	0.14	10.0	60	853	192,000	10,667	225	12.5	320	8.9	0.386
50	0.083	0.6	3.6	1.11	69,120	3840	62,500	3460	115	3.2	0.107
50	0.083	2.0	12	12.3	69,120	3840	5625	312	115	3.2	0.357
100	0.042	0.3	20	0.77	17,280	960	22,442	1247	28.8	0.8	0.430
Packed column											
10**	0.1	0.1	0.6	24	14,630	3659	610	152	24.4	3.1	204

* u_{min} for $k' = 0$.
** Particle size of silica.

In the first row in Table VIII the plate numbers for the inert peak n_0 and the effective plate numbers for $k' = 1$ (N_1) are extremely high, as are the number of plates generated per unit pressure drop ($n_0/\Delta P$ and $N_1/\Delta P$) and the speed of analysis (n_0/t_0 and $N_1/t_1 = N_1/2t_0$). The peak volume, however, is extremely small: $4\,\omega \approx 0.04$ μl. The cell volume of the detector has to be about ten times greater than $4\,\omega$ in order to avoid distortion of the peak.

To generate larger peak volumes, but to keep the efficiency and the speed of analysis constant, the column length must be increased by a factor of ten to 60 m and the linear velocity to $u = 10$ cm/sec. As shown in the second row in Table VIII, the peak volume is increased to $4\,\omega \approx 0.4$ μl (*i.e.*, a cell volume of 0.04 μl is acceptable), but at the same time the pressure drop increases to $\Delta P = 853$ bar. At the moment, it is hardly possible to generate inlet pressures of this order of magnitude with a micropump, and sample injection would also be difficult. In the second row the number of plates generated per unit pressure decreased dramatically. These values are comparable to or worse than those generated in routine LC with packed columns. Shorter columns of 30-μm I.D. and/or lower linear velocities would again result in intolerably small peak volumes.

The next two rows in Table VIII are the corresponding values calculated for open tubes of 50-μm I.D. The problem is again the peak volume. The N_1 and N_1/t_1 values in the fourth row are comparable (*i.e.*, the peak volume is acceptable) and the $N_1/\Delta P$ values are only twice as good as with packed columns.

In the fifth row in Table VIII the inner diameter of the ideal open tube is

100 μm. The number of plates generated per unit pressure drop is, of course, excellent. However, the efficiency and the speed of analysis are poor.

In the last row in Table VIII the corresponding parameters for packed columns are given. These values can easily be achieved experimentally using commercially available columns packed with irregular silica with a particle size of 10 μm. If the relevant parameters calculated for open tubes with acceptable peak volumes are compared with those generated experimentally using packed columns, the advantages of open tubes seem to disappear.

When comparing capillary and packed columns with the help of Table VIII, it has to be re-emphasized that the parameters for open tubes obtained experimentally must be worse than those obtained by calculation, because of the neglected mass transfer term and because of the experimental problems (*i.e.*, peak broadening caused by sampling and extra-column effects). The peak volumes in open tubes as determined experimentally will be greater than the calculated values. This would be advantageous, but the overall picture will remain unchanged, because of the poorer efficiencies.

One possibility for increasing the peak volume would be to inject the eluent continuously between the column outlet and the detector inlet. The standard deviation of Gaussian peak in time units would thereby remain constant. Unfortunately, the inlet and outlet turbulence in this inverse splitting system would decrease the resolution achieved in the column. Further, the tolerable sample sizes for capillary columns of 50-μm I.D. or less is of the order of magnitude of nanograms. If the peak volume is about 1 μl the concentration of the sample at the end of the column would be, on average, less than 1 ppm. Because of the noise level of the UV detector only samples with unusually high molecular extinction coefficients could be so diluted.

PRESSURE LIMITATIONS

Finally, u_{Min} increases with decreasing diameter of the capillary column, as shown in eqn. 3. Because $u \gg u_{\mathrm{Min}}$ and because the specific permeability, K, is proportional to d^2, the required pressure will increase sharply with decreasing inner diameter of the open tube. Although the figures in Table VIII are very impressive at low velocities, the pressure will become a limiting factor in LC in capillary columns, especially if micropumps have to be used.

CONCLUSIONS

The optimal parameters for separations by LC in capillary columns can be calculated if it is assumed that the dispersion of inert and retarded peaks can be described using only the $C_m u$ term of the Golay equation. This approximation is acceptable if (a) the inner diameter of the open tube is less than 100 μm, (b) the linear velocity is less than 1 m/sec, (c) the roughness of the wall is negligible, (d) the coil diameter is greater than 5 cm and (e) the flow is laminar. These conditions exclude secondary flow in the laminar region. Important parameters such as n, N, N/t_R, $N/\Delta P$ and peak volumes were calculated as a function of the inner diameter of the tubes, the capacity ratios of the compounds and the properties of the eluent. With the approximation given above, the speed of analysis, defined as the effective number of plates, N, generated per unit time, is independent of the velocity of the eluent. Further,

the number of plates generated per unit pressure drop is independent of the inner diameter of the capillary column. In LC, because of the high viscosity of the eluents and because of the small inner diameter of the open tubes, the $N/\Delta P$ values in capillary columns are comparable to the $N/\Delta P$ values generated in packed columns ($d_p = 10\ \mu m$).

It is shown that $0 < k' < 2$ is the most important range of capacity ratios if either simple or complicated mixtures are to be resolved. It contrast to packed columns in open tubes, h or n is an extremely sensitive function of k' in this range, and consequently only a narrow capacity ratio range gives optimal conditions for the separation.

The results achieved experimentally will always be worse than those calculated. It seems, therefore, that capillary columns with inner diameters greater than 30 μm will be of little practical interest in routine LC.

The extremely small peak volumes (*i.e.*, detection problems) and the required time of analysis will require long columns and high linear velocities. Consequently, the maximum available outlet pressure of the micropump will become a limiting factor. Micropumps are characterized by delivering the eluent at a small but constant flow-rate at high outlet pressures, as is required in LC with open tubes.

In LC with capillary columns, the peak dispersion can only be greater than calculated in this paper, because of the restricted rate of mass transfer in the stationary phase and because of instrumental difficulties. Consequently, it seems probable that open tubes cannot compete with packed columns in LC. This problem will be discussed in a following paper in which experimental results are compared for capillary and packed columns.

The above statement will not be true if columns are built in which the radial mass transfer in the laminar region is speeded up by secondary flow. This could be achieved in a geometrically deformed tube and/or by depositing the solid stationary thin layer in such a way that the wall of the open tube becomes rough. The smaller the inner diameter of the tube the less unevenness of the wall is required to generate secondary flow in a capillary column.

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SYMBOLS

B (cm^2/sec)	constant in the Golay equation, longitudinal diffusion
C_m (sec)	constant in the Golay equation, mass transfer in the mobile phase
C_s (sec)	constant in the Golay equation, mass transfer in the stationary phase
d (cm)	inner diameter of the open tube
D (cm^2/sec)	interdiffusion coefficient of the sample in the eluent
f	factor defined in eqn. 2

f^*	factor defined in eqn. 7
F'	factor defined in eqn. 9
F^*	factor defined in eqn. 17
h (cm)	height equivalent to a theoretical plate
h_0 (cm)	h at $k' = 0$
h_{min} (cm)	h for a retarded peak, if $B/u = C_s u = 0$
h_{Min} (cm)	h at the minimum of the h *versus* u curve
K (cm^2)	specific permeability
k'	$(t_R - t_0)/t_0$, capacity ratio
L (cm)	column length
n	$(4\ t_R/w)^2$, number of theoretical plates
n_0	n for the inert compound
n_{max}	n for a retarded sample, if $B/u = C_0 u = 0$
N	$(4\ t'_R/w)^2$, number of effective plates
N_{max}	N for a retarded sample, if $B/u = C_0 u = 0$
N_{ne}	$[4R\alpha/(\alpha - 1)]^2$, necessary number of effective plates defined in eqn. 10
N_1	N at $k' = 1$
P (dyne/cm$^2 \approx 10^{-6}$ bar)	pressure
ΔP (dyne/cm$^2 \approx 10^{-6}$ bar)	pressure drop over the column
r (cm)	radius of the open tube
R	$2(t_{R2} - t_{R1})/(w_1 + w_2)$, resolution
t (sec)	time
t_0 (sec)	hold-up time, or retention time of an inert sample
t_R (sec)	retention time of a retarded peak
t'_R (sec)	$t_R - t_0$, net retention time
u (cm/sec)	cross-section averaged linear velocity of the mobile phase
u_{Min} (cm/sec)	u at the minimum of the h *versus* u curve
$u_{min,0}$ (cm/sec)	u_{min} for the inert compound
w (sec)	peak width at baseline
α	t'_{R2}/t'_{R1}, relative retention
η (g/cm sec or poise)	dynamic viscosity
σ (cm)	standard deviation of a Gaussian peak
ω (cm^3)	$\sigma r^2 \pi \cdot$ peak volume/4 = standard deviation of a Gaussian peak
ω_0 (cm^3)	ω for the inert compound
ω_{min} (cm^3)	ω for a retarded peak, if $B/u = C_s u = 0$

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EVALUATION OF AMPEROMETRIC DETECTORS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: ANALYSIS OF BENZODIAZEPINES

WALTER LUND*, MERETE HANNISDAL and TYGE GREIBROKK
Department of Chemistry, University of Oslo, Box 1033 Blindern, Oslo 3 (Norway)
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SUMMARY

The design of amperometric detectors for high-performance liquid chromatography is discussed. A simple flow cell with interchangeable working electrodes made from glassy carbon, carbon paste and mercury is described, and its performance is compared with commercially available cells, using both constant-potential and pulse-measuring techniques.

The analysis of nitrazepam, diazepam and chlordiazepoxide was used as a model system; the detectors were used in the reduction mode and the mobile phase was methanol–water (60:40) containing 0.05 *M* ammonium acetate. The effects of various experimental parameters are reported. The detection limit was found to depend strongly on the reduction potential: at −0.93 V *vs.* Ag, 3 ng of nitrazepam could be detected, whereas at −1.30 V the detection limit was 30 ng, owing to the high background current at this potential. A potential more negative than −1.1 V must be used for the detection of diazepam and chlordiazepoxide; at −1.30 V the detection limit was 300 ng for these compounds.

INTRODUCTION

High-performance liquid chromatography (HPLC) with amperometric detection offers certain advantages over UV detection in terms of selectivity, sensitivity and cost[1]. A simple potentiostat with low-level current-measuring capabilities can easily be constructed[2]; however, the success of the whole system depends critically on the design of the electrochemical flow-through cell. Thus, preliminary experiments revealed that some commercial detectors did not function properly, and the reason was found to be the uncompensated resistance within these flow cells.

In this paper a simple detector cell is described in which there is negligible uncompensated resistance; the cell has a satisfactory response also when the mobile phase has a low electrical conductivity. The cell is equipped with interchangeable working electrodes made from glassy carbon, carbon paste and mercury, so that a relevant comparison of these electrode materials can be made. The analysis of benzodiazepines served as a model system; the detector was mainly operated in the

* To whom correspondence should be addressed.

reduction mode, but the anodic range of the detector was also tested. The reduction of oxygen, hydrogen ions and trace metals may interfere with the determination when the detector is used in the reduction mode, and it was of particular interest to ascertain the magnitude of these interferences. Normally, amperometric HPLC detectors are operated in the oxidation mode in order to avoid the above interferences.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) high-pressure liquid chromatograph, incorporating a U6K injector and a Model 6000 A solvent delivery system, was used. The column (250 × 2.6 mm I.D.) was packed with 5-μm Spherisorb ODS; the number of theoretical plates of the newly packed column was 3800. A Perkin-Elmer LC-55 UV detector was used in some of the experiments. For electrochemical detection, a Princeton Applied Research 174A Polarographic Analyzer and a Radiometer Servograph REC51/REA112 recorder were used.

The construction of the electrochemical flow-through cell is shown in Fig. 1. The cell is based on the "wall-jet" principle[3] with the flowing stream from the chromatographic column directed perpendicular to the working electrode surface. The cell was made from Teflon, a silver wire served as a quasi-reference electrode and a platinum wire or the stainless-steel exit tube was used as a counter electrode.

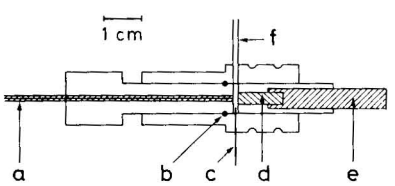

Fig. 1. Construction of the flow-through cell (Teflon). a = Stainless-steel inlet from chromatograph; b = O-ring; c = reference electrode, silver wire; d = working electrode, glassy carbon rod; e = brass rod; f = stainless steel exit tube, counter electrode.

The glassy carbon electrode was made by pressing a 3-mm rod (Tokai Electrode Mfg. Co. Ltd., Tokyo, Japan) into Teflon. The electrode mounted in a Plexiglass block was polished first with silicon carbide papers (No. 400 and 600), then with alumina suspensions (1 μm) and finally with diamond paste (1 and 0.25 μm) on rotating discs.

The carbon paste electrode was made from a Teflon rod with a 3-mm diameter well drilled in one end. The well was filled with a carbon paste prepared by mixing 5 g of graphite powder (Koch-Light, Colnbrook, Great Britain) and 3 ml of nujol as described by Adams[4]. Electrical connection was made via a platinum wire. The electrode surface was polished by rubbing the electrode against a smooth paper.

The same Teflon rod was also used as a support for the mercury pool electrode. The rod was placed in a vertical position and the well was filled with highly purified mercury.

The commercial cells were obtained from EDT Research (London, Great Britain) (type LC03, hereafter called the EDT cell) and Bioanalytical Systems (West

Lafayette, Ind., U.S.A.) (hereafter called the BS cell). The constructions of these cells have been described by Fleet and Little[3] and Kissinger *et al.*[2], respectively.

Reagents and solutions

The benzodiazepines, which were kindly supplied by A/S Apothekernes Laboratorium for Spesialpraeparater (Oslo, Norway), were dissolved in methanol. Methanol–water (60:40) containing 0.05 *M* ammonium acetate was used as the mobile phase; the salt served as a supporting electrolyte for the electrochemical detector. Oxygen was removed from the mobile phase by bubbling argon through the solution (1 l) for at least 4 h (normally overnight). The 10-ml samples were kept in 14-ml vials with plastic caps and deaerated for 5 min before the injection of 10-μl aliquots.

RESULTS AND DISCUSSION

Reduction of benzodiazepines

The polarographic reduction of nitrazepam, diazepam and chlordiazepoxide in aqueous solution is well documented[5,6]. However, no data were available for the particular medium used here, namely methanol–water (60:40) containing 0.05 *M* ammonium acetate (pH 7.25). The d.c. polarograms of the three benzodiazepines in this medium are shown in Fig. 2. The polarograms were recorded with a dropping mercury electrode in a quiescent solution. For chlordiazepoxide, three waves are observed with $E_{\frac{1}{2}}$ at −0.98, −1.24 and −1.62 V *vs.* Ag. For nitrazepam, two waves are observed with $E_{\frac{1}{2}}$ at −0.65 and −1.30 V *vs.* Ag (the first wave has a small maximum), whereas a single wave is obtained for diazepam with $E_{\frac{1}{2}}$ at −1.14 V *vs.* Ag. The wave heights indicate a 2:2:2 electron reduction of chlordiazepoxide, a 4:3 electron reduction of nitrazepam and a single 2-electron reduction of diazepam, in good agreement with the respective reduction mechanisms described by Oelschläger and co-workers[7–9]. The second wave of nitrazepam, which apparently corresponds to a 3-electron reduction, is probably a mixture of a 2- and a 4-electron reduction[8].

Voltammograms were also recorded using the glassy carbon and carbon paste electrodes which were subsequently used in the flow-through cells. For chlordiazepoxide no well defined waves were observed, but for the other two compounds the voltammograms were similar to the polarograms shown in Fig. 2; only the first wave of nitrazepam had the peaked shape characteristic of linear sweep voltammetry (see Fig. 4). On glassy carbon the "half-wave" potentials were −0.73 and −1.20 V for nitrazepam and −1.20 V for diazepam. The potentials varied slightly from day to day, probably as a result of changes in the condition of the electrode surface.

The effect of pH was studied by recording polarograms of the three benzodiazepines at different pH values; the pH was adjusted with acetic acid and ammonia solution. It was found that the half-wave potentials shifted slightly (less than 50 mV) towards more positive values when the pH was decreased from 7.7 to 5.8.

In all of the above experiments a silver wire was used as a quasi-reference electrode in order to reproduce the experimental conditions in the flow-through cell.

Separation of benzodiazepines

Chlordiazepoxide, nitrazepam and diazepam are well separated by HPLC on

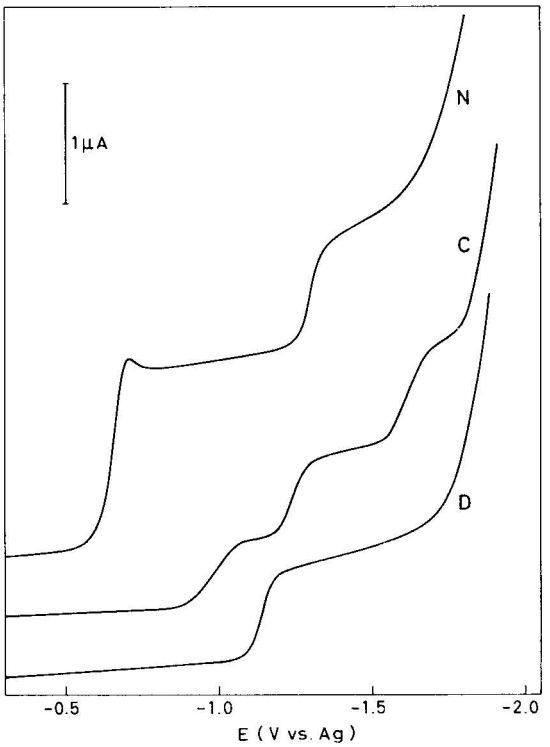

Fig. 2. Polarograms (d.c.) of 10^{-4} *M* nitrazepam (N), diazepam (D) and chlordiazepoxide (C) in methanol–water (60:40) containing 0.05 *M* ammonium acetate.

a reversed-phase column, using a mixture of methanol and water as the mobile phase. Methanol–water (60:40) containing 0.05 *M* ammonium acetate was used as mobile phase in this work; a typical chromatogram is shown in Fig. 3. Nitrazepam is eluted first, followed by chlordiazepoxide and diazepam; a similar result was obtained by Harzer and Barchet[10], whereas Knox and Pryde[11], who added ammonia to the mobile phase, reported a different sequence of the peaks. The separation depend on the methanol content of the mobile phase; with less methanol an inferior separation was obtained, and no separation was observed with methanol–water (40:60). Ammonium acetate, which gives a nominal pH of 7.25, serves as a supporting electrolyte for the electrochemical detector. Neither the separation nor the peak heights were improved when the pH was adjusted to higher or lower values with ammonia solution and acetic acid, respectively, in the pH range 5–8. The benzodiazepines are unstable in more strongly acidic and basic solutions, and the Spherisorb ODS packing of the column is unstable at pH values above 8.

Removal of oxygen

As shown in Fig. 3, the detector gives a signal for oxygen, which will interfere with the determination unless the concentration of oxygen is very low. Further, the solubility of oxygen is very high in a 60:40 methanol–water mixture[12]. For satisfactory detector performance in the cathodic region, it is essential that oxygen be

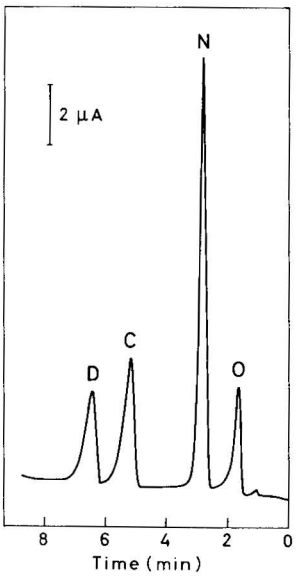

Fig. 3. Separation of 20 μg (0.07 μmole) of nitrazepam (N), 30 μg (0.1 μmole) of chlordiazepoxide (C), 28 μg (0.1 μmole) of diazepam (D) and trace amounts of oxygen (O) using amperometric detection. ODS column; mobile phase methanol–water (60:40) containing 0.05 *M* ammonium acetate; flow-rate 1 ml · min^{-1}; reduction potential −1.25 V *vs.* Ag.

removed from the mobile phase, as well as from the sample, prior to the analysis. Normally, the mobile phase was deaerated by bubbling argon through the 1-l container for at least 4 h (preferably overnight). Teflon tubing could not be tolerated in any part of the system, and stainless-steel tubing was used instead. The 10-ml samples were deaerated for 5 min; identical treatment of all samples was essential because of the high volatility of the solvent. Although a very thorough deaeration procedure was used, an oxygen peak was usually observed on the chromatograms.

Detector parameters

Constant potential and pulse measurements. For amperometric detection the working electrode is normally kept at a constant potential. Obviously, this is the most practical approach, because a very simple potentiostat can then be employed. It has, however, been claimed that a pulse technique would give better sensitivity and electrode stability when solid electrodes are used[13]. In this work, these advantages of the pulse technique were not confirmed. On the contrary, the use of a normal pulse technique was found to give much higher background currents and poorer signal-to-noise ratios than constant-potential measurements, and even the differential pulse technique offered little improvement in detection limits (see below). The fast changes in the applied potential (*i.e.*, pulses) give rise to slow solid-state redox reactions which alter the surface of any working electrode made from carbon, and the pulses also result in significant capacitive currents[14–16]. These effects are illustrated in Fig. 4 for the reduction of nitrazepam on glassy carbon.

The claimed beneficial effect of the pulse techniques on electrode stability is supposed to be a result of the working electrode being kept at an initial "cleaning"

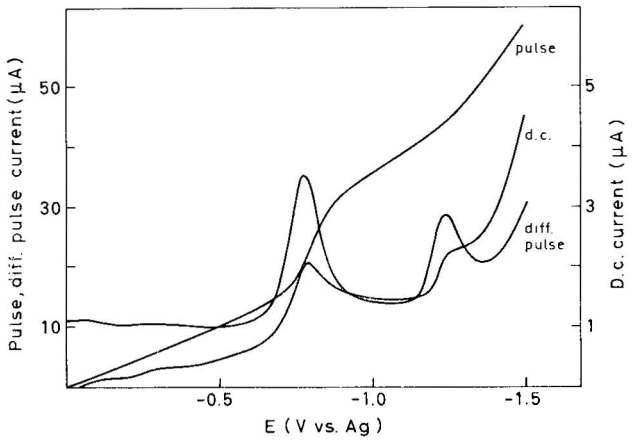

Fig. 4. Voltammograms of 10^{-4} *M* nitrazepam in methanol–water (60:40) containing 0.05 *M* ammonium acetate, obtained with a glassy carbon electrode and d.c., pulse and differential pulse techniques. Scan rate 5 mV · sec^{-1}; pulse repetition time 1 sec; modulation amplitude 50 mV.

potential between pulses, at which any unwanted electrode reactions cannot take place. Thus, the EDT detector incorporates a pulse potentiostat which is said to overcome the problems of adsorption and fouling of the electrode surface. However, in this work no improvement in electrode stability was observed when the pulse techniques were used. According to our experience, a poisoned electrode is best reactivated by mechanical cleaning (*i.e.*, polishing) of the electrode surface.

The long time constant of the memory circuits in the polarograph adds to the inconvenience of the pulse techniques; for narrow chromatographic peaks a low detector response must be expected.

No doubt differential pulse measurements at a constant initial potential will give a better selectivity than normal amperometric detection, because only species with half-wave potentials close to the initial potential will be detected. However, electrochemical detectors are selective in comparison with UV detectors for instance, and the additional selectivity offered by the differential pulse technique is therefore seldom required, unless the unknown compounds are very poorly separated by the column. The differential pulse technique is, however, useful for determining the "half-wave" potentials of unknown compounds from chromatograms recorded at different potentials, because it is simpler to locate the potential which corresponds to the maximum peak height than to construct the full d.c. voltammogram ($E_p = E_{\frac{1}{2}} - \Delta E/2$).

It has been pointed out[13,15] that the flow-rate dependence of the detector is minimized by using a pulse measuring technique. However, as will be shown below, the detector was found to have a very reproducible response when operated in the constant-potential mode, so again there should be little need for introducing the pulse approach, at least not for the type of liquid chromatograph used here.

Cell design. In order to avoid uncompensated resistance in any part of the flow-through cell, the three electrodes should be positioned as close as possible to each other. Of the various designs suggested in the literature, the wall-jet cell[3] is probably the best. The cell shown in Fig. 1, which is based on this principle, was

found to have a very satisfactory response. The outlet from the chromatographic column is directed perpendicular to the working electrode surface. A silver wire is used as a quasi-reference electrode; the electrode is positioned 1 mm from the working electrode. The stainless-steel exit tube, which is positioned 2 mm from the working electrode, serves as a mechanically robust counter electrode. In some experiments a platinum wire, positioned between the silver wire and the exit tube, was used as an alternative counter electrode; an identical detector response was observed in this instance. The working electrode shown in Fig. 1 is made from glassy carbon; the other working electrodes examined were made from carbon paste and mercury (pool). The characteristics of the different electrode materials are discussed below.

Normally, the volume of the flow cell is adjusted by altering the thickness of the thin layer of solution passing across the working electrode. For the wall-jet cells this is done by increasing the distance between the inlet tube and the working electrode. For the home-made cell a distance of 1 mm was mostly used; the effective cell volume (as defined by the area of the working electrode) was then 7 μl, and the total cell volume was 50 μl. As expected, a larger cell volume gave lower and broader chromatographic peaks, with marked tailing at the end. However, no significant increase in peak height was observed when the solution layer thickness was decreased below 1 mm.

The EDT cell was found to give results similar to those obtained with the home-made cell. However, at potentials more negative than -1.4 V a decrease in detector response was observed, which was probably due to uncompensated resistance within the cell; the counter and reference electrodes are positioned some distance from the glassy carbon working electrode. Further, only one of two reference electrodes tested was found to function properly.

The thin-layer cell designed by Kissinger *et al.*[2] and manufactured by Bioanalytical Systems was also tested, but it did not function properly under the experimental conditions used. In this cell the reference and counter electrodes are placed in a separate compartment far from the working electrode, and the electrical contact is made through a very thin layer of solution and thin tubing. Here a large uncompensated resistance cannot be avoided, particularly when a partly non-aqueous mobile phase is used. By making the connections between the electrodes shorter and wider, a better but still unsatisfactory response was obtained. No further use was therefore made of this cell.

Working electrodes. The ideal working electrode material should have large anodic and cathodic potential ranges with low background currents, and the properties of the electrode surface should not change with time. For practical reasons solid electrodes made from glassy carbon and carbon paste are more attractive than stationary or dropping-mercury electrodes. The EDT cell is equipped with a glassy carbon electrode, whereas the BS cell employs a carbon paste electrode. The cell shown in Fig. 1 was used with glassy carbon, carbon paste and mercury pool electrodes. A summary of our experience with these electrodes is given below.

The glassy carbon electrode must be polished very thoroughly in order to give a satisfactory voltammetric response. However, glassy carbon obtained from some manufacturers did not give a satisfactory background, irrespective of the polishing procedure. A poisoned or poorly polished electrode gives high background currents, and the reduction of the solvent appears at a relatively positive potential, whereas the "half-wave" potential of the electroactive compound is shifted in the

opposite direction owing to the low rate of the electrochemical charge-transfer reaction. The deactivation of the electrode was found to be much slower for a well polished than for a poorly polished electrode.

A residual wave was sometimes observed even on well polished electrodes at *ca.* −0.7 V. The wave disappeared when the electrode was kept at −1.0 V for some time, indicating that the residual wave is due to oxides present at the glassy carbon surface.

Background scans are shown in Fig. 5 for both a good and a bad glassy carbon electrode. The cathodic limit is determined by the reduction of hydrogen ions, and the anodic limit by the oxidation of the solvent. The voltammograms were obtained by scanning the potential from 0 V in the cathodic and anodic directions. It has been claimed that steady-state voltammetry would give much lower background currents than scanning voltammetry[14], but in the present work the two techniques gave comparable background currents in the potential region 0 to −1.0 V. At more negative potentials the steady-state currents were lower than those obtained in the scanning mode.

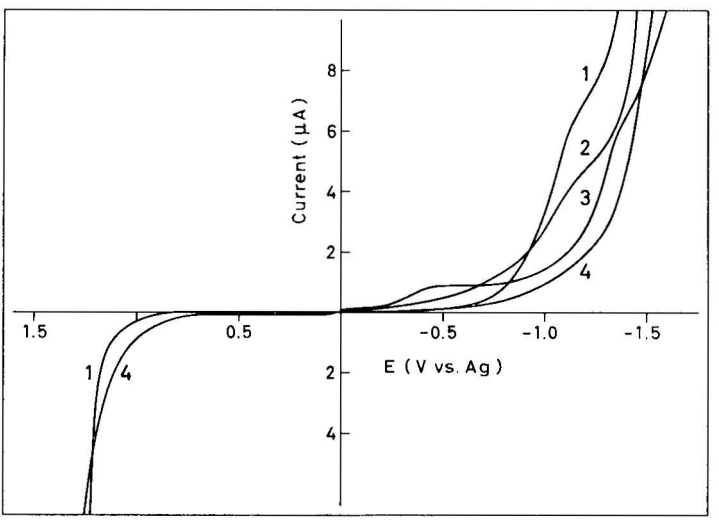

Fig. 5. Background voltammograms of the mobile phase for different working electrodes: 1 = carbon paste; 2 = glassy carbon, poorly polished; 3 = mercury pool; 4 = glassy carbon, well polished. Flow-rate 1 ml · min^{-1}; scan rate 5 ml · sec^{-1}.

For the carbon paste electrode the polishing procedure is relatively simple. After packing, the electrode surface is smoothed by rubbing the electrode across a glazed paper surface. A representative background scan is shown in Fig. 5, illustrating that a very good background is obtained in the anodic region, and also in the cathodic region below −0.6 V, but for more negative potentials an appreciable background is observed. The background was not improved by applying a potential of −1.0 V (or more negative) for some time, probably because trace amounts of oxygen are uniformly distributed throughout the paste and not only adsorbed on the electrode

surface. No problems with long-term stability were encountered on using methanol–water (60:40) as the mobile phase.

Glassy carbon and carbon paste electrodes are particularly well suited for detection in the anodic range. It is usually assumed that a mercury electrode would give a larger potential range and lower background currents than a carbon electrode in the cathodic region. Thus, a cathodic limit of *ca.* −1.8 V is indicated in Fig. 2. However, as can be seen from Fig. 5, the background for a mercury pool electrode was found to be similar to that of the carbon electrodes, probably because the stationary mercury surface is contaminated by metallic and/or organic impurities. Approximately the same cathodic limit was found by Rabenstein and Saetre[17] for their mercury pool detector. The residual wave in Fig. 5 at −0.3 V is probably due to trace amounts of oxygen within the system. The wave was not eliminated by applying a potential of −1.0 V for some time.

No doubt the use of a dropping mercury electrode would have extended the cathodic range of the detector (see Fig. 2). However, this electrode has obvious disadvantages; the current oscillations caused by the falling drops make the detection of narrow chromatographic peaks difficult, and the high charging currents give inferior detection limits (the detection limits given by Koen *et al.*[18] appear to be optimistic). According to our experience, a flow cell incorporating a dropping-mercury electrode is also a less reliable and practical detector for HPLC than cells with stationary electrodes. Finally, all cells with mercury electrodes have a very restricted anodic range.

The use of working electrodes made from platinum was not considered in this work as their potential range and background will be inferior to those of the carbon electrodes.

Choice of reduction potential. Normally, one would choose a potential near the cathodic limit of the detector in order to facilitate the determination of as many electroactive compounds as possible with a single setting of the detector. Because of the drastic rise in background current near the potential limit, a very negative potential cannot be used without some sacrifice in terms of the signal-to-noise ratio. Also, at a sufficiently negative potential the evolution of hydrogen may block the electrical contact between the electrodes. If a very selective detection is desired, the differential pulse technique may be used; in this instance the detector potential should be close to the half-wave potential of the compound in question.

The detection of nitrazepam is preferably carried out at −0.9 to −1.2 V using constant-potential amperometry, whereas −1.2 V or a more negative potential must be used for the detection of diazepam and chlordiazepoxide. When the chromatographic peak height of nitrazepam was drawn as a function of the detector potential, the "half-wave" potentials of the resulting voltammogram (which had no maximum) were found to correspond with those obtained using the same electrode and scanning voltammetry in a quiescent solution. This result confirms the absence of uncompensated resistance within the flow cell.

By studying the variation in peak height with potential a malfunctioning of the detector may be revealed. When this experiment was carried out with the EDT cell, a decrease in the peak heigh of nitrazepam was obtained for potentials more negative than −1.4 V.

Sensitivity and detection limits

The sensitivity of the HPLC technique depends on the chromatographic conditions, the detector design and the electrochemical behaviour of the compounds in question. For a given set of experimental conditions, the chromatographic peak heights depend on the retention time and the mechanism of the respective electrochemical reactions. Thus, the reason for the high sensitivity for nitrazepam which is indicated in Fig. 3 is that the reduction of nitrazepam involves seven electrons at −1.25 V (see Fig. 4), whereas only two electrons are involved in the reduction of diazepam and chlordiazepoxide at this potential. In addition, the short retention time of nitrazepam results in a large and narrow peak, with a width which is nearly half of that of the other two compounds.

As already mentioned, the magnitude of the background current depends on the potential chosen; at −1.3 V a marked drift and sudden shifts in the baseline were noticed, probably because of the evolution of hydrogen. At very negative potentials the detector also picked up some noise from the pumping system, as indicated in Fig. 6. Attempts to shield the detector from external electrical noise by placing it in a Faraday cage had little effect on the background.

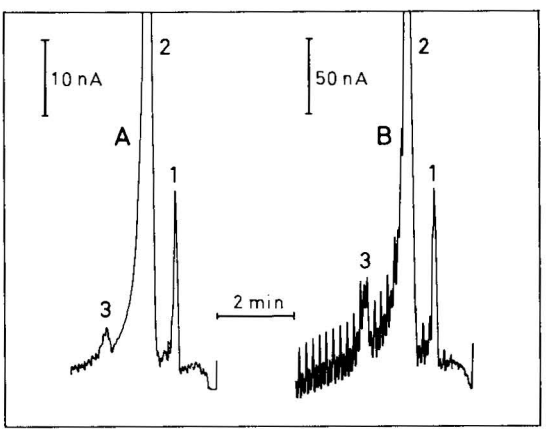

Fig. 6. Response of the detector to small amounts of nitrazepam. (A) 3 ng (10 µl) of nitrazepam detected at −0.93 V *vs.* Ag; (B) 30 ng (10 µl) at −1.30 V. Peaks: 1 = solvent front; 2 = oxygen; 3 = nitrazepam. Flow-rate 1 ml · min^{-1}.

As expected, the peak heights increased with flow-rate, and so did the background current; in the latter instance a linear dependence was observed. The results for nitrazepam are given in Table I.

In Table II the effect of various detector parameters on the detection limit (signal-to-noise ratio 2:1) is illustrated, using nitrazepam as the model compound. As expected, inferior detection limits are obtained for potentials near the cathodic limit. The use of the differential pulse technique is seen to give little improvement compared with the d.c. technique, except for the mercury pool electrode. The glassy carbon and carbon paste electrodes have similar detection limits. The mercury pool electrode appears to be inferior to the carbon electrodes at the more negative poten-

TABLE I

EFFECT OF FLOW-RATE ON PEAK HEIGHT, BACKGROUND AND RETENTION TIME FOR 30 μg OF NITRAZEPAM

Potential −1.2 V.

Flow-rate (ml · min^{-1})	*Peak height (μA)*	*Background (μA)*	*Retention time (min)*
0.7	8.8	11.5	11.2
1.5	9.7	17.0	5.2
2.0	12.3	20.5	3.9
2.5	16.8	24.5	3.1
3.0	18.8	28.3	2.6
3.5	20.5	32.7	2.2

TABLE II

DETECTION LIMITS FOR NITRAZEPAM, OBTAINED UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Working electrode	*Potential (V vs. Ag)*	*Technique*	*Detection limit (ng)*
Glassy carbon	1.30	D.c.	30
	0.93	D.c.	3
	0.78	Differential pulse	2
Carbon paste	1.30	D.c.	80
	0.93	D.c.	3
	0.78	Differential pulse	2
Mercury pool	0.83	D.c.	30
	0.68	Differential pulse	3

tials. When the EDT cell was used, detection limits identical with those given in Table II for the glassy carbon electrode were obtained. However, slightly different potentials were used for the EDT cell in order to compensate for the difference in the reference electrode potentials.

In Table III the detection limits for the three benzodiazepines under optimal electrochemical conditions are compared with those obtained using a UV detector. For nitrazepam the same detection limit was obtained for the amperometric and UV detectors, whereas higher detection limits were obtained for diazepam and

TABLE III

DETECTION LIMITS FOR NITRAZEPAM, DIAZEPAM AND CHLORDIAZEPOXIDE, USING AMPEROMETRIC (GLASSY CARBON) AND UV (254 nm) DETECTION

Injection volume 10 μl.

Compound	*Amperometric detection*		*UV detection limit (ng)*
	Potential (V)	*Detection limit (ng)*	
Nitrazepam	−0.93	3	3
Diazepam	−1.30	300	6
Chlordiazepoxide	−1.30	300	4

chlordiazepoxide using electrochemical detection, because of the highly negative potential needed for the detection of these compounds. However, owing to the high sensitivity for nitrazepam, this compound has a lower detection limit, also at −1.3 V (30 ng, see Table II), than diazepam and chlordiazepoxide.

A typical chromatogram of 3 ng of nitrazepam is shown in Fig. 6. At the high current sensitivity used, the small traces of oxygen in the solution give rise to a relatively large peak.

Calibration graphs and reproducibility. The electrochemical detector was found to have a linear response over a wide concentration range. The results for nitrazepam are given in Table IV. A glassy carbon electrode was used here, but similar results were obtained for the carbon paste and mercury pool electrodes, and also for the EDT cell. As shown in Table IV, the relative standard deviation for repeated injections was of the order of 1–3%.

TABLE IV

VARIATION IN PEAK HEIGHT WITH CONCENTRATION OF NITRAZEPAM

Potential −0.93 V *vs.* Ag; flow-rate 1 ml · min^{-1}.

Concentration (ppm)	*n**	*Peak height***		
		$\bar{x}$ (μA)	s (μA)	s_R (%)
1400	5	8.0	0.31	3.9
700	5	4.28	0.09	2.1
350	5	2.12	0.02	0.9
280	5	1.66	0.03	1.8
140	4	0.803	0.010	1.2
70	5	0.412	0.008	1.9
35	4	0.201	0.002	1.0
28	5	0.163	0.002	1.5
14	4	0.082	0.001	1.2
7	5	0.040	0.001	2.5

* n = number of 10-μl injections.

** $\bar{x}$ = mean; s = standard deviation; s_R = relative standard deviation.

CONCLUSIONS REGARDING AMPEROMETRIC DETECTION

Electrochemical flow cells used as detectors in HPLC should have a design that minimizes any uncompensated resistance within the cells. Not all commercial cells are equally satisfactory in this respect. A simple yet satisfactory cell design is shown in Fig. 1; the cell can be used with different types of working electrodes. Electrodes made from glassy carbon were found to have a relatively large cathodic range, but needed a cumbersome polishing procedure, while carbon paste electrodes exhibited a particularly low background in the anodic region, and a fresh electrode surface could be obtained by simply removing the top layer of the carbon paste.

Constant-potential amperometry was found to be preferable to normal potential pulse measurements; the latter technique gave high background currents when solid electrodes were used. The differential pulse technique was better in this respect, but this technique only detects compounds with half-wave potentials close to the initial potential chosen.

The detection limits were found to depend strongly on potential, because the reaction rate, number of electrons and background current were all a function of this parameter. Compounds which are reduced at a potential more positive than *ca.* −1.0 V have much better detection limits than those reduced at a potential close to the cathodic limit of the detector. Thus, the detection limit of nitrazepam was found to be 3 ng, whereas chlordiazepoxide and diazepam had inferior detection limits. Because the detector was used in the reduction mode, oxygen had to be removed completely from the mobile phase as well as from the sample solution prior to the analysis.

Amperometric detectors are cheaper than most other high-sensitivity detectors. When used in combination with UV detection, for instance, the electrochemical detector may provide additional selective information on the electroactive components of complicated mixtures.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CYTOKININS

ROGER HORGAN and MARGARET R. KRAMERS

Department of Botany and Microbiology, University College of Wales, Aberystwyth, Dyfed (Great Britain)

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SUMMARY

Details are given of reversed-phase, normal-phase, and adsorption high-performance liquid chromatographic systems suitable for the separation of a wide range of naturally occurring cytokinins related to zeatin. Particular attention has been given to the development of systems to separate cytokinins which are difficult to separate by more conventional means.

INTRODUCTION

High-performance liquid chromatography (HPLC) is being used increasingly for the isolation and estimation of plant growth substances. Abscisic acid[1] and 3-indolylacetic acid[2] have been detected and measured in plant extracts by this method. A recent publication[3] has reported the use of both analytical and preparative HPLC to achieve excellent separations of a wide range of gibberellins both as the free acids and as benzyl esters.

There have been several reports on the use of HPLC for the isolation and identification of cytokinins from plant material[4–6]. However, both the compounds separated and the methods used have been somewhat limited. In general, separations have been developed using only the limited range of cytokinins readily available from commercial sources and have been exclusively confined to reversed-phase HPLC systems. In addition the identifications of cytokinins based on their elution volumes from HPLC have not generally been confirmed by more rigorous means.

As part of our research into the biosynthesis and metabolism of cytokinins it became necessary to extend the available HPLC techniques to separate efficiently a wide range of naturally occurring cytokinins related to zeatin, and their metabolites. We deemed it of particular importance to develop HPLC techniques to separate compounds exhibiting close structural similarities, *e.g.*, dihydrozeatin, the *cis* and *trans* isomers of zeatin and their corresponding ribosides and glucosides. These are compounds which are particularly difficult to separate by more conventional means.

Most of the published work on HPLC of cytokinins has involved the use of ODS (C_{18}) reversed-phase systems. This is presumably because of the ease of operation of these systems under gradient-elution conditions and because reversed-phase

systems would be expected to have the greatest utility for the analyses of these relatively polar compounds. Published work[7] describing the separations of cytokinins on pellicular polyamide and various ion-exchange materials will not be discussed here as the efficiency and resolution achieved in these systems are too low to be considered in the context of present day HPLC.

The excellent resolution of certain cytokinins on thin-layer chromatography (TLC) suggests that with the choice of suitable solvent systems adsorption HPLC should be an equally powerful tool for cytokinin analysis. Possibly because of the difficulty of finding suitable solvent systems for the adsorption HPLC of polar compounds and the relative ease with which solvent systems can be chosen for reversed-phase HPLC this area has received little attention. In addition, when attempting to purify compounds in trace quantities from natural sources it is frequently advantageous to use techniques based on as widely different mechanisms of separation as possible. Thus we have explored the use of both adsorption and normal-phase HPLC as complementary techniques to reversed-phase HPLC for the separation of cytokinins.

This paper reports on the optimisation of reversed-phase HPLC for the separation of a wide range of cytokinins and also briefly discusses suitable solvent systems and stationary phases for adsorption and normal-phase HPLC of these compounds.

MATERIALS AND METHODS

Chemicals

All chromatographic solvents used were of reagent grade and were distilled in an all-glass apparatus before use.

Triethylammonium bicarbonate (TEAB) was prepared by saturating a 2.5 *M* solution of triethylamine with carbon dioxide.

All the cytokinins used in this study were synthesized by one of us (R.H.). Product identity was established by ultraviolet, mass spectrometric and nuclear magnetic resonance analyses.

Columns and packing materials

Columns (150 × 4.5 mm I.D.) of Hypersil, Hypersil ODS and Hypersil SAS (Shandon Southern, Runcorn, Great Britain) were prepared by slurry packing at 6000 p.s.i. using a pneumatic amplifier pump (Jones Chromatography, Llanbradach, Great Britain). Methanol was used as the slurry medium for the silica and isopropanol for the reversed-phase materials. Columns of Spherisorb, 5 μm (Phase Separations, Queensferry, Great Britain) were similarly prepared. Prepacked columns of Partisil 10 and Partisil 10 PAC (250 × 4.5 mm I.D.) were purchased from Whatman (Maidstone, Great Britain).

Chromatographic equipment

Chromatography under isocratic conditions was carried out using an Altex 110 pump (Altex Scientific, Berkeley, Calif., U.S.A.) and a Cecil CE 212 variable wavelength monitor (Cecil Instruments, Cambridge, Great Britain) operating at 265 nm and fitted with a 8-μl flow-through cell. Gradient-elution chromatography was carried out on a Pye LC3X system (Pye Unicam, Cambridge, Great Britain).

Sample introduction on both chromatographs was via an Altex 905-42 syringe loading sample injector. All injections were of 30 μl and were made into a 100-μl loop.

RESULTS

Reversed-phase HPLC

Two reversed-phase materials were used in the course of these investigations, Hypersil ODS, a 5-μm totally porous microspherical silica with a C_{18} bonded stationary phase, and Hypersil SAS, a similar material with a C_2 bonded phase.

Solvent systems investigated were all binary mixtures of methanol, ethanol or acetonitrile, and water. The effects of varying the pH and the ionic strength of the aqueous component were studied. Previously published work on reversed-phase HPLC of cytokinins has invariably involved the use of solvent systems at pH values of 2.5 to 3.5. The exact reasons for the use of these conditions are not clear. The pK_a values of most cytokinins are 4 in aqueous solution and these will be lowered somewhat in organic solvents, hence the pH of these solvent systems will be very close to the pK_a values of the cytokinins. Under these conditions maximum separation efficiencies will not be obtained. In addition the presence of a positive charge on all the compounds may tend to mask small polarity effects due to slight structural differences and lead to the inefficient separation of closely related compounds. In most cases the pH of the aqueous component of the mobile phase has been adjusted using acetic acid. It is possible that under these conditions ion-pairing effects may contribute to the separations observed. Some workers[4] have used acidic buffers as the aqueous components of their mobile phases to mask residual adsorption sites on the stationary phase. The use of acidic aqueous phases should however be avoided if possible since it has been observed in our laboratory that hydrolysis of cytokinin-O-glucosides can occur when these phases are removed by evaporation.

In view of the points made above and because the stationary phases used were virtually free of residual adsorption sites all the separations reported here were achieved at pH 7, at which pH the cytokinins are un-ionised. The use of pH values below 6 and above 8 gave inferior separations.

Since one of the objects of this study was to develop methods for the isolation of microgram quantities of cytokinins from natural sources it was considered essential to stabilise the pH of the aqueous component with a substance which could be easily and completely removed under the mildest possible conditions of temperature and pH. It was found that the addition of a few drops of 2.5 *M* TEAB to glass distilled water provided adequate stabilisation of the pH at 7. This material could be completely removed by co-evaporation with methanol at room temperature. Care must be exercised in the use of helium degassing systems with this buffer since a too vigorous purging with helium will cause the pH to rise due to the flushing out of carbon dioxide from the solvent. Provided mild and sporadic helium purging is used, the pH is stable for more than 12 h. Fig. 1 illustrates the separation of a complex mixture of zeatin-derived cytokinins on a 150 × 4.5 mm I.D. column of Hypersil ODS eluted with a gradient of increasing concentration of acetonitrile in water at pH 7 (adjusted with TEAB). The use of ethanol or methanol in place of the acetonitrile resulted in poorer separations. A similar separation was obtained for the corresponding dihydrozeatin compounds, but with each dihydrozeatin derivative

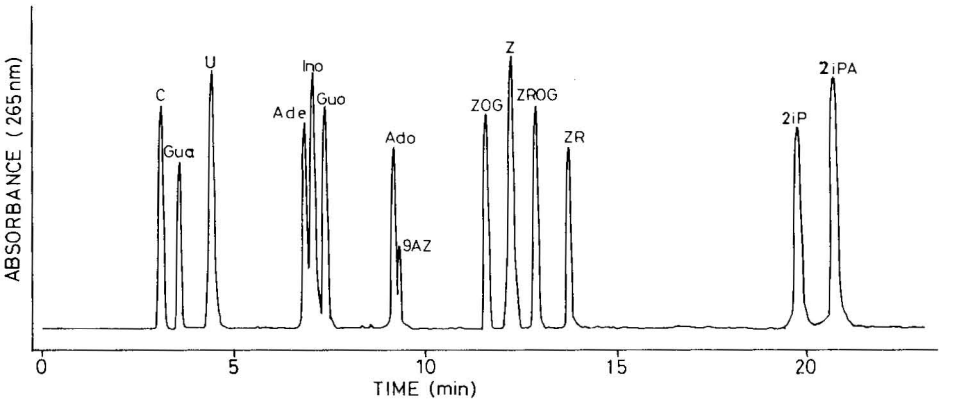
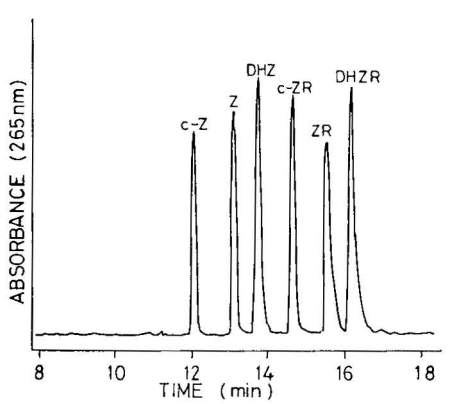


Fig. 1. Separation of a series of cytokinins related to zeatin and a number of common purines and pyrimidines by reversed-phase HPLC. Column, Hypersil ODS (150 × 4.5 mm I.D.); flow-rate, 2 ml/min; mobile phase, 3 segment linear gradient, water (pH 7 with TEAB) to 11% acetonitrile over 7 min, 11 to 20% acetonitrile over 9 min, and 20 to 40% acetonitrile over 15 min. Abbreviations: Ade = adenine; Ado = adenosine; C = cytidine; Gua = guanine; Guo = guanosine; Ino = inosine; U = uridine; Z = zeatin; ZR = ribosyl zeatin; 9AZ = 9-alanyl zeatin; ZOG = zeatin-O-glucoside; ZROG = ribosyl zeatin-O-glucoside (all zeatin compounds are *trans* unless otherwise stated and all glucosides are the β-D-glucopyranosides); 2iP = N^6-isopentenyl adenine; 2iPA = N^6-isopentenyl adenosine.

eluting slightly later than its zeatin counterpart. Figs. 2 and 3 illustrate the excellent resolving power of this system for mixtures of zeatin- and dihydrozeatin-derived compounds which are particularly difficult to separate by other methods. It is of



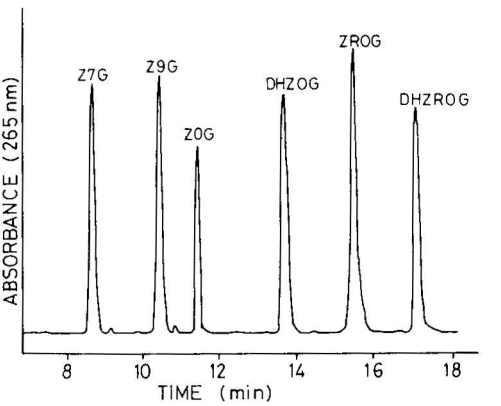


Fig. 2. Separation of a series of closely related zeatin derivatives by reversed-pahse HPLC. Separation conditions as in Fig. 1 except mobile phase, linear gradient of water (pH 7 with TEAB) to 30% acetonitrile over 30 min. Abbreviations: c-Z = *cis*-zeatin; c-ZR = ribosyl *cis*-zeatin; DHZ = dihydrozeatin; DHZR = ribosyl dihydrozeatin. Other abbreviations as in Fig. 1.

Fig. 3. Separation of a series of cytokinin glucosides by reversed-phase HPLC. Separation conditions as in Fig. 1 except mobile phase, linear gradient of 5% acetonitrile in water (pH 7 with TEAB) to 20% acetonitrile over 30 min. Abbreviations; Z9G = zeatin-9-glucoside; Z7G = zeatin-7-glucoside; DHZOG = dihydrozeatin-O-glucoside; DHZROG = ribosyl dihydrozeatin-O-glucoside. Other abbreviations as in Fig. 1.

interest to note that with the exception of 9-alanyl zeatin all the cytokinins elute later than the common purines.

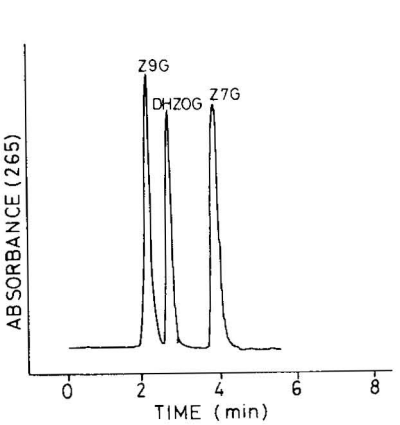
The separations achieved on Hypersil SAS were similar to those shown in Figs. 1, 2 and 3, although compounds eluted at a lower acetonitrile concentration and the resolution was somewhat inferior due to the greater amount of peak tailing exhibited by this phase. However, the magnitude of the separation between the cytokinins as a group and the common purines was greater on this material than on Hypersil ODS, and in addition 9-alanyl zeatin could be completely separated from adenosine.

We have used Hypersil SAS extensively in our laboratory for studies into the biosynthesis of cytokinins. Using a 150 × 4.5 mm I.D. column of this material and eluting isocratically with 5% acetonitrile in water (pH 7 with TEAB) it is possible to completely remove the common adenine metabolites, which usually constitute 99% of the labelled compounds extracted from tissue fed with radioactively-labelled compounds extracted from tissue fed with radioactively-labelled adenine, from the very minute quantities of cytokinins produced. In practice the column is eluted with the above solvent until the eluate reaches background radioactivity and the cytokinins are then eluted with pure methanol. We have found that a column of this size can efficiently effect this sort of group separation with sample sizes of up to 100 mg.

Adsorption HPLC

No detailed studies of cytokinins on adsorption HPLC have been published. The excellent resolution of quite complex mixtures of cytokinins by TLC indicates considerable potential for this technique although the solvent systems used are in most cases inappropriate for HPLC. However, as stated in the Introduction, when attempting to isolate small quantities of natural products in the pure state there is considerable advantage in the sequential utilisation of several separation techniques operating by as widely different mechanisms as possible. We have therefore conducted a preliminary investigation into the potential of adsorption HPLC for cytokinin analysis with a view to using it as a complementary technique to reversed-phase HPLC for the isolation and identification of cytokinins from plant sources.

The HPLC adsorbants investigated were Partisil 10, Spherisorb 5 μm and Hypersil. The utility of these materials was related to their specific surface areas. The most active of these, Partisil 10, with specific surface area of 400 m^2/g, was found to be useful only with solvent systems having a high proportion of a polar component (water, ammonium hydroxide or acetic acid). It was found to be particularly useful for the separation of closely related cytokinin glucosides using tetrahydrofuran–water mixtures, an example of which is shown in Fig. 4. Spherisorb 5 μm and Hypersil with specific surface areas of 300 and 200 m^2/g, respectively, exhibited similar properties but the lower activity Hypersil was found to be the most satisfactory material for the widest range of compounds. We attempted to develop a solvent system for HPLC which showed similar properties to the *n*-butanol–ammonia (sp.gr. 0.880)–water (6:1:2, upper phase) system which is useful in the TLC analysis of cytokinins. This work led to solvent systems based either on chloroform or acetonitrile with polar components of methanol and/or water and containing ammonium hydroxide. With solvent systems of this type all the compounds used in this investigation could be chromatographed satisfactorily. For example a mixture of zeatin-7- and zeatin-9-



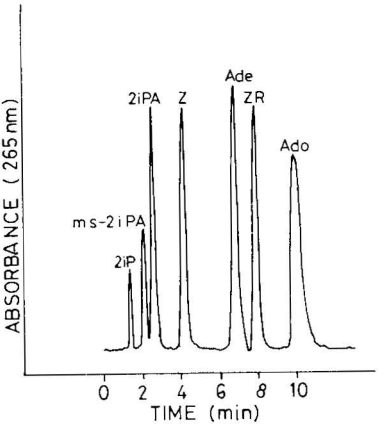


Fig. 4. Separation of three cytokinin glucosides by adsorption HPLC. Column, Partisil 10 (250 × 4.5 mm I.D.); flow-rate, 2 ml/min; mobile phase, tetrahydrofuran–water (9:1). Abbreviations as in Figs. 1 and 2.

Fig. 5. Separation of adenine, adenosine and a series of cytokinins by adsorption HPLC. Column, Hypersil (150 × 4.5 mm I.D.); flow-rate, 2 ml/min; mobile phase, ammoniacal chloroform (1 l of chloroform was satuarted by shaking with 250 ml of ammonia (sp.gr. 0.880) and allowed to stand overnight)–methanol–water (100:5:0.3). The column was eluted with 100 ml of mobile phase before starting the analysis. Abbreviations: ms-2iPA = 2-methylthio-isopentenyl adenosine. Other abbreviations as in Fig. 1.

glucosides could be well separated on a 150 × 4.5 mm I.D. column of Spherisorb 5 μm using a solvent system of acetonitrile–methanol–ammonium hydroxide (sp.gr. 0.880) (100:10:1). Fig. 5 illustrates an example of a cytokinin separation on Hypersil with a chloroform-based solvent system. This particular adsorbent/solvent combination has been used very successfully in our laboratory for the final purification of samples of zeatin and zeatin riboside after initial purification by reversed-phase HPLC. It should be pointed out however that when using solvent systems of this type that are effectively 100% water saturated, at least 20 column volumes should be allowed to pass through the column to ensure that stable capacity factors (k') are obtained.

The results presented above illustrate just a few of the solvent systems which we have found to be useful for the isolation and purification of cytokinins from natural sources. They do demonstrate however the potential of adsorption HPLC for cytokinin analysis and we hope will offer guidelines to other workers to develop systems to help solve their particular purification problems.

Normal-phase partition HPLC

No details of normal-phase partition HPLC of cytokinins have been published. We have briefly investigated the use of Partisil 10 PAC, a polar phase material consisting of cyanopropyl groups bonded to a microparticulate silica, for this purpose. This material appears to have good potential for the separation of the polar cytokinins which are more difficult to chromatograph on adsorption systems. Thus it could be used to complement reversed-phase chromatography for these compounds. Fig. 6 illustrates the separation of zeatin-7- and zeatin-9-glucosides on this material using a solvent system of acetonitrile–water (9:1). It should be noted that the separation of these compounds in this system is better than that achieved in any of the other HPLC systems studied in this investigation.

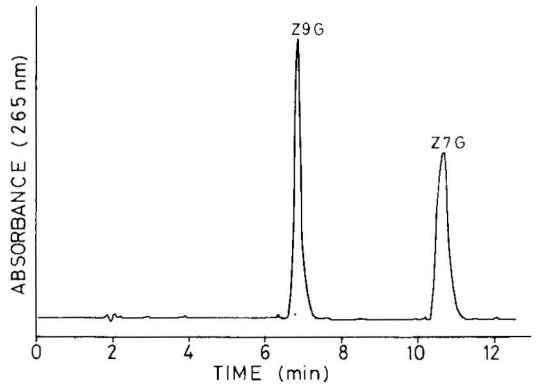


Fig. 6. Separation of isomeric zeatin N-glucosides by normal-phase partition HPLC. Column, Partisil 10 PAC (250 × 4.5 mm I.D.); flow-rate, 2 ml/min; mobile phase, acetonitrile–water (9:1). Abbreviations as in Fig. 1.

DISCUSSION

The results of this investigation show that reversed-phase HPLC is probably the most powerful single chromatographic technique for the purification of cytokinins from plant material. The suitability of reversed phases for operation under gradient-elution conditions with simple solvent systems, their high sample capacities under these conditions and their ability to separate a very wide range of compounds makes reversed-phase HPLC the obvious first step method for cytokinin purification. Reversed-phase HPLC has been viewed as a substitute for the more time consuming reversed-phase chromatography on Sephadex LH-20. In our view there are many situations where the two techniques should be considered complementary. With care, LH-20 columns can be constructed on almost any scale and can provide an initial purification step when large sample sizes make immediate preparative HPLC impracticable.

It should be borne in mind that although the results presented here demonstrate the excellent resolving power of Hypersil ODS for complex mixtures of naturally occurring cytokinins they do not necessarily indicate the usefulness of this material for the purification of cytokinins in plant extracts.

Preliminary work in our laboratory does however indicate that the reversed-phase system described in this paper performs well for the isolation of cytokinins from plant material. Using a 150 × 10 mm I.D. column of Hypersil ODS and the solvent system used in Fig. 1 it has been possible to isolate a pure sample of zeatin riboside from the ethyl acetate soluble, basic material obtained from 260 g fresh weight of *Vinca rosea* crown gall tissue. This was the only chromatographic step used in the procedure.

The elution order of the cytokinins on Hypersil ODS suggests that this material is not operating exclusively by a reversed-phase mechanism. In spite of the fact that the number of residual adsorption sites is minimal, evidenced by a k' value for nitrobenzene in heptane of <0.2 (ref. 8), the more polar ribosides elute later than the corresponding bases. This sort of anomaly has been noted for other reversed-phase materials[9].

As a result of our preliminary investigations into the adsorption and normal-phase HPLC of cytokinins, we believe that these techniques are of considerable value as second-stage methods to reversed-phase HPLC. They have received little attention to date, probably due to the difficulty of finding suitable solvent systems and the belief, mistaken we believe, that all the problems of cytokinin purification will be solved by the use of reversed-phase techniques. Obviously workers in this field will have to develop solvent systems suitable for their particular applications. However, we believe that the adoption of these techniques in combination with reversed-phase HPLC will eventually lead to a more efficient utilisation of HPLC as the major tool for the isolation of cytokinins from plant material.

ACKNOWLEDGEMENTS

We acknowledge the financial support of the Science Research Council, the Agricultural Science Research Council and the Royal Society for the purchase of the chromatographic equipment used during this investigation. The Science Research Council and Shell Research Ltd. are also acknowledged for the award of a CASE studentship to M.R.K. We thank Mr. I. Cantrell for his assistance with the adsorption HPLC and Dr. D. C. Walton for his helpful and constructive criticisms of this manuscript.

NOTE ADDED IN PROOF

Two recent publications by Andersen and Kemp[10] and Holland *et al.*[11] have described reversed-phase chromatography of cytokinins on reversed-phase materials other than those described here. The very different separations reported in these papers highlight the different properties of superficially similar reversed-phase materials. These are possibly due to minor differences in the extent of bonded phase coverage and the exact nature of the support material.

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DISSOCIATION OF METAL DITHIZONATES DURING GEL CHROMATOGRAPHY

TOSHIO DEGUCHI, RYOICHI TAKESHITA, ISAO SANEMASA and HIDEO NAGAI

Department of Chemistry, Faculty of Science, Kumamoto University, Kurokami, Kumamoto 860 (Japan)

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SUMMARY

Metal dithizonates in carbon tetrachloride were dissociated through gel chromatographic columns packed with Sephadex LH-20 or Merckogel OR-PVA 2000. The liberated metal ions were adsorbed on the surface of the gels, and the dithizone in its neutral form was eluted from the column. Detailed investigation of this phenomenon was carried out by batch methods. The proposed mechanism is the generation of hydrogen chloride caused by the interfacial reaction of the gels with carbon tetrachloride.

INTRODUCTION

In recent years, gel chromatography has been applied to the investigation of metal complexes[1-10]. We have previously[5,6] suggested that the gel chromatographic behaviour of EDTA complexes in aqueous media can be explained mainly by sieving effect. We have also investigated the behaviour of metal dithizonates on gels in non-aqueous media. When dithizonates of some heavy meta s were chromatographed on a Sephadex LH-20 column using carbon tetrachloride as eluent, copper dithizonate was immediately eluted from the column as a dithizonate, whereas the ions Cd^{2+}, Hg^{2+}, Pb^{2+} and Zn^{2+} were adsorbed at the top of the column and the liberated dithizone was easily eluted from the column[7]. This stripping-like phenomenon is obviously due to the dissociation of metal dithizonate and, in this respect, is very similar to the back-extraction process of dithizonate from organic phase to aqueous phase. This suggests that effects more complicated than the sieving effect may participate in the gel chromatographic behaviour of dithizonate in media such as carbon tetrachloride, and we have undertaken further studies to elucidate them.

In this paper, the behaviour of some heavy-metal dithizonates on gel phases is reported. Model experiments were designed in connection with gel matrixes, solvents and solutes, and were carried out by batch operations. Moreover, this phenomenon was applied to simplified purification of dithizone reagent, the concentration of trace metal ions and the fractionation of metal ions.

EXPERIMENTAL

Reagents and sample solutions

Dithizone, carbon tetrachloride and chloroform were purified by the JIS method[11], from which dithizone solutions in carbon tetrachloride (0.01 %, w/v) and chloroform (0.1 %, w/v) were prepared. Metal dithizonate solutions were prepared according to the JIS method: aqueous metal solutions were adjusted to the correct pH (see Table I) with buffer solutions and then extracted successively with the dithizone solutions until they showed no colour changes. These extracted solutions of the metal dithizonates were used as the sample solutions.

TABLE I

EXTRACTION pH OF SOME METAL DITHIZONATES

Metal	*Extraction pH*	*Buffer solution*
Silver(I)	7.0	barbital sodium + hydrochloric acid (primary dithizonate)
Cadmium(II)	12.0	sodium phosphate + sodium hydroxide (primary dithizonate)
Copper(II)	3.7	acetic acid + sodium acetate (primary dithizonate)
	12.0	sodium phosphate + sodium hydroxide (secondary dithizonate)
Mercury(II)	3.7	acetic acid + sodium acetate (primary dithizonate)
	12.0	sodium phosphate + sodium hydroxide (secondary dithizonate)
Lead(II)	12.0	sodium phosphate + sodium hydroxide (primary dithizonate)
Zinc(II)	8.9	barbital sodium + hydrochloric acid (primary dithizonate)

The other reagents used were, unless otherwise stated, commercially available analytical or chemical pure reagent grade.

Procedure

Column methods. Sephadex LH-20 (Pharmacia, Uppsala, Sweden), previously swollen with carbon tetrachloride or chloroform for 2 days, was packed in a glass tube (100 × 5.5 mm) to a height of *ca.* 5 cm. At the top of the column, a 1-ml portion of the sample was injected and then eluted with carbon tetrachloride or chloroform.

Batch method 1. This method was used to investigate the dissociation mechanism of the dithizonates. Sephadex LH-20 was swollen in a solvent for 2 days, extracted and air-dried. The dried gel (0.002–0.5 g) was weighed and shaken with a 10-ml portion of the sample solution for a few minutes. The mixture was filtered, and the filtrate was subjected to absorption spectrophotometry.

Batch method 2. This method was used to recover the metal ion adsorbed on the gel. Sephadex LH-20 was swollen in a solvent for 2 days, extracted and air-dried. The dried gel (0.2–0.4 g) was weighed and shaken with a 10-ml portion of the sample solution for a few minutes. The suspension was filtered, and the filtered gel was air-dried again. The dried gel was washed with redistilled water on the glass filter. The filtrate was subjected to atomic absorption spectrophotometry.

Apparatus

A Hitachi Model EPS 20 UV spectrophotometer was used to identify metal complexes. To determine the amount of metal ion adsorbed on the gel, a Hitachi Model 508 atomic absorption spectrophotometer was used. To determine the concentration of hydrogen ions and chloride ions liberated from the gel, a pH meter (Hitachi-Horiba F-5) and a chloride ion meter (Toa Ion Meter 1M1B) were used. The absorption spectrum of the gas released from the swollen gel was measured with a Nippon Bunko DS-403G IR absorption spectrophotometer.

RESULTS AND DISCUSSION

Column chromatographic behaviour of some metal dithizonates

At the top of the column, *ca.* 1 ml of the sample solution (metal dithizonate of $2\text{–}4 \cdot 10^{-5}$ *M* in carbon tetrachloride) was injected and then eluted with carbon tetrachloride at 20 ± 1°. When a sample solution of a zinc(II), cadmium(II), lead(II) or mercury(II) complex was chromatographed, the colour of the solution immediately turned greenish at the top of the column bed. This green band was easily eluted by successive elution with carbon tetrachloride. The visible absorption spectrum of the eluate was identical with that of dithizone in carbon tetrachloride solution. On the other hand, the dithizone complex of copper(II) was not affected by this treatment.

According to the selective adsorption on the gel column, metal ions can be easily separated into two groups: one is retained tightly in the column as a result of the dissociation of the dithizonates (zinc(II), cadmium(II), lead(II) and mercury(II)) and the other is eluted as the dithizonate (copper(II)). The metal ions retained in the column were eluted almost completely with prewashing (*ca.* 20 ml of acetone) and *ca.* 5 ml of aqueous EDTA solution. Experimental results of the percentage recoveries of the metal ions adsorbed on the gel are given in Table II.

TABLE II

RECOVERIES OF ADSORBED METAL IONS IN THE COLUMN METHOD

Metal ion	*Taken (μg)* *	*Found (μg)* **	*Recovery (%)*
Zinc(II)	9.50	8.50	89.5
	7.50	7.38	98.4
Cadmium(II)	5.03	4.74	94.2
	8.31	8.46	101.8
Lead(II)	28.8	22.8	78.2
	8.49	8.24	97.1

* Atomic absorption spectrophotometry after back-extracting sample solution with HCl (1 + 10).

** Atomic absorption spectrophotometry.

By this procedure, zinc(II), cadmium(II) and lead(II) present as impurities in commercial dithizone of analytical reagent grade were eliminated and were analysed by the atomic absorption method (zinc(II), 0.016%; cadmium(II), not detectable; lead(II), 0.004%). The method is applicable to the simple and rapid purification of the dithizone reagent by the removal of these contaminants.

Dissociation of metal dithizonates by batch method 1

Some characteristically coloured metal dithizonates were turned greenish during the gel chromatographic procedure, suggesting that dissociation occurs. Batch procedure 1 was used to study this phenomenon in more detail. A typical absorption spectra change for the dithizonate of lead (II) is shown in Fig. 1. The dithizonates of silver(I), cadmium(II) and zinc(II) showed the same pattern of dissociation. The dithizonates of copper(II) and mercury(II) showed different patterns of dissociation (Figs. 2 and 3).

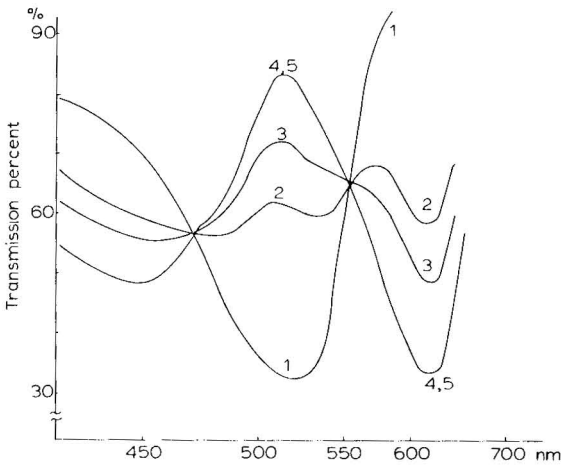

Fig. 1. Spectral change of lead(II) dithizonate on addition of swollen gel. Amounts (g) of swollen gel added in the dithizonate-carbon tetrachloride solution: 1, none; 2, 0.005; 3, 0.008; 4, 0.052; 5, 0.063.

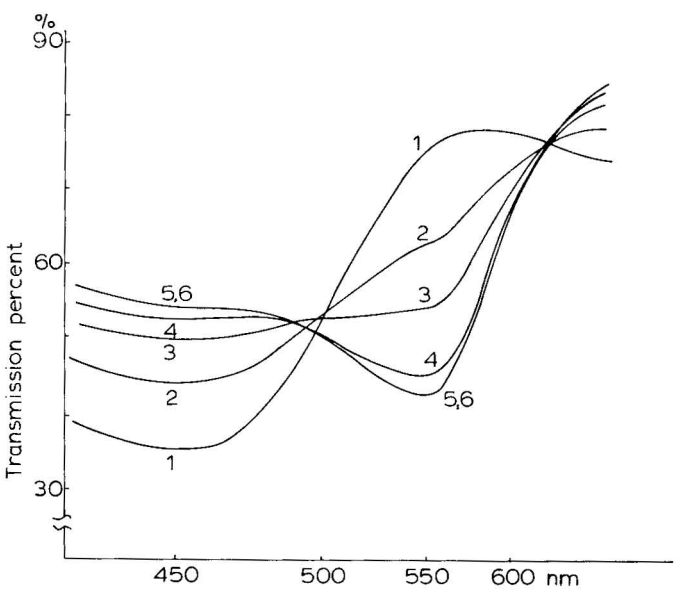

Fig. 2. Spectral change of copper(II) dithizonate on addition of swollen gel. Amounts (g) of swollen gel added in the dithizonate-carbon tetrachloride solution: 1, none; 2, 0.021; 3, 0.085; 4, 0.232; 5, 0.314; 6, 0.512.

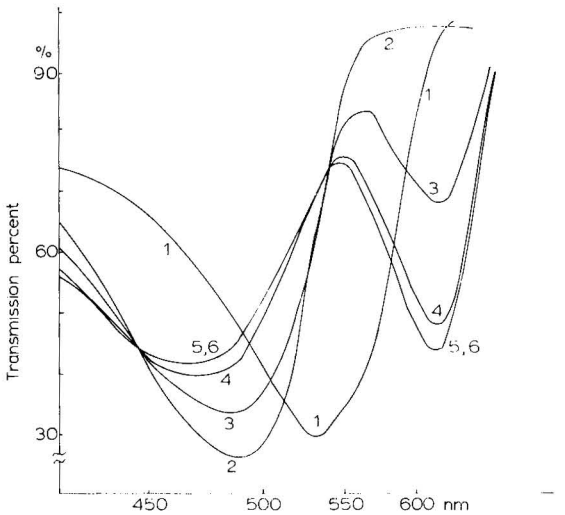

Fig. 3. Spectral change of mercury(II) dithizonate on addition of swollen gel. Amounts (g) of swollen gel added in the dithizonate–carbon tetrachloride solution: 1, none; 2, 0.045; 3, 0.081; 4, 0.091; 5, 0.118; 6, 0.130.

These dissociation patterns were classified in the following three categories.

(1) Primary dithizonate → metal ion + dithizone molecule

Silver(I), cadmium(II), lead(II) and zinc(II) belong to this group.

(2) Secondary dithizonate → primary dithizonate → metal ion + dithizone molecule

Mercury(II) belongs to this group.

(3) Secondary dithizonate → primary dithizonate

This dissociation does not proceed as far as the free ion and dithizone molecule. Copper(II) belongs to this group.

These three patterns show a good correlation with the back-extraction of metal dithizonates from organic solvents to aqueous media: the changes of the absorption spectra of these dithizonates suggest that addition of the gel swollen in carbon tetrachloride to metal dithizonate solutions corresponds to the acidification of the aqueous phase in the back-extraction procedure. The third category classified on the basis of absorption spectrometry seems to be in good agreement with the conventional experimental fact that the quantitative back-extraction of copper(II) dithizonate is difficult and incomplete even in highly acidic conditions[12,13].

Adsorbed metal ion and the recovery by batch method 2

Free metal ions were not detected either in the green effluent from the Sephadex LH-20 column or in the filtrate in the batch procedure. These facts suggest that the free metal ions liberated from their dithizonates are retained in the gel. In order to recover completely the metal ions adsorbed on the gel, batch method 2 was used. In the column method, the recoveries listed in Table II fluctuated too much and the procedure was somewhat difficult, perhaps owing to the different swelling properties of the gel in the presence of acetone and carbon tetrachloride. These difficulties are

avoided in the batch procedure. As mentioned in the experimental section, the gel adsorbing the metal ions was first dried and then washed on a sintered glass filter plate with various solutions, such as 0.1 *M* HCl, 0.01 *M* EDTA and redistilled water. It turned out that the redistilled water was just as effective as the other two. The data listed in Table III show that satisfactory recoveries were obtained by washing with redistilled water, except for mercury(II) dithizonate.

TABLE III

RECOVERIES OF ADSORBED METAL IONS IN THE BATCH METHOD

Metal ion	*Taken (μg)* **	*Found (μg)* **	*Recovery (%)*	*Gel amount added (g)*
Zinc(II)	8.76	8.63	98.5	0.2
Cadmium(II)	13.83	14.05	101.6	0.2
Lead(II)	10.6	10.5	99.1	0.2
Mercury(II)***	17.45	13.44	77.0	0.2
Mercury(II)***	11.23	10.03	89.3	0.4

* Atomic absorption spectrophotometry after back-extracting sample solution with HCl (1 + 10).

** Atomic absorption spectrophotometry.

*** Back-extraction with HCl (1 + 1).

Behaviour of metal dithizonates in carbon tetrachloride with Merckogel OR-PVA 2000

Merckogel OR-PVA 2000 (Merck, Darmstadt, G.F.R.), a cross-linked poly-(vinyl acetate) gel, showed the same dissociation phenomena with the metal complexes. Zinc(II) dithizonate in carbon tetrachloride, for example, was dissociated with the gel swollen in carbon tetrachloride (batch method 1). Fig. 4 shows the absorption spectra of the dithizonate from the two gels. The results suggest that the dissociative effect of Merckogel is somewhat less than that of Sephadex LH-20.

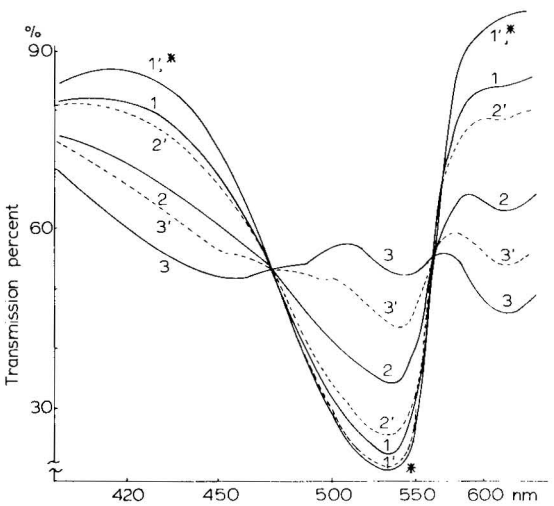

Fig. 4. Spectral change of zinc(II) dithizonate on addition of swollen gel. Dashed curves: Merckogel OR-PVA 2000; continuous curves: Sephadex LH-20. Amounts (g) of swollen gels added in the dithizonate–carbon tetrachloride solution: 1 and 1′, 0.02; 2 and 2′, 0.05; 3 and 3′, 0.10. The asterisk denotes the dithizonate–carbon tetrachloride solution.

The effect of solvent

The dissociation effects were studied in solvents other than carbon tetrachloride: chloroform, dichloromethane, acetone and cyclohexane. Sephadex LH-20 or Merckogel OR-PVA 2000 were swollen in these solvents for about 2 days, filtered and air-dried. In these solvents, the metal dithizonates used in this study showed no change with the air-dried gel (batch method 1), except that in chloroform a slight change occurred after *ca.* 5 days.

Investigation of the dissociation of metal dithizonates

Formation of hydrogen ions on the gel by carbon tetrachloride. As the dissociation of metal dithizonate in the carbon tetrachloride–gel system is very similar to the back-extraction procedure, it is reasonable to consider that hydrogen ions may play an important role in the dissociation. It could be assumed that hydrogen ions originate from the interaction between the solvent and the gel. To verify this assumption, qualitative tests of pH in the solvent–gel mixed system were undertaken. Each 10 ml of solvents was mixed with 0.3 g of gel (Sephadex LH-20), and the suspended solutions were left for about 2 days. Pieces of pH test-papers were directly immersed in the solution and in the solvent itself, and the colour change was compared with the reference. A distinct colour change, indicating the existence of hydrogen ions, occurred in the carbon tetrachloride solvent. No colour changes were observed for solvents such as dichloromethane, acetone, cyclohexane, toluene and dioxane. A slight colour change occurred in chloroform, indicating a very low hydrogen ion activity.

For the carbon tetrachloride solvent, more detailed tests using pH paper were carried out. The suspension was filtered through a sintered glass disk, and the filtrate and the swollen gel were checked with pH paper. The former did not contain any hydrogen ions, but the latter showed strong hydrogen ion activity. This fact clearly indicates that the hydrogen ions are located on the surface of the gel.

The hydrogen ions should be accompanied by some negative counter-ions. When the suspension of the gel in carbon tetrachloride was titrated with dilute $AgNO_3$ solution, a white precipitate (AgCl) was observed. The hydrogen ions on the surface of the gel were not detected in the filtrate, but they were eluted when the gel swollen in carbon tetrachloride was air-dried and washed with redistilled water. The quantitative determination of hydrogen ions and chloride ions was carried out by batch procedure. After the gel was swollen in carbon tetrachloride for 4–30 days, the swollen gel was air-dried and washed with 10 ml of redistilled water. The rinsed water was then used to determine both hydrogen ion and chloride ion. These ions were measured by pH meter and chloride ion selective electrode, respectively. The results are listed in Tables IV and V. These data suggest that the concentrations of the hydrogen ions and the chloride ions released from the gel are almost comparable and that their concentrations increase with the increasing amount of gel added to the solvent. The concentrations of these ions also increased, to some extent, with increased swelling time. After the swollen gel was air-dried, a small amount of the gel was sealed in the IR absorption cell (gas cell) and warmed; the characteristic rotation spectrum of hydrogen chloride was observed. The spectrum was positive proof of the existence of hydrogen chloride on the gel.

On the other hand, Sephadex LH-20 itself was somewhat decomposed by contact with carbon tetrachloride. The colour of the gel in carbon tetrachloride varied

TABLE IV

VARIATION OF HYDROGEN ION AND CHLORIDE ION CONCENTRATIONS IN THE PRESENCE OF DIFFERENT AMOUNTS OF GEL AFTER 5 DAYS SWELLING

Gel amount (g)	*$[H^+]$ value (M)*	*$[Cl^-]$ value (M)*
0.006	$4.90 \cdot 10^{-4}$	$2.41 \cdot 10^{-4}$
0.022	$1.32 \cdot 10^{-3}$	$9.26 \cdot 10^{-4}$
0.028	$1.62 \cdot 10^{-3}$	$1.23 \cdot 10^{-3}$
0.039	$2.04 \cdot 10^{-3}$	$1.69 \cdot 10^{-3}$
0.053	$3.02 \cdot 10^{-3}$	$2.76 \cdot 10^{-3}$

TABLE V

VARIATION OF HYDROGEN ION AND CHLORIDE ION CONCENTRATION AS A FUNCTION OF SWELLING TIME

Day	*$[H^+]/g$*	*$[Cl^-]/g$*	*Ratio*
4	$2.37 \cdot 10^{-2}$	$1.41 \cdot 10^{-2}$	1.68:1
5	$5.68 \cdot 10^{-2}$	$4.54 \cdot 10^{-2}$	1.25:1
30	$6.80 \cdot 10^{-2}$	$6.00 \cdot 10^{-2}$	1.13:1

from the normal white to light yellow after about a week and turned brownish after about a month. This coloration was very similar to the caramelization of the gel caused by warming the suspension of the gel in dilute HCl solution for *ca.* 30 min. Moreover, the gel swollen in carbon tetrachloride showed a positive sugar test with the tryptophan–sulphuric acid reaction or the phenol–sulphuric acid reaction. These results suggest that Sephadex LH-20 gel is gradually hydrolysed by hydrogen chloride formed during the swelling process in carbon tetrachloride.

Mechanism of dissociation of metal complexes in carbon tetrachloride with Sephadex LH-20 gel. The results of this study can be summarized as follows.

(1) Considerable amounts of hydrogen and chloride ions are formed when a gel such as Sephadex LH-20 is swollen in carbon tetrachloride.

(2) The hydrogen and chloride ions appear only at the interface of the gel phase and cannot be detected in the filtrate that is separated from the gel.

(3) Metal dithizonates, except for the copper(II) complex, are completely dissociated into metal ions and free dithizone in contact with the swollen gel, and the metal ions remain in the gel phase. The adsorbed metal ions are recovered almost completely by washing with redistilled water after drying the gel.

The results are very similar to those obtained in the back-extraction of metal complexes in organic phase into aqueous phase. The aqueous phase corresponds to the gel phase in the present carbon tetrachloride system. When viewed from the point of the effectiveness of back-extraction, the hydrogen ion activity arising from the swollen gel under the usual conditions may be insufficient to cause dissociation of copper(II) dithizonate. In practice, the dissociation of copper(II) dithizonate was observed when the gel was allowed to stand for about a month in contact with carbon tetrachloride before the copper(II) dithizonate was added to the mixture.

The formation of hydrogen chloride was not characteristic of gel matrices used, but was also observed in mixtures of high molecular ethers, such as ethylene glycol dimethyl ether, and carbon tetrachloride. This suggests that some of the functional

groups in the gel should play an important role in the generation of hydrogen chloride.

From the above results, the following mechanism for the dissociation of metal dithizonates can be proposed.

(1) The interfacial reaction at the gel between carbon tetrachloride and water sorbed in the gel matrix gives rise to the evolution of hydrogen chloride.

(2) The hydrogen chloride is adsorbed on to the surface of the gel, owing to its strong polarity.

(3) The dissociation of metal dithizonate into metal ion and free dithizone will be caused by the active hydrogen chloride on the surface of the gel in such a fashion that the metal ion bonded to dithizone is substituted by the hydrogen ion.

(4) The liberated dithizone molecules would not be further protonated unless the hydrogen ion concentration is very high, and then they are left in the carbon tetrachloride phase.

(5) The metal ions are adsorbed on to the surface of the gel, probably as chlorides.

If non-aqueous solvents such as carbon tetrachloride or chloroform, which contained chlorine, are used as eluents in chromatography with amphoteric gels such as Sephadex LH-20 or Merckogel OR-PVA 2000, then the generation of hydrogen chloride on the surface of the gels should be considered to be likely to occur.

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MAJOR AND MODIFIED NUCLEOSIDES IN tRNA HYDROLYSATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

GEORGE E. DAVIS, CHARLES W. GEHRKE and KENNETH C. KUO
Department of Biochemistry, University of Missouri, Columbia, Mo. 65211 (U.S.A.)
and
PAUL F. AGRIS
Division of Biological Sciences, University of Missouri, Columbia, Mo. 65211 (U.S.A.)
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SUMMARY

We describe a high-performance liquid chromatographic analytical method that can be readily placed in operation, and which is particularly well suited to scientists investigating tRNA structure, biosynthesis, and function, and for the determination of major and modified nucleosides of tRNA.

The method is characterized by the following features: (1) Sensitivity at the nanogram level; (2) High chromatographic resolution and selectivity; (3) Direct measurement of nucleosides with accuracy and precision; (4) Analysis is non-destructive and the high capacity of this chromatographic system allows easy isolation of pure nucleosides for further characterization; (5) Rapid separation and measurement in *ca.* 1 h after hydrolysis to nucleosides; and (6) Quantitation without use of radiolabeled compounds; however, labeled compounds are readily isolated and measured.

INTRODUCTION

Transfer ribonucleic acid (tRNA) has the most heterogeneous complex of nucleoside structures of all the nucleic acids[1–8]. Up to 25% of its *ca.* 76 nucleosides may be modified. The modifications, which number over 50, may be as simple as a methyl group or may be extremely complex. All modifications are achieved after the synthesis of the primary sequence by enzymes that are tRNA species, base, site, and sequence specific[1,8].

Realization of the tremendous biological significance of tRNAs has stimulated research directed at the elucidation of the many functional aspects of these complex macromolecules. Apart from their central role in protein biosynthesis and its regulation, tRNAs have been found to have many regulatory activities in RNA metabo-

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lism. These include their activities in regulating gene expression, priming reverse transcription, and possibly stimulating or inhibiting enzyme activities[1,2,8-13]. Further, tRNAs participate in the regulation of amino acid biosynthesis and transfer amino acids to cell wall structure. Another possible function now being intensively investigated is the action of tRNA and tRNA-modifying enzymes in the control of cellular development and differentiation[1,2,9,12,13]. Both tumor and embryonic tissue contain some altered isoaccepting tRNAs[14]. The control of the formation of these altered tRNAs may be the key to cancer prevention and treatment. It is likely that many more roles for tRNA will be found in the future as research progresses and better methods of analysis are developed. A sensitive, direct, rapid, and accurate method for the measurement of both major and modified nucleoside composition of often limited mammalian tissue samples of tRNA would advance our understanding of the biological significance of tRNA.

Earlier methods of analysis of nucleosides included separations from urine using cation-exchange isolation followed by silver nitrate precipitation of purines and two-dimensional paper chromatography[15], two-dimensional paper or cellulose thin-layer chromatography (TLC)[16], and anion-exchange isolation followed by two-dimensional paper chromatography or paper electrophoresis[17]. These methods are laborious and of relatively low sensitivity. More recent methods which have been applied to a variety of biological samples include various types of ion-exchange chromatography[18-26], gas–liquid chromatography (GLC)[27-34], TLC[35-38], and reversed-phase high-performance liquid chromatography (HPLC)[39-44]. The ion-exchange methods give good separation of many of the modified nucleosides; however, they lack the sensitivity necessary for analysis at low levels in limited samples and require up to 16 h for the chromatography. Gehrke and co-workers[28-30] have used GLC for separation of both nucleosides and bases. The method has good sensitivity and resolution, but requires extensive cleanup of the samples and an exacting derivatization of the compounds before chromatography.

The TLC method of Randerath's group[35] incorporates the exceptional sensitivity of post-hydrolysis tritium labeling of the periodate-oxidized ribonucleosides, which has made it the method of choice when the oligonucleotide sample is limited to a few micrograms. This method requires quantitative chemical conversion of all ribonucleosides to the corresponding tritiated triols, their subsequent TLC separation, autoradiofluorography, and finally liquid scintillation counting of the individual tritiated triols removed from the TLC plates. The total method is very lengthy, and has many operational steps. The extensive sample manipulation prior to the scintillation counting of the nucleosides reduces the reliability of the method, thus requiring a number of replicate analyses on each sample to achieve the reported precision[35]. Other TLC methods[37,38] do not have the sensitivity necessary for accurate measurement of small quantities of non-radiolabeled nucleosides.

Recently developed reversed-phase HPLC has been used for the separation and sensitive direct quantitation of nucleosides in biological fluids[40,42-44]. Our research investigations have centered on developing this chromatography and application of this versatile and powerful method to the analysis of nucleosides in many kinds of biological samples. This has resulted in the development of a rapid, sensitive, and reliable method for the direct determination of nanogram amounts of ribonucleosides in enzymatic hydrolysates of tRNA. More than 20 major and modi-

fied nucleosides can be quantitated in a 1-h run using a two-buffer step-gradient for the reversed-phase HPLC analysis of hydrolysates from 1 to 5 μg of tRNA.

This HPLC method is characterized by the following features: (1) Sensitivity at the nanogram level; (2) High chromatographic resolution and selectivity; (3) Direct measurement of nucleosides with accuracy and precision; (4) Analysis is non-destructive and the high capacity of this chromatographic system allows easy isolation of pure nucleosides for further characterization; (5) Rapid separation and measurement in *ca.* 1 h after hydrolysis to nucleosides; and (6) Quantitation without use of radiolabeled compounds; however, labeled compounds are readily isolated and measured.

We describe an HPLC analytical method that can be readily placed in operation, and which is particularly well suited to scientists investigating tRNA structure, biosynthesis, and function, and for the determination of major and modified nucleosides of tRNA.

EXPERIMENTAL

Apparatus

A modular HPLC system was used for the chromatographic studies consisting of a Model 6000A solvent delivery system, Model U6K universal injector, and a Model 440 two-channel absorbance detector (Waters Assoc., Milford, Mass., U.S.A.). The recorders used were a Honeywell Electronik 194 ABR and a Fisher Recordall Series 5000. The column consisted of two Waters μBondapak C_{18} 300 $\times$ 4 mm columns connected in series.

The temperature of the column was maintained using a constant-temperature circulating bath, Haake Model FJ (Saddle Brook, N.J., U.S.A.), connected to an aluminium column jacket[43].

Peak areas, retention times, and concentrations based on an external standard were calculated by a 3352B laboratory data system (Hewlett-Packard, Avondale, Pa., U.S.A.). The system consists of a 2100 computer with 24 K memory, 18652A analog-to-digital (A/D) converters, ASR33 teletype, and a 2748B high-speed photo reader.

The columns used for the boronate gel were glass, 150 $\times$ 5 mm (Fisher and Porter, Warminster, Pa., U.S.A.), modified by attachment of a 50-ml spherical reservoir to the top of the column.

The eluates from the boronate gel columns were lyophilized in Corex 25-ml screw-cap, round-bottom centrifuge tubes (Corning Glass Works, Corning, N.Y., U.S.A.) on a custom-built lyophilizer capable of maintaining a pressure of 0.05–0.1 mmHg with cold trap at $-60°$.

An Eppendorf Model 3200/30 microcentrifuge, Model 3300 rotary shaker, as well as various sizes of Eppendorf pipets (Brinkmann, Westbury, N.Y., U.S.A.) were used in the cleanup procedure.

A Micro Gram-Atic Balance (Mettler, Hightstown, N.Y., U.S.A.) was used to weigh milligram amounts of nucleosides for the calibration solutions.

Chemicals

The nucleosides, nucleotides, and nucleic acid bases were obtained from the

following sources: pseudouridine (ψ), cytidine (C), 3-methylcytidine (m^3C), inosine (I), 1-methylguanosine (m^1G), uridine 3′-monophosphate (3′UMP), guanosine 2′- and 3′-monophosphate (2′ and 3′GMP), adenosine 2′- and 3′-monophosphate (2′ and 3′AMP) (Sigma, St. Louis, Mo., U.S.A.); uracil (Ura), guanine (Gua), adenine (Ade), cytosine (Cyt), uridine (U), guanosine (G), adenosine (A), cytidine 5′-monophosphate (5′CMP), uridine 5′-monophosphate (5′UMP), adenosine 5′-monophosphate (5′AMP), guanosine 5′-monophosphate (5′GMP) (Mann Research Labs., New York, N.Y., U.S.A.); 5-methyluridine (m^5U), 4-thiouridine (s^4U), 4-acetylcytidine (ac^4C), 2′-O-methylcytidine (Cm), 2′-O-methyluridine (Um), 2′-O-methyladenosine (Am) (P-L Biochemicals, Milwaukee, Wisc., U.S.A.); 1-methyladenosine (m^1A), 5-methylcytidine (m^5C), 7-methylguanosine (m^7G), 1-methylinosine (m^1I), N^2-methylguanosine (m^2G), N^2,N^2-dimethylguanosine, (m^2_2G), and N^6-methyladenosine (m^6A) (Vega-Fox Biochemicals, Tucson, Ariz., U.S.A.).

Other chemicals were purchased from the following sources. Ammonium acetate and formic acid, ACS certified grade (Fisher Scientific, St. Louis, Mo., U.S.A.), ammonium hydroxide, analytical-reagent grade (Mallinckrodt, St. Louis, Mo., U.S.A.), ammonium dihydrogen phosphate (J. T. Baker, Phillipsburg, N.J., U.S.A.). Hydrazide Bio-Gel P–2, 200–400 mesh, lot no. 15569 (Bio-Rad Labs., Richmond, Calif., U.S.A.), *m*-aminophenylboronic acid hemisulfate, succinic anhydride, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) (Aldrich, Milwaukee, Wisc. 53233). All other chemicals were of the highest purity available. Methanol, distilled in glass (Burdick & Jackson, Muskegon, Mich., U.S.A.). All water used for the preparation of buffers and aqueous solutions was purified by a three-step process. The first step was reverse osmosis using an RO Pure apparatus (D0640 Barnstead Co., Boston, Mass., U.S.A.). A Nanopure D1794 four-cartridge water purification system was then used. A charcoal cartridge for adsorption of organics, two mixed-bed ion-exchange cartridges for removal of cations and anions, and a filtration cartridge for removal of all particles larger than 0.22 μm were used. Finally, the Nanopure water was distilled in a Corning all-glass still (Corning Glass Works).

Enzymes

Pancreatic ribonuclease, ribonuclease CB, ribonuclease T_2 (Calbiochem, San Diego, Calif., U.S.A.), and alkaline phosphatase, *E. coli* (Sigma) were used for the hydrolysis of tRNA samples.

HPLC buffers

A stock buffer concentrate was prepared as 2 l of a 2.0 *M* solution of $NH_4H_2PO_4$. This concentrate was then sterilized by filtering through a Millipore GS-22 filter (0.22 μm) and stored in glass at 4°. A 1-l volume of the working buffer was prepared daily by taking a 5.0-ml aliquot of the stock 2.0 *M* buffer solution and diluting it to 1.0 l with Nanopure distilled water in a volumetric flask. Then the pH was adjusted to 5.10 with a few drops of either a 1.0 *M* H_3PO_4 or 3.0 *M* NH_4OH solution. If methanol was to be added to the buffer, the appropriate volume was added after *ca.* 200 ml of water had been added to the buffer concentrate but before making to final volume with Nanopure distilled water. All buffers were sterilized by filtering through a Millipore GS-22 filter (0.22 μm) before use. Stored buffers were maintained

in a cold room at 4°. If stored 24 h or longer, the buffer was refiltered through a 0.22 μm filter.

Calibration standard solutions

Single compound stock solutions of nucleosides were exactly prepared to yield concentrations of *ca.* 1.00 μmole/ml in distilled Nanopure water. The exception to this concentration was for m^2G, which was made up at 0.25 μmole/ml owing to its low solubility. Standard solutions were stored at 4°, except for s^4U and ac^4C. These nucleosides were found to be relatively unstable and the solutions were frozen and stored at - 20°.

Calibration standards were made by dilution of aliquots of the single compound stock solutions to give a standard solution containing 200 μmoles/l C, U, G, and A; 20 μmoles/l m^1A and ψ; and 10 μmoles/l for each of the other modified nucleosides. A 25-μl volume of this solution was used to calibrate the chromatographic system.

Enzymatic hydrolysis of tRNA sample to ribonucleosides

A mixture of ribonucleases was made containing pancreatic ribonuclease (1 mg/ml), ribonuclease CB (500 units/ml), and ribonuclease T_2 (500 units/ml). tRNA samples were incubated with 5 μl of the ribonuclease mixture per 1 A_{260} (*ca.* 50 μg) of tRNA for 8 h at 37°. Following the ribonuclease digestion, 5 μl of a solution of alkaline phosphatase containing 12 mg/ml (144 units/ml) were added per 1 A_{260} of tRNA with enough 0.5 *M* Tris buffer, pH 7.8, to make the solution 0.05 *M* in Tris. The mixture was then incubated for 4 h at 37°. Following this treatment the solutions were diluted to accurately known concentrations and stored at −20° until used for HPLC analysis. Aliquots of these solutions were used for direct HPLC analysis without further treatment, or an isolation of the ribonucleosides was made with a phenylboronate substituted affinity gel (as described below) prior to HPLC analysis.

Phenylboronate substituted polyacrylamide affinity gel

An affinity gel with an immobilized phenylboronic acid functionality was used for isolation of ribonucleosides prior to HPLC separation and quantitation. The synthesis and use of this gel has been described in detail[40,43,44,46]. The tRNA enzymatic hydrolysate equivalent to 0.1–1.2 A_{260} (*ca.* 5–60 μg) adjusted to pH 8.8 with 0.25 *M* NH_4Ac buffer was placed on the 5 × 40 mm gel column. The column was washed sequentially with 1 × 1 ml, then 2 × 3 ml of 0.25 *M* NH_4Ac buffer (pH 8.8) and the nucleosides then eluted with 5 ml of 0.1 *M* HCOOH. The eluate was lyophilized to dryness, and redissolved in an accurately measured amount of distilled Nanopure water. Aliquots of this solution were then used for subsequent HPLC analysis.

RESULTS AND DISCUSSION

Reversed-phase HPLC of ribonucleosides found in tRNA

The relation of mobile phase pH, ionic strength, flow-rate, polarity and temperature to the resolution of the major and modified nucleosides using reversed-phase HPLC has been presented by Gehrke *et al.*[47]. These relationships were used to obtain a complete separation in 1 h of 18 ribonucleosides found in enzymatic hydrolysates

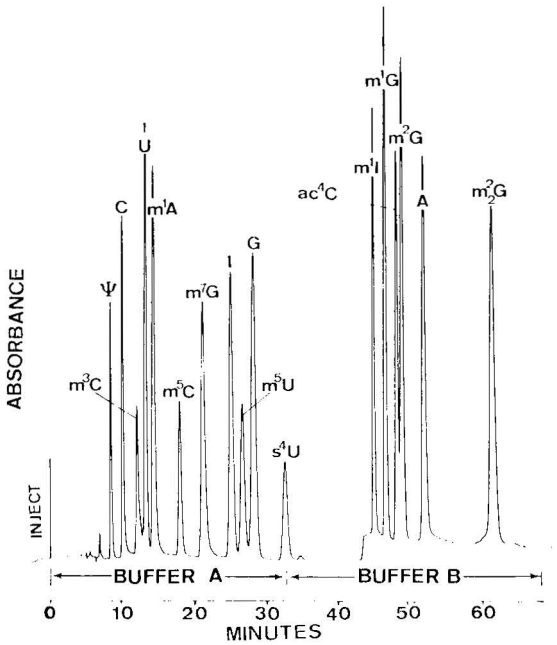

Fig. 1. Reversed-phase HPLC separation of standard nucleosides. Sample, 500 pmoles of each nucleoside; column, 600 × 4 mm μBondapak C_{18}; buffers: A, 2.5% (v/v) methanol in 0.01 *M* $NH_4H_2PO_4$, pH 5.10; B, 10% (v/v) methanol in 0.01 *M* $NH_4H_2PO_4$, pH 5.10; flow-rate, 1.0 ml/min; detector, 254 nm, 0.01 a.u.f.s.; temperature, 36.0°.

of tRNA (Fig. 1). A two-buffer step-gradient system and two 30-cm bonded C_{18} microparticulate columns in series were used to achieve the necessary resolution of early eluting compounds, as well as elution of more strongly retained nucleosides within 1 h. If thiouridine (s^4U) and 4-acetylcytidine (ac^4C) are not present, the buffer change may be made earlier and the elution accelerated without loss of sensitivity or resolution (Fig. 2).

Initial identification of the ribonucleosides was done on the basis of retention time. Stringent control of temperature as well as flow-rate gave excellent precision of retention times (Table I). Identification was further confirmed by comparison of the absorbance at 254 and 280 nm. The ratios obtained for standards and their precision are in Table II. The retention times, employing the chromatographic conditions detailed in Fig. 1, of 31 possible tRNA hydrolysis products are illustrated in Fig. 3. Although certain nucleotides and purine bases which may be formed by incomplete hydrolysis or cleavage of the ribose moiety from the nucleoside coelute with nucleosides, these possible byproducts can be detected by changes in the 254/280 nm absorbance ratio. Moreover, the 2′- and 3′-O-methylated nucleosides, 2′- and 3′-nucleotides and the nucleic acid bases can be removed by isolating the nucleosides on a phenylboronate gel column as described in the Experimental section. An enzymatic hydrolysis of tRNA to nucleosides may contain, after boronate gel cleanup, 5′-nucleotides as products of incomplete hydrolysis. Three of the four major 5′-nucleotides (5′CMP, 5′UMP, and 5′AMP) were separated from nucleosides found in

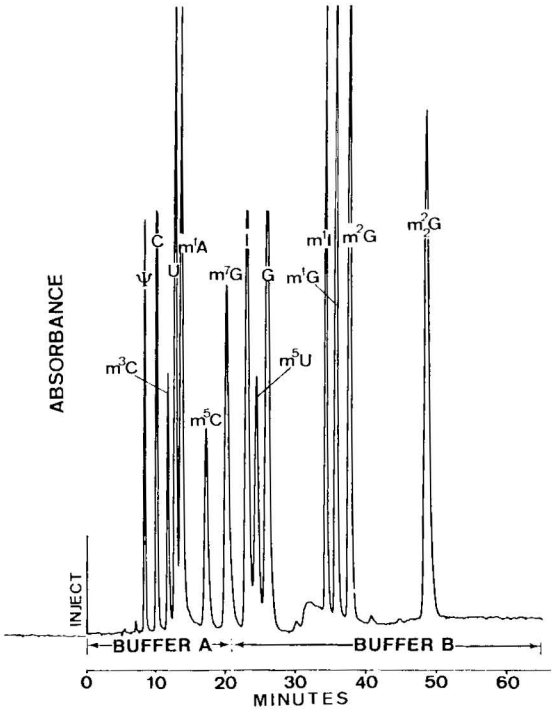

Fig. 2. Reversed-phase HPLC separation of standard nucleosides. Sample, 800 pmoles of each nucleoside; temperature, 37.6°; further conditions as in Fig. 1.

TABLE I

PRECISION OF REVERSED-PHASE HPLC RETENTION TIMES OF NUCLEOSIDES: TWO-BUFFER SYSTEM

R.S.D. = Relative standard deviation.

Nucleoside	*Retention time* (min)*	*R.S.D. (%)*
ψ	8.70	0.11
C	10.53	0.13
m^3C	12.53	0.10
U	13.83	0.17
m^1A	14.93	0.35
m^5C	18.86	0.20
m^7G	22.08	0.08
I	26.31	0.25
m^5U	27.72	0.14
G	29.58	0.24
s^4U	33.76	0.12
m^1G	45.86	0.15
m^2G	48.13	0.15
A	51.12	0.15
m_2^2G	60.16	0.13

* Each value is the average of four runs. Times are for 600 × 4 mm μBondapak C_{18} column, 35°, eluted at 1.0 ml/min for 33.0 min with 0.01 *M* $NH_4H_2PO_4$, pH 5.10, containing 2.5% methanol, then eluted with 0.01 *M* $NH_4H_2PO_4$, pH 5.10, containing 10% methanol. Times are not corrected for void volume time of 5.6 min.

TABLE II

ABSORBANCE RATIOS (254/280 nm) OF STANDARD NUCLEOSIDES

Nucleoside	*Retention time (min)*	*Peak area 254/280 nm**
ψ	8.70	1.77 ± 0.02
C	10.5	0.695 ± 0.002
m^3C	12.5	0.369 ± 0.005
U	13.8	2.190 ± 0.002
m^1A	14.9	3.36 ± 0.03
m^5C	18.9	0.61 ± 0.01
m^7G	22.1	1.36 ± 0.05
I	26.3	5.36 ± 0.03
m^5U	27.7	1.08 ± 0.01
G	29.6	1.763 ± 0.005
s^4U	33.8	1.83 ± 0.04
m^1G	45.9	1.66 ± 0.01
m^2G	48.1	1.75 ± 0.04
A	51.5	4.19 ± 0.02
m_2^2G	60.2	1.36 ± 0.02

* Each value is the average of three runs ± standard deviation.

tRNA, while 5′GMP coeluted with ψ. However, if none of the three well-separated 5′-nucleotides is found in the HPLC analysis of the hydrolysate, it would be highly unlikely that 5′GMP would be present.

Precision and accuracy of HPLC analysis

A standard mixture of 14 nucleosides was prepared containing the major nucleosides and 10 modified nucleosides at about the ratio which would be found if the modified nucleoside was present in a tRNA hydrolysate. Analysis of this mixture demonstrates the precision and accuracy obtainable with samples approximating 5 μg, 1 μg, and 200 ng of tRNA (Tables III–V). The relative standard deviations ranged from 0.1 to 1.6% at levels equivalent to 5 μg tRNA hydrolysate. At levels equivalent to 1 μg tRNA hydrolysate, the range for the relative standard deviation was 0.3–5%,

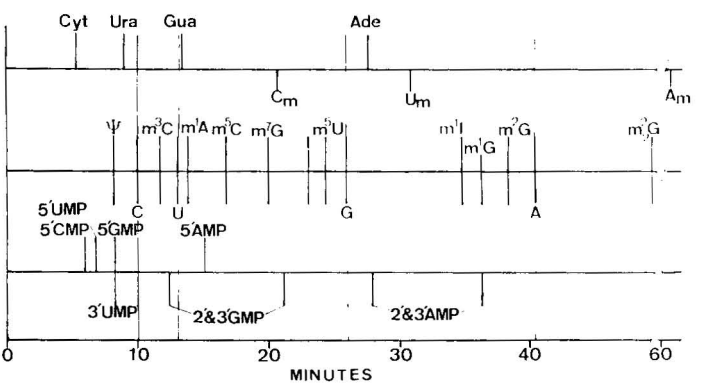

Fig. 3. Reversed-phase HPLC retention of tRNA hydrolysis products.

TABLE III

PRECISION AND ACCURACY OF HPLC ANALYSIS OF NUCLEOSIDE STANDARDS EQUIVALENT TO 5 μg tRNA HYDROLYSATE

Nucleoside	*Known value (pmoles)*	*Experimental value (pmoles)* *	*Difference (pmoles)*	*R.S.D. (%)*
C	5516	5518	+ 2	0.2
U	3492	3493	+ 1	0.1
G	4519	4514	− 5	0.2
A	5530	5542	+ 12	0.4
ψ	514	531	+ 17	0.4
m^1A	509	509	0	0.5
m^5C	251	251	0	0.5
m^7G	260	258	− 2	0.7
I	277	277	0	0.7
m^5U	250	251	+ 1	1.3
s^4U	466	463	− 3	1.6
m^1G	250	250	0	0.1
m^2G	250	250	0	1.4
m_2^2G	254	254	0	0.8

* Each value is the average of three runs at about the level found in 5 μg tRNA hydrolysate.

averaging 2.8% for the 10 modified and 0.9% for the major nucleosides. Even at levels equivalent to 200 ng of tRNA hydrolysate, an average precision of 4.2% was obtained for the major nucleosides and 6 of 10 modified nucleosides in the mixture.

The accuracy of the external standard method is shown in Table III–V. Differences between the experimental and known values ranged from 0 to 3% when the levels of nucleosides were equivalent to those found in 5 μg of tRNA. When the sample was equivalent to 1 μg of tRNA hydrolysate, all 14 measured values were still within

TABLE IV

PRECISION AND ACCURACY OF HPLC ANALYSIS OF NUCLEOSIDE STANDARDS EQUIVALENT TO 1 μg tRNA HYDROLYSATE

Nucleoside	*Known value (pmoles)*	*Experimental value (pmoles)* *	*Difference (pmoles)*	*R.S.D. (%)*
C	1103	1221	+ 118	0.8
U	698	719	+ 21	0.3
G	904	929	− 25	0.8
A	1106	1132	+ 26	1.6
ψ	103	96.4	− 6.6	1.5
m^1A	102	97.9	− 4.1	3.5
m^5C	50.2	51.1	+ 0.9	2.9
m^7G	51.9	53.7	+ 1.8	0.3
I	55.5	57.4	+ 1.9	0.2
m^5U	50.0	50.1	+ 0.1	3.9
s^4U	93.1	92.9	− 0.2	4.9
m^1G	50.0	52.4	+ 2.4	1.8
m^2G	50.1	53.0	+ 2.9	5.0
m_2^2G	50.8	54.1	+ 3.3	3.7

* Each value is the average of three runs at about the level found in 1 μg tRNA hydrolysate.

TABLE V

PRECISION AND ACCURACY OF HPLC ANALYSIS OF NUCLEOSIDE STANDARDS EQUIVALENT TO 200 ng tRNA HYDROLYSATE

Nucleoside	*Known value (pmoles)*	*Experimental value (pmoles)* *	*Difference (pmoles)*	*R.S.D. (%)*
C	221	234	+13	3.0
U	140	149	+ 9	2.5
G	181	196	+ 15	1.9
A	221	236	+ 15	1.8
ψ	20.6	19.0	- 1.6	0.9
m^1A	20.4	20.9	+ 0.5	1.0
m^5C	10.0	11.6	+ 1.6	4.0
m^7G	10.4	10.9	+ 0.5	5.4
I	11.1	11.1	0.0	5.4
m^5U	10.0	11.2	+ 1.2	8.5
s^4U	18.6	17.9	0.7	13.8
m^1G	10.0	9.7	0.3	2.3
m^2G	10.0	9.0	- 1.0	1.3
m_2^2G	10.2	10.1	0.1	7.1

* Each value is the average of seven runs at about the level found in 200 ng tRNA hydrolysate.

10% of the known value. Samples of standards equivalent to 200 ng of tRNA hydrolysate showed a greater variance from known values for three nucleosides, but this was for the analysis of only 10 pmoles of modified nucleoside and could probably be improved by standardization using mixtures of similar concentration.

Recovery of nucleoside standards taken through enzymatic hydrolysis procedure

A synthetic mixture of standards similar to the one used in the precision and accuracy experiments shown in Table III was used to demonstrate the stability of the nucleosides to the conditions employed in the enzymatic hydrolysis of tRNA samples. A 100-μl aliquot of a standard nucleoside mixture containing 14 nucleosides in the concentrations shown in Table VI was treated with a mixture of ribonucleases A, CB and T_2 for 8 h at 37° followed by pH adjustment to pH 7.8 with 0.05 *M* Tris buffer, then incubated with alkaline phosphatase (*E. coli*) 4 h at 37° as described in the Experimental section. A 25-μl aliquot of this mixture was then directly analyzed by HPLC. A comparison of the results before and after the enzymatic hydrolysis procedure and the recoveries is shown in Table VI. The values ranged from 91 to 107% for 13 of the 14 nucleosides. The recovery of s^4U was 63% with the remainder converted to U as noted by an increase in the concentration of U after hydrolysis equivalent to the loss of s^4U. Experiments are now underway to optimize the hydrolysis conditions and minimize this conversion. We found essentially no loss of other nucleosides (*e.g.* m^1A and m^7G) which are unstable to most conditions previously reported for hydrolysis and subsequent analysis.

Precision of HPLC analysis of nucleosides in tRNA hydrolysate

A direct HPLC analysis of a yeast $tRNA^{Phe}$ hydrolysate was performed; after demonstration of sensitivity, separation, identification, precision, accuracy, and recovery for analysis of mixtures. Approximately 50 μg (1.0 A_{260}) of purified $tRNA^{Phe}$ was

TABLE VI

RECOVERY OF STANDARD NUCLEOSIDES TAKEN THROUGH ENZYMATIC HYDROLYSIS PROCEDURE

Nucleoside	*Standards (nmoles/ml)*		*Recovery (%)*
	*Before hydrolysis**	*After hydrolysis***	
ψ	19.0	18.0	95
C	225	226	100
U	142	150	106†
m^1A	22.5	20.4	91
m^5C	10.8	11.6	107
m^7G	10.4	10.2	98
I	11.3	11.4	101
m^5U	9.90	9.96	101
G	182	182	100
s^4U	20.1	12.6	63†
m^1G	10.7	10.5	98
m^2G	14.6	14.8	101
A	228	230	101
m_2^2G	11.8	11.8	100

* Each value is the average of two independent runs from 25 μl injected.

** 100-μl standards incubated with a mixture of ribonucleases (A, CB, T_2) 8 h at 37°, pH raised to 7.8 with 0.05 *M* TRIS buffer, incubated 4 h at 37° with alkaline phosphate (*E. coli*), and then analyzed by HPLC without boronate gel cleanup.

† s^4U converted to U during hydrolysis.

hydrolyzed as described in the Experimental section. Aliquots of this hydrolysate equivalent to 0.1 A_{260} (*ca.* 5 μg) were analyzed. The precision for 11 nucleosides is given in Table VII. The relative standard deviations ranged from 0.6 to 3.5% for all nucleosides analyzed.

Boronate gel isolation of nucleosides from tRNA hydrolysate prior to HPLC analysis

In earlier chromatographic studies of nucleosides in biological fluids, we used

TABLE VII

PRECISION OF HPLC ANALYSIS OF NUCLEOSIDES IN tRNA

Nucleoside	*nmoles**	*R.S.D. (%)*
ψ	0.473	0.6
C	0.74	1.8
U	3.35	3.5
m^1A	0.11	2.7
m^5C	0.19	2.1
m^7G	0.07	0.9
m^5U	0.50	1.4
G	6.12	0.8
m^2G	0.27	1.1
A	4.77	1.0
m_2^2G	0.17	3.5

* Each value is the average of four independent runs. About 5 μg of tRNA hydrolysate used in each analysis. Yeast $tRNA^{Phe}$ from Dr. P. F. Agris. Analyzed without boronate gel cleanup.

a polyacrylamide gel containing immobilized phenylboronic acid substituents for the isolation of ribonucleosides prior to reversed-phase HPLC analysis[40,43,44]. This method (see Experimental) was used to obtain a ribonucleoside fraction from the tRNA hydrolysates that was free of protein and other compounds which do not contain the *cis*-diol configuration necessary for retention by the boronate gel column. The recovery for our phenylboronate gel was determined by addition of standard nucleosides to an *E. coli* tRNA hydrolysate. Approximately 8 nmoles of the four major nucleosides and 1 nmole each of 11 modified nucleosides were added to 50 μl of tRNA hydrolysate, which was placed on the boronate gel column, then washed, and the nucleosides eluted as described in Experimental. This eluate and corresponding ones for the unspiked tRNA hydrolysate were then analyzed by HPLC. The recoveries were essentially quantitative, ranging from 91 to 106%. These recoveries (Table VIII) are similar to those obtained for urine[43].

TABLE VIII

RECOVERY OF NUCLEOSIDES FROM *E. coli* tRNA HYDROLYSATE WITH BORONATE GEL ISOLATION

Nucleoside	*nmoles/ml in hydrolysate*				*Recovery (%)*
	tRNA + *Spike**	*tRNA**,**	*Spike recovered*	*Spike added*	
C	243	89.6	153	160	96
U	371	174	197	186	106
G	460	296	164	165	99
A	348	180	168	161	104
ψ	56.0	37.4	18.6	20.2	92
m^3C	20.6	1.0	19.6	20.6	95
m^1A	21.1	0.0	21.1	20.4	104
m^5C	20.2	0.3	19.9	20.1	99
m^7G	20.1	0.0	20.1	20.8	97
I	23.1	1.9	21.2	22.2	95
m^5U	18.8	0.0***	18.8	20.0	94
m^1I	19.2	0.5	18.7	19.8	94
m^1G	19.9	1.4	18.5	20.0	92
m^2G	18.8	0.0	18.8	20.0	94
m_2^2G	19.2	0.0	19.2	20.3	95

* Nucleosides in hydrolysates isolated on phenylboronate gel column, eluted, then analyzed by HPLC.

** Each value is the average of three independent runs.

*** tRNA used was from a ribo-T deficient mutant from Dr. P. F. Agris.

Chromatograms of yeast tRNA hydrolysates analyzed before and after boronate gel isolation of the ribonucleosides are shown in Figs. 4 and 5. Fig. 4 shows the separation of nucleosides from yeast tRNA hydrolysate before boronate gel isolation. Major peaks that were entirely or partially removed by initial isolation on the boronate gel are indicated by arrows.

A comparison of the data for analysis of yeast tRNA with and without boronate gel isolation is presented in Table IX. The affinity column isolation confirms the homogeneity of the nucleoside peaks and distinguishes minor nucleosides from artifacts of the isolation of tRNA and its hydrolysis.

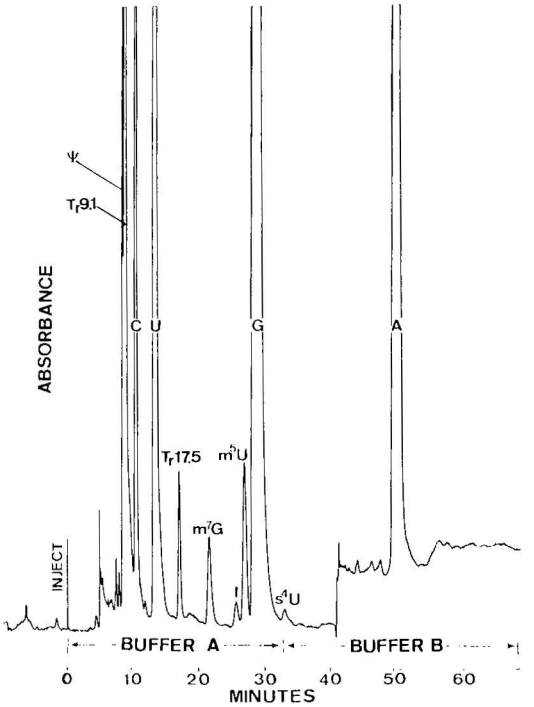

Fig. 4. Reversed-phase HPLC separation of nucleosides from tRNA hydrolysate before boronate gel isolation. Sample, 3 μg of yeast tRNA. Conditions as in Fig. 2.

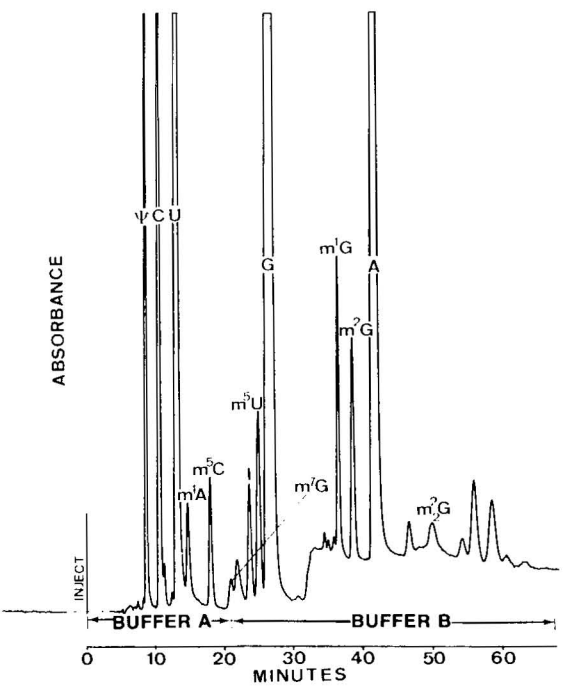

Fig. 5. Reversed-phase HPLC separation of nucleosides from tRNA hydrolysate after boronate gel isolation. Sample, 3 μg of yeast tRNA. Conditions as in Fig. 2.

TABLE IX

ANALYSIS OF NUCLEOSIDES IN YEAST tRNA HYDROLYSATE WITH AND WITHOUT INITIAL ISOLATION ON BORONATE GEL

Nucleoside	*nmoles/ml*	
	Without gel isolation	*With gel isolation*
ψ	52.7*	53.6*
C	70.9**	58.1**
U	205.6	190.1
m^1A	8.46	3.32
m^5C	8.91	8.78
m^7G	1.69	1.43
I	4.49	4.66
m^5U	14.8	15.7
G	225.5	225.5
m^1G	8.18	8.18
m^2G	7.29	7.03
A	165.6	164.6
m_2^2G	1.29	1.50

* Each value is for a single analysis of about 3 μg of yeast tRNA.

** Value for C is very low. It was evidently incompletely degraded on enzymatic hydrolysis. Subsequent hydrolyses show C is approximately equal to G in concentration.

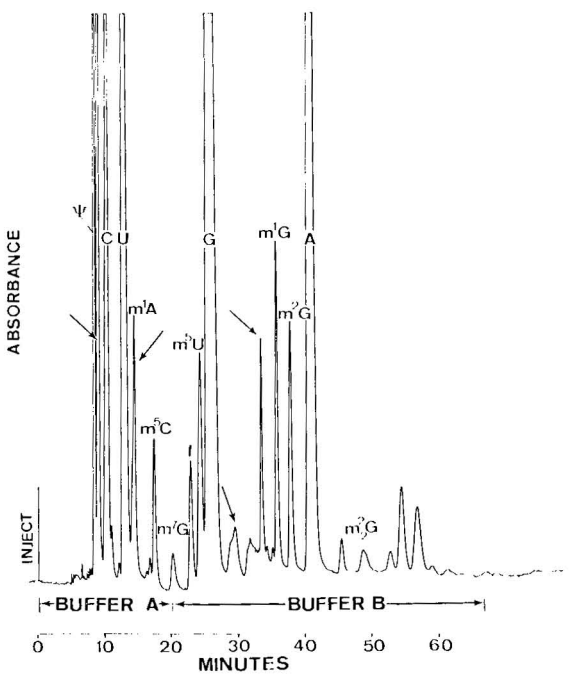

Fig. 6. Reversed-phase HPLC separation of nucleosides from tRNA hydrolysate. Sample, 5 μg of *E. coli* $tRNA_f^{Met}$; detector, 254 nm, 0.005 a.u.f.s.; further conditions as in Fig. 1.

Direct HPLC analysis of nucleosides in hydrolysates of purified isoaccepting tRNA

Samples of purified isoaccepting tRNAs were isolated and enzymatically hydrolyzed. These hydrolysates were analyzed directly by HPLC. Chromatograms of two of these samples are presented in Figs. 6 and 7. Approximately 5–10 μg (0.1–0.2 A_{260}) of tRNA were used for each analysis. Determination of the capacity of the tRNAs to accept phenylalanine or methionine demonstrated that the $tRNA^{Phe}$ sample was 50% pure while the $tRNA_f^{Met}$ had a purity of 80%. Peaks corresponding to unidentified compounds in the hydrolysates are designated by "Tr" followed by the retention time uncorrected and non-adjusted for void volume time.

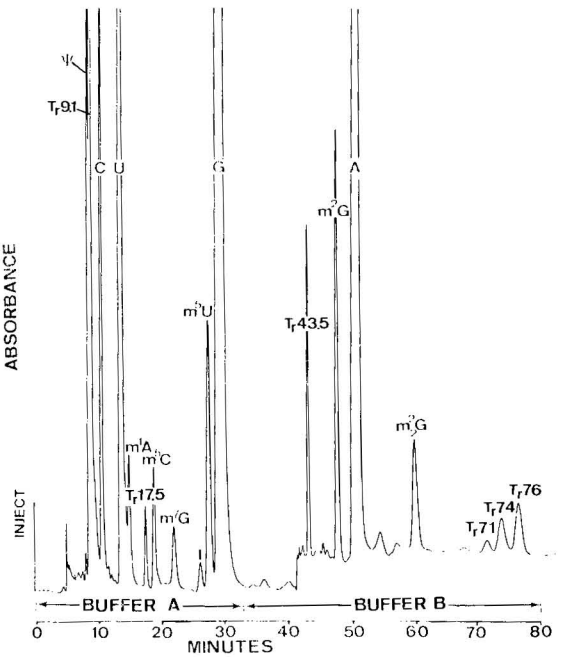

Fig. 7. Reversed-phase HPLC separation of nucleosides from tRNA hydrolysate. Sample, 10 μg of yeast $tRNA^{Phe}$. Conditions as in Fig. 1.

Direct HPLC analysis of nucleosides in hydrolysates of tRNA from Hodgkin's tumor and normal spleen

Enzymatic hydrolysates of tRNA isolated from normal human spleen and from Hodgkin's tumor were supplied by Dr. Ian Cooper of the Cancer Institute, Melbourne, Australia. Samples of four normal spleen tRNA hydrolysates, and four Hodgkin's tumor tRNA hydrolysates, were directly analyzed by HPLC for nucleosides. Chromatograms of two of these analyses are presented in Figs. 8 and 9. A comparison of the data from the tumor and normal samples is shown in Table X. No significant elevation of modified nucleosides was found in the tumor samples. This is consistent with the data obtained by Borek *et al.*[45] who suggest that a small subgroup of tumor-specific tRNA's having a very rapid turnover rate are responsible for the massive excretion of modified nucleosides by cancer patients, rather than a general hypermethylation of tumor tRNA.

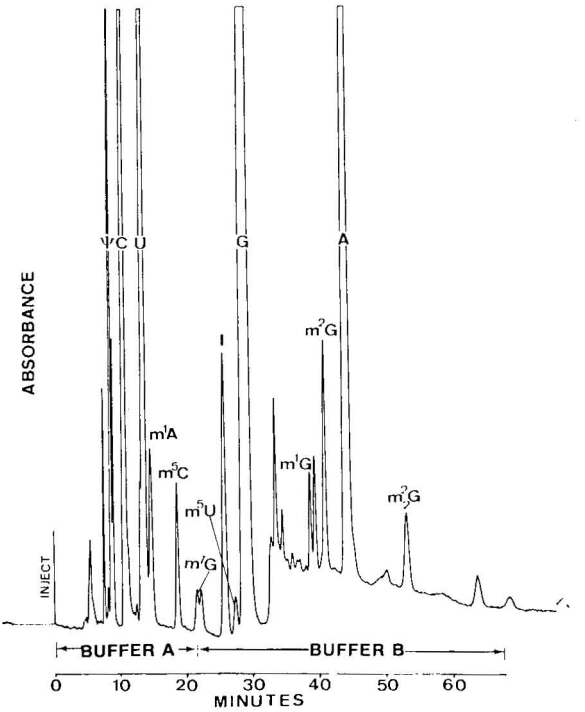

Fig. 8. Reversed-phase HPLC separation of nucleosides from tRNA hydrolysate. Sample, 20 μg of Hodgkin's tumor tRNA. Conditions as in Fig. 2.

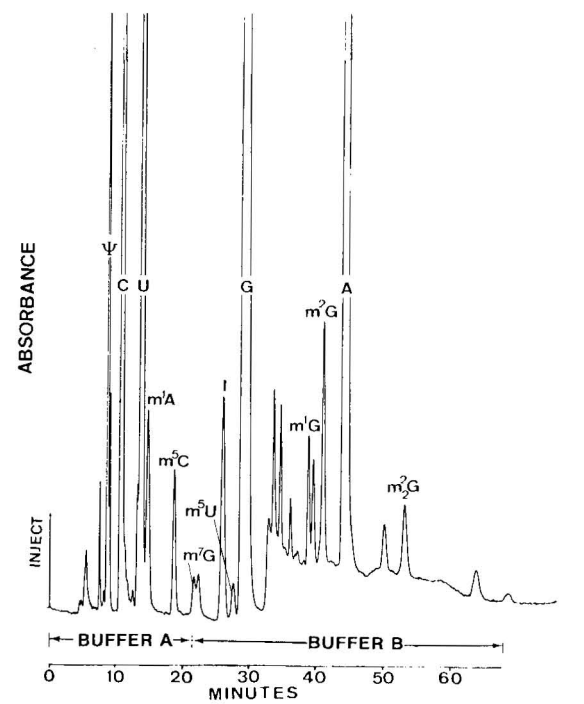

Fig. 9. Reversed-phase HPLC separation of nucleosides from tRNA hydrolysate. Sample, 10 μg of normal spleen tRNA. Conditions as in Fig. 2.

TABLE X

MODIFIED NUCLEOSIDES IN tRNA HYDROLYSATES OF NORMAL SPLEEN AND HODGKIN'S TUMOR

Nucleoside	*Hodgkin's tumor** *(moles/100 moles nucleoside)*	*Normal spleen (moles/100 moles nucleoside)*
ψ	1.55 ± 0.37	1.45 ± 0.47
m^1G	0.26 ± 0.09	0.31 ± 0.21
m^2G	0.65 ± 0.24	0.52 ± 0.32
m^2_2G	0.36 ± 0.19	0.25 ± 0.14

* Each value is the average of two analyses of a tRNA hydrolysate from each of four patients.

CONCLUSIONS

We have previously reported a reversed-phase HPLC method for the separation and sensitive direct quantitation of ribonucleosides in biological fluids[40,43,44]. This method has been improved and its application extended to the direct determination of nanogram amounts of ribonucleosides in enzymatic hydrolysates of tRNA. Emphasis in this study was placed on the quantitative chromatography of nucleosides and not the hydrolysis of tRNA. Our experience indicates that present enzymatic hydrolysis procedures are less than desirable for quantitative release of nucleosides. A systematic study is in progress to establish optimum conditions. More than twenty major and modified nucleosides have been quantitated in a 1-h run for the analysis of hydrolysates of from 1 to 5 μg of tRNA.

This chromatographic method is non-destructive, and does not require derivatization or radiolabeled compounds for chromatographic separation or detection. Precise analyses can be obtained from hydrolysates of microgram amounts of tRNA. The precision was determined by repeatedly analyzing 5 μg of tRNA hydrolysate (the relative standard deviations were in the range $< 1\%$ to $< 4\%$). No loss or alteration of nucleoside structure occurred when a 12-component mixture of nucleosides was subjected to a two-step enzymatic hydrolysis procedure. The accuracy of nucleoside chromatography was further established by demonstrating the separation of the major mononucleotides from the nucleosides, the removal of possible UV absorbing substances by boronate gel, and by absorbance measurements at 254 and 280 nm. Thus, false elevations of nucleosides by co-elution of other compounds are detectable.

These sensitive and selective methods allow the rapid analysis of trace levels of nucleosides in small samples of polynucleotide hydrolysates. They should serve as important tools in molecular biology and clinical research.

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CHARACTERIZATION OF NATIVE MULTICOMPONENT PROTEIN MIXTURES BY ONE- AND TWO-DIMENSIONAL GRADIENT ELECTROPHORESIS

K. FELGENHAUER

Neurologic University Clinic, Joseph-Stelzmann-Strasse 9, 5000 Köln 41 (G.F.R.)

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SUMMARY

An arrangement of one- and two-dimensional polyacrylamide gradients has been applied to the analysis of multicomponent mixtures of soluble proteins: (1) two-dimensional 3–25% slabs; (2) 3–40% cylinders; and (3) 20–50% cylinders. The run in the first dimension is performed in non-restrictive agarose gel to ascertain a "pure" charge abscissa. Gradient electrophoresis is performed over prolonged periods in order to reach or closely approach the exclusion (pore) limit, which is taken as an operational parameter for the hydrodynamic size of globular molecules irrespective of their frictional ratios. For each of the three gradients one to four reference mixtures were formulated that allow size evaluations of non-denatured proteins. Human serum again proved its usefulness as a universal reference mixture for newly developed techniques. The number of stainable components is in accordance with expectation based on the broad knowledge of serum proteins. Two-dimensional agarose/gradient electrophoresis seems to fulfil the one band–one protein strategy of high-resolution techniques.

INTRODUCTION

The retardation of protein–SDS (sodium dodecyl sulphate) complexes in polyacrylamide gel electrophoresis depends primarily on the molecular mass, because charge differences are diminished by the acidity of the detergent and the molecular weights of single constituents from complex protein mixtures can be easily determined[1–6]. Under the conditions usually applied, however, composite proteins that are kept together by non-covalent bonds dissociate into their subunits. As many biological fluids contain substantial amounts of high-molecular-weight proteins, their subunits then intermingle inseparably with the native low-molecular-weight proteins. An analogous technique that combines non-dissociating running conditions with the sieve effect of gel electrophoresis may therefore represent an attractive analytical tool.

After long running times on gel gradients, many proteins approach zero mobility[7,8] and the final position depends primarily on the hydrodynamic size[9].

Slowly migrating proteins, however, may never reach their exclusion (pore) limits within reasonable running times, thus leading to an overestimation of their molecular size. This source of error, which is unavoidable in one-dimensional techniques, can be overcome by a preceding free-zone electrophoresis, which sorts out all proteins that migrate below a limiting velocity.

The running position in agarose accurately reflects the free electrophoretic mobility, as the effective pore radius of a 1% gel is several times greater than the largest serum protein β-lipoprotein ($R = 12.4$ nm) and it is justified to consider the first dimension as the charge axis of a two-dimensional analytical grid. The non-restrictive agarose gel has many advantages. It can be brought into any convenient shape, allows two-directional separations, adheres tightly to the polyacrylamide gel surface and the difference of electroendosmosis in both gels can be overcome by appropriate running conditions.

Isoelectric focusing of multicomponent mixtures in synthetic ampholytes reveals microheterogeneities of many individual proteins that often cannot be clearly differentiated from true compositional heterogeneities. On application of human serum, which is the best known biological protein mixture, we were unable to localize precisely the position of several well known individual proteins, as any particular spot may represent either an individual protein or a charge isomer without functional significance.

The agarose/pore gradient map reveals a smaller number of spots, but each individual protein appears as a characteristic entity that can be confined by specific staining, immune techniques and analytical reasoning, thus taking advantage of the charge and size coordinates. The technique would therefore fulfil the "one spot–one protein" condition that remains the ultimate goal of high-resolution methods.

EXPERIMENTAL AND RESULTS

Separation according to size was performed on linear gel gradient slabs with total monomer concentrations from 3 to 25% ($T = 3$–25%). These withstand the long running times necessary to reach (or at least approach) zero mobility. Small-pore 5-mm cylinder gels with monomer concentrations up to 50% have been used for small molecular proteins with molecular weights above 3000 daltons. The total pore size range (3–50%) was then divided and for technical reasons the following compositions were chosen for standard experiments in glass tubes: large pore gradient, $T = 3$–40%, $C = 5$–10%; small pore gradient, $T = 20$–50%, $C = 10\%$ (T = total monomer concentration; C = portion of crosslinking monomer).

Micro-scale gradients of comparable low porosities have already been produced, utilizing density differences, capillary forces and lateral diffusion[10].

As in column chromatography the analytical validity of the method depends strongly on the calibration system utilized[11]. Each constituent of a universal reference mixture should fulfil the following requirements:

(1) overall globular conformation with frictional and axial ratios that are representative of the respective molecular size group[9];

(2) sufficient mobility to approach the exclusion limit with reasonable running times;

(3) high solubility in the running buffer to provide narrow starting zones;

(4) no interactions with other components of the mixture;

(5) commercial availability for a reasonable price in a highly purified state.

A limited polymerization tendency increases the number of values for the reference curve. After extensive studies, the proteins compiled in Table I and under *Calibration mixtures* were selected for calibration purposes. The finally adopted two-dimensional reference mixture contained lactoglobulin, albumin, transferrin, catalase and ferritin (Fig. 1). The following proteins of human serum are easy to recognize and can be used as additional references: prealbumin, α_2-macroglobulin and β-lipoprotein (Fig. 2). A representative calibration graph is shown in Fig. 3, and it is evident that the oligomers of lactoglobulin, albumin, transferrin and ferritin share the overall correspondence between hydrodynamic volume and molecular weight that is characteristic of "normal" globular proteins.

TABLE I

MOLECULAR DATA OF SELECTED REFERENCE PROTEINS

See also under *Calibration mixtures*. The values were compiled from refs. 12–16 and the literature sources cited in a previous paper[9]. MW = molecular weight; R = hydrodynamic radius; $\bar{R}$ = mass equivalent geometric radius for $\bar{v}$ = 0.73 g/ml; pI = isoelectric point; R_F = relative mobility as referred to bromophenol red in 0.2 M barbital buffer (pH 8.6); $R/\bar{R}$ = frictional ratio; $R/\bar{R}_r$ = frictional ratio representative of "normal" globular proteins of the respective size. Note the tendency to more space-occupying conformations with molecular size.

Protein	*MW*	*R (nm)*	*pI*	R_F	$R/\bar{R}$	$R/\bar{R}_r$
Ribonuclease	13,700	1.64	8.9		1.08	1.14
Whale myoglobin	16,890	1.86	8.2	19	1.11	1.16
Lactoglobulin-dimer	35,830	2.90	5.3		1.33	1.23
Ovalbumin	43,500	2.74	4.7		1.18	1.22
α-Amylase	48,600	2.67		27	1.11	1.24
Prealbumin	61,000	3.25	4.7	81	1.29	1.24
Albumin	69,000	3.58	4.9	67	1.27	1.26
Transferrin	81,000	3.67	5.9	31	1.30	1.27
Fumarase	206,000	5.27	7.7		1.27	1.31
Catalase	241,200	5.22	5.7	45	1.27	1.32
Ferritin	473,450	7.90	4.5	55	1.53	1.35
Thyroglobulin	669,000	8.60	4.5		1.48	1.36
α_2-Macroglobulin	798,000	9.35	5.4	44	1.49	1.36
β-Lipoprotein	2,239,000	12.40		33	1.46	1.42

Technical details of the two-dimensional procedure

First-dimensional electrophoresis of four samples is performed in a 1.5-mm layer of 1% agarose (Litex Co., Glostrup, Denmark) on a 10 × 10 cm slide. The pre-warmed samples, containing 0.3–0.8 mg of protein, are mixed 1:1 with 2% agarose at 52° and 1 μl of bromophenol red solution (saturated solution diluted 1:5). Gel slits of appropriate sizes 2 cm apart from the cathode edge are filled completely with 20–80 μl of the gel–sample mixtures. The running buffer is 0.2 M barbital buffer (pH 8.6).

Filter-paper wicks (Whatman No. 1) are applied as current bridges with contact zones of 1.1 cm at the cathode side and 1.7 cm at the anode side. A potential of 250 V is applied until the dye band has reached the edge of the filter-paper. The

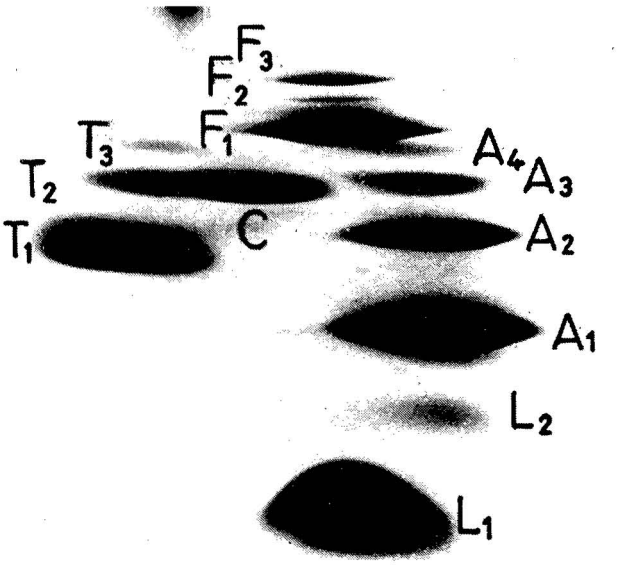


Fig. 1. Reference mixture for the two-dimensional technique. Relative mobilities of the monomers (R_F of bromophenol red = 100): albumin A = 67, lactoglobulin monomer L = 61, ferritin F = 55 (α_1), catalase C = 45 (α_2), transferrin T = 31 (β). The polymers of transferrin migrate increasingly faster than the monomer. Note a still faster migrating macromolecular transferrin aggregate at the large pore edge of the slab.

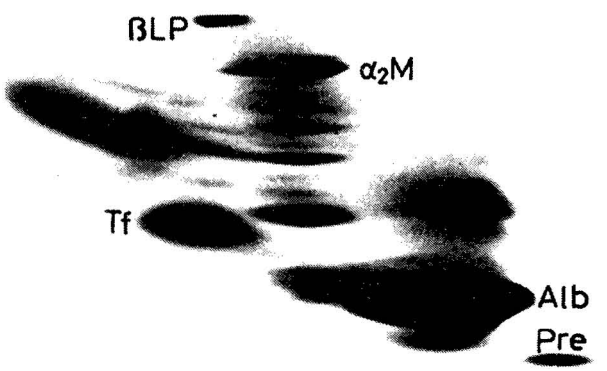


Fig. 2. Proteins of human serum that can be easily recognized for calibration purposes. The R_F values have been determined in agarose at pH 8.6 and the free mobilities m_0 ($cm^2/V \cdot sec$) on barbital buffer pH 8.6 (β-lipoprotein in phosphate buffer pH 7.8) were taken from Schultze and Heremans[17]. Prealbumin, R_F = 81 (m_0 = 7.6, pI 4.7); albumin, R_F 67 (m_0 5.9, pI 4.9); α_2-macroglobulin, R_F = 44 (m_0 = 4.2, pI 5.4); β-lipoprotein, R_F 33 (m_0 3.1); transferrin, R_F = 31 (m_0 = 3.1, pI = 5.9). Note the size-heterogeneous α_1-lipoprotein family above albumin and the comma-like appearance of the charge heterogeneous γ-immunoglobulins.

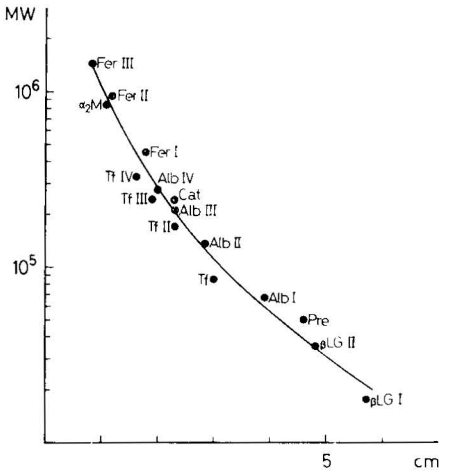


Fig. 3. Calibration graph for a representative two-dimensional slab gradient, T = 3–25%, C = 5%. The values were measured on slabs with the standard reference mixture and human serum. βLG = β-lactoglobulin; Pre = prealbumin; Alb = albumin; Tf = transferrin; Cat = catalase; Fer = ferritin; α_2M = α_2-macroglobulin.

gel is cut at this height, thus fixing the mobility reference (R_F = 100). Allowance for the endosmotic flow can be made by adding 20 μg of vitamin B_{12} to the sample. Individual strips (1.4 × 7.2 cm) are excised without touching the surface of the gel.

The residual fluid is carefully removed from the surface of the polyacrylamide gel with filter-paper. Flat air cushions between the glass and gel surface can be cautiously expelled by manual pressure from the outside. The agarose strip is slowly pushed into the gradient gel chamber with the help of a slide-rule, leaving a 1-mm gap. All remaining space is quickly filled with 1% agarose without entrapping air bubbles.

For the second-dimensional run a Pharmacia GE-4 chamber apparatus is used. Leaky gaskets are sealed with 1% agarose to prevent a current by-pass. The running buffer is 0.089 *M* Tris–0.082 *M* borate buffer (pH 8.6) containing 0.0025 *M* EDTA.

Electrophoresis is performed for 24 h with a constant current of 40 mA. The buffer, permanently circulated, is renewed after 12 h. The gradient chambers are placed for 5 min in an incubator at 80° to soften the adhesive (see below), immersed in water and opened with the help of a scalpel.

The staining solution is 10 g of Coomassie Brilliant Blue R 250 (Serva, Heidelberg, G.F.R.), 900 ml of water, 900 ml of methanol and 200 ml of glacial acetic acid. The solution is stirred overnight and filtered. The slabs are stained for 24 h and de-stained in 10% acetic acid–50% methanol with slight agitation. The de-staining solution is replaced several times. The gels are stored in air-tight plastic envelopes.

Preparation of the slab gradients

Plastic spacers (2 × 4 × 83 mm) are fixed on thoroughly cleaned 83 × 83 mm glass slides with a thermoplastic adhesive (UHU plus Endfest 300, UHU-Werk,

Bühl, G.F.R.). Eight gradient chambers are placed side-by-side in the Pharmacia GSC 8 casting apparatus and the monomer solution is introduced from the bottom. The single compartment volumes are determined with water, the following amounts being representative for an eight chamber experiment: 35 ml of water (agarose space), 88 ml of 3% acrylamide (half the chamber volume) and 103 ml of 25% acrylamide (half the chamber volume plus the dead volume).

Linear gradients are prepared with the Ismatec MP 13 GI-10 13-channel tubing pump (Ismatec SA, Zürich, Switzerland). The mixing chambers are kept in an ice-bath, the whole arrangement is protected from UV light and room temperature is kept between 20° and 22°. The initial pumping speed is set at 130 ml/h and increased stepwise to 220 ml/h after 15-min intervals so as to fill the chambers within 75 min. After a further 15 min at room temperature the apparatus is placed for 60 min in a water-bath at 27° in the dark for catalyst polymerization. Then a neon lamp is placed perpendicular above the casting chamber. After 3 h of mixed-catalyst photopolymerization the whole box is placed in a refrigerator and the single gradient chambers are dissected from the gel block prior to use.

Stock solutions

A: 36.3 g of Tris, 0.46 ml of N,N,N′,N′-tetramethylethylenediamine, 48 ml of 1 *N* hydrochloric acid, adjustment to pH 8.8, water to 100 ml.

B: 2.8% ammonium peroxodisulphate, freshly prepared 1 h before use and kept at 4°.

CI: 57 g of acrylamide, 3 g of N,N′-methylenebisacrylamide (BIS), water to 100 ml.

CII: 54 g of acrylamide, 6 g of BIS, water to 100 ml.

KI: 80 mg (KII = 800 mg) of $K_3Fe(CN)_6$ per 100 ml (maximum keeping time 4 weeks).

R: 8 mg of riboflavin per 100 ml (maximum keeping time 2 weeks).

G: 50 g of glucose per 100 ml, 0.1% sodium azide.

Slab gradient, T = 3–25%, C = 5%; 3% = 12.5 ml of A, 2.5 ml of B, 5 ml of CI, 7.5 ml of KI, 10.0 ml of R and 62.5 ml of water; 25% = 12.5 ml of A, 2.5 ml of B, 41.6 ml of CI, 7.5 ml of KI and 35.9 ml of G.

Cylindrical gradients

The same type of mixed polymerization was used for the 5-mm cylinder gels. The gradient preparation chamber, assembled from two glass plates (18 × 16 cm for 12 tubes, 18 × 32 cm for 24 tubes) and soft silicone spacer tubing (O.D. = 12 mm, wall thickness 2–2.5 mm) is held together with screw-clamps. The tubes, provided with 3–4-mm Parafilm collars at the upper edge, are held in place by a 12-(or 24-) hole frame which rests on the polymerization chamber. The monomer solutions are pumped into the chamber by a multichannel syringe distribution system as described earlier[18]. All other conditions are identical with those for the slab gradients.

Large-pore cylinder gradient, T = 3–40%, C = 5–10%; 3% = 12.5 ml of A, 2.5 ml of B, 5 ml of CI, 10 ml of KI, 4 ml of R and 66 ml of water; 40% = 12.5 ml of A, 2.5 ml of B, 67 ml of CII, 7.5 ml of KI and 10.5 ml of G.

Small-pore cylinder gradient, T = 20–50%, C = 10%; 20% = 12.5 ml of A,

2.5 ml of B, 33 ml of CII, 0.75 ml of KII, 3.75 ml of R and 47.5 ml of water; 50% = 12.5 ml of A, 2.5 ml of B, 83.0 ml of CII, 0.75 ml of KII and 1.25 ml of G.

Anode buffer (pH 8.5): 28.5 g of Tris, 136.8 g of glycine, water to 5000 ml (2 l per electrode chamber).

Cathode buffer (pH 8.5): 36.0 g of Tris, 172.8 g of glycine, water to 5000 ml (1 l per electrode vessel).

Sample volume: 0.2–0.5 ml, loaded with 50% glucose, 1 μl of bromophenol red.

Running conditions (constant voltage): 1st day, 75 V; 2nd and 3rd days, 150 V; 4th day, 175 V; 5th day, 225 V.

The buffer is replaced after 12-h intervals. The glass tubes are carefully cracked to liberate the gel cylinders.

Calibration mixtures

The following three protein mixtures were finally adopted for the large-pore cylinder gradients, T = 3–40% (Fig. 4).

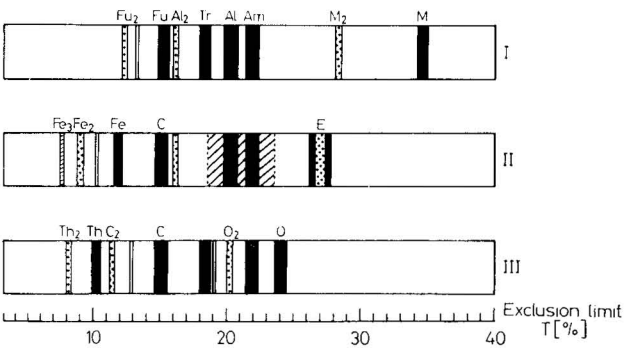


Fig. 4. Band patterns of the calibration mixtures for the large-pore cylinder gradients. The exclusion limits indicated are average values from numerous linear gradient experiments. Al = albumin; Am = α-amylase; C = catalase; E = egg trypsin inhibitor; Fe = ferritin; Fu = fumarase; M = myoglobin; O = ovalbumin; Th = thyroglobulin; Tr = transferrin.

I. 75 μg of whale myoglobin, carbamylated (Schuchardt, Darmstadt, G.F.R.), 150 μg of *Bacillus subtilis* α-amylase (Serva), 50 μg of human serum albumin (Serva), 50 μg of human serum transferrin (Behring-Werke, Marburg, G.F.R.) and 70 μg (35 μl) of pig heart fumarase (Boehringer, Mannheim, G.F.R.).

II. 200 μg of egg trypsin inhibitor, molecular weight 28,000 (Serva), 60 μg (3 μl) of bovine liver catalase (Boehringer), 145 μg (2 μl) of horse spleen ferritin (Miles, Kankakee, Ill., U.S.A.) and amylase and albumin as above.

III. 70 μg of ovalbumin (Serva), 450 μg of bovine thyroglobin (Sigma, St. Louis, Mo., U.S.A.) and amylase, transferrin and catalase as above.

The proteins are dissolved in 0.1 ml of saline. Human serum can be used as a supplementary reference mixture (Fig. 5). Some proteins (myoglobin, ribonuclease and insulin) do not approach their exclusion limits unless they have been acidified by thorough carbamylation in 2 *M* KCNO at 37° for 3 h. The reaction mixtures (50–100 μl) can be immediately applied together with the other reference proteins on to the gel gradient.

Fig. 5. Band pattern of human serum on large-pore cylinder gradients. The following proteins are easy to identify and can be used for calibration purposes: PA = prealbumin; Alb = albumin; Tf = transferrin; α_2M = α_2-macroglobulin; βLP = β-lipoprotein.

Calibration mixtures for the small-pore cylinder gradients, T = 20–50%, were as follows (Figs. 6 and 7).

(I) 100 μg of bovine insulin B-chain, molecular weight 3400 (Boehringer), 100 μg of ribonuclease A, carbamylated (Boehringer) and 50 μg of human serum albumin (Serva).

(II) 100 μg of β-lactoglobulin, molecular weight of the monomer = 17,900 (Serva), 50 μg of human serum transferrin (Behring-Werke) and insulin B-chain as above.

(III) 200 μg of insulin, carbamylated, molecular weight = 5700 (Serva), 100 μg of soya bean trypsin inhibitor, molecular weight = 21,500 (Serva) and 150 μg of egg trypsin inhibitor, molecular weight = 28,000 (Serva).

(IV) 300 μg of parathyroid hormone, molecular weight = 4100 (Beckman, Palo Alto, Calif., U.S.A.), 50 μg of whale myoglobin, carbamylated (Schuchardt) and 200 μg of acid α_1-glycoprotein, molecular weight = 44,100 (Behring-Werke).

SDS electrophoresis in the second dimension

Immediately after the first-dimensional run, the agarose strips are immersed in

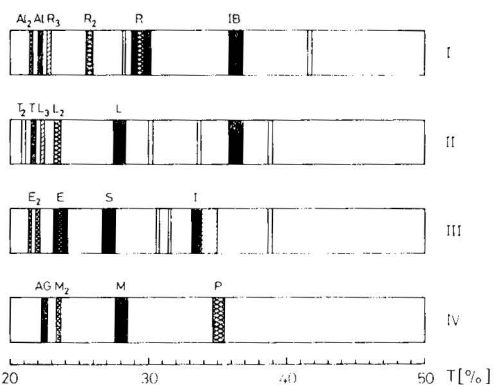


Fig. 6. Band patterns of the calibration mixtures for the small-pore cylinder gradients. The exclusion limits indicated are average values from eight linear gradient experiments. AG = acid α_1-glycoprotein; Al = albumin; E = egg trypsin inhibitor; I = insulin; IB = insulin B-chain; L = lactoglobulin; M = whale myoglobin; P = parathyroid hormone; R = ribonuclease; S = soya bean trypsin inhibitor; T = transferrin.

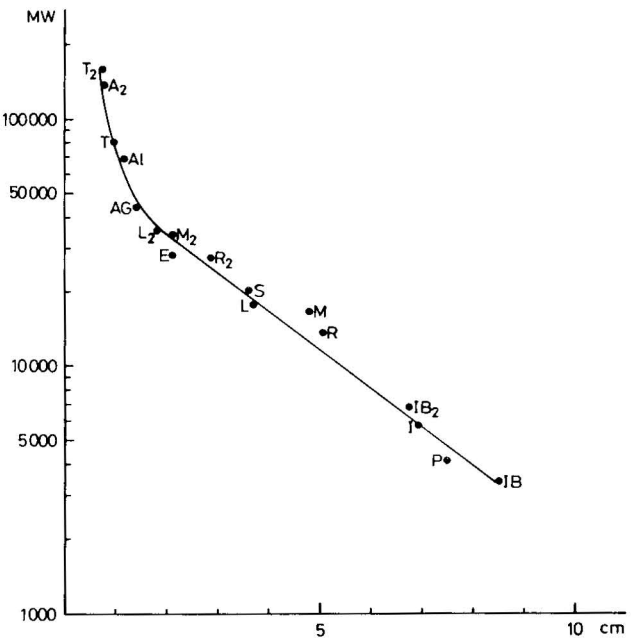


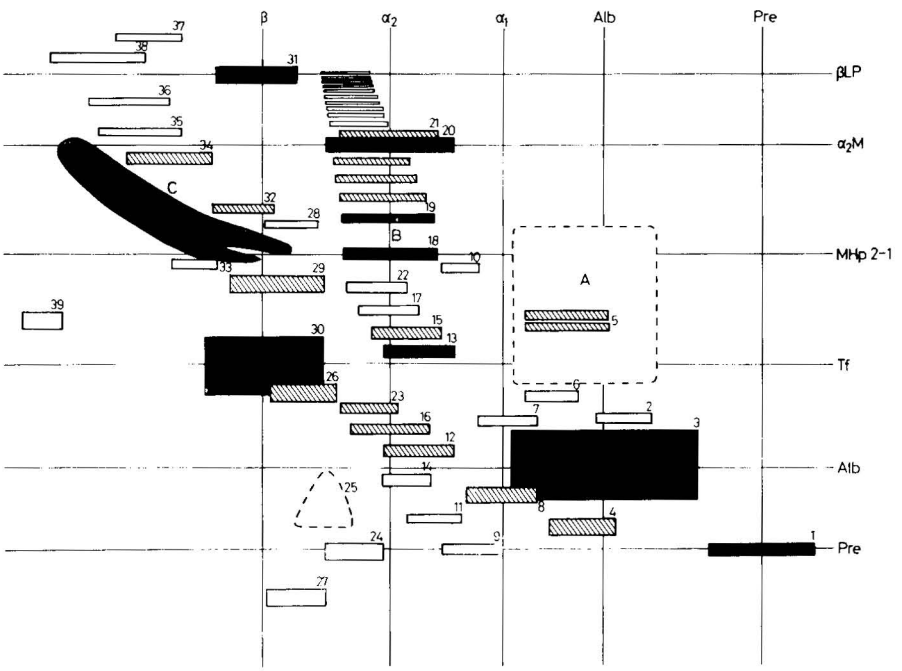
Fig. 7. Representative calibration graph for cylindrical gel gradients, T = 20–50%, obtained with the four calibration mixtures in Fig. 6.

1% SDS for 20 min at 45°. Separation in the second dimension can be performed either on isoporous gel slabs or on 3–25% gradients. As expected, the proteins of γ-mobility now migrate according to molecular weight. The strongly charged heterogeneous immunoglobulin family, which appears as a broad comma-like zone in the native state (Fig. 2), now forms a narrow band. The SDS treatment is not drastic enough to dissociate all composite reference and serum proteins, *e.g.*, the tetrameric prealbumin is only partially dissociated into the half-molecule, and both catalase and ferritin remain completely intact. Most oligomers, however, dissociate (*e.g.*, albumin), and some proteins vanish completely, *e.g.*, the high-density lipoproteins.

DISCUSSION

Two-dimensional techniques[19-27] that are able to single out the majority of individual constituents from a complex mixture of soluble proteins without the addition of extraneous substances such as urea, detergents or polymeric zwitterions, are attractive alternative analytical tools. Well known protein mixtures, *e.g.*, human serum, should be applied to compare the capacity and reliability of salient high-resolution techniques. The detection sensitivity of the two-dimensional agarose/PA gradient technique is *ca.* 1 μg with Coomassie blue staining, corresponding to a single protein level of about 10 mg per 100 ml. There are at least 31 thoroughly studied serum proteins with levels above 10 mg per 100 ml and it is unlikely that the number will increase substantially in the future. Twenty of these proteins have been localized, and there are a further seventeen well marked spots, which have not been or

have only tentatively been identified (see Fig. 8). Apart from these 37 components there are the oligomers of albumin and immunoglobulin G, the variable subfractions of the high-density lipoproteins (3–5) and the haptoglobin polymers (up to 15). It is unrealistic to expect more than 50–60 individual protein bands after conventional staining if one adheres to the "one spot–one protein" condition. The technique is virtually free of hazards, at least on application of serum. There is neither a precipitate left in the agarose gel nor a macromolecular aggregate detectable in the large-pore polyacrylamide gel, and there is no streak visible from the application slit downwards. Several other human body fluids have been tested, but none of them is more complex than serum, whether the fluid is primarily a product of filtration (*e.g.*, urine, cerebrospinal fluid, amniotic fluid, synovial fluid, cyst fluid) or secretion (*e.g.*, tears, colostrum,



1. Prealbumin
2. ?
3. Albumin
4. Acid α_1 - Glycoprotein
5. Albumin - Dimer
6. α_1T - Glycoprotein?
7. α_1 - Antichymotrypsin
8. α_1 - Antitrypsin
9. ?
10. Inter α_1 - Trypsininhibitor?
11. 3,8 S α_2 - Glycoprotein?
12. Gc - Globulin
13. Haptoglobin 1-1
14. Zn - α_2 - Glycoprotein?
15. Ceruloplasmin
16. α_2HS - Glycoprotein
17. ?
18. Monomeric Haptoglobin 2-1
19. Monomeric Haptoglobin 2-2
20. α_2 - Macroglobulin
21. ?
22. ?
23. ?
24. β_2 - Glycoprotein I ?
25. ?
26. Hemopexin
27. β_2 - Glycoprotein III ?
28. ?
29. β_1A - Globulin
30. Transferrin
31. Low Density Lipoprotein
32. β_1C - Globulin
33. Plasminogen ?
34. IgG - Dimer
35. IgG - Trimer
36. IgG - Tetramer
37. ?
38. Immunoglobulin M
39. ?

A High Density Lipoproteins
B Haptoglobin - Polymers
C Immunoglobulins A + G

Fig. 8. Schematic presentation of the normal two-dimensional serum pattern. The following proteins were chosen for constructing the charge/size network: prealbumin, albumin, α_1-antitrypsin, α_2-macroglobulin, β-transferrin and monomeric haptoglobin 2-1 and β-lipoprotein. The variable subfractions of the high-density lipoproteins have been omitted.

milk, seminal plasma). It remains to be seen whether extracts of soluble tissue proteins are more complex than serum.

If isoelectric gel focusing instead of electrophoresis is performed in the first dimension one may separate several hundred spots on application of serum[28], but it seems almost impossible to differentiate microheterogeneities from individual proteins. This is especially true for the numerous spots in the region of the medium-sized α_1/α_2-globulins. Some proteins, *e.g.*, transferrin, may separate into at least twenty charge and size heterogeneous spots, and it is difficult to interpret the frequent pearl necklace appearance of certain bands.

As the minimal resolving capacity on application of serum proteins is ten bands in the first (agarose) and twenty bands in the second dimension (gradient), one should be able to single out 100–200 individual components under optimal conditions. Serum proteins do not distribute evenly over the entire slab area. There are virtually no fast-moving high-molecular-weight proteins and only a few slow-moving small proteins, *e.g.*, γ-trace protein and the β_2-glycoproteins.

The most favourable two-dimensional technique would exploit "pure" molecular properties as isoelectric points, free electrophoretic mobilities, hydrodynamic sizes or molecular weights to obtain a two-axial grid over which the individual components can distribute statistically. Only then is each individual running position equivalent for a twin set of molecular parameters. Electrophoresis on restrictive media, *e.g.*, polyacrylamide gel[19,24], is unsuitable for this purpose as the running position depends on both size and charge. Even low-restrictive polyacrylamide gels prevent the attainment of a focusing equilibrium for the largest serum proteins, *e.g.*, α_2-macroglobulin, IgM and β-lipoprotein[24,29].

The resolving power of various electrophoretic techniques is generally evaluated by subjective standards, and it is desirable to develop some kind of objective parameter to compare various methods. One can use the maximal number of constituents that are clearly discernible within a certain migration path as a provisional criterion if a universally accepted reference mixture, *e.g.*, freshly obtained serum, preferentially of Hp-type 2-1, is applied. By this standard the discontinuous Ornstein–Davis polyacrylamide electrophoresis resolves 28 defined individual serum proteins with an average density of five bands per centimetre on 6.5% gel. The maximal band density decreases with the migration distance from 8 per centimetre in the macroglobulin region to 3 per centimetre in the region of medium-sized α-globulins.

The validity of the running position on gel gradients as a size parameter is based on the premise that the exclusion limit is reached or at least closely approached after long running times. The exclusion limit, as determined by a series of graded homogeneous polyacrylamide gels, can be correlated with the hydrodynamic size of proteins that have an overall globular shape[9]. For this group of "normal" proteins, the molecular weight can be estimated[30–32] provided that the frictional ratio is within the normal range. To give an example: the hydrodynamic volume of the very voluminous but globular immunoglobulin M molecule can be reliably evaluated by exclusion electrophoresis, but the molecular weight is considerably overestimated for its very space-occupying polyp-like structure[33], which explains the unusual high frictional ratio ($R/\bar{R} = 1.97$). Extended molecules such as fibrinogen are characterized by large frictional ($R/\bar{R} = 2.34$) *and* axial ratios (longest axis/shortest axis (a/c) = 7.3), but the error is reduced by molecular alignment during migration through the gel meshwork[9].

The separation principle of gel exclusion (or pore limit) electrophoresis contradicts the postulate that exact molecular weights of native proteins can be determined on gradients. As in partition chromatography, the proteins have to be denatured by urea, guanidine or SDS to eliminate differences in conformation and hydration. Therefore, SDS and exclusion electrophoresis cannot compete in molecular weight determinations, but the latter technique is accurate enough for evaluating the number of SDS-initiated subunits that comprise the native globular protein.

Similarly, the exclusion limit is a more relevant molecular parameter if a separation strategy has to be devised for a particular protein, as both partition and retardation coefficients depend primarily on the hydrodynamic volume and not on the molecular weight. The permeability behaviour of proteins *in vivo* is also controlled by hydrodynamic properties and not the molecular mass[34]. For all practical purposes, however, the molecular weight can be used as an operational size parameter for globular proteins if one considers the underlying restrictions.

The experimental determination of exclusion limits is rather cumbersome and can be replaced with sufficiently long running times on polyacrylamide gradients to obtain an equivalent experimental value. However, proteins of γ-mobility (p$I > 7$) still do not move fast enough to reach their pore limits within reasonable running times. As all standard proteins selected for the two-dimensional technique migrate faster than β-transferrin, proteins such as myoglobin, γ- and β-trace protein or the immunoglobulins cannot be evaluated accurately. This, however, can be overcome if charge differences are wiped out by SDS in the second dimension. In addition, the $T = 3$–25% gradients can still be used for proteins of molecular weight above 30,000, and homogeneous 20% gel slabs are appropriate for the range 5000–30,000 daltons. Even very alkaline proteins such as ribonuclease and cytochrome *c* can then be used as molecular weight markers.

Unfortunately, agarose gel cannot be heated to 100° and the comparatively mild conditions used (30 min at 45°) produce erratic effects on the individual components of a soluble protein mixture, which can be evaluated, however, by a band to band comparison of both patterns. Some proteins remain completely intact (*e.g.*, ferritin), some dissociate partially (*e.g.*, prealbumin) and others strongly. Some body fluids, *e.g.*, urine or milk, contain considerable proportions of low-molecular-weight proteins below 40,000 daltons that have to be differentiated from SDS-initiated subunits of larger molecules. If the nature of each spot has been established by comparison of the two-dimensional patterns, either the cylindrical small-pore $T = 20$–50% gradient or standard SDS electrophoresis may be the method of choice for further examinations.

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SEPARATION OF POLYMYXINS AND OCTAPEPTINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

SHIGERU TERABE*, RYUSEI KONAKA and JUN'ICHI SHOJI
Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku, Osaka 553 (Japan)
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SUMMARY

Seventeen decapeptide antibiotics of the polymyxin group and nine octapeptide antibiotics of the octapeptin group have been successfully separated on a commercial reversed-phase material with tartrate buffer–acetonitrile containing sodium 1-butanesulphonate and sodium sulphate as the mobile phase. All of the components of EM49 (a complex of octapeptins A and B) were preparatively separated by use of a large-diameter column, and the structures of two new components, named octapeptins A_4 and B_4, were deduced from the results of the fatty acid and amino acid analyses.

INTRODUCTION

Polymyxins (Table I), a group of polypeptide antibiotics produced by strains of *Bacillus polymyxa* and related species, have a general structure composed of a cyclic heptapeptide moiety and a side-chain consisting of a tripeptide with a fatty acyl residue. A large number of compounds, which are heterogeneous in acyl and/or amino acid residues, belonging to the polymyxin family have been reviewed by Vogler and Studer[1] and Shoji[2]. All of the polymyxins reported to date can be separated into single components by a counter-current distribution method[3] or thin-layer chromatography[4].

Octapeptins (Table II) have structures similar to those of polymyxins but the side-chains consist of only one amino acid with a fatty acyl residue. EM49[5] (later named octapeptin[6]) has been separated into four major components, EM49α, EM49β, EM49γ and EM49δ, on a CM-cellulose column, but both EM49α and EM49δ are still complexes[7,8].

It is considered that the complete separation of these peptides by high-performance liquid chromatography (HPLC) is very useful for identifying these antibiotics, for determining the relative contents of the components and for examining the purity. Recently a few papers have appeared on the separation of peptide anti-

* Present address: Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

TABLE I

STRUCTURES OF POLYMYXINS

FA → L-Dab → L-Thr → W → L-Dab → L-Dab → X → Y
↑
Z ← L-Dab ← L-Dab

Dab = 2,4-diaminobutyric acid.

Polymyxin	*W*	*X*	*Y*	*Z*	*FA**
M_1	L-Dab	D-Leu	L-Thr	L-Thr	a-C_9
M_2	L-Dab	D-Leu	L-Thr	L-Thr	i-C_8
D_1	D-Ser	D-Leu	L-Thr	L-Thr	a-C_9
D_2	D-Ser	D-Leu	L-Thr	L-Thr	i-C_8
C_1	Dab	Phe	Thr	Thr	a-C_9
C_2	Dab	Phe	Thr	Thr	i-C_8
S_1	D-Ser	D-Phe	L-Thr	L-Thr	a-C_9
E_1 (colistin A)	L-Dab	D-Leu	L-Leu	L-Thr	a-C_9
E_2 (colistin B)	L-Dab	D-Leu	L-Leu	L-Thr	i-C_8
B_1	L-Dab	D-Phe	L-Leu	L-Thr	a-C_9
B_2	L-Dab	D-Phe	L-Leu	L-Thr	i-C_8
B_3	L-Dab	D-Phe	L-Leu	L-Thr	n-C_8
F_1	(Dab (5), Thr (1), Leu (2), Ser (1), Ile (1))				a-C_9
F_2					i-C_8
F_3					n-C_8
T_1	L-Dab	D-Phe	L-Leu	L-Leu	a-C_9
T_2	L-Dab	D-Phe	L-Leu	L-Leu	i-C_8

* a-C_9 = 6-methyloctanoyl; i-C_8 = 6-methylheptanoyl; n-C_8 = octanoyl.

biotics by HPLC[9,10], and Tsji and Robertson[11] have reported the separations of polymyxin B_1 and B_2 and of E_1 and E_2 by reversed-phase chromatography with linear-gradient elution.

The characteristic feature of polymyxins and octapeptins in chromatography is their strong basicity because they have five or six 2,4-diaminobutyric acid (Dab)

TABLE II

STRUCTURES OF OCTAPEPTINS

FA → D-Dab → L-Dab → L-Dab → X → Y
↑
L-Leu ← L-Dab ← L-Dab

Dab = 2,4-diaminobutyric acid.

Octapeptin	*Synonym*	*X*	*Y*	*FA**
A_1	EM49β	D-Leu	L-Leu	a-$C_{11}h^3$
A_2	EM49α	D-Leu	L-Leu	i-$C_{10}h^3$
A_3		D-Leu	L-Leu	n-$C_{10}h^3$
A_4	—	D-Leu	L-Leu	i-$C_{11}h^3$
B_1	EM49δ	D-Leu	L-Phe	a-$C_{11}h^3$
B_2	EM49γ	D-Leu	L-Phe	i-$C_{10}h^3$
B_3		D-Leu	L-Phe	n-$C_{10}h^3$
B_4	—	D-Leu	L-Phe	i-C_9h^3
C_1	333-25	D-Phe	L-Leu	a-C_9h^3

* a-$C_{11}h^3$ = 3-hydroxy-8-methyldecanoyl; i-$C_{10}h^3$ = 3-hydroxy-8-methylnonanoyl; n-$C_{10}h^3$ = 3-hydroxydecanoyl; i-$C_{11}h^3$ = 3-hydroxy-9-methyldecanoyl; a-C_9h^3 = 3-hydroxy-6-methyloctanoyl.

residues. In this study ion-pair reversed-phase chromatographic conditions were employed with success, and the relationship between the order of elution and structure is discussed. Another purpose of this study was to resolve completely all of the components of EM49 and to deduce their structures.

EXPERIMENTAL

Reagents

Polymyxin M[12] was kindly donated by Dr. G. S. Katrukha of the A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry (Moscow, U.S.S.R.), and polymyxin F[13] and EM49[5] by Dr. E. Meyers of the Squibb Institute for Medical Research (Princeton, N.J., U.S.A.). Polymyxin E and B were purchased from Kayaku Antibiotics Research Laboratory (Tokyo, Japan) and Chas. Pfizer (Groton, Conn., U.S.A.), respectively. Other polymyxins[14,15] and octapeptin C_1[16,17] were prepared in the manner reported by Shoji *et al.*[14–17]. All of the polymyxins designated by the same capital letter but with different arabic numeral subscripts were used as mixtures, except for polymyxin T_1.

HPLC-quality acetonitrile (Wako, Osaka, Japan) and reagent-grade sodium 1-butanesulphonate (Eastman-Kodak, Rochester, N.Y., U.S.A.) were used. Water was purified by using an ionic-exchange column, reverse osmosis and finally single distillation.

Apparatus

The liquid chromatograph consisted of a Waters Model 6000A pump, a Rheodyne Model 7120 injector and a Japan Spectrooptics UVIDEC-100 variable-wavelength UV detector. The columns (20 cm × 4 mm I.D. for analytical purposes and 25 cm × 10 mm I.D. for preparative purposes) were packed with Nucleosil $5C_{18}$ (4-mm I.D. column) or $10C_{18}$ (10-mm I.D. column) (Machery, Nagel & Co., Düren, G.F.R.) by the technique recommended by Machery, Nagel & Co. with slurry solvent B (Machery, Nagel & Co.).

Procedure

The peptide antibiotics were dissolved in distilled water to a concentration of about 1 mg/ml for analytical work and 10 mg/ml for preparative work. The solutions were kept at about 4° when not in use. The amounts of sample injected were 5–50 and 500–1000 μg for analytical and preparative work, respectively. All experiments were run at room temperature. The flow-rates were 1.0 ml/min for the 4-mm I.D. column and 4.0 ml/min for the 10-mm I.D. column and the inlet pressures were 1800–2500 p.s.i. for the former and about 1000 p.s.i. for the latter. The detector was operated at 220 nm.

The mobile phase was prepared by mixing the following two solutions in an appropriate ratio to obtain the desired content of acetonitrile: solution A, tartrate buffer (0.005 *M*), pH 3.0, containing sodium 1-butanesulphonate (0.005 *M*) and sodium sulphate (0.05 *M*); solution B, a mixture of equal volumes of tartrate buffer (0.005 *M*), pH 3.0, and acetonitrile, containing sodium 1-butanesulphonate and sodium sulphate in the same concentration as in solution A. The mobile phase was filtered through a membrane filter (1 or 0.5 μm) and degassed prior to use. The con-

tent of acetonitrile in the mobile phase was adjusted in order to control the retention times of the samples. The contents of acetonitrile in the eluent agent are given with the chromatograms.

For the analyses of constituent fatty acids and amino acids of each component of octapeptin A and B, a total amount of about 3.5 mg of EM49 was injected several times and was fractionated into eight components. The pH of each fraction, concentrated to about 5 ml, was adjusted to 9.0 with sodium hydroxide solution and the solution thus obtained was extracted three times with *n*-butanol. The combined *n*-butanol extract was washed with water and evaporated to dryness. The residue was hydrolysed with constant-boiling hydrochloric acid at 110° for 1 h. The hydrolysate was extracted with three 1-ml portions of diethyl ether. The ethereal extract was dried over anhydrous sodium sulphate, evaporated to about 0.2 ml with a slow stream of nitrogen at 25° and treated with two drops of diazomethane solution in diethyl ether.

The methyl esters produced were analysed with a Shimazu GC-7AG gas chromatograph equipped with a hydrogen flame-ionization detector and a glass column (1.6 m × 3 mm I.D.) packed with 15% diethylene glycol succinate polyester on Chromosorb W (80–100 mesh) at 156°. The flow-rate of the carrier gas (nitrogen) was 45 ml/min. The aqueous layer left after the ether extraction was evaporated to dryness and hydrolysed with constant-boiling hydrochloric acid at 110° for 20 h. After evaporation of the hydrochloric acid, the hydrolysate was analysed with a Hitachi KLA-5 automatic amino acid analyser.

RESULTS AND DISCUSSION

The polymyxin group

As polymyxins have five or four unmasked Dab residues, they are strongly basic. Every attempt to separate polymyxins by reversed-phase liquid chromatography with a mixture of acetonitrile and 0.005 *M* phosphate buffer (pH 7.0) or with a mixture of acetonitrile and 0.01 *M* ammonium sulphate as the mobile phase failed to give good resolution because of peak tailing. In contrast, ion-pair reversed-phase liquid chromatography with a mixture of 0.005 *M* tartrate buffer (pH 3.0) and acetonitrile, containing 0.005 *M* sodium 1-butanesulphonate and 0.05 *M* sodium sulphate as the mobile phase, gave very good separations, as shown in Figs. 1 and 2. When sodium sulphate was not added to the mobile phase the resolutions were poorer and the peaks were broader.

Whereas polymyxin S_1[14] and T_1[15] were available as singly isolated compounds, the other polymyxins listed in Table I were obtained as mixtures having different fatty acyl residues. Three typical chromatograms of polymyxins are shown in Fig. 1. There is no doubt that the main peak in Fig. 1A should be assigned to polymyxin S_1. Mainly two peaks were observed in the chromatogram of colistin (polymyxin E) (Fig. 1B), as expected. The assignments of these two peaks were based on the relative peak areas and the relative retention times. Commercial colistin has been reported[18] to contain generally a larger amount of colistin A than colistin B. Colistin A (polymyxin E_1) has a 6-methyloctanoyl residue (a-C_9) and colistin B (polymyxin E_2) has a 6-methylheptanoyl residue (i-C_8), as shown in Table I. As there is no difference in the polypeptide moieties between the two, colistin B is expected to be eluted faster than colistin A. This relative elution order agrees well with that of polymyxin T_1 and T_2,

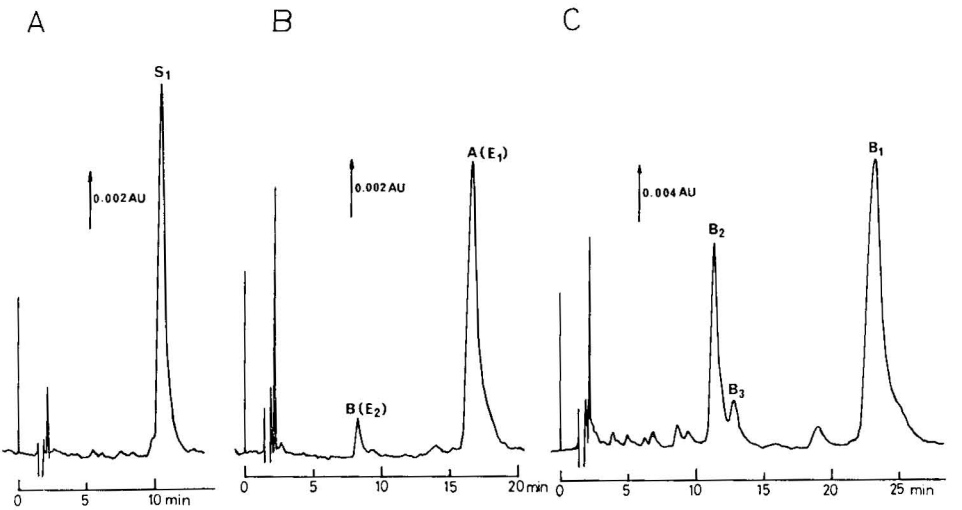

Fig. 1. Chromatograms of polymyxins. Content of acetonitrile in the mobile phase, 22.5%. Column: Nucleosil $5C_{18}$ (20 cm × 4 mm I.D.). Samples: (A) polymyxin S_1 (4.5 μg), (B) colistin (polymyxin E) (10 μg); (C) polymyxin B (33 μg).

whose elution order was confirmed by comparison of two chromatograms of polymyxin T_1 alone and of a mixture of polymyxin T_1 and T_2. Polymyxin B_1 and B_2[3] were similarly assigned as shown in Fig. 1C. Polymyxin B_3[19] is contained in a smaller

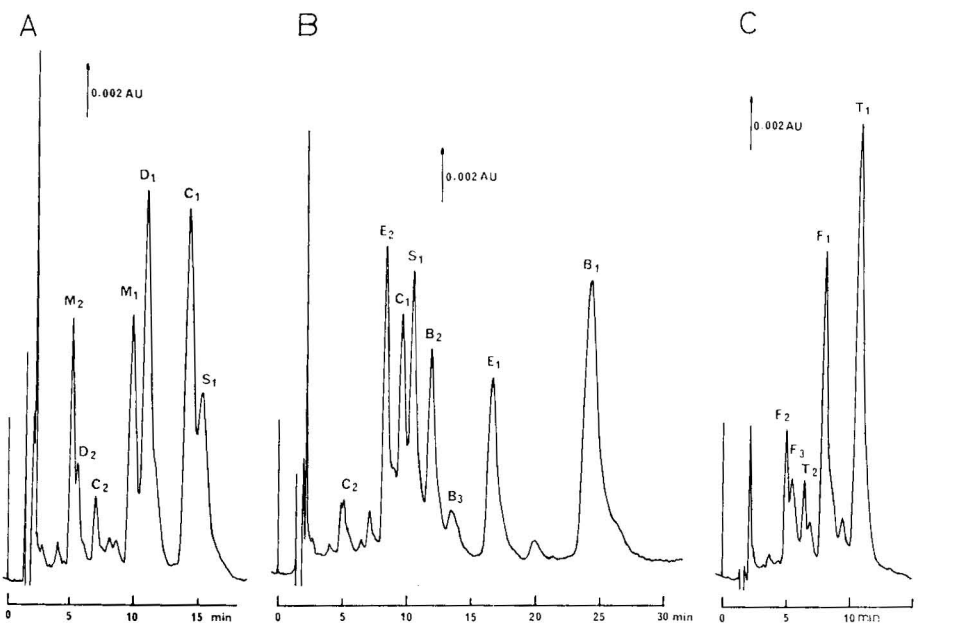

Fig. 2. Separation of polymyxins. Content of acetonitrile in the mobile phase: (A) 21%; (B) 22.5%; (C) 30%. Column: Nucleosil $5C_{18}$ (20 cm × 4 mm I.D.). Sample: (A) a mixture of polymyxin M, D, C and S; (B) a mixture of polymyxin C, E, S and B; (C) a mixture of polymyxin F and T.

amount than the other two and has an octanoyl residue (n-C_8), as shown in Table I. The peptide antibiotic having a 3-hydroxydecanoyl residue (n-$C_{10}h^3$) is eluted slower than that having a 3-hydroxy-8-methylnonanoyl residue (i-$C_{10}h^3$), as described later in the separation of octapeptins under similar chromatographic conditions. A similar result has also been observed in the HPLC of cerexins under reversed-phase liquid chromatographic conditions[20]. Taking into account the above, polymyxin B_3 was assigned as shown in Fig. 1C. Other polymyxins designated by the same capital letter but with different arabic numeral subscripts have similarly been assigned as shown in Fig. 2.

The retention times of the polymyxins listed in Table I varied widely when they were eluted under the same conditions. Therefore, the polymyxins investigated were divided into three groups so as to be eluted with moderate retention times. The three groups were chromatographed under different isocratic conditions and nearly complete separations of all polymyxins were obtained, as shown in Fig. 2. If a more complete resolution is required, the content of acetonitrile in the mobile phase can be reduced. The polymyxin E used to obtain Fig. 2 was of a different batch from that used to obtain Fig. 1B.

The retention times of the polymyxins increased in the order of fatty acyl residues i-C_8 < n-C_8 < a-C_9 if their polypeptide moieties were identical. The contribution of an amino acid residue to the order of elution can be evaluated based on a comparison of the retention times of various combinations of polymyxins with an identical fatty acyl group but different amino acid residues, *e.g.*, polymyxin M_1 and D_1, polymyxin E_1 and B_1, and polymyxin M_1 and E_1. It was concluded from the above considerations that the retention times increase in the order Dab < Ser and Thr < Leu < Phe. The latter order seems reasonable in view of the hydrophobicity of each amino acid residue. However, the former seems conflicting, because Dab is expected to be ion paired with 1-butanesulphonate under the chromatographic conditions used, whereas Ser has a hydroxy group. The most probable explanation of this behaviour is the effect of steric hindrance for ion pairing in the position where different amino acid residues are located, *viz.*, the position designated by W in Table I.

The octapeptin group

Octapeptins (Table II) have related structures to polymyxins and good separations were obtained when chromatographic conditions similar to those for polymyxins were employed. Octapeptin C_1, which has been reported to be a single entity[16], was eluted much faster than the other octapeptins when it was injected together with EM49. EM49 has been reported to consist of six components and to be separated into four major components[7]. However, the HPLC separation gave eight peaks, as shown in Fig. 3B, which was obtained under preparative conditions. The resolution of each peak was virtually identical with that obtained with an analytical column, and the amount injected could be increased to 1000 μg without a significant loss of resolution.

In order to identify the peaks, all of the components were fractionated and each fraction was analysed by a gas chromatograph and an amino acid analyser after hydrolysis (see Experimental). The methyl esters of fatty acids obtained from the acylpeptides were identified based on a comparison of the gas chromatographic retention times between the esters obtained and authentic esters[21]. The results are given in Table III. As the structures of octapeptins have been determined, the assignments of

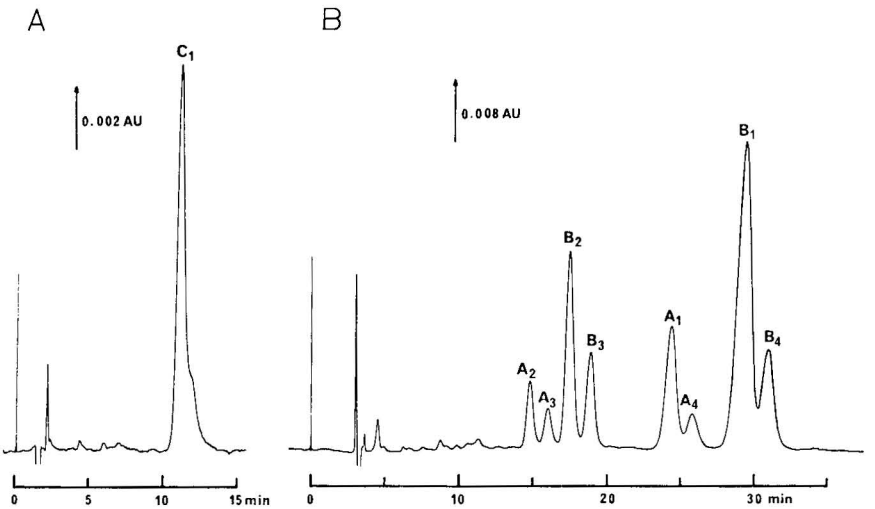

Fig. 3. Chromatograms of octapeptin C_1 and EM49. Content of acetonitrile: (A) 30%; (B) 31%. Column: (A) Nucleosil $5C_{18}$ (20 cm × 4 mm I.D.); (B) Nucleosil $10C_{18}$ (25 cm × 10 mm I.D.). Sample: (A) octapeptin C_1 (5.2 μg); (B) EM49 (a mixture of octapeptin A and B) (250 μg).

octapeptin A_1, A_2, A_3, B_1, B_2 and B_3 are straightforward from Table III. The structures of the remaining two, which were newly found, were deduced by analogy with other octapeptins as shown in Table III. The retention times increased in the order of fatty acyl residues i-$C_{10}h^3$ < n-$C_{10}h^3$ < a-$C_{11}h^3$ < i-$C_{11}h^3$ if the octapeptins had an identical peptide moiety. It can also be concluded that Phe is more hydrophobic than Leu on the basis of the discussion above regarding the polymyxin group. It should be noted that the difference in hydrophobic effects between C_{10} and C_{11} fatty acid residues is much more greater than that between Leu and Phe, *e.g.*, octapeptin B_2 and B_3 are eluted much faster than octapeptin A_1 and A_4.

TABLE III

FATTY ACID AND AMINO ACID ANALYSES ON SEPARATED COMPONENTS OF OCTAPEPTINS (EM49)

Peak *	*Fatty acid*	*Amino acid found (ratio)* **			*Identification*
		Dab	*Leu*	*Phe*	
1	i-$C_{10}h^3$	5.00	3.21 (3)	0.00	A_2
2	n-$C_{10}h^3$	5.00	2.49 (3)	0.00	A_3
3	i-$C_{10}h^3$	5.00	1.91 (2)	1.01 (1)	B_2
4	n-$C_{10}h^3$	5.00	1.76 (2)	0.72 (1)	B_3
5	a-$C_{11}h^3$	5.00	2.77 (3)	0.00	A_1
6	i-$C_{11}h^3$	5.00	2.65 (3)	0.00	A_4 (new)
7	a-$C_{11}h^3$	5.00	1.87 (2)	1.06 (1)	B_1
8	i-$C_{11}h^3$	5.00	1.91 (2)	1.01 (1)	B_4 (new)

* Numbered in order of elution.

** Values in parentheses represent rounded-off ratios.

CONCLUSION

It has been shown that all of the components of polymyxin M, D, C, S, E. B, F and T and octapeptin A, B and C can be separated successfully by use of a chemically bonded C_{18} stationary phase and a mobile phase consisting of a mixture of tartrate buffer and acetonitrile containing sodium 1-butanesulphonate and sodium sulphate. It should be emphasized that even groups of polymyxins and octapeptins that have an identical peptide moiety but isomeric fatty acyl residues can be separated successfully. Two new components, octapeptin A_4 and B_4, have been found in EM49 and their structures have been deduced by the gas chromatographic analysis for fatty acids and by analysis of the amino acids in the hydrolysate.

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CHROM. 11,737

GAS CHROMATOGRAPHY OF TRANSITION METAL DERIVATIVES OF THE SULPHUR ANALOGUES OF 4,4′-(ETHANE-1,2-DIYLDIIMINO)BIS-(PENT-3-EN-2-ONE)

E. PATSALIDES and B. J. STEVENSON

Research School of Chemistry, Australian National University, P.O. Box 4, Canberra 2600, A.C.T. (Australia)

and

SERGIO DILLI

Department of Analytical Chemistry, School of Chemistry, University of New South Wales, P.O. Box 1, Kensington 2033, N.S.W. (Australia)

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SUMMARY

The thermoanalytical and gas chromatographic behaviour of the copper(II), nickel(II), zinc(II), cadmium(II), palladium(II) and platinum(II) chelates of 4,4′-(ethane-1,2-diyldiimino)bis(pent-3-ene-2-thione) and the copper(II) and nickel(II) chelates of 4-[(3-oxo-1-methylbut-1-enyl)aminoethylamino]pent-3-ene-2-thione are reported here for the first time. With the exception of the cadmium chelate, the derivatives elute as sharp, symmetrical peaks at microgram levels without extensive decomposition. Detection limits with flame ionization detection were in the range 0.2–1.5 ng of metal. A nearly complete separation of five chelates of the first ligand is demonstrated. The use of this reagent for the simultaneous determination of copper, nickel and zinc has been examined.

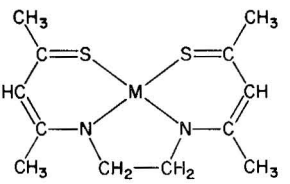
INTRODUCTION

Following the extensive gas chromatographic (GC) studies of metal β-diketonates and their useful but limited applications in quantitative analysis, attention has focussed on the derivatives of other ligands. Among those studied[1] are the chelates of dialkyldithiocarbamic and dialkyldithiophosphoric acids, mono- and dithio-β-diketones, bi- and tetradentate β-keto enamines and salicylaldimines. These classes of ligands, however, have not been extensively studied and their potential as gas chromatographic reagents has still to be clearly established.

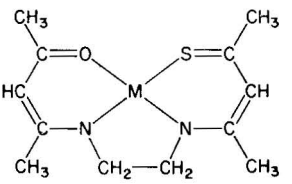
At present, the most promising reagents for analytical purposes, apart from the β-diketones, are the tetradentate β-keto enamines, particularly those with fluoroalkyl substituents such as 1,1,1,1′,1′,1′-hexafluoro-4,4′-(ethane-1,2-diyldiimino)bis-(pent-3-en-2-one). A wide variety of such ligands and their chelates have been prepared and studied by GC[1–4]. The application of these selective reagents has, furthermore,

extended the range of elements determinable at 10^{-9} to 10^{-12} g to include copper, nickel, palladium and vanadium. Their value, thus, is supplementary to the β-diketones which are suited to the determination of elements such as beryllium, aluminium, chromium, cobalt and rhodium. Despite the excellent GC characteristics of the β-keto enamine chelates, relatively little has appeared on analytical applications although several studies[5-7] have established the value of these reagents in quantitative trace analysis.

In this paper an assessment of the sulphur-containing ligands 4,4′-(ethane-1,2-diyldiimino)bis(pent-3-ene-2-thione) and 4-[(3-oxo-1-methylbut-1-enyl)aminoethylamino]pent-3-ene-2-thione as GC derivatizing reagents is presented. Their chelates (I and II, respectively) are related to the β-keto enamine derivatives by the replacement of one or both of the oxygen donor atoms with sulphur.



I



II

M = Cu(II), Ni(II), Zn(II), Cd(II), Pd(II), Pt(II)

Although tetradentate β-keto enamines have been known for a long time[8], the analogous β-thiono enamines have only recently[9-11] been prepared. Few of the properties of such compounds and their chelates have been reported and this paper is an initial study of both the thermoanalytical and gas chromatographic properties of chelates of I and II. The potential of the β-thiono enamines lies in their selectivity for derivatives of b-class ions such as palladium(II) and platinum(II) so that this type of ligand may be valuable in the extraction and gas chromatographic determination of these ions. As a group these may also be suitable reagents for zinc(II) and cadmium-(II) which cannot at present be determined by GC at the nanogram level.

EXPERIMENTAL

Preparative

The ligands 4,4′-(ethane-1,2-diyldiimino)bis(pent-3-ene-2-thione), DT-AAED, and 4-[(3-oxo-1-methylbut-1-enyl)aminoethylamino]pent-3-ene-2-thione, MT-AAED, were isolated from products of the reaction using 4,4′-(ethane-1,2-diyldiimino)-bis(pent-3-en-2-one), AAED, in a method originally described[11] for DT-AAED. The ligands were separated on a column (25 × 5 cm O.D.) of silica gel (Merck type 60 for TLC) using 4-g lots of crude reaction product. DT-AAED was eluted with benzene–ethyl acetate (9:1, v/v) and MT-AAED with benzene–ethyl acetate (1:1, v/v). After removal of solvent each residue was purified by recrystallizing twice from methanol. The ligands are characterized as follows. DT-AAED: bright yellow needles, corrected m.p. 150° (lit.[11] 140–141°). $C_{12}H_{20}N_2S_2$ requires C 56.2, H 7.9, N 10.9, S 25.0%; found C 56.0, H 7.9, N 10.8, S 24.9%. mol. wt.: calculated 256.4; found 251 (by vapor

pressure osmometry in acetone at 25°). MT-AAED: bright yellow needles, corrected m.p. 112°. $C_{12}H_{20}N_2OS$ requires C 60.0, H 8.4, N 11.7, S 13.3%; found C 60.0, H 8.4, N 11.6, S 13.2%. mol. wt.: calculated 240.0; found 241.

The copper and nickel chelates of AAED and MT-AAED and the zinc, copper, nickel, cadmium and palladium chelates of DT-AAED were prepared and purified by methods similar to those described[3,10] in the literature. The platinum(II) chelate of DT-AAED, which has not been reported previously, was obtained in less than 10% yield as orange needles (m.p. 348°) by the reaction of potassium tetrachloroplatinate-(II) with ligand in methanol. The chelate was separated from other products on a column of silica gel and recrystallized from a mixture of dichloromethane and tetrachloromethane. The copper(II) and nickel(II) chelates of MT-AAED, which also have not been reported previously, were obtained as brown needles (m.p. 188°) and green needles (m.p. 242°), respectively, whereas the zinc(II) derivative could not be obtained using the above methods. The identity of all chelates was confirmed by mass spectrometry and the purity established by analysis for carbon, hydrogen, nitrogen, sulphur and metal content.

Instrumentation

Thermoanalytical data were obtained on a Rigaku Denki Thermoflex Model M8076 combined DTA/TG instrument using a temperature scan rate of 5°/min. A quantity of 7.5 mg of each compound in an aluminium cup was used for each run. Gas flow (nitrogen) through the inner silica furnace tube was fixed at 100 ml/min.

A Varian Model 1700 gas chromatograph equipped with a flame ionization detector (FID) and a Hewlett-Packard 3380A integrator was used. Dry, commercial high-purity nitrogen was employed as carrier gas (30 ml/min). The temperatures of the injection block, column and detector were 240°, 230° and 240°, respectively, unless stated otherwise.

The columns employed were borosilicate glass coils (3 ft. × $\frac{1}{4}$ in. O.D.) packed with Chromosorb 750 or Gas-Chrom Q (80–100 mesh, AW DMCS) coated with 3% w/w SE-30 and OV-101, respectively. Columns were conditioned for 2 days at 250° then silylated with both HMDS and BSTFA at 180° (200 μl each) and conditioned at 250° for a further day before use. Chelate solutions were injected onto the columns through a borosilicate glass insert. To minimize interaction of chelate vapors with hot metal surfaces the connection between the column outlet and detector was replaced with one which was glass-lined.

Chelate solutions

Each chelate or ligand (5 mg) was prepared initially in dichloromethane (10 ml) as a 0.05% solution. Further dilution with carbon disulphide provided solutions containing 0.5, 0.05, 0.005 μg chelate for each injection (1 μl). Detection limits, tailing indices and calibrations were obtained by making serial injections beginning with the most concentrated solution.

Examination of chelates eluted from the GC column

To determine whether chelates eluting from the column were decomposing at 230° samples were collected at the detector. For this purpose the detector flame was extinguished and the collector (anode) removed. A borosilicate glass tube

(15 cm × 3 mm O.D.) was inserted over the detector jet by means of a PTFE sleeve. The tube, extending above the detector, was cooled with dry-ice pellets. Chelate was injected onto the column (10 × 10 μg) and any condensed material washed into a small vial with several drops of dichloromethane. The solution was examined by chromatography on silica gel layers with ethyl acetate–benzene (1:9, v/v) and by mass spectrometry.

Procedure for simultaneous extraction of zinc, copper and nickel ions as DT-AAED derivatives

A solution containing 10 μg/ml each of zinc, copper and nickel was prepared by dilution of stock solutions (1000 μg/ml). For construction of a calibration curve 0.0, 2.0, 4.0, 8.0 and 10.0-ml aliquots of the substock solution were transferred to 50-ml beakers, evaporated nearly to dryness and treated with 10% (w/v) sodium acetate in methanol (3.0 ml) and 1% (w/v) DT-AAED in acetone (2.0 ml). The solutions were then heated on a hot plate for 20 min at 50° and adjusted to a final volume of 5 ml with methanol. After cooling, distilled water (25 ml) was added and the solutions extracted by shaking for 2 min with carbon disulphide (4.0 ml) containing 0.002% w/v of triacontane as internal standard. Suitable aliquots of "unknown" solutions were processed using the same procedure. Extracts were analysed directly by gas chromatography at 230° on a column of 3% OV-101 on Gas-Chrom Q.

RESULTS AND DISCUSSION

Thermoanalytical properties

The characteristic feature of MT-AAED, DT-AAED and several of their chelates was a low thermal stability suggesting that such compounds were not suitable for GC. In contrast to AAED, for example, which volatilizes without appreciable decomposition[3] at temperatures above 300°, MT-AAED and DT-AAED decomposed

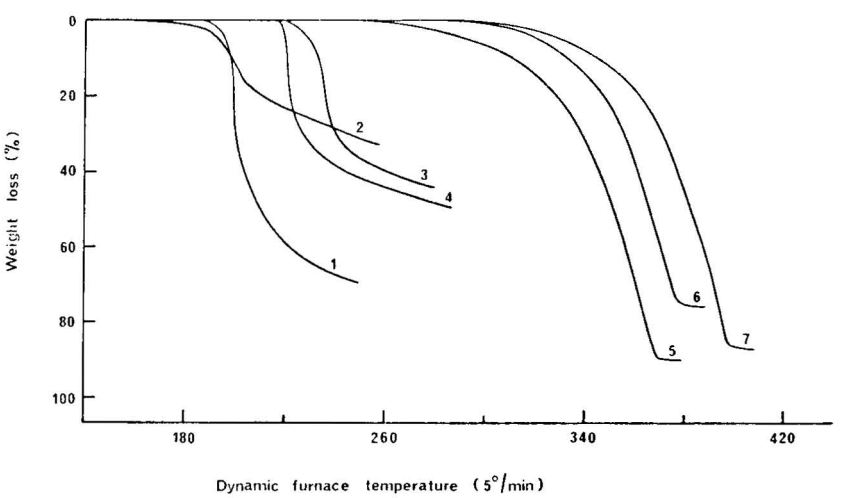


Fig. 1. Thermogravimetric curves for DT-AAED (1) and its chelates with Cd(II) (2), Zn(II) (3), Cu(II) (4), Ni(II) (5), Pd(II) (6) and Pt(II) (7).

exothermally at 212° and 198°, respectively, without volatilizing. From the thermoanalytical data in Fig. 1 and Table I, it is evident that the DT-AAED compounds examined fall into two distinct groups with respect to thermal stability. In the first, the nickel, palladium and platinum chelates are stable to 300° whereas in the second group, the free ligand and the zinc, cadmium and copper chelates are considerably less stable and decompose exothermally at temperatures in the range 185–200°. In the latter group, the chelates decompose on heating to give volatile organic products and dark solids as residues. Above 300° the nickel, palladium and platinum chelates give orange or red-orange sublimates which do not resemble the original compounds. Examination of these sublimates on silica gel layers revealed that, in addition to predominantly undecomposed chelate, an intense red band was also obtained in each case. Mass spectral examination of this band after isolation from silica gel layers showed no evidence of dehydrogenated chelate[3,12] but contained organic pyrolysis products* whose identity could not be readily established.

TABLE I

PHYSICAL AND CHEMICAL TRANSFORMATIONS IDENTIFIED BY DTA

Abbreviations: f = fusion; v = volatilization; d = decomposition; exo = exothermic; endo = endothermic.

Compound	*Temperature (°C)*
MT-AAED	113f, 212 d (endo)
Copper chelate	188f, 235 d (exo)
Nickel chelate	242f, 240–310 (v)
DT-AAED	154f, 198 d (exo), 200 d (endo)
Copper chelate	187? 198f, 220 d (exo), 224 d (endo)
Nickel chelate	265f, 260 370 v (d)
Zinc chelate	236 d (exo)
Cadmium chelate	187 d (exo)
Palladium chelate	316f, 300–380 v (d)
Platinum chelate	348f, 320–400 v (d)

The contrast in thermal stabilities suggests a fundamental difference in the chemical bonding of the two groups and this, in turn, may be related to differences in the nature of the sulphur–metal σ and d_π–d'_π bonds. Features which may serve to lower thermal stability in chelates or ligands containing sulphur in place of oxygen are the greater size and polarizability[13] of the former. Thus, due to its greater nucleophillic character compared to oxygen, sulphur may more readily initiate pyrolytic decomposition in compounds. In addition, sulphur forms extraordinarily stable sulphides with b-class metal ions, which may be produced when the chelates are heated to high temperatures. Evidence for the production of sulphides in DT-AAED and its copper, zinc and cadmium chelates is suggested in the highly exothermic nature of the

* As an example, in the platinum chelate of DT-AAED the extract of the red band gave a mass spectrum with intense peaks at m/z 306, 223, 206, 149 and 91, absent in the spectrum of the pure chelate. Since these peaks did not have an isotopic pattern suggesting the presence of platinum these must be due to organic degradation products.

decompositions. In contrast, MT-AAED, which contains only one sulphur atom per molecule, decomposes endothermally although its copper chelate decomposes exothermally (see Table I). Similar highly exothermic decompositions have been observed with certain β-keto enamine chelates[3] in which the β-diketone moiety contains halogen substituents in the α-positions, and in monothio β-diketonates[14,15]. In these compounds decomposition also appears to be initiated by elimination of a very stable compound. For the nickel, palladium and platinum DT-AAED chelates stabilization may result[13] from strong sulphur–metal bonding due largely to enhanced d_π–d'_π bonding which could lower the tendency for the formation of metal sulphides and decrease the likelihood of decomposition. These chelates, in fact, do not undergo exothermic decompositions although some decomposition occurs during volatilization, albeit at temperatures above 300°.

Assuming a lower dipole moment for the sulphur-containing chelates it can be anticipated that replacement of sulphur for oxygen in AAED would enhance the volatility of the corresponding chelates of MT-AAED and DT-AAED. However, as is clearly shown in Fig. 2, the reverse trend was obtained in the case of the nickel chelates. Furthermore, volatility decreases stepwise as the number of sulphur atoms in the chelate increases. Comparative data for other chelates containing sulphur is not available although evidence[1] suggests that a similar trend occurs in derivatives of mono- and dithio-β-diketones. Since the chelates cannot depart greatly from a square-planar geometry, it is possible to explain volatility effects largely in terms of the high polarizability of the sulphur atom. Sulphur can directly affect chelate volatility by participating in intermolecular metal–sulphur interactions similar to the metal–oxygen interactions previously[3] invoked. Moreover, it may also increase electron density in the π-resonance systems of the chelate rings and thereby increase overall polarizability in the chelate molecules and lower volatility. Such considerations suggest that bulky or electron-withdrawing substituents in the chelate would give derivatives of greater volatility more suited to GC.

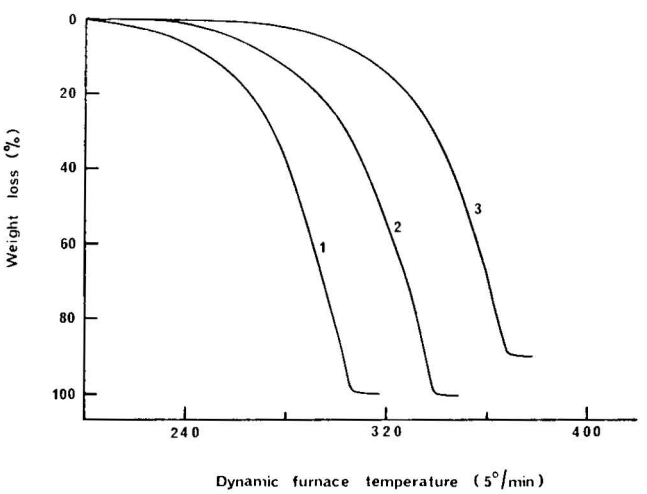


Fig. 2. Thermogravimetric curves for the Ni(II) chelate of AAED (1), MT-AAED (2) and DT-AAED (3).

Gas chromatographic behaviour

In addition to a lower volatility, the thio-chelates had greater retention on SE-30 than the corresponding AAED derivatives. The relative retention of the copper and nickel chelates of the three ligands is shown in Fig. 3. For the DT-AAED chelates column temperatures of 230–240° were generally required to maintain retention times below 20 min. At such temperatures, although rapid decomposition was expected for the less stable derivatives (see Table I), the zinc, copper, nickel, palladium and platinum chelates of DT-AAED eluted from the column without apparent decomposition. As is shown in Fig. 4a, the peaks at the 5-μg level were relatively sharp and had minimal tailing. Nearly complete separation of the five chelates was obtained on SE-30, however, the copper and nickel chelates were only partly resolved ($R = 0.7$) on this stationary phase. In the case of the cadmium chelate (see Fig. 5) decomposition was evident from the chromatogram. The broad double peak obtained for this compound indicated that an on-column reaction was occurring during its elution.

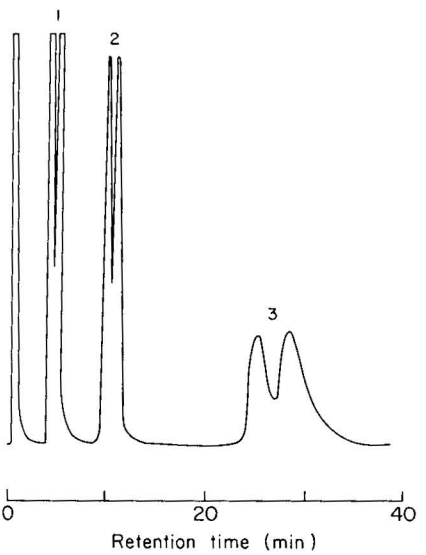


Fig. 3. Chromatogram of a mixture of the Cu(II) and Ni(II) chelates of AAED (1), MT-AAED (2) and DT-AAED (3). For each ligand the copper derivative elutes in advance of the correspondingly nickel(II) derivative. Column, 3% SE-30 on Chromosorb 750; column temperature, 220°.

The reactive nature of the chelates was revealed in their gas chromatographic behaviour. Although several columns gave sharp, symmetrical peaks others, inexplicably, gave broadened, tailing peaks. The repeated introduction of palladium or platinum chelates onto the column also caused a gradual deterioration in the shape and resolution of the peaks as illustrated in Fig. 4b. Furthermore, this deterioration appeared to be irreversible since improvement could not be achieved by re-silylation and conditioning at high temperature. In one instance, the injection of small quantities of crude extract of a platinum chelate permanently destroyed the quality of the column. This behaviour, which has serious implications for possible analytical applications, can be interpreted in terms of decomposition of the chelates to metallic palladium or

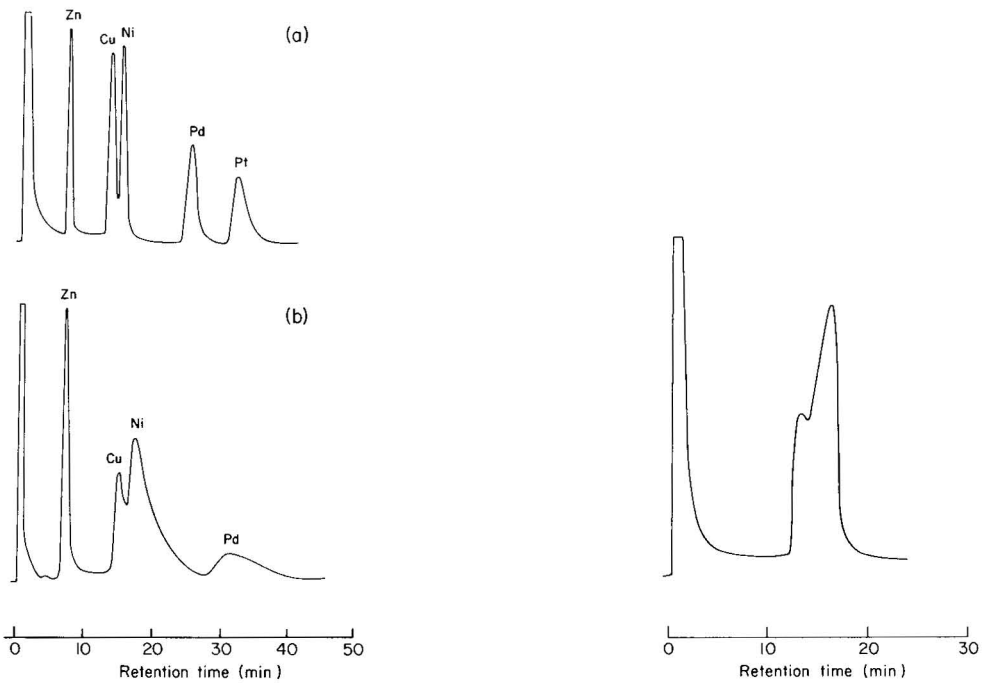


Fig. 4. Chromatogram of a mixture of the Zn(II), Cu(II), Ni(II), Pd(II) and Pt(II) chelates of DT-AAED. a, Obtained on a fresh column; b, the irreversible deterioration in peak shapes resulting from the introduction of palladium or platinum chelates onto the column. Column, 3% SE-30 on Chromosorb 750; column temperature, 230°.

Fig. 5. Chromatogram of the Cd(II) chelate of DT-AAED. Conditions as in Fig. 4.

platinum which may then catalyse the decomposition of chelates subsequently injected into the column. Indeed, the ability of these metals to promote the dehydrogenation of β-keto enamine chelates has been demonstrated[3,12] and it is possible that similar reactions occur with the more reactive β-thiono enamine analogues. Consistent with this interpretation when only zinc, copper and nickel chelates were introduced into the columns, no deterioration in the peak shapes was obtained even after extensive use of the column.

Recovery of chelates eluted at 230° established that decomposition occurred to some extent even for the more stable nickel, palladium and platinum chelates. Examination of recovered zinc, copper, nickel, cadmium and palladium chelates on silica gel layers revealed numerous additional compounds. For example, the zinc chelate, normally only a yellow band with R_F 0.45, gave a red band (R_F 0.75), a yellow band (R_F 0.64) and a brown band (R_F 0.0), also. In the mass spectrum of this material intense peaks not present in the spectrum of the pure chelate were observed at m/z 362, 360, 314, 312 and 310.

It was possible to detect nanogram quantities of DT-AAED chelates eluting from the column. Detection limits given in Table II were not limited by adsorption but by the detector noise at high sensitivity, however, a linear response was obtained in the range 5–0.005 μg for each of the chelates in Table II. The degree of peak tailing

depended markedly on the quality of the column but little difference was observed between the various chelates. Tailing indices[16] obtained for the zinc and nickel derivatives with SE-30 on Chromosorb 750 as support are shown in Table III and indicate a considerable degree of tailing at the lower level of 0.05 μg. Little or no tailing was obtained at this level on the column with OV-101 on Gas-Chrom Q and this was used for analytical purposes.

TABLE II

DETECTION LIMITS (FID) FOR CHELATES OF DT-AAED

Detection limits are based on a twice noise level criterion.

Metal	*Detection limit (ng)*	
	Chelate	*Metal*
Zinc	5	1
Copper	5	1
Nickel	1	0.2
Palladium	5	1.5

TABLE III

TAILING INDICES FOR THE ZINC AND NICKEL CHELATES OF DT-AAED

Metal	$T^{5}_{0.1}$	$T^{0.5}_{0.1}$	$T^{0.05}_{0.1}$
Zinc	1.0	1.8	20
Nickel	1.2	6	18

Simultaneous extraction and analysis of zinc, copper and nickel

In ascertaining the feasibility of using GC for determining zinc, copper and nickel simultaneously, solutions containing known concentrations of each metal in the range 10–100 μg/ml were prepared and analysed following conversion to the DT-AAED derivatives. Derivatization involved reaction of the metal ions with ligand in methanol containing excess sodium acetate. Following the addition of water and extraction with carbon disulphide, the extracts, found to be stable for up to 6 days, were analysed directly by GC (see Fig. 6). Comparison of peak areas obtained for the extracts, with those of solutions of the purified, solid chelates showed that greater than 90% recovery was attained for each ion.

Various difficulties were experienced in the analysis of the metal ions. When the concentrations of the copper and nickel derivatives differed by greater than a factor of about five it became difficult to obtain accurate area measurements for the minor component. Tailing of the ligand peak also interfered with the determination of zinc. This interference appeared difficult to avoid since excess ligand was required to ensure complete derivatization of the ions. Furthermore, unlike many β-diketones, DT-AAED is insufficiently acidic to be removed from the organic extract by washing with aqueous sodium hydroxide. For the chromatogram shown in Fig. 6, ligand concentration 50-fold in excess of the total ion concentration was employed. It is important that the ligand should not exceed 100-fold excess concentration because of the difficulty in measuring the areas of early peaks. At the sensitivity used in the analyses, inter-

ference was also experienced from the tailing peaks of solvents such as dichloromethane, nitromethane and ethyl acetate. To avoid this, it was convenient to use carbon disulphide which gave both a low FID response and satisfactory extraction.

Fig. 7 shows that a linear calibration curve can be obtained in the range 0–25 μg/ml of metal provided a fresh column was used for each chelate. The concentrations of the metals found by GC shown in Table IV, however, were in poor agreement with the actual concentrations and re-examination of the procedure revealed an unacceptable reproducibility for the copper chelate peak which became more erratic with continued use of the column. Since each concentration was expressed as a percentage of the total peak area, this also probably explains the results obtained for nickel and zinc. As observed also in studies[16] of the fluorinated β-diketonates of oxovanadium(IV), peaks corresponding to the copper derivative were obtained when solutions of the pure ligand were injected onto the column. This clearly suggested that reactions were occurring in the injection port and, indeed, inspection of the column revealed a black deposit on the glass wool plug at the inlet. Evidently the deposit, formed from pyrolysis of the copper chelate, was reacting with excess ligand in the extracts subsequently injected onto the column, regenerating the chelate which then eluted with the analyte to give an erroneous result.

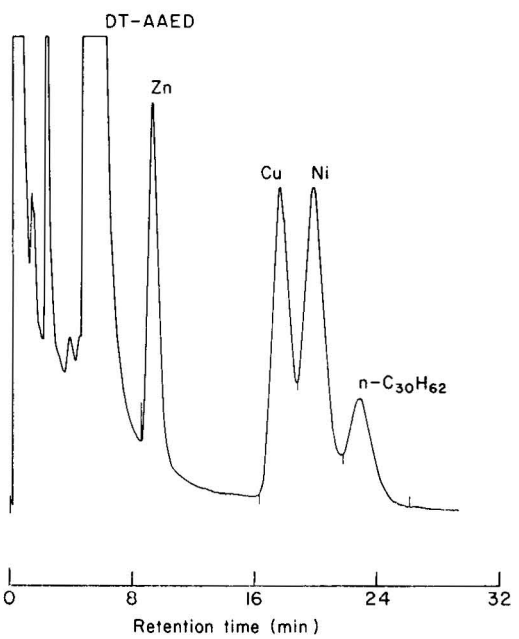


Fig. 6. Chromatogram of the Zn(II), Cu(II) and Ni(II) chelates of DT-AAED following derivatization and extraction with carbon disulphide. Peaks for each chelate correspond approximately to 20 ng of metal. Column 3% OV-101 on Gas-Chrom Q. Conditions as in Fig. 4.

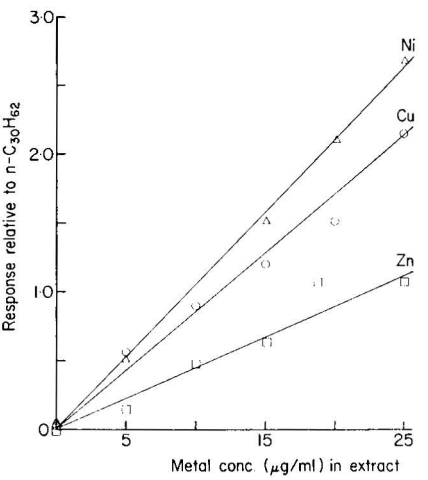


Fig. 7. Calibration curves for zinc, copper and nickel extracted as the DT-AAED derivatives for concentrations in the range 0–25 μg/ml of each ion in the carbon disulphide extract. Column and conditions as in Fig. 6.

TABLE IV

GAS CHROMATOGRAPHIC ANALYSIS OF SOLUTIONS CONTAINING ZINC, COPPER AND NICKEL IONS

Solution	*Actual concn. (μg/ml)*			*Found by GC (μg/ml)*		
	Zn	*Cu*	*Ni*	*Zn*	*Cu*	*Ni*
1	40.0	20.0	20.0	38.6	17.8	27.0
2	20.0	50.0	10.0	23.0	54.8	4.0
3	10.0	30.0	50.0	4.0	20.0	30.0

As this problem is not readily overcome the above procedure cannot, at this stage, be recommended as a practical method for the simultaneous determination of the three metals although it may be useful for the analysis of nickel and zinc in the absence of copper. Moreover, the use of MT-AAED as an alternative reagent whose derivatives are more volatile and elute at lower column temperatures seems handicapped. Like AAED, MT-AAED does not appear to produce a zinc chelate and a peak corresponding to this derivative was not observed in the extracts even through the copper and nickel compounds appeared to elute normally. Currently, investigations of other tetradentate β-thiono enamines are progressing with a view to identifying more suitable reagents.

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URINE ANALYSIS OF PLATINUM SPECIES DERIVED FROM *cis*-DICHLORODIAMMINEPLATINUM(II) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOLLOWING DERIVATIZATION WITH SODIUM DIETHYLDITHIOCARBAMATE

STEVE J. BANNISTER, LARRY A. STERNSON* and A. J. REPTA

Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kan. 66045 (U.S.A.)

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SUMMARY

A clinically useful method is described for the quantitative analysis of platinum species derived from *cis*-dichlorodiammineplatinum(II) in urine. The drug and its biodegradation products are derivatized directly in urine by reaction with sodium diethyldithiocarbamate (DDTC) to form a common product, a 2:1 DDTC–platinum adduct. This complex is stable and can be quantitatively extracted into 0.1 volumes of chloroform. An aliquot of the chloroform layer is then subjected to high-performance liquid chromatography on a μBondapak CN column and the eluent monitored spectrophotometrically at 254 nm. At this wavelength the DDTC–platinum adduct has a molar absorptivity of 43,000, and platinum levels of 25 ng/ml of urine can be detected with a precision of ± 2.5% and an accuracy of ± 4%.

INTRODUCTION

cis-Dichlorodiammineplatinum(II) (CDDP) has generated ever increasing interest as an anti-tumor agent since Rosenberg *et al.*[1] demonstrated its cytotoxic effects on *Escherichia coli*. CDDP is now regarded as an effective agent in the treatment of a wide variety of malignant solid tumors including testicular, ovarian, osteosarcoma, head and neck and other solid neoplasms[2,3].

Plasma levels of free circulating platinum decline rapidly after i.v. administration of the drug as it is cleared by the kidneys or alternatively becomes tightly bound to plasma protein[4,5]. Methods for measurement of urinary platinum excretion were sought by this laboratory as part of an effort to elucidate the human pharmacokinetics of CDDP after intravenous administration.

Non-flame atomic absorption spectrometry (NFAAS) is currently the most widely used technique[6,7] for determining non-radiolabelled platinum in biological samples and responds to trace (parts per 10^9) levels of platinum. The accuracy and precision of NFAAS, however, is highly dependent on the sample matrix. We have

* To whom correspondence should be addressed.

similarly found that wavelength dispersive X-ray fluorescence measurements[8] are subject to dramatic matrix effects. Since urine is highly variable in its physical properties and chemical content, the analysis of total platinum in untreated urine is subject to variance and irreproducibility in analytical results. The present study was directed toward the development of a clinically useful analytical method for platinum in urine with the high sensitivity of NFAAS but with a broader linear range and less interference from and dependence on the sample matrix.

Initially, the extractability of CDDP and its *in vivo* reaction products from urine into water-immiscible organic solvents was investigated as a means for circumventing the matrix effects. This approach was concluded to be unfeasible, however, due to the poor partition characteristics of the analytes between water and common organic solvents and their instability in many of these solvents. Accordingly, a reagent was sought which, when added to urine, would react quantitatively with CDDP and any other platinum species formed from CDDP *in vivo* to form a single stable platinum-containing product which would be extractable into a water-immiscible solvent. Such a product would subsequently be separated from co-extracted potential interferents by high-performance liquid chromatography (HPLC) and should have properties permitting its detection at low ng/ml levels by common detection devices which could be interfaced with the chromatograph.

EXPERIMENTAL

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000-A solvent delivery system, Model U6K injector and Model 440 absorbance detector operated at 254 nm. Detection at wavelengths other than 254 nm was done using a Varian (Palo Alto, Calif., U.S.A.) Vari-Chrom variable-wavelength liquid chromatography detector. Columns (30 cm × 1/4 in. O.D.) were all obtained from Waters and included μBondapak C_{18}, μBondapak alkyl phenyl, μBondapak NH_2 and μBondapak CN.

UV–Visible spectrophotometry was performed using a Cary (Palo Alto, Calif., U.S.A.) 219 spectrophotometer and 1-cm quartz cells. NFAAS was carried out with a Varian/Techtron 175 atomic absorption spectrometer with CRA-90 carbon-rod atomizer.

Materials

cis-Dichlorodiammineplatinum(II) was obtained from the National Cancer Institute (Bethesda, Md., U.S.A.) and was used without further purification. Sodium diethyldithiocarbamate (DDTC) was purchased from Sigma (St. Louis, Mo., U.S.A.) and was washed with chloroform, filtered under nitrogen, dried under reduced pressure and stored at −10°. Chloroform was Fisher spectral grade (Fisher Scientific, Fair Lawn, N.J., U.S.A.). Methanol, isopropanol and heptane were all Fisher HPLC Grade. Water was distilled after mixed-bed deionization and had a specific conductance of 1.0–1.3 μS. Human urine was obtained from volunteers not undergoing CDDP therapy. McIlvaine buffer (0.2 *M*; pH 7.5) was used in studies parallelling those carried out in urine.

Synthesis of diethyldithiocarbamate derivative of CDDP

CDDP (0.17 mmol; 50 mg) was dissolved in 200 ml water. Sodium diethyldithiocarbamate (DDTC) (*ca.* 23 mmol; 4 g) was added and the solution stirred for 24 h. The resulting precipitate was removed by filtration and recrystallized from methanol–chloroform (9:1). Elemental analysis (%) of the yellow crystals: calculated for $Pt(DDTC)_2$, C 24.43, H 4.10, N 5.70, S 25.09, Pt 39.68; found, C 24.88, H 4.65, N 5.35, S 25.87, Pt 39.18.

Derivatization of platinum in urine

Urine or buffer (9 ml) containing 0.25–250 μg of CDDP was placed in a 15-ml conical centrifuge tube. One milliliter of a 10% (w/v) solution of diethyldithiocarbamate prepared in 0.1 *N* sodium hydroxide solution and 2 ml of an aqueous, saturated solution of sodium nitrate were added to the tube, which was sealed thoroughly, mixed and allowed to stand at room temperature (*ca.* 23°) for 1 h.

Extraction of the platinum–DDTC adduct

The urine or buffer mixture (10 ml) was extracted with 1 ml of water-saturated chloroform for 3 min. The sample tube was then centrifuged for 5 min at 1200 *g*, and after brief vortex mixing to disrupt a congealed third phase appearing between the aqueous and organic layers, the tube was centrifuged for an additional 10 min. The aqueous layer and the stiff emulsion layer above the chloroform were removed and discarded.

Chromatography

In all runs, 30-μl aliquots of the chloroform solution containing the platinum–DDTC adduct were injected onto a μBondapak CN column (two 30-cm columns coupled in series by 0.009 in. I.D. tubing). Components were eluted isocratically from the column using heptane–isopropanol (82:18) as mobile phase at a flow-rate of 2 ml/min.

Non-flame atomic absorption spectrometry

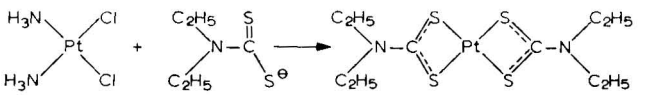
Chloroform samples (5 μl) containing the platinum–DDTC adduct were injected slowly into an atomizer held at 100°. After deposition of the sample, the atomizer was held at 100° for 45 sec, 1000° for 10 sec and 2300° for 1 sec. The temperature ramp rate from 1000° to 2300° was 600° sec^{-1}. The platinum absorbance at 265.95 nm was monitored using an element lamp current of 10 mA. Hydrogen continuum background correction was used. Nitrogen was passed through the system at 4.25 l/min.

RESULTS AND DISCUSSION

CDDP is a square planar molecule that undergoes bimolecular substitution reactions (with preservation of stereochemistry) at platinum, similar to reactions observed at the electrophilic carbon atoms of other alkylating agents[9]. Accordingly, a nucleophilic reagent was sought which would react with CDDP and its *in vivo* reaction products directly in urine to form a single, stable product that would meet the analysis criteria delineated above. We have previously demonstrated[10] that sodium diethyldithiocarbamate (DDTC), 2, reacts readily and quantitatively with the epoxide, dianhydrogalactitol, directly in blood plasma to form a stable product, which is

significantly more hydrophobic than the parent and with pronounced UV absorbance at 254 nm.

To determine the applicability of this reagent to CDDP analysis, CDDP was mixed with DDTC in aqueous buffer (pH 7.5) solution. A yellow precipitate formed rapidly and was isolated by filtration. The recrystallized product was shown by elemental analysis to represent the addition of 2 moles of DDTC to 1 mole of CDDP and is presumed to be structure 3. Similar chelates have been shown to form between DDTC and a variety of metal ions, including platinum(II)[11], and have proven useful in chromatographic separation of metals[12]. The adduct(s) strongly absorbs UV light with wavelength maxima at 254 nm (a_m = 43,000 M^{-1} cm^{-1}) and 347 nm (a_m = 22,000 M^{-1} cm^{-1}).



1 2 3

The platinum–DDTC chelate is a neutral species, that is nearly insoluble in water, acid (pH 1) and base (pH 13) but freely soluble in chloroform. Thus it was presumed that the chelate, 3, would partition favorably into chloroform from an aqueous phase. Using a 10:1 ratio of McIlvaine buffer (pH 7.5) to chloroform, the adduct was quantitatively retained in the chloroform phase after vigorous agitation of the mixture for 1 h. Partitioning efficiency was pH-independent, *i.e.*, quantitative retention of 3 in chloroform was observed when the pH of the aqueous phase was varied between 1 and 13. The affinity of the adduct for chloroform and its incompatibility with water allows for the ten-fold concentration of the analyte (3) which is achieved by extraction.

The reaction was also found to proceed directly in urine containing 25–15,000 ng of CDDP per ml, in which the product, 3, was isolated by extraction into chloroform. Extraction of 3 from urine by chloroform was shown to be quantitative, when the ratio of urine to chloroform was 10:1. Larger aqueous:organic phase ratios could not be used because of emulsification problems occurring at the interface after extraction, which hampered sampling of the organic phase.

The nature of the derivatization was investigated further in urine in order to optimize reaction conditions. The formation of 3 from 1 is apparently an A $\xrightarrow{k_1}$ B $\xrightarrow{k_2}$ C process, where B represents the product(s) formed from the addition of 1 mole of DDTC to 1 and C is compound 3 which will ultimately be monitored spectrophotometrically. The reaction was carried out in urine containing 0.025–15 μg of CDDP per ml, by the addition of a 10% solution of DDTC (prepared in 0.1 M sodium hydroxide solution) to give a final concentration of 1% DDTC in the urine; and the formation of 3 was monitored as a function of time by HPLC. At 25°, in urine, CDDP was quantitatively converted to 3 in 10 min. When the reaction was repeated in McIlvaine buffer (0.2 M; pH 7.5), the formation of 3 with time was slower than that observed in urine and followed apparent first-order kinetics with a half-life of < 10 min. The apparent simple first-order behavior for formation of 3 indicates that one of the rate constants is much greater than the other, since no lag phase is observed. The yield of 3 obtained in 1 h at room temperature decreased with decreasing pH (Table I).

TABLE I

EFFECT OF pH OF REACTION MEDIA ON THE OVERALL RECOVERY AND ANALYSIS OF CDDP FROM BUFFER

The recovery of platinum was measured spectrophotometrically at 254 nm, as $Pt(DDTC)_2$, using the procedure described in the text. McIlvaine buffers made from combinations of 0.2 *M* Na_2HPO_4 and 0.1 *M* citric acid solutions were used. Ionic strength uncontrolled. The buffer solutions were spiked with three different concentrations (5.1, 20.3, 50.6 μg/ml) of CDDP. Each solution was prepared in duplicate.

pH of buffer	*Recovery of platinum from buffer (%)*	r^2 *
5.29	86	0.991
6.46	92.4	0.998
7.05	94.8	0.999
8.08	95.8	0.995

* Regression coefficient for platinum response.

Accordingly, reaction conditions were established wherein 1 ml of reagent solution [containing 10% DDTC (w/v) in 0.1 *N* NaOH] was added to 9 ml of urine and the reaction allowed to stand at room temperature for 1 h. Sodium hydroxide was present in the reagent to elevate the pH above 7, where maximum and reproducible yield of 3 was achieved. Precipitation of inorganic salts occurs with some urine samples upon the addition of base, however, this sediment does not affect the yield of 3 obtained. After the 1-h reaction period, the adduct, 3, was quantitatively extracted from the urine into 0.2 volumes of chloroform.

Compound 3 showed no detectable deterioration in chloroform over 2 months and no breakdown in urine (containing 1% DDTC) was observed after more than 72 h.

Chromatography

The high molar absorptivity of the adduct lends itself to spectrophotometric detection. To unsure selective monitoring of 3 in the chloroform extract, the mixture was fractionated by HPLC. The objectives of the separation step were: (1) to resolve the peak resulting from 3 from peaks arising from urine constituents and products formed from decomposition of DDTC which are co-extracted into chloroform; (2) to allow monitoring of the column effluent at 254 nm rather than 347 nm to achieve greater sensitivity, since the molar absorptivity of 3 at 254 nm is twice that observed at 347 nm and the light intensity generated by a mercury vapor lamp at 254 nm is significantly greater than that achievable at 347 nm (with either a mercury vapor or deuterium lamp); and (3) to achieve symmetrical, well-shaped peaks with capacity factors (k') in the range 3–6.

Attempts to achieve these goals were unsuccessful using reversed-phase (C_{18} or alkyl phenyl) μBondapak columns. However, using a μBondapak NH_2 or CN column these aims could be realized. An evaluation of various stationary phases is summarized in Table II. Unfortunately, the μBondapak NH_2 column is not an inert phase and after repeated injection of chloroform extracts (*ca.* 200) irreproducible peak heights, loss in peak symmetry (tailing) and increased retention volumes (V_r) for 3 were observed. Column performance could be partially regenerated by successive washes with acetic acid (0.1 *N*; 100 ml), water (200 ml), isopropanol (100 ml) and

TABLE II

SUMMARY OF COLUMN PERFORMANCE FOR THE ANALYSIS OF PLATINUM IN URINE AS $Pt(DDTC)_2$

The analysis was carried out as described in Experimental. Analytes were detected spectrophotometrically at 254 nm, unless otherwise indicated. μBondapak columns (30 cm × 3.9 mm I.D.) were used. The mobile phase was optimized by solvent-flow programming (Waters Model 660 solvent programmer), adjusting the composition of the indicated binary mixtures from 0 to 100%.

Stationary phase	*Mobile phase (composition)*	k'	N[c]	R_s[d]	*Comments on performance*
C_{18}	methanol–water (70:30)	3.1[a]	1070	[e]	[f,g]
Alkyl phenyl	methanol–water (70:30)	4.8[a]	1700	[e]	[f,g]
	methanol–phosphate buffer (0.005 *M*; pH 3) (70:30)	4.3[a]	1100	0.5	[f,h]
NH_2	heptane–isopropanol (87:13)	4.1[b]	490	0.7	[f,i,j]
CN	heptane–isopropanol (82:18)	3.1[b]	810	0.9	[f,j]
CN (two columns)	heptane–isopropanol (82:18)	3.0[b]	1600	1.3	[k]

[a] Capacity factor for $Pt(DDTC)_2$ calculated as $(t_r - t_0)/t_0$ where t_r is the retention time of $Pt(DDTC)_2$ and t_0 is the time for elution of a nonretained component (determined from a injection of 30 μl of chloroform).

[b] Capacity factor for $Pt(DDTC)_2$ calculated as $(t_r - t_0)/t_0$ where t_0 was determined with an injection of 75 μl of heptane.

[c] Plate count calculated from an injection of $Pt(DDTC)_2$ (16 μg Pt per ml) in chloroform as $16\,(t_r/t_w)^2$ where t_w is the width of the $Pt(DDTC)_2$ peak at the baseline and t_r is its retention time.

[d] Resolution of the $Pt(DDTC)_2$ peak from its closest neighboring peak, calculated as $2(t_2 - t_1)/(w_1 + w_2)$ where t_1 and t_2 are the retention times of bands 1 and 2 and w_1 and w_2 are their baselines widths defined by the tangents to the inflection points of a given curve.

[e] Grossly inadequate ($R_s \leq 0.5$).

[f] Inadequate resolution of analyte from co-extracted materials.

[g] Very complex chromatogram.

[h] Chromatogram simplified; analyte can be detected spectrophotometrically at 347 nm. Detection limit: 1 μg CDDP per ml.

[i] Rapid column degeneration.

[j] Platinum can be quantitated spectrophotometrically at 254 nm as $Pt(DDTC)_2$ to levels approaching 350 ng of CDDP per ml of urine.

[k] Good performance; platinum can be quantitated as $Pt(DDTC)_2$ to levels of 25 ng CDDP per ml of urine.

then reintroduction of mobile phase (200 ml), which is consistent with azomethine formation. However, this procedure is time-consuming and does not completely restore chromatographic characteristics. These column performance problems could be circumvented by using a μBondapak CN column. To achieve minimum analysis time with adequate resolution of components, a mobile phase for isocratic separation on a CN column was selected by starting with pure isopropanol and adding sufficient heptane to increase k' to 3–6 (Fig. 1) while maintaining the resolution of 3 from contaminating peaks. Complete resolution could only be achieved with columns 60 cm long. A mobile phase of isopropanol–heptane (18:82) was selected in which 3 elutes with $V_r = 24$ ml, $k' = 3$ and the number of theoretical plates, N, is 1600 (based on 3).

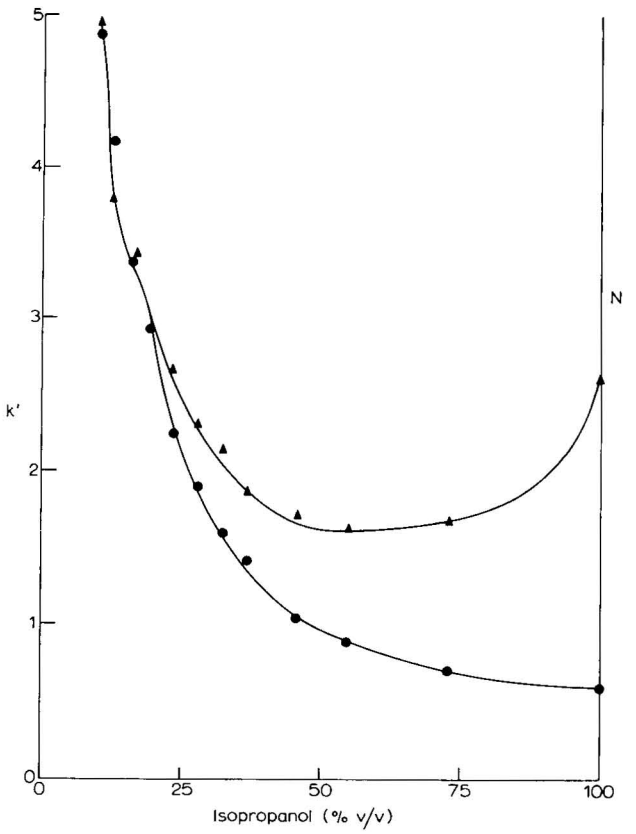


Fig. 1. The effect of variation in isopropanol–heptane mobile phase composition on the capacity factor (k', ○) and column efficiency (N, △) for separation of the CDDP–DDTC adduct (3) (16 μg Pt/ml) on a μBondapak CN column (60 cm × 3.9 mm I.D.). Flow-rate: 2 ml/min.

A minimum is observed in the curve describing the change in plate count (N) with mobile phase composition. At high isopropanol concentrations, the large plate count apparently results from minimum retention of 3 by the stationary phase and therefore minimum band broadening. The increasing plate count also observed at low isopropanol concentrations may result from the decreased viscosity (η) of the mobile phase ($\eta_{\text{heptane}} = 0.386$ cP; $\eta_{\text{iso-propanol}} = 1.811$ cP at 25°) produced by the addition of the heptane.

Fig. 2 shows the analysis of 25 ng of CDDP per ml of urine as its DDTC adduct. Injection volumes greater that 50 μl of chloroform changed the retention characteristics slightly, accelerating the elution of 3.

Standard curves and sensitivity

A standard curve was prepared by analyzing twelve urine samples to which CDDP had been added at different concentrations ranging from 25 to 500 ng/ml. Over this concentration range, chromatogram peak height was linearly related to CDDP concentration, and is described by the line $y = 0.65x + 2.15$ (with a zero-

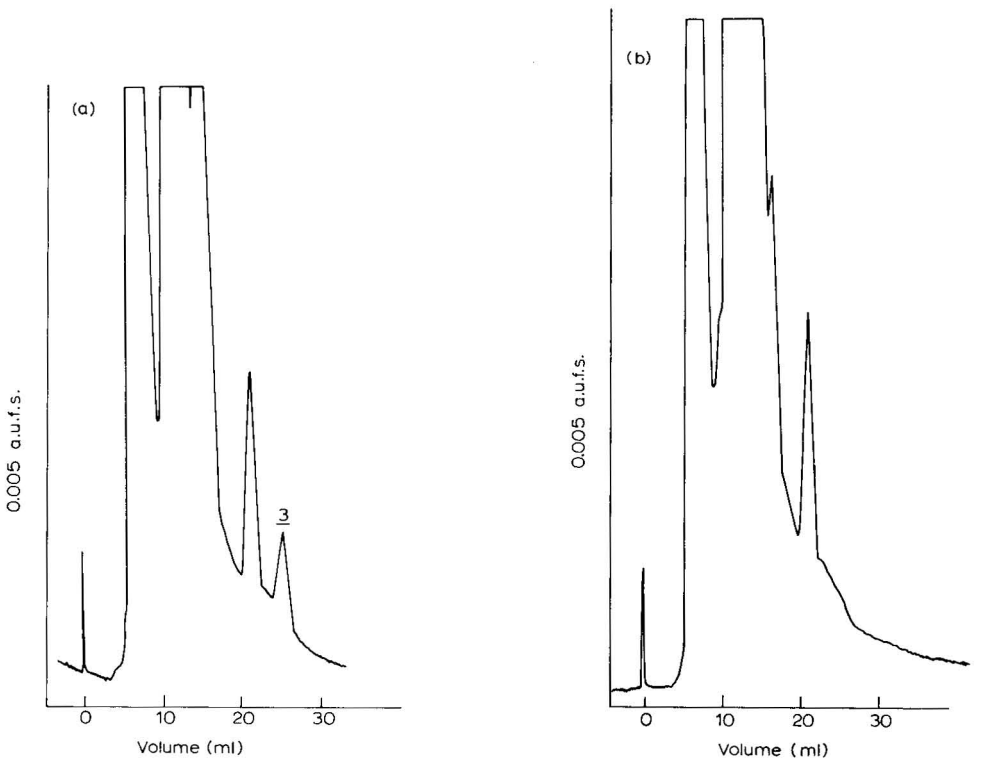


Fig. 2. Chromatograms of the CDDP–DDTC adduct (3) obtained by carrying out the methodology described in the text on a 9-ml urine sample containing 25 ng of CDDP (a) and of a drug-free urine sample subjected to the assay (b).

order correlation coefficient of 0.99). Total recovery of CDDP from urine was $> 97\%$ over this range, and measurements could be made with an accuracy of $\pm 4\%$ and precision of $\pm 2.5\%$. The minimum amount of platinum detected by the method was approximately 25 ng/ml of urine. The identical standard curve was obtained for analysis of platinum immediately after CDDP addition to urine and for samples allowed to remain untreated in urine for 36 h prior to the addition of DDTC. Thus, although CDDP is unstable in urine, it must be converted to species which react with DDTC in a manner similar to CDDP to form 3. Immediate processing of samples is therefore not necessary.

There is still some uncertainty with regard to the spectrum of platinum complexes that react with DDTC to form 3. We have found that CDDP, CDDP incubated with urine for 35 h and the biodegradation products of CDDP which have been isolated from plasma[13] (but not yet identified) respond identically with the reagent to form 3. Furthermore, Fackler *et al.*[11] have shown that 3 "reacts only slowly (hours) with diphenylmethylphosphine", a reagent known to react very rapidly with other divalent platinum complexes, supporting the contention that the complex 3 is stable and DDTC is able to displace most ligands bonded to platinum.

Atomic absorption detection

Alternatively, an aliquot of the chloroform extract containing 3 can be introduced directly into the graphite furnace of an atomic absorption spectrophotometer and the platinum levels measured. By this detection method, platinum levels approaching 10 ng/ml of urine can be followed quantitatively. Unfortunately, the imprecision and instrumental variability associated with this technique made it unreliable and thus less advantageous than the HPLC method.

CONCLUSION

A clinically-useful method has been developed for measuring non-radiolabelled platinum in urine following administration of CDDP to patients, while circumventing the matrix effects associated with other analytical methods (X-ray fluorescence and atomic absorption spectrometry). By taking advantage of the electrophilicity of the divalent platinum atom and the lability of its ligands, it is possible to convert CDDP as well as a number of its biodegradation products to a common compound with hydrophobic character, permitting its isolation from biological fluid by extraction which coincidently provided a ten-fold concentration of the sample. Further separation of the platinum adduct was afforded by HPLC. The platinum–DDTC complex strongly absorbs UV light ($\lambda_{max.}$ 254 nm; a_m 43,000) and could be detected in the column effluent to levels approaching 25 ng of platinum per ml of urine with a precision of $\pm$ 2.5% and accuracy of $\pm$ 4%. The chromatographic step was essential in providing sufficient specificity to permit spectrophotometric monitoring of the adduct at either 254 or 347 nm since other absorbing species are also extracted from urine by chloroform. Attempts to measure platinum in urine by other techniques fell far short of these statistics, apparently due to sample matrix effects and instrumental variability. The method is rapid, inexpensive, requires minimum sample manipulation and is suitable for automation to accommodate large numbers of samples.

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DETERMINATION OF FOOD PRESERVATIVES AND SACCHARIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

U. LEUENBERGER, R. GAUCH and E. BAUMGARTNER
Kantonales Laboratorium, P.O. Box, CH-3000 Berne 9 (Switzerland)
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SUMMARY

The quantitative analysis of benzoic and sorbic acid, methyl, ethyl and propyl esters of *p*-hydroxybenzoic acid and saccharin in foodstuffs is described. These compounds are quantitatively extracted with disposable clean-up columns packed with Extrelut® and simultaneously determined by high-performance liquid chromatography on reversed-phase columns. Complicated matrices such as cheese, cake, ketchup and chocolate were tested and recoveries were generally better than 95% in the concentration ranges normally used in the food industry.

INTRODUCTION

The existing approaches for determining quantitatively compounds in a complicated matrix generally consist of two steps: extraction and quantification. The isolation of acidic compounds such as benzoic and sorbic acid, the esters of *p*-hydroxybenzoic (PHB) acid and saccharin may be time consuming. The common method of isolating these compounds from substances that might interfere in the quantitative determination is extraction either with a Soxhlet apparatus or manually with a separating funnel[1,2]. As such extractions are common in the routine work of almost every analytical laboratory, an alternative, easier method of extraction has been devised, namely the use of disposable Extrelut® clean-up columns (Merck, Darmstadt, G.F.R.), which simplifies considerably the isolation of organic compounds, especially when working with complex matrices that tend to form emulsions[3,4]. This approach is used extensively in forensic and clinical chemistry and has recently been applied in food chemistry to extract the mycotoxin patulin from apple and grape juice[5].

The second step, the quantitative determination of the extracted compounds, can be performed either by chromatography [gas–liquid chromatography (GLC)[6], thin-layer chromatography (TLC)[7] or high-performance liquid chromatography (HPLC)[8–12]] or by measuring the UV absorbance of the total extract[13]. Whereas the GLC determination requires derivatization prior to injection, commercially available pre-coated thin-layer plates lack the separation power for sorbic and benzoic acid. These problems can be eliminated by using reversed-phase systems and HPLC. This

chromatographic approach has great advantages over those involving the use of silica gel in the ease of equilibration and in cleaning the column from accumulated contaminants by simply flushing with methanol. HPLC is not only rapid, but shows also a greater linear calibration range than TLC. One gradient and two isocratic systems are proposed for the simultaneous identification of the above-mentioned compounds.

EXPERIMENTAL

Materials and chemicals

Extrelut pre-packed columns (Art. 11737) and an Extrelut refill pack (Art. 11738) were obtained from Merck.

The chemicals used were diethyl ether, acetone, chloroform and ethanol (all distilled in glass), pure methanol (Art. 6008, Merck), isopropanol (pro analysi) (Art. 9634, Merck), 0.1 *N* sodium hydroxide solution, 0.5 *N* sulphuric acid, 5 *N* sulphuric acid (140 ml of concentrated acid diluted to 1000 ml with distilled water), aqueous solution of concentrated ammonia, aqueous saturated solution of sodium chloride and ethyl acetate (distilled).

Phosphate buffer solution was prepared by dissolving 2.5 g of $K_2HPO_4 \cdot 3H_2O$ (Art. 5099, Merck) and 2.5 g of KH_2PO_4 (Art. 4873) in doubly distilled water and passing the solution through a 0.45-μm filter (for instance, a "solvent clarification kit", Art. 85122, Waters Assoc., Milford, Mass., U.S.A.).

Standard solutions

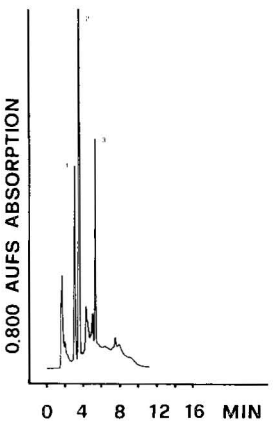
A 25-mg amount of each of the following compounds was dissolved together in 25 ml methanol and diluted to 50 ml in a volumetric flask: saccharin (Art. 38, Fisher Scientific, Zürich, Switzerland), benzoic acid (p.a.) (Art. 136, Merck), sorbic acid (Art. 662, Merck), methyl *p*-hydroxybenzoate (Art. 6757, Merck), ethyl *p*-hydroxybenzoate (Art. 887, Merck) and propyl *p*-hydroxybenzoate (Art. 7424, Merck).

This stock solution was diluted 1:10 for the HPLC method for PHB esters alone and the two acids plus saccharin (see *Chromatography*) resulting in a concentration of 0.05 μg/μl per compound. For the determination of the five preservatives (see *Chromatography*) the undiluted parent solution had to be applied.

Experimental procedures

Direct analysis. Fruit juices such as orange juice, wines and other aqueous media generally need no clean-up. In any case, a filtration (0.45-μm pore diameter, for instance waters sample clarification kit, Art. 26870) is recommended for eliminating any particulate matter. The detection limit for benzoic acid, which has the smallest absorption coefficient of the compounds analysed, is far lower than the concentrations normally used in the food chemistry. Fig. 1 shows such a chromatogram of commercial orange juice containing 300 ppm each of benzoic and sorbic acid and methyl PHB. The baseline shift is due to the gradient used. Fig. 2 shows a similar analysis of white wine spiked with 170 ppm of sorbic acid.

Samples containing mainly triglycerides and similar matrices such as butter and margarine. Extremely fatty samples such as margarine have to be extracted by the



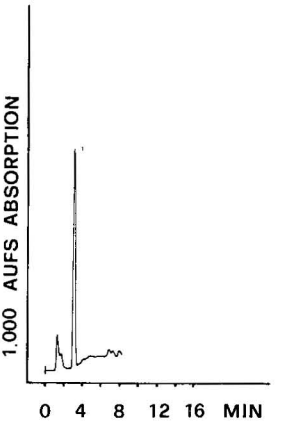


Fig. 1. Orange juice spiked with 300 ppm of benzoic acid (1), sorbic acid (2) and methyl *p*-hydroxybenzoate (3).

Fig. 2. White wine spiked with 170 ppm of sorbic acid (1).

following procedure. A 5-g sample is dissolved in 50 ml of diethyl ether and extracted twice with 10 ml of 0.1 *N* sodium hydroxide solution in a separating funnel. The basic aqueous extracts are acidified with 1 ml of 5 *N* sulphuric acid in a 50-ml volumetric flask and diluted to volume with methanol.

This solution is filtered as in described under *Direct analysis* and analysed.

Extrelut clean-up. This method is suitable for most other foodstuffs such as cheese, cakes, yoghurts and other samples that tend to form emulsions during the extraction. The pre-packed or refilled Extrelut column in a plastic tube consists of a wide-pore Kieselguhr of grainy structure and is characterized by a high pore volume. The tube is capped at both ends with screens containing a filter disc. The elution rate is controlled by the diameter of a cannula stuck on to the effluent cone. A 5–20-g sample is homogenized for 3 min in 50 ml of 0.5 *N* sulphuric acid using a beaker and a high-speed blender. The homogenate is transfered quantitatively into a 100-ml volumetric flask and diluted to volume with water. When analysing material that sediments quickly, it is homogenized again in order to obtain a representative aliquot. A 20-ml volume of this pre-treated sample is pipetted on to the Extrelut column and allowed to filter in for at least 15 min. It is important to replace the original filter disc by some glass-wool when working with samples of thicker consistency. Subsequently it may also be necessary to cover the retained particulate matter again with some glass-wool.

The absorbed preservatives and saccharin can now be eluted with 350 ml of chloroform–isopropanol (9:1) using a closed 500-ml separating funnel as a solvent reservoir above the column (Mariott flask). The eluate is collected in a 500-ml round-bottomed flask and evaporated carefully nearly to dryness. The last few millilitres of isopropanol are removed with a gentle flow of nitrogen in order to prevent substantial losses of benzoic and sorbic acid, which have relatively high vapour pressures. The residue is transferred with methanol into a 10-ml volumetric flask and diluted to volume with methanol. To speed up the dissolution, the use of an ultrasonic bath is recommended. The filtered extract is now ready for analysis.

Instrumental

An Ultra Turrax high-speed mixer (type TP 18/2, IKA Werk, Stauffen im Breisgau, G.F.R.) was used.

For HPLC, a rheodyne Model 7105 injector (Perkin-Elmer, Norwalk, Conn., U.S.A.), two Altex Model 100 pumps (Altex Scientific, Berkeley, Calif., U.S.A.), a Model 420 microprocessor programmer (Altex), a μBondapak C_{18} column (Waters), a Uvikon UV detector (Kontron, Zürich, Switzerland) with a variable-wavelength detector, Model LCD 725 (wavelength used, 235 nm) and an SP 4000 integration system (Spectra-Physics, Santa Clara, Calif., U.S.A.) were employed.

Chromatography

Determination of the five preservatives. In order to analyse the PHB esters in the same run as the two acids, it is necessary to use a gradient. Pump A delivers phosphate buffer solution as described under *Materials and chemicals*, whereas pump B delivers methanol. The initial chromatographic conditions were 20% B, a linear gradient from 20% to 80% B within 1 min, and flow-rate 2 ml/min. Fig. 3 shows a calibration chromatogram with an injection of a 10-μl standard (5 μg of each compound). The same chromatographic conditions were used in obtaining Figs. 1 and 2.

PHB esters alone. Isocratic elution was carried out with 60% methanol in phosphate buffer (for preparation see *Materials and chemicals*), flow-rate 1.2 ml/min. A 10-μl volume of the standard (0.05 μg/μl, see *Materials and chemicals*) was used for calibration.

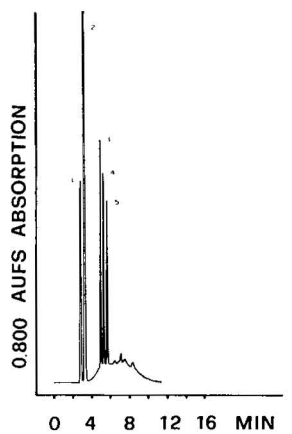
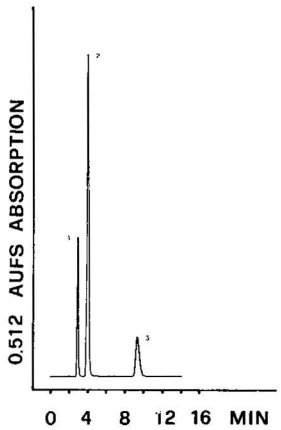


Fig. 3. Standard chromatogram of benzoic acid (1), sorbic acid (2), methyl (3), ethyl (4) and propyl *p*-hydroxybenzoate (5) (5 μg of each).

The two acids plus saccharin. The isocratic use of pure buffer solution (for preparation see *Materials and chemicals*) and a rapid flow-rate of 2.8 ml/min led to the separations shown in Figs. 4 and 5.

RESULTS AND DISCUSSION

As shown above, the isolation and extraction of preservatives and saccharin by using the disposable clean-up column for routine analysis offers considerable



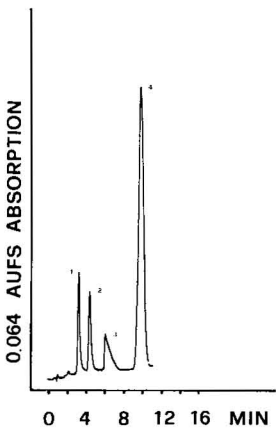


Fig. 4. Standard chromatogram of benzoic acid (1), sorbic acid (2) and saccharin (3) (4 μg of each).

Fig. 5. Standard chromatogram of benzoic acid (1; 0.4 μg), sorbic acid (2; 0.4 μg), cyclamate (3; 91 μg) and saccharin (4; 1 μg). Detection at 207 nm.

savings in time and materials, especially when dealing with samples that tend to form emulsions. The advantages are great enough to predict further applications in routine extractions, especially in trace analysis.

Typical recoveries found with this rapid extraction mode are summarized in Table I. The detection limits of all six compounds are at least ten times below those commonly used in applications of preservatives and saccharin.

The extracts from this clean-up column are generally so clean and free from interfering compounds that not even matrix peaks can be observed in the chromatogram, although some of the neutral fraction is eluted together with the acidic compounds.

TABLE I

RECOVERIES FROM FOOD SAMPLES FOLLOGING EXTRELUT CLEAN-UP

Sample	*Recovery (%)*				
	Benzoic acid	*Sorbic acid*	*PHB esters*		*Saccharin*
			Methyl	*Propyl*	
Peppermint candies	—	—	—	—	98.0
Milk chocolate	95.2	87.6		—	97.0
Chestnut paste	98.3	90.7	93.6	92.9	96.0
Toothpaste	—	—	98.0	100.0	91.0
Marmalade	96.7	92.7	97.4	99.9	98.5
Ketchup	99.8	86.2	99.5	97.7	98.7
Fruit cocktail	99.8	80.0	93.0	93.3	100.4
Canned Russian salad	96.7	98.3	95.9	99.2	95.8
Canned mixed vegetables	87.4	87.7	100.9	101.1	98.5
Mixed pickles	92.0	—	100.5	100.8	96.3
Yoghurt (plain)	98.5	97.9	100.5	100.5	101.4
Cake	—	—	—	—	99.7
Cheese	96.3	95.3	99.3	96.6	—

Preliminary approaches to load the Extrelut column with alkaline samples in order to elute first the basic and neutral fractions were found to be unnecessary, and later esters were partially saponified. Although it is easy to change the column to a basic pH, problems arise on acidification as both organic and inorganic acids obviously are adsorbed on the column. Gaseous HCl is also strongly adsorbed, changes the filling material and is not suitable for routine work.

The detector wavelength used of 235 nm is a compromise made in order to detect all of the compounds concerned simultaneously (absorption maxima: benzoic acid 227 nm, saccharin 207 nm, sorbic acid and PHBs 250–260 nm). The detection limit can be greatly enhanced for a specific analysis by using the appropriate absorption maximum.

The simultaneous determination of benzoic acid, sorbic acid and saccharin as shown in Fig. 4 or the quantification of all five preservatives (Fig. 3) within one run makes this analysis faster than with previously described methods earlier.

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MEASUREMENT OF TESTOSTERONE WITH A HIGH-PERFORMANCE LIQUID CHROMATOGRAPH EQUIPPED WITH A FLOW-THROUGH ULTRAVIOLET SPECTROPHOTOMETER

ROGER C. COCHRAN, KIRWIN J. DARNEY, Jr. and LARRY L. EWING

Division of Reproductive Biology, Department of Population Dynamics, The Johns Hopkins University School of Hygiene and Public Health, 615 N. Wolfe Street, Baltimore, Md. 21205 (U.S.A.)

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SUMMARY

A technique is described for measuring nanogram amounts of testosterone using high-performance liquid chromatography with detection by a flow-through spectrophotometer. The addition of a non-radioisotopic internal standard (4-androsten-11β-ol-3,17-dione) to the biological specimen automatically corrects for testosterone losses due to extraction and non-quantitative sample injection into the high-performance liquid chromatograph. This new method, which can be performed by inexperienced personnel, is shown to be rapid, precise, accurate and specific for testosterone.

INTRODUCTION

Testosterone is the major androgen synthesized and secreted by the mammalian testis[1]. Techniques developed to measure the concentration of steroids in biological fluids generally fall into three categories: (a) radioligand assays, which include both competitive protein binding assays[2–4] and radioimmunoassays[5–7]; (b) physicochemical methods, which include double isotope dilution[8–10] or gas–liquid chromatography (GLC) with flame-ionization, electron-capture or mass spectrometer detectors[11–13] and (c) enzymatic measurement of steroids[14–16].

The measurement of testosterone by double isotope dilution and gas–liquid chromatography is tedious, time consuming and requires considerable technical skill. Radioligand assays of testosterone, while sensitive and less tedious, are subject to non-specific interference from unknown sources. The enzymatic measurement of any steroid requires access to highly purified, stable and steroid specific enzymes. Unfortunately, such an enzyme is not available for testosterone.

Recent advances in the separation of steroids by high-performance liquid chromatography (HPLC) followed by the measurement of light absorbance at 240 nm with a flow-through spectrophotometer led to the development of a new method to measure dexamethasone in urine[17]. Briefly, this method involved the addition to urine samples of a non-radioisotopic internal standard which chromatographed

differently than dexamethasone in an HPLC system; extraction of the sample with an organic solvent; and finally, injection of an aliquot of the solvent residue into the HPLC system.

We adopted the principle of this method to develop a similar, simple and specific method for determining the concentration of testosterone in spermatic venous effluent from the rabit testis perfused *in vitro*.

MATERIALS AND METHODS

Materials

A Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A chromatography pump coupled with a U6K injector and a μBondapak C_{18} column were used for HPLC. The mass of the Δ^4-3-keto steroids was measured by monitoring the absorbance of light at 240 nm with a spectroflow Model 770 UV analyser (Schoeffel, Westwood, N.J., U.S.A.) interfaced with an Autolab System IV integrator computer (Spectra-Physics, Stirling, N.J., U.S.A.). A Packard Model 420 gas–liquid chromatograph with a 3% OV-210 on 80–100 mesh Gas-Chrom Q column, flame-ionization detector, and interfaced with the Autolab System IV integrator–computer was used for estimation of testosterone mass by a previously published gas–liquid chromatographic method[18].

Testosterone (4-androsten-17β-ol-3-one), testosterone acetate (4-androsten-17β-ol-3-one-acetate) and 4-androsten-11β-ol-3,17-dione (11β-DIONE) were obtained from Steraloids (Wilton, N.H., U.S.A.) and recrystallized to constant melting point before use. [^{3}H]Testosterone was purchased from New England Nuclear (Boston, Mass., U.S.A.) and purified by thin-layer chromatography (TLC) on pre-coated plates (250 μm, Analtech, Newark, Del., U.S.A.) prior to use. Solvents were acetic anhydride, pyridine, carbon disulfide, ethyl ether (reagent grade; Mallinckrodt, St. Louis, Mo., U.S.A.), benzene, ethylacetate (Nanograde; Mallinckrodt), Photrex reagent (specially denatured ethanol; J. T. Baker, Phillipsburg, N.J., U.S.A.) and spectrophotometric-grade acetonitrile (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). Water was double-distilled in glass.

New Zealand white rabbits (12 months of age), purchased from Bunnyville (Altoona, Pa., U.S.A.), were housed in an air-conditioned (20°) and light-controlled (14 h light: 10 h dark) room and supplied with 120 g Rabbit Checkers (Ralston Purina, St. Louis, Mo., U.S.A.) per day and water *ad libitum*. Testes were perfused *in vitro* with a medium constituted of Krebs-Ringers bicarbonate solution with 3% (w/v) fraction V bovine serum albumin (KRB–BSA), and 25% bovine red blood cells as described previously[18,19].

HPLC testosterone assay

A 400-ng amount of 11β-DIONE in benzene was pipetted into the bottom of 12-ml disposable, glass, screw-cap tubes using a micromedic automatic pipette (Micromedic Systems, Huntsville, Ala., U.S.A.). For the standard curve, different amounts of testosterone in benzene were next added to the appropriate test tubes. The benzene was evaporated under nitrogen. Two drops of ethanol followed by 1 ml of KRB–BSA, or testicular venous effluent were added to each tube. A 10-ml volume of ice-cold diethyl ether from a freshly opened can was added next. The mixture was shaken

vigorously for 1 min, centrifuged at 1500 *g* for 10 min. The aqueous layer was snap-frozen in a dry-ice–acetone bath and the ether decanted into another disposable glass test tube. The ether extract was evaporated under nitrogen and the residue dissolved in 50 μl of Photrex reagent. Approximately one-half of this solution was placed in the U6K injector of the chromatograph. The internal standard (11β-DIONE) and testosterone were eluted differentially from the μBondapak C_{18} column with acetonitrile–water (40:60, v/v) at 2 ml/min. The areas of the resultant peaks of UV absorbance were integrated and the quantity of testosterone present calculated automatically by the Spectra-Physics integrator computer according to the formula

$$\text{Testosterone quantity} = \frac{\text{Area of T peak}}{\text{Area of 11}\beta\text{-DIONE peak}} \times KF \times 100\text{ ng 11}\beta\text{-DIONE}$$

where *KF* is the ratio of the absorbance peak area for 100 ng of 11β-DIONE to that of 100 ng testosterone. Estimates of *KF* were performed with each series of testosterone assays.

GLC testosterone assay

Testosterone measurement by the GLC technique was described in detail and validated in an earlier report[18].

RESULTS

General

Testosterone was readily separated from the 11β-DIONE internal standard on the μBondapak C_{18} column with acetonitrile–water (40:60, v/v) at a flow-rate of 2 ml/min. Testosterone and 11β-DIONE eluted at 480 and 270 sec, respectively.

The partition coefficient for testosterone and 11β-DIONE between diethyl ether and the aqueous sample were similar. This was tested in the following experiment. One hundred ng each of testosterone and 11β-DIONE internal standard were pipetted into ten test tubes. These test tubes were divided into two groups of five tubes each. An aliquot from each of the first five tubes was injected directly into the chromatograph. The absorbance of light at 240 nm by the T and 11β-DIONE peaks was measured and the 11β-DIONE/T ratio computed. The second group of five samples were extracted with 10 volumes of diethyl ether, aqueous phase snap-frozen in dry ice and acetone, ether extract decanted, evaporated to dryness and an aliquot injected into the HPLC system. The absorbance of light at 240 nm by the T and 11β-DIONE peaks was measured and the 11β-DIONE/T ratio computed. Analysis of variance showed that there was no difference in the ratio (11β-DIONE/T) of light absorbed at 240 nm for the unextracted and extracted samples (0.93 ± 0.01; $\bar{x} \pm$ S.E.M. and 0.91 ± 0.03, respectively).

Sensitivity

The smallest amount of testosterone contained in a biological sample which could be measured consistently by the HPLC assay was 10 ng. Biological samples containing 5 ng of testosterone failed to generate a detectable testosterone peak at 240 nm.

Accuracy

Recrystallized testosterone, from 10 to 2000 ng, was added to 1-ml portions of KRB–BSA. Five replicates at each of seven concentrations were measured by the HPLC method (Fig. 1). Regression analysis showed the estimated amounts of testosterone to be linearly correlated with the mass added. The highly significant correlation coefficient was 0.99. The slope of the regression line and the y intercept were 1.0 and 1.5, respectively. The measured testosterone values were identical to results obtained when the same amounts of standard testosterone were injected directly into the HPLC system without prior extraction.

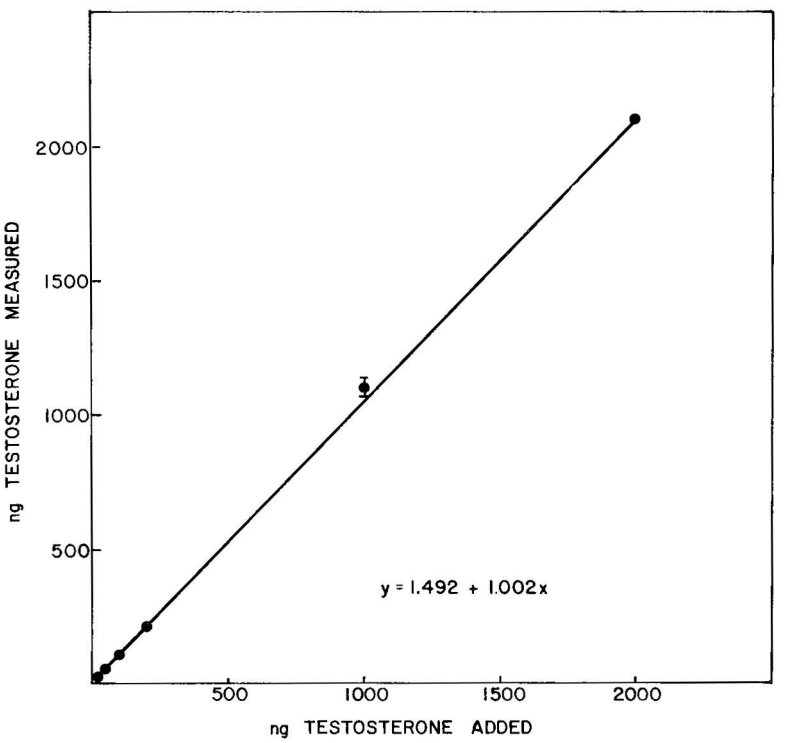

Fig. 1. Accuracy of HPLC for measuring testosterone using regression analysis.

Precision

The precision of the HPLC method was examined using a two-way analysis of variance to measure the intra- and inter-assay variability. The data required for this analysis was collected by measuring the testosterone concentration in five 1-ml aliquots taken from each of 3 samples, which contained 75, 150, or 300 ng T/ml, on 3 different days. The results in Table I show that the estimates of testosterone in each pool were not significantly different ($P > 0.25$) on any given day (intra-assay variance) or from day to day (inter-assay variance). The intra-assay coefficient of variation (standard deviation/mean $\times$ 100%) was 3.2% and the inter-assay coefficient of variation was 3.6%.

TABLE I

RESULTS OF HPLC TESTOSTERONE ASSAY FOLLOWING EXTRACTION OF ADDED STANDARD FROM KRB–BSA

Five samples were taken from each of 3 different pools on 3 different days.

Testosterone added	*Testosterone estimated (mean ± standard error of mean, ng)*		
	Day 1	*Day 2*	*Day 3*
75	75.6 ± 1.6	73.6 ± 4.5	72.4 ± 3.6
150	141 ± 4.3	147 ± 5.4	145 ± 2.4
300	284 ± 3.4	291 ± 9.9	295 ± 8.1

Specificity

The specificity of testosterone measurement by the HPLC method described above was due to three physico-chemical properties of testosterone. First, the differential extraction of the biological sample with ethyl ether assured the separation of testosterone and other lipophilic molecules from polar hydrophilic molecules. Secondly, the large extinction coefficient for light absorbance at 240 nm by testosterone is unique to Δ^4-3-ketosteroids. Thirdly, the retention time of testosterone, eluted from a μBondapak C_{18} column with acetonitrile–water (40:60), is unlike that of any other testicular Δ^4-3-ketosteroid.

Experimental verification of specificity was obtained by comparing the testosterone secretion rate of rabbit testes perfused *in vitro* as described previously[19]. The venous effluent was collected for 1 h. Triplicate testosterone determinations were made on aliquots of spermatic venous effluent from each of the five perfusions by both HPLC and GLC. The mean ± the standard error of the mean for testosterone secretion (μg T/h) was 7.3 ± 0.7 and 7.8 ± 0.9 when measured by HPLC and GLC, respectively. A two-way analysis of variance revealed no significant difference ($P > 0.25$) between testosterone secretion of perfused rabbit testes measured by the HPLC and GLC methods. In contrast, there was a highly significant difference ($P < 0.001$) between the amount of testosterone secreted by the individual rabbit testes.

DISCUSSION

Progress in studying the synthesis and secretion of testosterone by mammalian testes has been hampered by the lack of a simple, rapid, yet specific method for measuring testosterone in biological samples containing complex mixtures of steroids.

Discovery of an internal standard (11β-DIONE) which partitions similarly to testosterone between ethyl ether and an aqueous phase but which elutes differently than testosterone from a μBondapak C_{18} column allowed us to develop a HPLC method to measure testosterone in biological samples. The diagram in Fig. 2 shows that the HPLC method for testosterone measurement requires only an extraction followed by HPLC. In contrast, the GLC technique used routinely in our laboratory requires extraction, derivative formation, TLC, liquid scintillation spectrometry and GLC. The sensitivity, accuracy, precision and specificity of this new HPLC testosterone measurement method compares favorably with the GLC technique used

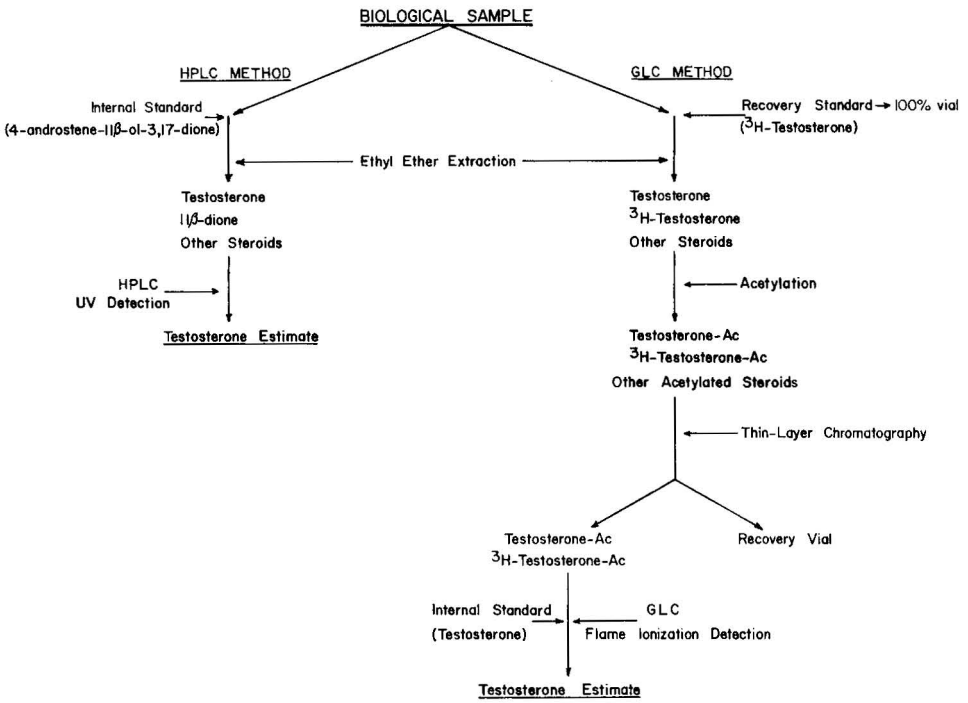

Fig. 2. Comparison of the new HPLC technique for measuring testosterone with an established GLC method[18] routinely used in our laboratory.

routinely in our laboratory[18]. Thus, for measuring testosterone, the HPLC method can be operated reliably by inexperienced personnel to make determinations rapidly on large numbers of samples.

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CHROM. 11,751

POST-COLUMN COMPLEXATION TECHNIQUE FOR THE SPECTROPHOTOMETRIC DETECTION OF POLY(OXY-1,2-ETHANEDIYL) OLIGOMERS IN STERIC EXCLUSION CHROMATOGRAPHY

CHARLES R. WARNER, SAMI SELIM and DANIEL H. DANIELS
Food and Drug Administration, Division of Chemistry and Physics, Washington, D.C. 20204 (U.S.A.)
(Received January 24th, 1979)

SUMMARY

A solid-phase complexation column (SPCC), which consists of ammonium cobaltothiocyanate (ACTC) adsorbed on a solid support, has been developed as part of a selective detection technique for molecules bearing one or more poly(oxy-1,2-ethanediyl) oligomers (POEs). Due to the ion–dipole attraction between the ammonium ion and the negative dipoles of the polyether oxygen atoms, a quantity of ACTC is solubilized in the mobile phase as the POE–ACTC complex. The complexed ACTC is readily quantitated at 320 or 620 nm because of the strongly absorbing cobaltothiocyanate anion. The detection technique is approximately 100 times more sensitive than the differential refractive index detector for this class of compounds. This detector is used with steric exclusion chromatography on Sephadex LH-20 and μStyragel columns.

INTRODUCTION

Because of needs generated by the great variety of scientific and industrial applications of poly(oxy-1,2-ethanediyl) oligomers (POEs), many analytical methods have been developed. The procedures for POEs fall into two categories: procedures for specific substances that are well characterized structurally and techniques for the determination of the oxyethylene content of the sample. One example of the former is the thin-layer chromatographic (TLC)[1] procedure reported by Hodda[2]; the latter is exemplified by the hydrogen bromide cleavage of the polyoxyethylene ether linkages to yield one equivalent of 1,2-dibromoethane for each mole of ethylene oxide incorporated into the molecule[3].

Polysorbates, commonly used in food, have been quantitated by gas chromatographic analysis of the fatty acid moieties[4], TLC[1] and co-precipitation with barium phosphomolybdate[5]. Siggia *et al.*[6] developed a titrimetric procedure which is based on the reaction of POEs with hydriodic acid to yield iodine. Ehrenberger[7] used the hydriodic acid reaction and quantitated the resulting alkyl iodides by gas chromatography. In his review, Longman[8] discussed colorimetry and co-precipitation with heteropoly acids. Other methods for this class of compounds include high-performance

liquid chromatography[9-14] and nuclear magnetic resonance spectroscopy[15,16]. All of these techniques were considered in our search for a liquid chromatographic procedure for determining polyethylene glycols and polyethylene glycol sorbitan esters, such as polysorbates 20, 60, 65 and 80.

Steric exclusion chromatography (SEC) was selected as the method of choice, as the retention volumes of all sample components lie within a range limited by the interstitial volume and interstitial plus pore volumes of the column. Unfortunately, the previously published liquid chromatographic procedures rely upon differential refractive index (RI) or ultraviolet spectral (UV) detectors, neither of which is applicable because of the POEs and the sample matrices under consideration. POEs such as polysorbates, which are permitted as direct food additives[17], and polyethylene glycols do not absorb within the readily accessible region of the UV spectrum and, therefore, UV detection was not considered. As the ultimate goal of this work was to quantitate POEs in complex matrices, the RI detector would not be suitable because of its lack of selectivity and relative insensitivity. In view of these considerations, work was initiated on the development of a detector technique that would take advantage of a specific property common to these polyethers.

The propensity to form ion–dipole complexes with cations is characteristic of a variety of POEs. Because of optimal stereochemical and electronic factors, some crown ethers (cyclic POEs) react with potassium thiocyanate to form crystals of known stoichiometry with melting points higher than that of each of the components[18]. Recent work[19] has demonstrated that non-cyclic POEs will also form crystalline adducts. POEs that do not have the conformations required to form isolatable crystalline complexes can be shown to exhibit substantial ion–dipole complexation energies through phase transfer studies.

Phase transfer, in this context, refers to a marked change in the solubility or partion coefficient of a substance due to complexation. Most POEs will function as phase transfer agents for selected salts and this fact has been utilized in analytical method development. Of the large number of available colorimetric procedures based upon the phase transfer principle[8], the ammonium cobaltothiocyanate (ACTC) technique has emerged as the most generally useful[20]. The method employs a two-phase system consisting of an organic solvent, such as dichloromethane or toluene, and an aqueous solution of ACTC. In the absence of phase transfer agents, ACTC does not partition into the organic layer; however, the portion of the salt that is complexed by the POE in the sample will be transferred to the organic solvent after the two layers are thoroughly mixed to achieve equilibrium. Because of the strongly absorbing cobaltothiocyanate anion, spectrophotometry of the organic layer at 320 or 620 nm will, with suitable calibration, provide quantitation of the POE content.

As even hydrophilic POEs will solubilize certain salts in organic solvents if there is no aqueous layer present[21], we concluded that interaction of a POE with a solid phase source of ACTC, as depicted in Fig. 1, would ensure phase transfer of ACTC by the widest possible variety of POEs.

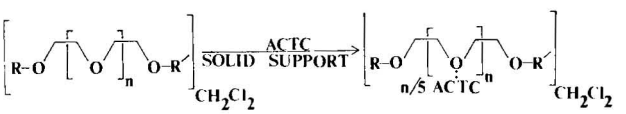

Fig. 1. Interaction of a POE with a solid-phase source of ACTC.

In this paper, the preparation and properties of a solid-phase complexation column (SPCC) are described. The SPCC in combination with a photometer has proved to be a very selective detector, producing linear responses. For POEs in the molecular weight range 200–1540 it is approximately two orders of magnitude more sensitive than the RI detector.

EXPERIMENTAL

Materials and apparatus

The following reagents were used as received: polysorbate 60 [polyoxyethylene (20) sorbitan monostearate] from Sigma (St. Louis, Mo., U.S.A.); polysorbate 80 [polyoxyethylene (20) sorbitan monooleate], Carbowax PEG 400, ammonium thiocyanate (ACS certified grade), and cobalt nitrate (ACS certified grade) from Fisher Scientific (Fair Lawn, N.J., U.S.A.); Brij 35 [polyoxyethylene (23) lauryl ether] from Aldrich (Milwaukee, Wisc., U.S.A.); Carbowax 20M permanently bonded packing, Super Pak 20M, from Analabs (New Haven, Conn., U.S.A.). All solvents were of Distilled-in-Glass quality (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). Sephadex LH-20 was purchased from Pharmacia (Piscataway, N.J., U.S.A.). The 500 Å μStyragel column was manufactured by Waters Assoc. (Milford, Mass., U.S.A.). Carbowax 400 distearate was purchased from Polysciences (Warrington, Pa., U.S.A.).

A Model 3500 (Spectra Physics, Santa Clara, Calif., U.S.A.) liquid chromatograph was used with a variety of injectors and detectors. A Model 725 automatic sample injector (Micromeritics, Norcross, Ga., U.S.A.) with a 10-μl sampling loop or a U6K injector (Waters Assoc.) was used. A Spectra Physics Model 8200 detector with the 312-nm filter as well as a Model SF/70 spectroflow monitor (Schoeffel, Westwood, N.J., U.S.A.) provided photometric detection. A "guard column", which consisted of a 60 mm × 6.4 mm O.D. (2.8 mm I.D.) stainless-steel tube, was equipped with 50-μm frits at each end (Whatman, Clifton, N.J., U.S.A.). The frits were secured with 1/4-to-1/16 in. reducing unions. Peak areas were determined with a Spectra Physics Minigrator. The LH-50 column was contained within a 60 cm × 11 mm O.D. glass column equipped with glass frits at each end.

Solid-phase complexation column

A 4.5-ml portion of a solution, prepared by dissolving 29.3 g of ammonium thiocyanate and 14.0 g of cobalt nitrate in 50 ml of deionized water, was added dropwise to 1 g of Super Pak 20M packing. The resulting slurry was shaken gently until all of the packing was thoroughly wetted by the solution. The slurry was transferred to a funnel with a glass frit. Dichloromethane was repeatedly forced through the packing bed until all obvious traces of water were removed. The resulting blue packing was exposed to the atmosphere until it was a free-flowing powder. The guard column was filled by simply pouring the ACTC–Super Pak 20M reagent into the column with a frit affixed to one end. The other frit was then positioned and secured. The SPCC was readied for use by connecting it to the chromatographic system and flushing with dichloromethane until no blue color was visible in the effluent. The outlet of the SPCC was then connected to the photometric detector.

Detector linearity studies

The relationship between peak area and amount injected was studied by injecting known amounts of each of the test compounds, Brij 35, Carbowax 400 and polysorbate 80, directly into the SPCC. The automatic sample injector was used with the Model 3500 liquid chromatograph. The Model 8200 detector was used with the 312-nm filter. Peak areas were determined with the Minigrator. Dichloromethane was used as the mobile phase at a flow-rate of 1.2 $ml \cdot min^{-1}$.

RESULTS AND DISCUSSION

In practice, the SPCC was used as shown diagrammatically in Fig. 2. Dichloromethane was selected as the mobile phase because there is no significant elution of ACTC from the SPCC in the absence of a phase transfer agent in the column effluent. However, as each POE emerges from the SEC column and passes through the SPCC, a portion of ACTC, which is complexed by ion–dipole interaction, is transferred to the mobile phase. The eluate from the SPCC is monitored at 320 or 620 nm to quantitate the strongly absorbing cobaltothiocyanate anion. The reaction (Fig. 1) is driven to completion because the eluate continuously comes in contact with additional ACTC as it proceeds through the SPCC. The ratio of one molecule of ACTC to five oxyethylene units is a weighted average derived from a review of the colorimetric reactions described in the literature[22].

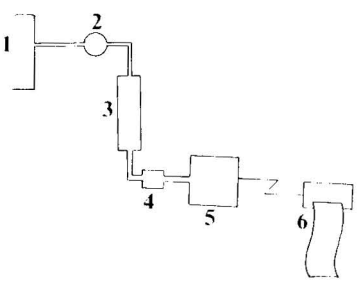

Fig. 2. Schematic representation of chromatography–detection system. 1 = Pump and dichloromethane reservoir; 2 = U6K injector; 3 = 60 cm × 11 mm I.D. LH-20 column; 4 = SPCC; 5 = UV/visible photometer; 6 = recorder.

The performance of the SPCC exceeded our expectations. The linearity is excellent over a concentration range of two orders of magnitude (a limitation imposed by the dynamic range of the photometer). The coefficient of correlation for the responses to the three substances given in Fig. 3 is greater than 0.999 for each substance. The data were obtained by direct introduction of the POE into the SPCC (see Experimental). The reproducibility is excellent, as indicated by Fig. 4. The use of the detector with a Sephadex LH-20 column produced the results shown in Fig. 5.

Figs. 6 and 7 illustrate the use of the SPCC–spectrophototometric detector at two different wavelengths with comparable data for the RI detector. With monitoring at 320 nm the SPCC is approximately two orders of magnitude more sensitive than the RI detector. The use of 620 nm for monitoring decreases the sensitivity of

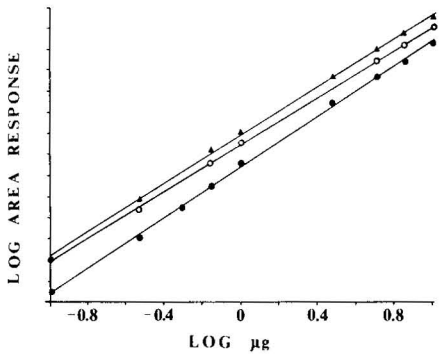

Fig. 3. Relationship of peak area response to amount injected directly on SPCC. △, Carbowax 400; ○, Brij 35; ●, polysorbate 80.

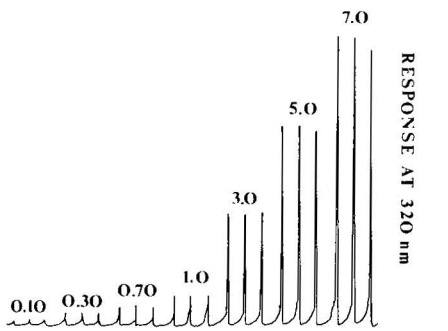

Fig. 4. Multiple injections of dichloromethane solution of Carbowax 400 introduced directly into the SPCC (see Experimental). The number above each set corresponds to micrograms of Carbowax 400.

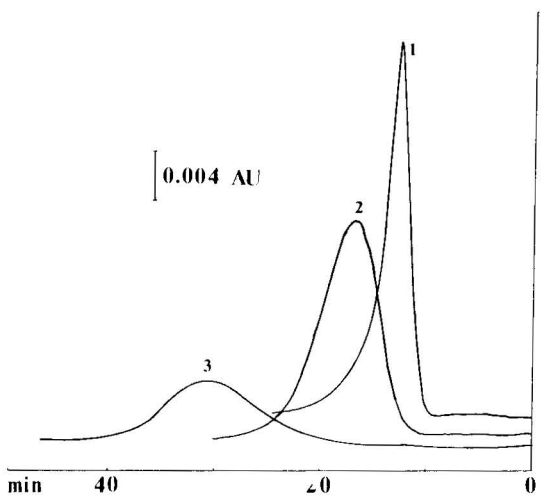

Fig. 5. Elution curves for 5.0 µg each of polysorbate 60 (1), Carbowax 600 (2) and Carbowax 200 (3). A system consisting of a 60 cm X 11 mm I.D. Sephadex LH-20 column with SPCC and photometric detection at 320 nm was used with dichloromethane as the mobile phase.

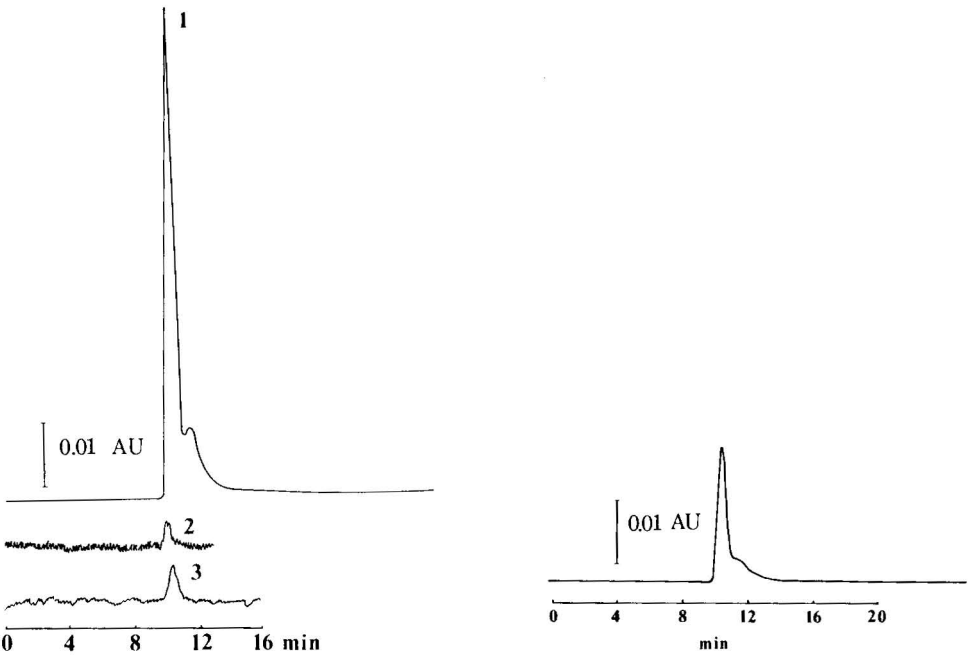

Fig. 6. Chromatography of Carbowax 400 distearate with a single 500 A μStyragel column and dichloromethane at 0.8 ml · min^{-1} as the mobile phase. A, 12 μg on-column with SPCC and spectrophotometric response at 320 nm (0.1 absorbance unit full scale); B, 12 μg on-column with the RI detector; C, 0.12 μg on-column with SPCC and spectrophotometric response at 320 nm (0.01 absorbance unit full scale).

Fig. 7. Chromatography of Carbowax 400 distearate with SPCC and spectrophotometric response at 620 nm; 12 μg on-column with same chromatographic conditions described in Fig. 6.

the SPCC–spectrophotometric technique by a factor of four but adds immeasurably to the selectivity.

CONCLUSION

The concept of a post-column reactor with an adsorbed reactant that will complex with selected components in the eluate has led to the development of a convenient detector. The sensitivity for this class of compounds exceeds that of the RI detector by two orders of magnitude. The SPCC is easily constructed and added to existing chromatographic systems. The technique is applicable, in combination with SEC, as a screening technique to detect intermediate molecular weight POEs in complex matrices such as food.

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AFFINITY CHROMATOGRAPHY ON CONCANAVALIN A–SEPHAROSE OF ANTIGENIC FRACTIONS OF HUMAN SEMINAL PLASMA

MARÍA N. MAZZINI and ALBERTO S. CEREZO*

Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Pabellón 2, Ciudad Universitaria, Buenos Aires (Argentina)

and

JOSEFINA M. S. DE CEREZO

Catedra de Biología, Facultad de Farmacia y Bioquímica, Buenos Aires, and Centro de Investigaciones en Reproducción, Facultad de Medicina, Piso 10, Buenos Aires (Argentina)

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SUMMARY

Elution diagrams obtained on affinity chromatography show that the antigenic fractions of human seminal plasma studied, namely (a) the non-dialyzable components of human seminal plasma and (b) its trichloroacetic acid-soluble fraction, (c) the trichloroacetic acid-soluble fraction of whole human seminal plasma and (d) the pronase digested human seminal plasma, are complex mixtures of glycoproteins with minor amounts of polysaccharides. Some of these glycoproteins contain significant percentages of carbohydrates while others contain only trace amounts. Most of the glycoproteins carry non-reducing end-chain groups comprising α-D-glucopyranosyl, α-D-mannopyranosyl or sterically related residues.

INTRODUCTION

Concanavalin A (Con A)–Sepharose is known to interact specifically with branched polysaccharides and glycoproteins, the non-reducing end chains of which comprise α-D-glucopyranosyl, α-D-mannopyranosyl, β-D-fructofuranosyl and sterically related residues[1]. The fractionation of branched-chain polysaccharides adsorbed on Con A is thought to depend on the differences in the degree of branching. Restrictions imposed by the immobilization of the lectin on the matrix may modify this operation. Thus, in the immobilized case, molecules may be fractionated according to differences in the length of the branches or the distribution of branch points which are not apparent when the complexing is carried out in solution[2].

In previous studies[3,4] four antigenic substances were isolated by gel filtration through Sephadex G-100 of the trichloroacetic acid-soluble fraction of human seminal plasma. The substances were then examined by chemical and physicochemical methods

* To whom correspondence should be addressed.

and identified as three glycopeptides (G-1, G-2 and G-4) containing *ca.* 10% protein, and a polysaccharide (G-3). All contained glucose as the main sugar component and their rotatory powers suggested the presence of glycosidic linkages of the α-D-type[4]. They form insoluble complexes with Con A[4,5].

It was of interest to analyze the macromolecular components of human seminal plasma and some of its fractions possessing antigenic activity by use of affinity chromatography on Con A–Sepharose, and to study the distribution of the molecules according to the configurations of their non-reducing end chains and, in those cases which gave adequate complexing with Con A, according to the differences in the degree of branching.

MATERIALS AND METHODS

Seminal plasma was prepared from whole normal semen obtained from healthy donors, as described previously[3]. Pooled normal human seminal plasma was used as starting material. Con A, covalently bound to Sepharose 4B by the cyanogen bromide method[6], was supplied by Pharmacia (Uppsala, Sweden) as a suspension containing 10 mg/ml of Con A in 0.1 *M* sodium acetate buffer (pH 6.0) with 1 *M* sodium chloride, 1 m*M* calcium chloride, 1 m*M* magnesium chloride, 1 m*M* manganese chloride and 0.01% merthiolate.

Other chemicals and reagents used were as noted previously[3,4].

Non-dialyzable fraction of human seminal plasma

The human seminal plasma prepared as above was dialyzed against 0.15 *M* sodium chloride at 4° for 24 h, centrifuged, dialyzed against distilled water under the same conditions and then freeze-dried.

Trichloracetic acid-soluble fraction of human seminal plasma

This was prepared as described[3].

Pronase-digested human seminal plasma

The enzyme was suspended in 1/15 *M* phosphate buffer, pH 7.0 (1 mg/ml), and a portion of this suspension was mixed with nine times its volume of human seminal plasma. The latter was obtained by centrifugation of whole semen in a Sorvall centrifuge (rotor SS-34 at 12,000 *g*) followed by dialysis against 1/15 *M* phosphate-buffered saline (pH 7.1). The mixture of the enzyme and plasma was incubated at 37° for 90 min with constant stirring. The enzyme was centrifuged off, and the solution that remained was dialyzed until free from salts and then freeze-dried.

Trichloroacetic acid-soluble fraction of non-dialyzable human seminal plasma

The human seminal plasma was dialyzed against 0.15 *M* sodium chloride for 24 h at 4°, centrifuged at 12,000 *g* for 10 min and the trichloroacetic acid-soluble fraction then prepared as above.

Affinity chromatography

An aqueous slurry of adsorbent (52 ml of swollen gel) containing *ca.* 520 mg of Con A was packed into a column (107 × 0.8 cm) and washed with 0.1 *M* sodium

phosphate buffer–1 *M* sodium chloride (pH 7.2) at 20–25° and 0.176 ml/min until a steady state was reached. Solutions (10 mg/ml) of the fractions described above were applied at the top of the column and eluted with the same buffer. When all the non-bound or weakly bound material had been eluted, the eluent was changed to 0.01 *M* sodium borate buffer (pH 6.0), followed by a 0.1 *M* solution of the same buffer. The strongly adsorbed carbohydrates were finally eluted with a 2.0% solution of methyl α-D-glucopyranoside in 0.1 *M* sodium phosphate buffer (pH 7.2).

The eluate was continuously monitored for carbohydrate by the phenol–sulphuric acid reaction[7], and for proteins by UV spectrophotometry at 280 nm[8]. Since the same amounts of products were passed through the column in all cases, the elution diagrams in Fig. 1 and 2 are directly comparable.

The elution volumes of the macromolecules which are not retained by the Con A–Sepharose were determined by chromatography of a polysaccharide (a galactomannan having a branched molecule, the non-reducing end-chain groups of which are α-D-galactopyranosidic residues[9], a protein (bovine serum albumin) and a glycoprotein (ovalbumin[10]). These products gave no precipitate with Con A in solution[4].

RESULTS

Fig. 1 shows the elution patterns of the galactomannan, the bovine serum albumin and the ovalbumin. All were eluted with phosphate buffer; the polysaccharide and the protein gave only one peak each but the ovalbumin showed more than one peak in agreement with its heterogeneity[10]. The galactomannan eluted first (elution

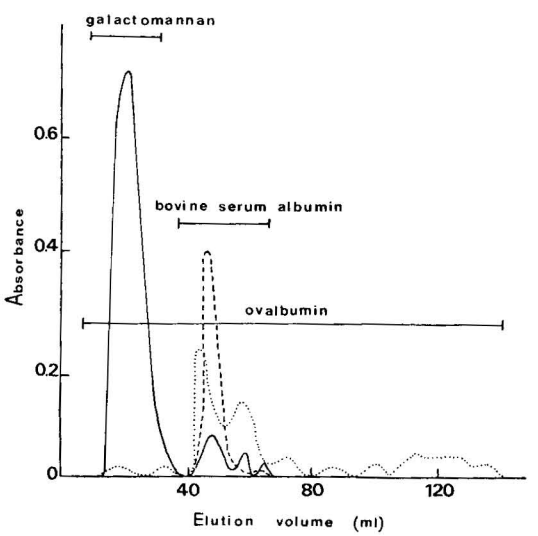

Fig. 1. Elution patterns on Con A–Sepharose of macromolecules (galactomannan, bovine serum albumin and ovalbumin) which do not interact with Con A. The elution was carried out with 0.1 *M* sodium phosphate buffer–1 *M* sodium chloride (pH 7.2) at 20–25° and 0.176 ml/min. Galactomannan: ———, carbohydrate. Bovine serum albumin: – – – –, protein. Ovalbumin: . . ., protein; ———, carbohydrate.

volume 17–34 ml) followed by the protein and the glycoprotein at the same elution volume (40–60 ml, carbohydrate peaks for the ovalbumin).

Fig. 2 shows the elution patterns of (a) the non-dialyzable fraction of human seminal plasma, (b) the trichloroacetic acid-soluble fraction of the non-dialyzable part of the human seminal plasma, (c) the trichloroacetic acid-soluble fraction of human seminal plasma and (d) the pronase-digested human seminal plasma. The four elution diagrams are similar in that they indicate the existence of three different types of substances in the mixtures analysed, according to the eluents used, namely those which were eluted with phosphate buffer, compounds which were not eluted by phosphate buffer but are eluted by borate buffer and finally those which interact strongly with the lectin and are eluted only by the use of a molecular hapten inhibitor of low molecular weight (methyl α-D-glucopyranoside).

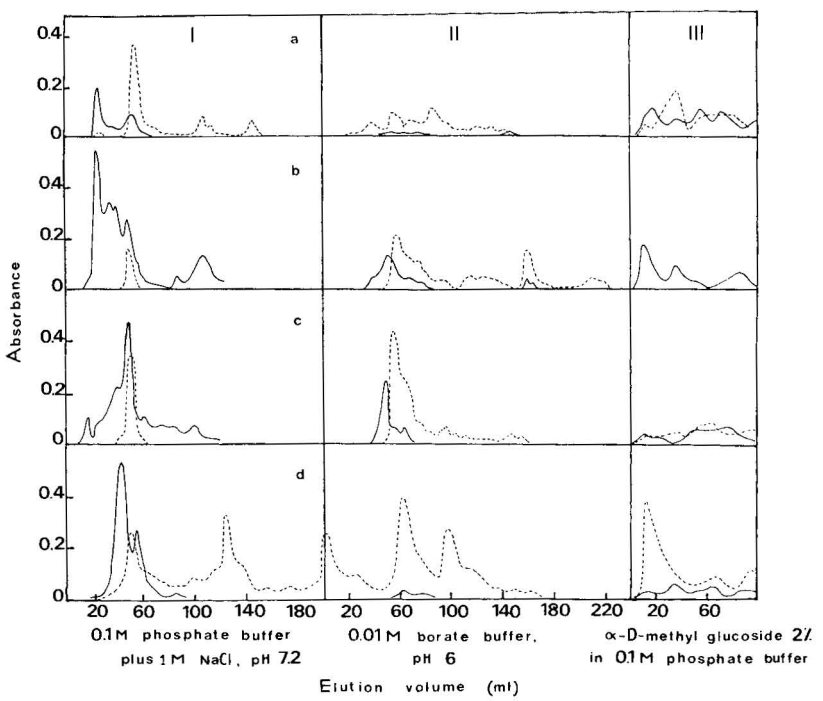

Fig. 2. Elution patterns on Con A–Sepharose of some antigenic fractions of human seminal plasma. a = Non-dializable fraction of human seminal plasma; b = its trichloroacetic acid-soluble fraction (in III the proteins were not determined); c = trichloroacetic acid-soluble fraction of whole seminal plasma; d = pronase-digested human seminal plasma. I = Elution with 0.1 *M* sodium phosphate buffer–1 *M* sodium chloride (pH 7.2); II = elution with 0.01 *M* sodium borate buffer (pH 6.0); III = elution with 2% methyl α-D-glucopyranoside in 0.1 *M* sodium phosphate buffer (pH 7.2). – – –, Protein; ———, carbohydrate.

In the case of the pronase-digested human seminal plasma, small amounts of compounds, which absorb at 280 nm as do proteins but do not react as carbohydrates, were not eluted with phosphate or 0.01 *M* borate buffers, but were displaced by borate 0.1 *M* buffer (these compounds are not shown in Fig. 2).

Fig. 2 also shows that in all cases the major quantities of carbohydrates were eluted by phosphate buffer I, while those products containing minor amounts of sugars were separated from the lectin by the borate buffer II. Proteins of the non-dialyzable (a) and the pronase-treated (d) human seminal plasma were eluted in similar quantities by the three eluents, but those of the trichloroacetic acid-soluble fractions (b and c) were mainly eluted by borate buffer and only very little by phosphate.

The superposition of sugars and proteins in the elution diagrams of Fig. 2 suggests the presence of three types of compounds. First, those formed by carbohydrates (I; b and c), which may contain a little protein (I; a). They are displaced by phosphate buffer and their elution volumes (15–40 ml) are similar to that of the galactomannan or higher (60–120 ml) (I; b and c). Secondly, glycoproteins which are eluted by both buffers, those displaced by phosphate having elution volumes similar to that of ovalbumin or bovine serum albumin. All the material eluted by the methyl α-D-glucopyranoside solution belongs to this type of compounds. Finally, there are peaks (I; a and d) (II; d) and zones (II; b and c) which react only as proteins. No carbohydrates were detected in these.

DISCUSSION

Human seminal plasma is a very complex mixture of macromolecules which has been analyzed by several procedures[11–17]. The immunological methods[12,13,18–20] are based on the interaction of the antigens with their specific antibodies, although the chemistry of these interactions is often unknown.

The use of immobilized Con A permits an analysis of the high-molecular-weight components of human seminal plasma or of fractions derived from it on the basis of specific interactions, the chemistry and structural aspects of which are known (see Introduction).

It is noteworthy that only carbohydrates (with a very small amount of protein in fraction a) were eluted at the elution volume of the galactomannan, while superposition of peaks of protein and carbohydrates was found at the elution volume of the ovalbumin or bovine serum albumin (Fig. 2). It was concluded that human seminal plasma contains polysaccharides (with a very small amount of protein in fraction a) (I, Fig. 2) and glycoproteins (I, Fig. 2) which are linear molecules or, if branched, their non-reducing end-chains do not contain α-D-glucopyranosyl, α-D-mannopyranosyl or its 2-amino-2-deoxy derivatives and β-D-fructofuranosyl residues.

Since the immobilization of the lectin on the matrix introduces a geometrical factor in its reaction with carbohydrates[2] it is possible that the above macromolecules contain adequate end-chain residues for interaction with Con A but that the distance between them precludes the reaction.

Only non-interacting glycoproteins (or mixtures of polysaccharides and proteins) were found in the non-dialyzable fraction of human seminal plasma treated with pronase (d, Fig. 2). Further elution with phosphate buffer (I, Fig. 2) eluted some weakly adsorbed products. These react as carbohydrates in fractions b and c and as proteins in fractions a and d. Since proteins do not interact with Con A[21] it is supposed that they contain a very small amount (not detectable) of carbohydrates which are responsible for the interaction.

Compounds which interacted with Con A were eluted with 0.01 *M* borate

buffer (II, Fig. 2). Superposition of protein and carbohydrate peaks occurred in the four cases, and also compounds were eluted which react only as proteins. It is concluded that two types of glycoproteins which contained major amounts of protein were eluted in this step, one type containing only traces of carbohydrates, the other containing measurable amounts of sugars.

After elution with 0.01 *M* borate, a more concentrated borate buffer (0.1 *M*)[2] was passed through the column. Only a small amount of substances appeared on chromatography of fraction d, and none with the other fractions. These substances are glycoproteins which must contain traces of carbohydrates.

Only glycoproteins were eluted by the methyl α-D-glycopyranoside.

In summary, the results of affinity chromatography on Con A–Sepharose of different fractions of human seminal plasma suggest the following.

(a) The use of protein-removal techniques, such as treatment with a broad spectrum protease (pronase) or precipitation with trichloroacetic acid solution, does not produce mixtures of macromolecules simpler than the original human seminal plasma. The precipitation of human seminal plasma or its non-dialyzable fraction with trichloroacetic acid solution increases the relative content of carbohydrates, but, unexpectedly, treatment of the non-dialyzable fraction with pronase reduces the carbohydrate content.

(b) The polysaccharides and some glycoproteins do not interact (or do so weakly) with Con A and so they do not contain the sugar residues and/or the geometrical requirements for the interaction. On the other hand, most of the glycoproteins interact, some very strongly, with Con A–Sepharose. These results are in accord with the composition of some of the antigens isolated from the trichloroacetic acid-soluble fraction of human seminal plasma[4].

(c) In terms of the strength of the interaction with the insoluble Con A, compounds might be divided into those which do not interact or weakly interact and are eluted with phosphate buffer, those which bind to the protein and are not eluted with phosphate but are eluted with borate buffer and finally those which are strongly adsorbed on the lectin and are displaced only by a solution of methyl α-D-glucopyranoside. This fractionation reflects the differences in the structures of the polysaccharides or in the structures of the carbohydrate portion of the glycoproteins, namely in the identity of the end-chain residues, the amount of branching and the length and distribution of the branches. Further research is necessary to elucidate these structural features.

(d) The elution diagrams show that the fractions of human seminal plasma studied are complex mixtures of glycoproteins with lesser amounts of polysaccharides. Some of these glycoproteins contain significant amounts of carbohydrates while others contain only trace amounts.

ACKNOWLEDGEMENT

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CHROM. 11,743

FRACTIONATION OF HISTONES ON A METAL ION EQUILIBRATED CATION EXCHANGER

I. CHROMATOGRAPHIC PROFILES ON AN AMBERLITE IR-120 (Al^{3+}) COLUMN

A. M. DIWAN and P. N. JOSHI
Department of Chemistry, University of Poona, Pune 411 007 (India)
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SUMMARY

Amberlite IR-120, a polystyrene sulphonate type of cation exchanger, equilibrated with Al^{3+} ions, has been employed for the fractionation of whole histone. This adsorbent permits the quantitative and reproducible recovery of whole histone in six fractions.

INTRODUCTION

The fractions of whole histone are closely similar in size and charge, and the chromatographic techniques available for their separation are not adequate for their resolution[1–4]. Further, these techniques are subject to operational limitations and irreversible binding. For these reasons, methods based on alternative properties are desirable for the separation of histones. This paper deals with histone fractionation on a cation-exchange column.

EXPERIMENTAL AND RESULTS

Whole histone was isolated from buffalo liver by acid extraction. The whole histone was characterized by high-resolution acrylamide gel electrophoresis as described by Paniym and Chalkley[5]. The electrophoretic pattern of buffalo liver histones was comparable with those of rat liver and calf thymus histones (Fig. 1A). The absence of non-histone proteins was ascertained by gel electrophoresis at alkaline pH. The absence of DNA and RNA was ascertained by colorimetric methods[6]. The whole histone was 98% pure as protein, with bovine serum albumin as the standard protein.

An Amberlite IR-120 (Al^{3+}) column was prepared from Amberlite IR-120 (Na^{+}) by treatment with Al^{3+} ions as described by Shankar and Joshi[7]. Acetate buffer of pH 4.0 (50 m*M*) was prepared according to Gomori[8]. The aluminium ions were determined by Sandell's procedure[9]. The basic amino acids were analysed according to Moore and Stein[10].

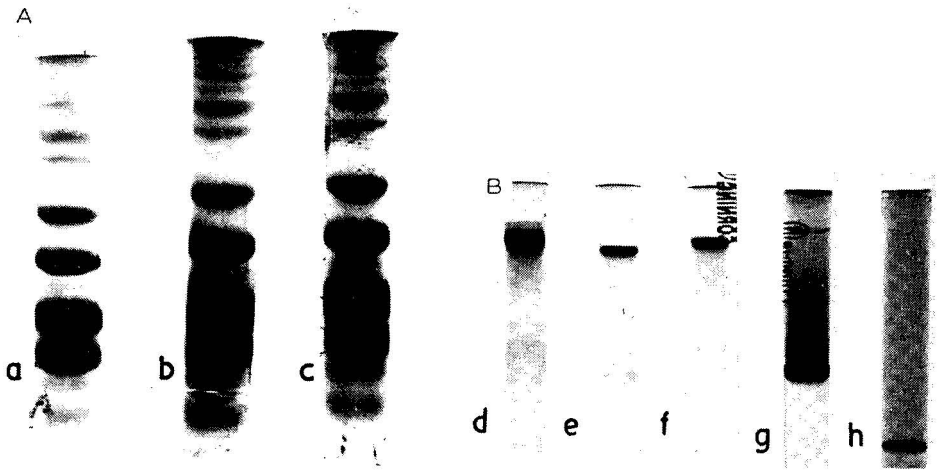

Fig. 1. (A) Gel electrophoretic patterns of (a) rat liver, (b) calf thymus and (c) buffalo liver whole histone. Electrophoresis was carried out according to Paniym and Chalkley[5]. (B) SDS gel electrophoretic pattern of fractions from the IR-120 (Al^{3+}) column. (d)–(h): fraction I–V, respectively.

Typical chromatographic profiles of whole histone

Buffalo liver whole histone was dissolved in acetate buffer (pH 4.0) and the solution was applied to the column containing 5 g of IR-120 (Al^{3+}), having 6 mequiv. of Al^{3+} previously equilibrated with the above buffer. The solution was allowed to percolate through the column. The column was operated at a flow-rate of 30 ml/h at 25°. The effluent was collected and the column was washed with four bed volumes of the buffer to remove loosely retained species of histones.

The adsorbed histone was then eluted with a discontinuous gradient of ethanol–hydrochloric acid. Fractions of 10 ml were collected and assayed for protein content by the method of Lowry *et al.*[11] or by measuring the UV absorption at 280 nm.

The percentages of total histone adsorbed and eluted are given in Table I. Fig. 2 shows a typical profile of histone on the IR-120 (Al^{3+}) column.

TABLE I

CHROMATOGRAPHIC PROFILES OF BUFFALO LIVER AND RAT LIVER WHOLE HISTONE ON AN IR-120 (Al^{3+}) COLUMN

Retention (%)	*Elution by ethanol–hydrochloric acid (%)*						*Total elution of fractions I–VI (%)*
	I	*II*	*III*	*IV*	*V*	*VI*	
100	17.1	18.12	14.89	18.12	19.28	12.46	100
Molarity of acid in 50% ethanol	0.1	0.3	0.5	0.6	0.7	1.0	

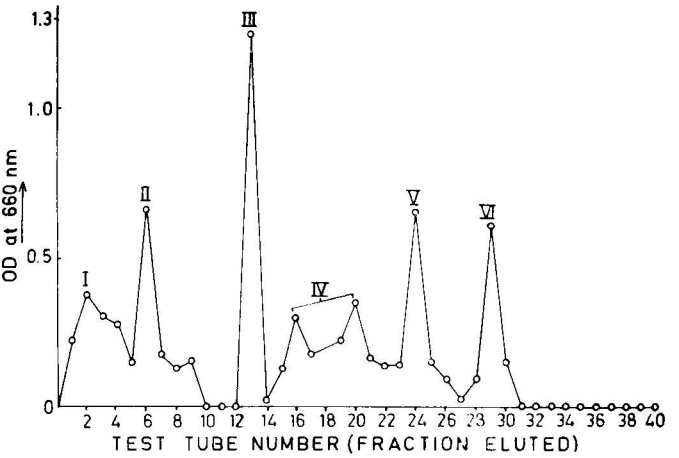

Fig. 2. Typical chromatographic elution profiles of buffalo liver whole histone on an IR-120 (Al^{3+}) column.

Re-chromatography

The behaviour of a fraction when subjected to re-chromatography is an important aspect of any chromatographic technique. Reproducible re-chromatographic behaviour can be regarded as confirming the homogeneity of the material eluted in a specific fraction.

Fraction I obtained by elution with 0.1 *M* hydrochloric acid–ethanol was re-chromatographed on a fresh IR-120 (Al^{3+}) column. The percentages adsorbed and eluted are given in Table II. Fig. 3 depicts the elution profile.

TABLE II

RE-CHROMATOGRAPHY OF FRACTION I ON AN IR-120 (Al^{3+}) COLUMN

Retention (%)	*Acid concentration in hydrochloric acid–ethanol eluting agent (M)*	*Elution (%)*
100	0.1	60
	0.3	10
	1.0	10
		Total: 80

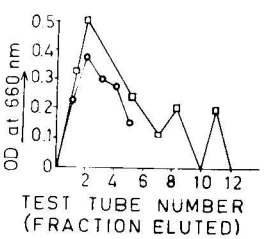

Fig. 3. Re-chromatography of fraction F_1 on an IR-120 (Al^{3+}) column. ○, Chromatography; □, re-chromatography.

Characterization of fractions

The histone fractions obtained from the IR-120 (Al^{3+}) column were characterized by basic amino acid analysis and by gel electrophoresis.

Amino acid analysis. The appropriate fractions from the IR-120 (Al^{3+}) column were concentrated and hydrolysed with 6 *N* hydrochloric acid. The fractions were evaporated to remove excess of acid and then diluted with buffer of pH 5.28. The amino acids were analysed on a 15-cm column essentially according to Moore and Stein[10]. Amino acids such as arginine, histidine and tyrosine were determined by specific colour reactions[12], the results being expressed as moles of individual amino acid per 100 moles of amino acids (Table III).

TABLE III

AMINO ACID CONTENT OF CHROMATOGRAPHIC FRACTIONS FROM AN IR-120 (Al^{3+}) COLUMN

Fraction	*Lysine**	*Arginine**	*Lys/Arg*	*Phenylalanine**	*Tyrosine**	*Histidine**	*Tentative identification of fractions*
I	28.3	1.7	16.6	0.4	0.3	0	F_1
II	16.9	6.3	2.5	1.4	3.7	2.2	F_{2b}
III	12.5	9.2	1.4	1.6	2.2	2.8	F_{2a2}
IV	9.6	13.8	0.7	2.2	3.4	1.9	F_{2a1}
V	10.1	13.6	0.9	2.0	2.1	2.3	F_3
VI	10.0	10.2	1.0	2.1	2.5	2.0	Aggregate

* Expressed as total moles of individual amino acid per 100 mole of total amino acid.

Electrophoresis. The histone fractions obtained were subjected to polyacrylamide gel electrophoresis using 15% acrylamide in 6.25 *N* urea according to Paniym and Chalkley[5]. Electrophoresis in 7.5% acrylamide was carried out according to the method of Wray and Stubblefield[13]. However, in all instances low mobilities and hence poor resolutions were observed for the IR-120 (Al^{3+}) fractions. Even the method of John and Forrester[14] did not give satisfactory results. Histones are eluted as histone–Al^{3+} complexes from IR-120 Al^{3+} column and these complexes could not be resolved satisfactorily under various conditions of electrophoresis. Therefore, sodium dodecyl sulphate (SDS) electrophoresis was carried out according to Shapiro *et al.*[15]. The order of mobility of the fractions was 5 > 4 = 3 > 2 > 1 (Fig. 1B). This only indicates that fraction I has the highest molecular weight and fraction V the lowest molecular weight. As the histone fraction of highest molecular weight is the very lysine-rich histone F_1, it can be concluded that very lysine-rich histone elutes first from the IR-120 (Al^{3+}) column, and this conclusion is supported by the results of amino acid analyses.

DISCUSSION

The failure of the IR-120 (Na^+) column to adsorb histone and the large increase in retention on the IR-120 (Al^{3+}) column show that interaction on the IR-120 (Al^{3+}) is the basis of adsorption. Dissociation of the resin Al^{3+}–histone complex and its subsequent removal could be the basis for resolution by elution.

Reagents such as sodium chloride, sodium fluoride, sodium citrate, sodium tartrate, hydrochloric acid alone or ethanol alone fail to elute significant amounts of histones from the IR-120 (Al^{3+}) column. Acid–ethanol was the only eluting agent that was successful. Different fractions of IR-120 (Al^{3+}) contain various concentrations of Al^{3+} ions, and this may indicate that different fractions have different binding capacities with immobilized Al^{3+}. A pH of 4.0 was selected for adsorption mainly because (1) the maximal concentration of Al^{3+} was found to be achieved at pH 4.0 and (2) the tendency for aggregation is minimal at pH 4.0 and increases with increasing pH. Low ionic strength buffers were employed in order to minimize the tendency for aggregation.

As indicated in Table III, the lysine to arginine ratio decreases with increasing elution volume. The tentative identifications of the first five IR-120 (Al^{3+}) fractions are F_1, F_{2b}, F_{2a2}, F_{2a1} and F_3, respectively. The sixth fraction was a unique fraction, in which the lysine content was approximately equal to the arginine content. This fraction might have resulted from aggregation. The fractionation on IR-120 (Al^{3+}) seems to be qualitatively related to the amino acid composition. The first fraction had the highest lysine content, which gradually decreased until the fourth fraction and for the fifth and sixth fractions remained virtually constant. On the other hand, the arginine content was low for the first fraction and gradually increased until the fourth fraction.

The order of elution from Amberlite IRC-50 for whole histone, using a gradient of guanidium chloride, is F_1, F_{2a2}, F_{2b}, F_3 and F_{2a1}. This is slightly different from the order on IR-120 (Al^{3+}). The results also agree with those on Amberlite IRC-50, in which lysine-rich histones are eluted earlier than arginine-rich histone. Another advantage of the IR-120 (Al^{3+}) column is that all of the fractions are clearly separated.

On comparison of the results on IR-120 (Al^{3+}) with those obtained by CM-cellulose chromatography as described by Senshu and Iwai[3] using ethanol–formic acid for elution, it can be seen that in the latter instance arginine-rich histones are eluted first, followed by moderately lysine-rich histones and finally very lysine-rich histones. The order of elution of whole histone from CM-cellulose corresponded to their extractability from nucleoprotein with ethanol–hydrochloric acid and the reverse of their extractability with aqueous acids. Here it was concluded that the interaction of histone fractions with CM-cellulose seems to be similar to the interaction with deoxyribonucleoprotein. Using similar systems, with IR-120 (Al^{3+}) lysine-rich histones are eluted first, followed by arginine-rich histones. The order is therefore different from that in CM-cellulose chromatography and may indicate a difference in the mechanism of operation of IR-120 (Al^{3+}) and CM-cellulose as far as histones are concerned.

The reported amino acids analysis suggests that fraction I is very lysine-rich histone which is heterogeneous. The known chromatographic systems are unable to resolve whole histone into individual fractions or into sub-fractions in one operation and therefore the sub-fractionation that occurs during re-chromatography is not totally unexpected. During re-chromatography, in the absence of other fractions, further resolution seems possible. In fact, chemically fractionated histones are known to be better suited for the resolution of histones by chromatographic means. Therefore, sub-fractionation during re-chromatography of F_1 is to be expected.

The validity of the chromatographic method for achieving satisfactory resolutions on IR-120 (Al^{3+}) of proteins[16], enzymes[17] and RNA[7] has been demonstrated

by several workers. During re-chromatography, as reported in Table II, fraction I was resolved into three peaks. Most of the elution occurred with 0.1 *M* hydrochloric acid–ethanol reagent, which is also the eluting agent for the fraction during the earlier elution. The other two peaks are very small in comparison with the major peak.

Quantitative elution and satisfactory re-chromatography suggest that the fractionation procedure is reliable. IR-120 (Al^{3+}) fractionates histones according to amino acid composition and to some extent according to molecular weight.

CONCLUSIONS

These studies were aimed mainly at examining the chromatographic behaviour of histones. It can be stated that the described fractionation procedure does not appear to be of the conventional ion-exchange chromatographic type because Al^{3+} ions are also involved in the adsorption.

The investigation has shown that the use of an IR-120 (Al^{3+}) column is a promising technique for the fractionation of whole histone. Further, IR-120 (Al^{3+}) is reasonably stable at elevated temperatures and even at high salt concentrations and the technique offers a simple, reproducible and inexpensive system for the fractionation of proteins. In addition, certain factors such as concentration of protein, flow-rate and ageing of histones do not have significant effects on the chromatographic behaviour of histones on the IR-120 (Al^{3+}) column.

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CHROM. 11,745

ENZYMATIC REACTIONS ON THIN-LAYER CHROMATOGRAPHIC PLATES

II. PHOSPHOLIPASE A_2 HYDROLYSIS OF PHOSPHATIDYLCHOLINE AND SEPARATION OF THE PRODUCTS ON A SINGLE PLATE

JYOTIRMOY DUTTA and ARUN Kr. DAS
Department of Chemistry, Bose Institute, Calcutta 700 009 (India)
and
ANURADHA BISWAS
Department of Physiology, University College of Science, Calcutta 700 009 (India)
(Received December 8th, 1978)

SUMMARY

A procedure for the phospholipase A_2 hydrolysis of phosphatidylcholine on a thin-layer chromatographic plate and subsequent separation of the products on the same plate is described. A 0.2–0.8-mg amount of Russell's viper venom (phospholipase A_2) in 0.2 ml of 0.005 *M* calcium chloride solution was applied on a 0.5-mm silica gel G plate as a band over which 2–5 mg of egg phosphatidylcholine in 0.2 ml of diethyl ether containing 5% of methanol was evenly applied. After the reaction had proceeded for 15–20 min in a diethyl ether-saturated chamber at 25°, the plate was developed with chloroform–methanol–water (65:25:4). The bands were identified and their contents extracted. The extent of hydrolysis under different reaction conditions was evaluated from the amount of lysophosphatidylcholine formed. Approximately 74.6% (maximum) conversion was obtained within 15 min at 25° using a substrate to enzyme ratio of 4:1. The acyl group distributions in the 1- and 2-positions of hen egg phosphatidylcholine obtained from the gas–liquid chromatographic analysis of the methyl ester corresponding to the lyso and free fatty acid band agreed with those obtained by the method of Wells and Hanahan. The method is also applicable to phosphatidylethanolamine.

INTRODUCTION

Studies over the last decade have revealed that phospholipids of membranes from different animal tissues consist of molecular species differing in the fatty acid distributions at the 1- and 2-positions of the diacylglycerophosphatide molecules[1]. It has been suggested that the specific distributions of the fatty acid components at these two positions not only reflect the biosynthetic capabilities of the tissue membrane

but also are the products of a regulatory mechanism for maintaining the appropriate lipid–lipid or lipid–protein interactions as required for optimal functioning of the particular membrane concerned[2]. Thus an understanding of the nature and distributions of different fatty acyl groups in phospholipids is assuming increasing importance. Hydrolysis of the acyl group attached at a specific position by stereospecific phospholipase, *viz.*, phospholipase A_2 [3-6], separation of the products and evaluation of the compositions of fatty acids in the lysophospholipid and free fatty acid fractions is the basis of finding the distributions of acyl groups in the 1- and 2-positions of phospholipids[7-10].

Recently we developed a method[11] in which hydrolysis of the acyl group attached to the 1- and 3-positions of triglycerides by pancreatic lipase and separation of the reaction products were carried out on a single thin-layer chromatographic (TLC) plate. This paper deals with the development of a similar method in which phospholipids are hydrolysed at the 2-position by phospholipase A_2 (Russell's viper venom) and the reaction products are separated on the same TLC plate. The products of hydrolysis can easily be isolated and their fatty acid compositions can be evaluated by gas–liquid chromatography (GLC).

The extent of hydrolysis in relation to different reaction conditions has been measured. The method has been used to establish the distributions of the acyl groups at 1- and 2-positions of phosphatidylcholine from hen egg. An extent of hydrolysis comparable to that of phosphatidylcholine under optimal conditions has also been observed with phosphatidylethanolamine.

EXPERIMENTAL

Solvents

All solvents were of analytical-reagent grade, and were dried and redistilled. Diethyl ether was freed from peroxide before being dried and distilled.

Reference lipids

Phosphatidylcholine (PC) (egg), lysophosphatidylcholine (LPC) (egg) and phosphatidylethanolamine (bovine), all of 99% purity, were purchased from Applied Science Labs. (State College, Pa., U.S.A.).

Reagents

Source of phospholipase A_2 (E.C. 3.1.1.4). Lyophilized venom of Russell's viper (*Vipera russelli*) was purchased from the CSIR Centra for Biochemicals (University of Delhi, New Delhi, India).

Preparation of venom solution, determination of its protein content and assay of the activity of phospholipase A_2 in the solution. The lyophilized venom was shaken with the appropriate volume of 0.005 *M* calcium chloride solution ($CaCl_2 \cdot 2H_2O$, G.R. grade; E. Merck, Darmstadt, G.F.R.) (2 mg of venom per millilitre). The venom dissolved completely in the calcium chloride solution. This solution was used for all experiments. The protein content per millilitre of the solution was determined according to Lowry *et al.*[12] and the activity of phospholipase A_2 was assayed according to the method of Magee and Thompson[13] as modified by Dawson[14]. Venom was found to be composed solely of soluble protein. The activity of phospholipase A_2 was found

to be 1.2 units per milligram of protein (1 unit will hydrolyse 1 μmole of phosphatidylcholine to lysophosphatidylcholine per minute at pH 7.2 and 30°).

Plate preparation

Glass plates (20 × 14 cm) were coated with 0.5-mm layers of silica gel G (E. Merck), activated at 110° for 1 h and stored in a desiccator. Before use the plates were pre-developed in diethyl ether to remove any organic contaminants to the top of the plate, from where they were removed by scraping off a narrow band of the adsorbent.

Phospholipase A_2 hydrolysis of egg phosphatidylcholine and resolution of the products on the same TLC plate

The required volume of venom solution (0.2–0.4 ml) containing 0.4–0.8 mg of venom protein was applied as a band on the preparative TLC plate 2 cm from one of the shorter edges. The plate was held horizontally in the air draught from an electric fan for 10 min to remove most of the water from the band. The required amount of egg PC (2.5–5 mg) in peroxide-free diethyl ether containing 5% of methanol was applied as evenly as possible over the enzyme band. The plate was immediately placed in a TLC chamber saturated with diethyl ether vapour (a beaker containing the solvent was placed in the chamber for this purpose) and kept at a specific temperature in order to prevent the evaporation of ether from the application zone. After a stipulated time (15–20 min) the plate was transferred quickly into another saturated TLC chamber and developed to 14 cm from the line of application with chloroform–methanol–water (65:25:4). The different bands on the developed chromatogram were located with iodine vapour (Fig. 1), identified by comparing their R_F values with those

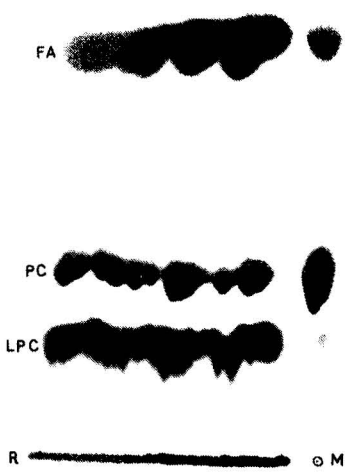

Fig. 1. Hydrolysis of egg PC by phospholipase A_2 and resolution of the reaction products on the same TLC plate. TLC plate: 14 × 20 cm, 0.5-mm silica gel layer. Reaction conditions: 0.4 mg of venom (Russell's viper) equivalent to 0.48 unit (see text) of phospholipase A_2 applied as band over which 4.8 mg of egg PC were evenly applied; reaction temperature, 25°; reaction time, 15 min; developing solvent, chloroform–methanol–water (65:25:4); detection, treatment with iodine vapour followed by spraying with 0.5% starch solution. R = Reaction zone; M = mixture of reference LPC, PC and linoleic acid.

TABLE I

RECOVERY OF LPC FROM THE REACTION ZONE

Set of experiments	*Materials applied in sequence on the chromatographic plate*		*LPC recovered (μmole)*		*LPC recovered (R − L) (μmole)*	*Recovery of LPC (%)*
	Left-hand half	*Right-hand half*	*From left-hand half (L)*	*From right-hand half (R)*		
1st	0.40 mg of lipase, 2.06 μmole of egg PC	0.40 mg of lipase, 2.06 μmole of egg PC, 4.60 μ mole of LPC	1.53 ± 0.02	6.09 ± 0.10	4.56 ± 0.10	99.13 ± 2.17
2nd	0.40 mg of lipase, 2.06 μmole of egg PC	0.40 mg of lipase, 2.06 μmole of egg PC, 9.30 μmole of LPC	1.52 ± 0.03	10.79 ± 0.24	9.27 ± 0.24	99.68 ± 2.58
3rd	0.40 mg of lipase, 2.06 μmole of egg PC	0.40 mg of lipase, 2.06 μmole of egg PC, 13.90 μmole of LPC	1.53 ± 0.04	15.22 ± 0.07	13.69 ± 0.08	98.49 ± 0.58

of known standards and marked. The LPC band was then scraped off the plate, transferred to a mini-column and the lipids were recovered from the adsorbent by extraction with three 3-ml portions of chloroform–methanol–water (1:1:0.2). The solvents were evaporated under reduced pressure, removing the last traces by flushing with nitrogen. The lipids were finally dissolved in a known volume of the same solvent mixture for the determination of LPC. Where determinations of fatty acid (FA) composition were required, the FA bands were extracted in a similar manner with diethyl ether. The solvent was removed under nitrogen and the lipids were redissolved in a known volume of chloroform and kept under nitrogen in the cold for further analysis.

Determination of LPC

LPC was estimated spectrophotometrically as micromoles of phosphorus according to Ames[15]. From this result the molar percentage hydrolysis of PC was calculated.

Isolation of PC from hen egg

Yolks from fresh hen eggs were collected, homogenized with half their volume of distilled water and the lipids extracted by the procedure of Bligh and Dyer[16]. Phospholipids were isolated by silicic acid (100 mesh; Mallinckrodt, St. Louis, Mo., U.S.A.) column chromatography[17]. Pure PC was isolated from hen egg phospholipids by preparative TLC using chloroform–methanol–water (65:25:4) as the developing solvent. Bands were detected with iodine vapour, the R_F values were compared with those of known standards and the lipids were recovered from the adsorbent by elution with chloroform–methanol–water (1:1:0.2). The isolated material produced a single spot of phospholipid with an R_F value identical with that of reference PC spotted on the same TLC plate.

Identification of FA components by GLC

The FA composition of the phospholipids and the products of their on-plate lipolysis by phospholipase A_2 were determined by the GLC analysis of the corresponding mixed methyl esters prepared by the methanol–sulphuric acid method[18,19]. A dual-column F & M Model 700R analytical gas chromatograph with a flame-ionization detector (FID) was used. Chromatograms were obtained on 6 ft. × 1/8 in. stainless-steel columns packed with 10% EGSS-X coated on 100–120-mesh Gas-Chrom W (Applied Science Labs.) at 160°. The carrier gas (nitrogen) flow-rate was kept at 40 ml/min. Components were identified by comparing their retention times with those of authentic standards. Compositions were calculated from peak areas obtained by the triangulation method and corrected by multiplication by the appropriate calibration factors.

RESULTS

The results in Table I show the recovery of LPC from the reaction zone. Twelve chromatographic plates divided into three equal sets were used in these experiments. Each plate was divided into two halves and on each half 0.4 mg venom was applied as bands. On the left-hand half of all the plates 2.06 μmole of egg PC were applied over the enzyme bands, but on the right-hand half of the plates of the first, second

and third sets, in addition to 2.06 μmole of egg PC, 4.6, 9.3 and 13.9 μmole of LPC were added over the enzyme bands. Reactions were carried out at 25° for 15 min. The contents of all the LPC bands were measured. The amounts of LPC obtained from the left-hand halves were due to the hydrolysis of 2.06 μmole of PC and those from the right-hand halves from the hydrolysis of 2.06 μmole of PC and the added LPC. The difference between these two results was obviously the amount of LPC recovered. The results show that up to at least 13.9 μmole (*ca.* 7.22 mg) of LPC added, the removal from the reaction zone was almost quantitative. As LPC was the most polar of all the lipid components on the chromatogram, it can be assumed that other products were also removed quantitatively from the reaction zone.

The results of the time course of on-plate lipolysis (Fig. 2) show that at a substrate to enzyme ratio of 12:1, between 2.5 and 15.5 min the extent of hydrolysis increased from 17.0 ± 1.0 to 56.1 ± 0.5% and remained steady at about this level (56.3 ± 0.5%) up to 30.5 min. The conversion can be increased by decreasing the substrate to enzyme ratio, as is clear from Fig. 3. The maximal conversion of approximately 74.6% can be achieved with the present enzyme sample when this ratio is 4 or less. When the method is to be used for either the preparation of pure lyso compound or the determination of acyl group distributions in phospholipids, a lower ratio is obviously to be preferred, but care should be taken not to overload the TLC plate.

The extents of lipolysis within 15 min using 0.4 mg of venom and 6.19 μmole of egg PC at different reaction temperatures between 5 and 35° were measured. The results (Fig. 4) show that the extent of hydrolysis increased rapidly up to 20°, at which temperature *ca.* 53.0 ± 0.2% of egg PC was hydrolysed within 15 min. On a further increase in temperature up to 30°, this value increased very slowly to 56.1 ± 0.5%, probably owing to an increased rate of evaporation of diethyl ether from the reaction

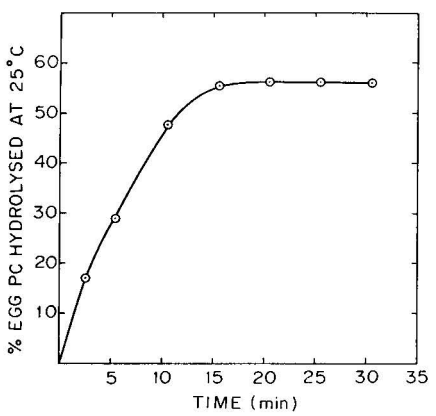

Fig. 2. Time course of on-plate lipolysis of egg PC. Venom from Russell's viper (0.4 mg) was applied as a band over which 4.8 mg of egg PC (*ca.* 6.19 mole) were added throughout the band as quickly as possible. Reactions at 25° were carried out for 2.5, 5.5, 10.5, 15.5, 20.5, 25.5 and 30.5 min, the time being calculated from just after the complete addition of PC on the enzyme band to the complete coverage of the reaction zone by the developing solvent. The amounts of PC hydrolysed were evaluated from those determined for LPC. Each point on the curve is the mean of the results of four experiments; the deviation from the mean for all the points was within ±0.8.

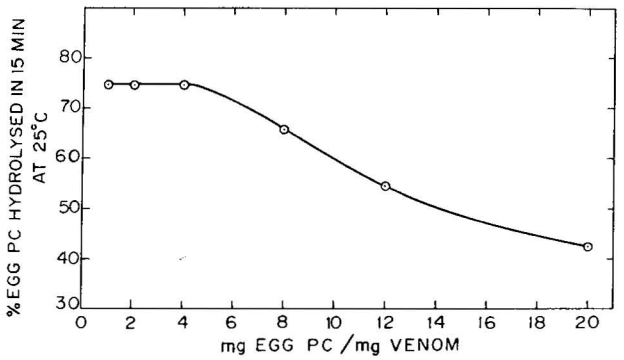

Fig. 3. Effect of substrate concentration per milligram of venom on the extent of lipolysis. On each of six sets (four in a set) of TLC plates 1 mg of venom was applied. The amounts of egg PC applied were 1, 2, 4, 8, 12 and 20 mg. The reaction was allowed to proceed for 15 min at 25° and the amounts of PC hydrolysed were measured from those determined for LPC. Each point on the curve is the mean of the results of four experiments; the deviation from the mean for all the points was within ± 0.9.

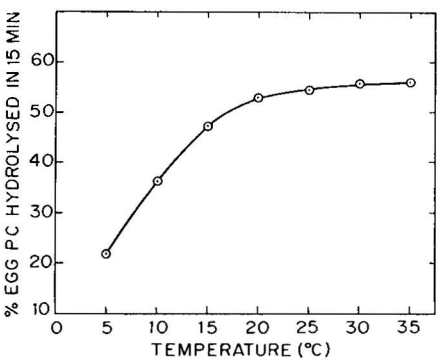

Fig. 4. Effect of temperature on the extent of hydrolysis. Venom (0.4 mg) and egg PC (4.8 mg) (*ca.* 6.19 mole) were applied, in sequence, as a band on each of seven sets of TLC plates. The reaction was carried out for 15 min at 5°, 10°, 15°, 20°, 25°, 30° and 35° from the first to the seventh set. The amounts of PC hydrolysed were calculated from those determined for LPC. Each point on the curve is the mean of the results of four experiments; the deviation from the mean for all the points was within ±0.6.

zone. It was shown by Hanahan[20] that during this enzymatic reaction an ether-soluble enzyme–substrate complex is formed.

The positional distributions of fatty acids in hen egg PC were determined. Hen egg PC was subjected to on-plate enzymatic hydrolysis (1 mg of venom per 5 mg of substrate) and the reaction products were separated and isolated. The fatty acid compositions of LPC and free FA were determined by GLC and the positional compositions are given in Table II. The same compositions were also determined by the GLC analysis of the products obtained by the lipolysis of hen egg PC according to the method of Wells and Hanahan[6], and are reported in Table II for comparison. The results show that the compositional data obtained by the two methods were in close agreement. The major molecular species of hen egg PC can be calculated from the compositions in Table II assuming a 1-random–2-random distribution of acyl groups.

TABLE II

POSITIONAL DISTRIBUTION OF FATTY ACIDS IN HEN EGG PC

Position	*Method of calculation*	*Fatty acid composition*							
		14:0	*16:0*	*16:1*	*18:0*	*18:1*	*18:2 (n − 6)*	*20:4 (n − 6)*	*22:6*
PC*	—	1.2	31.5	2.4	16.6	34.4	10.5	3.4	tr**
1	This work	1.7	58.7	tr**	31.7	6.1	1.2	0.6	—
	Ref. 6	1.5	58.3	tr**	32.5	5.8	1.0	0.9	—
2	This work	0.1	1.7	4.1	0.6	65.2	21.4	6.9	tr**
	Ref. 6	0.1	1.2	3.3	tr**	66.4	22.8	6.2	tr**

* Initial phosphatidylcholine.
** tr = less than 0.1 %.

It was found that phosphatidylethanolamine can be effectively subjected to on-plate hydrolysis by snake venom phospholipase A_2. Experiments with 2.4 mg of phosphatidylethanolamine and 0.2 mg of venom at 25° showed that 56.5 ± 1.1 % of the lipids can be hydrolysed within 15 min.

DISCUSSION

A number of methods have been established for enzymatic deacylation at the 2-position in phospholipids[3–6]. Although good results for the positional distribution of acyl groups can be obtained by these methods, they involve a number of steps. For example, those of Haverkate and Van Deenen[5] and Wells and Hanahan[6] require vigorous shaking of the reaction mixture, evaporation of the solvents under nitrogen, separation of the products by TLC and evaluation of acyl group distributions by GLC. The time required is several hours.

In the present method a number of steps have been avoided by performing the enzymatic reaction and the separation of the products on the same TLC plate. Thus the time required is considerably reduced and the losses due to transfers are diminished.

The close agreement between the positional distributions of acyl groups in hen egg PC obtained by the present on-plate method and those obtained by Wells and Hanahan[6] indicates that the on-plate method is adequate for the determination of above distribution values. This method can also be used for milligram-scale preparations of pure lyso compounds.

ACKNOWLEDGEMENTS

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Note

Evaluation of dead space in chromatographic systems

W. K. AL-THAMIR, J. H. PURNELL and C. A. WELLINGTON
Department of Chemistry, University College of Swansea, Swansea, Wales (Great Britain)
and
R. J. LAUB
Department of Chemistry, Ohio State University, Columbus, Ohio 43210 (U.S.A.)
(Received December 28th, 1978)

The determination of dead space, V_A, or dead time, t_A, in chromatographic systems is required both for the evaluation of specific retention volumes or of partition coefficients and of capacity factors (k'), these being of significance in that, amongst other things, they provide a basis for numerical definition of relative retention and, hence, of separation.

The most commonly employed method of assessing t_A is the elution of a non-sorbed solute. With most detectors in gas–liquid chromatography (GLC) this is, typically, air or inert gas, for which there is little danger of error as these gases are essentially insoluble in liquid stationary phases at the most useful working temperatures. In gas–solid (GSC) or liquid chromatography (LC), on the other hand, it is difficult to ensure that the chosen eluate is genuinely non-sorbed or completely insoluble; protracted procedures have, as a result, been developed over the years for these techniques and are still a matter of current concern, as is comprehensively described in the most recent publications[1–4]. Further, with flame-ionization detection, even GLC provides problems as the sample size of non-combustible gas required to yield a detector response may well cause much flow variation due to viscosity effects while methane cannot be guaranteed to have a sufficiently small capacity factor to offer a truly accurate result.

An alternative approach to the above is to take advantage of the well known fact that, in both GC and LC, the true retention volumes (V'_R) or retention times (t'_R) of members of homologous series are often connected via an equation of the form

$$\log t'_R = a'n + b' \tag{1}$$

where $t'_R = t_R - t_A$, a' and b' are series constants and n is a number representing the size of the homologous unit. As only the gross retention time, t_R, can be determined directly, the usual approach is to plot a graph of $\log t_R$ against n, extrapolate linearly the data for the highest values of n, use the intercept at $n = 0$ to correct values of t_R for all data points and re-plot the new data. The procedure must be reiterated to achieve linearity of the data according to eqn. 1, normally at least five times to achieve 5% accuracy of V_A. Complete accuracy, of course, demands an infinite num-

ber of iterations. If members very high in the homologous series are used, experiments become very time consuming but the number of iterations is reduced markedly.

We show here that all of the foregoing problems can be solved by a simple numerical variant of the second procedure.

Re-writing eqn. 1:

$$\ln(t_R - t_A) = an + b \tag{2}$$

we have

$$t_R = t_A + \exp(an + b) \tag{3}$$

Hence, for two successive homologues ($n = n$ and $n = n-1$),

$${}^{n}t_R = t_A + \exp(an + b)$$

$${}^{n-1}t_R = t_A + \exp[(n-1)a + b]$$

Thus

$${}^{n}\Delta t_R = {}^{n}t_R - {}^{n-1}t_R = (1 - e^{-a}) \exp(an + b) \tag{4}$$

For the pair $n = n - 1$ and $n = n - 2$, correspondingly,

$${}^{n-1}\Delta t_R = (1 - e^{-a}) \exp[a(n-1) + b] \tag{5}$$

Hence

$${}^{n}\Delta t_R / {}^{n-1}\Delta t_R = e^{a} \tag{6}$$

Having evaluated a, it is a simple matter to evaluate b via eqn. 4 or 5. Hence t_A can be evaluated for all homologues employed and a consistent result will confirm its validity.

Table I illustrates application of the method to data for elution of C_1–C_4 alkanes from a GSLC column (alumina + squalane). The data here are reported simply in terms of chart millimetres. Application of our method yielded the values $a' = 0.617$ and $b' = -0.430$, as they apply in eqn. 1.

TABLE I

RETENTION DATA (CHART mm) FOR ELUTION OF C_1–C_4 ALKANES FROM A GSLC (Al_2O_3 + SQUALANE) COLUMN

Log $t'_R = 0.617n - 0.430$.

Alkane	t_R	t'_R	t_A
Methane	21.5	1.50	20.0
Ethane	26.5	6.40	20.1
Propane	46.5	26.40	20.1
n-Butane	129.5	109.10	20.4

We can see the excellent consistency of the data.

Table II shows a set of data, in terms of volumes, for elution of both *n*-alkanes and 1-alkenes from a 2% (w/w) column of dinonyl phthalate (DNP) on Chromosorb G. The relevant equations are quoted in the Table.

TABLE II

RETENTION DATA (ml) FOR *n*-ALKANE AND 1-ALKENE ELUTION FROM A COLUMN OF 2% DNP ON CHROMOSORB G AT 50°

Compressibility factor, $j = 0.375$.
Alkanes: $\log t'_R = 0.471n - 1.082$.
Alkenes: $\log t'_R = 0.405n - 0.777$.

Compound	V_R	V'_R	V_A
Methane	19.05	0.25	18.80
Ethane	19.68	0.73	18.95
Propane	21.10	2.15	18.95
n-Butane	25.29	6.34	18.95
Ethene	19.68	1.08	18.60
Propene	21.35	2.75	18.60
Butene-1	25.59	6.97	18.60

Table II further confirms the validity of the method but establishes further its power in that it works admirably when $V_A \gg V'_R$, that is, we are able to achieve a highly accurate result even when retention is almost trivial. Two further points can be drawn out in addition. Firstly, the measurement of V_A attains greater credibility when, as here, the same value is derived from two different homologous series. Secondly, to evaluate V_A truly, retention volumes at the retention temperature must be used and the average then multiplied by the compressibility factor, j. Thus, the correct result from Table II is $V_A = 0.375 \times 18.78 = 7.04$ ml.

Finally, we show one of a number of successful examples we have drawn from the literature of high-performance liquid chromatography (HPLC)[5]. This relates to the analysis of a coal tar pitch cyclohexane extract run on a 25-cm Partisil 5 column. Consideration of the structures of the large number of compounds cited in this

TABLE III

RETENTION DATA[5] (ml OF *n*-HEXANE) FOR ELUTION FROM A PARTISIL 5 COLUMN

Compound	V_R	V'_R	V_A
Pyrene*	16.0	11.4	4.4
Benzo(*a*)pyrene	27.7	23.3	4.4
Dibenzo(*ah*)pyrene	51.2	46.8	4.4
Pyrene	16.0	12.2	3.8
Benzo(*a*)/benzo(*e*)pyrene**	28.5_5	24.7_5	3.8
Dibenzo(*ae*)pyrene	54.0	50.2	3.8
Anthracene*	16.8	13.1	3.7
Benzo(*a*)anthracene	27.3	23.6	3.7
Dibenzo(*aj*)anthracene	46.2	42.5	3.7
Phenanthrene	16.2	11.5	4.7
Benzo(*a*)anthracene	27.3	22.6	4.7
Dibenzo(*ah*)anthracene	49.1	44.4	4.7

* Detailed consideration of the data and chromatograms indicate that the listed retention volumes for these two compounds have been overestimated, probably due to the incomplete resolution attained. The values here are our best estimate of the correct calues.

** Data are taken as the average of those for elution of benzo(*a*)pyrene and benzo(*e*)pyrene.

admirable work reveals the possibility of four groups of three compounds each of which might fulfil the requirements of eqn. 1. These are as listed in Table III which contains also the data and calculated values of column dead volume.

The average value over the four groups is $V_A = 4.15$ ml. The authors[5] quote 4.2 ml. Despite this very satisfactory agreement we, on balance, prefer the lower value of 3.7–3.8 ml since 4.2 ml looks rather a large dead volume for a column of the quoted dimensions.

It is important to emphasise here that any three data points in which $\Delta_2 > \Delta_1$ will give a consistent value of V_A. If, therefore, only three points are available the quality of the data must be unquestioned. Even a quite small error in one will have profound effect on the value of V_A derived. On account of this, the use of several groups of related solutes is much to be recommended since, if all give a similar result, this can be accepted with confidence since coincidence of erroneous results is most unlikely.

Finally, it is worth emphasising that the method has one major advantage over most others, the exact value of n required in the calculations is unimportant, it is only necessary that the values used be in the correct ratio. In the first group of Table III, for example, the C numbers are 16, 20, 24 but the calculations can be carried out with $n = 4$, 5, 6, or any other multiples. We are thus relieved of the problems of (a) identifying the compound of $n = 0$ and (b) extrapolating over a long range of n, which characterise most other methods.

We believe the above to be a useful method and both more dependable and simpler than alternatives when direct determination of V_A by elution of totally non-sorbed materials is precluded or uncertain. It obviously has particular potential in HPLC, where the determination of k' is notoriously a problem.

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CHROM. 11,726

Note

Evaluation of selected stationary phases suitable for the gas–liquid chromatographic analysis of triglycerides

R. ANEJA, A. BHATI, R. J. HAMILTON, F. B. PADLEY and D. A. STEVEN*

Department of Chemistry, Liverpool Polytechnic, Liverpool (Great Britain) and Unilever Research Laboratory, Colworth (Great Britain)

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High-temperature gas–liquid chromatography (GLC) is an established technique for the separation of triglycerides according to molecular weight[1–4]. Because of the low vapour pressure of triglycerides, present methods are based upon the use of short columns of low stationary phase loadings and using high temperatures. Excellent reviews by Litchfield[5] and earlier by Pierce[6] optimise conditions relating to the quantitative analysis of triglyceride mixtures.

Many compounds have been reported as suitable for use as stationary phases in GLC, but little information is available as to their applicability to triglyceride analysis. Polyesters of succinic acid, a cyanopropyl phenyl siloxane[7] (Silar 5CP) and more recently a polar siloxane[8] (Silar 10C) have been used for the resolution of saturated and unsaturated diacylglycerols. Unfortunately, they have only moderate thermal stability and similar separations using triglycerides cannot be satisfactorily effected. Silar 10C showed some resolution of triglycerides by degree of unsaturation, but the columns could only be used for a few months at 250–270°.

The major criterion in selecting a stationary phase suitable for triglyceride analysis is its thermal stability with only those which can operate at temperatures of 325° or greater being considered suitable. Hence OV-1, OV-101, OV-17, OV-22, SE-30, SE-52, JXR, Dexsil 300 and PmPE have been the stationary phases of choice, but all tend to be non-polar compared to the polyesters which are used in fatty acid analysis.

In the present study, selected stationary phases were evaluated as to their resolution and quantitative recovery of model monoacid triglyceride mixtures, consisting of saturated and unsaturated triglycerides. The stationary phases evaluated were OV-17, OV-22, Dexsil 300GC, PmPE (high polymer) and a polyphenyl ether sulphone phase (Poly-S-179). The analyses enabled the phases to be placed in order of their selectivity to quantitatively resolve triglyceride mixtures. The upper thermal limits for the stationary phases are given in Table I.

* To whom correspondence should be addressed at the following address: Department of Chemistry, Liverpool Polytechnic, Liverpool, Great Britain.

TABLE I

RECOMMENDED UPPER THERMAL LIMITS FOR THE STATIONARY PHASES

Stationary phase	*Type of phase*	*Recommended thermal limit*
OV-17	50% methyl phenyl silicone	375°
OV-22	35% methyl phenyl silicone	350°
Dexsil 300GC	carborane methyl silicone	500°
PmPE (high polymer)	polyphenyl ether	450°
Poly-S-179	polyphenyl ether sulphone	400°

MATERIALS AND METHODS

Materials

Dexsil 300GC, OV-17 and OV-22 were purchased from Phase Separations (Queensferry, Great Britain). PmPE (high polymer) was purchased from Varian Aerograph, Walnut Creek, Calif., U.S.A.. Poly-S-179 was purchased as a 2% (w/w) column packing on Supelcoport (100–120 mesh) from Supelco (Bellefonte, Pa., U.S.A.). Supelcoport (100–120 mesh) was purchased from Supelco (chemically treated to reduce adsorption effects). Trilaurin, trimyristin, tripalmitin, tristearin, triolein and trilinolein (all 99% purity) were purchased from Sigma (St. Louis, Mo., U.S.A.).

Gas–liquid chromatography

A Pye Unicam Model 104 gas chromatograph equipped with flame ionisation detectors was used for the analyses.

Column packings were prepared by coating the stationary phases onto the solid support, from chloroform solution, by the solvent evaporation technique[9]. Glass columns 0.45 m × 2.5 mm I.D. were filled with the column packings with the aid of a vacuum pump and a vibrator to ensure closely packed columns. The columns were satisfactorily conditioned by heating at 350° for 8 h with a nitrogen flow-rate of 80 ml/min.

The injection port temperature and detector were maintained at 350°. A reference saturated triglyceride mixture was prepared (see Table II) and analysed on each column. A temperature program in the range 220–350° at 3–4°/min with a nitrogen flow-rate of 80 ml/min was used to elute the triglycerides.

Evaluation methods

(a) The recovery of a model saturated monoacid triglyceride mixture (Table II)

TABLE II

COMPOSITION OF THE SATURATED MONOACID TRIGLYCERIDE MIXTURE

An aliquot of 5 μl was used for the analyses (~ 34.7 μg) to determine response factors.

Triglyceride	*Weight (g) taken to 100 cm³*	*Weight %*	*Mole %*
Trilaurin	0.1763	25.39	29.92
Trimyristin	0.1728	24.91	25.94
Tripalmitin	0.1708	24.62	22.96
Tristearin	0.1740	25.08	21.18

was evaluated by determining the weight and molar response factors for the eluted triglycerides. Peak areas were measured by triangulation, quantitative weight response factors (F_w) and molar response factora (F_m) for individual triglycerides were calculated by the internal normalisation technique. Here F_w = actual wt.%/area % and F_m = actual mole%/area %. A value of 1.00 was assigned to F_w and F_m for trilaurin (primary standard) which was assumed to be completely recovered from the column. The primary standard was then included in all calibration mixtures so that the calibration factors from all GLC runs were comparable.

(b) The resolution of the triglyceride peaks was measured by determining the separation factor ΔC (ref. 10), defined as the minimum carbon number difference between two adjacent triglycerides that can be separated with baseline resolution (see Fig. 1).

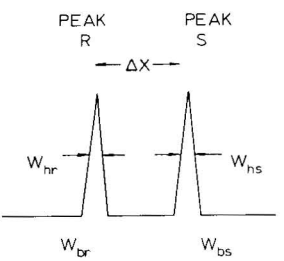

Fig. 1. Calculation of separation factor (ΔC). Any two triglyceride peaks R and S with a difference in carbon number may be chosen. The average baseline width ($W_{b\,rs}$) of triglyceride peaks in this region was found using the relationship $W_{b\,rs} = 1.6(W_{hr} + W_{hs})$. Then the distance ($\Delta X$) between the apices of the two peaks was measured. The maximum number of peaks (M) which can be separated with baseline resolution is: $M = \Delta X/W_{b\,rs} = \Delta t/1.6\,(W_{hr} + W_{hs})$. The minimum carbon number difference which can be resolved with baseline resolution (ΔC) is $6/M$, therefore $\Delta C = 9.6\,(W_{hr} + W_{hs})/\Delta X$.

(c) The resolution of saturated and unsaturated triglycerides of the same carbon number was determined by analysing a reference mixture of tristearin, triolein and trilinolein. Response factors for triolein and trilinolein were only calculated for stationary phases that resolved the reference mixture.

RESULTS AND DISCUSSION

The response of the flame ionisation detector is known to vary with load levels[11]. In the present study, no attempt was made to ascertain this variation or to correct for it. Instead, a constant weight of the reference triglyceride mixture was used to evaluate the stationary phases. The response factors given in Table III relate the actual composition of the test mixture to the area response recorded on the chromatogram. These response factors give an accurate indication of sample recovery.

On short columns, (0.45–0.60 m) OV-17, OV-22, Dexsil 300GC and PmPE separate triglycerides only on the basis of molecular weight (carbon number). Good resolution, combined with good recovery, is obtained for most of these stationary phases in an acceptable analysis time of less than 45 min. However, PmPE gave unacceptably high response factors, indicating large losses of the higher molecular weight triglycerides, and is therefore unsuitable for use in the quantitative analysis

TABLE III

EXPERIMENTALLY DETERMINED WEIGHT AND MOLAR RESPONSE FACTORS ON 2% LOADINGS OF THE STATIONARY PHASES IN 0.45 m × 2.5 mm I.D. GLASS COLUMNS

Stationary phase	*Weight calibration factors (F_w)*						*Molar calibration factors (F_m)*					
	*36***	*42*	*48*	*54*	*$54^{3=}$****	*$54^{6=}$*	*36*	*42*	*48*	*54*	*$54^{3=}$*	*$54^{6=}$*
Theoretical*	1.00	0.97	0.95	0.93								
OV-17	1.00	0.99	1.08	1.24			1.00	0.88	0.85	0.93		
OV-22	1.00	0.99	1.11	1.26			1.00	0.87	0.87	0.92		
Dexsil 300GC	1.00	1.01	1.24	1.55			1.00	0.89	0.97	1.10		
PmPE	1.00	1.25	1.97	4.17			1.00	1.12	1.56	3.00		
Poly-S-179	1.00	0.78	0.85	1.24	1.70	2.63	1.00	0.89	0.88	1.14	1.22	1.90

* Theoretical F_w response factors for specific triglycerides, calculated by assuming that all of the injected sample reaches the detector and that the flame ionisation detector response is proportional to the hydrocarbon content of each triglyceride.

** Total number of carbon atoms in the fatty acid acyl groups of each triglyceride, referred to as the carbon number.

*** Refers to the total number of alkenic linkages in each triglyceride. Therefore, for monoacid triglycerides, $54^{3=}$ is triolein.

of triglyceride mixtures. Losses could be due to pyrolysis during vapourisation of the sample, on-column degradation, adsorption or condensation. Since the experimental conditions were the same for all of the stationary phases, then adsorption of triglycerides onto the stationary phase is the most likely cause of sample loss. No low-molecular-weight fragments could be detected which would indicate pyrolysis or degradation.

The ΔC values are a more practical guide than the number of theoretical plates in determining if a specific column would yield a desired separation. Litchfield *et al.*[10] defined an empirical ΔC value equal to the minimum carbon number difference between two triglycerides that could be separated with baseline resolution in the C_{42-48} region of the chromatogram. In the present study, ΔC values in both the C_{42-48} and C_{48-54} regions of the chromatogram were calculated since most natural fats contain primarily C_{48}, C_{50}, C_{52} and C_{54} triglycerides. A linear relationship exists between retention time and the number of carbon atoms in a homologous series of compounds, for temperature programmed operation. However, because of a continuously decreasing difference in volatility between higher molecular weight pairs of

TABLE IV

EXPERIMENTALLY DETERMINED SEPARATION FACTORS (ΔC VALUES) IN THE C_{42-48} AND C_{48-54} REGIONS OF THE CHROMATOGRAM

All the stationary phases were used as 2% (w/w) coatings on Supelcoport (100–120 mesh).

Stationary phase	ΔC_{42-48}	ΔC_{48-54}
OV-17	1.9	2.1
OV-22	2.2	2.6
Dexsil 300GC	1.9	2.1
PmPE	1.9	2.1
Poly-S-179	2.1	2.2

triglycerides, they are eluted at progressively closer intervals and so the linear relationship exists only over a limited range of the triglyceride series. This is shown by an increase in the ΔIC_{48-54} value compared to the $\Delta I\,C_{42-48}$ value for all of the stationary phases evaluated. The results are given in Table IV. The low separation factors obtained on the OV-17 column, combined with almost quantitative recovery of the sample, make it one of the stationary phase of choice when resolving and quantitatively analysing triglyceride mixtures on the basis of molecular weight.

For all of the stationary phases a loading of 2% (w/w) was found to be opti-

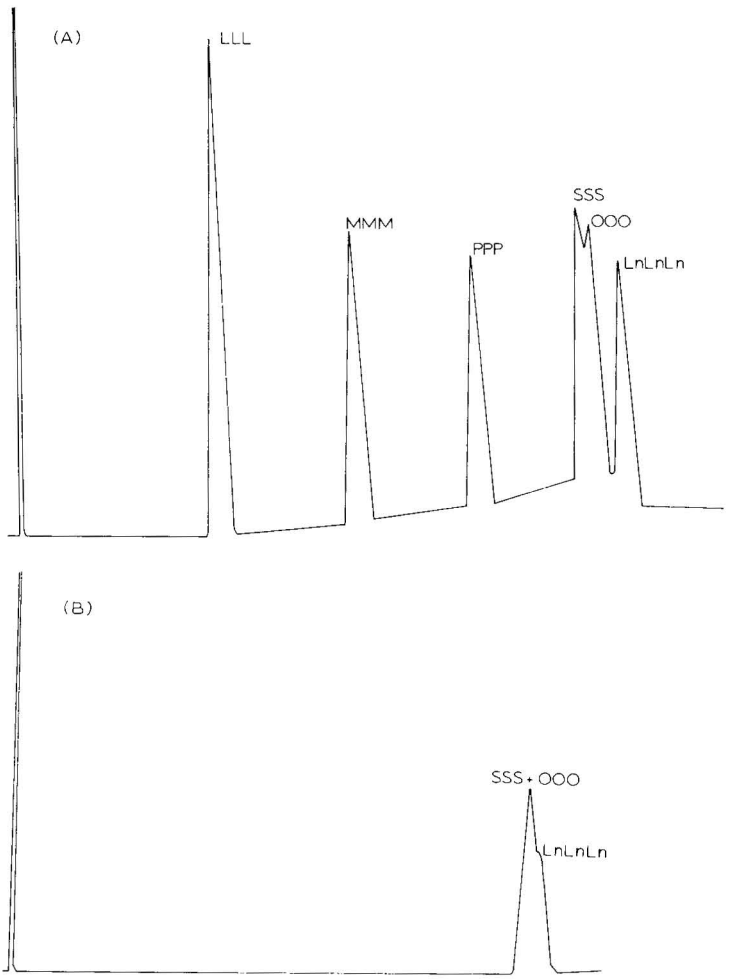

Fig. 2. (A) Chromatogram of a synthetic mixture of trilaurin (LLL), trimyristin (MMM), tripalmitin (PPP), tristearin (SSS), triolein (OOO) and trilinolein (LnLnLn). Conditions: 0.45 m × 2.5 mm I.D. glass column packed with 2% Poly-S-179 on Supelcoport (100–120 mesh). Temperature programmed between 220–335° at 3°/min with a nitrogen flow-rate of 80 ml/min. Injection port and detector maintained at 350°. (B) Attempted resolution of tristearin (SSS), triolein (OOO) and trilinolein (LnLnLn) on OV-17 stationary phase. Conditions: 0.45 m × 2.5 mm I.D. glass column packed with 2% OV-17 on Supelcoport (100–120 mesh). Temperature programmed in the range 230–350° at 3°/min with a nitrogen flow-rate of 80 ml/min. Injection port and detector maintained at 350°.

mum, as it gave good resolution and recovery of triglycerides without having to utilise temperatures beyond 350° to elute the higher molecular weight samples.

Poly-S-179 was the only stationary phase to resolve triglycerides of the same carbon number, but differing in degree of unsaturation, on a 0.45 m column. Fig. 2A shows a typical chromatogram for the resolution of a mixture of saturated and unsaturated triglycerides; the triglycerides are eluted in order of increasing molecular weight and increasing degree of unsaturation. Attempts to increase the resolution of the C_{54} triglycerides by moving to longer column lengths met with only partial success, as the increase in resolution was accompanied by a marked increase in the amount of on-column degradation. On a 1.06 m column containing 2% Poly-S-179, tristearin and triolein were completely resolved with baseline resolution but the chromatogram showed marked amounts of broad ill-defined material eluting immediately before the tristearin peak making a quantitative analysis troublesome. This on-column degradation places a practical limit on column length. Weight and molar response factors were calculated for triolein and trilinolein on the Poly-S-179 phase and these are given in Table III.

Fig. 2B shows the attempted resolution of tristearin, triolein and trilinolein on a 0.45 m column containing 2% OV-17. The saturated and unsaturated triglycerides were not resolved with trilinolein distorting the descending edge of the main C_{54} peak.

The reference mixture of simple saturated monoacid triglycerides was eluted from the 2% Poly-S-179 column at a temperature approximately 25° below that on the other stationary phases. This is probably due to the low affinity of the triglycerides, which have a non-polar character, for the polar stationary phase. The response factors for the unsaturated triglycerides increased with the degree of unsaturation, indicating an increase in irreversible adsorption onto the column and an increase in on-column degradation.

The present study is to be extended by utilising high-resolution capillary GLC columns coated with Poly-S-179. Lower elution temperatures and increased resolution should enable valuable separations of synthetic and natural triglyceride mixtures to be effected, based on both molecular weight and degree of unsaturation.

In conclusion, it can be said that a polyphenyl ether sulphone (Poly-S-179) stationary phase has sufficient polarity to permit improved separations of triglycerides based on the degree of unsaturation and sufficient thermal stability to allow its application without deterioration.

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CHROM. 11,742

Note

Identifizierung seltener Aminosäuren durch Mikrodansylierung

HARTMUT LAATSCH

Organisch-Chemisches Institut der Universität Göttingen, Tammannstrasse 2, D-3400 Göttingen (B.R.D.)

(Eingegangen am 8. Dezember 1978)

Durch zweidimensionale Dünnschichtchromatographie ihrer fluoreszierenden 5-Dimethylaminonaphthalin-1-sulfonyl-(Dansyl-)derivate lassen sich Aminosäuren mit freien Amino- oder phenolischen Hydroxylgruppen besonders an Polyamid schnell und mit grosser Treffsicherheit identifizieren. Die Dansylierung gehört deshalb inzwischen zu den Standardmethoden der Aminosäure- und Peptidanalytik, wie zahlreiche Publikationen und mehrere Zusammenfassungen zeigen[1–3].

Da die bisherigen Arbeiten über Dansylierungen überwiegend physiologisch-chemisch orientiert sind, wurden fast ausschliesslich Aminosäuren untersucht, wie sie in höheren Organismen vorkommen ("physiologische" Aminosäuren). Bei der Analyse mikrobieller Peptide ist dagegen auch mit ungewöhnlichen Säuren zu rechnen, deren Dansylderivate selbst in ausführlicheren chromatographischen R_F-Karten[4,5] nicht enthalten sind. Bisher war deshalb die chromatographische Analyse z.B. eines Peptidantibiotikums wenig aussagekräftig, da die Gefahr von Fehlinterpretationen durch mangelnde Kenntnis des chromatographischen Verhaltens seltener Aminosäuren gross war.

Mit der vorliegenden Untersuchung über die Dansylierung von 90 Aminosäuren und 11 weiter möglicher Analysen-Bestandteile (z.B. Aminozucker) wird begonnen, diese Lücke zu schliessen.

EXPERIMENTELLES

Methodik

Für zweidimensionale Trennungen an Polyamid besonders bewährt hat sich ein von Schulze und Neuhoff[6,7] angegebenes Laufmittelsystem, das mehr als 60 der hier untersuchten Dansylderivate schon an 3 × 3 cm Polyamidfolien nach zweimaliger Entwicklung reproduzierbar auftrennt (Fig. 1 und 2) und neben der Materialersparnis durch das kleine Folienformat den Vorteil kurzer Entwicklungszeiten bietet.

Experimente

100 μl der zu untersuchenden Aminosäuremischung (jeweils etwa 0.5 mM in 0.05 M $NaHCO_3$-Puffer, pH 10.0) wurden mit 250 μl einer frischen Dansylchloridlösung in Aceton (2.7 mg/ml) vermischt und 30 min bei 37° aufbewahrt. Von dieser Lösung wurden 0.1–0.5 μl zur Chromatographie mit einer dünn ausgezogenen Glas-

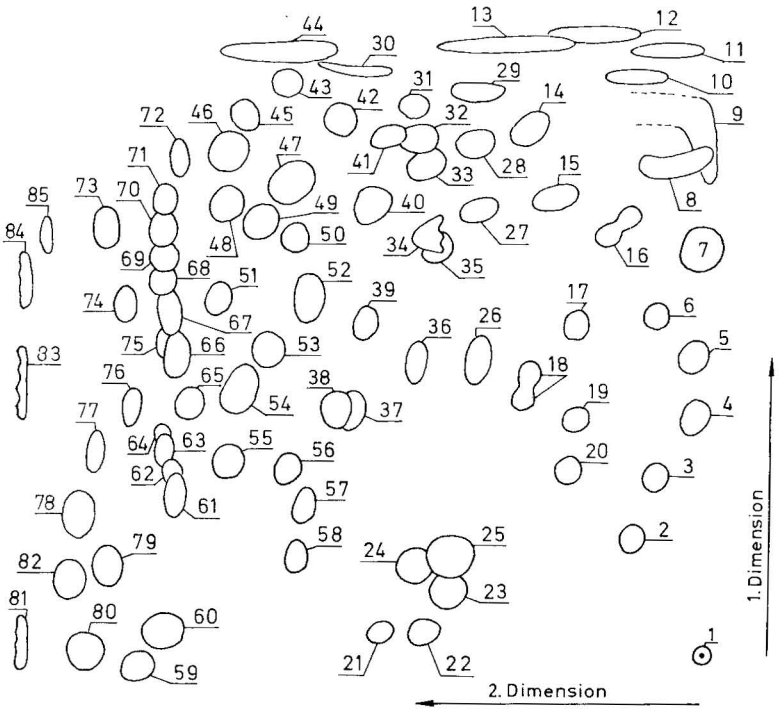

Fig. 1. Fleckkarte der Dansylderivate. Chromatographie in der 1. Dimension in 4 Vol.-% Ameisensäure in Wasser, in der 2. Dimension in 20 Vol.-% Eisessig in Benzol. 1 = Start, 2 = Cystin, 3 = 5-Hydroxytryptophan*, 4 = Taurin, 5 = Cysteinsäure, 6 = *meso*-Diaminobernsteinsäure, 7 = Dansylsulfonsäure (blau), 8 = β-Aminoäthan-phosphonsäure, 9 = Dansylsulfinsäure (blau), 10 = α-Aminoäthanphosphonsäure, 11 = Arginin, Canavanin, Cystin*, *meso*-Diaminobernsteinsäure*, Diaminopimelinsäure*, Glucosamin, δ-Hydroxylysin*, Mannosamin, 12 = *erythro*- und *threo*-2,3-Diaminobuttersäure*, Diaminopropionsäure*, Histidin*, 13 = 1-Methylhistidin, 14 = Serin, 15 = Asparaginsäure, 16 = 3,4-Dihydroxyphenylalanin* (gelb), 3,4-Dihydroxyphenyl-α-methylalanin* (gelb), 17 = Carboxymethylcystein, 18 = Diaminopimelinsäure, 19 = Tyrosin*, 20 = Cystathionin, *allo*-Cystathionin, 5-Hydroxytryptophan* (gelb), 21 = 5-Hydroxytryptophan (gelb), 22 = 3,5-Dijodtyrosin, 23/24 = 3,4-Dihydroxyphenylalanin* (gelb), 25 = Tryptophan, 26 = δ-Hydroxylysin, 27 = Glutaminsäure, 28 = Citrullin, Methionindioxid, Homoserin, Threonin, *allo*-Threonin, 29 = Asparagin, 30 = Histidin* (gelb), 31 = Glutamin, 32 = Methylthreonin, 33 = 4-Hydroxyprolin, 34 = Tyrosin* (gelb), α-Methyl-*p*-tyrosin* (gelb), α-Methyl-*m*-tyrosin (gelb), 35 = Methylglutaminsäure, 36 = Diaminopropionsäure, 37 = 2,4-Diaminobuttersäure, *threo*-2,3-Diaminobuttersäure, 38 = Ornithin, 39 = β-Hydroxyleucin, 40 = Glycin, 41 = γ-Amino-β-hydroxybuttersäure, 42 = Hydroxyvalin, 43 = Methioninoxid, 44 = 3-Methylhistidin, 45 = Ethalolamin, 46 = O-Methylserin 47 = Ammoniak (blau), 48 = β-Alanin, 49 = Alanin, 50 = β-Aminopimelinsäure, 51 = α- und γ-Aminobuttersäure, 52 = *erythro*-2,3-Diaminobuttersäure, 53 = Pyroglutaminsäure** (gelb), 54 = Methionin, 55 = Phenylalanin, 56 = α-Phenylglycin, 57 = Lysin, 58 = 3,4-Dihydroxyphenylalanin* (gelb), 3,4-Dihydroxyphenyl-α-methylalanin (gelb), 59 = 3,4-Dihydroxyphenylalanin (gelb), 60 = Tyrosin (gelb), 61 = Histidin (gelb), Norleucin, 62 = Leucin, 63 = Isoleucin, 64 = *allo*-Isoleucin, 65 = Norvalin, 66 = Valin, 67 = δ-Aminovaleriansäure, 68 = β-Aminoisobuttersäure, Sarkosin, 69 = N-Methylalanin, 70 = β-Aminobuttersäure, α-Aminoisobuttersäure, 71 = O-Methylvalin, 72 = Diethanolamin, γ-Aminobuttersäureamid*, 73 = α-Methyl-*p*-tyrosin (blau), 74 = Prolin, 75 = Isovalin, 76 = ε-Aminocapronsäure, 77 = Pipecolinsäure, 78 = N-Methylvalin, 79 = N-Methylphenylalanin, 80 = α-Methyl-*m*-tyrosin (gelb), 81 = 5-Methoxytryptophan (blau), 82 = N-Methylisoleucin, N-Methylleucin, 83 = Diethylamin, γ-Aminobuttersäurelactam**, 84 = Dimethylamin, 85 = Methylamin. Keine Dansylderivate wurden erhalten aus: 4-Aminobenzoesäure, 2-Aminobenzoesäure, γ-Aminobuttersäurelactam**, Kreatin, Kreatinin, Picolinsäure, Pyroglutaminsäure**, Pyrrolcarbonsäure und Valerolactam. * = Teildansyliert; ** = Derivat bildet sich nur über die Dansyl-aminosäure.

kapillare in der rechten unteren Ecke (je 2 mm vom Rand entfernt) einer 3 × 3 cm Polyamidfolie (Mikropolyamid F 1700; Schleicher und Schüll, Dassel, B.R.D.) punktförmig aufgetragen. Die Entwicklung erfolgte in der ersten Dimension in 4 Vol.-% Ameisensäure in Wasser, und nach Zwischentrocknung in der zweiten Dimension mit 20 Vol.-% Essigsäure in Benzol. Die Chromatogramme wurden nach dem Trocknen im UV-Licht bei 366 nm ausgewertet.

ERGEBNISSE UND DISKUSSION

Da aus mehrfach dansylierbaren Aminosäuren mit Reagenzunterschuss Gemische aus teil- und perdansylierten Verbindungen entstehen können, waren aus den hier untersuchten Substanzen mehr als 120 Dansylderivate zu erwarten. Die Teildansylierung kann daher zu erheblich unübersichtlicheren Chromatogrammen

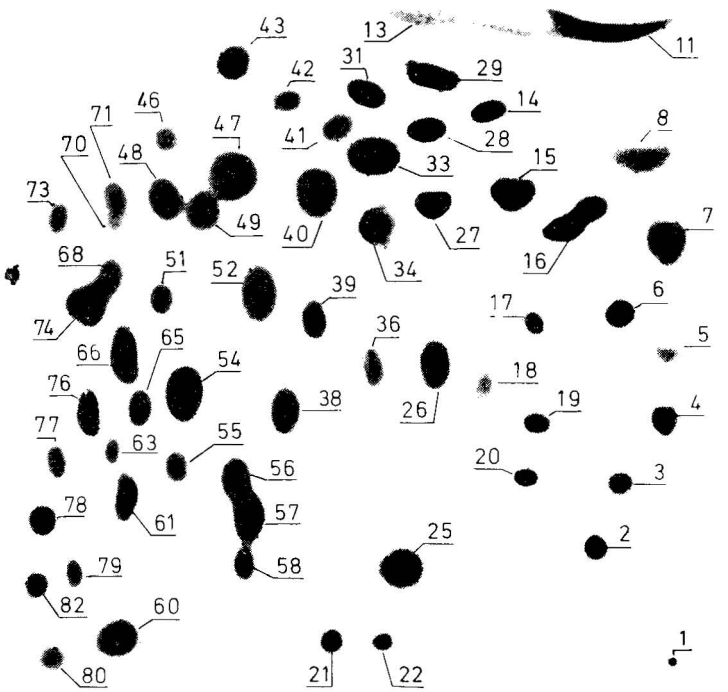

Fig. 2. Chromatogramm einer teildansylierten Mischung aus 52 Komponenten an 3 × 3 cm Mikropolyamid. Bezifferung wie in Fig. 1.

als die Untersuchung ausdansylierter Mischungen führen. So sind z.B. durch Teildansylierung von 3,4-Dihydroxyphenylalanin sieben Dansylderivate zu erwarten, von denen sich sechs nachweisen liessen. Die Reaktion mit Reagenzüberschuss führt dagegen zu einem einzigen Produkt. Die Chromatographie teildansylierter Proben kann dennoch sinnvoll sein, da sich bestimmte Aminosäuren (z.B. Histidin, 5-Hydroxytryptophan, Tyrosin) eindeutig anhand ihrer charakteristisch angeordneten, geformten (Fleck 34, Fig. 1), oder in bestimmten Farben fluoreszierenden Monodansylflecken zuordnen lassen.

Trotz der grossen Trennschärfe war eine vollständige Unterscheidung aller untersuchten per-Dansylaminosäuren selbst auf 5 × 5 cm Folien nicht möglich und es ist fraglich, ob die Trennung bei der hohen Fleckdichte im Chromatogramm durch andere Laufmittelsysteme wesentlich verbessert werden kann. So sind z.B. die polaren hydrophilen Aminosäuren im rechten oberen Quadranten des Chromatogramms (Fig. 1) nur zum Teil unterscheidbar: mit gleichem R_F-Wert wie Threonin laufen Homoserin, Methioninoxid, Citrullin und *allo*-Threonin, von denen letzteres sich auch im Aminosäureanalysator ("physiologischer Lauf") wie Threonin verhält.

In der zweiten Dimension zeigen 11 Aminosäuren (61–72) gleichen R_F-Wert und werden nur unvollständig getrennt.

Die annähernd sichere Identifizierung eines Fluoreszenzflecks im Chromatogramm gelingt daher nur durch folgende Arbeitsweise.

(a) Der zu untersuchenden Dansyl-Aminosäuremischung werden mehrere bekannte Dansyl-Aminosäuren mit ähnlichem chromatographischen Verhalten als Bezugssubstanzen zugemischt, wobei nach Chromatographie ein Muster von Referenzpunkten entsteht, das einem Ausschnitt der Fig. 1 entsprechen muss*.

(b) Die nun mit Hilfe der R_F-Karte zugeordnete Säure der Untersuchungssubstanz muss im Gemisch mit einem authentischen Vergleichspräparat bei Identität dessen Fluoreszenzfleck verstärken und völlig überdecken.

(c) Bei R_F-Werten, denen mehrere Säuren zugeordnet werden können, ist der entsprechende Fluoreszenzfleck auszukratzen und sein Inhaltsstoff in einem zweiten Laufmittelsystem zu rechromatographieren[8].

Während die zweifelsfreie Identifizierung einer Aminosäure durch Chromatographie ihres Dansylderivates schwierig ist und die Untersuchung weiterer, hier noch nicht aufgeführter Säuren und neuer Laufmittelsysteme verlangt, können andererseits alle diejenigen Aminosäuren mit grosser Sicherheit ausgeschlossen werden, für die an entsprechender Stelle im Chromatogramm keine Fluoreszenz beobachtet wird. Dies begründet den besonderen Wert der Dansylmethode bei Untersuchungen auf seltene Aminosäuren.

DANK

Den Herren Prof. Dr. H. Brockmann, Doz. Dr. A. Zeeck, Dr. K. Frobel (Universität Göttingen), Prof. Dr. V. Neuhoff und Dr. E. Schulze (Max-Planck-Institut für Experimentelle Medizin, Göttingen) danke ich für Proben seltener Aminosäuren. Herr H.-G. Zimmer hat freundlicherweise das Chromatogramm (Fig. 2) photographiert[9].

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* In Abhängigkeit von der Folienchargе, Schwankungen der Temperatur oder Laufmittelzusammensetzung (Verdampfungsverluste) können sich gegenüber Fig. 1 abweichende R_F-Werte ergeben. Die relative Lage der Flecken zueinander bleibt jedoch konstant; siehe auch Lit. 1.

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Note

Separation of the metabolites of [26,27-^{3}H]25-hydroxycholecalciferol in plasma extracts by high-pressure liquid chromatography on a preparative column

B. PELC and A. L. HOLMES

M.R.C. Mineral Metabolism Unit, The General Infirmary, Great George Street, Leeds LS1 3EX (Great Britain)

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Several high-pressure liquid chromatographic (HPLC) systems have been developed recently to separate metabolites of vitamin D_2 (calciferol) and vitamin D_3 (cholecalciferol) in human and animal plasma[1–11] and to identify vitamins D_2 and D_3 in pharmaceutical preparations[12–14]. The first described method was reversed-phase liquid–liquid chromatography using aqueous methanol for elution from a column packed with octadecylsilane permanently bonded to glass beads[1]. This chromatographic system has been shown to be useful in the analysis of pharmaceutical forms[13], but has been found less useful in the analysis of plasma extracts due to a minimal resolution of 1α,25-$(OH)_2$-D_3 from 25,26-$(OH)_2$-D_3* (see ref. 2).

Small porous silica gel columns have found wide application in the HPLC separation of the metabolites of vitamins D_2 and D_3 in plasma. Two 25 cm × 2.1 mm Zorbax-Sil columns in series, eluted with 1–20% isopropanol in Skellysolve B have been used for the separation of vitamins D_2 and D_3 and their metabolites[2]. A recent communication has described the separation of synthetically prepared vitamin D derivatives, on a Zorbax-Sil column, using dichloromethane with 2% methanol[3]. The separation of 1α,25-$(OH)_2$-D_2 and 25-OH-D_3 has been achieved on a Porasil column (30 × 0.4 cm), eluted with hexane containing 5% absolute ethanol[9] and on two Porasil columns in series eluted with dichloromethane containing 2% isopropanol[10].

Recently we have studied the metabolism of intraveneously injected [^{3}H]25-OH-D_3 and investigated the concentrations of labelled metabolites in human plasma. The separation of labelled metabolites on Sephadex LH-50 columns indicated, as expected, the formation of the polar metabolites [^{3}H]24,25-$(OH)_2$-D_3 and of a mixture of [^{3}H]1,25- and 25,26-$(OH)_2$-D_3[15]. We found that Merckosorb SI 60, dry packed in a 1 m × 5.5 mm preparative high pressure column separated the three important polar metabolites of [^{3}H]25-OH-D_3 in human plasma extracts without preliminary chromatographic separation of lipids; a procedure necessary when analytical and semipreparative silica gel columns have been used[2,4–6,8,10].

* Abbreviations: 25-OH-D_2 — 25-hydroxycalciferol; 25-OH-D_3 — 25-hydroxycholecalciferol; 1α,25-$(OH)_2$-D_3 — 1α,25-dihydroxycholecalciferol; 24,25-$(OH)_2$-D_3 — 24,25-dihydroxycholecalciferol; 25,26-$(OH)_2$-D_3 — 25,26-dihydroxycholecalciferol.

MATERIALS

[26,27-^{3}H]25-OH-D_3, specific activity *ca.* 10,000 mCi/mmol was purchased from the Radiochemical Centre, Amersham, Great Britain. It was found to be about 99% pure by HPLC in a 1 m × 5.5 mm Merckosorb SI 60 column, eluted with dichloromethane containing 3% methanol.

[26,27-^{3}H]24,25-$(OH)_2$-D_3 and [26,27-^{3}H]25,26-$(OH)_2$-D_3 were prepared by chick kidney homogenate hydroxylation of tritiated 25-OH-D_3 by the method of Tanaka *et al.*[17]. Tritiated 25,26-$(OH)_2$-D_3 was further identified by co-chromatography with unlabelled 25,26-$(OH)_2$-D_3 (a gift from Dr. J. Redel, Hôpital Cochin, Paris, France) on a 25 cm × 4.6 mm Zorbax-Sil column. [26,27-^{3}H]1α,25-$(OH)_2$-D_3 was a gift from Dr. M. Uskoković, Hoffmann-La Roche, Nutley, N.J., U.S.A.

A Varian 8500 chromatograph (Varian, Palo Alto, Calif., U.S.A.) was used for HPLC. A 1 m × 5.5 mm I.D. column was dry packed with 15 g of Merckosorb SI 60, mean particle size 20 μm. Methanol (analytical reagent grade) and dichloromethane distilled over a 100-cm column (fraction 40–41°) were used. The scintillator contained 2,5-diphenyloxazole (PPO, 15 g) and 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP, 500 mg) in 2.5 l toluene. Radioactivity was measured in a Beckman LS-233 liquid scintillation counter.

METHODS

Separation of [^{3}H]25-OH-D_3 and its metabolites by HPLC

About 5000 cpm of [^{3}H]25-OH-D_3, 3500 cpm of [^{3}H]24,25-$(OH)_2$-D_3, 1700 cpm of [^{3}H]25,26-$(OH)_2$-D_3 and 4000 cpm of [^{3}H]1α,25-$(OH)_2$-D_3 in 100 μl dichloro-

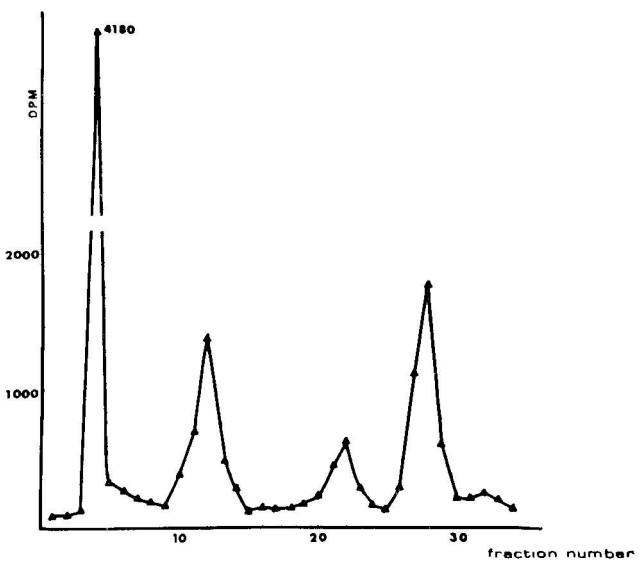

Fig. 1. Separation of tritiated 25-OH-D_3 and its metabolites on a 1 m × 5.5 mm I.D. column packed with Merckosorb SI 60, mean particle size 20 μm. Eluted with dichloromethane containing 3% methanol, 10-ml fractions collected, evaporated and counted. Fractions 4–7: 25-OH-D_3; fractions 11–15: 24,25-$(OH)_2$-D_3; fractions 21–23: 25,26-$(OH)_2$-D_3 and fractions 26–29: 1α,25-$(OH)_2$-D_3.

methane was injected into a 1 m × 5.5 mm column, and eluted with dichloromethane, containing 3% methanol. 10-ml fractions were collected (flow-rate, 200 ml/h) and thereafter the whole fractions were evaporated and counted (Fig. 1).

Extraction of metabolites from human plasma

Patients were injected with 10 μCi of [^{3}H]25-OH-D_3 which was dissolved in 0.5 ml ethanol and diluted to 10 ml with a saline solution shortly before injection. Blood samples (10–20 ml) were collected 24 h later and the plasma separated. A trace of ascorbic acid (as antioxidant) was added and lipids and metabolites were extracted with 40 ml ethanol, vortexed for 3 min and centrifuged. The second extract was obtained after suspension of the protein cake in 20 ml ethanol, vortexing and centrifuging. Pooled extracts were evaporated under vacuum at 40°. The residue was dissolved in dichloromethane (10 ml) a small quantity of insoluble material was filtered off, the filtrate concentrated under CO_2, redissolved in 0.2 ml dichloromethane and transferred quantitatatively onto the column. The tube was washed with an additional 0.2 ml dichloromethane, transferred to the column and the radioactive derivatives eluted with dichloromethane, containing 3% methanol. Whole fractions were counted (Fig. 2).

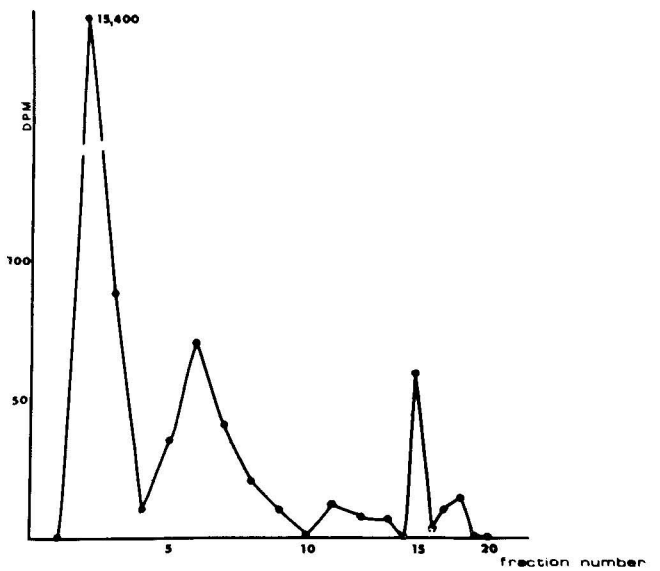

Fig. 2. Separation of metabolites, extracted from 8.7 ml of human plasma. For convenience 20-ml fractions (fraction 1–13) and 10-ml fractions (fractions 14–20) were collected. Fractions 2–3: 25-OH-D_3; fractions 5–8; 24,25-$(OH)_2$-D_3; fractions 11–12: 25,26-$(OH)_2$-D_3 and fraction 15: 1α,25-$(OH)_2$-D_3.

Separation of [^{3}H]25,26-$(OH)_2$-D_3 and [^{3}H]1α,25-$(OH)_2$-D_3 isolated first on a Sephadex LH-20 column

A mixture of the two metabolites was eluted from a Sephadex LH-20 column as a 50-ml fraction (between 250–300 ml) using a chloroform–hexane (2:1) mixture

according to ref. 15. On chromatography on a 1 m × 5.5 mm column packed with Merckosorb SI 60 it was resolved into two peaks, corresponding to the two metabolites (Fig. 3).

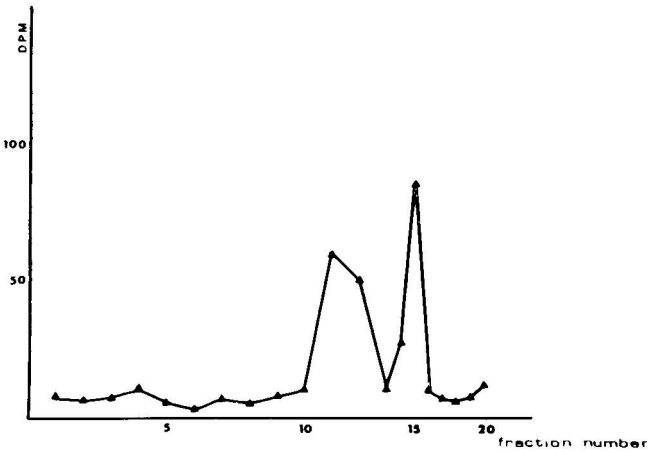

Fig. 3. Separation of a synthetic mixture of [^{3}H]25,26- and [^{3}H]1α,25-(OH)$_2$-D$_3$, isolated first on a Sephadex LH-20 column. For convenience 20-ml fractions (fractions 1–13) and 10-ml fractions (fractions 14–20) were collected. Fractions 11–12: 25,26-(OH)$_2$-D$_3$ and fraction 15: 1α,25-(OH)$_2$-D$_3$.

RESULTS AND DISCUSSION

Since the introduction of Sephadex LH-20 in the chromatography of metabolites of vitamin D (ref. 15) only one communication to our knowledge has described the metabolism of labelled cholecalciferol in man[16]. The results of this study were limited by a very low conversion of cholecalciferol to the final active metabolite 1α,25-(OH)$_2$-D$_3$. Using labelled 25-OH-D$_3$ we tried to improve the precision of calculation of the conversion of [^{3}H]25-OH-D$_3$ to the 1α,25-dihydroxy derivative. Porous silica gel columns in HPLC are capable of resolving the polar metabolites of cholecalciferol[2,4,6,8–10]. A prepurification step has been found necessary to remove a large quantity of lipids from the plasma. We achieved a good separation of metabolites of 25-OH-D$_3$ on a 1-m preparative column without preliminary separation of lipids. A synthetic mixture of 25,26-(OH)$_2$-D$_3$ and 1α,25-(OH)$_2$-D$_3$, isolated first on a Sephadex LH-20 column, was resolved into two peaks (Fig. 3).

Two similar chromatographic systems have recently been published[3,10]. The former used a mixture of dichloromethane with 2% methanol, the latter dichloromethane with 2% isopropanol, in both cases on analytical columns. We believe that our chromatographic system represents a reasonable compromise between Sephadex LH-20 and microporous analytical columns, packed with 5–10 μm size particles. Our method is especially useful when larger quantities of human or animal plasma extract have to be used.

In our study of the metabolism of labelled 25-OH-D$_3$ in man we were limited by two factors: the permitted dose of tritium and the size of blood samples. Blood samples of 20 ml were collected, giving usually 8–10 ml plasma. In one of the patients (K.P., body weight 40 kg, demonstrated in Fig. 2) an attempt was made to evaluate

the conversion of tritiated 25-OH-D_3 to other metabolites. The extract from 8.7 ml plasma gave, after chromatography, 15,500 dpm of [^{3}H]25-OH-D_3. This corresponds to $3.56 \cdot 10^6$ dpm (1.6 μCi) in the whole plasma. Calculations were based on the assumption that total plasma content is equal to 5% of the body weight, *i.e.* 16% of the injected dose was circulating in plasma at that time. The whole plasma content of tritiated 24,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-D_3 was calculated to be 38,000 dpm and 13,600 dpm respectively. This would correspond to 0.17% conversion of tritiated 25-OH-D_3 to 24,25-dihydroxy derivative and 0.06% conversion to 1α,25-dihydroxy derivative.

Recently published mean levels of vitamin D (D_2 + D_3) and its important metabolites in human plasma[5] showed that the quantity of 24,25-$(OH)_2$-D is about 8.3% of that of 25-OH-D and that for 1α,25-$(OH)_2$-D the corresponding quantity is about 0.14%. This would mean that in our patient there is a feedback control limiting production of both labelled polar metabolites of 25-OH-D_3 (refs. 18 and 19).

ACKNOWLEDGEMENTS

Our thanks are due to Dr. M. Peacock for discussion and useful comments, Drs. J. Redel and M. Uskoković for samples of vitamin D_3 metabolites and to Mr. G. Taylor for technical assistance (kidney homogenate hydroxylation).

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CHROM. 11,739

Note

Gas–liquid chromatographic separation of methyl ethers of L-rhamnose as their methyl glycosides, trifluoroacetylated L-rhamnitols and acetylated L-rhamnononitriles

FRANTIŠEK JANEČEK, RUDOLF TOMAN, ŠTEFAN KARÁCSONYI and DUŠAN ANDERLE

Institute of Chemistry, Slovak Academy of Sciences, 809 33 Bratislava (Czechoslovakia)

(Received January 16th, 1979)

L-Rhamnose is a constituent sugar of many polymers, especially in the plant kingdom[1,2]. The mode of linkage and the distribution of L-rhamnose residues in these macromolecules has a considerable influence on their size and shape[3]. Among the methods used in the investigation of these residues, methylation analysis is still of fundamental importance. After methylation and subsequent hydrolysis, the resulting mixture of methylated sugars is converted into volatile derivatives and analyzed by gas–liquid chromatography (GLC) or combined GLC–mass spectrometry (MS). Several types of derivatives have been used, *e.g.*, methyl glycosides[4], acetates[5], alditol acetates[6] and aldononitrile acetates[7,8]. Nevertheless, a prerequisite for the unambiguous qualitative and quantitative evaluation of the complex mixtures, the knowledge of retention data and response factors of the individual derivatized sugar methyl ethers, is presumed.

The separations of all possible O-methyl derivatives of L-rhamnose as their corresponding partially methylated alditol acetates have already been described[9]. We now extend these investigations to the corresponding methyl glycosides, trifluoroacetylated alditols (TFAA) and acetylated aldononitriles (AAN) which appeared more suitable for GLC and GLC–MS analyses, through their better separation, ease of preparation, duration of analyses, etc.

EXPERIMENTAL

Derivatives

Methyl ethers of methyl α-L-rhamnopyranoside were prepared as described by Toman *et al.*[10]. Hydrolysis with 90% formic acid for 3 h at 100° and subsequent purification by thin-layer chromatography on silica gel G using chloroform–methanol (9:1) and light petroleum (b.p. 35–50°)–acetone (5:2) afforded the corresponding methyl ethers of L-rhamnose. A small amount (1–3 mg) of each derivative was reduced with sodium borohydride (3–10 mg) and worked-up in the usual way. The dry residue was then trifluoroacetylated with 0.3 ml of trifluoroacetic anhydride in the presence of 3 μl of dry pyridine. The reaction mixture was stirred vigorously for *ca.* 1 min and left at room temperature for 1 h.

L-Rhamnononitrile acetates were prepared as follows. A sample of methylated L-rhamnose (1–3 mg) was dissolved in a solution (150 μl) of hydroxylamine hydrochloride in pyridine (100 mg in 1 ml) and heated at 110° for 15 min. Acetic anhydride was added (200 μl) and the reaction mixture was kept at 110° for 30 min. The mixture was then cooled and, after addition of water (1 ml), evaporated to dryness. Chloroform (100 μl) was added and the resulting solution was directly injected into the column.

Apparatus

GLC of methyl ethers of methyl α-L-rhamnopyranoside and of the corresponding TFAA was performed with a Hewlett-Packard Model 5711 A chromatograph equipped with a flame ionization detector (FID). The separations of O-acetyl-O-methyl-L-rhamnononitriles were carried out on a Hewlett-Packard Model 5754 G instrument with an FID.

Columns and operating conditions

The following columns were used:

(A) 3% ECNSS-M on Chromaton N AW DMCS (80–100 mesh) at a programmed temperature range of 110° (8 min) to 150° at 2°/min, flow-rate 25 ml/min;

(B) 10% DEGS on Chromosorb W AW DMCS (80–100 mesh) at 180°, flow-rate 34 ml/min;

(C) 5% BDS on Gas-Chrom Z (80–100 mesh) at 135°, flow-rate 41 ml/min;

(D) 4% XE-60 on Gas-Chrom Z (80–100 mesh) at 130°, flow-rate 24 ml/min;

(E) 3% OV-225 on Chromosorb W AW DMCS (80–100 mesh) at 110°, flow-rate 39 ml/min;

(F) 20% SF-96 on Chromosorb W AW DMCS (60–80 mesh) at 120°, flow-rate 29 ml/min;

(G) 3% QF-1 on Gas-Chrom Q (100–120 mesh) at 165°, flow-rate 8 ml/min;

(H) 1% XE-60 on Gas-Chrom Q (100–120 mesh) at 165°, flow-rate 6.1 ml/min;

(J) 5% NPGS on Gas-Chrom Z (80–100 mesh) at 165°, flow-rate 9.8 ml/min.

The columns (stainless steel) had the following sizes: 200 × 0.316 cm O.D. (A–F); 300 × 0.316 O.D. (G, H); and 180 × 0.316 O.D. (J). Nitrogen was used as carrier gas throughout.

RESULTS AND DISCUSSION

As mentioned above, the GLC separation of all possible O-acetylated O-methyl-L-rhamnitols is relatively poor, and does not allow a proper quantitative evaluation. This fact, together with our need to perform the methylation analysis on oligomers containing high proportions of L-rhamnose residues, prompted us to search for other derivatives more suitable for GLC analyses.

Glycosidation (methanolysis) of methyl ethers of L-rhamnose gives rise to α-anomers only and in addition, when the reaction is performed under more vigorous conditions (heating with 7% methanolic hydrogen chloride for 8 h), the content of furanosides is negligible. Hence, the separation is much simplified, since each derivative gives only one peak on GLC, and might be useful in methylation analysis of certain types of oligo- and polysaccharides.

TABLE I

RELATIVE RETENTION TIMES $t'_{R,2\text{-O-MeRh}}$ OF METHYL O-METHYL-α-L-RHAMNOPYRANOSIDES AND O-TFA-O-METHYL-L-RHAMNITOLS

Positions of O-methyl groups	*Columns*					
	Methyl α-L-rhamnosides				*TFA L-rhamnitols*	
	A	*B*	*C*	*D*	*E*	*F*
2, 3, 4	0.11	0.11	0.10	0.21	1.06	2.17
2, 3	0.47	0.41	0.41	0.56	1.00	1.43
2, 4	0.36	0.32	0.31	0.51	1.56	1.72
3, 4	0.26	0.27	0.25	0.39	1.17	1.53
2	1.00	1.00	1.00	1.00	1.00	1.00
3	1.12	1.05	1.12	1.09	1.17	0.65
4	1.22	1.18	1.30	1.40	1.50	0.91
$t'_{,2\text{-O-MeRh}}$ (min)	13.46	17.48	31.26	16.46	5.67	11.42

The best separation of all possible methyl ethers of methyl α-L-rhamnopyranoside has been achieved on column A (Table I). In this case, the difference in relative retention times (Δt) is not lower than 0.1 which enables, using columns of suitable efficiency, a convenient quantitative estimation. If $\Delta t < 0.1$, the peaks are not well resolved. This situation occurred when columns B, C and D were used for the separations. In this case, a combination of two different columns is necessary to obtain the desired resolution.

O-Trifluoroacetyl (TFA)-O-methyl-L-rhamnitols chromatographed on column F (Table I) also provide an unambiguous qualitative and quantitative evaluation. Somewhat poorer results, but still comparable with the corresponding alditol acetates[9], have been obtained using column E.

Finally, attempts have been made to separate the methyl ethers of L-rhamnose as AAN. As is evident from Table II, separations also enabling quantitative estimation have not been achieved, since several compounds form pairs separable only on the

TABLE II

RELATIVE RETENTION TIMES $t'_{R,2\text{-O-MeRh}}$ AND RETENTION INDICES *I* OF O-ACETYL-O-METHYL-L-RHAMNONONITRILES

Positions of O-methyl groups	*Columns*					
	$t'_{R,2\text{-O-MeRh}}$			*I*		
	G	*H*	*J*	*G*	*H*	*J*
2, 3, 4	0.33	0.39	0.46	2025	2055	2133
2, 3	0.60	0.64	0.80	2117	2122	2208
2, 4	0.64	0.70	0.80	2129	2133	2208
3, 4	0.92	0.93	1.00	2184	2173	2238
2	1.00	1.00	1.00	2198	2183	2238
3	1.30	1.28	1.42	2239	2216	2286
4	1.27	1.21	1.50	2235	2209	2293
	1.56	1.39	1.70	2267	2228	2310
$t'_{R,2\text{-O-MeRh}}$ (min)	28.35	43.07	27.48			

highly efficient columns. The retention indices were also determined for these derivatives. Such indices have not yet been routinely used in GLC identification of sugar derivatives.

Obviously, for both qualitative and quantitative investigations of mixtures of methyl ethers of L-rhamnose, the methyl glycosides and TFA derivatives are more advantageous than alditol acetates. Better separations are obtained, GLC requires less time and in the case of methyl glycosides the preparation of samples is very simple compared with the alditol acetates. AAN of methylated L-rhamnoses are separated almost as well as the corresponding alditol acetates. Moreover, the reaction can be performed in one step and, after evaporation and dissolution in a suitable solvent, the reaction mixture is directly injected into the column.

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Note

High-performance liquid chromatography of decamethrin

D. MOUROT, B. DELÉPINE, J. BOISSEAU and G. GAYOT
Laboratoire National des Médicaments Vétérinaires, Javené, 35300 Fougères (France)
(Received January 5th, 1979)

Decamethrin (DECIS, NRDC 161), (*S*)-α-cyano-3-phenoxybenzyl-*cis*-(1*R*,3*R*)-2,2-dimethyl-3-(2,2-dibromovinyl)cyclopropane carboxylate is a newly developed pyrethroid insecticide; it is the most potent insecticide currently known[1].

Ultraviolet techniques cannot be used directly because decamethrin exhibits an absorption maximum at 230 nm and many other organic substances present in the formulations absorb strongly at this wavelength. The separation of decamethrin from its photolysis degradation products using thin-layer chromatography or gas–liquid chromatography has been reported[2], but has not been applied to quantitation of the active constituent.

High-performance liquid chromatography (HPLC) offers an alternative for the analysis of pyrethroid insecticides[3,4] and this paper describes the HPLC analysis of decamethrin in reversed- and normal-phase modes.

EXPERIMENTAL

Chromatographic system

A Varian LC 8500 high-pressure liquid chromatograph with a variable-wavelength UV detector operating at 230 nm was used. The two chromatographic columns were a LiChrosorb RP-8 (Merck, Darmstadt, G.F.R.) column (10 cm × 4.7 mm I.D.), particle size 10 μm, and a LiChrosorb Si-60 (Merck) column (15 cm × 4.7 mm I.D.), particle size 5 μm. The flow-rates were maintained at 70 and 80 ml/h, respectively (column pressure 500 p.s.i.).

Chemicals

The reference sample of decamethrin was a generous gift from Roussel Uclaf–Procida (Paris, France). All solvents were of analytical-reagent grade and *n*-hexane and diisopropyl ether were anhydrous materials.

Analytical method

In the normal-phase system, isocratic elution was carried out with *n*-hexane–diisopropyl ether (93:7); for the RP-8 column, isocratic elution was carried out with acetonitrile–1% sulphuric acid (70:30).

RESULTS AND DISCUSSION

Decamethrin, being a well defined optical isomer of a group of eight possible isomers, appears as a single peak in both reversed- and normal-phase systems.

On the Si-60 column (see Fig. 1a), the order of elution was internal standard (diphenylamine) and decamethrin, the retention times being 4.5 and 7.6 min, respectively. However, replicate injections of decamethrin led to decreasing retention times, as described previously for sumicidin[4]. Pumping of pure diisopropyl ether through the column for 2 min followed by stabilization was also necessary in order to obtain good reproducibility of retention times.

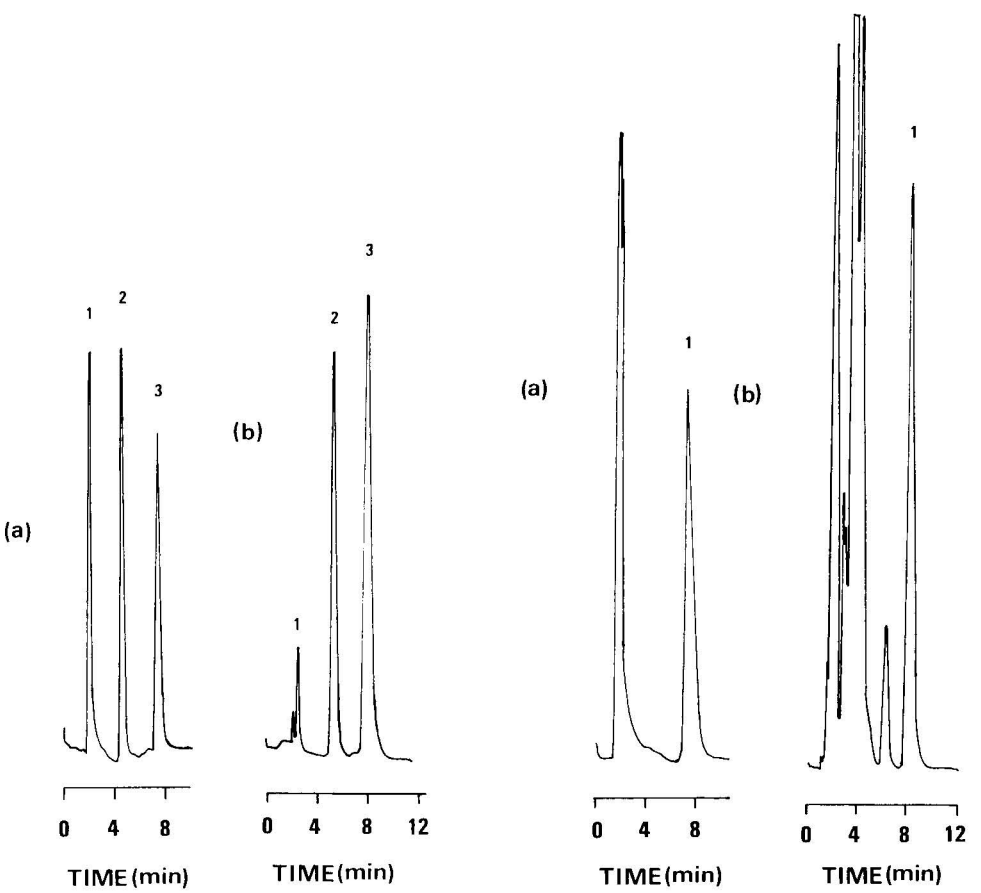

Fig. 1. HPLC of decamethrin in reversed- and normal-phase modes. (a) LiChrosorb Si-60 column; mobile phase, *n*-hexane–diisopropyl ether (93:7); flow-rate, 80 ml/h; detector sensitivity, 0.5 a.u.f.s. Peaks: 1 = injection artefact; 2 = diphenylamine (internal standard); 3 = decamethrin (0.5 g/l in chloroform). (b) LiChrosorb RP-8 column; mobile phase, acetonitrile–1% sulphuric acid (70:30); flow-rate, 70 ml/h; detector sensitivity, 0.5 a.u.f.s. Peaks: 1 = injection artefact; 2 = pentachlorobenzene (internal standard); 3 = decamethrin (1 g/l in methanol).

Fig. 2. HPLC scan of decamethrin formulation in reversed- and normal-phase modes. (a) LiChrosorb Si-60 column; (b) LiChrosorb RP-8 column. Conditions as in Fig. 1. Peak 1 = decamethrin.

However, in the reversed-phase system, excellent reproducibility of retention times was attained and, as shown in Fig. 1b, the order of elution was pentachlorobenzene (internal standard) and decamethrin, with retention times of 5.3 and 7.7 min, respectively.

Fig. 2 shows the chromatograms of a decamethrin formulation in reversed- and normal-phase modes. In each instance, the decamethrin peak was well defined without any significant interference encountered with other substances present in the formulation; however, on the RP-8 column, the internal standard peak was overlapped by a substance present in this insecticidal preparation. By modification of the acetonitrile to sulphuric acid ratio (*ca.* 60:40) and the flow-rate (*ca.* 90 ml/h) a good separation was achieved and quantitation by the internal standard method was still possible. Another formulation tested (as a powder) was quantitated according to the initial conditions.

CONCLUSION

This simple, rapid separation (less than 10 min) allows the presence of decamethrin in commercially available insecticidal preparations to be ascertained. It appears that both methods give excellent results and that the analysis is not affected by the presence of other substances if certain precautions are taken. The choice between the two is dependent on the composition of the formulation: oily-based formulations on the Si-60 column and aqueous formulations on the RP-8 column.

The application of this HPLC method to develop a screening test for the identification of pyrethrin or pyrethroid insecticide residues is now in progress and will be described in a subsequent publication.

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Note

Chromatography of non-human albumins on Cibacron Blue–agarose

Application to the separation of albumin from rat alpha-fetoprotein

PHILIP C. KELLEHER and CAROL J. SMITH

Department of Medicine, University of Vermont College of Medicine, Burlington, Vt. 05405 (U.S.A.)

and

RALPH PANNELL*

Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vt. 05405 (U.S.A.)

(Received December 8th, 1978)

A major difficulty in the purification of many serum proteins is their separation from albumin. Human, rat and mouse alpha-fetoproteins (AFP) are physicochemically similar to the albumins of the respective species so that separation by conventional physical and chemical techniques is difficult[1]. Travis *et al.*[2] described the removal of albumin from human serum by affinity chromatography on a column of agarose–Cibacron Blue conjugate. We attempted to apply their method as a step in the purification of non-human AFP and found a far lower affinity of rat, mouse and bovine albumin for Cibacron Blue than of human albumin under the same conditions. This paper describes conditions for chromatography of murine and bovine albumin on Cibacron Blue–agarose.

EXPERIMENTAL

Cibacron Blue–agarose[2] was packed in a 240 × 9 mm column (bed volume, 15 ml) and equilibrated with one of the following buffers: buffer 1, 10 m*M* Tris–HCl, pH 7.5; buffer 2, 50 m*M* Tris–HCl, pH 7.5, 10 m*M* NaCl; buffer 3, 50 m*M* Tris–HCl, pH 7.5, 50 m*M* NaCl; buffer 4, 50 m*M* Tris–HCl, pH 7.5, 500 m*M* NaCl. All buffers contained 10 m*M* NaN_3. Buffers 3 and 4 have been used to prepare albumin-free human serum proteins[2,3].

Pooled one-day-old newborn rat or mouse sera, pooled hepatoma-bearing rat sera containing AFP, pooled normal adult mouse sera, partially purified rat AFP preparations, or culture media containing rat AFP and fetal bovine serum were dialyzed against one of the above buffers prior to chromatography. Single donor normal adult human sera were dialyzed against buffer 1 prior to chromatography. The equilibrating buffer was used to elute by descending flow the proteins which did

* Present address: Department of Microbiology, University of St. Louis Medical School, St. Louis, Mo. 63104, U.S.A.

not bind to Cibacron Blue. The albumin bound to Cibacron Blue was eluted with 50 m*M* Tris–HCl, pH 7.5, containing 200 m*M* NaSCN. The column was regenerated with two column volumes of the equilibrating buffer.

Some of the rat serum samples were treated with activated charcoal[4] prior to dialysis against the equilibrating buffer for chromatography.

Murine AFP and murine and human albumins in the sera and chromatographic fractions were quantified by radial immunodiffusion[5,6]. Bovine albumin was quantified by the method of Lowry *et al.*[7] after precipitation with 5% trichloroacetic acid and solubilization with 95% ethanol[8]. Proteins eluted from the Cibacron Blue–agarose column were monitored by the procedure of Lowry *et al.*[7].

RESULTS

All of the rat and mouse AFP applied to the Cibacron Blue–agarose column was recovered in the unbound fraction at all buffer concentrations. The binding capacities of rat, mouse and bovine albumins for Cibacron Blue, however, were affected by the composition of the equilibrating buffer. The amount of albumin bound to the Cibacron Blue was inversely proportional to the ionic strength of the buffer (Table I). The relative proportions of AFP and albumin in the samples did not affect the binding of albumin to Cibacron Blue. Recoveries of rat, mouse, bovine and human albumins (unbound and bound fractions) from the column were 95–100%. Repeated use of the Cibacron Blue–agarose column did not result in a decreased binding of albumin. Seventy five percent of the bound rat albumin was eluted when 50 m*M* Tris–HCl buffer, pH 7.5, containing 2 *M* NaCl rather than 200 m*M* NaSCN was used.

TABLE I

EFFECT OF BUFFER COMPOSITION ON BINDING OF RAT, MOUSE AND BOVINE ALBUMINS TO CIBACRON BLUE–AGAROSE

Aliquots (0.25 ml) of rat, mouse and human sera and culture media were chromatographed as described in the text.

Buffer	*Composition*	*Albumin bound (%)*			
		Rat	*Mouse*	*Bovine*	*Human*
1	10 m*M* Tris–HCl	98–100*	99**	100**	100**
2	50 m*M* Tris–HCl, 10 m*M* NaCl	69– 82	93	—	—
3	50 m*M* Tris–HCl, 50 m*M* NaCl	54– 61	—	60	—
4	50 m*M* Tris–HCl, 500 m*M* NaCl	39– 46	51	18	—

* Range of values from six determinations.
** Average of values from three determinations.

The binding capacities (mg albumin/ml column bed volume) of human, rat, mouse and bovine albumins for Cibacron Blue were 2.0, 4.8, 0.83 and 0.79, respectively, when chromatographed using buffer 1.

Treatment of rat serum samples with activated charcoal prior to Cibacron Blue affinity chromatography did not improve the binding of rat albumin to Cibacron Blue with buffer 3. In addition, charcoal treatment resulted in a loss of 10 to 24% of the AFP after Cibacron Blue affinity chromatography.

DISCUSSION

Albumins from various species are physicochemically similar. Species variability in the affinity of various albumins for Cibacron Blue is not surprising, however, in view of differences found between human and bovine albumins in the binding of sulfonphthalein dyes[9] and in other conformational parameters which affect ligand binding[10].

Travis and Pannell[3] found the use of the high-ionic-strength buffer 4 necessary to prevent the blue dextran–agarose from behaving as a cation exchanger. Gold *et al.*[11] utilized the high-ionic-strength buffer in the purification of human AFP; they removed 79% of the albumin present in the sample and recovered 75% of the AFP. Young and Webb[12], using a buffer ionic strength of 160 m*M* in the chromatography of fetal human serum, removed 97% of the albumin and recovered 91% of the AFP.

With buffer conditions of low ionic strength we removed over 98% of rat and mouse albumins from all AFP-containing preparations by Cibacron Blue affinity chromatography. In most instances no albumin could be detected in the AFP preparations. Rat albumin present in newborn serum and tumor-bearing adult serum had the same chromatographic behavior on Cibacron Blue–agarose columns. Purification of rat or mouse AFP by negative immunoadsorbent affinity chromatography is facilitated by removal of albumin since albumin is the major contaminating normal serum protein against which a high-titer antiserum must be prepared[13].

Cultures of the AFP-secreting rat hepatoma cell line UVM-RH777 are grown in nutrient mixture F12 supplemented with 10% fetal bovine serum in this laboratory. We also demonstrated that the quantitative removal of bovine serum albumin from rat AFP-containing culture media necessitated the use of low-ionic-strength buffer in Cibacron Blue affinity chromatography. Passage of these culture media over Cibacron Blue–agarose allowed us to visualize the rat AFP in various electrophoretic procedures without additional purification.

Albumins exist in serum unbound and bound to bilirubin, fatty acids and drugs. Activated charcoal treatment, which has been used to defat albumin[3], was attempted to increase the unbound fraction for binding to Cibacron Blue. The fact that Cibacron Blue binding of rat albumin was not improved by charcoal treatment but by lowering the buffer ionic strength suggests that endogenous ligands were not interfering with Cibacron Blue binding but that the affinity of rat albumin for Cibacron Blue is sufficiently low for buffers of moderate ionic strength to decrease the protein–dye interaction.

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Note

Determination of sulphur-containing amino acids by quantitative ion-exchange thin-layer chromatography

ANDRÁS VÁRADI and SÁNDOR PONGOR

Enzymology Department, Institute of Biochemistry, Hungarian Academy of Sciences, Budapest (Hungary)

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Difficulties in the determination of sulphur-containing amino acids in biological materials are mainly due to oxidative losses during acidic hydrolysis. To overcome this problem, methionine and cysteine + cystine are usually converted into stable oxydized derivatives prior to hydrolysis. Methionine sulphone ($MESO_2$) and cysteic acid ($CYSO_3H$) can be separated by ion-exchange column chromatography (for a review, see ref. 1) or thin-layer chromatography (TLC)[2,3]. Detection is usually carried out by means of the ninhydrin reaction. Non-hydrolytic methods are also available, such as reaction with cyanogen bromide combined with gas chromatography[4–7] for methionine, and hydrazinolysis and colorimetry[3,8] for cysteine + cystine.

A procedure combining ion-exchange TLC and video-densitometric quantitation has been developed as a convenient method for the determination of sulphur-containing amino acids. A comparison with other methods was carried out by analysing different legume seeds in which methionine and cysteine + cystine are the nutritionally critical (limiting) amino acids.

EXPERIMENTAL

Sample preparation

A 200-mg amount of pulverized plant material was oxidized with 2 ml of ice-cold performic acid according to Hirs[9]. After oxidation, the samples were diluted with 6 ml of distilled water and lyophilized. Hydrolysis was carried out with 1.5 ml of 6 *N* hydrochloric acid at 105° for 48 h. The hydrolysates were filtered and the clear filtrates were used directly for the determination of cysteic acid. With soybean samples methionine sulphone can also be determined in this solution but, because of the lower protein concentration in beans, peas and lentils, the hydrolysates of these samples have to be concentrated. The filtrates were evaporated to dryness under vacuum at 40–50° and the residue was redissolved in 400 μl of distilled water, the solutions obtained being used for the determination of methionine sulphone.

A 2-μl volume was applied on to the chromatosheet for cysteic acid and 6 μl for methionine sulphone.

Chromatography of methionine sulphone

Li^+ (0.6 *M*)–citrate (0.1 *M*) buffer (pH 2.50 ± 0.05) was prepared by mixing lithium citrate tetrahydrate (28.20 g), lithium chloride (12.70 g) and 37% hydrochloric acid (20.1 ml) and diluting to 1000 ml with deionized water. Strongly acidic cation-exchange chromatosheets (20 × 20 cm, Fixion 50 X8 Li^+, Chinoin, Nagytétény, Hungary, or Ionex 25 SA-Li^+, Macherey, Nagel & Co., Düren, G.F.R.) were used. The chromatosheets were equilibrated by continuous development for 24 h with the eluting buffer diluted 10-fold. The chromatography was carried out at 4° by continuous development for 16 h.

Chromatography of cysteic acid

For the separation of cysteic acid an anion-exchange TLC method, described by Ferenczi and Dévényi[10], was used with modification of the eluting buffer. Chromatosheets coated with a layer of anion-exchange resin (Fixion 2-X8 Ac^-, Chinoin, or Ionex, Macherey-Nagel & Co.) were used. The sheets were equilibrated by continuous development for 24 h with 0.01 *N* acetic acid. The eluting buffer was Na^+ (0.02 *M*)–acetate (0.2 *M*) (pH 3.7) prepared from 10 ml of 0.2 *M* sodium acetate solution and 90 ml of 0.2 *M* acetic acid.

Staining

The chromatograms were stained with ninhydrin spray reagent consisting of 100 ml of solution A, 20 ml of solution B and 3 ml of pyridine, where solution A is 1 g of ninhydrin in 100 ml of acetone (freshly prepared) and solution B is 10 g of cadmium acetate in 150 ml of glacial acetic acid plus 100 ml of distilled water.

The addition of pyridine increased the colour yield about 3-fold on the chromatograms developed with low pH buffers in comparison with the original spray[11]. No further increase was observed on chromatosheets developed with a buffer of pH 5.1, illustrating that this effect is due to the shift of pH. After staining, the sheets were dried and kept overnight in the dark.

Densitometry and computation of results

Densitometry was carried out with an automatic video-densitometer[11] (Telechrom OE-976, Eurolab, Munich, G.F.R.). The measurement is based on the video-scanning principle followed by a high-speed integration. The chromatograms were evaluated in the reflectance mode. Two density integrals were determined for each sample: the value for the individual amino acid ($MESO_2$ or $CYSO_3H$) and the total density of ninhydrin-positive material. The latter was used as the total amino acid content. The measured values were transferred to an on-line Hewlett-Packard HP-97 programable calculator and the results were expressed as the percentage by weight of the total amino acid content. The computation of the results included the calculation of a calibration constant for each chromatogram. This constant was determined from a standard sample chromatographed on each chromatosheet. The time required for the quantitative evaluation was about 4 sec per sample, including calculation and printing.

For comparison, methionine was determined by gas–liquid chromatography (GLC) as methylthiocyanate formed in the cyanogen bromide reaction[6], using a Hewlett-Packard 5720A gas chromatograph. Cysteine + cystine was determined as cysteic acid[12] on a Beckman Unichrom amino acid analyser.

RESULTS AND DISCUSSION

In our previous work[13] we dealt with the cation-exchange TLC separation of aspartic acid, threonine, serine, asparagine, glutamine and glutamic acid. It was found that their separation can be achieved at low pH using an "Li system", *i.e.*, sheets coated with resin in the Li^+ form, with lithium citrate buffers for elution. As the chromatographic behaviour of methionine sulphone is similar to that of the above amino acids, we tested the "Li system" for separation.

The pH of the eluting buffer has a marked influence on the separation of $MESO_2$ from these amino acids (Fig. 1). It can be seen that $MESO_2$ cannot be separated satisfactorily from Thr + Ser + Asp at lower pH (2.3–2.4) or from Glu at higher pH (2.6–2.7). Fig. 2 shows the separation of $MESO_2$ (pH 2.5) (as described under Experimental) in hydrolysates of different performic acid-treated samples. It can be seen that even the relatively small amount of $MESO_2$ is well separated from the large excess of Asp + Thr + Ser and Glu present in legume hydrolysates. In the present separation, ninhydrin-positive degradation products of plant materials do not interfere with $MESO_2$. This interference is a source of difficulties in separating $MESO_2$ by silica gel TLC[3]. Linear response curves were obtained in the ranges important for determining sulphur-containing amino acids in plant materials (2–12 μg of $MESO_2$ and 0.2–1.2 μg of $CYSO_3H$ per spot).

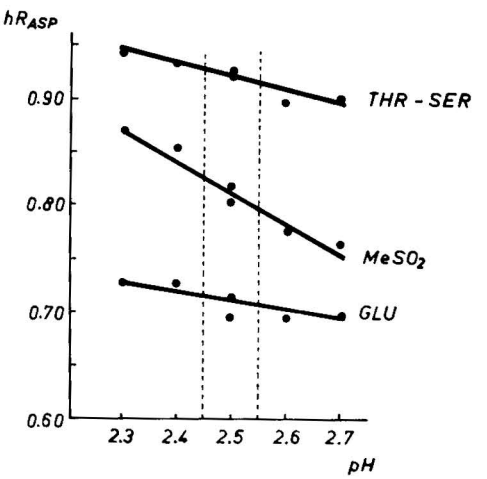

Fig. 1. Effect of the pH of the eluting buffer on the separation of methionine sulphone. Eluting buffer, Li^+(0.6 *M*)–citrate (0.1 *M*); continuous development at 4° for 16 h. The results are expressed as migration distances relative to that of Asp (hR_{Asp}).

The molar ratio of Glu to Met was determined in two synthetic peptides. The separations are shown in Fig. 2. In ACTH (1–14) a ratio of 1.1:1 was measured and in ACTH (1–32) a ratio of 3.3:1 was found, in agreement with the theoretical values (1:1 and 3:1, respectively).

The $MESO_2$ and $CYSO_3H$ contents of plant materials were expressed as percentage by weight of the total amino acid content. As these percentage contents

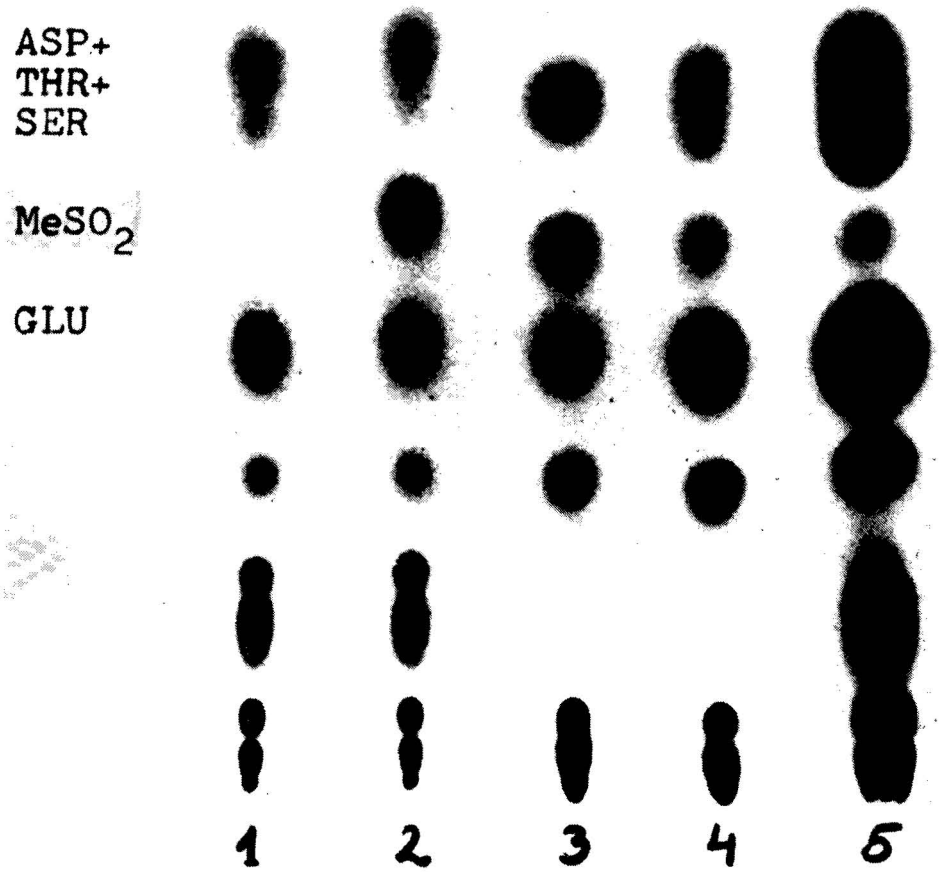

Fig. 2. Separation of methionine sulphone. Samples: 1 = calibration mixture of amino acids without methionine sulphone; 2 = calibration mixture containing methionine sulphone; 3 = hydrolysate of oxidized ACTH (1–14) fragment; 4 = hydrolysate of oxidized ACTH (1–32) fragment; 5 = hydrolysate of oxidized field bean.

are derived from the ratio of two amounts determined on the same sample, it could be expected that they are independent of the sample size in a given range. The "useful range" was found to be 1.5–3.0 mg of plant seed meal for $MESO_2$ and 0.2–0.33 mg for $CYSO_3H$ (per spot). Although these ranges may vary somewhat with the protein content, it can be stated that a sufficiently broad interval exists in which the percentage content can be determined without an exact knowledge of the sample size. Therefore, approximate weighing can be used, which is an important simplification in large-scale routine work.

The reproducibility of the method was determined by analysing amino acid calibration mixtures containing $MESO_2$ (or $CYSO_3H$) and hydrolysates of performic acid-treated plant materials. Standard deviations were determined from 35 parallel measurements (five parallel runs on seven chromatograms, six samples being run on

each chromatosheet, one of them being used for the calculation of the conversion constant). The results are given in Table I. Although the standard deviations are always higher with plant samples, these deviations (less than 6% of the mean) permit one to detect relative differences of 15%.

Table II gives the results for the methionine contents of 16 legume samples.

TABLE I

REPRODUCIBILITY OF TLC-VIDEODENSITOMETRIC METHOD

Each value is the average ± standard deviation calculated from five determinations.

Experiment No.	*Methionine (%)*		*Cysteine/cystine (%)*	
	Standard amino acid mixture (N.V. 5.00)***	*Field beans (N.V. 1.18)****	*Standard amino acid mixture (N.V. 2.00)***	*Peas (N.V. 0.86)§*
1	5.01 ± 0.09	1.16 ± 0.05	1.96 ± 0.06	0.88 ± 0.04
2	5.03 ± 0.07	1.19 ± 0.04	1.99 ± 0.03	0.85 ± 0.06
3	5.13 ± 0.16	1.23 ± 0.05	2.03 ± 0.06	0.82 ± 0.03
4	5.09 ± 0.13	1.25 ± 0.06	2.00 ± 0.02	0.83 ± 0.03
5	5.09 ± 0.12	1.16 ± 0.03	2.02 ± 0.04	0.86 ± 0.03
6	5.12 ± 0.08	1.18 ± 0.04	1.97 ± 0.04	0.85 ± 0.04
7	4.94 ± 0.08	1.24 ± 0.05	2.01 ± 0.05	0.87 ± 0.05
Coefficient of variation (%)	1.4–3.1	2.5–5.0	1.6–3.6	2.6–5.6

* N.V. = nominal value.

** $MESO_2$ and $CYSO_3H$ were added to standard amino acid mixture in the concentrations given. Values expressed as % (w/w) of the total amino acid content.

*** Determined by the cyanogen bromide GLC method and expressed as % (w/w) of the protein content (calculated as Kjeldahl N × 6.25).

§ Determined by amino acid analyser as cysteic acid and expressed as % (w/w) of the protein content.

TABLE II

METHIONINE CONTENT OF PLANT SEED SAMPLES AS DETERMINED BY DIFFERENT METHODS

Results expressed as % (w/w) of methionine in the protein.

Sample	*BrCN GLC method*	*Video-densitometry*
Field beans	1.18	1.1
Bengal gram	1.33	1.2
Urd beans	1.63	1.7
Mong beans	1.04	1.1
Kidney beans	1.19	1.3
Beas	0.72	0.8
Cowpeas	0.63	0.7
Pigeon peas	1.15	1.1
Lentils (J-11)	0.87	1.0
Lentils (J-17)	0.83	0.8
Lentils (J-19)	0.85	0.9
Soybeans (Wells)	1.53	1.6
Soybeans (Altona)	1.08	1.2
Soybeans (Wilkin)	1.17	1.3
Soybeans (Traverse)	1.21	1.2

For comparative purposes, the samples were also analysed by the cyanogen bromide GLC method, and the results were expressed in percentage by weight of the protein content (Kjeldahl nitrogen value × 6.25).

The results of the cystine determination are compared with amino acid analyser results in Table III.

TABLE III

CYSTEINE + CYSTINE CONTENT OF PEA SEED SAMPLES DETERMINED AS CYSTEIC ACID BY DIFFERENT METHODS

Results expressed as % (w/w) of the total amino acid content.

Sample	*Amino acid analyzer (%)*	*Video-densitometry (%)*
J-63	1.35	1.3
J-64	0.86	0.8
J-66	0.85	0.9
J-74	1.11	1.0
J-80	1.16	1.1
J-83	0.99	0.9

The samples were selected to represent different methionine and cystine contents, respectively. It can be seen that the results of the procedure described here were in generally good agreement with those of the other methods examined for the analysis of crude samples such as plant seeds.

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Note

Méthode densitométrique de dosage de la patuline dans les jus de fruits

M. G. SIRIWARDANA et P. LAFONT

I.N.S.E.R.M., S.C.R. 8, Microbiologie Appliquée à l'Alimentation et à la Nutrition, 44 Chemin de Ronde, B.P. No. 40, 78110 Le Vesinet (France)

(Reçu le 22 janvier 1979)

La patuline est un métabolite commun à plusieurs espèces d'*Aspergillus*, de *Penicillium*, de *Gymnoascus* et à *Byssochlamys nivea*[1]. Ce produit, qui fut d'abord étudié pour ses propriétés bactériostatiques, s'est avéré être un toxique: par injection sous-cutanée, il induit la formation de tumeurs[2]; d'autre part ses propriétés mutagènes ont été mises en évidence chez *Saccharomyces cerevisiae*[3].

Les micromycètes producteurs de patuline sont largement représentés dans la mycoflore de denrées alimentaires diverses. Au cours des dernières années, la mycotoxine a été mise en évidence dans du jus de pomme[4,5], dans des fruits (pommes, poires, pêches, abricots, raisins, bananes, ananas)[6]. Différentes méthodes analytiques ont été proposées permettant de définir les taux de contamination[1,7]; celle retenue par l'*Association of Official Analytical Chemists* a fourni, pour 20 laboratoires travaillant sur les mêmes échantillons, des résultats hétérogènes[8]. Cette observation nous a conduits à élaborer une méthode de dosage de la patuline utilisant un solvant organique miscible à l'eau, l'alcool isopropylique, et, comme agent de révélation sur chromatographie sur couche mince (CCM), l'acétate d'aniline.

MATÉRIEL ET MÉTHODES

Échantillons

La méthode a été appliquée à l'analyse de jus de pomme soit industriels, soit préparés par nous-mêmes à partir de fruits sains ou altérés, et à celle de jus de raisin.

Le rendement du procédé d'extraction a été établi en utilisant des jus de pomme artificiellement contaminés par de la patuline cristallisée (Serva Feinbiochem, Heidelberg, R.F.A.).

Extraction

50 ml du jus de pomme sont additionnés de 50 ml du mélange isopropanol–acétate d'éthyle (60:40, v/v). Après une agitation de quelques minutes, on ajoute 10 g de chlorure de sodium et on poursuit l'agitation 3 min à température du laboratoire. L'adjonction de chlorure de sodium provoque une séparation de la phase organique et de la phase aqueuse. La première est soutirée, desséchée sur sulfate de sodium anhydre, concentrée, sous volume de 2 ml, sous vide en chauffant à 50° au maximum, puis reprise par 25 ml d'acétate d'éthyle et 75 ml de benzène.

Purification de l'extrait

Cette purification, par passage sur colonne de silice, est effectuée dans les conditions décrites par Scott[8] (colonne de 15 g de silica gel 60, 200 mesh, Merck, Darmstadt, R.F.A.; élution par benzène–acétate d'éthyle (75:25, v/v); récupération de l'effluent correspondant à un volume d'élution compris entre 100 et 300 ml; concentration sous vide à 50°; solubilisation dans 500 ml de chloroforme).

Chromatographie sur couche mince

L'extrait et des dilutions successives d'une solution de patuline cristallisée à 1 μg/ml en chloroforme sont déposés sur couche mince de silice (silica gel 60). Une première élution est réalisée avec le mélange pentane–acétate d'éthyle (96:4, v/v); après évaporation, une seconde élution, dans la même direction, utilise le mélange éther isopropylique–pentane–éthanol–pyridine (84:12:4:0.8, v/v) en cuve à atmosphère non saturée. Après développement, les chromatogrammes sont laissés 1 h, à température ambiante, dans une pièce ventilée obscure, pour assurer l'élimination des solvants. Ils reçoivent ensuite, en pulvérisation, le réactif, préparé extemporanément: acide acétique glacial (0.9 ml), eau distillée (8.1 ml), aniline (1 ml). Les chromatogrammes sont chauffés 10 min à 80° et examinés sous irradiation UV (lampe Philips HPW 125). L'intensité de la fluorescence jaune-verdâtre de la patuline peut être appréciée à l'oeil nu, ou mesurée par densitométrie.

Dosage densitométrique

Il a été réalisé sur un fluorodensitomètre PH I 5, Vernon (Paris, France), pourvu d'une source "Mercure" (longueur d'onde de la lumière d'excitation: 256 nm; filtre Wratten 4A).

RÉSULTATS

La fiabilité de la méthode d'extraction par le mélange isopropanol–acétate d'éthyle a été étudiée en utilisant des jus de pomme commerciaux ou préparés au laboratoire et des jus de raisin, additionnés de patuline à des taux variant de 10 à 200 μg/l. Les résultats enregistrés au cours de cinq essais figurent dans le Tableau I. Dans l'ensemble de notre expérimentation la moyenne du pourcentage de récupération de la mycotoxine a été supérieure à 91 % (les résultats d'un certain nombre d'essais figurent dans le Tableau I).

La détection en CCM de la patuline par le réactif que nous employons correspond vraisemblablement à la formation d'une base de Schiff[9] sous l'action de l'acétate d'aniline. La plus faible quantité de mycotoxine visible, dans ces conditions, sous irradiation UV, à l'oeil nu, est 0.004 μg.

Le dosage fluorodensitométrique apparait possible dans les limites de concentration où la loi de Beer s'applique; ainsi que l'indiquent les valeurs portées dans le Tableau II, ces limites sont 0.010 et 0.1 μg.

Les mesures fluorodensitométriques doivent être effectuées dans l'heure suivant l'application du réactif sur le chromatogramme; pour pouvoir les faire ultérieurement, il convient de conserver le chromatogramme à l'obscurité, la couche mince étant couverte par une autre plaque de verre. En l'absence de ces précautions, une couleur brune se développe.

TABLEAU I

POURCENTAGE DE RÉCUPERATION DE LA PATULINE

Moyenne du pourcentage de récupération: 91.2; écart type: 5.2.

Quantité de toxine ajoutée (µg/l)	*Quantité de toxine extraite (exprimée en % de la quantité de toxine ajoutée)*				
	Jus de pomme commercial		*Jus de pomme, non clarifié, fabriqué au laboratoire*		*Jus de raisin commercial*
	1	*2*	*1*	*2*	
10	90	90	90	95	85
20	88	90	95	88	92
50	92	85	87	84	90
75	83	90	93	87	104
100	92	102	90	95	85
200	95	100	88	95	98

TABLEAU II

DOSAGE DENSITOMÉTRIQUE DE LA PATULINE

Quantité (Q) de patuline déposée (µg)	*Moyenne (M) de surface de pic (mm^2) (n = 6)*	*Coefficient de variation (%)*	$\frac{Q}{M}$
0.010	7.8	16.6	1.28
0.015	11.8	9.3	1.27
0.020	15.8	3.2	1.26
0.025	20.2	3.9	1.24
0.050	39.1	3.3	1.27
0.060	47.9	2.9	1.25
0.080	64.0	3.0	1.25
0.100	80.1	3.9	1.25
0.150	107.7	4.5	1.39
0.200	140.5	4.0	1.42

Différents solvants ont été utilisés pour développer les chromatogrammes sur couche mince. Ainsi que l'indiquent les données rassemblées dans le tableau III, le mélange éther isopropylique–pentane–éthanol–pyridine (84:12:4:0.8, v/v) présente l'avantage de séparer nettement la patuline de l'acide pénicillique; or ce dernier métabolite, produit par de nombreuses espèces fongiques, risque d'être présent dans des jus de fruit et réagit avec l'acétate d'aniline comme la patuline. D'autre part ce solvant d'élution ne permet qu'une migration chromatographique limitée de substances absorbantes en UV normalement présentes dans l'extrait de jus de pomme.

TABLEAU III

VALEURS R_F DE LA PATULINE ET DE L'ACIDE PÉNICILLIQUE

Systèmes utilisés: 1, éther isopropylique–pentane–éthanol–pyridine (84:12:4:0.8); 2, toluène–acétate d'éthyle–90% acide formique (50:40:10); 3, chloroforme–acétone (90:10); 4, chloroforme–méthanol (95:5); 5, pentane–acétate d'éthyle (96:4).

	Système				
	1	*2*	*3*	*4*	*5*
Patuline	0.32	0.39	0.42	0.35	0
Acide pénicillique	0.23	0.41	0.40	0.35	0

DISCUSSION

Dans la méthode décrite ci-dessus, la concentration de l'extrait organique de jus de fruit est réalisée sans chauffage à une température supérieure à 50°. Dans des séries d'essais nous avons observé que le respect de telles conditions expérimentales évite la formation de substances fluorescentes sous irradiation UV, pouvant perturber le dosage de la patuline, et une perte apparente, partielle, de cette dernière. Il est vraisemblable que ces deux phénomènes, liés l'un à l'autre, correspondent à une réaction d'addition entre la mycotoxine à fonction lactone et des composés aminés, une réaction de ce type étant favorisée par l'élévation de la température[10].

L'extraction par le mélange isopropanol–acétate d'éthyle permet de recouvrer 91 % de la patuline introduite dans un jus de fruit; d'autre part la répétition des essais a fourni des résultats quantitativement homogènes. Il semble qu'en matière de rendement cette méthode d'extraction est meilleure que celle préconisée par l'*Association of Official Analytical Chemists*[8].

Reiss[11], ultérieurement Scott[8], ont utilisé le chlorure de 3-méthyl-2-benzothiazolinone-hydrazone pour mettre en évidence la patuline en CCM.

La limite inférieure de détection de la mycotoxine, avec ce réactif, est de 0.010 μg, alors qu'avec l'aniline en milieu acétique elle atteint 0.004 μg. L'utilisation de ce dernier réactif permet d'autre part de ne pas avoir de coloration de la couche mince de silice ce qui facilite un dosage de la mycotoxine par densitométrie. Nous avons d'autre part noté que sous irradiation par un rayonnement UV non filtré, le spot de patuline présente une absorption après vaporisation d'aniline; cette absorption est visible en superposant le chromatogramme et une plaque de cellulose. Certaines observations nous conduisent à estimer que ce phénomène pourrait servir de base à un mode de confirmation de la présence de patuline dans un produit alimentaire.

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* Voir aussi U. Leuenberger et R. Gauch, *J. Chromatogr.*, 161 (1978) 303 (*Rédacteur Journal of Chromatography*).

Journal of Chromatography, 173 (1979) 429–434

CHROM. 11,725

Note

Retention of Cu^{2+} on glass beads coated with chelating agent

NOBORU OKUBO, TOSHIHIKO HANABUSA and MOTOICHI MIYAZAKI
Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa (Japan)
(Received January 8th, 1979)

Recently, the study of the preconcentration of trace amounts of metals in environmental samples prior to their analysis has been extended[1–16].

A preliminary report on a promising method using glass beads coated with dibenzoylmethane (DBM) for the retention of micro-amounts of Cu^{2+} has appeared[12]. In this communication, the method was further investigated by using some chelating agents such as 8-hydroxyquinoline (HQ), 2-thenoyltrifluoroacetone (TTA) and benzoyltrifluoroacetone (BFA).

EXPERIMENTAL

Apparatus and conditions

A glass chromatographic column (40 × 1.0 cm I.D.) was packed with glass beads coated with chelating agent. A Mitsumi SJ-1210 peristaltic pump (Mitsumi Scientific, Tokyo, Japan) was used for maintaining a constant flow-rate of eluate from the column. A Toyo fraction collector SF-160 K (Toyo Kagaku Sangyo, Tokyo, Japan) was used to collect the eluates. A Hitachi-Horiba M 7 meter (Hitachi, Tokyo, Japan) was used for measuring the pH of the sample solution and the eluates. The chelating agents HQ, TTA and BFA were of analytical grade and were obtained commercially. Acids, alkalis, $CuSO_4 \cdot 5H_2O$ and Na_2-EDTA of analytical grade were purchased from Wako (Tokyo, Japan). Organic solvents were of commercial reagent grade and were purified in the usual manner. Glass beads were prepared by Nihon Chromat Works (Tokyo, Japan) Water was purified by distilling twice deionized water in all-glass vessels. Apiezon L for precoating the glass beads was obtained from AEI (Manchester, Great Britain), and metal indicator and the reagents used for EDTA titration were of analytical grade.

The preconcentration system is the same one as previously reported[12] (Fig. 1), and the chelating agents used are listed in Table I.

Preparation of Cu^{2+} solution

A 1000 ppm Cu^{2+} solution was prepared by dissolving a known amount of $CuSO_4 \cdot 5H_2O$ in water and used as a stock solution. Solutions of 5, 10, and 30 ppm Cu^{2+} were made by diluting the stock solution with water just before use.

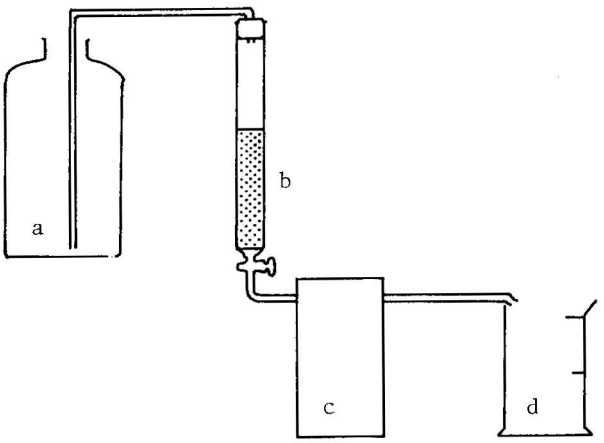

Fig. 1. Preconcentration system. a = Reservoir; b = column containing glass beads coated with chelating agent; c = peristaltic pump; d = measuring cylinder.

TABLE I
CHELATING AGENTS USED FOR COATING GLASS BEADS

Chelating agent	*Name*	*Formula*	*M.p. (°C)*
Dibenzoylmethane (DBM)	1,3-diphenylpropane-1,3-dione	$C_{15}H_{12}O_2$	80
Oxine (HQ)	8-hydroxyquinoline	C_9H_7NO	76
Thenoyltrifluoroacetone (TTA)	4,4,4-trifluoro-1-(2-thienyl)-butane-1,3-dione	$C_8H_5F_3O_2S$	44–46
Benzoyltrifluoroacetone (BFA)	4,4,4-trifluoro-1-phenylbutane-1,3-dione	$C_{10}H_7F_3O_2$	39–41

Preparation of a column with glass beads coated with chelating agent

The Apiezon L-precoated glass beads were coated with 3% HQ, TTA, and BFA, respectively, and the coated glass beads were packed into a glass column. The procedure is summarized in Fig. 2.

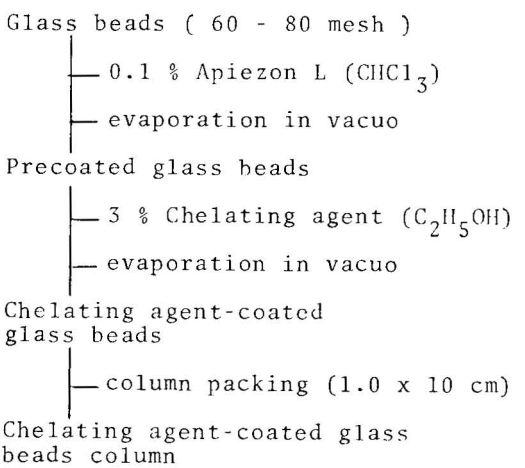

Fig. 2. Preparation of a column containing glass beads coated with chelating agent.

Elution profiles

The elution profiles for the retention of Cu^{2+} were obtained from the TTA and BFA columns. To the top of the column, 1.5 l of 10 ppm Cu^{2+} solution was applied, and the flow-rate of the eluate was maintained at 5 ml/min by using a peristaltic pump.

Each 50-ml fraction of the eluates which had not reacted with the chelating agent on the glass beads and, therefore, was not retained in the column, was determined by titrating the eluate with 0.005 *M* EDTA solution at pH 4.5 using pyrocatechol violet as a metal indicator. The coating of the glass beads with HQ was found insufficient to perform the retention study of Cu^{2+} owing to its relatively high solubility in water, and its poor adhesiveness on the surface of the Apiezon L-precoated glass beads. As seen in Fig. 3, the retention profile of the TTA-coated glass beads column showed that TTA hardly retained Cu^{2+} at all and is apparently unsuitable for the preconcentration of Cu^{2+}, probably owing to the relatively high solubility of TTA in water and hence a significant loss of TTA from the column during the elution procedure. The retention of Cu^{2+} on BFA-coated glass beads was found to be quantitative until the total eluates amounted to 0.5 l in volume, when the Cu^{2+} solution had a concentration below 10 ppm (Fig. 4).

The elution pattern of the BFA system was similar to that of DBM system which had been reported previously at low concentration of Cu^{2+}.

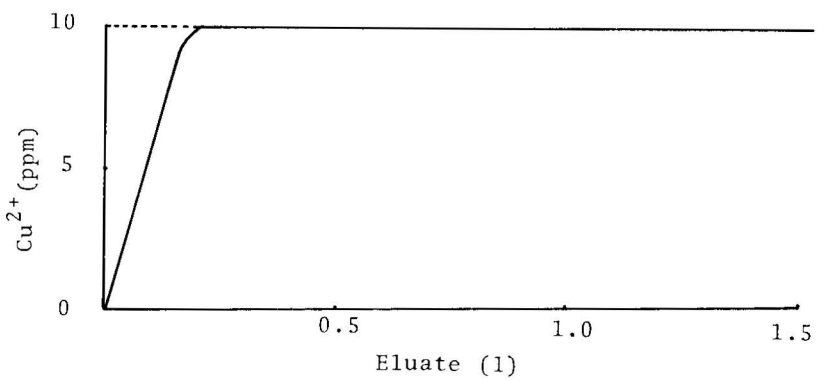

Fig. 3. Retention profile of Cu^{2+} by TTA-coated glass beads column. Column, 3% TTA-coated glass beads (60–80 mesh); flow-rate, 5 ml/min; Cu^{2+} concentration, 10 ppm.

Dissolution of the retained copper chelate on the glass beads column

The dissolution of the retained copper, which was present as the copper chelate on glass beads coated with chelating agent, was examined by using various organic solvents on the DBM and BFA glass beads.

It was shown that the dissolution of CuDBM chelate on the glass beads from the column was not easy with benzene, ethyl acetate, chloroform or methyl isobutyl ketone (MIBK), and generally needed a large amount of the solvent, whereas the dissolution of CuBFA chelate from the column was found to proceed more readily and required a relatively small amount of the solvent. Therefore, the dissolution of CuBFA chelate from the column was investigated more thoroughly.

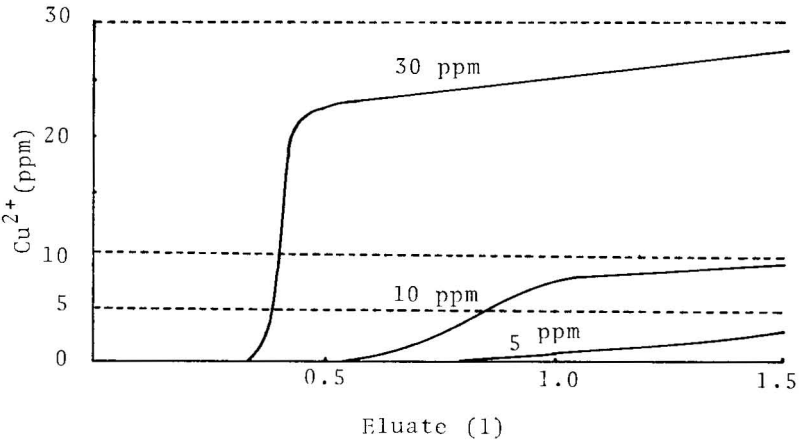

Fig. 4. Retention profile of Cu^{2+} by BFA-coated glass beads column. Column, 3% BFA-coated glass beads (60–80 mesh); flow-rate, 5 ml/min; Cu^{2+} concentrations, 5, 10 and 30 ppm.

Extraction of Cu^{2+} from the CuBFA chelate solution

To determine the proper conditions to extract Cu^{2+} from the CuBFA chelate solution, four solvent systems at various pH were used with a CuBFA chelate that was synthesized in the author's laboratory. The results are shown in Table II.

A 5-ml volume of CuBFA chelate solution, which contained 8 ppm Cu, was mixed with 5 ml of hydrochloric acid solution and Cu^{2+} was back-extracted into the acid solution twice and shaken for 2 min on a mechanical shaker. The two aqueous phases were combined and Cu^{2+} in the solution was determined by EDTA titration. Table II shows that quantitative extraction of Cu^{2+} from the CuBFA chelate solutions is attained at pH 0.8 with benzene, ethyl acetate and MIBK.

TABLE II

RECOVERIES OF Cu^{2+} ON ACIDIC BACK-EXTRACTION FROM THE $Cu(BFA)_2$ CHELATE SOLUTION AT VARIOUS pH

Organic solvent	*Extraction (%)*			
	pH 2.9	*pH 2.3*	*pH 1.5*	*pH 0.8*
Benzene	91	96	99	100
Ethyl acetate	24	47	99	100
MIBK	45	57	82	100
$CHCl_3$	67	72	72	69

Recovery test of Cu^{2+} from the BFA glass beads column

The recovery test of Cu^{2+} from the BFA glass beads column was carried using 0.5 l of 5 ppm Cu^{2+} solution. After the elution, the column was air-dried and treated with 10 ml of benzene to dissolve the CuBFA chelate. A 1-ml volume of the benzene solution was diluted five-fold with benzene and the resulting solution was mixed with 5 ml of 0.16 *N* HCl (pH 0.8) to back-extract Cu^{2+} twice from the solution. The hydrochloric acid phases were combined and subjected to EDTA titration for the determination of Cu^{2+}. The procedure and the results are given in Fig. 5 and Table III.

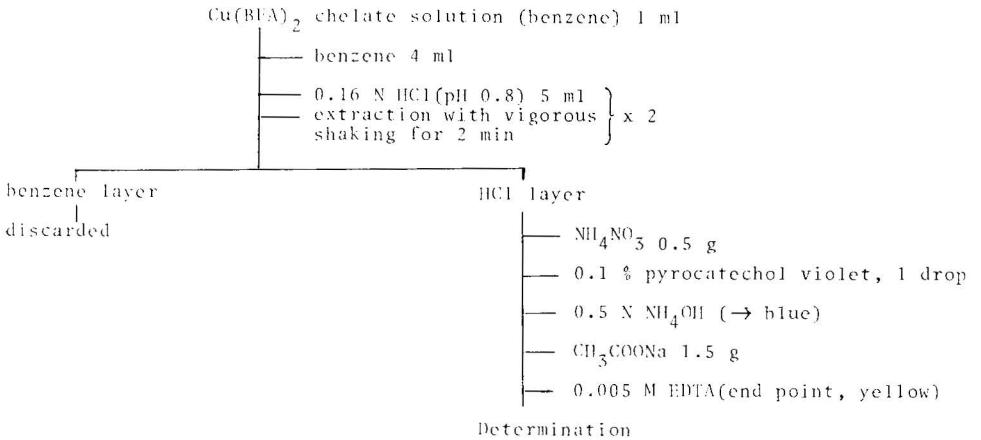

Fig. 5. Procedure for determination of Cu^{2+} (back-extraction method).

TABLE III

RECOVERIES OF Cu^{2+} AT LOW CONCENTRATION USING A BFA-COATED GLASS BEADS COLUMN

Column No.	*Taken Cu^{2+} (mg)*	*Recovered Cu(mg)*	*Recovery (%)*
1	2.50	2.48	99 } mean 99
	2.50	2.48	99 }
2	2.50	2.45	98 } mean 98
	2.50	2.45	98 }
3	2.50	2.68	107 } mean 108
	2.50	2.70	108 }

Using three separately prepared BFA glass beads columns and eluates amounting to 0.5 l from 5 ppm Cu^{2+} solution, the recovery of Cu^{2+} from the original solution was within the range 98–108% (average 103%).

CONCLUSION

Retention of Cu^{2+} on a coated glass beads column was effected by using benzoyltrifluoroacetone (BFA) as a chelating agent for coating. Complete dissolution of the retained CuBFA chelate from the surface of the glass beads was achieved using an organic solvent such as benzene. The resulting benzene solution was treated with diluted hydrochloric acid to back-extract Cu^{2+} for determination. Thus the BFA glass beads column system appears to be a promising one for the preconcentration of micro-amounts of Cu^{2+} in aqueous samples, such as environmental materials.

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journal of chromatography news section

MEETING

FOURTEENTH INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY
"CHROMATOGRAPHY '79"

The Fourteenth International Symposium on Advances in Chromatography will be held September 25–29, 1979, at the Aula of the Ecole Polytechnique Fédérale de Lausanne in Lausanne, Switzerland.

A total of 89 papers will be presented at the Symposium representing contributions from 17 countries. A special feature of the meeting will be an exposition of the latest instrumentation and books. There will also be informal discussion groups held during the course of the Symposium.

Registration should be made in advance. The programs, registration forms and hotel reservation cards can be obtained from:

Prof. E. sz. Kováts
Department de Chimie
EPFL-Ecublens
1015 Lausanne
Switzerland

or

Prof. Albert Zlatkis
Chemistry Department
University of Houston,
Central Campus
Houston, Texas 77004, U.S.A.

The detailed program of the Symposium is given below.

Monday, September 24, 1979

Morning

9:00 · E. sz. Kováts
· Welcome from the city of Lausanne
· Welcome from the president of EPFL
♫ Musical Interlude
· Presentation of Tswett Chromatography Award
♫ Musical Interlude

CONTEMPORARY CHROMATOGRAPHY

L.S. Ettre, presiding

9:45 Gas Chromatographic and Mass Spectrometric Measurement of Pollutants: Selective Electron Capture Sensitization. **R.E. Sievers**, R.M. Barkley, Jr., D.W. Denney, J.M. Roberts, R.S. Hutte, M.J. Bollinger, M.A. Wizner and M.P. Phillips (University of Colorado, Boulder, Colo., U.S.A.) and F.C. Fehsenfeld (Aeronomy Lab, Boulder, Colo., U.S.A.)

10:15 Flip-Flop Chromatography. A.J.P. Martin (University of Houston, Houston, Texas, U.S.A.) and **I. Halász**, H. Engelhardt and P. Sewell (Universität des Saarlandes, Saarbrücken, G.F.R.)

10:45 Intermission

11:00 A Study of High Efficiency Packed Column with Micro Packings in Gas Chromatography. **Lu Peichang**, Zhou Liangmo, Wang Chinghai, Wang Guanghua, Xia Aizu and Xu Fengbao (Dalien Institute of Chemical Physics, Dalien, Liaoning, People's Republic of China)

11:30 Separation of Optical Isomers with Chiral Solvents. P.E. Hare (Carnegie Institution, Washington, D.C., U.S.A.) and **E. Gil-Av** (Weizmann Institute of Science, Rehovot, Israel)

Afternoon: Lecture Hall A

THE ENVIRONMENT

R.E. Sievers, presiding

2:00 Direct Aqueous Injection Gas Chromatography for the Analysis of Trace Organics in Water. **P.G. Simmonds** and E. Kerns (Ringwood, Hants, Great Britain)
2:20 Isotopic Evaluation of Chromatographic Sorbents Used in Air Pollutant Studies. **E. Pellizzari** (Research Triangle Institute, Research Triangle Park, N.C., U.S.A.)
2:40 The Elucidation of Geomatrices by Laser Pyrolysis/Gas Chromatography and Pyrolysis/Mass Spectrometry. **C.E.R. Jones** and N.E. Vanderborgh (Los Alamos Scientific Laboratory, Los Alamos, N.M., U.S.A.)
3:00 Application of Glass (WCOT) Capillary Chromatography with MS, SIM, and FID Detection: Analysis of Weathered and Unweathered Southern Louisiana Crude Oil. **D.J. Hallett** and D.B. Peakall (Canadian Wildlife Research Centre, Ottawa, Ontario, Canada) and F.I. Onuska and M.E. Comba (Canada Centre for Inland Waters, Burlington, Ontario, Canada)
3:20 GLC–MS Studies on Extracts and Distillates from Posidonomia Shales (FRG). **W. Heller**, M. Schallies and K. Schmidt (Institut für Organische Chemie, Tübingen, G.F.R.)
3:40 Intermission

DETECTION SYSTEMS

C.F. Poole, presiding

3:50 Detection of Germanium Compounds by Surface or Gas-Phase Luminescence. C.G. Flinn and **W.A. Aue** (Dalhousie University, Halifax, Canada)
4:10 Determination of Kováts Indices with a Capillary Column and Electron Capture Detection. Application to an Assay of the Enzymatic Conversion of 3,4-Epoxy-1-butene into Diepoxybutane. E. Malvoisin, **E. Evrard**, M. Roberfroid and M. Mercier (University of Louvain, Brussels, Belgium)
4:30 Application of Glass Capillary/Electron Capture GC to Determination of Organochlorine Compounds in Environmental Samples. **R.J. Norstrom** and D.J. Hallett (National Wildlife Research Centre, Ottawa, Ontario, Canada)

Afternoon: Lecture Hall B

GENERAL GAS CHROMATOGRAPHY

E. Grushka, presiding

2:00 Utility of High-Temperature Thermotropic Liquid Crystals as Stationary Phases for Novel Gas–Liquid Chromatographic Separations. **W.L. Zielinski, Jr.** and G.M. Janini (National Bureau of Standards, Washington, D.C., U.S.A.)
2:20 *In Situ* Loading and Reloading of Gas Chromatographic Columns with Stationary Liquids. R.J. Jonker and **H. Poppe** (University of Amsterdam, Amsterdam, The Netherlands) and J.F.K. Huber (University of Vienna, Vienna, Austria)
2:40 Influence of Different Carrier Gases and Temperatures on the Overall Performance of a Packed Column. **L. Rohrschneider** and E. Pelster (Chemische Werke Hüls, Marl, G.F.R.)
3:00 Poly(α-olefins) – Novel Non-Polar Stationary Phases for Gas Chromatography. **F.I. Onuska** (National Water Research Institute, Burlington, Ontario, Canada)
3:20 Vapor Phase Silylation of Alcohols for Air Analysis. M. Osman, **H.H. Hill, Jr.**, M. Holdren and H. Westberg (Washington State University, Pullman, Wash., U.S.A.)
3:40 Intermission

E. Cremer, presiding

3:50 Information Content of Multidimensional Switching Systems in Gas Chromatography. **J. Ševčík** (Packard-Becker B.V., Delft, The Netherlands)
4:10 A Comprehensive Study on the Polymer Beads and Pyrolytic Carbon Beads as Chromatographic Column Packings. Zhu An and **Deng Li-Ru** (Institute of Chemistry, Academia Sinica, Peking, People's Republic of China)
4:30 Polyfunctional Polymer Sorbents. **K. Sakodynskii**, L. Panina and V. Boeva (Institute of Physical Chemistry, Moscow, U.S.S.R.)
4:50 The Determination of Traces of HCl by Derivatization with Epoxides. B. Vierkorn-Rudolph, M. Savelsberg and **K. Bächmann** (Technische Hochschule Darmstadt, Darmstadt, G.F.R.)
5:10 A Comparison Between Conventional and Cross-Correlation Gas Chromatography for Evolved Gas Analysis. M. Kaljurand and **E. Küllik** (Institute of Chemistry, Estonian SSR, Tallinn, U.S.S.R.)

Tuesday, September 25, 1979

Morning: Lecture Hall A

CHROMATOGRAPHY AND THE ANALYTICAL LABORATORY

A mini symposium on the role of chromatographic methods in major analytical laboratories and their requirements for future development

P. Javet, presiding

9:00 The Role of Chromatographic Methods at Ciba-Geigy. **W. Büchler** (Ciba-Geigy AG, Basel, Switzerland)
9:40 The Role of Chromatography in Analysis within Unilever. **A. McKinnon** (Unilever Research, Sharnbrook, Great Britain)
10:20 The Role of Chromatography in the Analytical Laboratories of BASF. **W. Huber** (BASF AG, Ludwigshafen, G.F.R.)
11:00 The Role of Chromatographic Methods in a Forensic-Law Enforcement Laboratory. **A.C. Moffat** (Central Research Establishment, Home Office, Aldermaston, Great Britain)

Afternoon: Lecture Hall A

BIOMEDICAL APPLICATIONS OF GAS CHROMATOGRAPHY

E.C. Horning, presiding

2:00 Quantitative Analysis of Corticosteroids in Adrenal Cell Cultures by Capillary Column Gas Chromatography and Combined Mass Spectrometry. **B.F. Maume**, C. Millot, D. Mesnier, D. Patouraux, J. Doumas and E. Tomori (Université de Dijon, Dijon, France)
2:20 A Distribution Study of Volatile Halogenated Organic Compounds Between Rat Blood Serum and Adipose Tissue using a Purge/Trap Procedure. **C.D. Pfaffenberger**, A.J. Peoples and H.F. Enos (University of Miami School of Medicine, Miami, Fla., U.S.A.)
2:40 Combined High-Pressure Liquid Chromatography and Radioimmunoassay Method for the Quantification of Δ^9-Tetrahydrocannabinol Metabolites in Human Urine. P.L. Williams, **A.C. Moffat** and L.J. King (Home Office Central Research Establishment, Berkshire, Great Britain)
3:00 Volatile Pheromones in Insects by Gas Chromatography Detection and Structure Analysis. **E.D. Morgan** and R.C. Tyler (University of Keele, Staffordshire, Great Britain)
3:20 Synthesis and Properties of a Novel Optically Active Stationary Phase for the Resolution of Amino Acids Enantiomers. T. Saeed, **P. Sandra** and M. Verzele (University of Ghent, Ghent, Belgium)
3:40 Intermission

C.J.W. Brooks, presiding

3:50 High Efficiency Glass Capillary Column Gas Chromatography and Gas Chromatography–Mass Spectrometry of Oxygenated Metabolites of Arachidonic Acid (Hydroxylated Fatty Acids, Prostaglandins and Thromboxanes). **M. Rigaud**, P. Chebroux, A. Soustre, J. Durand, H. Rabinovitch and J.C. Breton (Medical University, Limoges, France)

4:10 On the Silylation of Tryptamine and 5-Hydroxytryptamine. Kinetic Aspects and Practical Analytical Implications. E. Martinez and **E. Gelpi** (Instituto de Biofisica y Neurobiologia, Barcelona, Spain)

4:30 Microliter Extraction and Extract Concentration for Biomedical Trace Analysis with Glass Capillary Gas Chromatography. **W. Dünges** and K. Kiesel (Pharmakologisches Institut der Universität Mainz, Mainz, G.F.R.)

4:50 Gas Chromatography/Mass Spectrometry of Urinary Acids from Patients with Abnormal Fat Metabolism. **H.M. Liebich** and U. Stierle (Medizinische Universitätsklinik Labor, Tübingen, G.F.R.)

5:10 Analysis of Therapeutic and Commonly Abused Drugs in Serum and Urine by Gas–Liquid Chromatography using a Photoionization Detector. L.F. Jaramillo and **J.N. Driscoll** (HNU Systems, Newton, Mass., U.S.A.)

Afternoon: Lecture Hall B

HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

R.E. Kaiser, presiding

2:00 Microprocessor Controlled Automatic HPTLC–TLC–Spectroscopy for Routine Analysis. **U. Hezel** (Carl Zeiss, Oberkochen, G.F.R.)

2:20 A New Approach to Sample Application for TLC. **D.C. Fenimore** (Texas Research Institute for Mental Sciences, Houston, Texas, U.S.A.)

2:40 Problems and Applications of Reversed-Phase Thin-Layer Chromatography. A.M. Siouffi and T. Wawrzynowicz (Faculté des Sciences et Techniques de Saint-Jerome, Marseille, France), F. Bressolle (Laboratoire de Chimie Analytique, Montpellier, France) and **G. Guiochon** (Ecole Polytechnique, Palaiseau, France)

3:00 TLC of Slightly Soluble Substances at Elevated Temperature. **G. Szekely** (Ciba-Geigy, Basel, Switzerland)

3:20 Intermission

GENERAL CHROMATOGRAPHY

R.D. Schwartz, presiding

3:30 Metabolic Profiles of Panaeid Shrimp: Dietary Lipids and Ovarian Maturation. **B.S. Middleditch**, S.R. Missler and H.B. Hines (University of Houston, Houston, Texas, U.S.A.), J.P. McVey and A. Brown (National Marine Fisheries Service, Galveston, Texas, U.S.A.) and D.C. Ward and A.L. Lawrence (University of Houston, Houston, Texas, U.S.A.)

3:50 Analysis of Potable Water Contaminants in Kuwait by GC/MS and Their Potential Health Hazards. **F. Shunbo**, Y.Y. Al-Sultan and M.M. Helmy (Kuwait Institute for Scientific Research, Kuwait, Kuwait)

4:10 An Automatic System for the High Resolution GC Analysis of Gasoline-Range Hydrocarbon Mixtures. **E.R. Adlard**, A.W. Bowen and D.G. Salmon (Shell Research Ltd., Chester, Great Britain)

4:30 Gas Chromatographic/Mass Spectrometric Analysis of Neutral Lipids from Methanogenic, Halophilic and Thermoacidophilic Bacteria. **G. Holzer** and J. Oró (University of Houston, Houston, Texas, U.S.A.) and T.G. Tornabene (Colorado State University, Fort Collins, Colo., U.S.A.)

4:50 Application of Head Space Analysis to the Study of Volatile Organic Impurity Concentrates: Gas Chromatographic Determination of Aromatic Hydrocarbons in Atmospheric Air. **B.V. Ioffe**, A.G. Vitenberg and I.A. Tsibul'skaya (Leningrad State University, Leningrad, U.S.S.R.)

Wednesday, September 26, 1979

Morning: Lecture Hall A

GLASS CAPILLARY COLUMNS IN GAS CHROMATOGRAPHY

S.R. Lipsky, presiding

9:00 "Selective" Sampling to Capillary Columns for High-Resolution Gas Chromatography in Multi-dimensional Systems Involving a Packed Precolumn. **G. Schomburg**, H. Behlau, I. Hazai and F. Weeke (Max Planck Institut für Kohlenforschung, Mülheim/Ruhr, G.F.R.)

9:20 Non-Extractable Methyl Silicone Gums as Stationary Phases for Glass Capillary Gas Chromatography. **L. Blomberg** and T. Wännman (University of Stockholm, Stockholm, Sweden)

9:40 A Simple and Versatile All Glass Splitless Sample Introduction System for On- and Off-line Trace Analysis with Capillary Gas Chromatography. **J.A. Rijks** (Eindhoven University of Technology, Eindhoven, The Netherlands) and J. Drozd and J. Novák (Czechoslovak Academy of Sciences, Brno, Czechoslovakia)

10:00 Glass Capillary Adsorption Columns for Gas Chromatography. R.G. Mathews, J. Torres and **R.D. Schwartz** (Pennzoil Company, Shreveport, La., U.S.A.)

10:20 A New Approach to Chromatographic Optimization with Glass Capillary Columns. **W. Jennings** and J.A. Settlage (University of California at Davis, Davis, Calif., U.S.A.) and R.J. Miller (American Hoechst Corp., Leominster, Mass., U.S.A.)

10:40 Intermission

D. Reymond, presiding

10:50 Application of Fused Quartz GC Capillary Columns to Biomedical and Environmental Problems. **R.D. Dandeneau**, P.F. Bente, III, and D. Smith (Hewlett-Packard Company, Avondale, Pa., U.S.A.)

11:10 Optimization of the Separation Process in Analysis of Complex Mixtures by Capillary Gas Chromatography. **J. Krupčík**, A. Šimová, J. Mocák, G. Guiochon and J. Garaj (Slovak Technical University, Bratislava, Czechoslovakia)

11:30 A Capillary GLC Injection System for Large Volumes. **W. Vogt**, K. Jacob, A.-B. Ohnesorge and H.W. Obwexer (Institut für Klinische Chemie am Klinikum Grosshadern der Universität München, Munich, G.F.R.)

Morning: Lecture Hall B

THEORETICAL AND PRACTICAL ASPECTS OF GAS CHROMATOGRAPHY

C. Horváth, presiding

9:00 Chromatographic Peak Shape Analysis III. The Determination of Adsorption–Desorption Constant. S.D. Mott and **E. Grushka** (State University of New York at Buffalo, Buffalo, N.Y., U.S.A.)

9:20 Retention Characteristics of Polymers in Thermal Field-Flow Fractionation. **J.C. Giddings** (The University of Utah, Salt Lake City, Utah, U.S.A.)

9:40 Mixed Retention Mechanisms at the Surface of Column Packing. **F. Riedo** and E. sz. Kováts (Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland)

10:00 A Study of the Adsorption Effects at the Surface of Poly(Ethylene Glycol) Column Packings. **D.F. Fritz**, A. Sahil and E. sz. Kováts (Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland)

10:20 Evaluation of a Thermally Stable Hydrocarbon as a Non-polar Base Stationary Phase for Use in Rohrschneider–McReynolds Type Schemes. **J.K. Haken** (The University of New South Wales, New South Wales, Australia) and F. Vernon (The University of Salford, Lancashire, Great Britain)

10:40 Intermission

J.H. Knox, presiding

10:50 Analysis of Kinetics and Concentration Dependence in Electron Capture Detectors. **W.E. Wentworth** and E.C.M. Chen (University of Houston, Houston, Texas, U.S.A.)
11:10 Polystyrene Bonded Silica Particles in Gas Chromatography: Separation Mechanisms. **B. Monrabal** (Dow Chemical Iberica, S.A., Tarragona, Spain)
11:30 Characterization and Selection of Stationary Phases for Gas–Liquid Chromatography by Pattern Recognition Methods. **J.F.K. Huber** and G. Reich (University of Vienna, Vienna, Austria)
11:50 Estimation of Energy of Intermolecular Interactions in GC from Retention Index Values. **R. Golovnya** and T. Misharina (Academy of Science, U.S.S.R., Moscow, U.S.S.R.)

Thursday, September 27, 1979

Morning: Lecture Hall A

BIOMEDICAL APPLICATIONS OF LIQUID CHROMATOGRAPHY

R.W. Frei, presiding

9:00 The Identification and Quantitation of Nucleosides, Bases and Other UV-Absorbing Compounds in Serum Using Reversed-Phase High-Performance Liquid Chromatography. I. Chromatographic Methodology. **R.A. Hartwick**, S.P. Assenza and P.R. Brown (University of Rhode Island, Kingston, R.I., U.S.A.)
9:20 The Identification and Quantitation of Nucleosides, Bases and Other UV-Absorbing Compounds in Serum, Using Reversed-Phase High-Performance Liquid Chromatography. II. Evaluation of Human Sera. R.A. Hartwick, A.M. Krstulovic and **P.R. Brown** (University of Rhode Island, Kingston, R.I., U.S.A.)
9:40 Ligand Exchange Chromatography of Small Peptides on Copper(II) Modified Silica Gel. Application to the Study of the Enzymatic Degradation of the Methionine-Enkephalin. A. Guyon, F. Guyon, A. Foucault and **M. Caude** (Ecole Supérieure de Physique et de Chimie Industrielles de Paris, Paris, France)
10:00 A New Approach to the Analysis of Quaternary Ammonium Compounds and Basic Drugs, Based on Ion-Pair Adsorption HPLC. **J.E. Greving**, H. Bouman, J.H.G. Jonkman, H.G.M. Westenberg and R.A. de Zeeuw (State University, Groningen, The Netherlands)
10:20 A Stability-Indicating Assay for Azathioprine and 6-Mercaptopurine by Reversed-Phase High-Performance Liquid Chromatography. **A.F. Fell** and S.M. Plag (Heriot-Watt University, Edinburgh, Great Britain) and J.M. Neil (Western General Hospital, Edinburgh, Great Britain)
10:40 Intermission

P.R. Brown, presiding

10:50 Reversed-Phase Ion-Pair HPLC of Drugs and Related Compounds using Underivatized Silica as Stationary Phase. **J. Crommen** (Université de Liège, Liège, Belgium)
11:10 Non-polar Strong Cation Exchangers for Separation of Steroids in Mixed Chromatographic Systems. M. Axelson and **J. Sjövall** (Karolinska Institutet, Stockholm, Sweden)
11:30 Diagnosis of Neural Crest Tumors by Reversed-Phase High-Performance Liquid Chromatographic Determination of Urinary Catecholamine Metabolites. **A.M. Krstulovic** (Manhattanville College, Purchase, N.Y., U.S.A.), K. Lohse (Schoeffel Instrument Division, Westwood, N.J., U.S.A.) and M. Zakaria (University of Rhode Island, Kingston, R.I., U.S.A.)
11:50 A High-Performance Liquid Chromatographic Assay for Vitamin K in Human Serum. **M.F. Lefèvere**, A.P. de Leenheer and A.E. Claeys (University of Ghent, Ghent, Belgium)

Morning: Lecture Hall B

THEORETICAL ASPECTS OF LIQUID CHROMATOGRAPHY

J.C. Giddings, presiding

9:00 1. The HETP of an Unretained Component of Fluid Flow in a Coiled Round Tube. 2. The Spreading of a Solute in Poiseuille Flow Immediately after Injection. **M.J.E. Golay** (Perkin-Elmer, Norwalk, Conn., U.S.A.)

9:20 Evaluation of Association Constants for Complexes by HPLC. **C. Horváth**, W.R. Melander and A. Naham (Yale University, New Haven, Conn., U.S.A.)

9:40 Factors Influencing the Reversed-Phase Separation of Tetracyclines by HPLC. **J.H. Knox** and J. Jurand (University of Edinburgh, Edinburgh, Great Britain)

10:00 Investigations on the Mechanisms of Ion-Pair Chromatography. **B.A. Bidlingmeyer** (Waters Associates, Milford, Mass., U.S.A.) and S.N. Deming, W.P. Price, Jr., B. Sachok and M. Petrusek (University of Houston, Houston, Texas, U.S.A.)

10:20 Numerical Analysis of Chromatographic/Spectrometric Data. Purity of Partially Resolved Peaks and Extraction of the Pure Component Spectra. **J.M. Halket** (Institute for Reproductive Biology and Medicine, Hamburg, G.F.R.)

10:40 Intermission

H. Engelhardt, presiding

10:50 HPLC Phase Characterization: Particle Size, Surface Area, Mean Pore Diameter, Amount of Bonded Phase and Activity. **M. Verzele** and J. Lammens (University of Ghent, Ghent, Belgium)

11:10 Evaluation of Ion-Pair Mechanisms in Reversed-Phase Liquid Chromatography by a Recycle System. **J.L.M. van de Venne** and A.P. Konijnendijk (Eindhoven University of Technology, Eindhoven, The Netherlands)

11:30 Instrumental Optimization in HPLC: Theory and Practical Results. **R.E. Kaiser** and R.I. Rieder (Institut für Chromatographie, Bad Dürkheim, G.F.R.)

11:50 Preparative Liquid Chromatography: Sample Volume Overload. B. Coq, G. Cretier, J.L. Rocca and **M. Porthault** (Université Claude Bernard Lyon I, Villeurbanne, France)

Afternoon: Lecture Hall A

INFORMAL DISCUSSIONS

2:00 Liquid Chromatography
Chairman: **I. Halász**
Starter: **R.P.W. Scott**

3:30 Glass Capillary Columns Gas Chromatography
Chairman: **G. Schomburg**
Starter: **J. Rijks**

Afternoon: Lecture Hall B

INFORMAL DISCUSSIONS

2:00 High-Performance Thin-Layer Chromatography
Chairman: **D.C. Fenimore**
Starter: **R.E. Kaiser**

3:30 Chromatography and the Environment
Chairman: **P.G. Simmonds**
Starter: **E. Pellizzari**

Friday, September 28, 1979

Morning: Lecture Hall A

LIQUID CHROMATOGRAPHY – GENERAL

I. Halász, presiding

9:00 The Application of Microbore Columns for Fast Liquid Chromatographic Analysis. **R.P.W. Scott** and M. Munroe (Hoffman-La Roche, Inc., Nutley, N.J., U.S.A.)
9:20 Design and Selective Application of a DME Amperometric Detector in Column Liquid Chromatography. H.B. Hanekamp, P. Bos and **R.W. Frei** (Free University, Amsterdam, The Netherlands)
9:40 The On Line Coupling of HPLC and NMR. **E. Bayer** and K. Albert (University of Tübingen, Tübingen, G.F.R.)
10:00 Anion Chromatography with Low-Conductivity Eluents. D.T. Gjerde, **J.S. Fritz** and G. Schmuckler (Iowa State University, Ames, Iowa, U.S.A.)
10:20 Exclusion Chromatography of Water-Soluble Polymers. **H. Engelhardt** and D. Mathes (Universität des Saarlandes, Saarbrücken, G.F.R.)
10:40 Intermission

L.S. Ettre, presiding

10:50 Techniques of Capillary Liquid Chromatography. Y. Hirata and **M. Novotný** (Indiana University, Bloomington, Ind., U.S.A.)
11:10 Application of Droplet Counter-Current Chromatography in Natural Products Isolation. **K. Hostettmann**, M. Hostettmann-Kaldas and O. Sticher (Eidgenössische Technische Hochschule Zürich, Zürich, Switzerland)
11:30 Improved Detectability of Barbiturates in High-Performance Liquid Chromatography by Pre-Column Labelling and Ultraviolet Detection. **A. Hulshoff** (Rijksuniversiteit Utrecht, Utrecht, The Netherlands) and H. Roseboom (Rijksinstituut voor de Volksgezondheid, Utrecht, The Netherlands)
11:50 Liquid Chromatographic Resolution of Enantiomers on Chiral Amide Bonded-Silica Gel Normal Phase. **S. Hara** and A. Dobashi (Tokyo College of Pharmacy, Tokyo, Japan)
12:10 Closing of Symposium

NEW INTENSIVE SHORT COURSES

September 22–23, 1979

The following courses will be given on the Friday and Saturday prior to the symposium.

1. Open Tubular (Capillary) Gas Chromatography
   Directed by: Dr. G. Schomburg
   Dr. L.S. Ettre
2. High-Performance Liquid Chromatography
   Directed by: Prof. Cs. Horváth
   Dr. I. Molnár
3. Gas Chromatography/Mass Spectrometry
   Directed by: Prof. C.J.W. Brooks
   Prof. B.S. Middleditch
   Dr. W.J.A. VandenHeuvel
4. High-Performance Thin-Layer Chromatography
   Directed by: Dr. U. Hezel
   Dr. D.C. Fenimore
5. Maintaining and Troubleshooting Chromatographic Systems
   Directed by: Mr. J.Q. Walker
   Mr. M.T. Jackson
   Dr. M.P.T. Bradley
6. Sequential Simplex Optimization
   Directed by: Prof. S.N. Deming
   Dr. S.L. Morgan

For details and registration forms write to:
Prof. Albert Zlatkis
Chemistry Department
University of Houston, Central Campus
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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.

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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.

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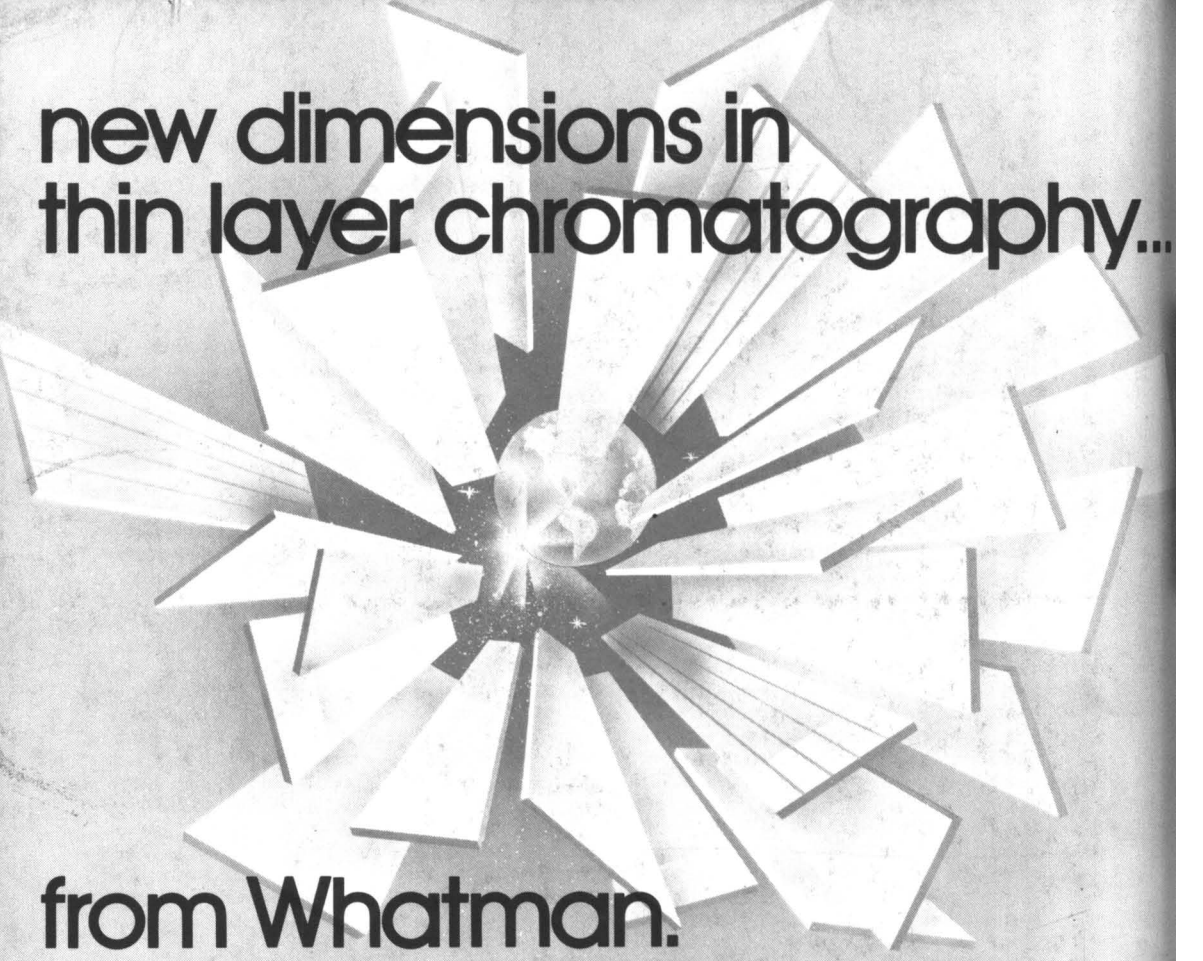

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